INDEXED

FIRST INTERNATIONAL
CONFERENCE ON

VACCINES

AGAINST VIRAL AND RICKETTSIAL DISEASES OF MAN

PAN AMERICAN HEALTH ORGANIZATION
Pan American Sanitary Bureau, Regional Office of the
WORLD HEALTH ORGANIZATION

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FIRST INTERNATIONAL CONFERENCE ON VACCINES AGAINST VIRAL AND RICKETTSIAL DISEASES OF MAN

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Washington, D.C. 20037, U.S.A.

Preface

A survey of opinion among members of the scientific community, conducted late in 1965, made clear the desirability of convening an international conference to summarize present knowledge and future needs in the field of vaccines against viral and rickettsial diseases of man, and to consider chemotherapeutic and other approaches in the control of these diseases. The Conference was organized by the Pan American Health Organization and the World Health Organization and was held at the PAHO Headquarters in Washington, D.C., from 7 to 11 November 1966. It was structured to serve as a forum for the presentation of individual papers by acknowledged authorities from all parts of the world, and for prepared discussions and open debate on those presentations.

The current and new information on practically all important aspects of the subject, presented by nearly 300 distinguished scientists from 27 nations, is recorded in the following pages.

We wish to express our gratitude to all those who contributed their efforts to ensure the success of the Conference and the early publication of the proceedings. We are particularly indebted to the Program Committee for the excellent content of the agenda, and to the participants for the high quality of their presentations, which have made a contribution of inestimable value to the body of knowledge in this field.

ABRAHAM HORWITZ
Director, Pan American Sanitary Bureau
Regional Office, World Health Organization

Editorial Note

The present volume contains all the papers submitted by the participants at the Conference, arranged in the order of their presentation, by session, together with the statements presented by the discussants on each topic. The transcripts of the general discussions have been edited for the sake of brevity and consistency of style; all statements relating to the opening or closing of sessions, and other non-substantive remarks, have been deleted in order to condense the presentation as far as possible.

The texts of the papers have been reproduced as submitted by the authors, with a minimum of editorial changes. For the most part, illustrations have been reproduced directly from the original material submitted. This has made possible the early publication of the proceedings but precluded the possibility of standardizing format in all the papers.

TABLE OF CONTENTS

SESSION X. GENERAL DISCUSSION

PARTICIPANTS

Dr. Francis R. Abinanti
National Institutes of Health
Bethesda, Maryland, USA

Sir Christopher H. Andrewes
Overchalke, Coombe Bisset
Salisbury, Wilts.
England

Dr. O. G. Andzaparidze
Research Institute of Virus Preparations
Moscow, USSR

Dr. M. S. Artenstein
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Karl Bakos
National Veterinary Institute
Stockholm, Sweden

Dr. James Banta
National Institutes of Health
Bethesda, Maryland, USA

Dr. Samuel Baron
National Institutes of Health
Bethesda, Maryland, USA

Dr. O. V. Baroyan
Gamaleya Institute of Epidemiology and Microbiology
Moscow, USSR

Dr. John D. Bauer
The Wellcome Laboratories of Tropical Medicine
Beckenham, Kent, England

Dr. P. L. Bazeley
The Salk Institute for Biological Studies
La Jolla, California, USA

Dr. Alan J. Beale
Virus Vaccine Department
Glaxo Laboratories, Ltd.
Buckinghamshire, England

Dr. Earl S. Beck
National Institutes of Health
Bethesda, Maryland, USA

Dr. Walter B. Becker
National Institutes of Health
Bethesda, Maryland, USA

Dr. J. Frederick Bell
Rocky Mountain Laboratory
Hamilton, Montana, USA

Dr. Joseph A. Bell
National Institutes of Health
Bethesda, Maryland, USA

Dr. Abram S. Benenson
Jefferson Medical College
Philadelphia, Pennsylvania, USA

Dr. Christel M. Benitz
Medical Research
Cyanamid International
Pearl River, New York, USA

Dr. Sanford Berman
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Barbara C. Bernheim
National Institutes of Health
Bethesda, Maryland, USA

Dr. A. J. D. A. Billiau
Rega Institute
University of Louvain
Louvain, Belgium

Dr. Leonard N. Binn
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. James L. Bittle
Infectious Disease Research
The Dow Chemical Company
Zionsville, Indiana, USA

Dr. Dionyz Blaškovič
Institute of Virology
Bratislava, Czechoslovakia

Dr. Henry H. Bloom
Microbiological Associates, Inc.
Bethesda, Maryland, USA

Dr. Vladimir M. Bolotovsky
Central Institute of Epidemiology
Moscow, USSR

Dr. Otto Bonin
Paul Ehrlich Institute
Frankfurt am Main, Germany

Dr. F. L. Bowling
Office of the Surgeon General
Department of the Air Force
Washington, D.C., USA

Dr. F. Marilyn Bozeman
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Robert G. Brackett
Virus Research
Parke, Davis & Company
Detroit, Michigan, USA

Dr. Jacob A. Brody
National Institutes of Health
Bethesda, Maryland, USA

Col. Edward L. Buescher
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Eugene B. Buynak
Merck Institute for Therapeutic Research
West Point, Pennsylvania, USA

Dr. Victor J. Cabasso
Virus Immunological Research
Lederle Laboratories
Pearl River, New York, USA

Dr. J. Campbell
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Edward A. Carbrey
National Animal Disease Laboratory
Ames, Iowa, USA

Dr. Jordi Casals
Yale University and Rockefeller Foundation
New Haven, Connecticut, USA

Dr. Gabriel Castellano
Microbiological Associates, Inc.
Bethesda, Maryland, USA

Dr. Robert M. Chanock
National Institutes of Health
Bethesda, Maryland, USA

Dr. Proctor Child
Armed Forces Institute of Pathology
Washington, D.C., USA

Dr. Mikhail P. Chumakov
Institute of Poliomyelitis and Viral Encephalitides
Moscow, USSR

Dr. W. Chas. Cockburn
World Health Organization
Geneva, Switzerland

Dr. Kenneth W. Cochran
The University of Michigan
Ann Arbor, Michigan, USA

Dr. Hans Cohen
Rijks Instituut voor de Volksgezondheid
Utrecht, The Netherlands

Dr. Lewis L. Coriell
South Jersey Medical Research Foundation
Camden, New Jersey, USA

Dr. Herald R. Cox
Viral and Rickettsial Research
Lederle Laboratories
Pearl River, New York, USA

Dr. E. J. Cunningham
Medical Division
VISTA
Washington, D.C., USA

Dr. Edward C. Curnen
Columbia University
New York, New York, USA

Dr. Arthur C. Curtis
Public Health Division
U.S. Agency for International Development
Washington, D.C., USA

Dr. Robert T. Cutting
U.S. Army Medical Research and Development Command
Washington, D.C., USA

Dr. Peter Dans
National Institutes of Health
Bethesda, Maryland, USA

Dr. Harry E. Dascomb
Louisiana State University
New Orleans, Louisiana, USA

Dr. Fred M. Davenport
The University of Michigan
Ann Arbor, Michigan, USA

Dr. Dorland J. Davis
National Institutes of Health
Bethesda, Maryland, USA

Dr. Albert Dawkins
The Johns Hopkins University
Baltimore, Maryland, USA

Dr. F. W. Deinhardt
Presbyterian-St. Luke's Hospital
Chicago, Illinois, USA

Dr. P. De Somer*
Rega Institute
University of Louvain
Louvain, Belgium

Dr. George W. A. Dick
Bland-Sutton Institute
London, England

Dr. John H. Dingle
Western Reserve University
Cleveland, Ohio, USA

Dr. Zvonimir Dinter
University of Uppsala
Uppsala, Sweden

Dr. A. W. Downie
The University of Liverpool
Liverpool, England

* Unable to attend.

Dr. Christopher C. Draper
The Wellcome Research Laboratories
Beckenham, Kent, England

Dr. Harold A. Drayton
University of Guyana
Georgetown, Guyana

Dr. T. D. Dublin
U.S. Agency for International Development
Washington, D.C., USA

Dr. John A. Dudgeon
The Hospital for Sick Children
London, England

Dr. James T. Duff
National Institutes of Health
Bethesda, Maryland, USA

Dr. H. Bruce Dull
Communicable Disease Center
Atlanta, Georgia, USA

Dr. Geoffrey Edsall
Massachusetts Institute of Laboratories
Boston, Massachusetts, USA

Dr. D. G. ff Edward
The Wellcome Research Laboratories
Beckenham, Kent, England

Dr. Bennett L. Elisberg
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Juan E. Embil
Dalhousie University
Halifax, N.S., Canada

Dr. John F. Enders
The Children's Hospital Medical Center
Boston, Massachusetts, USA

Dr. J. Å Espmark
M.D. Anderson Hospital and Tumor Institute
Houston, Texas, USA

Dr. Alfred S. Evans
Yale University
New Haven, Connecticut, USA

Dr. Jacqueline Fabia
International Children's Center
Paris, France

Dr. Akinyele Fabiyi
National Institutes of Health
Bethesda, Maryland, USA

Dr. Raymond Fagan
Virology Research
Wyeth Laboratories
Radnor, Pennsylvania, USA

Dr. Egisto Falchetti
Istituto Sieroterapico e Vaccinogeno Toscano
Sclavo
Siena, Italy

Dr. Harry A. Feldman
State University of New York
Syracuse, New York, USA

Dr. Paul Fenje
Connaught Medical Research Laboratories
Toronto, Ontario, Canada

Dr. Mary Fink
National Institutes of Health
Bethesda, Maryland, USA

Dr. Paul Fiset
University of Maryland
Baltimore, Maryland, USA

Dr. Allan L. Forbes
Life Sciences Division
Department of the Army
Arlington, Virginia, USA

Dr. John P. Fox
University of Washington
Seattle, Washington, USA

Dr. Thomas Francis, Jr.
The University of Michigan
Ann Arbor, Michigan, USA

Dr. Harald Frederiksen
Office of Technical Cooperation and Research
U.S. Agency for International Development
Washington, D.C., USA

Dr. David A. Fucillo
National Institutes of Health
Bethesda, Maryland, USA

Dr. George J. Galasso
The University of Virginia
Charlottesville, Virginia, USA

Dr. Sven Gard
Karolinska Institute
Stockholm, Sweden

Dr. Ross L. Gauld
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. A. Giovanardi
Universitá di Milano
Milan, Italy

Dr. Gary L. Gitnick
National Institutes of Health
Bethesda, Maryland, USA

Dr. Paulo de Góes
Instituto de Microbiologia
Universidade do Brasil
Rio de Janeiro, Brasil

Dr. Francis B. Gordon
Department of Microbiology
Naval Medical Research Institute
Bethesda, Maryland, USA

Dr. Arthur N. Gorelick
Virus and Rickettsial Department
U.S. Army Biological Center
Frederick, Maryland, USA

Dr. Alan Gray
Bacterial and Viral Vaccines
Merck, Sharp & Dohme
West Point, Pennsylvania, USA

Dr. J. Thomas Grayston
University of Washington
Seattle, Washington, USA

Dr. Herschel E. Griffin
Preventive Medicine Division
Office of the Surgeon General of the Army
Washington, D.C., USA

Dr. Norman R. Grist
University of Glasgow
Glasgow, Scotland

Dr. Richard Grunert
Pharmaceutical Research
E. I. du Pont de Nemours & Co.
Wilmington, Delaware, USA

Dr. Richard Haas
University of Freiburg
Freiburg, Germany

Dr. William McD. Hammon
University of Pittsburgh
Pittsburgh, Pennsylvania, USA

Dr. Dorothy M. Hamre
University of Chicago
Chicago, Illinois, USA

Dr. V. R. Harrison
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Leonard Hayflick
The Wistar Institute
Philadelphia, Pennsylvania, USA

Dr. R. Heberling
National Institutes of Health
Bethesda, Maryland, USA

Dr. Alfred Hellman
Oak Ridge National Laboratory
Oak Ridge, Tennessee, USA

Dr. Werner Henle
The Children's Hospital of Philadelphia
Philadelphia, Pennsylvania, USA

Dr. Walter A. Hennessen
Behringwerke, AG.
Marburg/Lahn, Germany

Dr. Caspar W. Hiatt
National Institutes of Health
Bethesda, Maryland, USA

Dr. Maurice R. Hilleman
Merck Institute for Therapeutic Research
West Point, Pennsylvania, USA

Dr. Monto Ho
University of Pittsburgh
Pittsburgh, Pennsylvania, USA

Dr. Horace L. Hodes
The Mount Sinai Hospital
New York, New York, USA

Dr. Ariel C. Hollinshead
The George Washington University
Washington, D.C., USA

Dr. Jacob C. Holper
Infectious Diseases Research Division
Abbott Laboratories
Chicago, Illinois, USA

Dr. H. E. Hopps
National Institutes of Health
Bethesda, Maryland, USA

Dr. Richard Hornick
The University of Maryland
Baltimore, Maryland, USA

Dr. Dorothy Horstmann
Yale University
New Haven, Connecticut, USA

Dr. Abraham Horwitz
Pan American Health Organization
Washington, D.C., USA

Dr. Robert J. Huebner
National Institutes of Health
Bethesda, Maryland, USA

Dr. Robert N. Hull
The Lilly Research Laboratories
Indianapolis, Indiana, USA

Dr. David L. Huxsoll
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Howard J. Igel
National Institutes of Health
Bethesda, Maryland, USA

Dr. Drago Ikić
Institute of Immunology
Zagreb, Yugoslavia

Dr. Imam Z. E. Imam
Virus Research Center
Production Laboratories
Agouza, Cairo, UAR

Dr. George G. Jackson
The University of Illinois
Chicago, Illinois, USA

Dr. Leon Jacobs
National Institutes of Health
Bethesda, Maryland, USA

Dr. Keith E. Jensen
Medical Research Laboratories
Chas. Pfizer and Company, Inc.
Groton, Connecticut, USA

Dr. Erik Johansson
Hem Research Incorporated
Bethesda, Maryland, USA

Dr. Donald W. Johnson
Animal Health Division
U.S. Department of Agriculture
Hyattsville, Maryland, USA

Dr. Harald N. Johnson
Arthropod-Borne Virus Studies
The Rockefeller Foundation
Berkeley, California, USA

Dr. Karl McK. Johnson
Middle America Research Unit
Balboa Heights, Canal Zone

Dr. William S. Jordan, Jr.
The University of Virginia
Charlottesville, Virginia, USA

Dr. J. Mehsen Joseph
Maryland State Department of Health
Baltimore, Maryland, USA

Dr. François A. Kalabus
Ministère de la Santé
Quebec, Canada

Dr. Albert Z. Kapikian
National Institutes of Health
Bethesda, Maryland, USA

Dr. Martin M. Kaplan
World Health Organization
Geneva, Switzerland

Dr. Julius A. Kasel
National Institutes of Health
Bethesda, Maryland, USA

Dr. Samuel L. Katz
The Children's Hospital Medical Center
Boston, Massachusetts, USA

Dr. Herbert E. Kaufman
The University of Florida
Gainesville, Florida, USA

Dr. C. Henry Kempe
The University of Colorado Medical Center
Denver, Colorado, USA

Dr. Ruth L. Kirschstein
National Institutes of Health
Bethesda, Maryland, USA

Dr. Morton Klein
Temple University
Philadelphia, Pennsylvania, USA

Dr. Hilary Koprowski
The Wistar Institute
Philadelphia, Pennsylvania, USA

Dr. Saul Krugman
New York University
New York, New York, USA

Dr. René Labusquière
Organization for Coordination and Cooperation in the Control of Major Endemic Diseases for the Countries of Central Africa
Yaoundé, Republic of Cameroon

Dr. Nicholas M. Larin
Biologicals Research Department
The Pfizer Group
Kent, England

Dr. Edwin H. Lennette
Viral and Rickettsial Disease Laboratory
State of California Department of Public Health
Berkeley, California, USA

Dr. Pierre R. Lepine
Institut Pasteur
Paris, France

Dr. Stanley Levin
Kaplan Hospital
Rehovot, Israel

Dr. L. James Lewis
Sterling-Winthrop Research Institute
Biological Division
Rensselaer, New York, USA

Dr. Florence Lief
University of Pennsylvania
Philadelphia, Pennsylvania, USA

Dr. Royce Z. Lockart, Jr.
Central Research Department
E. I. du Pont de Nemours & Co.
Wilmington, Delaware, USA

Dr. William T. London
National Institutes of Health
Bethesda, Maryland, USA

Dr. Clayton G. Loosli
University of Southern California
Los Angeles, California, USA

Dr. Joseph P. Lowenthal
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Lauri Luoto
National Institutes of Health
Bethesda, Maryland, USA

Dr. Ronald B. Mackenzie
Rockefeller Foundation
Bogotá, Colombia

Dr. Frederick O. MacCallum
Virus Laboratory
The Radcliffe Infirmary
Oxford, England

Dr. Donald R. E. MacLeod
Connaught Medical Research Laboratories
University of Toronto
Toronto, Ontario, Canada

Dr. Herdis von Magnus
Statens Seruminstitut
Copenhagen, Denmark

Dr. Barrie P. Marmion
Monash University
Melbourne, Australia

Dr. Salvador Martín
Instituto de Vacunas y Biológicos
México, D.F., México

Dr. Mauricio Martins da Silva
Pan American Health Organization
Washington, D.C., USA

Dr. Minoru Matumoto
The University of Tokyo
Tokyo, Japan

Dr. Gordon Meiklejohn
University of Colorado Medical Center
Denver, Colorado, USA

Dr. Joseph L. Melnick
Baylor University
Houston, Texas, USA

Dr. Harry M. Meyer, Jr.
National Institutes of Health
Bethesda, Maryland, USA

Dr. Jack W. Millar
Bureau of Medicine and Surgery
Washington, D.C., USA

Dr. John D. Millar
Communicable Disease Center
Atlanta, Georgia, USA

Dr. William S. Miller
U.S. Army Biological Center
Frederick, Maryland, USA

Dr. Milan V. Milovanovic
World Health Organization
Rio de Janeiro, Brazil

Dr. William J. Mogabgab
Tulane University
New Orleans, Louisiana, USA

Dr. S. B. Mohanty
University of Maryland
College Park, Maryland, USA

Dr. J. Anthony Morris
National Institutes of Health
Bethesda, Maryland, USA

Dr. Maurice A. Mufson
University of Illinois
Chicago, Illinois, USA

Dr. Daniel Mullally
National Institutes of Health
Bethesda, Maryland, USA

Dr. Edward S. Murray
Harvard University
Boston, Massachusetts, USA

Dr. Roderick Murray
National Institutes of Health
Bethesda, Maryland, USA

Dr. Samuel L. Musser
Research Department
Philips Roxane, Inc.
St. Joseph, Missouri, USA

Dr. Robert W. McCollum
Yale University
New Haven, Connecticut, USA

Dr. Stewart J. McConnel
U.S. Army Medical Research Unit
Frederick, Maryland, USA

Dr. J. Corbett McDonald
McGill University
Montreal, Canada

Lt. Col. Robert W. McKinney
U.S. Army Medical Research Unit
Frederick, Maryland, USA

Dr. I. William McLean, Jr.
Microbiological Research
Parke, Davis & Company
Detroit, Michigan, USA

Dr. Frederick P. Nagler
Virus Laboratories
Department of National Health and Welfare
Ottawa, Ontario, Canada

Dr. Neal Nathanson
The Johns Hopkins University
Baltimore, Maryland, USA

Dr. Charles F. Needy
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Thieu L. Nchiem
Rubella Vaccine Program
Microbiological Associates
Bethesda, Maryland, USA

Dr. Erling C. Norrby
Karolinska Institute
Stockholm, Sweden

Dr. Fernando C. Ottati
Medical Research and Development
Cyanamid International
Pearl River, New York, USA

Dr. John R. Overman
National Institutes of Health
Bethesda, Maryland, USA

Dr. Paul D. Parkman
National Institutes of Health
Bethesda, Maryland, USA

Dr. Armando S. Parodi
Universidad de Buenos Aires
Buenos Aires, Argentina

Dr. Robert H. Parrott
Children's Hospital of the District of Columbia
and Georgetown University
Washington, D.C., USA

Dr. Vytautas Pavilanis
University of Montreal
Montreal, Quebec, Canada

Dr. Anthony M.-M. Payne
World Health Organization
Geneva, Switzerland

Dr. Gerald V. Peacock
U.S. Department of Agriculture
Hyattsville, Maryland, USA

Dr. F. Bruce Peck
The Lilly Clinical Research Laboratories
Indianapolis, Indiana, USA

Dr. R. O. Peckinpaugh
Naval Medical Research Unit
Great Lakes, Illinois, USA

Dr. H. G. Pereira
National Institute for Medical Research
London, England

Dr. Frank T. Perkins
National Institute for Medical Research
London, England

Dr. John C. Perkins
National Institutes of Health
Bethesda, Maryland, USA

Dr. Cornelius B. Philip
Rocky Mountain Laboratory
Hamilton, Montana, USA

Dr. Charles E. Phillips
National Animal Disease Laboratory
Ames, Iowa, USA

Dr. Margaret Pittman
National Institutes of Health
Bethesda, Maryland, USA

Dr. Stanley A. Plotkin
The Wistar Institute
Philadelphia, Pennsylvania, USA

Dr. Marius F. Polak
Rijks Instituut voor de Volksgezondheid
Utretch, The Netherlands

Dr. Bernard Portnoy
University of Southern California
Los Angeles, California, USA

Dr. Bosko Postic
University of Pittsburgh
Pittsburgh, Pennsylvania, USA

Dr. Abel L. Prinzie
Rega Institute
University of Louvain
Louvain, Belgium

Dr. Robert H. Purcell
National Institutes of Health
Bethesda, Maryland, USA

Dr. Manuel Ramos Alvarez
Hospital Infantil de México
México, D.F., México

Dr. Karel Raska
World Health Organization
Geneva, Switzerland

Dr. A. F. Rasmussen
University of California
Los Angeles, California, USA

Dr. Frank Rauscher
National Institutes of Health
Bethesda, Maryland, USA

Dr. Robert C. Reinsinger
Animal Health Division
U. S. Department of Agriculture
Hyattsville, Maryland, USA

Dr. Andrew J. Rhodes
University of Toronto
Toronto, Ontario, Canada

Dr. Lionel E. Rhuland
Department of Virus Research
The Upjohn Company
Kalamazoo, Michigan, USA

Dr. Wilton A. Rightsel
Virus Research Department
Parke, Davis & Company
Detroit, Michigan, USA

Dr. Harris D. Riley
The University of Oklahoma Medical Center
Oklahoma City, Oklahoma, USA

Dr. Conrado Ristori
Servicio Nacional de Salud
Santiago, Chile

Dr. Frederick Robbins
Western Reserve University
Cleveland, Ohio, USA

Dr. Roslyn Q. Robinson
Communicable Disease Center
Atlanta, Georgia, USA

Dr. Eugene I. Rosanoff
Virology Research Department
Wyeth Laboratories
Philadelphia, Pennsylvania, USA

Dr. Max J. Rosembaum
Department of the Navy
Medical Research Unit
Great Lakes, Illinois, USA

Dr. C. E. van Rooyen
Dalhousie University
Halifax, N.S., Canada

Dr. Albert B. Sabin
University of Cincinnati
Cincinnati, Ohio, USA

Dr. Samuel Saslaw
The Ohio State University
Columbus, Ohio, USA

Dr. Morris Schaeffer
Bureau of Laboratories
The New York City Department of Health
New York, New York, USA

Dr. Anton J. Schwarz
Pittman-Moore Research Center
Zionsville, Indiana, USA

Dr. Thomas McNair Scott
The Children's Hospital of Philadelphia
Philadelphia, Pennsylvania, USA

Dr. John R. Seal
National Institutes of Health
Bethesda, Maryland, USA

Dr. Edward S. Seligmann
National Institutes of Health
Bethesda, Maryland, USA

Dr. A. A. Selivanov
Institute of Experimental Medicine
Leningrad, USSR

Dr. John L. Sever
National Institutes of Health
Bethesda, Maryland, USA

Dr. E. D. Shaw
Armed Forces Institute of Pathology
Washington, D.C., USA

Dr. Alexis Shelokov
National Institutes of Health
Bethesda, Maryland, USA

Dr. Ralph C. Singer
Communicable Diseases Branch
Office of the Surgeon General of the Army
Washington, D.C., USA

Dr. Thomas J. Smith
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. A. A. Smorodintsev
Institute of Experimental Medicine
Leningrad, USSR

Dr. R. M. Sohier
Faculté de Medecine
Lyon, France

Dr. V. D. Soloviev
Gamaleya Institute of Epidemiology and Microbiology
Moscow, USSR

Dr. Fred L. Soper
Pan American Health Organization
Washington, D.C., USA

Dr. James V. Sorrentino
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Leslie P. Spence
Trinidad Regional Virus Laboratory
Port-of-Spain, Trinidad

Dr. Edith Stanley
University of Illinois
Chicago, Illinois, USA

Dr. Alex J. Steigman
Mount Sinai Medical College and Hospital
New York, New York, USA

Dr. Ljubinko Stojkovic
Serbian Institute of Public Health
Belgrade, Yugoslavia

Dr. Joseph Stokes
The Henry Phipps Institute
Philadelphia, Pennsylvania, USA

Dr. Juraj Strauss
National Institutes of Health
Bethesda, Maryland, USA

Dr. Charles H. Stuart-Harris
The University of Sheffield
Sheffield, England

Dr. Eugenio Suárez
Pan American Health Organization
Washington, D.C., USA

Dr. Benjamin H. Sweet
Gulf South Research Institute
New Orleans, Louisiana, USA

Dr. Lubos Syrůček
Institute of Epidemiology and Microbiology
Prague, Czechoslovakia

Dr. K. J. Taylor
Microbiological Screening Service
Cutter Laboratories
Berkeley, California, USA

Dr. Helen L. Tepper
National Institutes of Health
Bethesda, Maryland, USA

Dr. William J. Thomas
Virology Research
National Drug Company
Swiftwater, Pennsylvania, USA

Dr. William D. Tigertt
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Howard Tint
Biological and Chemical Development
Wyeth Laboratories
Philadelphia, Pennsylvania, USA

Dr. Y. Togo
University of Maryland
Baltimore, Maryland, USA

Dr. John J. Trentin
Baylor University
Houston, Texas, USA

Dr. David A. Tyrrell
National Institute for Medical Research
Salisbury, Wilts, England

Dr. Joseph Ungar
Swiss Serum and Vaccine Institute
Berne, Switzerland

Dr. Jacobus D. Verlinde
University of Leiden
Leiden, The Netherlands

Dr. Marina Voroshilova
Institute of Poliomyelitis and Viral Encephalitides
Moscow, USSR

Dr. John C. Wagner
National Institutes of Health
Bethesda, Maryland, USA

Dr. R. R. Wagner
The Johns Hopkins University
Baltimore, Maryland, USA

Dr. Robert Ward
University of Southern California
Los Angeles, California, USA

Dr. Thomas G. Ward
Microbiological Associates, Inc.
Bethesda, Maryland, USA

Dr. Joel Warren
Biologics Research Department
Chas. Pfizer and Company
Terre Haute, Indiana, USA

Dr. Robert E. Weibel
University of Pennsylvania
Philadelphia, Pennsylvania, USA

Dr. Thomas H. Weller
Harvard University
Boston, Massachusetts, USA

Dr. Herbert A. Wenner
University of Kansas School of Medicine
Kansas City, Kansas, USA

Dr. Earle F. Wheelock
Western Reserve University
Cleveland, Ohio, USA

Dr. Carrie E. Whitmire
Biological Development and Production
Winthrop Laboratories
Rensselaer, New York, USA

Dr. T. J. Wiktor
The Wistar Institute
Philadelphia, Pennsylvania, USA

Dr. Robert J. Wilson
Connaught Medical Research Laboratories
Toronto, Ontario, Canada

Dr. Charles L. Wisseman, Jr.
University of Maryland
Baltimore, Maryland, USA

Dr. Ruth G. Wittler
Walter Reed Army Institute of Research
Washington, D.C., USA

Dr. Albert E. Woeltjen
Clinical Research Department
Pfizer International, Inc.
New York, New York, USA

Dr. Allen F. Woodhour
Merck Institute for Therapeutic Research
West Point, Pennsylvania, USA

Dr. Theodore Woodward
University of Maryland
Baltimore, Maryland, USA

Dr. Robert L. Woolridge
National Institutes of Health
Bethesda, Maryland, USA

Dr. Charles J. York
Institute for Comparative Biology
San Diego, California, USA

Dr. Julius S. Youngner
University of Pittsburgh
Pittsburgh, Pennsylvania, USA

Dr. Karel Zăcek
Institute of Epidemiology and Microbiology
Prague, Czechoslovakia

Dr. Victor M. Zhdanov
Institute of Virology
Moscow, USSR

Program Committee

Dr. W. Chas. Cockburn
World Health Organization
Geneva, Switzerland

Dr. Sven Gard
Karolinska Institute
Stockholm, Sweden

Dr. Werner Henle
The Children's Hospital of Philadelphia
Philadelphia, Pennsylvania, USA

Dr. M. R. Hilleman *(Consultant)*
Merck Institute for Therapeutic Research
West Point, Pennsylvania, USA

Dr. M. Martins da Silva *(Conference Secretary)*
Office of Research Coordination
Pan American Health Organization
Washington, D.C., USA

INTRODUCTORY REMARKS

ABRAHAM HORWITZ

Director, Pan American Sanitary Bureau,
Regional Office of the World Health Organization
for the Americas

DR. HORWITZ: I wish to express, in the name of the Pan American Health Organization and the World Health Organization, our deep gratitude for your presence in this house during this week. For you are going to benefit us—and through us, millions of human beings all over the world—with your knowledge and experience in a field of deep significance for science and for the well-being of man.

We consider this Conference to be a direct continuation of the dialogue started a few years ago, under our sponsorship, on live poliovirus vaccines. The presence of many of you today who also attended the two conferences on live poliovirus vaccines is in the tradition of Aristotle when he stated that a community is viable when everyone knows the face of his neighbor. We, of this Organization, are anxious to create a true intellectual community of the sciences and arts of health as the most essential force in fostering progress in the well-being of people.

A few years ago, in my introductory remarks to the First PAHO/WHO Conference on Live Poliovirus Vaccines, I declared that "in the evolution of ideas in search for those truths which bear on the lives of many people it is indispensable, from time to time, to pause and to analyze what is known, what still remains to be learned, and to determine the course which must be followed to reach the original objectives." Thus we are assembled here to analyze the past and the present and to project the future of vaccines against viral and rickettsial diseases. The program for the week ahead is ambitious, but we feel certain that the eminence and high competence of the speakers and participants are full guarantee that the Conference's objectives will be attained.

If there is a characteristic common to all or most of the subjects to be discussed, it is their complexity—complexity in the sense that nature still keeps many secrets to be unravelled. The more we know, the greater the stimulus for those especially endowed with an inquisitive mind and great imagination to penetrate to the very essence of each process in order to interpret those forces revealing the existence of new relationships which require new principles and laws. Ortega y Gasset wisely observed that facts "cover" reality and that the role of science is precisely to "uncover" it. He symbolized science as "construction" and judged it, as it related to matters corporeal or spiritual, as much a product of imagination as of observation, the latter not being possible without the former. He added: "This characteristic, in part at least, the imaginative element of science, makes of her a sister of poetry."*

In keeping with the best of their postulates, both the Pan American Health Organization and the World Health Organization are eager to learn from your experience what knowledge is ready to be applied to prevent or cure disease, as well as to learn of those concepts that will stimulate further investigation. The history of the recent past is rich in accomplishments and justifies these new explorations. We need only to mention vaccines against yellow fever, smallpox, and poliomyelitis. Where the vaccines have been applied in a systematic way, these diseases

* Ortega y Gasset, José. *En torno a Galileo—Esquema de las crisis*, page 17, Espasa-Calpe, S.A., Madrid, 1965.

have been brought to comparative inconsequence and we look forward to similar success in the most recent initiative of Governments in their agreement to undertake a world-wide effort to eradicate smallpox, coordinated by the World Health Organization.

In view of such background and purpose, it seems fitting that this international scientific conference is being held in our new house and is devoted to viral and rickettsial diseases and to measures that have been or are being developed for their prevention. We would hope to be able in the future to continue this dialogue so as to consolidate some of the experiences, as well as to explore others, such as those related to chronic and degenerative diseases, neoplasia, autoimmune and other maladies of long-term occurrence, which only now are beginning to demonstrate or to suggest a viral etiology.

Since the days of Jenner and Pasteur, who used intact animals, to our days in which cell-culture procedures are commonly employed, great progress has occurred in virus vaccine production. It is to be regretted that the same accomplishment has not been registered in their application for human betterment, even in the case of those vaccines whose preventive capacity is solid and long-proven and whose cost, through industrial production, is relatively small. As a consequence, millions of human beings living in the so-called underdeveloped world are deprived of the benefits of immunization and specific diseases still show high incidence and mortality. Under the circumstances, the question has been raised as to whether these countries should be concerned with advanced research and its effects, or whether they should concentrate on the traditional procedures for the solution of basic health problems.

Colm has stated that development derives from "growth and change" involving the economy, the organization and administration of services, and social welfare. All of them are preconditions to the application of modern technology. In this complex interplay of forces, health care is one of the essential components because, jointly with education, it contributes to the development of each personality and, through this process, to productivity and production. Progress is mainly the result of the spirit of creation, inventiveness and ingenuity, as well as a recognition of responsibility and of the need for decision on the part of those endowed with a sense of national purpose. All other elements, capital investment included, are complementary. Development will not be realized unless increasing social opportunities are offered within each society, so that each human being "will be more, knowing more."

In the field of health, people do not accept discrimination with regard to the quality of curative and preventive medicine to which they aspire. They want immediately for themselves and for their children that which is modern as soon as it becomes practical and is known. Governments must satisfy these aspirations, as a moral imperative and as a fundamental activity for the future of the country. For this very important purpose, scientists and the other elements of the intellectual community should keep in active association with Governments, regardless of currents of political opinion, because it is the well-being of the people that is at stake. International organizations, intergovernmental in nature and in objective, such as the World Health Organization and the Pan American Health Organization, should contribute their efforts to spread modern knowledge, to assist in its application, and to create the appropriate atmosphere for the best minds in each discipline to coordinate and to systematize their thoughts and experiences. In this way, old concepts and techniques are modernized; new ideas are explored. As in any attempt to explain all phenomena of the real world, the process is never ending.

Under these conditions, we gather here this week. You can be certain that the facts and the proposals that you are going to leave with us after your discussions—which we hope will be both productive and lively—will be spread throughout the world. You can count on the interest of the Governments, regardless of their stage of development, and on our decision to provide them with the technical assistance needed to organize programs of immunization against the diseases included in the agenda of this Conference, when circumstances will justify and permit such action. In doing this, we interpret the role of science as so well defined by our Advisory Committee on Medical Research: "The long-range goal is to promote the upgrading of the community in its most human aspects through the cultivation of science. Indeed, science, if

understood properly as a form of culture, is a means of eventually providing the whole community with an objective awareness of the proper context of man; it gives a holistic view of the universe, in keeping with man's intellectual nature; it will eventually provide a basis for mutual understanding; and it is in any case a proper basis on which to build education."*

* *Report to the Director*. First Meeting, PAHO Advisory Committee on Medical Research, 18-22 June 1962, Washington, D. C.

The opening address for this Conference will be presented by Dr. Stuart-Harris, who is Professor of Medicine at the University of Sheffield, England. Dr. Stuart-Harris has had many years of experience in virology and rickettsiology as teacher, laboratory experimenter, clinician, and author. He is gifted with farsightedness tempered by practical reality. We are pleased and privileged to have him with us today to present the keynote address on present accomplishments and future needs for viral and rickettsial vaccines.

KEYNOTE ADDRESS:

PRESENT ACCOMPLISHMENTS AND FUTURE NEEDS

C. H. STUART-HARRIS

Department of Medicine, University of Sheffield
Sheffield, England

DR. STUART-HARRIS: The infections that will be considered by this Conference attack the populations of all countries throughout the world to a greater or lesser degree. Their control by means that will be both safe and effective is the dream of those charged with responsibility for the health of the people. Yet the very success of some of the weapons forged in the laboratory and applied in the field has served but to highlight the failures in other directions. Sometimes the failure has been attributed to the weapons themselves or to the characteristics of the infectious agent; sometimes it has been in the method of application of the prophylactic to the population. Nevertheless, as it is my task to ring up the curtain on this Conference, I prefer to deal first with success rather than failure, in the hope that the principles that have led to success may also guide us in the consideration of future needs.

The Achievements

It is now a little over 200 years since Edward Jenner embarked on the career that made him the pioneer in the control of disease by artificial immunization. From the time of the first vaccination in a boy with lymph from the vesicle of a milkmaid's cowpox, immunization against smallpox has had a chequered career. When Jenner died in 1823, his work was only partly recognized by his countrymen, and it was bitterly criticized after his death. Variolation with material from cases of variola continued to be practiced in Britain until 1840 in spite of protests and a firm recommendation for Jennerian vaccination by the Royal College of Physicians of London. As vaccination gradually came to be adopted during the latter half of the 19th century, so did variola decline in Europe and North America.

Today, however, the situation is different. There remain foci of virulent smallpox in Asia and elsewhere that threaten the rest of the world where variola major has ceased to occur. In Britain the rate of acceptance for vaccinial immunization in babies and infants, is now only about 30 per cent unless a special effort is made. Thus every importation of variola is a cause of public alarm and perhaps mass vaccination campaigns, with their attendant risk of severe reactions and hazards for eczematous babies. Only recently has the World Health Assembly decided to devote large resources in an effort to vanquish smallpox at its source. Truly, the achievement so far obtained could have been infinitely greater had the necessary effort been made.

But Jenner's torch lit the path for Pasteur, and it was Pasteur who showed the way to attenuate microbial virulence artificially. The principles enunciated by Pasteur in his work on fowl cholera, anthrax, and rabies have stood the test of time and are still in use today. They were the inspiration for Theiler and his colleagues of the Rockefeller Foundation in their successful cultivation of the 17D strain of yellow fever virus. They led also to the growth and development of immunology, which today has become a key scientific discipline in its own right.

The control of disease by artificial immunization based on Pasteurian principles is of course most strikingly instanced in our time by poliomyelitis. The fact that in this vast country of

the United States paralytic poliomyelitis has become reduced from a disease numbered in thousands of cases annually to a mere 61 in 1965 is an astonishing and solid achievement directly attributable to immunization. The fact that this has been brought about by the deployment of two entirely different potent vaccines—those of Salk and of Sabin—is also remarkable. Had these weapons not been employed in dramatic mass campaigns in both the United States and the USSR, the course of history might well have been different. The lesson that a vaccine must be not only safe and effective but also acceptable to the people should by now have been well and truly learned.

It is not for me to adjudicate upon the respective merits of different forms of poliovirus vaccines, and I know that all those present would prefer that I honor the one to whom the modern work on poliomyelitis can be traced—John Enders. Not only did he and his co-workers at the Boston Children's Hospital provide the tissue-culture tools requisite for the preparation of virus vaccines in bulk, but he himself has since tamed the measles virus and provided the world with yet another potent weapon. Nor does the story end here, for already a tissue-culture line of rubella virus has been established by Parkman and others at the National Institutes of Health, which may provide another safe and effective prophylactic against a disease that is trivial for the child but hazardous indeed for the fetus. The application of both measles and rubella vaccines for the attempted control of their respective diseases is an unfinished story, but no one can fail to be amazed at the pace of development in the past ten years.

Present Challenges

It is time now to turn to the challenges presented to us by the unconquered infections of our several populations in order to assess the present needs. The commonest, if not the most deadly, infections in all parts of the world are still the acute infections of the respiratory tract. The fact that it is more than 30 years since the recovery of the influenza virus in the laboratory, and that influenza is still an undefeated disease, is surely a sobering thought. Is it because our weapons are too blunt or inadequately applied, or are we defeated by the changing virus whose antigenic variation is so sharp a contrast to the stability of the viruses of smallpox, yellow fever, poliomyelitis and measles? Probably all three reasons are valid but the debate about to begin may serve to assess the matter.

Then there are the hordes of cases of common colds, of pharyngitis, and of the acute lower respiratory tract infections that press upon people of all ages and whose rule is one of etiological diversity. The serological complexity of certain species such as the rhinoviruses make the immunological problem enormous. The possibility of formulating polyvalent vaccines made up of several species is to be discussed. If only some common product of the virus particles existed that would immunize against different serotypes, the task might seem less formidable. Moreover, the need to define the respiratory virus problems at different ages and in different groups of the population is still outstanding. It is perhaps peculiarly my prerogative in this Conference to declare that immunization must begin by defining its objective. Unless there is a clinical problem whose etiology has been established, then there is no basis for formulating and using a vaccine.

Let us turn to the still relatively poorly understood group of enterovirus infections. Among these one would select aseptic meningitis as the most serious clinical condition, but the diversity of viruses concerned makes illness a challenging problem indeed. There are those who believe in the possible harmful effects of Coxsackie and echoviruses in obscure conditions of the nervous system. Others point to the cardiac effects of certain enteroviruses or to their role in gastrointestinal disease. The sum total of clinical hazards of this ubiquitous group of viruses is hard to fathom. How much these remarks may apply also to the complex arbovirus field, others more competent than I must judge. But the challenge of virus encephalitis is one that must be felt in many widely separated countries throughout the world.

The conquest of disorders in which there is an acarine or insect vector raises the possibility of control measures other than immunization. The rickettsial infections illustrate this problem as well as, if not better than virus diseases. Those of us who witnessed the historical success of insecticides in the control of louse-borne epi-

demic typhus in Naples in 1944 are not likely to forget the value of vector destruction. Immunization against epidemic typhus is now feasible by more than one method, yet other rickettsiae such as those of scrub typhus and Q fever are more recalcitrant. The clinical need for control measures has to be established before the worthwhileness of immunization can be assessed. Wartime epidemics revealed the existence of *Rickettsiae orientalis*, but the endemic importance of this infection to populations of Southeast Asia and elsewhere has still to be assessed. Much the same can be said of tick-borne and Q fever rickettsiae that flourish in the animal kingdom in many countries all over the world. Action against the numerous animal species concerned seems difficult to effect. The existence of satisfactory chemotherapeutic agents against the rickettsial infections has taken away much of the urgency of prophylaxis. Does the experience of the fight against the rickettsiae have a wider message? The tantalizing prospect of chemotherapy against the true viruses is now doubly attractive in view of the problems just outlined. Chemoprophylaxis which has a better theoretical chance of success is already being explored against smallpox. The future possibility of extending this method, particularly for epidemic control, must clearly now be taken into account.

Present Needs

There is at the present time much talk of the possible eradication of disease. Coming as I do from an island that has successfully eradicated at least one virus disease—rabies—by the simple expedient of quarantine of potentially infected animals, it might be thought that I would speak strongly in favor of this concept. On the contrary, the word "eradication" seems to me to foster an illusion. It is perfectly possible to eradicate a particular infection from one particular country or area, but the reality is that the world is an epidemiological unit and that other areas may continue to foster the infection long after eradication has been achieved elsewhere. The existence of animal reservoirs of infection in any case makes eradication an impossibility for certain of the virus diseases now to be discussed.

I should like to propose that the primary aim of preventive immunization may be stated quite simply as the artificial induction of resistance to infection so that children may be born healthy and attain adult life without the risk of repeated illnesses to which they are now subject. Where the infectious agent is ubiquitous—as in the case of the polioviruses, measles, and rubella—it is necessary to immunize each generation anew. Where the agent persists in localized areas, as for instance, in the case of yellow fever or variola, the induction of resistance by immunization campaigns in these areas may be successful in quenching the fire of infection at its source. This is essentially the technique of epidemic control. We need at the present time to take stock of the diseases caused by the major groups of the virus infections of man. We need to define our objectives anew. This may demand further detailed clinical and epidemiological studies combined with an assessment of the virus flora. Only then can it be stated whether the problem is best met by basic immunization of children or by some form of epidemic control.

Second, there is a great need for work on the immunizing materials themselves with a view to improving their purity and antigenic effectiveness. As was pointed out by the WHO Scientific Group on Human Viral and Rickettsial Vaccines* some virus vaccines honored by tradition fail to conform to the standards set for the more recently developed vaccines. Smallpox and rabies vaccines are examples of this, and even yellow fever and live influenza vaccines need to be cleansed of accompaniments such as the fowl leukosis complex. The existence of known extraneous agents in living tissue and the possibility of further as yet unknown contaminants frightens many workers and greatly complicates the problems of safety and control of live attenuated vaccines. The preparation of such vaccines in cell strains that can be subjected to defined criteria and experimental tests is an attractive method that is already being explored as an alternative to cultures from animal tissues.

Even where inactivated vaccines furnish a good alternative to living vaccines, from the standpoint of protection there is much to be learned in regard to the methods of administration, precise composition, and number of doses. Take, for example, the question of influenza.

* *WHO Technical Reports Series* **325**, 1966.

Inactivated saline vaccines exert so transient an effect that they are only protective for relatively short periods of time and injections must be practiced annually. The oily adjuvants hitherto used afford an excellent chance of prolonging the antibody response, yet give troublesome local reactions in a small number of recipients. The recent introduction of purified hemagglutinin, which avoids the pyrogenic effect of whole virus vaccine, may therefore be the best approach to the influenza problem. But will this afford merely basic immunization for certain groups of the population, and ought reliance for epidemic control be placed on an attenuated living vaccine? Many in my own country feel unable to accept the latter mode of immunization until a laboratory method of test for attenuation of influenza virus is formulated which will provide the equivalent for influenza to the monkey nervous system for poliomyelitis virus.

Third, there is need to develop new vaccines from etiological agents shown to be responsible for a significant amount of illness. The respiratory virus field is one that cries out for exploration and that can only be approached on the basis of a vaccine containing many constituents. Will the number of doses required for an inactivated polyvalent respiratory vaccine be then too great for acceptability, or will needle-less methods of administration soothe away the sting and fear of injections?

Fourth, we need to obtain a far greater degree of cooperation from the population in the future. The extension of immunization demands this, unless we are merely to substitute one vaccine for another. In my country, where drugs are not only tolerated but requested, vaccination still conjures up an unpleasant image of a procedure bringing with it the hazard of reactions and only the negative benefit of freedom from a disease which might never happen. Fear is, however, not the basic reason for the low rates of acceptance of vaccination. A recent example may be given of the effect of improving the administration of immunization by the installation of a computer system for supervising the call of parents to the clinic at the appropriate time. In one area in England where the rate of immunization of children against smallpox was formerly 30 per cent, computer control raised the figure to more than 80 per cent.

Perhaps this Conference will also give some attention to the basic need for supervision or surveillance of the population so that a watch can be kept not only on the state of immunity but on all possibly harmful reactions or illnesses attributable to immunization. This will extend the present form of control exerted primarily on the manufacture and test of the vaccines themselves.

Conclusion

The famous exhortation sent to Jenner by John Hunter—"Why think? Why not try the experiment?"—was written about Jenner's work on hedgehogs. It has been taken out of its context and used repeatedly to uphold the experimental method. This Conference is not concerned with the hibernation of hedgehogs but with man's conflict with his environment. Even so, it would do well to take heed of Hunter's comments in the unfinished business of the prophylactic immunization against infectious disease. May I paraphrase this by saying: "We need to think. We need also to try the experiment."

SESSION I

CONTROL OF ACUTE RESPIRATORY DISEASES

Monday, 7 November 1966, 8:30 a.m.

CHAIRMAN

DR. THOMAS FRANCIS, JR.

RAPPORTEUR

DR. EDWIN H. LENNETTE

Section A.

Influenza Virus Vaccines

Presentation of Papers by:

Dr. Fred M. Davenport
Dr. Victor M. Zhdanov
Dr. Lubomir Syrůček
Dr. Werner Henle

Discussants:

Dr. H. G. Pereira
Dr. Roslyn Q. Robinson
Dr. Anatoli A. Smorodintsev
Sir Christopher H. Andrewes
Col. Edward L. Buescher
Dr. R. Sohier

Section B.

Respiratory Syncytial and Parainfluenza Virus Vaccines

Presentation of Papers by:

Dr. Robert H. Parrott
Dr. Z. Dinter

Discussants:

Dr. Robert M. Chanock
Dr. S. B. Mohanty

SECTION A. INFLUENZA VIRUS VACCINES

PRESENT STATUS OF INACTIVATED INFLUENZA VIRUS VACCINES *

FRED M. DAVENPORT

Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Michigan, U.S.A.

DR. DAVENPORT (*presenting the paper*): There can no longer be any reasonable doubt that a high degree of protection against influenza A and B will be achieved by administration of inactivated influenza virus vaccines of appropriate antigenic composition and potency. The largest body of data to support this statement is that developed by the Commission on Influenza of the Armed Forces Epidemiological Board in close and active collaboration with the military.

Figure 1 reproduces the results of a continuous series of carefully controlled field trials on vaccine efficacy carried out between 1943 and 1960. In most years a high degree of protection ranging from about 70 per cent to more than 90 per cent was demonstrated. The principal exceptions were in 1947 and 1955 when the A-prime and B-prime strains emerged. These experiences provided convincing demonstrations of the significance of major strain variation in relation to vaccine-induced immunity. The success achieved by incorporating the newly emergent subtypes in subsequent vaccines gave an equally clear demonstration that vaccine effectiveness was not seriously impaired by minor strain variations. The total experience until 1957 afforded a firm scientific basis for decisions necessitated by the recycling of A_2-like viruses in that year. A high degree of protection was observed in 1957 and 1960 when potent Asian virus vaccines were employed. This impressive record of vaccine effectiveness does not stand alone; as is summarized elsewhere, it is supported by data from independent field trials conducted by the British Medical Research Council (MRC) Committee on Influenza and by others (12, 8).

In recent years the Commission on Influenza has been testing a different method for monitoring vaccine efficacy. Since annual service-wide vaccination is carried out in the military, while coverage of the civilian higher risk groups is spotty and by the most optimistic estimate does not exceed 25 per cent (13), it seems reasonable to use as an indicator of vaccine performance the comparative experience of the military and civilian populations when challenged by epidemics.

Table 1 reproduces the Army's experience with respiratory disease from 1956 through 1965.† The data shown are incidence rates of common respiratory disease and influenza and of influenza alone per 1000 average strength. The advent of Asian influenza in 1957 caused a marked rise in admissions for respiratory illness. Delivery of vaccine containing the Asian strain could not be obtained before many of the troops, especially those stationed abroad, had been exposed. The year 1957, then, represents the low point for vaccine coverage in the military and the high point of respiratory morbidity. From 1957 through 1965 the rates for common respiratory disease and influenza and for influenza itself fell almost progressively despite the

* A portion of the investigations reported was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, U. S. Army, Washington, D. C.

† The writer is indebted to Colonel Herschel E. Griffin, Chief, Preventive Medicine Division, Office of the U.S. Surgeon General, for these data.

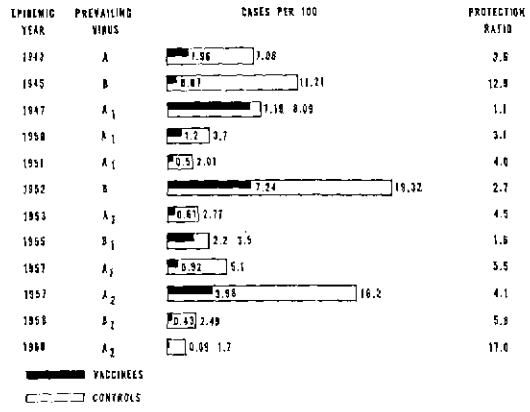

Fig. 1. Results of vaccine field trials (Commission on Influenza).

occurrence of sharp outbreaks of Asian influenza among civilians in 1960, 1963, and 1965 and of influenza B in 1962. The Navy and Air Force figures show the same trends. Moreover, close annual surveillance of the etiologic agents responsible for acute respiratory diseases is maintained at five recruit-training centers that bracket this country from California to New Jersey and from Illinois to Mississippi, and during the period in question it was shown that influenza did not gain a foothold at these bases. Clearly the difference in the military and civilian experience with influenza during the four epidemics provides compelling evidence that the inactivated polyvalent influenza virus vaccines used in the military continued to afford a high degree of protection against influenza A and B.

TABLE 1. INCIDENCE RATES—CRD&I* AND INFLUENZA† PER 1000 AVERAGE STRENGTH AMONG AD ARMY PERSONNEL AT ARMY FACILITIES

Year	CRD&I	Influenza
1956.....	74.46§	1.33§
1957.....	195.41§	52.95§
1958.....	146.78	14.13
1959.....	113.19	6.98
1960.....	108.12	7.98
1961.....	83.88	4.70
1962.....	87.27	4.59
1963.....	80.05	4.15
1964.....	60.10	3.12
1965.....	71.57	2.84

*CRD&I = Common respiratory disease and influenza.
†CRD&I figures are total; influenza figures are separate.
§Army at all facilities prior to October 1957; thereafter, Army at Army facilities only.
Source: Statistical Summary Reports.

Since 1957 the high prophylactic value of influenza virus vaccines has also been confirmed by the results of several studies carried out in civilian population groups, even though limitations on the design and conduct of such vaccine trials tend to yield an underestimate of protection rates (8, 15, 21). Yet Langmuir *et al.*, on the basis of preliminary reports, suggested that vaccine efficacy in 1963 was only 20 to 25 per cent at best and claimed that there was little evidence that recent vaccines had afforded significant protection (13). However, Moffet *et al.* subsequently described their findings in 122 vaccinated and 145 unvaccinated children observed from 3 January through 4 April 1963 (17). They reported a crude relative reduction in febrile illness of 30 per cent in favor of the vaccinees. By the application of Stille's formula an intrinsic vaccine efficacy (IVE) of at least 68 per cent was calculated (20). By definition, intrinsic vaccine efficacy is the clinical relative reduction rate divided by the infectivity rate in nonimmunized control patients, and the use of this device shuns the question whether vaccination has created an antibody ceiling that hampers serodiagnosis in the vaccinees. Actually it has been repeatedly shown that the masking of serodiagnosis in vaccinated subjects is not a serious problem in the conduct of field trials of influenza virus vaccine efficacy. Nevertheless, the IVE formula does provide an independent parameter of protection. From Moffet's serologic data an 80 per cent measurement of vaccine efficacy can be derived, which is in fair agreement with the IVE estimate.

In another study Bashe *et al.* observed 90 vaccinated and 90 control children resident in two separate institutions in Ohio during February and March 1963 (1). A crude relative reduction in "influenza-like disease" of 24 per cent was recorded for the vaccinated subjects. At that point the authors discarded their serologic data on theoretical grounds and did not check their final negative conclusions by estimating intrinsic vaccine effectiveness. However, their published data show that the calculated intrinsic vaccine effectiveness was 53 per cent and the serologic measurement of protection was 59 per cent.

Monto *et al.* reported that the 1963 military vaccine was effective in preventing dissemination (18). It is evident that the military data and the findings in the 1963 civilian trials refute the gloomy opinion that recent vaccines have not afforded significant protection against influenza.

Now, despite the fact that the record of vaccine effectiveness since 1943 is impressive, there is no justification for complacency. Control of influenza has not been achieved except in limited segments of the population such as the military. Clearly the application of protective vaccines has lagged far behind their development.

To date, the needs for vaccine have always exceeded the supply. In 1957–1958, the year of peak effort, 65 million doses were issued in the United States. Yet there are in our population over 50 million persons in the high-risk groups alone (2), and about 10 million doses are needed for the protection of persons in strategic occupations. In addition, there is a popular demand by persons who just do not want to be sick if they can help it. Problems in the allocation and distribution of vaccine persist even when the volume available is high (6).

On the basis of information collected by the Communicable Disease Center, Atlanta, Georgia, about 44.4 million doses were used in 1963, but only 9.7 million in 1964 and 10.5 million in 1965. This decline in vaccine use is deplorable, since in 1963 only about 20 to 25 per cent of persons over 65 received vaccine (13), and in the subsequent years of low promotional activity the high-risk groups were virtually unprotected. It is no surprise then, in view of the recurrent vaccine shortages, inadequate vaccine coverage, and apathy in application, that we have not made an appreciable dent in the number of excess deaths encountered in epidemic years.

Unfortunately, concentration of thought and effort on the high-risk groups has diverted attention away from serious attempts to control the morbidity of influenza by application of sound epidemiological principles. It is well known that at all times the attack rate is highest in school-age children and that such children are the principal means of introducing infection into families, whence the disease may spread to factories and to other places where adults congregate (7). The results of many field trials have unequivocally demonstrated that inactivated vaccines can provide a high degree of protection in childhood. Consequently one would anticipate that influenza could be as effectively controlled in schoolchildren as in the military if uniform vaccination were carried out. Vaccination of schoolchildren and their families would not only benefit the recipients, it would also provide a shield against dissemination to the rest of the community. The shield could be enlarged and strengthened by moving systematically up the age scale, with the attack rate progressively declining until herd immunity is established.

Adoption of the policy of vaccinating schoolchildren on a broad scale has been hampered in part by concern over vaccine reactions, even though in this age group such reactions are infrequent and are easily controlled by salicylates. The development, then, of the purified hemagglutinin vaccines that do not induce febrile toxic reactions even in infants constitutes a practical step toward meeting some of the objections raised about vaccinating children. Since it has been shown that the hemagglutinin vaccines induce antibody levels as high as or higher than intact virus vaccines of comparable strength do, there are adequate reasons to recommend their use at all ages, including the school ages (5, 11). Several large lots of experimental hemagglutinin vaccines have been prepared commercially, and it may be hoped that this type of product will now move through the orderly steps needed for licensing. About 50 million doses would be needed for schoolchildren alone (6).

Another deterrent to the broader use of influenza virus vaccines is the requirement for annual revaccination. This deterrent can be met by the use of mineral-oil-in-water adjuvant vaccines, since it has been shown that high antibody levels persist for 8 to 9 years after a single dose (4). Moreover, febrile toxic reactions to vaccine are eliminated even in children, and the dose of either virus or purified hemagglutinin in adjuvant can be reduced to about one tenth of that needed to yield comparable antibody levels with aqueous products. Experience with mineral-oil adjuvant vaccines in the military extends from 1951 to the present. Success in application has led to the recommendation that such preparations be adopted by the armed services for routine immunization against influenza. This recommendation has been accepted by the military, and the final data requested prior to certifying the safety of the product are

now being gathered. Mineral-oil adjuvant influenza virus vaccines have been employed extensively in the United Kingdom and in continental Europe, and their many advantages recommend widespread adoption.

Just as there are unresolved problems in the application of vaccines, so too are there problems in the formulation of vaccines. A recurrent question is, When should a strain change be made? There is general agreement that when a new subtype of influenza A or B emerges it is crucial for maximal effectiveness that the new variant be incorporated in the vaccine as rapidly as possible. But how frequently should strain substitutions be made in response to the occurrence of minor antigenic variations within a subtype? Frankly, there are no absolute criteria. One clue comes from the experiences encountered in field trials. Table 2 shows protection data on the various strain components of the vaccine by year of isolation of the strains, indicating in each case the vintage interval, which is defined as the number of years elapsed between the date of isolation of the most recent vaccine strain and the challenge. Inspection reveals that the level of protection was not conspicuously higher in the two instances (1943 and 1957) when it was possible to incorporate the same year's virus in the vaccine. Indeed, in several cases—1950, 1953, and 1960, for example—a high level of protection was obtained against influenza A prime or Asian influenza using vaccine strains isolated three years previously. The vintage interval in the studies of Moffet (17), Monto (18), and Bashe (1) was six years. The MRC Committee on Influenza reported a successful field trial using an eight-year-vintage vaccine (14). To date the champion is the Lee strain of 1940, which afforded good protection for twelve years—through 1952.

TABLE 2. VACCINE EFFECTIVENESS AND VACCINE STRAINS

Epidemic year	Prevailing virus	Protection rates	Vaccine strains	Vintage* interval
1943.....	A	3.6	PR8/34, WEISS/43	0
1945.....	B	12.9	LEE/40	5
1947.....	A_1	1.1	PR8/34, WEISS/43	4
1950.....	A_1	3.1	FM1/47	3
1952.....	B	2.7	LEE/40	12
1953.....	A_1	4.5	FM1/47, CUPPETT/50	3
1955.....	B_1	1.6	LEE/40	15
1957.....	A_1	5.5	AA/56	1
1957.....	A_2	4.1	JAPAN 305/57	0
1960.....	A_2	17.0	JAPAN 305/57	3

*Years between date of most recent vaccine strain and the epidemic.

Another clue comes from observations on the breadth of antibody response of vaccinated humans and from knowledge of the relation between circulating antibody levels and resistance to influenza. Table 3 illustrates the first consideration. Twenty-six subjects of military recruit age were bled before and two weeks after vaccination with a monovalent aqueous vaccine containing 200 CCA units of B/Taiwan/2/62. Hemagglutination-inhibition antibody levels were measured with prototype-B strains in paired specimens. The results are expressed as geometric mean titers and as the proportion of

TABLE 3. HI ANTIBODY RESPONSES TO B/TAIWAN/2/62 MONOVALENT VACCINE

Test strain	Serum specimen	Response to aqueous vaccine	
		Geo. mean titer	Ratio*
B/Lee/40	Pre	17	25/26
	Post	90†	
B/GL/54	Pre	58	24/26
	Post	230	
B/MD/59	Pre	38	25/26
	Post	179	
B/Taiwan/62	Pre	42	25/26
	Post	203	
B/LA/65	Pre	54	23/26
	Post	276	

*Proportion of subjects showing twofold rise or greater.
†Geometric mean HI antibody levels before and two weeks after vaccination.

subjects exhibiting an antibody increase two-fold or greater. It will be recalled that antigenic analysis of the Taiwan strain using the sera of lightly stimulated animals suggested that this virus was only remotely related to its predecessors. However, the broader antibody response of humans clearly identifies the Taiwan isolate as another member of the B-prime subtype not too distantly related to other strains, including the Lee strain of 1940. Similar findings with antigenic variants of A, A-prime and A_2 subtypes have been observed (3).* An important component of the success achieved in using vintage vaccines, then, is the fact that high levels of antibody are induced to all members of the same subtype, even to those that have not yet appeared.

Since the incidence of influenza in vaccinated subjects is inversely related to the level of circulating antibody capable of reacting with the epidemic strain, and since the levels of vaccine-induced antibody measured with different strains of the same subtype are of different heights (Table 3), the question recurs, How high is high enough? From direct correlations of levels of vaccine-induced antibody and resistance (19, 16), and from empirical observations made in the course of the field trials, it would appear that the induction of a minimal HI antibody level of 1 to 128 does confer a uniformly high degree of protection. Observation of levels below that threshold creates concern about the sureness of predictions of vaccine efficacy. However, one cannot be certain of failure, since low avidity of the test strain or destruction of antibody by harsh treatment of sera may be dominant in the serologic results. The results of tests with the sera of birds or animals are more difficult to employ for predictive purposes unless the findings indicate a fairly close relationship between vaccine and epidemic strains. Clearly more information is needed to provide a firmer basis for decision, since changes in strain composition can create serious problems in the production and testing of influenza virus vaccines and thereby contribute to vaccine shortages.

Concerning the question of whether it is worthwhile to retain in the vaccine strains of subtypes not recently prevalent, such as swine, PR8, A prime, and Lee, there are two views—one for, one against. To find a person who is undecided is rare. The scientific background for the development of the concept that it is advantageous to create a vaccine containing a pool of antigens derived from viruses of the known subtypes of influenza A and B has recently been reviewed (8), and time does not permit a restatement in detail. Basically, a prime objective is to develop by vaccination at all ages a broad composite of antibodies that simulates the characteristic of the older segments of the population, whose resistance, acquired at the expense of repeated natural exposures, is at all times the highest despite the occurrence of major or minor antigenic shifts. It has been shown that a broad composite of antibodies can be induced early in life by the use of polyvalent vaccines and that vaccination can establish a base upon which enhanced responses can be built through booster injections (10). The thesis recognizes the importance of heterologous as well as homologous antibody coverage. It also recognizes that, owing to the wide sharing of the common antigens of influenza A or B, both within and between subtypes, the opportunity exists to create resistance against strains of future prevalence by the administration of a compound vaccine. The demonstration by Meiklejohn *et al.* that a PR8 vaccine provided mild protection against influenza A prime (16) and by Gundelfinger *et al.* that a swine and FM1 vaccine provided moderate protection against Asian influenza (9) are findings that provide direct support to the basic concepts. It would seem prudent, then, to retain the older strains not only for present benefits but also for their potential usefulness as a safeguard against the possible abrupt appearance of a new subtype moving so rapidly that specific strain coverage cannot be employed for protection. Whether the currently available coverage is broad enough to meet future needs adequately is a question that can only be resolved by further investigation.

In summary, the available data re-emphasize the fact that inactivated influenza virus vaccines have a long and excellent record of efficacy. Control of influenza by annual vaccination has been demonstrated in the military. Problems remain in the supply, allocation, and distribution of vaccine that must be resolved by administrative action if the benefits are to be extended to

* Unpublished observations.

the rest of the population. Adherence to epidemiologic principles would appear to offer the surest guide for directing vaccination programs. The development of improved inactivated vaccines should stimulate a renewed effort to gain effective control of influenza by vigorous application of accumulated knowledge. Certain questions concerning the formulation of vaccines have been mentioned and certain answers suggested. The need for further information is apparent. Ignorance, however, is not our major handicap in the control of influenza.

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SECTION A. INFLUENZA VIRUS VACCINES

PRESENT STATUS OF LIVE INFLUENZA VIRUS VACCINES

V. M. ZHDANOV

Institute of Virology
USSR Academy of Medical Sciences, Moscow, USSR

DR. ZHDANOV (*presenting the paper*): Live influenza vaccines have been known for the past 30 years—since 1937, when Burnet (1) and, almost simultaneously, Smorodintsev's and Zilber's collaborators (2, 3) employed influenza A virus after passages in ferrets and white mice to immunize volunteers through the upper respiratory tract. The history of the problem has been expounded in our reviews (4, 5) and hence need not be dwelt on in detail here. Why the seemingly rapid progress in this field was followed by a deadlock that has not yet been overcome merits serious attention.

In principle, intranasal immunization with a live attenuated influenza virus should be recognized as the most promising method of prophylaxis of this infection, since herd immunity is a regulator of influenza epidemics and intranasal immunization makes it possible to vaccinate large masses of population rapidly.

However, scientific and technical difficulties not yet overcome are hampering the development of this method. Probably the main difficulty in solving the immunization problem with either killed or live influenza vaccines has to do with alteration in the antigenic structure of influenza viruses during an epidemic or during circulation among the population; as a result, each new epidemic is caused by a new variant of the virus against which old strains provide only a partial defense. This is no longer disputed, though the mechanisms are still under discussion. Data obtained from study of the influenza A_2 viruses that caused the 1957 pandemic and the subsequent epidemics in 1959, 1962, and 1965, may be used by way of illustration.

Figure 1 represents these epidemics as curves on the graph of monthly influenza cases in Moscow per 100,000 population; Table 1 summarizes the data on the antigenic correlation between influenza A_2 strains isolated during the epidemics in tests of cross hemagglutination-inhibition reaction with rat antisera. Even a brief analysis of these data convinced us that the viruses isolated during the epidemics differed in antigenic structure not only from the preceding A_1 and A_0 viruses but from each other as well. The viruses isolated in 1957 and 1959 are an exception—the tests revealed no distinct differences. A possible explanation might be that the 1957 and 1959 epidemics were waves of one and the same pandemic, or the method employed to differentiate the antigenic structure of the strains studied may not have been sensitive enough.

The immunologic importance of these differences is demonstrated in Table 2. Mice were infected with A_2 Bar/57 strains isolated during the pandemic, and tests for immunity were conducted by means of a challenge infection both with the same virus and with A_2 16/65 virus isolated during the 1965 epidemics. Since this latter virus was avirulent for mice, the two strains were used as variants adapted to chick embryos. The results of these experiments suggest stable enough homologous immunity and rather weak heterologous immunity. Thus, the loss of cross-immunity between corresponding viruses is related to alteration of antigenic structure within the limits of the same subtype A_2 viruses, in the present case.

Because of this peculiarity of the viruses and the fact that influenza immunity is gradually lost even after a natural infection, live virus vac-

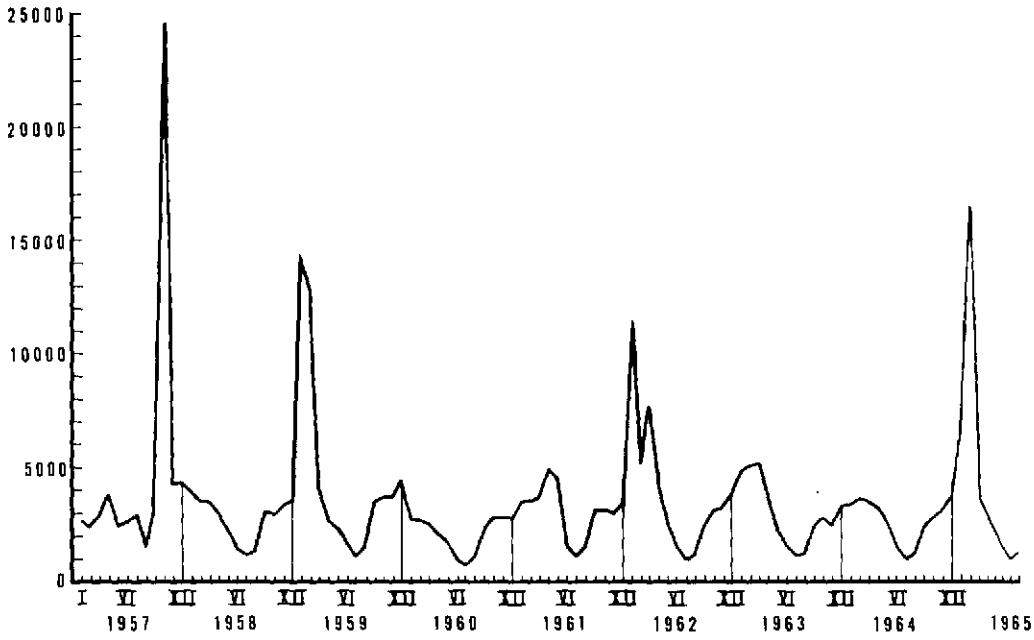

Fig. 1. Monthly incidence of influenza and acute respiratory diseases in Moscow per 100,000 population.

cines—made as they are from strains isolated during previous epidemics—cannot be relied upon to confer effective protection.

Broad application of the live virus vaccines is hindered by a second serious obstacle—the genetic instability of influenza viruses. The well-known degradation of laboratory virus strains during passages in chick embryos may serve as an illustration. Serial passages of influenza viruses isolated from patients bring about a relatively rapid loss of their pathogenic and immunogenic properties for man. These properties are lost at different times and in different degrees. Pathogenicity is the first to go, which is why most laboratory strains of influenza virus cannot provoke a typical case of influenza even after administration in considerable amounts, though they can still multiply in the human nasopharynx and cause mild local and sometimes general lesions (rhinitis, fever) together with an immunologic reaction manifested as a rise in the antibody titer. Later they lose this

TABLE 1. ANTIGENIC RELATIONSHIPS BETWEEN INFLUENZA-VIRUS STRAINS IN RECIPROCAL HEMAGGLUTINATION-INHIBITION TITERS

(0=*less than* 10)

No.	Strains	Sera						
		1	2	3	4	5	6	7
1	A_0 WS/33	320	20	0	0	0	0	0
2	A_1 FM/47	40	320	0	0	0	0	0
3	A_2 Sing 1/57	0	0	640	320	80	20	10
4	A_2 Mow V/59	0	0	320	320	40	20	10
5	A_2 Mow K/62	0	0	80	80	320	40	20
6	A_2 Eng 12/64	0	0	40	40	80	160	160
7	A_2 Mow 51/65	0	0	40	40	80	160	320

TABLE 2. CROSS-PROTECTION TESTS IN MICE BETWEEN TWO STRAINS OF A_2 INFLUENZA VIRUS

Primary infection	Challenge viruses	Titers of virus in lungs (hours after infection)				
		1	24	72	96	144
A_2 Bar/57	A_2 Bar/57	1.0	0	0	0	0
A_2 Bar/57	A_2 16/65	4.0	6.0	2.0	0	0
Noninfected	A_2 Bar/57	2.0	6.1	4.3	4.0	2.3
Noninfected	A_2 16/65	4.0	6.3	6.0	4.0	2.0

property too, becoming nonimmunogenic. The results of our experiments are summarized in Table 3. It should be noted, in connection with this table, that dissociation of pathogenic and immunogenic properties in A viruses occurs most often between passages 4 and 8, whereas the loss of ability to multiply in the mucous membrane and to induce antibody production is recorded between passages 12 and 16. These rates might vary, within rather broad limits, in different virus strains.

So far nobody has succeeded in elucidating the reasons for these phenomena. They are likely to be connected with the fact that viria are composed of host antigenic components. Rovnova and Kosyakov (6) revealed in influenza viria at least three such components: Forssman antigen, group A antigen, and species-specific antigen. Naturally, the last two of these are lost after the first passage in chick embryos and are replaced by antigens of this species. But the role they play in the formation of influenza immunity remains obscure. On the other hand, natural strains of influenza virus are known to represent as a rule a heterogeneous population (7), and it is quite appropriate to speculate that in the process of passage in chick embryos a selection of variants occurs, with loss of pathogenicity and immunogenicity for man. This is mere supposition, based at present only on indirect experimental findings.

TABLE 3. PATHOGENIC AND IMMUNOGENIC PROPERTIES OF INFLUENZA VIRUS A STRAIN AFTER SERIAL PASSAGES IN CHICK EMBRYOS

No. of passages	Multiplication in nasal mucosa (per cent of re-isolation)	Antibody response (per cent)	Fever above 37.5° C (per cent)	Local symptoms (per cent)
2-4	60	70	10	50
8-12	20	50	0.5	20
20	10	40	0.1	20
29-30	2	25	0.1	2

Dissociation of the pathogenic and immunogenic properties of influenza viruses during their serial passages outside a human organism is the principal method of obtaining vaccinal strains, but the imperfection of the method lies in this very fact: each new passage results in an alteration of their pathogenic and immunogenic properties. So far nobody has worked out a method of stabilizing these properties at a given level, though such attempts have been made (8, 9), chiefly by means of maintaining the virus vaccinal strain during a certain passage. I need hardly say that genetic instability of the virus is a serious drawback of the live influenza virus vaccine.

Aggravating this drawback has been the absence of stable genetic laboratory markers for estimating the pathogenic and immunogenic properties of influenza virus, as, for example, with polioviruses. Thus far, attempts to obtain such markers have failed. As a result, the testing of vaccinal strains in volunteers remains for the present the principal method of selecting them. Three groups of tests are routinely used to evaluate the quality of strains: reactivity (local and general), ability of the virus to multiply in the mucosa of the nasopharynx (reisolation of the virus in vaccinated persons), and immunogenicity. It is quite obvious that the selection and testing method is not perfect—it is very laborious; it is not standardized; and groups of volunteers, even if selected for low antibody titers, cannot be similarly homogeneous. Table 4, which shows the results of tests of the same

strains conducted according to the standard technique in three different laboratories, clearly illustrates this situation.

Since the live virus vaccines employed up to now are prepared in chick embryos, one more difficulty in their production should be mentioned: the danger of contamination of chick embryos by avian leukosis viruses. This difficulty is primarily a technical one, since embryos can be supplied from leukosis-free farms and methods have been worked out to reveal contaminant viruses both in this case and in other live vaccines prepared in tissue cultures.

These circumstances have proved a serious obstacle to broad application of live influenza virus vaccines, which were looked upon so hopefully in the early fifties. The period of high hopes gave place to one of great disappointment, the bitterness of which is still felt. Nevertheless, interest in live vaccines has recently been revived. Investigations are in progress in the USSR, in England, and in Japan (10, 11). At our Institute we have undertaken research directed toward solving the problems I have mentioned, and at the same time we have continued field tests of the available live virus vaccines. I should like to report on these tests here.

In Smolensk, a city of nearly 200,000 inhabitants, and in neighboring small towns, our collaborator Dr. A. N. Slepushkin carried out two sets of field tests during two recent seasons: a detailed study of a coded series of vaccines and placebo in a limited contingent and mass vaccination of half the population.

In 1964–1965 a vaccine containing separate components A and B was used. Vaccination was carried out three times, and part of the population was immunized twice with both components. Table 5 summarizes the data obtained in the coded experiments. It can be seen that despite the positive serological conversion, the effectiveness of component A (which had caused the outbreak) was low and the index of effectiveness did not exceed the 1.6 manifested during mass immunization (Table 6). The explanation should be sought in the strains themselves: the virus used was A_2, which had been isolated in 1959 and which by 1965, as was shown above, had undergone two changes in its antigenic structure—in 1962 and again in 1965. It is curious that two vaccinations were almost as effective as three (Table 7). Identical results were obtained in Moscow, but lack of time does

TABLE 4. SURVIVAL AND IMMUNOGENICITY OF CODED VACCINAL STRAINS

Strain and code	Institution*	Survival	Fourfold or greater increase in antibody			
		No. of persons	Per cent	No. of persons	Per cent	Mean increase in titer
No. 4	A	11/20†	55	12/19	63	6.5
A_2	B	6/19	31	13/21	62	7.1
Moscow	C	20/22	91	8/18	44	1.8
21/65	D	7/19	37	9/17	53	3.2
No. 7	A	8/20	40	9/19	47	3.3
A_2	B	9/27	33	6/18	33	3.1
Leningrad	C	18/21	85	7/19	37	6.2
151/65	D	0/18	0	3/15	20	1.8
No. 1	A	10/21	50	3/16	19	2.3
B/Rumania	B	9/31	29	7/8	87	4.4
1/63	C	15/21	71	4/19	21	2.6
	D	2/20	10	1/10	10	1.5

*Numerator—number of positive results.
Denominator—number of investigated.
†A. Institute of Viral Preparations.
B. Institute of Virology, USSR Academy of Medical Sciences.
C. Dnepropetrovsk bacteriological plant.
D. Institute of Vaccines and Sera and Experimental Medicine, USSR Academy of Medical Sciences, Leningrad.

TABLE 5. EFFECTIVENESS OF IMMUNIZATION WITH LIVE INFLUENZA VACCINE, BASED ON THE MORBIDITY DATA OF TOTAL ACUTE RESPIRATORY DISEASES AND SEROLOGICAL DIAGNOSIS OF INFLUENZA

Findings	Groups of workers of flax plant and Voskhod plant	Morbidity during February–April 1966						
		No. in group	Morbidity per 100 persons	Per cent of patients serologically surveyed	Decrease of morbidity		Mean error of difference	P
					Multiplicity	Percentage difference		
Influenza and acute respiratory diseases	Not immunized..	1883	10.8	48.8				
	Immunized.....	4633	3.5	80.0	3.0	7.3	0.65	0.001
Influenza morbidity (serologically diagnosed)	Not immunized..	1883	4.3					
	Immunized.....	4633	1.06	—	4.0	3.24	0.38	0.001

TABLE 6. COMPARISON OF INFLUENZA AND OTHER ACUTE RESPIRATORY INFECTIONS MORBIDITY IN THE TOWNS WITH VACCINATED AND NONVACCINATED POPULATION

(1962–1965 EPIDEMICS)

Town		Vaccination data	Morbidity during epidemics (per cent)	Percentage difference 1962/1965	Morbidity (per cent)		Weekly morbidity at peak of epidemics (per cent)
					Children	Adults	
Kaluga	1962	No vaccination	23.4	1.3	—	—	—
	1965		23.1		26.3	19.8	8.1
Vitebsk	1962	No vaccination	27.2	15.1	—	—	—
	1965		22.1		40.5	17.0	7.1
Smolensk	1962	No vaccination	19.0	32.6	—	—	6.3
	1965	40.5% of population vaccinated	12.8		23.8	8.7	3.6
Roslavl	1962	No vaccination	18	13.6	—	—	—
	1965		15.5		35.6	14.9	5.8
Safonov	1962	No vaccination	15.7	10.9	—	—	—
	1965		14.0		19.1	13.6	6.9
Yartsevo	1962	No vaccination	18.3	23.5	—	—	—
	1965	51% of population vaccinated	14.0		21.4	10.0	4.3

TABLE 7. EFFECTIVENESS OF LIVE INFLUENZA VACCINE A_2B (1965 EPIDEMIC)

Vaccination data	No. of persons in groups	Morbidity (per cent)	Mean duration of illness (per cent)	Protection ratio
Nonvaccinated	7,989	20.0	5.7	—
Vaccinated twice	7,286	14.5	6.0	1.4
Vaccinated three times	12,927	12.5	5.1	1.6

TABLE 8. EFFECTIVENESS OF LIVE INFLUENZA VIRUS VACCINATION, 1964 AND 1965–1966

Vaccination data	No. of persons	Morbidity, influenza and acute respiratory diseases, February–April 1966					
		Per cent	Mean duration of illness (days)	Decrease		Mean error of difference	P
				Protection ratio	Percentage difference		
Not vaccinated 1965	6,749	7.7	4.8				
Vaccinated twice 1965	18,656	4.0	4.3	1.8	3.7	0.31	0.001
Not vaccinated 1964 or 1965	4,113	9.4	4.8				
Not vaccinated 1965, vaccinated twice or three times 1964	2,607	4.6	4.8	2.0	4.8	0.64	0.001
Not vaccinated 1964, vaccinated twice 1965	6,660	4.7	4.5	2.0	4.7	0.49	0.001
Vaccinated twice 1964 and 1965	12,002	3.5	4.2	2.7	5.9	0.40	0.001

not permit me to consider these parallel experiments.

During the next season, 1965–1966, we selected for vaccination a strain isolated in 1964 that in antigenic structure corresponded in most respects to the 1965 strains. Approximately the same contingents were immunized in Smolensk and neighboring towns, and the same coded experiments were carried out among groups of workers at the Flax plant and the Voskhod plant. The results are shown in Table 8. It is worth mentioning that during 1965–1966 there was no influenza A_2 epidemic and only a moderate rise in influenza A and B morbidity was recorded. This may explain the limited effect of the influenza virus vaccine.

As can be seen, the results of the first two years of our scheduled five years of observations have not been promising. But they have taught us a lesson—that vaccinal strains of influenza viruses, especially A virus, need to be renewed periodically, preferably every year.

In short, many years' study of live influenza virus vaccines in the USSR have not yet overcome the scientific and technical difficulties nor made it possible to obtain standard preparations that might be recommended for broad practical application. In order to solve the problem of specific influenza prophylaxis both further studies of live virus vaccine and new approaches, based on study of the fundamental properties of these viruses and their behavior in the human organism, are required.

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SECTION A. INFLUENZA VIRUS VACCINES

ANIMAL RESERVOIRS OF INFLUENZA

L. SYRŮČEK

Institute of Epidemiology and Microbiology
Prague, Czechoslovakia

DR. SYRŮČEK (*presenting the paper*): As the evidence of recent decades shows, the influenza viruses are agents of infection both in man and in animals. It is not surprising, therefore, that many authors assign to animals an important role as reservoirs of human influenza. This view could explain the repeated shifts in the antigenic and biological properties of the human influenza virus—especially Type A, and possibly Type B also. Theories have been advanced propounding the development of new subtypes of human influenza viruses in an unknown animal source, and still other theories explain the sudden emergence of a new subtype by genetic interaction between a known human strain and an animal strain.

Elucidation of the role of animal reservoirs of human influenza is dependent on studies of antigenic, pathogenic, and genetic relations between representatives of human and animal influenza strains. Studies of the ecology and circulation of these viruses in nature are no less important.

Influenza viruses were isolated successively from birds, swine, man, and horses. Antibodies to influenza viruses have been detected in other animal species as well, as I shall mention below, but the interpretation of these findings is as yet incomplete. As Table 1 shows, more animal than human subtypes of Type A influenza virus are known.

The first group of studies establishing a relationship between animal and human influenza viruses is concerned with the antigenic aspect of the matter.

Animal influenza viruses themselves show very interesting interrelations, even without regard to the animal species from which they were isolated. A clear bilateral antigenic relationship was demonstrated between strain Turkey Canada/64-Wilmot and strain Duck England/62, or strain A-Equi 2. Interestingly, A-Equi 1 is antigenically quite different from A-Equi 2. Strains Duck Czechoslovakia/56, which are antigenically related to the English duck strains, show no relationship to strains A-Turkey Canada/64 or A-Equi 2 (Tůmová, personal communication, 1966).

Far more interesting, however, are the direct and indirect relations between the antigenic structure of human influenza strains and of animal strains.

Persons in the older age groups have repeatedly been found to possess hemagglutination-inhibition, complement-fixation, and virus-neutralization antibodies to strain A-Swine. This phenomenon is explained in two ways.

Lief, Henle, and co-workers (1) have demonstrated that guinea pigs and white mice exposed repeatedly to experimental infection with human influenza A_0, A_1, or A_2 virus develop complement-fixation and virus-neutralization antibodies to swine influenza (A-S-15) (Fig. 1, Table 2). This is probably due to the presence in human influenza A viruses of a small amount of antigenic substance similar to the swine antigen. This explanation is supported by the finding of Vobecký (personal communication, 1966) that the inhabitants of isolated, far-off localities in Mongolia where there has been no contact with swine for centuries possess anti-swine antibodies (Fig. 2).

Other authors interpret these findings by assuming that elderly persons have gone through past influenza pandemics caused by viruses now

TABLE 1. TYPE A INFLUENZA VIRUSES

Subtype	Representative variants	Former designation
Hominis 0	A/hominis 0/WS/33	A/WS
	A/hominis 0/PR/8/34	A/PR8
Hominis 1	A/hominis 1/FM/1/47	A1/FM1
	A/hominis/Netherlands/36/56	A/Dutch/36/56
Hominis 2	A/hominis 2/Singapore/1/57	A2/Singapore/1/57
	A/hominis 2/Netherlands/65/63	A2/Netherlands/65/63
	A/hominis 2/England/12/64	A2/England/12/64
Suis 1	A/suis 1/Shope/15/31	Shope/15
	A/suis 1/Cambridge/4/39	A/Cam.
Equi 1	A/Equi 1/Czechoslovakia/1/56	A/Equi/Prague/56
Equi 2	A/Equi 2/USA/1/63	A2/Miami/1/63
?	A/Galli 1/Indonesia/1/27	Fowl plague virus
	A/Galli 1/N/Germany/49	Virus N
	A/Meleagris 1/England/1/63	Turkey virus
	A/Galli 2 Sterna/Scotland/1/59	"Smith" virus
	A/Galli 2 Sterna/South Africa/1/61	Tern virus
	A/Anatis 2/England/1/56	A/Anatis/England/56
	A/Anatis 1/Czechoslovakia/1/56	A/Anatis/Czech/56
	A/Anatis 1/England/1/62	A/Duck/England/62
	A/Turkey/Canada/64 (Wilmot)	—
	A/Turkey/Italy/65	—
	A/Duck/Italy/65	—
	etc.	

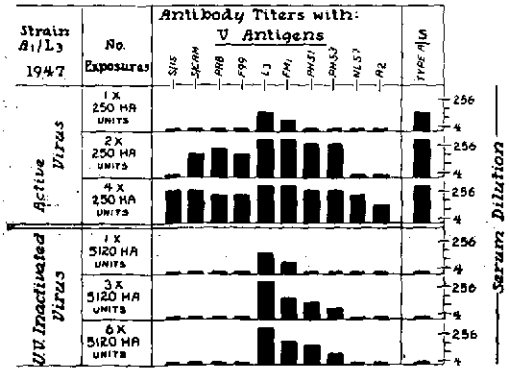

Fig. 1. Comparison of antibody responses of guinea pigs on multiple exposure to an active versus inactive A_1 virus (Lief *et al.*).

present only in animals. Persons displaying A-Swine antibodies are held to have survived the 1918–1919 pandemic, and those with A-Equi 2 antibodies to have been involved in that of 1890–1891. This would imply that the antigenic similarity is not accidental but the result of a direct etiological relation between the animal strains mentioned and human disease (2–7).

On the basis of present-day knowledge neither of the interpretations of these serological findings can be confirmed or disproved. The possibility cannot be excluded moreover, that both are valid and that the two phenomena exist in nature side by side.

On the other hand, antibodies to human influenza strains of the A and B groups have been detected in the sera of numerous animal species. The findings of antibodies to the human A_2 virus in horses seem to confirm the possibility of inapparent infection with human virus in this animal species (8). Other serological findings

TABLE 2. RESISTANCE OF MICE TO CHALLENGE WITH VARIOUS STRAINS OF INFLUENZA VIRUS FOLLOWING FOURFOLD EXPOSURE TO A SINGLE A_1 STRAIN

Challenge virus			No. dead in 10 days		Lesion score	
Strain	Dose		Test mice	Control mice	Test mice	Control mice
S/15	100	LD_{50}	0/5	5/5	1.0	5.0
A/PR8	100	LD_{50}	2/5	5/5	3.0	5.0
A_1/FM$_1$	100	LD_{50}	0/5	5/5	<1.0	5.0
A_2/Phila.'58	100	LD_{50}	0/5	5/5	<1.0	5.0
B/Lee	1–10	LD_{50}	4/5	5/5	4.2	5.0

suggestive of infection in horses, calves, sheep, ducks, dogs, and even wolves with A and B human viruses cannot be looked upon as conclusive at this time (9). The question of the infection of monkeys during human influenza epidemics also remains open. There have been some negative findings (10), and no further observations along these lines have been published to date.

Difficulties associated with the nonspecificity of serological reactions have led many workers to direct their attention to natural infection of animals with human influenza viruses, and vice versa. Furthermore, experimental infection of both animals and human volunteers has been undertaken.

In 1957 Patočka and associates were successful in eliciting experimental infection in pigs with the A_2 virus (11). Natural infection, but without clinical signs, was also observed in this animal species (8). This year Tůmová and Menšík infected 45 young pigs with the A-S-15 virus. The virus was repeatedly recovered from sick animals. An interesting point is that one of the six persons tending the infected pigs became infected without developing clinical signs (Tůmová, personal communication, 1966).

In 1961 Kluska and co-workers described a case of laboratory infection with A-Swine virus in a woman and transmission of the infection to other members of her family (13). Tůmová and co-workers attempted to infect human volunteers with the A-Equi strain in 1958, but without success.

In the past two years the results of Kasel and associates have attracted attention. These workers infected human volunteers with the A-Equi 2 strain and succeeded in eliciting infection in 22 out of 27 subjects, obtaining a manifest, though mild, influenza-like disease in 4 (14, 15).

Hence it seems that the infection of man with animal strains is possible, but probably exceptional.

An important phase in the study of the relationship between human and animal influenza viruses is the genetic research that has demonstrated the possibility of genetic interaction between the two groups. Of substantial importance here is the work of Tůmová and Pereira, who were successful in reactivating a strain of the classical fowl plague virus by a number of human, swine, equine, and avian influenza serotypes. Interesting are the hybrids obtained through interaction between fowl plague and human A_2 influenza viruses (15). This has provided a theoretical premise for a possible explanation of the development of new influenza variants in the human population.

It should be stressed, however, that despite considerable progress in this field there exists no definite proof of the spread of human influenza from an animal source. The ample results obtained by serological studies in animal and human populations are difficult to interpret,

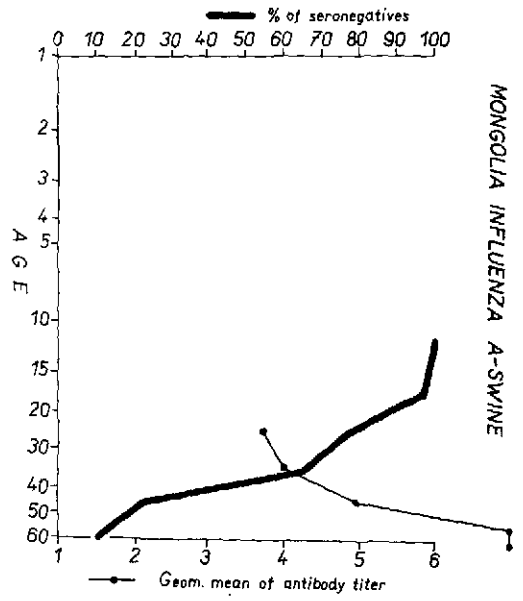

Fig. 2. Hemagglutination-inhibition antibodies to A-Swine influenza virus in Mongolian population, 1966.

particularly because of the nonspecific reactions of most of the tests in use. Only the very recent virus-neutralization reaction in monkey kidney tissue culture provides a means of largely eliminating these nonspecific reactions.

The greatest attention, however, should be devoted to attempting to isolate further influenza virus strains both from man and from animals—especially now, when the emergence of a new subtype of human influenza virus and the development of a new influenza pandemic can be expected.

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SECTION A. INFLUENZA VIRUS VACCINES

PROBLEMS OF ANTIGENIC VARIATIONS AND BROADENING OF IMMUNOLOGIC RESPONSE *

WERNER HENLE †

The Children's Hospital of Philadelphia
Philadelphia, Pennsylvania, U.S.A.

DR. HENLE (*presenting the paper*): The capacity of influenza A and B viruses to undergo variations is too well known to require detailed discussion. New variants arise and rapidly replace their predecessors. In the case of influenza A, major antigenic changes, equivalent to a new subtype, appear every 10 or more years and are, as a rule, ushered in with a pandemic. Minor changes are noted subsequently at shorter intervals during the reign of each subtype. Similar variations occur among influenza B viruses, but farther apart.

The frequent changes in antigenic patterns have prevented dependable protection of human populations by means of immunization, since the viruses are apt to keep ahead of the latest revision in the composition of the vaccine. While reasonably effective vaccines can be prepared in sufficient time and quantity for second and further rounds of a given subtype, this can be achieved during its first pandemic spread only by extraordinary effort and then merely in countries that enjoy an extended period of grace before the new virus is introduced and becomes widely disseminated. It is this situation that requires particular attention and the exploration of approaches to its prevention.

The answer to the problem would be relatively simple if it should actually turn out, as has been suggested, that the number of possible major antigenic variations is limited (6, 7) and that earlier subtypes will in time reappear after absences of many decades (20, 1). In that case, a composite vaccine including all major antigens looms in the foreseeable future. Furthermore, in combination with acceptable adjuvants such a vaccine may then afford an extended period of protection and reduce the present need of revaccination in advance of every influenza season. These expectations, if fulfilled, could well eliminate the frustrations of the past. It is not possible, however, to say whether all major antigens are known at present or whether the number will still increase—and, for that matter, whether it will turn out to be finite. Only the future can provide a final answer, possibly a few years hence when the reign of the current A_2 subtype is expected to terminate. It will then become evident whether its successor corresponds to an earlier subtype or constitutes a hitherto unknown A_3 variant.

In the present state of uncertainty it seems still justified to consider steps to meet the possible emergence of further major antigenic variants. One approach, being explored by Dr. Florence S. Lief, is based upon the broadening of antibody spectra observed in man and animals in response to multiple exposures to live influenza A viruses (11–13, 15, 18).

Study of paired sera collected from patients during A_1 and A_2 epidemics by strain-specific complement-fixation tests (17) have established a number of facts. In infants experiencing their very first attack by a given type of influenza virus, the antibody response is, as a rule, strictly specific for the infecting strain or family of strains. Antibodies to other subtypes may occasionally be formed, however, when the infec-

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tion is especially severe. In second or further attacks patients not only develop antibodies to the infecting virus but also recall antibodies to the strains with which they had previous experience. Indeed, they respond most rapidly, and often to highest titers, with antibodies to the family of strains responsible for the earliest infections. This is in agreement with the doctrine of original antigenic sin (7).

In addition, patients may form antibodies to subtypes with which they could not have had previous contact—that is, to viruses that circulated before they were born or that had not yet arisen at the time of the illnesses under study. Thus many children born after 1947 responded during the Asian pandemic with antibodies to A and swine influenza family strains in addition to the expected antibodies to A_1 and A_2 viruses. More intriguing, a considerable proportion of sera from A_1 patients ranging in age from 16 to 60 years revealed, on restudy after A_2 antigens became available, the presence of antibodies to this new subtype. This observation agrees with an earlier report that human γ globulins processed well in advance of the A_1 influenza period already contained antibodies to this family of strains (2).

There is no clear-cut evidence to show that multiple immunization of man with inactivated influenza vaccines *per se* leads to similarly broadened antibody responses. To be sure, monovalent vaccines not only induce homologous antibodies but also raise the levels of pre-existing antibodies to other subtypes (3, 4, 14, 21). These heterologous responses are undoubtedly conditioned by previous experience with other homotypic strains and by the establishment of broad antibody spectra in the course of past infections. Seldom if ever do heterologous antibodies appear *de novo* after the administration of inactivated vaccines (15, 18). As will be discussed below, repeated injections of animals with inactivated virus produce essentially only subtype-specific antibody responses.

The broadening of antibody spectra by multiple infections can readily be reproduced in experimental animals. This was noted early in rabbits with swine and A family strains, which, however, share some readily detectable common antigens (9, 22). Experiments carried out in guinea pigs by Dr. Lief and her co-workers (12, 13) have given the following results: A first intranasal inoculation always produced a strain-specific antibody response. When in serial exposures at two- to three-month intervals strains of several subtypes were used in the historical order of their appearance, beginning with swine influenza, the second and third exposures to A family strains yielded some antibodies to A_1 strains; after a fourth exposure, which was to an A_1 strain, some of the animals formed antibodies to the A_2 subtype. Such broad spectra of antibodies could be elicited also by multiple exposures of guinea pigs to a single strain of virus. Thus, with repeated exposures to an A_1 strain the spectrum ultimately extended to earlier and later subtypes; with a swine influenza strain it moved all the way forward to the A_2 family; and with an A_2 strain it reached all the way back to swine influenza virus. The spectra failed to include antibodies to equine or avian influenza strains, and repeated exposures to an influenza Equi-1 strain elicited no antibodies to human or porcine strains.

In contrast to the results with live virus, intraperitoneal injections of large doses of inactivated viruses or of ether-separated hemagglutinin components repeated six to eight times yielded complement-fixing antibodies only for strains of the given subtype (5, 12, 13).

The broad antibody spectra obtained after multiple exposures to live virus were measurable not only by the complement-fixation but also by hemagglutination-inhibition and neutralization tests in mice, employing mouse-adapted prototype strains. They also afforded protection against infection, as was demonstrated by Drs. Lief and Syrůček, who exposed mice repeatedly to egg-adapted strains of influenza virus by the airborne route. After the fourth exposure to a single subtype—e.g., A_1 or A_2 strains—the animals resisted infection by mouse-adapted prototype strains of other subtypes, as was evident from reduced pulmonary lesions and survival.

The results of these animal experiments suggested the possibility of inducing in man a broad spectrum of antibodies, including antibodies to future subtypes, by multiple exposures to attenuated live viruses. After such a conditioning one may expect that the whole spectrum of antibodies can be recalled rapidly in time of need by revaccination with presumably any available homotypic vaccine whether live

or inactivated. Several observations tend to support this suggestion.

That a broad antibody spectrum in man may also reflect some corresponding protection is suggested by epidemiological observations made during the first dissemination of new subtypes. The attack rates in these instances are generally found to be lower among persons over forty years of age than in schoolchildren or young adults (8). While this difference might be due to different degrees of exposure, it might equally be explainable on the basis of an increasing frequency of broad antibody spectra with advancing age.

It has also been reported that vaccines against "outgoing" subtypes, while on the whole ineffective against the "incoming" subtype, nevertheless induced slight though significant reductions in attack rates of the new viruses both in 1947, when the A_1 virus was introduced (19), and again in 1957, with the appearance of A_2 (10). These results might well be due to a recall of broad antibody spectra. The protective effects of heterologous, homotypic vaccines could conceivably be more pronounced if the development of a broad antibody spectrum were not left to chance.

The problems of strain selection, production, and evaluation of live influenza vaccines have already been discussed (see Zhdanov, pp. 9–15). The approach under discussion here presents merely an extension of the use of live vaccines to multiple exposures at yet-to-be-determined intervals in order to achieve, if possible, a broadened antibody response. Thus, its exploration depends upon the development of safe, potent virus preparations that can be given without significant ill effects to all age groups, especially children, and that call forth high titers of antibodies. While a suitable single strain of virus may, after appropriately spaced multiple exposures, be capable of producing as broad an antibody spectrum in children as was observed in animals, the successive use of different subtypes is likely to be more efficient and faster because it may permit exposures at shorter intervals, at least initially, when the antibodies elicited are expected to be still largely subtype-specifically oriented.

Studies toward this goal are now being actively pursued by Dr. Lief who has kindly given permission to include some of her recent observations in this discussion. As a first step it seemed to her essential to segregate variants from individual strains of virus by appropriate manipulations; to obtain a profile of the substrains so selected with respect to various biological activities; and to compare cloned viral populations with significantly different properties as immunizing agents in small groups of volunteers. The results of such comparative tests might ultimately permit the choosing of strains suitable for live vaccine preparations on the basis of laboratory tests rather than by trial and error. The limited experience gained thus far tends to support the soundness of this approach.

By relatively simple procedures (16) variants were segregated from parent A_1 or A_2 virus populations that differed in their susceptibility to nonspecific inhibitors in sera and, more important, in human nasal secretions. Paired S+ (sensitive) and S— (insensitive) lines, after cloning by the limiting dilution technique, were evaluated as to their antigenic patterns and their capacities to induce not only homologous antibody responses but also broad spectra of antibodies in guinea pigs and mice in response to multiple exposures. The substrains were additionally evaluated in terms of pathogenicity and toxicity for mice and chick embryos, neuraminidase activity and other pertinent properties. Of several pairs of S+ and S— variants, one derived from the A_2/2946/1957 strain was selected for the preparation of trial vaccines. These were produced from the fourteenth chick-embryo passages by Wyeth and Company according to current safety requirements and tested in small groups of 18-to-20-year-old female prisoners who volunteered for these studies and were housed in strict isolation.

It has thus far been shown that 10^7 EID_{50} of either strain, when administered by spray into nose and throat, was noninfective, as evidenced by failures to reisolate virus and to detect antibody responses. However, when doses of about $10^{8.7}$ EID_{50} were given it appeared that it is the inhibitor-sensitive (S+) rather than the inhibitor resistant (S—) variant that is most likely to infect and that is recoverable from the upper respiratory tract over a period of five to six days after exposure. S+ virus was reisolated from every one of 11 subjects exposed to it and all the volunteers responded with antibodies measurable both by hemadsorption-neutralization and by strain-specific complement-fixation tests. Furthermore, exposure to the S+ variant of the

1957 strain was found to induce antibodies to more recent A_2 viruses as well as to earlier subtypes. None of the volunteers developed clinical influenza, but three presented evidence of a mild, afebrile coryza. In contrast, the S— variant was reisolated from only 4 out of 12 exposed individuals and the period of excretion was shorter than that seen with S+ virus. One of these volunteers developed an afebrile rhinitis. Preliminary serological tests thus far suggest that the antibody responses, if they occur, are measurable mainly by hemadsorption-neutralization tests and that they may be essentially restricted to the A_2 antigens.

These observations, if confirmed by more extensive tests, may well explain the irregular results of some of the earlier live virus vaccine trials in which predominantly S— strains were employed. The data obtained by Dr. Lief with S+ virus are certainly sufficiently encouraging for second and further exposures of the same groups of volunteers to be planned in the near future. Their sera will of course be preserved for further study if and when an A_3 variant should arise. It may turn out, however, that the cycle is indeed closing and that earlier subtypes will make a reappearance. In that event the problem under discussion will find a more rapidly attainable solution than that proposed here.

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SECTION A. INFLUENZA VIRUS VACCINES

DISCUSSION

CHAIRMAN FRANCIS: Thank you, Dr. Henle. Our formal discussion of these papers opens with Dr. H. G. Pereira of the National Institute for Medical Research at Mill Hill, London.

DR. PEREIRA: Antigenic variation is one of the most important factors to be considered in connection with influenza vaccination. A worldwide surveillance of influenza viruses is therefore required in order to detect the emergence of new antigenic variants or subtypes as early as possible. This is the main objective of the long-established influenza program of the World Health Organization.

In the case of influenza Type A antigenic changes occur either abruptly, giving rise to new subtypes, or gradually, during the periods of prevalence of given subtypes. The study of such antigenic changes is of value in the choice of strains to be used for vaccination and, sometimes, in the forecasting of epidemics. However, the only occasion on which such forecasts can be made with reasonable accuracy is when a new influenza A subtype emerges. With influenza B, the situation is more complicated—first, because all antigenic variants are directly or indirectly related to each other; second, because occasional antigenic variants may fail to spread, and third, because different antigenic variants sometimes circulate concurrently. In this connection, I would like to ask Dr. Davenport a question. Several times he mentioned B_1 or B prime, to use his term. I should like to ask what criterion he uses to divide influenza B into subtypes comparable to A. I find this extremely difficult because I am not able to detect a sharp demarcation between influenza B strains if enough strains are compared at one particular time. The transition is gradual, and it is difficult to delineate subtypes of influenza B. With A, however, this is clear, at least when human strains are considered.

After a new influenza A subtype is discovered, urgent measures should be taken toward the production of large amounts of vaccine. Under these circumstances a live vaccine is unlikely to be obtained quickly enough. In the present state of our knowledge the most practicable objective to be attempted is the preparation of an inactivated monovalent vaccine containing an adequate amount of the new virus subtype. The difficulties in producing enough vaccine in time to control the spread of a pandemic are considerable and the best we can hope for is to reduce the impact of the pandemic.

The origin of new influenza A subtypes remains to be discovered. One possibility is that such subtypes originate from animals. Influenza A viruses have been found causing natural infections in pigs, horses, and birds. The number of antigenically distinct influenza A viruses of avian origin is rapidly increasing. When only a few of these avian viruses had been compared, it appeared that they also could be classified into subtypes analogous to those of human origin. Recent work with additional strains reveals, however, a continuous antigenic chain without clear delineation of subtypes.

CHAIRMAN FRANCIS: Thank you, Dr. Pereira. The next discussant is Dr. Roslyn Q. Robinson, Director of the Virology Section, Communicable Disease Center, Atlanta, Georgia.

DR. ROBINSON: The papers presented this morning covered very well our current concepts regarding influenza immunization. They also discussed recent advancements that hold considerable promise for improvements in the future. Immunization, whether with inactivated or live virus vaccines, is beset with the same problem of antigenic variation among viruses. This problem was recognized in the papers of Dr. Davenport and Dr. Zhdanov, and a potential

method of overcoming it was presented by Dr. Henle. The studies proposed by Dr. Syrůček may, in the future, shed light on the origin of new variants.

With Type A viruses we are quite aware of the consequences of the emergence of a strain of novel antigenic character bearing no resemblance to strains isolated in the past. Under such circumstances I think we would all agree that if we are to protect the population through immunization the vaccines should contain a virus that resembles the infecting strain as closely as possible. What is more difficult to agree upon is the significance of less marked changes that occur every two or three years during interpandemic periods. Perhaps even more difficult to agree upon is the significance of minor antigenic changes that occur among Type B viruses, which appear to be more variable but show distinct relationships to earlier strains. However, with Type B influenza, where epidemics are not generally associated with excess mortality, the factors leading to a decision to immunize a population may be different from those considered when Type A influenza is in question.

Information collected by the World Health Organization through a worldwide network of laboratories shows that Type A epidemics occur with some regularity in a particular geographic area. In interpandemic periods the viruses involved in each successive epidemic show a certain degree of antigenic deviation from viruses isolated in a previous epidemic, although by conventional techniques a great deal of similarity does exist. Various procedures have been used for antigenic comparison of influenza viruses. The magnitude of difference among strains varies widely, some techniques tending to accentuate and others tending to minimize the differences. If antigenic variation is among the important factors in the cause of periodic epidemics of influenza, then what is needed is a much clearer translation of these differences into terms that describe their potential in causing disease even in the face of related antibody.

It has been suggested that if antisera are prepared in roosters with the virus currently used for vaccine preparation and it is subsequently found in hemagglutination-inhibition tests that contemporary strains react to a titer eightfold or more lower than the homologous titer, then little protection can be expected from that vaccine and consideration should be given to changing of the vaccine formula. Perhaps Dr. Hilleman would like to comment on this further during the open discussion. Unfortunately, not many data so conveniently relating antigenic differences to vaccine efficacy have been accumulated. This kind of yardstick could be extremely useful in providing vaccines of greatest value in the future.

CHAIRMAN FRANCIS: Thank you, Dr. Robinson. The next discussant is Professor Anatoli A. Smorodintsev, Chief of the Department of Virology, Institute of Experimental Medicine, USSR Academy of Medical Sciences, Leningrad.

DR. SMORODINTSEV: Active immunization against influenza by intrarespiratory inoculation of attenuated virus was first recommended in a paper of ours 30 years ago. At that time, we described the favorable clinical and laboratory results obtained in more than 100 volunteers. After this paper was published, some American authors stressed the possible epidemiological danger of such vaccinations for susceptible adults and children.

While this kind of side effect was rarely observed when well-attenuated live vaccine was used, other complications arose because of the instability of influenza infection etiology and also because of technological difficulties such as lack of preparation standards and imperfect vaccination technique.

The well-known periodic changes in the antigenic structure of influenza viruses require that specific new A_2 strains be introduced into vaccine production every five to ten years. Inoculation by aerosol spray also demands more detailed standards for equipment and dosage. It is also important to search for substances that can increase the interaction of avirulent vaccines with the susceptible membranes.

It would be particularly desirable to stimulate greater immunogenic activity of the live vaccine by using larger doses than at present—not 10 to 50,000 units but approximately one to 500,000 units. This requires considerable improvement in the technique of virus production in developing embryos and an increase in the concentration of live viruses through reduction of losses dur-

ing the freeze-drying process and subsequent storage of the vaccine.

Also to be stressed is the fact that present methods of assessing the effectiveness of live vaccine are not more than 50 to 70 per cent accurate. This is because of the considerable number of incorrect clinical diagnoses of influenza, even in epidemics, which makes it very difficult to assess the real effectiveness of a preparation on the basis of routine diagnosis.

However, even in its present imperfect state live influenza vaccine can make a substantial difference in the immunological structure of the population and the course of an epidemic—especially in children, who are particularly susceptible to respiratory immunization with avirulent preparations in comparison with adults.

The live vaccine does stimulate regular changes in the morbidity of the population that has been vaccinated, even though, as I have said, quantitative evaluation is hampered by the great number of incorrect clinical diagnoses.

I think that the future of active immunization against influenza will be with live vaccines because they are much simpler to apply, much cheaper, and much more effective if specific and standardized.

The accompanying figures illustrate the epidemiological effectiveness of live vaccine as demonstrated by scientific observations in Leningrad, made together with Drs. G. J. Alexandrova and B. A. Mikutzkaya. Figure 1 shows the results in 2,000 vaccinated children and in a

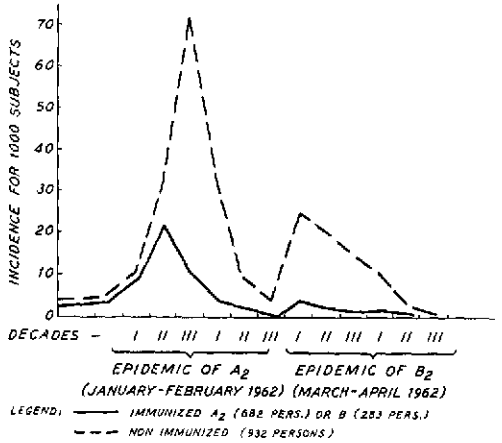

Fig. 1. Incidence of influenza A_2 and B in immunized and nonimmunized preschool children during the epidemic of A_2 and B_2 type in 1962.

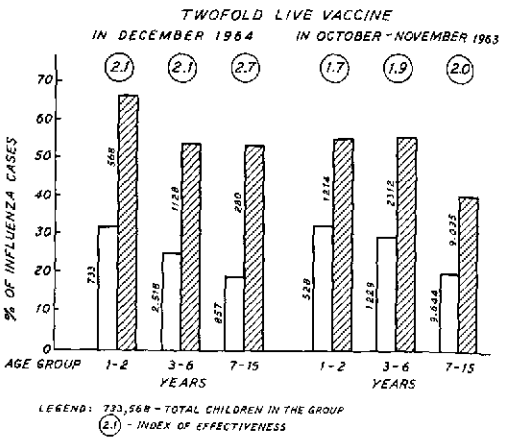

Fig. 2. Incidence of influenza during the epidemic of type A_2 in January-February 1965 in different age groups vaccinated in the same child institutions.

control group, observed during a wave of influenza A_2 and a second wave of B; Figure 2 presents similar data for the 1965 epidemic and makes very clear the effectiveness of vaccination in all three age groups, quite independent of the lapse of time between the vaccination and the outbreak.

CHAIRMAN FRANCIS: Thank you, Dr. Smorodintsev. The next discussant is Sir Christopher H. Andrewes, of Salisbury, England.

SIR CHRISTOPHER ANDREWES: The antigenic changes that take place in influenza A virus from time to time afford its most fascinating and at the same time tantalizing characteristics.

The broad immunogenic response that Dr. Henle told us about can be explained in one or other of two ways. It may be simply a reflection of a general immunological law that after repeated inoculations, antigenic response tends to get broader and broader.

Dr. Davenport and Dr. Francis would probably explain it otherwise, as a reflection of the fact that all the viruses that Dr. Henle was working with contained, unbeknown to him, antigenic components reacting with the strange antibodies that turned up.

Now, as many of you know, there are two prevailing theories about antigenic variation in the influenza virus. One is the cyclical theory of Francis and Davenport, which supposes that

there are a limited number of antigens—A, B, C, D—and that each of these takes its turn in becoming the dominant antigen, relegating the other components to a relatively unimportant position. The second view, which I think is more widely held in Britain, is that the variation may be not circular but progressive, the virus being an adaptive, opportunistic creature that varies antigenically according to how it is being inhibited by the herd immunity it is confronted with.

The late Professor J. Mulder showed that people who are 70 or 80 years old have antibodies against the A_2 viruses, and this was interpreted as meaning that the 1889–1890 virus was related to the A_2 virus of 1957. Dr. Davenport very naturally upheld this as supporting the cyclical theory: it was time that that antigen came around again.

I would suggest that, besides the circle and the straight line, there is still another possibility. The antigenic variation may be wavy, perhaps quite complicated—the virus exploiting any possibility for varying in any particular direction.

It could be that, while this is going on, the virus occasionally hits on an antigen it produced in a previous age; we would then get the sort of result that Dr. Mulder suggested.

It does not follow that all the components that the virus had produced along the road are always incorporated in it. Nor does it follow that it will continue in the same cycle as before. It can go on varying in an unpredictable way.

I do not know whether this idea is any better than the circle or the straight-line theory. But I do maintain that while theories are in considerable doubt, we should not place too much emphasis on them. I should like to take the role of an uncoupling enzyme that would uncouple some of these theoretical speculations from our practical immunization procedures until it has been definitely shown that the theories are all right. And then we can recouple again.

CHAIRMAN—FRANCIS: Thank you, Sir Christopher. The next discussant will be Colonel Edward L. Buescher, of the Walter Reed Army Institute of Research in Washington, D. C.

COL. BUESCHER: My comments will be concerned with the epidemiology of the disease influenza. You will recall that Dr. Stuart-Harris emphasized two basic aims for immunization. The first, he said, was to protect against overt disease and disability resulting from natural infection; the second was to suppress the epidemic dissemination of infective agents. Dr. Davenport suggested that there are two target populations in which the control of influenza is of primary importance. Immunization against influenza in these groups illustrates Dr. Stuart-Harris' two basic aims.

In the primarily urban communities of Scandinavia, Europe, the United States, and large portions of the Orient, influenza epidemics occur and are perpetuated primarily in susceptible persons in the younger age groups: children of preschool and school age. Their ecological association with one another during the winter months especially in schools makes these children ideally suited for disseminating influenza virus in their communities. Clinical manifestations of infection in this age group are generally mild, and rarely does the infection threaten life. It is these infected youngsters, however, who possess the maximal potential for transmission to those in the population for whom influenza poses particularly great risk to morbidity and mortality: the aged, persons with organic heart disease, and pregnant women. Clearly optimal control of epidemic dissemination resides in rendering the greater proportion of susceptible children immune. For both professional and administrative reasons, this has not been possible in the United States and other countries. Conventional methods for communicating the desirability of immunization have little impact on parents, whose interest is required to make any pediatric immunization program successful. It is appropriate, therefore, to suggest that in addition to engaging in our scientific efforts to improve vaccines we address ourselves to the problem of improving communication, bringing scientific facts to practical application in medicine, and making such improved practice not only palatable but actually sweet to the community that we serve.

CHAIRMAN FRANCIS: Thank you, Col. Buescher. Our final discussant on the program is Dr. R. Sohier, of the Virology Laboratory, School of Medicine, Lyon, France.

DR. SOHIER: To be efficient, inactivated influenza vaccines must contain an adequate amount of each of the current A and B strains and also a strain closely related or identical to the virus prevalent at the time of use.

For this reason, it is necessary to isolate and quickly identify the strains as soon as the first cases appear and even more important to carry out serological surveys of the immunological situation of a population.

The World Health Organization, which is aware of the problems that must be solved, has set up a network of international, regional, and national centers to deal with the prevention of influenza. The activities of one National Center are examined here to determine what may and what must be done in the future.

Since 1957 the Center has been trying to conduct a continuous survey, performing complement-fixation tests with soluble antigens A and B in all sera obtained from acute respiratory infections. This serological reaction was chosen because it permits detection of most of the recent influenza cases and follow-up of the evolution of the illness. We have been able to prove that complement-fixing antibodies appear soon after the beginning of the infection and disappear during the following weeks in such a way that ninety days after the beginning of the illness the antibody titer is generally ≤ 8. The CF test fails only in a few cases in babies.

From 1957 to 1966 we have been able to detect first cases of influenza quickly and to follow the course of the epidemic. Most of these epidemics appeared to begin in the same period of the year (January-February) and to end in March (Fig. 1). Some sporadic cases were detected during the intervals between epidemics.

As soon as the first cases were notified, or detected by the continuous serological survey (CF test), the Center tried to isolate strains from samples collected in communities where influenza appeared or in institutions and hospitals.

The lability of influenza virus made necessary the use of suitable methods to transport the samples. Isolation has been improved by the use of double-walled containers packed with dry ice, and by providing hospital departments regularly with tissue culture bottles renewed once or twice a week and sent to the laboratory a few hours after harvesting. As soon as they arrive at the laboratory large quantities of antibiotics are added to the bottles.

A comparative study on the best method of isolation showed the superiority of monkey kidney tissue cultures (*Cercopithecus aethiops*) over embryonated eggs for both A and B viruses, but especially for B. The isolated strains

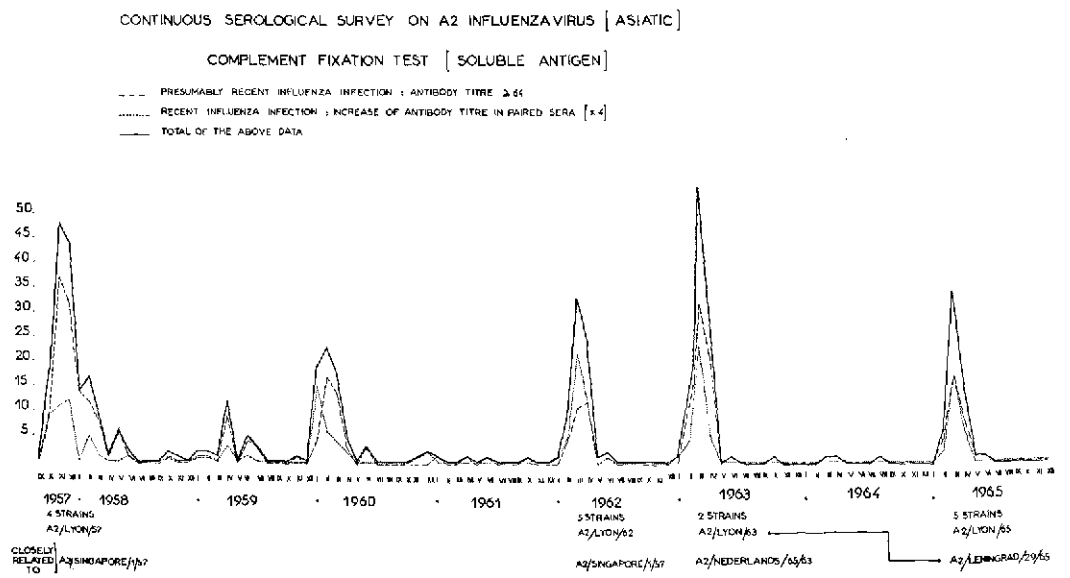

Fig. 1. Complement serological survey of A_2 influenza virus (Asiatic). Complement fixation test (soluble antigen).

were immediately identified by the CF test performed with the culture medium of tubes showing CPE used as antigen, and two immune sera containing antibodies against soluble antigens A and B respectively. Subtypes or variants were then identified by means of the HI test, the CF test with viral antigen prepared from the isolated strain, and recently by the neutralization test in tissue cultures.

All strains were sent as soon as possible to the World Influenza Center. Since 1965 inoculations in ferrets have been performed with the first strains isolated at the beginning of an outbreak.

The results can be summarized as follows:

Influenza A_2. Strains were isolated in 1957, 1962, 1963, and 1965. From observations made in our laboratory and at the World Influenza Center they appeared to be antigenically related to those isolated at the same time or shortly before in other European countries (Fig. 1).

It may be useful, however, to draw attention to a strain isolated in 1963 ("Ruguet" A_2/France/51/63), which seemed at first to be closely related to the strain A_2/Netherlands/65/63. Later, when a major epidemic occurred in the USSR and various other European countries (Sweden, Poland, Hungary, Switzerland) we as usual compared the strains isolated during previous years with the new ones isolated in 1965 in France and in the USSR. Using the HI test and the CF test with viral antigen we established, and Dr. Pereira of the World Influenza Center confirmed, that A_2/France/51/63 is similar to A_2/Leningrad/29/65.

It seems, therefore, that the new variant A_2 that in 1965 brought on a major epidemic, especially in Europe, had appeared in France (and perhaps in other countries) as early as 1963.

Influenza B. From 1951 to 1966 a few sporadic cases were detected by means of serological tests. Because there was no epidemic it was impossible to isolate strains.

In 1966 an outbreak mainly among children was studied by the CF test in the continuous survey and by the isolation of 22 strains. The

TABLE 1. CYTOPLASMIC INCLUSIONS PRODUCED BY INFLUENZA VIRUS TYPE B

Strain No.	Date of isolation	No. of passages	Nature of inoculum	Days after inoculation													
				1		2		3		4		5		6		7	
				CPE (2)	I (3)	CPE	I	CPE	I	CPE	I	CPE	I	CPE	I	CPE	I
BO 1539	1966	1	MK(1)	—	0	—	0	+	●●●								
BO 1613	—	4	—							+	●●						●
BO 1588	—	2	—	±	0	±	●●●	±		+							●
BO 1602	—	2	—	—	●	±	●●●				●●●						●
BO 1639	—	3	—	—	●			++	●●●								
BO 1543	—	3	—					+++	●●●								
—	—	(1)(4)+2	ALL					+++	● (1)								
BO 1604	—	2	MK					+	●●●								
—	—	(1)+2	ALL					+++	●●●●								
BO 1603	—	2	MK					+	●●●								
BO 1615	—	2	—					+	●●●								
BO 1651	—	3	—							+++	●						
BO 1650	—	3	—									+++	●●				
BO 1524	—	3	—			+	●●●										
BO 1612	—					++	●●●										
B TAIWAN	1962	1	MK									+	0			+	0
		11	ALL			+++	0			+++	0						
A PR8	1934	22	ALL			+	0			+++	0						
A1/LYON/132	1957	14	ALL			++	0			++	0						
A2/SINGAPORE/1	1957	21	ALL			++	0					+	0				
A2/1536	1966	5	MK									+	0				

(1) MK = Monkey kidney.
(2) CPE = Cytopathic effect (+ = 25%; ++ = 50%; +++ = 75%).
(3) I = Inclusions (0 = absence; ● = presence).
(4) (1) *One passage on tissue culture before two passages in eggs.

TABLE 2. COMPARATIVE STUDY OF INFLUENZA VIRUS STRAINS TYPE B USING NEUTRALIZATION IN MONKEY TISSUE CULTURE AND HEMAGGLUTINATION INHIBITION TESTS

Strains	B "Lee"/5394		B "Bon"		B/Johannesburg/33/58		B Taiwan/4/62		B/Lyon/7/66	
Year	1940		1943		1958		1962		1966	
Doses	130		30		30		30		10	
Sera	HI	NT	HI	NT	HI	NT	HI	NT	HI	NT
B Lee/5394/*1940* Chicken	1536	32–64	48	<4	96	4	48	<4	10	<4
B "Bon"/*1943* Ferret	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
B Johannesburg/33/*1958* Ferret	<12	<4	<12	<4	1536	≥128	<12	4	10	32
B Taiwan/4/*1962* Ferret	<12	4	<12	<4	12	<4	1536	128	20	8
B/Lyon/7/*1966* Ferret	<10	4	<10	<4	40	4	<10	4	640	128

B "Bon" = Strain "Corre", type "Bon" B/Lyon/114/56.
HI = Hemagglutination inhibition.
NT = Neutralization test.
nd = Not done.

superiority of tissue cultures over embryonated eggs for strain isolation was indisputable.

The importance of research on cytoplasmic and sometimes nuclear inclusions in cells infected with B virus was confirmed by the study of 15 strains. These characteristic inclusions were detected 14 times between the second and the fourth day. They were not seen in cells infected with A_2 influenza viruses—APR 8, A_1 1957, A_2 1957, and A_2 1966 (see Table 1).

A variety of immunological techniques was employed for identification of the antigenic characters of strains. Since results obtained with the HI test are sometimes unreliable, the CF test was also used, with viral antigens prepared from one of the 1966 strains (B/Lyon/7/66) and prototype strains and antiviral sera prepared by inoculation of guinea pigs with virus suspensions treated with ether in order to eliminate the internal soluble antigen. Because some antiviral sera react very slightly with a soluble antigen, research is being carried out to determine whether this is a specific reaction and whether the viral suspension used for the preparation of immune sera still contains internal soluble antigen.

The neutralization test was done on *Cercopithecus aethiops* primary kidney cells using titrated suspensions of various B viruses and sera prepared in ferrets with a single intranasal inoculation, the blood sample being taken two weeks later. The results appear in Table 2.

As the comparative results show, the antigenic characteristics of most of the B viruses isolated up to 1965 are more clearly distinguished from those of the 1966 strains by neutralization tests than by any other serological test.

The meeting of Directors of WHO Reference Centers (Arbovirus and Respiratory and Enterovirus) held from 19–22 July in Moscow suggested that in vaccination programs the state of

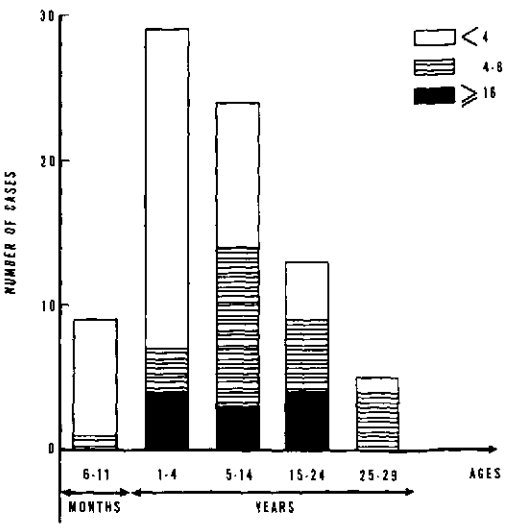

Fig. 2. Titer of neutralizing antibodies against influenza virus B/Lyon/7/66 (80 human sera collected before the onset of the 1966 epidemic).

immunity of the population be taken into account. Vaccination may be indicated if the recently isolated virus differs greatly in its antigenic scheme from those previously isolated and if the immunity level of the population is low.

We carried out a serological survey of 80 sera using the neutralization test on culture cells with the B virus strain isolated in 1966 (B/Lyon/7/66). The results are shown in Figure 2. Neutralizing antibodies were present in sera collected before the beginning of the outbreaks (between March and December 1965) among a small number of patients with low titers. A titer of ≥ 16 was found among subjects 1 to 24 years old, but not among those younger or older. This survey is still in progress on a larger number of sera.

Summary

Rational preparation of vaccines is possible only if we have continuing information on (a) the antigenic characteristics of the virus involved in interpandemic epidemics and in pandemics, and (b) the state of immunity of the population. Consequently, laboratories capable of collecting all this information have an important role to play.

For this reason WHO has set up a network of national centers for influenza surveillance and for the collection of immunological and virological data. The work of one of these has been examined here.

A continuous serological survey using the CF test with soluble antigens for all sera collected in cases of acute respiratory disease made possible continuous knowledge of the evolution of A_2 and B influenza. During each epidemic, strains were isolated, identified, and quickly sent to the World Influenza Center. Changes in the antigenic characters of A_2 strains were ascertained several times. They were closely related to those isolated in various European countries. A strain isolated in France in 1963 proved to be antigenically identical to those that caused important European outbreaks in 1965.

Methods of sampling and isolating strains have been improved through better transport of samples, through the provision of tissue culture bottles to hospitals, and through the replacement of embryonated eggs by tissue cultures.

Identification was made by the HI test and the CF test for A_2 strains, and by the study of characteristic cell inclusions and the neutralization test for B strains. The classification of B strains seems more precise with the neutralization test and the CF test than with the HI test. Henceforth, these three tests will have to be used for serological surveys.

CHAIRMAN FRANCIS: Thank you very much, Dr. Sohier. This completes the presentations by the discussants on the program; however, since time permits, it will be possible to hold a general discussion.

DR. SOLOVIEV: In papers on influenza and vaccination a central place has been occupied by the changeability of virus and the appearance of new variants, which creates considerable difficulties in preparing potent vaccines in good time. In this connection, I should like to call attention to an investigation carried out in our laboratories. Over a period of six years we collected sera from healthy people between twenty and forty years of age residing in the same locality. Altogether, we investigated nearly 800 sera with two strains of influenza virus A_2: an old strain, Singapore, isolated in 1957; and a new strain, Moscow 21, isolated eight years later during the 1965 epidemic. For the detection of influenza antibodies we used the hemagglutination-inhibition test. All sera were processed with carbon dioxide.

The figure shows the results of this work, illus-

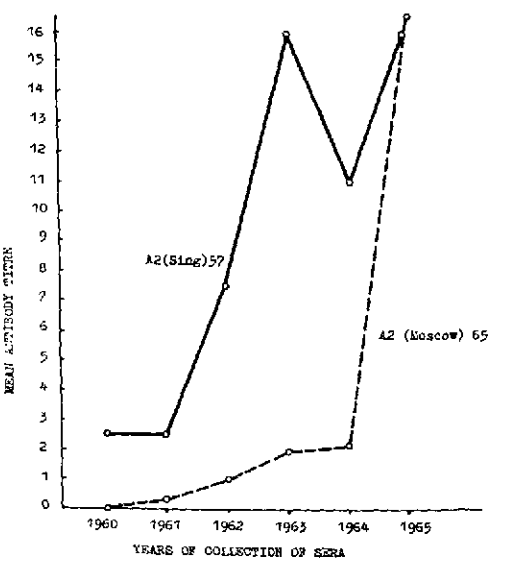

Fig. 1. Antibodies to influenza A_2 viruses.

trating the sharp difference between the two variants of virus. For the old strain high titers of antibodies were observed during all six years. For the new strains antibodies were either absent or low. Only after the epidemic outbreak in 1965 did the level of antibodies to the new strain increase sharply, reaching the same level that had been observed for the old strain.

All this leads to the following two conclusions: (1) different antigenic variants of influenza virus A_2 created herd immunity that varied greatly, which should be kept in mind in the manufacture of influenza vaccine; and (2) serological prognosis of an influenza epidemic can be misleading if virus strains inappropriate to the epidemic are used as antigens for the detection of antibodies.

CHAIRMAN FRANCIS: I should like to call on Dr. Davenport again, to reply to Dr. Pereira's question regarding Type B and also for an additional comment on the animal part.

First, I should like to say, however, that Sir Christopher Andrewes spoke as if it were our notion that there was a fixed cycle of recurrence of strains and I should like to correct that view. I think we have always considered the movement more likely to be random, with the recurrence in the rearrangement of antigens rather than in a fixed cycle.

A second matter I might mention is the interpretation of antibodies in older age groups. What impresses us is that the pattern is not fixed according to age and that antibodies to old strains develop only because of repeated exposure, but that the pattern of distribution of these dominant antibodies move with the passage of time. The point is not that after age 40 a person develops antibodies to this or that, but that these are definite patterns with specific time relations; they move as time passes. What you find in a 1940 population is quite different from what you find in a 1950 population. And this chronological alteration can be observed through repeated study which again emphasizes the fact this is not just a natural broadening by virtue of general experience alone.

DR. DAVENPORT: As Dr. Pereira knows, I would agree with him that there is wide sharing of the antigens of B strains isolated from 1940 to the present and that the divisions are nowhere near as sharp as with the A strains. It is convenient to use the term B prime in presentation because it saves time in spelling out precisely the year, the strain, and so forth.

One of the criteria used in support of the idea of there being a B prime is the fact that in 1955 there was virtually no protection in field trials using the Lee strain of 1940. And this was one of the original criteria used in developing the A-prime nomenclature. With reference to the following table, I should like to comment on it very briefly and state that, as we have demonstrated earlier, there is a high percentage of positive antibody titers against the 1963 horse strains in the sera of persons over 70 years of age. These antibodies are neutralizing antibodies. They react specifically in the photometric test and were interpreted by us as indicating another period of past prevalence of influenza A strains.

The absence of antibody below the age of 70 virtually indicated to us that horses obviously did not constitute an active reservoir of human influenza.

Recently, we have been examining the same collection of sera for antibody against the avian strains. With three avian strains—Tern South Africa, Duck England/56, and Turkey England/61—no antibody was found in any age group. However, with Duck England/62 isolate, antibody began to appear at the age of about 73 and was present in most of the age groups from 73 through 97.

TABLE 1. DISTRIBUTION OF EQUINE-2 AND DUCK/ENG./62 HI ANTIBODIES IN SERA OF HUMANS

Age range	No. of specimens	Per cent + Milford/63	Per cent + Live Duck/62	Per cent + Formalized Duck/62
89–97	17	65	12	24
87–88	20	75	0	15
85–86	25	76	16	24
83–84	25	44	16	16
81–82	25	52	0	4
79–80	25	60	4	8
77–78	25	56	12	12
75–76	25	40	0	4
73–74	24	29	4	4
71–72	25	16	0	0
69–70	25	20	0	0
66–68	25	4	0	4

The frequency of Duck/62 antibody was always lower than that found with the horse strains. The titer values were also always lower. And in no case have we found a serum that contains Duck virus antibody that did not also contain horse virus antibody. Even when the antigen is formalized, which increases the sensitivity of the reaction, the percentage remains below that found with the horse isolate. So far we have failed to demonstrate the presence of neutralizing antibody using the live Duck virus as test reagent.

Our working hypothesis, then, is that this antibody detected with the live Duck England virus is heterologous and its detection is dependent upon the presence in the same serum of Horse/63 virus antibodies. Antigen-sharing between Duck/62 and Horse/63 viruses has been demonstrated by Tůmová and Pereira and by Lief.

DR. KAPLAN: Dr. Zhdanov mentioned that the use of avian-leukosis-free eggs for vaccine purposes was essential in the potential development and use of living attenuated influenza vaccines prepared in chick embryos. This applies equally to any other vaccine—for example, 17D for yellow fever, parainfluenza, and measles—using chick embryos for culture of the virus. I should like to point out the considerable doubt recently cast on the COFAL test as a means of ascertaining leukosis-free eggs and poultry flocks by Trager and Rubin.

As you know, for several years the World Health Organization has been coordinating studies on animal influenzas and their possible relation to human disease and epidemics. Reports of this work are issued periodically by WHO to collaborating laboratories. The most recent report is of interest with respect to natural and artificial infection with influenza B in pigs.

Farkas, Romvary, and Takatscy have reported from Hungary the presence of antibody specific for influenza B by the HI and neutralization tests in serological surveys of swine herds following the B epidemic in humans in that country in 1965. Experimental infections with B/Hungary/65 were carried out in a small group of pigs. Clinical and HI and SN antibody responses were demonstrated, as was a spread to uninoculated controls kept in an adjoining pen separated by iron bars from the experimentally infected group. These results indicate that swine are, or may become, sources of influenza B for man. Shope showed this to be true with respect to influenza A in the late 1930's. Monkeys have already been shown to be susceptible to natural infection with influenza B.

CHAIRMAN FRANCIS: Thank you, Dr. Kaplan. Dr. Robinson asked whether Dr. Hilleman would comment briefly on his questions.

DR. HILLEMAN: It seems important from the practical aspect of influenza vaccine use to arrive at some criterion, based on reliable laboratory data, to ascertain the significance of antigenic change in currently circulating influenza A and B viruses in relation to the strains that are in the current vaccine. The available data suggest that when an antiserum prepared in chickens against the strain in the vaccine gives an eightfold or less hemagglutination-inhibiting antibody titer against the current virus than against the homologous vaccine strain, then the vaccine will afford only minimal or borderline protection against the natural disease challenge. We found this to be the case in studies we carried out in vaccine evaluation studies in children in the Philadelphia area during 1964–1965. It was shown that the B/Md/1/59 antigen used in the vaccine afforded only slight protection against the 1964–1965 influenza B virus circulating in the population at that time. Serologic analysis of strains showed that the titer of B/Md/1/59 antiserum prepared in chickens was eightfold less in tests with the isolates from the epidemic than in tests with the homologous B/Md strain. This same general relationship was noted previously with influenza A viruses. In interpreting the data and the significance of such analyses, it is necessary to take into account the variable avidity of virus strains for antibody as measured by the hemagglutination-inhibition procedure.

DR. STUART-HARRIS: I have the following question for Dr. Zhdanov: It was reported that the Russian influenza epidemic of 1965 was forecast in the USSR even though the 1965 virus strain was not available before the epidemic. Is this true and how was it done?

DR. ZHDANOV: The method is founded on observation of the level of antibodies for the 12 variants of the virus A_2, including the one that provoked the last epidemic, but one.

If the low level of the antibodies for this is compared to the high level for previous strains, conclusions can be drawn. I refer you to the publications of our laboratory.

In the history of the study of influenza, as in the study of other problems, there are three periods. First, we start with the assurance of ignorance. Then we proceed through a stage of lack of assurance, due to increasing knowledge. And then we come to a third phase—assurance again, but this time based on knowledge. I hope we are getting to that stage.

CHAIRMAN FRANCIS: We shall now proceed with Section B of the program, which deals with respiratory syncytial and parainfluenza virus vaccines.

SECTION B. RESPIRATORY SYNCYTIAL AND PARAINFLUENZA VIRUS VACCINES

EXPERIENCE WITH INACTIVATED RESPIRATORY SYNCYTIAL AND PARAINFLUENZA VIRUS VACCINES IN INFANTS *

ROBERT H. PARROTT, HYUN WHA KIM, JULITA O. ARROBIO, JOSÉ G. CANCHOLA, CARL D. BRANDT, JOSEPH L. DEMEIO, KEITH E. JENSEN, AND ROBERT M. CHANOCK †

DR. PARROTT (*presenting the paper*): Because parainfluenza and respiratory syncytial (RS) virus infections are among the most prevalent causes of severe respiratory tract illness among infants and children, these agents have received a high priority in vaccine development (1). Since there have been no genetic markers or animal hosts for indicating virulence and possible attenuation of these viruses, immediate efforts have been directed toward the development of inactivated vaccines.

Most children in previously reported studies on potential inactivated vaccines against these agents have possessed naturally acquired neutralizing antibody for homotypic viruses before vaccine injection. Nonetheless, several inactivated parainfluenza vaccines are reported to have effected an increase in antibody titer in such children (2–6).

Over the past two years we have tested the immunogenicity in infants with respect to a variety of inactivated potential vaccines for parainfluenza virus Types 1, 2, and 3 and the respiratory syncytial virus.

We have concentrated our studies on the response to these vaccines during the first 12 months of life, since primary infection and severe illness occur commonly during this period. Furthermore, to be completely effective these virus vaccines should be capable of stimulating neutralizing antibody in infants without prior homotypic experience.

METHODS

The vaccines. The potential immunizing agents tested included the following:

1. Two parainfluenza Type 2 vaccines derived from a monkey kidney tissue culture (MKTC) isolate from a human infant, grown in vervet MKTC and formalin-inactivated. The viral antigen was then prepared as an alum-adsorbed vaccine at fourfold concentration and as an alum-adsorbed vaccine emulsified in Drakeol-Arlacel at final twofold concentration of original antigenic material.

2. Two parainfluenza Type 3 vaccines derived from a MKTC isolate from a human infant, passed in embryonated eggs and then grown in chicken embryo tissue culture (CETC) and formalin-inactivated. The viral antigen was then prepared as an alum-adsorbed vaccine at fourfold concentration and as an alum-adsorbed vaccine emulsified in Drakeol-Arlacel at final twofold concentration of original antigenic material.

3. A group of vaccines for parainfluenza virus Types 1, 2, and 3 derived from monkey kidney isolates from human subjects and, after passage in MKTC, propagated in embryo-

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† Drs. Parrott, Kim, Arrobio, Canchola, and Brandt (Research Foundation of the Children's Hospital of the District of Columbia and Georgetown University School of Medicine, Washington, D. C.); Dr. Chanock (the National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland); Dr. DeMeio (the National Drug Company, Philadelphia, Pennsylvania); and Dr. Jensen (Pfizer Laboratories, Groton, Connecticut).

nated hens' eggs(7). For parainfluenza Type 1 vaccine allantoic fluid harvests were concentrated 20 times; for parainfluenza Type 2 and 3 amniotic fluid was used without concentration. The egg propagated virus suspensions were inactivated with formalin and tested without addition of an adjuvant.

4. A parainfluenza Type 1 virus vaccine derived from a primary human embryonic kidney tissue culture isolate from a human infant, which was propagated in human embryonic kidney, then in vervet MKTC culture, and harvested from the latter. After millipore filtration and formalin inactivation, the viral antigen was concentrated 100 times by centrifugation and then precipitated in alum.

5. Two respiratory syncytial virus vaccines, both derived from the Bernett strain, one grown in cynomolgus MKTC and the other in vervet MKTC. Both were formalin-inactivated, concentrated four times, and alum-adsorbed.

6. Another respiratory syncytial virus vaccine derived from the Bernett strain and grown in vervet MKTC. After millipore filtration and formalin inactivation, viral antigens were concentrated 100 times by centrifugation and alum precipitation.

All the vaccines were safety-tested according to standards of the Division of Biologics Standards of the National Institutes of Health.

Neutralization Tests. Neutralization tests for parainfluenza virus were carried out in rhesus MKTC tubes using hemadsorption as the indicator system. Neutralization tests for respiratory syncytial virus were carried out in HEp-2 roller tubes tissue culture. In later studies, a modification of the neutralization tests employing microtiter plates was used (C. B. Smith and J. G. Canchola, unpublished data). In both types of test the results were essentially the same.

All the serums tested for neutralizing antibody against parainfluenza Type 1 and 3 viruses and the respiratory syncytial virus were inactivated for 30 minutes at 56°C, whereas those tested against parainfluenza Type 2 virus were not heat-inactivated. All serum titers are expressed as the final dilution. The lowest serum dilution tested was 1:4 or 1:8.

Clinical Methods. After it was determined that there were no untoward local or systemic reactions in adult volunteers, a group of infants between 3 and 12 months of age were selected for study (with parental consent). The infant subjects were from families of relatively low socioeconomic status, primarily Negro. They lived at home.

Upon admission to the study, each infant was given a complete physical examination, after which he was administered an intramuscular injection of 0.5 ml of one of the vaccines (0.25 ml of Drakeol-Arlacel preparations). No infant had fever at the time of vaccination.

One month after the first injection of the vaccine, another physical examination was carried out and another 0.5 ml intramuscular injection of the same vaccine was given. Most of the infants received a third, or "booster," injection of the vaccine approximately three months after the second injection. The exceptions were those infants receiving the parainfluenza Type 2 vaccine prepared in chick embryo, about half of the ones receiving the fourfold concentrated RS vaccine, and miscellaneous subjects who underwent natural infection with virus of the same serotype as that in the vaccine before completing the series of injections.

To determine possible intercurrent respiratory tract illness in these infants and their families, careful and frequent clinical and virological surveillance was carried out for eight months by the members of the vaccine study team. This surveillance included home visits and telephone calls to determine possible reactions or illness and the collection of throat and anal swab specimens for virus and *Mycoplasma* studies at least weekly and during any reported illness.

Infection with the agent against which the vaccine was directed did not occur in vaccinees or in the controls or other children in the community except in the cases specifically mentioned below.

Serum specimens were obtained before the injection of vaccine, one month after the first injection, two to three weeks after the second, and just prior to and two to three weeks after the booster injection.

An additional "control" for the possibility that intercurrent natural infection might influence serological results was introduced in the trials with 100 times concentrated parainfluenza Type 1 and respiratory syncytial vaccines. Each of these vaccines was administered to infants

selected alternately from the same population during the same time period.

CLINICAL RESULTS

Untoward Reactions. Essentially no unusual pain, tenderness, erythema, fever, or other immediate or delayed local reactions or systemic reactions have been observed in infants receiving these vaccines.

Immunogenicity. Although not all studies of vaccines against a particular virus type were carried out concurrently, we are presenting the results by virus type in order to permit comparison among the various methods of preparation.

We have assumed that antibody present in these infants at the outset was passive, transplacentally transmitted antibody. Therefore, not only a fourfold rise in antibody but also a persistent level of antibody four times higher than the lowest dilution of serum tested (i.e. 1:4 or 1:8) was considered evidence of vaccine immunogenicity if there had been no natural infection.

Parainfluenza Type 1. In the case of both preparations of parainfluenza Type 1—the 20X chick embryo vaccine and 100X MKTC vaccine—slightly less than half the infants responded after only two injections but most responded after the third, delayed injection (Table 1). With both vaccines there was a sixfold increase in geometric mean antibody titer at the end of the study period in infants that had no demonstrable neutralizing antibody at the outset. Therefore, either of these vaccines can produce serum antibody responses in infants who have had no prior natural experience with the agent.

Parainfluenza Type 2. There appeared to be limited immunogenicity with parainfluenza Type 2 vaccines grown in MKTC (Table 2). The alum preparation was estimated to have antigenic mass four to six times greater than that of Drakeol-Arlacel preparation and it induced antibody response in two thirds of the infants, although usually only after the third injection.

Drakeol-Arlacel seemed to be of no value as an adjuvant in the infants; only one infant receiving the vaccine with this potential adjuvant showed a significant antibody response. Clearly the aqueous, chick-embryo-grown vaccine was immunogenic even after only two injections. There was a sixfold increase in geometric mean antibody titer of infants who had no demonstrable serum antibody to the agent when they first received this vaccine.

Parainfluenza Type 3. Similarly, the alum-precipitated, tissue-culture-grown parainfluenza Type 3 vaccine (60 per cent response) was more effective than the Drakeol-Arlacel preparation (42 per cent response) (Table 3), and the aqueous, chick-embryo-grown vaccine induced a response in all the subjects tested. After three injections of this last vaccine, there was a sixteenfold rise in mean geometric antibody titer in infants without passive antibody at the outset.

In brief, among the parainfluenza virus vaccines tested, the aqueous chick embryo cavity fluid preparations seemed to have the most pre-

TABLE 1. IMMUNOGENICITY OF PARAINFLUENZA VIRUS TYPE 1 INACTIVATED VACCINES

Parainfluenza Type 1 vaccine preparation	Passive antibody prior to injection	No. of infants tested	Fourfold rise or persistent level fourfold over baseline†	
			After 2 injections	After 3 injections
(20X) Chick embryo	Present	2	2	2
	Absent*	8	2	7
	Total	10	4	9 (90%)
(100X) MKTC alum	Present	1	0	0
	Absent*	13	5	10
	Total	14	5	10 (71%)

*Neutralizing antibody not detected in 1:4 or 1:8 dilution of serum.
†Baseline refers to lowest dilution of serum tested (1:4 or 1:8).

TABLE 2. IMMUNOGENICITY OF PARAINFLUENZA VIRUS TYPE 2 INACTIVATED VACCINES

Parainfluenza Type 2 vaccine preparation	Passive antibody prior to injection	No. of infants tested	Fourfold rise or persistent level fourfold over baseline†	
			After 2 injections	After 3 injections
(4X) MKTC alum	Present	1	0	0
	Absent*	11	2	8
	Total	12	2	8 (67%)
MKTC (2X) Drakeol Arlacel	Present	0	—	—
	Absent*	12	0	1
	Total	12	0	1 (8%)
(1X) Chick embryo	Present	2	2	—
	Absent*	8	7	—
	Total	10	9 (90%)	—

*Neutralizing antibody not detected in 1:4 or 1:8 dilution of serum.
†Baseline refers to lowest dilution of serum tested (1:4 or 1:8).

dictable immunogenicity. We have no information yet on the protective efficacy of serum antibody produced by any of these vaccines. We are currently evaluating a trivalent vaccine preparation of the egg-grown parainfluenza Type 1, 2, and 3 antigens.

Respiratory Syncytial. The fourfold concentrated respiratory syncytial virus vaccines grown in cynomolgus or in vervet MKTC were clearly not sufficiently immunogenic in infants—whether or not passive antibody was present—to be considered for further study (Table 4). In fact, during our studies with these agents we observed that 20 (38 per cent) of the 53 infants who received two or three injections experienced natural RS virus infection during the subsequent period of high prevalence. Nine of these infections were associated with severe lower respiratory tract illness requiring hospitalization. Three of the hospitalized infants had antibody levels at the time

TABLE 3. IMMUNOGENICITY OF PARAINFLUENZA VIRUS TYPE 3 INACTIVATED VACCINES

Parainfluenza Type 3 vaccine preparation	Passive antibody prior to injection	No. of infants tested	Fourfold rise or persistent level fourfold over baseline†	
			After 2 injections	After 3 injections
(4X) CETC alum	Present	4	0	2
	Absent†	6	2	4
	Total	10	2	6 (60%)
CETC (2X) Drakeol Arlacel	Present	10	2	4
	Absent†	2	0	1
	Total	12	2	5 (42%)
Chick embryo	Present	4	4	4
	Absent†	12	4	12
	Total	16	8	16 (100%)

*Baseline refers to lowest dilution of serum tested (1:4 or 1:8).
†Neutralizing antibody not detected in 1:4 or 1:8 dilution of serum.

TABLE 4. IMMUNOGENICITY OF RESPIRATORY SYNCYTIAL VIRUS INACTIVATED VACCINES

Respiratory syncytial vaccine preparation	Passive antibody prior to injection	No. of infants tested	Fourfold rise or persistent level fourfold over baseline*	
			After 2 injections	After 3 injections
R.S. (4X) MKTC alum	Present	16	8	—
	Absent†	7	1	—
	Total	23	9 (39%)	—
R.S. (4X) MKTC alum	Present	20	9	5
	Absent†	10	0	0
	Total	30	9 (30%)	5 (17%)
R.S. (100X) MKTC alum	Present	10	10	10
	Absent†	0	—	—
	Total	10	10 (100%)	10 (100%)

*Baseline refers to lower dilution of serum tested (1:4 or 1:8).
†Neutralizing antibody not detected in 1:4 or 1:8 dilution of serum.

of their illness, which reflected an immunogenic effect of the RS virus vaccine.

The immunogenicity of the 100X concentrated respiratory syncytial vaccine has been more encouraging. Ten infants completed a series of three injections without interruption by natural infection (virus recovery or fourfold rise in antibody). Five of the ten developed a fourfold or greater rise in antibody, and at the end of the vaccination period all ten had an antibody titer four times higher than the lowest serum dilution tested.

The group of RS vaccinees and a group of infants who had received parainfluenza Type 1 vaccine over the same period had comparable geometric mean RS antibody titers at entry into the study. This titer represented maternal antibody (Table 5). The titer rose gradually in the RS vaccinees from whom RS virus had not been recovered, and it fell in the comparable parainfluenza vaccinees. Since all RS vaccinees had detectable antibody at the end of the vaccination period, and their mean titer was six times that of the parainfluenza Type 1 vaccinees, it appeared that the vaccine induced an immunogenic response in most if not all of the young infants tested.

During a period of high RS virus prevalence that overlapped and followed the test period for

TABLE 5. RS VIRUS SERUM NEUTRALIZING ANTIBODY IN INFANTS WHO RECEIVED RS VIRUS OR PARAINFLUENZA TYPE 1 VIRUS VACCINE AND FROM WHOM RS VIRUS WAS NOT RECOVERED DURING IMMUNIZATION

Vaccine	No. of infants	Geometric mean RS virus neutralizing antibody titer* (reciprocal) at indicated time				
		Preinjection	1 Month after 1st injection	1 Month after 2nd injection	Before booster injection†	After booster injection‡
RS Vaccine (Lot 100)	12	16	16	32	48	48
Parainfluenza Type 1 virus vaccine (Lot 23)	12	16	12	12	8	8

*<1:4 considered as 1:2 for purpose of determining geometric mean titer.
†Three months after second injection.
‡Three weeks following booster injection.

this vaccine we observed two infants in whom antibody that was apparently vaccine-induced did not protect against illness (Table 6).

Infant A was hospitalized with severe bronchiolitis about 36 days after the second vaccine injection. Prior to the illness the infant had registered a greater than fivefold rise of RS virus neutralizing antibody after two 0.5 ml injections of the vaccine. After infection the patient developed an additional tenfold increase in antibody titer, although RS virus was not recovered.

Infant B was hospitalized with severe bronchiolitis 17 days after the second vaccine injection. RS virus was recovered on three occasions from throat swabs over a period of 12 days during hospitalization. This infant had developed a fivefold increase in neutralizing antibody after the second vaccine injection. This development of vaccine-induced antibody appeared to precede the onset of the patient's illness.

The findings in infant A and infant B suggest that an RS virus neutralizing antibody titer of 1:32 or 1:64 induced by vaccine did not provide protection from severe RS illness. We have previously observed that low to moderate levels of maternal antibody did not protect young infants against RS virus illness. It has also been reported that naturally acquired serum antibody at levels of 1:32 to 1:64 do not provide effective protection against infection (1).

We have noted that the respiratory tract illnesses associated with RS virus infection in RS vaccinees were more severe than those associated with RS virus infection in the group of infants who received the parainfluenza Type 1 virus vaccine. Four of five RS vaccinees who were infected with RS virus required hospitalization, whereas none of five parainfluenza Type 1 vaccinees infected with RS virus was admitted to the hospital. These observations raise the question of a paradoxical vaccine effect. Possibly RS vaccines of low potency in some way "sensitize" an individual or potentiate the host's response to natural RS infection. Such a phenomenon has been noted previously with rickettsial vaccines (8), trachoma vaccine (9), and *M. pneumoniae* vaccine (10).

Thus our findings indicate that it is possible to stimulate homotypic serum neutralizing antibody in young infants with inactivated parainfluenza or RS vaccines but they introduce some doubt as to the protective value of such antibody in at least RS infection. Most serum antibody levels attained after use of these vaccines were lower than those registered after natural infection. Recent studies suggest that antibody in respiratory tract secretions is of signal importance in the prevention of infection, at least against the parainfluenza Type 1 virus (11).

Clearly, if inactivated paramyxovirus vaccines are to be effective against respiratory tract illness, either they must provide a massive antigenic stimulus which induces serum antibody levels similar to those following natural infection, or some method must be developed whereby they can stimulate antibody in respiratory tract secretions.

TABLE 6. EVIDENCE FOR FAILURE OF VACCINE-INDUCED SERUM ANTIBODY TO PROTECT AGAINST RS VIRUS INFECTION AND BRONCHIOLITIS

Infant	Antibody tested	Reciprocal of antibody titer at indicated time after injection of RS virus vaccine			
		1st injection ↓ 0	2nd injection ↓ 1 month	2 months	3 months
A	Neut. antib.	6	8	32	384
	CF antib.			32	256 or >
				↑ Bronchiolitis* RS virus not recovered	
B	Neut. antib.	24	12 64		
			↑ Bronchiolitis† RS virus recovered		

*Onset of illness six days after 2 mo. blood obtained.
†Onset of illness 17 days after 1 mo. blood obtained.

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SECTION B. RESPIRATORY SYNCYTIAL AND PARAINFLUENZA VIRUS VACCINES

PARAINFLUENZA TYPE 3 VACCINE IN CATTLE

Z. DINTER

Department of Virology, Institute of Medical Microbiology, University of Uppsala, Uppsala, and National Veterinary Institute, Stockholm, Sweden

DR. DINTER (*presenting the paper*): Parainfluenza Type 3 virus (PIV-3) is widespread in cattle and is apparently a frequent cause of respiratory disease in calves. Two types of PIV-3 vaccines for cattle have been developed. One consists of inactivated virus with or without the addition of adjuvant, the other of a live attenuated strain.

The present report summarizes the mode of preparation and assay of these vaccines and also the results of field trials. A short description of the pathogenicity of PIV-3 and its role in the etiology of respiratory disease in cattle is given first.

THE ROLE OF PIV-3 IN DISEASE

The results obtained in transmission experiments and serological and histological studies must be treated separately from observations made during natural outbreaks of the disease, where agents other than PIV-3 might be causally involved.

Betts *et al.* (4) delivered calves by hysterotomy and reared them without colostrum. After intratracheal and intranasal inoculation with a freshly isolated PIV-3 strain, all the calves reacted with fever and most showed signs of respiratory disease. When killed five and seven days after inoculation, all showed extensive pneumonia. The virus was recovered from the respiratory tracts and regional lymph nodes of calves killed on the fifth day but not from calves killed on the seventh. Bacterial infection could be excluded. Working with colostrum-deprived calves and two strains of PIV-3, Dawson *et al.* (8) found the clinical response to inoculation mild. Although lung involvement with one of the strains was extensive, there was no clinical evidence of pneumonia. According to the experiences of the British workers (26), PIV-3 produces characteristic and consistent lesions. The pathognomonic features associated with presence of the virus are a syncytial giant cell formation in the bronchiolar and alveolar epithelial cells with pleomorphic, eosinophilic, and phloxinophilic cytoplasmic and nuclear inclusions (26). A superinfection with pasteurellae, mostly supported by stress (17, 19), may change this proliferative pneumonia into an exudative type, resulting in a syndrome that in the United States is often called shipping fever.

The respiratory disease seen in the field and presumed to be caused by PIV-3 varies in severity. In some European countries a mild form is prevalent. A transient cough and anorexia are most commonly observed. These signs are frequently accompanied by diarrhea, particularly in young animals. Prolonged coughing is considered a sign of complication. The disease affects calves much more than adult cattle. Appreciable weight loss and increased mortality among calves were noticed on several Swedish farms where outbreaks of such a "pneumoenteritis" are common during the cold season. The more severe form, shipping fever, appears to be prevalent in the United States, but cases of pneu-

moenteritis in young calves (13) and outbreaks of pneumonia (30), apparently caused mostly by PIV-3 alone, have also been reported recently from that country.

PARTICIPATION FREQUENCY OF PIV-3 IN INFECTION AND DISEASE

Antibodies to PIV-3 have been found in a large proportion of both sick and healthy cattle by several workers from various countries. Table 1 shows the distribution of antibody titers in three selected groups of Swedish cattle. A large proportion of healthy calves three to eight weeks old had low antibody titers or none at all, whereas a high incidence of intermediate to high titers was found in old healthy cattle and in cattle examined after recent outbreaks of respiratory disease (2).

Pette *et al.* (27) analyzed serum samples from 595 cattle of various ages in Bavaria. According to this analysis, the number of seropositive animals was highest in calves one to nine weeks old. Up to one year, the number decreased with increasing age. However, in cattle older than one year the number of seropositive animals was again high. This fluctuation reflects changes from an early status of passive immunity, mediated by maternal antibodies, to a transitory status in which the antibodies vanish, to be replaced by active immunity acquired by infection (27). In the United States a higher incidence of seropositive animals was encountered during the cold season, when shipping fever is prevalent (1).

The serological studies have further shown that silent infections or very mild disease, and also reinfections, occur frequently. Using the rise in antibody titer as a criterion, the British workers (26), found that PIV-3 had been involved in 24 per cent of 150 outbreaks of respiratory disease occurring in the United Kingdom. In a histological analysis, lesions characteristic of PIV-3 pneumonia, including giant cell bronchiolitis, were seen in lung samples from 16 per cent of the 125 outbreaks investigated (26).

It is thus obvious that a PIV-3 vaccine would be of value in preventing pneumonia, whose complicated forms may cause severe losses in the calf stock.

TABLE 1. DISTRIBUTION OF ANTIBODY TITERS TO PIV-3 IN THREE SELECTED GROUPS OF SWEDISH CATTLE

Group	No. of cattle tested	HI titer* and number of cattle in per cent				
		<8-8	16-32	64-128	256-512	>512
A.....	144	53	19	19	3	6
B.....	154	30	9	27	28	6
A+B....	298	41	14	23	16	6
C.....	244	18	13	29	33	7

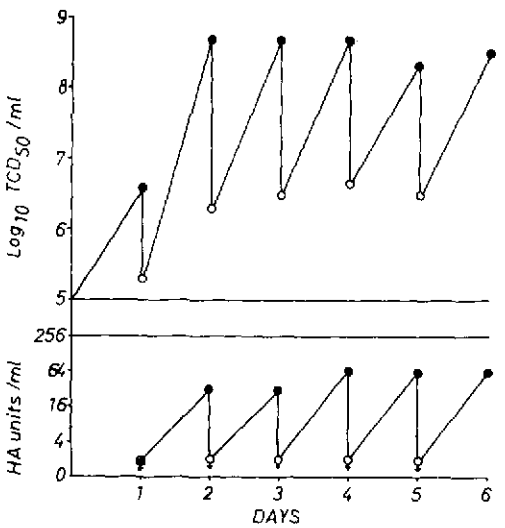
*Reciprocal of serum dilution.
A—Slaughterhouse calves 3 to 8 weeks old.
B—Slaughterhouse cattle over 2 years old.
C—Cattle of various ages on 41 farms with recent outbreaks of respiratory disease.
Source: K. Bakos and Z. Dinter, "Antiköperreaktion des Rindes auf die Infektion mit dem Virus der Parainfluenza 3," *Zentralbl Bakteriol* 1. Orig. **180:** 1-11, 1960.

INACTIVATED VACCINE

It was early shown (24) that formalin-inactivated PIV-3 is a potent antigen, particularly when incorporated into a mineral-oil adjuvant.

Preparation. PIV-3 is usually grown in primary cultures of calf kidney cells. The culture fluid is harvested when the titers of infectivity and hemagglutinin reach maximum levels. The Swedish workers (unpublished data) used the PIV-3 strain 23. Cultures in stationary flasks, infected with this strain, showed only slight cytopathic changes but produced high daily yields of virus from the second to at least the sixth day after inoculation (10), as is shown in Figure 1. It was found later (20) that no interferon is produced during the growth of strain 23 in calf kidney cell cultures. From a flask culture containing a 50 ml medium the virus could thus be harvested five times, resulting in a final yield of 250 ml virus suspension per culture. No difference in antigenic potency was noticed between vaccines prepared with a pool of such daily harvests and the total yield taken the sixth day after the inoculation.

In experiments of McClelland *et al.* (24) the virus was inactivated by formalin in a final concentration of 1:4000 at 37°C and pH 6.8. The Swedish workers used 1:2000 formalin at 26°C and pH 7.2. No differences in antigenic potency were seen between the virus inactivated for 16 hours at 26°C and that inactivated for four days



Titer of infectivity or hemagglutinin before (●) and after (○) change of medium.
Source: Unpublished data.

Fig. 1. Daily yields of PIV-3 strain 23 in primary calf kidney cell cultures.

at 4°C. Table 2 shows the rates of inactivation at both these temperatures. The final product of McClelland *et al.* (24) was an emulsified mixture of equal parts of the aqueous vaccine and an Arlacel A (10 per cent)/mineral oil (90 per cent) emulsion, whereas the final product of the Swedish workers was the aqueous vaccine.

Laboratory assay. The antigenic potency of PIV-3 vaccines can be measured on guinea pigs (24). In the experiments of Swedish workers the extinction limit titer of the vaccine was estimated according to Gard *et al.* (11). The vaccine and serial tenfold dilutions were inoculated twice intramuscularly at three-week intervals into eight to ten guinea pigs per dilution. Serum samples were obtained from these animals eight to ten days after the second inoculation. Each serum was tested for neutralizing antibodies against 1000 TCD_{50} virus, and also for hemagglutination-inhibiting (HI) antibody. Table 3 shows the results obtained with two lots of aqueous vaccine. The results suggest that the extinction limit titers can be roughly correlated with mean HI titers estimated on serum samples from eight to ten guinea pigs per undiluted vaccine. The two vaccine lots, although prepared in the same way, showed different antigenic potencies, whereas no differences were seen in this respect between viable and inactivated virus of the same batch. In similar experiments of McClelland *et al.* (24) the mineral-oil adjuvant vaccine proved to be superior to the aqueous one.

Evaluation of the vaccine in calves. In the experiments of Hamparian *et al.* (18), calves devoid of measurable antibodies to PIV-3 and held in isolation were inoculated once with the mineral-oil adjuvant vaccine intramuscularly into the anterior cervical region. In response, HI antibodies appeared first, followed by complement-fixing and neutralizing antibodies. The highest levels of neutralizing antibody were found between days 33 and 66 after vaccination; they were then significantly higher than the antibody titers encountered in groups of calves that had recovered from a natural or experimental infection with PIV-3. A control group of

TABLE 2. INACTIVATION OF PIV-3 STRAIN 23 BY FORMALIN 1:2000 AT 4°C AND 26°C AT pH 7.4

Inactivation time at 4° C (in days)	at 26° C (in hours)	Amount of inactivated virus in $\log_{10}$ units TCD_{50} 4° C*	26° C†
1	3	1.8	1.8
2	6	3.8	3.9
3	9	5.4	5.7
4	12	6.6	≥6.8

*Mean of three experiments.
†Mean of 14 experiments.
Source: Unpublished data.

TABLE 3. ANTIGENICITY OF VIABLE AND INACTIVATED PIV-3 STRAIN 23 FOR GUINEA PIGS

Vaccine lot	Inoculated	Vaccine or virus dilution and HI titer* 10^{-0}	10^{-1}	10^{-2}	10^{-3}	Extinction limit titer/ml† (log10)
9/6	Twice	2048	1024	128	<8	2.7
Control‡	Twice	2048	512	256	<8	2.5
10/9	Once	32	32	<8	<8	1.7
Control	Once	32	32	<8	<8	1.5
19/9	Twice	256	256	16	<8	2.1
Control	Twice	512	512	64	<8	2.1

*Reciprocal of serum dilution; mean obtained on serum samples from 8 to 10 guinea pigs per dilution.
†Estimated according to Gard *et al.* (11).
‡Viable virus of the vaccinal batch withdrawn prior to inactivation.
Source: Unpublished data.

calves devoid of antibodies to PIV-3 was added to these and the vaccinated animals. All four groups were exposed to challenge with a PIV-3 strain aerosol into one of the nostrils. No signs of disease appeared as a result of this challenge. However, there was no postchallenge rise of neutralizing antibodies in the majority of vaccinees, whereas all the other animals responded with a significant antibody rise. The virus was recovered from all control calves but not from the vaccinees. Excretion of virus occurred in calves recovered from natural infection and having a prechallenge neutralizing antibody titer of 1:5 or less, but it was of short duration.

In a study by Mohanty and Lillie (25) the calves were inoculated with the mineral-oil adjuvant vaccine of the Merck group. After a single dose of this vaccine the neutralizing antibodies developed slowly, reaching their highest levels after six or seven weeks. These animals and a control group were then subjected to the stress of hauling and exposure during inclement weather on two consecutive days. The following day all the calves were inoculated intranasally with the vaccinal PIV-3 strain SF-4, and two days later they were all inoculated intratracheally with *Pasteurella multocida*. All the unvaccinated calves responded with a respiratory illness of varying severity. The virus and pasteurellae were recovered from the unvaccinated animals, and their antibody titers against PIV-3 showed a significant rise. Among the vaccinated animals no signs of illness were observed, but a rise of antibody titers was noted. The virus was not recovered but pasteurellae were isolated from all the animals. Thus, a vaccine directed against PIV-3 was adequate in preventing the disease caused by a combined effect of PIV-3 and pasteurella. However, Hamdy and Trapp (16) had to vaccinate against both PIV-3 and pasteurellae in order to protect their experimental calves from disease, and the challenge pasteurella strain had to be of the same serotype as the strain used for preparing the bacterin.

Quite recently an exhaustive study on calfhood immunization against shipping fever was published by Matsuoka *et al.* (23). The adjuvant vaccine they used contained three formalin-inactivated agents: PIV-3, *Pasteurella multocida*, and *Pasteurella hemolytica*. The calves were vaccinated at four to ten weeks of age and revaccinated at weaning age. Seven days after revaccination these calves and unvaccinated control calves were subjected to stress and exposed to challenge with an aerosol containing the three agents. The observation time after challenge was 14 days. Clinical signs of disease were seen in a few animals within five days. The temperatures were higher and persisted longer in the control calves than in the vaccinated calves. One out of 17 vaccinated calves and 4 out of 14 unvaccinated calves died during the observation time. The vaccinated calves showed a rapid rise of HI titers within seven days after challenge, whereas the control calves showed only a slow rise. It should be added that low titers of maternal antibodies to PIV-3 were found at the time of the first vaccination. Before challenge there had been only slight increases in antibody titer or none at all. PIV-3 was isolated from both control and vaccinated calves. However, shedding of the virus persisted longer in the unvaccinated calves than in the vaccinees. Fourteen days after challenge the animals were sacrificed. The lungs showed lesions of a severe pneumonia in 18 per cent of the vaccinated calves and in 54 per cent of the control calves. The lungs of the remaining calves were normal or only slightly changed.

ATTENUATED VACCINE

The use of a viable, attenuated PIV-3 as a vaccine for calves has been recommended by Bögel and Liebelt (5) in West Germany.

Preparation and laboratory assay. A strain Sd-2 of PIV-3 was passaged 65 times in primary cultures of calf kidney cells and then tested for innocuity by intranasal or intranasal and intravenous inoculation into colostrum-deprived young calves held in isolation (5). No signs of illness appeared within 14 days after inoculation. Serum samples of these animals, which had had no antibodies prior to inoculation, showed HI antibodies on day 14 after inoculation. The virus was recovered from nasal swabs taken from all the animals several days after inoculation. Later the animals were exposed to a challenge infection by intranasal and intramuscular inoculations with a PIV-3 strain having a low number of passages in cell cultures. No signs of illness were observed, and the virus could not be recovered from the nasal swabs.

Field assay. After 27 or 28 more passages in calf kidney cell cultures the strain Sd-2 was tested as a vaccine in the field. Large groups of calves were inoculated under varying environmental conditions, and the strain was found to be inoffensive. The emphasis of the studies (6, 7) was on the influence of pre-existing antibodies on the antigenic stimulus of an intranasal or a simultaneous intranasal and intramuscular vaccination. The rise of HI titer was used as a criterion for the immunizing effect of vaccination throughout the experiments. No significant difference was noticed between the effects of intranasal and of intranasal and intramuscular inoculation. By this combination the authors tried to differentiate a pre-existing passive immunity from a pre-existing active immunity. After vaccination by both routes, the vaccinal virus was excreted in calves with low titers of maternal antibodies. The antibody rise was nil or insignificant. On the other hand, the vaccinal virus was not recovered from calves with low titers of antibodies acquired by previous natural infection. The majority of these calves showed a significant antibody rise. From these and similar studies it was concluded (7) that infection of the nasal mucosa with the vaccinal strain would guarantee the development of a local immunity, since infection is not inhibited by low titers of maternal antibodies. Further data on this point have been presented by Pette *et al.* (27). According to these authors, the vaccinated calves showed a general decline in antibody titers beginning two or three months after vaccination. A second vaccination was therefore recommended. The second vaccination would not only reinforce the immunity but also include calves that at the time of first vaccination were protected by maternal antibodies and hence were insusceptible to infection with the vaccinal strain (27).

EXPOSURE OF VACCINATED CATTLE TO NATURAL DISEASE

The earliest attempt to immunize calves with PIV-3 vaccine against field outbreaks of respiratory disease yielded inconclusive results. An aqueous vaccine was used, mostly in a single dose. The number of calves that became ill during the observation time after vaccination was small, but it was found to be approximately the same in vaccinated calves and in unvaccinated controls. Further, a single dose of an aqueous vaccine was shown to be a very poor antigenic stimulus when compared to a single dose of an adjuvant vaccine (24). Later the attempts to immunize calves were continued using adjuvant vaccines. In a trial of Woods *et al.* (29), 96 beef calves were vaccinated with a single dose before weaning; 180 unvaccinated calves served as controls. Ten calves—five vaccinated and five unvaccinated—developed acute respiratory disease 30 days after weaning. One of the difficulties encountered in the field trials was that shipping fever, when desired as a "challenging epidemic," apparently struck only a few animals in a herd under observation.

Two attempts to immunize calves against shipping fever were carried out by Hamdy *et al.* (14, 15). Since these attempts were similarly organized and showed similar results, the more recent one (14) will be related here. An adjuvant PIV-3 vaccine was inoculated into shipped and native calves in three doses: the first dose three weeks prior to shipment or weaning, the second at the time of shipment or weaning, and the third at the time of arrival in the feedlot or one week after weaning. The PIV-3 vaccine was given in a combination with one, two, or three other vaccines containing either inactivated *Pasteurella spp.* or attenuated infectious bovine rhinotracheitis virus. The observation time was three weeks after arrival or weaning. There were 125 vaccinated calves and 175 unvaccinated control calves. Clinical signs similar to those of shipping fever were observed in 17 vaccinated calves (13.5 per cent and in 25 unvaccinated controls (14.8 per cent). Nasal swabs were collected from all affected calves. PIV-3 was recovered from 36 calves, *Escherichia coli* from 35, *Pasteurella hemolytica* from 13, and a mycoplasma from 2. Attempts to isolate other viruses or miyagawanellae were unsuccessful. While these results must be regarded as discouraging, Matsuoka *et al.* (23) recently announced that their vaccine, described above in the section on inactivated vaccines, has given good protection against a natural outbreak of shipping fever.

Vaccinations with two doses of an aqueous PIV-3 vaccine were introduced on five Swedish beef-raising farms in 1960. These farms had their own breed but also imported young calves from other farms. The vaccinations were ad-

ministered either in September and October or in January and March, depending on when a new supply of calves arrived. Vaccine lots showing a potency similar to that of lot 9/6 (Table 3) were used. Calves under six weeks of age received 10.0 ml; older calves received 20.0 ml. The vaccine was administered subcutaneously and the vaccination was repeated four to six weeks after the first inoculation. The veterinarians in charge of these farms reported that after the vaccination campaign had started pneumoenteritis occurred in only a few calves and the mortality became lower (Bakos, personal communication). The mean figures available for the five farms were 20 per cent average mortality before and 5 per cent after vaccination.

Thousands of calves were vaccinated with the attenuated PIV-3 strain Sd-2 in West Germany, although the use of this vaccine was not much publicized (Bögel, personal communication). Unfortunately, no reports are available showing the effect of vaccination as a controlled attempt to immunize calves against outbreaks of respiratory disease.

As of this date, no PIV-3 vaccine for cattle is in use in the United Kingdom (Lamont, personal communication).

DISCUSSION

There is at present no explanation for the contradictory results of several attempts to immunize calves against experimental and natural shipping fever. Reisinger stated (28) that the "pathogenesis of shipping fever includes various etiological agents which may appear singly or in combination, plus various environmental stressors." In one of the field trials (14) the vaccinated calves had high titers of circulating antibodies to PIV-3 at the time of the outbreak, but even so, several vaccinated calves showed signs of shipping fever, and no other virus than PIV-3 was isolated. It is posible that the reduced resistance was due to a stressor not present during experimental exposure. The role of *Pasteurella spp.* in the pathogenesis of shipping fever has long been recognized, and therefore a vaccine against this disease usually contains both PIV-3 and *Pasteurella spp.* In the field trial mentioned above, *Escherichia coli* was also found to be associated with the disease. However, its role in the syndrome of shipping fever is at present unknown (14).

The possible existence of variants of PIV-3 was discussed by Hamdy and Trapp (14, 15). However, the variations in PIV-3 with respect to cytopathogenicity (10, 22), hemagglutinating activity (12, 21), heat-inactivation (12, 22), and virulence (4, 8) have not been found to be associated with striking antigenic divergencies between the variant strains.

Quite recently Matsuoka *et al.* (23) reported that a vaccine containing PIV-3 and *Pasteurella spp.* has given good protection against both the experimental disease and a natural outbreak of shipping fever. These authors followed a calfhood vaccination schedule, whereas other workers mostly vaccinated older calves. It is possible that the causal agents of a natural outbreak may be incidentally homologous to those of the experimental disease.

Common to all trials was the observation that after experimental or natural exposure to disease the majority of vaccinated calves showed a rise in antibody to PIV-3. In some trials a short term shedding of PIV-3 was also observed following exposure. Both findings indicate that infection had taken place. It is conceivable that, depending on the nature and severity of stress and the type or types of bacteria involved, the infection might be converted into disease.

Discussing the attenuated PIV-3 strain as a vaccine, the German authors (5, 7, 27) pointed out the importance of a "local immunity" induced, they suggest, by intranasal inoculation with the strain. This mode of vaccination would hence be superior to the parenteral administration of inactivated virus. However, no direct proof was presented to support these suggestions.

The basis for a preliminary vaccination program in Swedish farms resulted from a review showing that about 50 per cent of calves three to eight weeks old were devoid of measurable titers of antibody to PIV-3 (2, see Table 1). In farms that followed the vaccination schedule, the pneumoenteritis common prior to vaccination occurred in only a few calves after vaccination, and the mortality decreased appreciably. Since no calves were left unvaccinated, the beneficial effect of vaccination is at present only presumptive. Even so, the vaccine will now be prepared on a large scale so that successively more farms can be vaccinated.

It is well established that other viral or nonviral agents—for example, infectious bovine rhinotracheitis virus or miyagawanellae—do participate in the etiology of respiratory disease in cattle. Quite recently it was shown by the British workers (26) that adenovirus and reovirus participate, each with a frequency approximately similar to that of PIV-3. Two new serotypes of bovine adenovirus isolated in Hungary are cytopathogenic for calf testicular cells but not for calf kidney cells (3) and may be overlooked if only the latter type of cells is used for isolation. One of them was found to prevail among agents of pneumoenteritis in calves (Bartha, personal communication). Furthermore, concurrent infections with two viruses were also found to occur, as for instance with PIV-3 and the agent of mucosal disease, during outbreaks of a severe respiratory-enteric disease in 1958 in Sweden (9). A significant increase in antibody titers against more than one virus was found in paired serum samples from 22 out of 107 outbreaks of respiratory disease in calves in the United Kingdom (26).

It is thus obvious that a vaccination program for respiratory disease in cattle should be based on an analysis of the prevalence of the causal agents similar to that carried out by the British workers.

SUMMARY

Parainfluenza Type 3 virus (PIV-3) is widespread in cattle and is often a cause of respiratory disease. The pattern of disease varies. In some places—northern Europe, for example—the disease is usually mild, although severe forms with high mortality may occur, especially among young calves. In the United States a more serious form—"shipping fever"—is observed, which is often precipitated by severe transport stress. This latter form is aggravated by bacterial superinfection, mostly by pasteurellae, which effect a change from the characteristically proliferative pneumonia into an exudative form.

Both inactivated and attenuated vaccines have been developed. Vaccines against shipping fever usually contain inactivated PIV-3 and pasteurellae in oil adjuvant.

The results of the immunization experiments are rather unclear. One report shows that inactivated PIV-3 vaccine protected against both experimental shipping fever induced by stress and infection with PIV-3 and pasteurellae, while another claims that vaccine against pasteurellae has to be included to obtain this effect. In several trials, however, both these types of vaccines failed to protect against natural outbreaks of shipping fever. This can, of course, be due to differences between the infecting agents and those used in the vaccine. In Sweden, where the milder form of disease predominates, the use of an inactivated PIV-3 vaccine has reduced the average mortality from 20 per cent to 5 per cent in an uncontrolled study.

In West Germany an attenuated PIV-3 vaccine administered nasally was shown to be safe and to provide protection against experimental disease. No data have been supplied to show the efficacy of this vaccine against natural outbreaks of respiratory disease.

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SECTION B. RESPIRATORY SYNCYTIAL AND PARAINFLUENZA VIRUS VACCINES

DISCUSSION

CHAIRMAN FRANCIS: We will turn now to the discussion of these papers. The first discussant is Dr. Robert M. Chanock, Chief of the Respiratory Virus Unit, Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

Dr. Chanock presented a summary of a paper entitled "Resistance to Parainfluenza and Respiratory Syncytial Virus Infection—Implications for Effective Immunization and Preliminary Study of an Attenuated Strain of Respiratory Syncytial Virus," which appears in full as an annex to this section on pages 53–61.

CHAIRMAN FRANCIS: The next discussant on the program is Dr. S. B. Mohanty, Department of Veterinary Science, University of Maryland, College Park, Maryland.

DR. MOHANTY: As Dr. Dinter pointed out, there is indeed inconsistency in the results of vaccination against shipping fever in the United States. There is no doubt that the interaction of viral and bacterial multiplication along with a stressor makes the so-called "shipping fever" of cattle a complex disease. It has been successfully induced in calves exposed to myxovirus parainfluenza Type 3, *Pasteurella spp.*, and stress.

In the United States inactivated parainfluenza Type 3 vaccine, with or without *Pasteurella* bacterin, has been used against shipping fever. While fairly comparable results are achieved under experimental conditions, variable results have been obtained under field conditions. The serologic responses of cattle are usually in general agreement in these reports, but discrepancies have been noted in prevention of the disease. The vaccines have failed to protect cattle against outbreaks of shipping fever despite their high circulating antibodies.

This inconsistency in vaccine efficacy may be explained by the possible existence of variants of myxovirus parainfluenza Type 3, the presence of other viruses in this disease complex, and the fact that the neutralizing antibody titers in the nasal mucosa of the vaccinated cattle are much lower than the corresponding circulating antibodies. Stress factors undoubtedly play an important role in the dissemination of shipping fever. Variations in the stress conditions to which cattle are exposed may explain the difference in the incidence of the disease. The cattle are vaccinated, shipped to a variety of locations with different weather conditions, and observed after their arrival. Recurrent parainfluenza Type 3 infections are also not uncommon in man and cattle.

Furthermore, we have noticed that a multiplicity of viruses are involved in the bovine respiratory disease complex. We have induced respiratory disease syndrome in calves experimentally infected with a bovine adenovirus and reovirus Type 1 of human origin. These clinical syndromes are usually indistinguishable from those of shipping fever.

It is evident that the vaccine should be modified to protect cattle against naturally occurring cases of shipping fever. We feel that efforts should be made to modify the vaccine to increase the nasal antibody titers in vaccinated cattle. Nasal antibodies are, perhaps, more important than the circulating antibodies—for protecting cattle against shipping fever. I understand that a pharmaceutical company is in the process of marketing a shipping fever vaccine in this country. It will be interesting to follow the results of field trials of this vaccine.

CHAIRMAN FRANCIS: Thank you, Dr. Mohanty. We have a few minutes that can be used for

further discussion. Are there any comments or questions?

DR. MCCOLLUM: I have a question for Dr. Chanock. I would like to know about nasal antibody, its source, the significance of the levels measured, and whether these levels may be taken as indications of antibody levels at other points lower down in the respiratory tract.

DR. CHANOCK: The nasal antibody that we described in the discussion presentation was characterized by Dr. Joseph Bellanti, Georgetown University, Washington, D.C., and was found to be immunoglobulin Type A—that is, IgA. The serum antibody in the same individual, however, was found to be IgG. This indicates that the nasal and the serum antibodies for parainfluenza Type 1 are different immunoglobulin species.

We also found that volunteers experimentally infected with parainfluenza Type 1 virus developed nasal secretion antibody with high frequency and this antibody persisted in the nasal secretions for at least six to eight months in most of the infected individuals. This finding suggests to us that the antibody that develops in the nasal secretions is not the result of filtration of antibody from serum but rather an antibody that is produced in a pararespiratory tract location or possibly within the respiratory tract itself.

This is consistent, I believe, with information available from studies on rhinoviruses and other respiratory tract agents. It seems quite clear that we are dealing with an antibody in the respiratory tract that is different from that in serum. Even though there is a rough correlation between level of antibody in serum and level of antibody in nasal secretions, it should be stressed that these are different antibodies.

DR. KLEIN: Dr. Chanock pointed out that the passive antibody from the mother does not protect against RS in the infant. We do know that of the immunoglobulins only gamma G gets past the placenta. Therefore, the infant gets only gamma G and does not get gamma A from the mother. Since gamma A may well be the crucial neutralizing antibody in exudate, as the only one that gets over, might the failure of passive antibody be due to the fact that the infant only has gamma G and not gamma A?

DR. CHANOCK: This was the theme, actually, of my presentation—namely that the RS virus antibody transferred from the mother to the infant's serum does not protect the respiratory tract.

Another point of interest is that the IgA in nasal secretions is thought by many observers to be quite different from the IgA that is present in serum. Thus the simple filtration of antibody from serum is not the source of antibody in nasal secretions. Nasal secretion antibody develops in response to an antigenic stimulus that is presented to the respiratory tract.

CHAIRMAN FRANCIS: There is no time to go into this matter further because it is a very complicated one. There are other perfectly logical procedures that could also be used to identify the nasosecretions, but that would take us too far afield.

DR. RASMUSSEN: Does Dr. Chanock or any of the other discussants have a hypothesis to explain the apparent greater susceptibility in the infants with higher levels of antibody?

DR. CHANOCK: The infant between one and four months of age has the greatest risk of developing a serious RS virus lower respiratory tract illness. During an RS virus epidemic there is an even greater number of infants between the ages of 6 and 15 months of age in the population, but such older infants experience a lower incidence of RS lower respiratory tract illness.

One can explain this disparity in two ways: (1) the antibody that passes from the mother to the newborn infant in some way plays a role in the pathogenesis of illness; or (2) the difference is related to the diameter of the respiratory air passages, which varies with age. At present we do not know which of these two explanations is correct. Possibly both factors are involved.

DR. K. M. JOHNSON: I believe that the evidence brought out by Dr. Parrott and the reanalysis of the ecology of RS infection presented

by Dr. Chanock, whatever its subsequent elucidation, already find potentially strong parallel in a rather exotic situation elsewhere in the world. This is the situation with respect to so-called "dengue shock syndrome" (hemorrhagic fever) in Southeast Asia. Dr. Scott Halstead and his colleagues have now, I personally believe, gone a very long way in showing that this clinically severe disease, which is almost completely restricted to small children, may be associated with secondary rather than primary dengue virus infection.

Thus it is possible that maternal antibodies described here may be operating in a similar way, and that severity of disease is somehow related to unusual antigen-antibody reactions. If further study reveals that this is so, new problems of a very fundamental nature will have to be faced in development of potent and *safe* vaccines for certain agents.

ANNEX TO SECTION B. RESPIRATORY SYNCYTIAL AND PARAINFLUENZA VIRUS VACCINES

RESISTANCE TO PARAINFLUENZA AND RESPIRATORY SYNCYTIAL VIRUS INFECTION—IMPLICATIONS FOR EFFECTIVE IMMUNIZATION AND PRELIMINARY STUDY OF AN ATTENUATED STRAIN OF RESPIRATORY SYNCYTIAL VIRUS

R. M. CHANOCK, C. B. SMITH, W. T. FRIEDEWALD, R. H. PARROTT, B. R. FORSYTH, H. V. COATES, A. Z. KAPIKIAN, AND M. A. GHARPURE *

DR. CHANOCK (*presenting the paper*): The most severe illnesses produced by the parainfluenza and respiratory syncytial (RS) viruses are generally associated with primary infection, which usually occurs during the first three to five years of life with the former viruses and during the first two years with the latter. Thus, RS virus bronchiolitis or pneumonia is characteristically a disease of the first or second year of life, and croup or pneumonia caused by a parainfluenza virus most commonly occurs before age five (4, 5). When reinfection occurs at a later time the associated respiratory tract disease is usually less severe than that occurring with the first infection.

The patterns of illness just described suggest that primary infection confers upon the host a relative resistance to severe illness associated with parainfluenza or respiratory syncytial virus infection. The importance of serum neutralizing antibody in such resistance, however, is not completely clear, particularly in RS virus infection in young children and parainfluenza virus infection in adults. A better definition of the role of serum antibody has obvious implications in the area of immunoprophylaxis, since parenterally administered inactivated vaccines act primarily to stimulate the development of antibody in serum.

In this presentation we will re-examine the epidemiology of the respiratory "paramyxoviruses" for clues to the protective effect of serum antibody. We will also present new information indicating that antibody in respiratory tract secretions plays a larger role than serum antibody in resistance to infection. After discussing the relevance of these findings to "paramyxovirus" immunoprophylaxis we will describe preliminary observations with an attenuated strain of RS virus.

RS VIRUS

Effectiveness of serum neutralizing antibody during early infancy. It has been shown by Beem that serum neutralizing antibody is transferred quantitatively from the mother to the newborn infant (1). Using a relatively insensitive neutralization technique, Beem found that seven of eight infants aged four to six months still possessed serum neutralizing antibody, presumably of maternal origin. Canchola, using a microtiter neutralization technique, tested 29 normal infants three to six months of age and found that each possessed serum neutralizing antibody at a level of 1:4 to 1:64 (2). When the sera of these infants were tested by the more sensitive plaque-reduction technique (60 per cent plaque-reduction endpoint), serum

*Drs. Chanock, Smith, Friedewald, Forsyth, Coates, Kapikian, and Gharpure (Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.); Dr. Parrott (Children's Hospital, Washington, D. C., U.S.A.).

titers of 1:25 to 1:1280 or greater were found (7). The plaque-reduction serum titers for normal infants between three and five months of age are shown in Figure 1.

Low to moderate levels of neutralizing antibody are commonly present in the serum of young infants just prior to or at the time of onset of RS virus lower respiratory tract disease (3). In 1961 we found that 20 of 27 infants aged two to six months had serum neutralizing antibody titers of 1:4 to 1:16 (as measured in roller tube cultures of HEp-2) when they were admitted to the hospital for RS bronchiolitis or pneumonia. Using the sensitive plaque-reduction technique, we recently found that 12 of 13 infants aged three to five months had serum neutralizing antibody in titers (based on 60 per cent plaque reduction) ranging from 1:40 to 1:680 at the time RS bronchiolitis or pneumonia was first diagnosed during the 1965–1966 epidemic. It is unlikely that such acute phase serum antibody represents a response to concurrent infection, since the incubation interval between exposure to RS virus and development of lower respiratory tract disease appears to be four days (11). Thus, the neutralizing antibody present in acute-phase serum is probably passively acquired antibody of maternal origin. As is shown in Figure 1, the normal infants generally had higher serum neutralizing antibody titers than did infants in the acute phase of RS virus lower respiratory tract disease. However, five of the infants with RS virus illness had moderate levels of antibody—1:210 to 1:680—in their acute-phase sera.

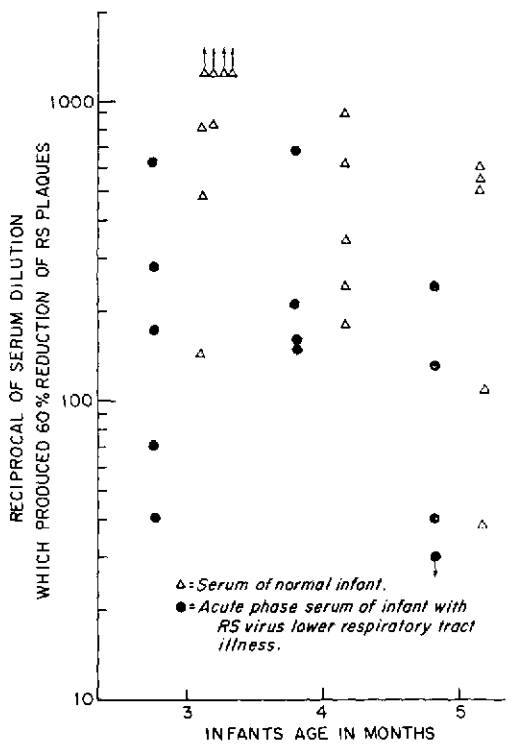


Fig. 1. Titer of neutralizing antibody in sera of normal infants and in acute phase sera of infants with RS virus bronchiolitis or pneumonia as determined by plaque-reduction technique.

The observations just described suggest that low to moderate levels of serum neutralizing antibody do not provide effective resistance to RS-virus-induced lower respiratory tract disease. Possibly in the absence of serum antibody RS virus illness would occur more often and be more severe. However, it is also possible that passively acquired antibody may contribute to the pathogenesis of RS virus illness by combining with virus in the respiratory tract to produce a local anaphylactic reaction.

The age distribution of RS virus bronchiolitis and pneumonia is unique for the known respiratory tract pathogens in that this virus is most important as a cause of lower respiratory tract disease during the first six months of life (4). A further age analysis of bronchiolitis and pneumonia illnesses (Fig. 2) indicates that RS-virus-induced disease occurs commonly during the first and second months of life; in fact it occurs more often during this period than during the fifth, sixth, seventh, and eighth months. This age-illness relationship is consistent with the hypothesis that passively acquired neutralizing antibody may be an essential ingredient in the pathogenesis of RS virus bronchiolitis. Furthermore, the pattern of RS virus infection and illness shown in Figure 2 supports the contention that maternally transmitted neutralizing antibody does not provide effective protection. If such antibody were protective, relatively little RS-virus-induced illness would be observed during the first few months of life when maternally transmitted antibody is at its highest level.

Effectiveness of serum neutralizing antibody during childhood. First infection with RS virus does not appear to provide complete protection against the effects of a second infection occur-

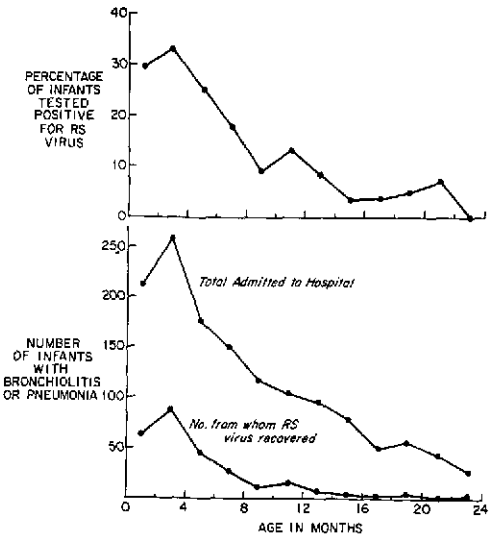


Fig. 2. Relation of age to recovery of RS virus from infants with bronchiolitis or pneumonia. Children's Hospital, Washington, D. C., June 1959 to June 1966.

ring within one to three years. In such instances it is difficult to evaluate the protective effect of serum antibody alone since other immune mechanisms, such as local antibody production in the respiratory tract, may also play a role in resistance. In the young infants described previously this complication did not arise since serum antibody of maternal origin was present without the associated local responses stimulated by infection.

The most complete information so far on the protection afforded by primary infection was obtained during an outbreak of RS virus infection in infants and young children eight months to four years of age that occurred in a welfare nursery (Junior Village) where a longitudinal study of illness and microbial infection was under way (11). Serum specimens collected prior to the time the outbreak occurred were available from each of the nursery occupants. The outbreak was explosive: it lasted only three weeks and over 90 per cent of the nursery residents developed serologic evidence of infection; 40 per cent developed clinical signs of pneumonia. Children with low to moderate levels of serum neutralizing antibody—1:4 to 1:16—developed pneumonia as often as those who lacked detectable antibody (Table 1). High levels of serum neutralizing antibody—1:32 or greater—were associated with resistance; even so, the high-level group showed only a twofold reduction in pneumonia incidence. A similar relationship was seen in the group of nursery occupants from whom the virus was recovered: serum neutralizing antibody was associated with only partial resistance. The lower respiratory tract illnesses seen in the nursery outbreak, although definite, were less severe than those that occur in primary infection of infancy. That this was due to host factors such as age and not to antibody was shown by the finding that children who had low to moderate levels of RS antibody before the outbreak developed lower respiratory tract illnesses comparable in severity to those observed in children without such antibody. The pattern and type of illness seen in the nursery outbreak

TABLE 1. EFFECT OF PREINFECTION SERUM NEUTRALIZING ANTIBODY ON THE OCCURRENCE OF PNEUMONIA DURING A NURSERY OUTBREAK OF RS VIRUS INFECTION

(JUNIOR VILLAGE 1960)

Reciprocal of preoutbreak serum neutralizing antibody titer	Includes all nursery residents		Includes those residents from whom RS virus recovered	
	No.	No. who developed pneumonia during outbreak	No.	No. who developed pneumonia associated with RS virus*
<4	29	13 (45%)	13	11 (85%)
4–16	28	15 (54%)	4	2
32 or >	23	5 (22%)	3	1
		20 (39%)		3 (43%)

*Onset of pneumonia occurred up to three days before, day of, or day after initial RS virus isolation.

suggests that primary infection is not highly effective in conferring resistance to subsequent RS virus illness.

One can only speculate about the capacity of RS virus to successfully evade the host's resistance mechanisms and to produce significant illness during second infection. The successful evasion of host defense mechanisms may possibly be explained by the fact that this virus characteristically induces the formation of syncytia in tissue culture (6), plus the fact that it enters the body, infects, and produces its pathogenic effect in the same tissue (the respiratory tract epithelium). Direct attack on respiratory tract epithelium minimizes the protective role of serum neutralizing antibody. Syncytium formation permits incorporation of uninfected cells into the disease process under conditions that minimize the effectiveness of serum or locally produced antibody in halting the extension of infection and tissue damage.

Effectiveness of serum neutralizing antibody in adults. RS virus infection and associated upper respiratory tract disease occur under natural conditions in adults despite the presence of moderate to high levels of serum neutralizing antibody (8, 9). Similarly, adult volunteers given RS virus become infected and develop mild illness even though serum antibody is present (10). Infection and illness in volunteers are not related to the level of serum neutralizing antibody. With each of three different strains of virus we have infected and produced mild respiratory tract illness in 50 to 90 per cent of the volunteers (10).

PARAINFLUENZA VIRUSES

Effectiveness of serum neutralizing antibody during infancy and childhood. Information on the protective effect of Type 3 virus antibody during late infancy and early childhood, was obtained during a series of Type 3 virus outbreaks occurring in a welfare nursery (Junior Village) that was under longitudinal surveillance for microbial experience and illness (5). Serum neutralizing antibody correlated with resistance to infection; this resistance was not complete, however, since 33 per cent of the children with high levels of antibody became reinfected (Table 2). Sequential infection of children during successive outbreaks was actually demonstrated. Although reinfection occurred, serum antibody still exerted an effect: the period of virus shedding was shorter than during primary infection. Serum neutralizing antibody also correlated with resistance to febrile illness and lower respiratory disease. However, febrile illness occurred in children with moderate levels of antibody (1:8 to 1:32) approximately 40 per cent as often as in children undergoing first infection. Similarly, lower respiratory tract involvement during Type 3 virus infection occurred less often during reinfection—approximately 20 per cent as often as during first infection. In these studies strict criteria were used in analyzing the association between virus infection and the occurrence of illness (5).

The correlations just described are similar to those observed with RS virus with the difference that parainfluenza Type 3 virus neutralizing

TABLE 2. EFFECT OF PREINFECTION SERUM NEUTRALIZING ANTIBODY ON INFECTION WITH TYPE 3 PARAINFLUENZA VIRUS AND ITS CONSEQUENCES, JUNIOR VILLAGE, 1957–1959

Reciprocal of pre-infection serum neutralizing antibody	Observations on all nursery residents				Observations on residents from whom virus recovered*			
	Neutralizing antibody rise†		Virus recovery‡		No. in group	Median No. of days virus recovered from throat	% with Type 3 virus associated illness	
	No. tested	% positive	No. tested	% positive			Febrile	Pneumonia or bronchitis
<8	46	100	27	96	54	8	78	33
8–32	22	91	15	67	18	3	33	7
64–1024	50	34	37	33	43	1	19	7

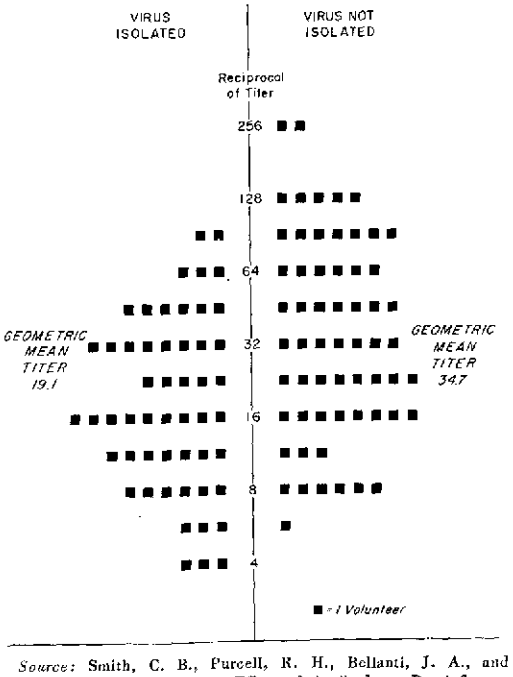
*Outbreaks Nos. 2 and 3.
†Outbreaks Nos. 1 and 2.
‡Outbreak No. 2.

antibody exhibits a stronger correlation with resistance than does RS virus antibody. This finding is in keeping with the epidemiology of these two viruses. Like RS virus, parainfluenza Type 3 virus is ubiquitous, however, unlike RS virus, it does not produce an inordinately large number of illnesses during the first six months of life at a time when maternally transmitted neutralizing antibody is present in serum.

Effectiveness of serum neutralizing antibody in adults. In adult volunteers a poor correlation was observed between serum neutralizing antibody and resistance to experimental Type 1 virus infection as measured by virus recovery. Each of the volunteers in the study reported in Figure 3 possessed neutralizing antibody prior to challenge with Type 1 virus (13). The reciprocal geometric mean serum titer of those who resisted infection was 34.7, whereas the mean titer of those who were infected was 19.1. This difference was statistically significant ($P < 0.01$). However, the variation in titers in the two groups was wide, and it was difficult to predict an individual's response to Type 1 virus challenge on the basis of his serum antibody level.

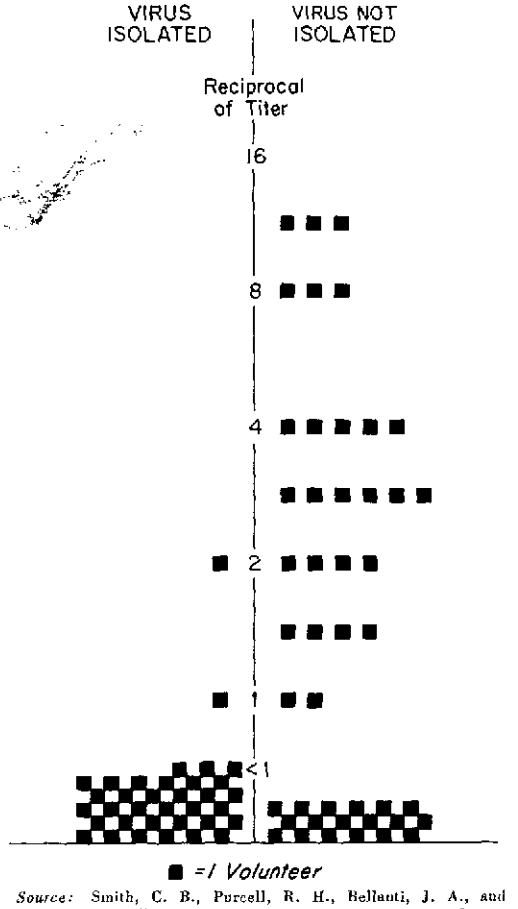
Effectiveness of neutralizing antibody in nasal secretions of adults. A more impressive correlation between neutralizing antibody and resistance to experimental Type 1 virus infection was observed when the data for neutralizing activity in nasal secretions were examined.

As Figure 4 shows, only 2 of 29 volunteers with antibody in nasal secretions became infected, whereas on 33 of 51 occasions subjects who lacked such antibody became infected after challenge (13). Nasal secretions from 34 of the volunteers were concentrated 10 times in order to detect low levels of neutralizing activity. Concentration led to detection of neutralizing activity



Source: Smith, C. B., Purcell, R. H., Bellanti, J. A., and Chanock, R. M. "Protective Effect of Antibody to Parainfluenza Type 1 Virus." *New Eng J Med* **275**:1145–1152, 1966.

Fig. 3. Serum neutralizing antibody titer prior to challenge with parainfluenza Type 1 virus.



Source: Smith, C. B., Purcell, R. H., Bellanti, J. A., and Chanock, R. M. "Protective Effect of Antibody to Parainfluenza Type 1 Virus." *New Eng J Med* **275**:1145–1152, 1966.

Fig. 4. Neutralizing activity of nasal secretions prior to challenge with parainfluenza Type 1 virus.

in 10 of the secretions that were originally negative. The reciprocal geometric mean titer of neutralizing activity in the group that resisted infection was 11.8, whereas that of the group from whom virus was recovered was 1.6—a highly significant difference statistically.

The protective effect of serum and nasal neutralizing antibody were analyzed separately by a matching technique (Table 3). The subjects with high levels of nasal secretion neutralizing activity (1:3 and higher) were infected less often than those with low levels, regardless of whether they had high or low levels of serum antibody. In contrast, when the volunteers were matched according to level of nasal secretion antibody there was no difference in the rate of infection between those with low and high levels of serum antibody. Thus the protective effect of neutralizing antibody in nasal secretions was demonstrable regardless of serum antibody titer. Furthermore, when the effects of nasal neutralizing antibody were matched out no protective effect of serum antibody was demonstrable. Thus the level of neutralizing antibody in nasal secretions was a better index of host resistance to reinfection than was the level of serum antibody.

A subsequent study showed that the antibody found in nasal secretions after Type 1 virus infection was predominantly IgA, whereas the antibody in serum was predominantly IgG (12). Type 1 neutralizing antibody persisted in the nasal secretions of 11 out of 17 men for a period of six to eight months. This finding, plus the fact that there is a difference between nasal secretion and serum Type 1 virus immunoglobulins, suggests that antibody in nasal secretions is produced in or near the respiratory tract and does not represent a simple filtrate of serum.

TABLE 3. COMPARISON OF PROTECTIVE EFFECT OF TYPE 1 PARAINFLUENZA VIRUS NEUTRALIZING ACTIVITY IN SERUM AND NASAL SECRETIONS OF VOLUNTEERS

Neutralizing activity in nasal secretions*	No. of volunteers infected/No. in group		
	Serum neutralizing antibody titer <1:48	Serum neutralizing antibody titer >1:48	
<1:3.....	10/13	3/4	P>.3†
>1:3.....	1/9	0/8	P>.3†
	P<.01‡	P<.05‡	

*Nasal secretions and serum collected from 34 volunteers prior to intranasal and oropharyngeal instillation of $10^{4.5}$ TCD_{50} of Type 1 parainfluenza virus. Nasal secretions were concentrated 10 times before being tested for neutralizing activity.

†No significant difference between low and high serum antibody groups matched according to nasal secretion neutralizing activity (Fischer exact test).

‡Significant difference in incidence of infection between groups with low and high levels of nasal secretion neutralizing activity when matched according to serum antibody titer (Fischer exact test).

Contrast between immune response to infection and parenteral administration of inactivated vaccine. When 35 men who had serum neutralizing antibody for Type 1 virus were experimentally infected with this virus, only 51 per cent developed a fourfold or greater rise in serum antibody, whereas 80 per cent of those who received two injections of inactivated Type 1 virus vaccine developed a serologic response (Table 4). Furthermore, the geometric mean serum antibody titers were higher after vaccination than after infection. In contrast, the infected volunteers developed antibody in their nasal secretions with greater frequency and to a higher mean titer than did the vaccinees.

Nine men (not shown in Table 4) who received inactivated vaccine No. 1 and who developed a fourfold or greater rise in serum antibody were subsequently challenged with Type 1 virus; six became infected. This response was similar to that of the volunteers who lacked neutralizing antibody in nasal secretions. Thus an inactivated vaccine that stimulated serum neutralizing antibody more effectively than antibody in nasal secretions failed to provide protection against reinfection. In contrast, infection did not occur in 20 volunteer challenges involving men who were infected previously and who possessed antibody in their nasal secretions.

IMPLICATIONS FOR IMMUNOPROPHYLAXIS OF PARAINFLUENZA AND RS VIRUS INFECTIONS

To summarize, serum neutralizing antibody does not appear to be highly effective in providing resistance to RS-virus-induced severe lower respiratory tract disease in early infancy. Serum antibody for Type 3 parainfluenza virus is somewhat more effective in conferring protection, but its protective effect is only partial.

TABLE 4. COMPARISON OF ANTIBODY RESPONSE OF VOLUNTEERS TO INFECTION WITH PARAINFLUENZA TYPE 1 VIRUS OR IMMUNIZATION WITH INACTIVATED VACCINES

Group	No. of men	Serum neutralizing antibody			Nasal secretion antibody*	
		No. of men with fourfold or greater rise	Reciprocal geometric mean titer		No. of men who developed antibody†	Reciprocal geometric mean titer after 5 weeks‡
			Pre-vaccination or pre-challenge	Five weeks after vaccination or challenge		
Virus infection§	35	18 (51%)	35	48	29 (83%)	5.2
Inactivated vaccine #1 ‖	14	11 (79%)	36	87	3 (21%)	1.4
Inactivated vaccine #2 ¶	11	9 (82%)	40	96	0	0

*Nasal secretions tested without prior concentration.
†Subjects who lacked antibody and developed detectable antibody or who had antibody and developed fourfold or greater rise.
‡Adjusted for protein concentration of 100 mg%.
§Virus infection indicated by isolation of Type 1 virus.
‖National Drug Co. lot 100.
¶ Pfizer Drug Co. lot 23.

In adults antibody in nasal secretions was found to be a better index of host resistance to Type 1 parainfluenza virus infection than was serum antibody. Immunization with an inactivated vaccine was more effective in stimulating serum antibody than antibody in nasal secretions; failure to stimulate the latter antibody effectively was correlated with failure of vaccination to provide protection against reinfection. In contrast, infection stimulated the development of nasal secretion antibody in most volunteers, and such men were found resistant to subsequent re-challenge.

From these findings we deduce that the measurement of antibody in nasal secretions may provide more useful information concerning the potential effectiveness of "paramyxovirus" vaccines than does the measurement of serum antibody. Furthermore, efforts to develop live attenuated vaccines for these viruses would appear indicated, since infection with parainfluenza Type 1 virus stimulated higher levels of nasal secretion antibody than did an inactivated vaccine.

Older children and adults who are undergoing reinfection probably constitute the major source of infection for susceptible infants and young children. For this reason it would be desirable to develop immunoprophylactic procedures for the prevention of such reinfection as well as for the prevention of primary infection in early life.

PRELIMINARY EXPERIENCE WITH AN ATTENUATED STRAIN OF RS VIRUS

Stimulated by the considerations just described, particularly the fact that low to moderate levels of serum neutralizing antibody fail to provide effective protection against RS virus illness in early infancy, we have pursued an active search for an attenuated variant of RS virus suitable for use in a live virus vaccine. About two and a half years ago we began a systematic attempt to select such a variant. Initially we cultivated the A_2 strain of RS virus (kindly supplied by Drs. Lewis and Ferris of Melbourne, Australia, in its first human embryonic kidney tissue culture passage) in bovine embryonic kidney tissue culture at 34°C for 21 passages. Five of the six volunteers given this material by the intranasal and oropharyngeal routes became infected and developed minor upper respiratory tract illness (Table 5). Each of these men, like all the other adults we have tested, possessed serum neutralizing antibody prior to challenge. The response of the volunteers to the twenty-first bovine embryonic kidney culture passage material resembled that of subjects who were given other strains of RS virus in early tissue culture passage. This sug-

TABLE 5. EFFECT OF CULTIVATION OF A2 STRAIN OF RS VIRUS AT LOW TEMPERATURE UPON INFECTIVITY AND VIRULENCE FOR ADULT VOLUNTEERS

Tissue culture passage history	Temperature of cultivation	Number of men		
		Given 10^4 to 10^5 $TCID_{50}$	Infected with RS virus*	With upper respiratory tract illness associated with RS virus infection
HEK† 5..... BEK‡ 21	34° C	6	5	5
+BEK 12.....	28° C	9	8	5
+BEK 19.....	26° C	Study #1 6 Study #2 30 } 45 Study #3 9	1 7 } 9 1	0 1§ } 1 0

*As indicated by virus recovery and/or a rise in neutralizing and/or CF antibody.
†Human embryonic kidney culture.
‡Bovine embryonic kidney culture.
§Volunteer had concurrent infection with rhinovirus type 29.

gested that the twenty-first passage virus was not demonstrably attenuated.

Subsequently, the A_2 strain was cultivated for 12 passages at 28°C in bovine embryonic kidney tissue culture. This material was given to nine volunteers; eight men were infected and five developed a common-cold-like illness. These findings suggested that cultivation at 28°C did not affect the emergence of an attenuated variant.

The A_2 strain was then terminally diluted three times at 28°C and cultivated for 16 passages at 26°C in bovine embryonic kidney tissue culture. This material was given to six volunteers in one study and to a total of 45 volunteers in three separate studies. A total of nine men were infected and only one volunteer developed minor upper respiratory tract illness. This may not have been caused by RS virus, since the subject developed a rise in neutralizing antibody for Type 29 rhinovirus. The latter virus was present in three of the volunteers of the second study prior to administration of RS virus (Table 5). The response of volunteers in the three studies suggests that the 26°C virus was both less infectious and less virulent for adults than the parent virus.

We plan to continue our investigation of the 26°C adapted A_2 strain by a cautious step-by-step evaluation of the virus in progressively younger age groups. If in each instance the virus infects but fails to produce illness it will then be tested in the next younger age group. Ultimately, if each of the graduated studies is successful, the low-temperature strain will be evaluated in young infants—the host for whom an effective RS virus vaccine is most needed.

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SESSION II

CONTROL OF ACUTE RESPIRATORY DISEASES (*continued*)

Monday, 7 November 1966, 2:00 p.m.

CHAIRMAN
SIR CHRISTOPHER H. ANDREWES

RAPPORTEUR
DR. C. H. STUART-HARRIS

Section C.
Adenoviruses

Presentation of Papers by:
Dr. Maurice A. Mufson
Dr. Robert J. Huebner

Discussants:
Dr. Robert M. Chanock
Dr. Julius A. Kasel
Dr. John J. Trentin
Dr. H. G. Pereira
Dr. A. A. Selivanov

Section D.
Rhinoviruses

Presentation of Papers by:
Dr. Herbert A. Wenner
Dr. David A. Tyrrell
Dr. William S. Jordan, Jr.

Discussants:
Dr. William J. Mogabgab
Dr. Dorothy M. Hamre

Section E.
Mycoplasma Vaccines

Presentation of Papers by:
Dr. D. G. ff Edward
Dr. William J. Mogabgab
Dr. Robert M. Chanock

Section F.
Combined Respiratory Virus Vaccines

Presentation of Paper by:
Dr. Maurice R. Hilleman

Discussants (Sections E. and F.):
Dr. Leonard Hayflick
Dr. B. P. Marmion
Dr. Keith E. Jensen

SECTION C. ADENOVIRUSES

EFFICACY OF KILLED AND LIVE ADENOVIRUS VACCINES

MAURICE A. MUFSON

Department of Medicine, University of Illinois College of Medicine; University of Illinois Medical Division; and Kektoen Institute for Medical Research of the Cook County Hospital, Chicago, Illinois, U.S.A.

DR. MUFSON (*presenting the paper*): During the 13 years since the discovery of the adenoviruses in 1953 by Rowe and co-workers and Hilleman and co-workers, cumulative epidemiologic studies have demonstrated the major importance of adenovirus Types 4 and 7, and to a lesser extent Type 3, as etiologic agents of acute respiratory tract disease among military recruits (3, 5, 15, 29–31, 34, 39, 45, 48). The control of adenovirus infections by immunoprophylaxis presents a continuing problem. Experimental inactivated adenovirus Types 3, 4, and 7 vaccines, introduced soon after the recognition of the major etiologic role of these viruses, proved effective in reducing specific adenovirus respiratory tract disease (7, 11, 16, 26, 27, 42, 43). Although large-scale studies among military recruits confirmed the over-all efficacy of killed adenovirus vaccines, these vaccines vary in effectiveness (30, 40). Recently, an enteric-administered live adenovirus Type 4 vaccine was introduced for the immunoprophylaxis of adenovirus Type 4 infection with encouraging results (4, 6, 9, 33).

This report summarizes the available information on the immunogenicity, efficacy, and safety of killed and live adenovirus vaccines.

Importance of Adenovirus Infections

The importance of adenovirus Types 3, 4, and 7 as etiologic agents of acute respiratory tract disease in military recruits was established in epidemiologic studies in the United States and the Netherlands (3, 15, 29, 31, 39, 45, 48). The contribution of adenoviruses in acute respiratory disease, assessed in a number of military-recruit populations, is summarized in Table 1: in the United States, adenovirus Types 4 and 7 infections predominate; in the Netherlands, Types 14 and 21 have also been associated with outbreaks of acute respiratory disease (45).

In military recruits, adenoviruses are associated with about 10 to 50 per cent of severe febrile upper respiratory tract disease; these illnesses frequently necessitate hospitalization. Pneumonia illnesses due to adenoviruses are less extensive (3, 34). However, about 70 to 90 per cent of military recruits become infected during training.

In children and civilian adults, adenovirus Types 3, 4, and 7 cause few respiratory illnesses. Commonly occurring adenovirus serotypes in childhood infections are Types 1, 2, 3, 5, 6, and 7 (2, 5, 46). In a large cross-sectional epidemiologic study of respiratory tract disease in children conducted between 1957 and 1961 in Washington, D. C., only 5 per cent of 4,605 ill children showed evidence of adenovirus-associated disease (5). Adenovirus infections were detected in 1 per cent of children with upper respiratory tract illnesses and, depending upon the clinical syndrome, in 2 to 4 per cent of lower respiratory tract illnesses. In civilian adults, the proportion of respiratory tract illnesses due to adenoviruses is usually less than 5 per cent (5). These findings contrast sharply with the high adenovirus infection rates among

TABLE 1. ADENOVIRUS INFECTION IN ACUTE RESPIRATORY DISEASE AMONG MILITARY RECRUITS

Location	Study interval	Testing method	Acute respiratory disease			Ref.
			Illness type	No. tested	Percentage adenovirus positive	
Great Lakes, Ill.	1954–1955	Serol.	Upper respiratory	1,013	40	48
Great Lakes, Ill.	1954	Serol. and isol.*	Afebrile	222	8.1	39
			Febrile	351	13.1	
Fort Dix, N.J.	1954–1955	Serol.	All	704	50.0	15
The Netherlands	1958–1962	Serol. and isol.†	Upper respiratory	1,540	29.7	45
Keesler Air Force Base, Miss.	1958–1959	Serol.	Febrile	232	20	31
Keesler Air Force Base, Miss.	1959–1960	Serol.	Afebrile	491	21	31
Great Lakes, Ill.	1958–1959	Isol.‡	Febrile	859	52	29
Camp Lejeune, N.C.	1959–1963	Isol.‡	Febrile	975	44.0	3
		Isol.‡	Afebrile	1,775	11.5	
Parris Island, S.C.	1959–1963	Serol. and isol.‡	Afebrile	367	0.5	3
			Febrile	668	29.6	

*Type 4 recovered.
†Types 4, 7, 14 and 21 recovered.
‡Types 4 and 7 recovered.

military recruits and focus attention on the importance of immunoprophylaxis among the recruits.

Ecology of Adenovirus Infections

Among military recruit populations, the ecologic characteristics of adenovirus infection include endemic, little seasonal variation of adenovirus Types 3 and 7, winter predominance of adenovirus Type 4, high infection rates, and moderately rapid person-to-person spread. At most recruit training camps, adenovirus infections prevail at high levels throughout the year (5, 34, 45). Types 3 and 7 show little seasonal variation, but Type 4 infection can occur predominantly in the winter. Among recruits undergoing advanced training at Camp Lejeune, North Carolina, studies conducted between 1959 and 1963 detected annual sharply circumscribed winter epidemics of acute respiratory tract disease due to adenovirus Type 4 (3). Each outbreak began during January and lasted through March or April. During the remaining months of the year the recruit population was almost completely free of adenovirus infection.

Among recruits in general, almost all susceptible individuals become infected before the completion of basic training—usually during the first two to three weeks after arrival and start of training. Among the recruits at Parris Island, South Carolina, however, the level of adenovirus infection is low, but a significant proportion of acute respiratory tract disease occurring during the winter months was due to adenovirus Type 4 or Type 7 infection (3).

Immunoprophylaxis with Inactivated Adenovirus Vaccines

Because of the major etiologic importance of Types 3, 4, and 7 in acute respiratory disease occurring among military recruits, immunoprophylaxis has been restricted to these types. The major experience is with inactivated bivalent (Types 4 and 7) and trivalent vaccines prepared in monkey renal cells (1, 7, 11–14, 16–18, 25–28, 42, 43, 47). Inactivated vaccines induce a homotypic antibody response, which reaches a peak in about 14 days (8). Types 3 and 7, which share antigens, also produce heterotypic antibody responses.

Initial studies with inactivated adenovirus vaccines demonstrated high protection rates and encouraged wider use of these vaccines (7, 11, 16, 26, 27, 42, 43). The results of several vaccine trials conducted at a number of military recruit camps during the past decade are com-

pared in Table 2. Immunization with inactivated bivalent or trivalent adenovirus vaccines effectively lowers the incidence of total respiratory disease occurring in military recruits. Depending upon the study, the reduction in total respiratory tract disease ranged between 15 and 81 per cent. When these data are analyzed with respect to adenovirus-positive respiratory disease, the specific protection rates are much higher—usually greater than 90 per cent and as high as 98 per cent, in several early studies.

Later, vaccines prepared on a large scale were less effective. Sherwood and co-workers pointed out that immunization with commercially prepared adenovirus vaccines failed to achieve the effectiveness previously demonstrated with experimental vaccines prepared in small batches (40). These investigators observed only a 52 per cent reduction in hospital illness due to adenovirus infection. In an extensive re-evaluation of the efficacy of inactivated trivalent adenovirus vaccine, conducted in three phases during 1962 and 1963 at Great Lakes, Illinois, protection rates were discouragingly low (30). During the first phase of the study, the protection rate for adenovirus-positive respiratory disease was 86 per cent, but in the second and third phases the reduction in adenovirus-specific respiratory tract disease was much lower—70 and 43 per cent, respectively. No differences in antigenicity were detected by administering inactivated adenovirus vaccines intramuscularly or by "jet" hypospray.

Oncogenic Potential of Certain Adenovirus Serotypes

Recently, the safety of parenteral adenovirus vaccines was challenged by the isolation of latent simian viruses from monkey renal cells; by the demonstration of oncogenic potential in hamsters of certain adenovirus serotypes, including Types 3, 7, 12, 14, 18, 21, and 31; and by the discovery of a "hybrid" or transcapsidation of Simian Virus 40 (SV_{40}) and adenovirus Type 7 vaccine strain (L.L.). Trentin and co-workers first demonstrated that adenovirus Type 12 produced tumors in suckling hamsters (44). Subsequently, Huebner and co-workers (21, 23), Girardi and co-workers (10), and Pereira and co-workers (32) confirmed these observations and extended them to other adenovirus types, including 3, 4, 7, 18, 21, and 31. Suckling hamsters inoculated with these serotypes develop tumors, and these tumors contain neoantigen (or T antigen), which reacts with antibody in the sera of tumor-bearing hamsters. The neoantigen is not part of the virion, but it is also produced early in the cytolytic cycle in tissue culture (20). The tumors are transplantable.

TABLE 2. EFFICACY OF KILLED ADENOVIRUS TYPES 3, 4, AND 7 VACCINE

Location	Vaccine*	Dose (ml)	No. of men receiving		Percentage reduction in†		Ref.
			Vaccine	Placebo	Total resp. disease	Adenovirus specific disease	
Great Lakes, Ill.	Tri	2	2,713	11,309	65	—	11
Fort Dix, N.J.	Bi	2‡	311	313	81	98	42
Fort Ord, Calif.	Tri	1	1,306	3,049	43	70	7
Fort Ord, Calif.	Tri	1	801	1,972	55	93	26
Fort Wood, Mo.	Bi	1.5	2,471	5,767	55	90	16
San Diego, Calif.	Tri	1	1,203	1,240	15	72	27
Fort Ord, Calif.	Tri	1	822	463	58	90	43
Bridgenorth, Eng.	Tri	1	402	661§	40	70	47
Great Lakes, Ill.	Tri	1	3,283	2,930	55	86	30
Great Lakes, Ill.	Tri	1	2,126	2,202	49	70	30
Great Lakes, Ill.	Tri	1	1,847	2,741	52	43	30

*Tri = Trivalent; Bi = Bivalent (Types 4 and 7).
†Calculated excluding respiratory illness occurring during the first 10 to 14 days after immunization.
‡Two 1 ml doses, 6 to 9 day interval.
§Individuals observed but not administered placebo.

In later studies, a "hybrid" particle of SV_{40} and the adenovirus Type 7 vaccine strain (L.L.) was discovered (22, 35, 36, 38). The L.L. strain of adenovirus Type 7 had undergone 22 serial passages in primary rhesus renal cells, and during its twenty-third and twenty-fourth passage in cell culture—in green monkey renal cells—the cultures were treated with antibody to SV_{40}; a later passage of the virus was employed for vaccine production (22). Infectious SV_{40} virus was not detected in subsequent passages. Tumors induced in hamsters after inoculation with the hybrid virus contained the SV_{40} neoantigen, but infectious SV_{40} was not detected in the tumors. The studies of Rowe and Pugh suggest that the SV_{40} DNA and a portion of the adenovirus Type 7 DNA are contained in the same capsid (37).

The L.L. strain adenovirus Type 7 was employed in inactivated adenovirus vaccines administered to military recruits. There is no evidence, however, that any adenovirus serotypes, including this one, are oncogenic for man. The "hybrid" strain failed to elicit antibody against SV_{40} or neoantigen of SV_{40} tumors. Therefore, the oncogenic SV_{40} genome is neither functional nor immunogenic in an inactivated preparation. Nonetheless, because of an undetermined risk associated with the use of inactivated adenovirus vaccines, inactivated adenovirus Types 3, 4, and 7 vaccines were recently withdrawn from general use by the Division of Biologics Standards of the National Institutes of Health.

Development of Live Adenovirus Type 4 Vaccine

To circumvent the unassessable hazards associated with adenovirus vaccine prepared in monkey renal cells and administered parenterally, Chanock and co-workers described in 1963 enteric immunization with a live adenovirus Type 4 strain, which lacked oncogenic potential (4, 6). The virus was grown in fetal human diploid fibroblast cell strains and given in enteric coated capsules. The immunogenicity of this vaccine was investigated in volunteer studies; the results are summarized in Table 3. In the first two studies, the virus was propagated in human embryonic kidney and African green monkey renal cells, respectively, but in the second two studies the virus was grown in fetal human diploid fibroblast cells. All volunteers in each of the four studies were free of homotypic neutralizing antibody prior to virus feeding. Fourfold or greater homotypic neutralizing antibody responses developed in the majority of volunteers. The geometric mean antibody titers ranged between 1:10 and 1:20. Most of the men shed virus in the stool, but in no instance was virus recovered from the oropharynx. Essentially similar findings in volunteers were reported by Smorodintsev using a live attenuated adenovirus Type 4 strain (41).

Alternate Methods of Immunization

Alternate methods of immunization with adenovirus Types 1, 3, and 7 have also been investigated (Table 4). Hitchcock and co-workers demonstrated that a live attenuated adenovirus Type 7 strain administered oropharyngeally induced high levels of homotypic neutralizing antibody in four individuals free of neutralizing antibody (19). Of four individuals receiving oropharyngeal adenovirus Type 7, three shed virus in the oropharynx. Only two out of six indi-

TABLE 3. IMMUNOGENICITY OF ENTERIC-ADMINISTERED LIVE ADENOVIRUS TYPE 4 VACCINE IN VOLUNTEERS

Strain	Cell passage*	Dose TCD_{50} (Log_{10})	Antibody status†	No. tested	Fourfold or greater antibody rise	Geometric mean titer	No. who shed virus	Ref.
CL38558	HEK(4)	6.2	<1:4	3	3	1:20	3	6
WRAIR	AGMK(16)	6.0	<1:4	3	2	1:10	3	4
CL68578	FHDC(7)	5.4–6.5	<1:4	22	18	1:18	20	4
CL68578	FHDC(11)	5.2–6.5	<1:4	18	13	1:13	17	4
Attenuated	‡	2.0–3.0	‡	12	7	‡	‡	41

*Figure in parenthesis = passage level. HEK = Human embryonic kidney; AGMK = African green (*Cercopithecus*) monkey kidney; FHDC = Fetal human diploid fibroblast cell strains.
†Neutralizing antibody.
‡No data.

TABLE 4. EXPERIMENTAL METHODS OF IMMUNIZATION OF VOLUNTEERS WITH ADENOVIRUSES TYPES 1, 3, OR 7

Serotype	Route administered	Cell passage*	Dose TCD_{50} (Log_{10})	Antibody status	No. tested	Four-fold or greater antibody rise†	Geometric mean titer	No. who shed virus	Ref.
Type 7	Oropharyngeal	PK(7 or 17)	2.7–5.7	<1:4	4	4	1:136	3	19
				>1:4	6	2	1:137	2	
Type 7	Enteric capsule	HEK(4)	5.8–6.2	<1:4	15	12	1:37	14	6
Type 7	Enteric capsule	‡	2.0–3.0	‡	10	6	‡	1	3
Type 3	Enteric capsule	‡	2.0–3.0	‡	9	5	‡	2	3
Type 1 specific antigen	Intramuscular	HEK(4)	CF titer 1:128	<1:4	6	6	1:20	—	24
Type 1 group antigen	Intramuscular	HEK(4)	CF titer 1:64	<1:4	3	2	1:5	—	24

*Figure in parenthesis = passage level; HEK = Human embryonic kidney; PK = Pig kidney.
†Neutralizing antibody.
‡No data.
CF = Complement fixing.

viduals with preinoculation antibody developed fourfold or greater antibody responses. The geometric mean antibody titers were comparable for the two groups, about 1:137.

Adenovirus Types 3 and 7 are also antigenic when administered in enteric capsules (6, 41). Twelve out of fifteen volunteers administered adenovirus Type 7 vaccine developed fourfold or greater antibody responses. The geometric mean neutralizing antibody titer was 1:37. These data suggest that enteric immunization might also be employed for adenovirus Type 3 or 7.

Kasel and co-workers investigated the feasibility of immunizing with purified adenovirus antigens, as an alternative to employing live or inactivated virions (24). Type 1 specific antigen administered intramuscularly to six volunteers without neutralizing antibody induced moderate levels of antibody in all; the geometric mean titer of antibody was 1:20. The Type 1 group antigen produced much lower levels of neutralizing antibody in two out of three individuals. These findings suggest that immunization with type-specific antigens constitutes an alternative method of immunization for adenovirus. Additional studies are required to explore this approach fully.

Field Trials with Live Adenovirus Type 4 Vaccine

From the results of volunteer studies, enteric administration of live adenovirus Type 4 appeared to provide a method for safe and successful control of acute respiratory tract disease due to this virus. In 1964 limited studies among military recruits were carried out by Edmondson and co-workers at Camp Lejeune, North Carolina (9), and in 1965 a second pilot study was conducted among naval recruits at Great Lakes, Illinois (33). The results of these studies are summarized in Table 5.

Over-all enteric immunization with adenovirus Type 4 proved highly effective; protection rates were high, and few if any secondary infections occurred in placebo-treated individuals as a result of spread from fecal contamination. At Camp Lejeune, North Carolina, febrile respiratory disease due to adenovirus Type 4 was reduced 97.6 per cent in the vaccine group compared to the placebo group (9). Afebrile respiratory tract disease due to adenovirus Type 4 was reduced 70 per cent.

Homotypic neutralizing antibody developed in all recruits free of neutralizing antibody who were fed adenovirus Type 4. The geometric mean neutralizing antibody titer was 1:37. Antibody developed as early as the tenth day after

TABLE 5. EFFICACY OF ENTERIC ADMINISTERED LIVE ADENOVIRUS TYPE 4 VACCINE

Location	Time	Testing method	Antibody status	No. of men receiving		Percentage reduction in: Febrile respiratory disease		Adenovirus 4 pos. disease		Ref.
				Vaccine	Placebo	Hosp.	Outpatient	Febrile	Afebrile	
Camp Lejeune, N.C.	1964	Isol.	<1:4	89	90	*	*	97.6	70.0	9
			>1:4	36	38	*	*	—	15.3	
Great Lakes, Ill.	1965	—	All	386	386	43	11	*	*	33

*No data.

feeding of the virus capsule. None of the recruits in the placebo group developed neutralizing antibody or shed virus; secondary infection was not detected in antibody-free recruits fed placebo capsules.

In the Great Lakes study, hospitalization for acute respiratory disease was reduced 43 per cent in the vaccine group and outpatient febrile respiratory tract disease was reduced 11 per cent (33). It is believed that the reduction in acute respiratory disease due to adenoviruses specifically was much higher.

Future Prospects

Initial field trials of live adenovirus Type 4 vaccine suggest that this vaccine may answer the need for successful control of one type of adenovirus infection among military recruits and other semiclosed populations. More extensive field trials are obviously needed and are under way in several recruit camps. It is important to ascertain the effectiveness of the vaccine when administered to large populations, the likelihood of fecal-oral spread, the occurrence of possible shifts in the ecologic pattern of adenovirus infections in military recruits, and the impact on the occurrence of respiratory tract disease due to other viruses. Since volunteer studies indicate the feasibility of enteric immunization using monovalent like adenovirus Types 3 and 7 vaccines, enteric-capsule vaccines should also be developed for these types. Perhaps polyvalent live adenovirus vaccines will eventually be used for the rapid, safe, and effective control of adenovirus infections.

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SECTION C. ADENOVIRUSES

THE PROBLEM OF ONCOGENICITY OF ADENOVIRUSES *

ROBERT J. HUEBNER

Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health, Bethesda, Maryland, U.S.A.

DR. HUEBNER (*presenting the paper*): Eight of 31 human, and 6 of 18, simian adenoviruses have been shown to cause tumors in hamsters (8, 14, 19); strains of avian, bovine, and canine adenoviruses have caused similar tumors (3, 29, 30). Hamster and rat embryo cells have been transformed *in vitro* by adenovirus Type 12 (21, 22, 24), and Type 12 has also produced tumors in rats and mice (18, 20).

Representative strains of the human serotypes have been on test in newborn hamsters for sufficiently long periods (300 to 400 or more days) to permit a tentative grouping of most of them into three categories according to their oncogenic potentials: a highly oncogenic group, A, a moderately oncogenic group, B, and a nononcogenic group, C (6, 9, 14, 23) (see footnote to Table 2).

Antigenic Determinants of Adenovirus Oncogenesis

The hamster and rat tumors induced by the A and B groups of adenoviruses were found to be completely devoid of infectious virus; however, the viral specificity of the tumors induced by each of the eight oncogenic human adenoviruses was confirmed by the demonstration of virus-specific complement-fixing (CF) and fluorescent antibody (FA) antigens in the tumor cells (18, 24). The properties of these newly recognized antigens are given in Table 1. The sera of the tumored hamsters contained antibodies not only to antigens in the tumors (8, 18) but also to the very similar T antigens produced by adenoviruses in infected tissue cultures (13, 18) (Tables 2 and 3). The T antigens were particularly interesting because they represented a newly recognized group of virus-in-

TABLE 1. PROPERTIES OF VIRUS-SPECIFIC BUT NONVIRION TUMOR CF ANTIGENS IN HAMSTER AND OTHER MAMMALIAN TUMOR CELLS INDUCED BY ADENOVIRUSES

1. Persist indefinitely in cancer cells propagated *in vivo* (by tumor transplants) or *in vitro* (tissue cultures), despite absence of infectious virus.
2. Are not part of the virion and are distinct from the "C" subunit of adenoviruses found in some adenovirus tumors.
3. Are soluble, i.e., are not sedimented at gravitational forces that sediment virus particles.
4. Are in part resistant and in part susceptible to ether, and are reduced in titer by genetron. Some are heat labile (56° to 60°C for 30 minutes); others are stable.
5. Are present only in cancer cells; are absent from adjacent normal tissue.
6. Serum antibodies progressively increase in titer in relation to size of tumor and time of exposure to tumor.
7. Surgical removal of tumor results in marked decrease in serum antibody titers, which rebound to high levels if and when tumor recurs.
8. Virus neutralizing antibodies are generally not induced, except when tumor cells also contain virion structural subunits (e.g., C antigen of adenovirus).
9. Are specific for inducing virus when present in cells of different hosts. However, there is sharing of antigens between members of at least two oncogenic groups of adenoviruses (groups A and B).

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TABLE 2. ANTIBODY TITERS OF SERA FROM TUMORED HAMSTERS VS. 4-8 UNITS OF ADENOVIRUS-INDUCED AND FSA_3 TUMOR ANTIGENS

Tumor antigens	Serum titers vs. designated antigens								
	Adenovirus serotypes								
	12	18	31	3	7	14	16	21	FSA_3
Group A*									
Adeno 12.....	160	40	80	<20	<20	<20	<20	<20	<20
Adeno 18.....	160	40	80	<20	<20	<20	<20	<20	<20
Adeno 31.....	80	20	40	<20	<20	<20	<20	<20	<20
Group B									
Adeno 3.....	<20	<20	<20	80	80	40	†	40	20
Adeno 7	<20	<20	<20	40	160	40	40	160	<20
Adeno 14.....	<20	<20	<20	20	80	20	<20	40	<20
Adeno 21.....	<20	<20	<20	20	160	<20	40	160	<20
Isoantigen									
FSA_3.....	<20	<20	<20	<20	<20	<20	<20	<20	40–80

*Forty to 100 per cent of newborn hamsters given maximum infectious titers of Group A viruses developed tumors within 100 days. Group B viruses, on the other hand, almost never produced tumors in less than 100 days; maximum virus titers induced tumors between 100 and 200 days in 5–50 per cent of the newborn hamsters injected (6).
†Not tested.

duced antigens—they were non-virion in nature yet virus-specific (7, 13, 24). In infected cells the T antigens appeared within a few hours after inoculation with high multiplicities of input virus, reaching peak titers prior to the assembly of new infectious particles. Like the tumor antigens (Table 1), the T antigens were soluble; that is, they were not sedimentable with the virus particles (18). Sera of hamsters carrying Types 12, 18, and 31 tumors contained antibodies to heat-stable and heat-labile T antigens (6, 13, 14, 18), whereas sera from hamsters carrying Types 3 and 7 tumors reacted only with a heat-labile antigen (15); the heat-labile

TABLE 3. ANTIBODY TITERS OF SERA FROM TUMORED HAMSTERS VS. 4-8 UNITS OF TISSUE-CULTURE-GROWN ADENOVIRUS T ANTIGENS

T antigens	Serum titers vs. designated antigens							
	Adenovirus serotypes							
	12	18	31	3	7	14	16	21
Group A								
Adeno 12.....	>80	>80	>80	<20	<20	<20	<20	<20
Adeno 18.....	20	>80	>80	<20	<20	<20	<20	<20
Adeno 31.....	20	80	>80	<20	<20	<20	<20	<20
Group B								
Adeno 3.....	<20	<20	<20	40	>80	80	>80	80
Adeno 7.....	<20	<20	<20	80	>80	>80	>80	>80
Adeno 14.....	<10	<10	<10	10	10	<10	10	10
Adeno 16.....	<20	<20	<20	80	40	<20	80	40
Adeno 21.....	<20	<20	<20	20	80	<20	>80	20

adenovirus tumor and T antigens resembled those described for SV_{40} (2) and polyoma (10) viruses.

The tumor and T antigens of the oncogenic adenoviruses fell into two serologically distinct categories (A and B) corresponding to the oncogenic potentials of the eight recognized oncogenic adenoviruses (14) (Tables 3, 4, and 5). Thus, the highly oncogenic adenoviruses, Types 12, 18, and 31, shared the same complement-fixing T antigens, while the moderately oncogenic serotypes—3, 7, 14, 16, and 21—shared another quite distinct T antigen. Two adenoviruses, Types 4 and 11, which shared T antigens with several representatives of the B group, have not produced tumors in hamsters, rats, or mice, despite extensive tests (6). As is shown in Table 4, the remaining 21 serotypes,

TABLE 4. REACTIONS OF T ANTIGEN CELL PACK PREPARATIONS FROM 31 HUMAN ADENOVIRUSES GROWN IN KB OR HEK CELLS

Oncogenic adenovirus group	Adenovirus		Human anti-virion sera	A group			B group				
	Serotype of			Tumored hamster sera (4 units of antibody with homologous T antigen)							
	T antigen*	Strain		12	18	31	3	7	14	16	21
	12	14510	>128	128	8	32	0	0	0	0	0
A	18	D.C.	>128	>128	>128	8	0	0	0	0	0
	31	1781/62	>128	>128	>128	32	0	0	0	0	0
	3	G.B.	>128	0	0	0	>32†	32	16	8	8
	7	Gomen	>128	0	0	0	32	32	8	8	16
B	14	DeWit	>128	0	0	0	32	16		0	
	16	Ch. 79	>128	0	0	0	>32	32	32	8	32
	21	1645	>128	0	0	0	32	16	8	0	32
	1	Ad. 71	128	0	0	0	0	0	0	0	0
	2	Ad. 6	128	0	0	0	0	0	0	0	0
	4	38558	>128	0	0	0	8	8	0		8
	5	Ad. 75	128	0	0	0	0	0	0	0	0
	6	Ton. 99	>128	0	0	0	0	0	0	0	0
	8	Trim	>128	0	0	0	0	0	0	0	0
	9	Hicks	>128	0	0	0	0	0	0	0	0
	10	JJ	>128	0	0	0	0	0	0	0	0
Nononcogenic	11	Slobitski	>128	0	0	0	32	8	0	0	0
C	13	A.A.	>128	0	0	0	0	0	0	0	0
	15	Ch. 38	>128	0	0	0	0	0	0	0	0
	17	Ch. 22	>128	0	0	0	0	0	0	0	0
	19	3911	>128	0	0	0	0	0	0	0	0
	20	931	>128	0	0	0	0	0	0	0	0
	22	2711	>128	0	0	0	0	0	0	0	0
	23	2732	16	0	0	0	0	0	0	0	0
	24	3153	128	0	0	0	0	0	0	0	0
	25	BP-1	>128	0	0	0	0	0	0	0	0
	26	BP-2	>128	0	0	0	0	0	0	0	0
	27	BP-4	128	0	0	0	0	0	0	0	0
	28	BP-5	>128	0	0	0	0	0	0	0	0
	29	BP-6	64	0	0	0	0	0	0	0	0
	30	BP-7	>128	0	0	0	0	0	0	0	0

*Approximately 10 per cent packed cells to media; prepared by Dr. Klaus Schell.
†Reciprocal of dilution.
0 = No reaction at 1:8.

all negative in tests for oncogenesis in hamsters, failed to produce CF antigens which were cross-reactive with those induced by the A and B oncogenic groups. It was interesting that the A and B groups of oncogenic adenoviruses fell into Rosen's hemagglutination (HA) groups 4 and 1, respectively, whereas for the most part the nononcogenic types fell into groups 2 and 3 (Table 5).

Genetic Determinants of Adenovirus Oncogenesis

The oncogenic and antigenic determinants of the adenoviruses described above were found by Pina and Green (23) and Green (9) to correspond to consistently reproducible differences in DNA base compositions (G+C content) of the 31 purified adenoviruses (Table 5). These very interesting studies revealed that the DNA's of the three highly oncogenic adenoviruses (group A) were each characterized by relatively low base compositions of 48–49 per cent G+C, whereas the DNA's of the five moderately oncogenic serotypes had intermediate G+C contents of 50–53 per cent (23). Type 11, which shares T antigens with the B group but so far has not produced tumors in hamsters (only one strain has been tested), also fell into the intermediate G+C group (Table 2). The remaining 22 nononcogenic adenoviruses, including Type 4, were found to have significantly higher (56–60 per cent) G+C contents (9, 23). DNA-DNA homology studies have shown similar relationships and differences between the adenoviruses (9).

Recently Fujinaga and Green (4, 5, 9) reported that the polyribosome fractions of adenovirus-induced tumors and transformed cells, pulse-labeled with H^3 uridine, yielded messenger RNA (mRNA) that hybridized in highly specific fashion with the corresponding purified DNA's, and with the DNA's of adenoviruses belonging to the same oncogenic, antigenic and base composition categories (Table 5). Thus, mRNA derived from tumor cells induced by Type 12 reacted to very much higher levels with the DNA's of Types 12 and 18 than with DNA's

TABLE 5. ADENOVIRUS ONCOGENESIS IN RELATION TO DNA, mRNA AND ANTIGENIC DETERMINANTS

T antigen group	Type	Oncogenicity*	HA group†	DNA composition G+C content‡	mRNA§
A	12	High (A)	4	Low (47–49%)	A
A	18	"	4	"	A
A	31	"	4	"	A
B	3	Moderate (B)	1	Intermediate (50–53%)	B
B	7	"	1	"	B
B	14	"	1	"	B
B	16	"	1	"	B
B	21	"	1	"	B
B	11	Negative (C)	1	"	Unclassified ‖
B	4	"	3	High (56–60%)	"
Unclassified ¶	1,2,5,6	"	3	"	"
"	8–10, 13, 15, 17, 19, 22–24, 26, 27, 29, 30	"	2	"	"
"	20, 25, 28	"	1‡	"	"

*Based on tests in newborn hamsters observed for 400 or more days, except for Types 8, 9, 10, and 27, which have been on test between 250 and 350 days (6).
†Hemagglutinin groupings (26).
‡Guanine + cytosine content (23).
§Messenger RNA isolated from ribosomal fraction of hamster tumors induced by the respective viruses grouped according to hybridization with purified DNA of various adenoviruses (4, 5).
‖ Messenger RNA's from tumors induced by Types 12 (mRNA group A) and by Type 7 (group B) were found nonreactive with DNA's of Types 2 and 4 (4, 5).
¶Not in T antigen group A or B.

of the nononcogenic Types 2 and 4, or of the intermediately oncogenic Types 7, 16, or 21. On the other hand, the mRNA of tumor cells induced by Types 3, 7, 14, and 16 hybridized with the DNA's of the moderately oncogenic B group (Types 3, 7, 14, 16, and 21), but not with the DNA's of Types 12 and 18 or with DNA's of Types 2 and 4 (Table 5).

Significance for Studies of Human Cancer

Despite the fact that both adenovirus infections and cancer are highly prevalent, adenoviruses have so far not been shown to cause cancer in man; but the evidence against such a possibility is equally deficient.

The current evidence, however, is clearly in favor of the view that adenovirus oncogenesis is a highly specific virus-directed phenomenon. Thus the genes responsible for neoplastic alterations have been found to be linked with certain antigenic and biochemical determinants that are found in some but not all representatives of the adenoviruses. Furthermore, these uniquely specific antigenic and molecular fingerprints suggest at least two promising approaches for assessing the possible role of adenoviruses in human cancer.

One of these is a systematic search in human tumors for T antigens specific for adenoviruses and also for antibodies to T antigens in the sera of cancer patients. Fortunately, acute adenovirus infections rarely if ever induce antibodies to the nonvirion T antigens. A research program designed to explore this approach is now being sponsored by the National Cancer Institute and is also supported in part by the Collaborative Research Program of the National Institute of Allergy and Infectious Diseases.

Another technique for detecting manifestations of specific adenovirus genes in virus-free tumor cells is that described by Fujinaga and Green cited above (4, 5, 9). They propose to examine fresh human cancer cells pulse-labeled with radioactive RNA precursors for the presence of adenovirus-specific mRNA molecules. This can be detected by testing for DNA-RNA complexes using the purified DNA's of the 31 human adenoviruses. The same procedure could presumably be used to test mRNA reactivities with purified DNA's of other DNA viruses as well. This procedure has the advantage of not requiring the numerous specific serological reagents needed in the search for the T antigens and antibodies.

Viral Capsids and Outer Envelopes as Determinants of Oncogenesis

Recently my associates and I (16, 27, 28, 32) and others (25) reported the incorporation of genes responsible for the neoplastic and antigenic activities of SV_{40} virus within various adenovirus infectious particles including those of strains used for vaccines. Infection of monkey kidney cells with these "hybrid" viruses depended on two-hit kinetics involving a "hybrid" adenovirus-SV_{40} particle and a "helper" nonhybrid adenovirus particle (27, 28). A potentiation effect was also noted in studies of malignant transformation *in vitro* (1). The induction of SV_{40}-like tumors and of tumor and T antigens having the characteristics of those induced by SV_{40} and by adenovirus depended entirely upon hybrid adenovirus particles, since the tumors were prevented by specific antisera to adenovirus but not by antisera to SV_{40} (16, 32). The tumors induced in hamsters by the various hybrid viruses appeared much earlier and more frequently than those induced either by SV_{40} or by the respective adenoviruses alone (16, 32). This potentiation suggested that the protein coat or capsid may be an important determinant of DNA oncogenesis (14).

A similar concept was also proposed recently for Rous sarcoma virus (RSV) by Hanafusa and Hanafusa (11), who found that the outer envelopes of the defective RSV furnished by various avian leukosis viruses constituted major determinants of RSV oncogenesis in newborn hamsters, a concept suggested earlier by Vogt for RSV oncogenesis in chickens (33).

Sarma, Vass, and Huebner (31) reported the transfer of the defective Bryan strain RSV genome from virus-free hamster tumor cells to chick embryo cells, which when transplanted into chickens produced virus-free tumors that served as rich sources of nonproducer (NP) sarcoma cells. These NP cells readily yielded infectious pseudotype RSV's when avian leukosis viruses were added to them (29, 31). Recently my associates and I (12, 17) found a similar relationship between the defective Moloney (murine) sarcoma virus (MSV) and

various murine leukemia viruses. By using virus-free but genome-positive hamster tumor cells induced by MSV, we succeeded in producing a number of MSV pseudotype viruses by substituting other murine leukemias for the Moloney virus envelope. Thus "new" sarcoma viruses with the immunologic characteristics of Rauscher, Friend and Gross leukemias (17) were produced.

In all instances the sarcomas produced by the adenovirus-SV_{40} hybrids and the sarcoma-leukemia complexes were specific for the respective genomes. Thus the adenovirus-SV_{40} hybrid produced tumors with the antigenic and cytologic properties characteristic of both viruses. The pseudotype RSV's and MSV's induced tumors with the characteristics of the original sarcoma genomes. The outer capsids and envelopes, however, played an important role in oncogenesis: they appeared to determine the relative efficiency with which the sarcoma genomes were able to gain entry to susceptible cells. These observations may be quite relevant in relation to naturally occurring cancer and leukemia.

Oncogenic Viruses in Relation to Vaccines

The problem of the oncogenic adenoviruses in relation to vaccines cannot be considered altogether separately from other oncogenic viruses known or suspected to occur in viral vaccines, or from the general question of their possible role in human cancer. There are several reasons for this. First of all, there are three distinct categories of viruses causing neoplasms in animals that have been found in viral vaccines produced in the tissues and/or cells of chick embryos, mice, and monkeys. These viruses are (1) the avian leukosis viruses (ALV), which are members of the myxovirus family; (2) SV_{40} and polyoma viruses, members of the papovavirus group; and (3) various simian and human adenoviruses. Secondly, various recombinants and hybrids have been reported as occurring between certain of these viruses. Thirdly, additional oncogenic members of these categories of virus are known or suspected to occur in the tissues of additional animal species now being used for vaccine development and production, particularly the canine and the bovine species. Finally, should any of these viruses that are oncogenic in animals be found to cause cancer in man, our present efforts to eliminate them from the current vaccines might well be replaced by even more determined efforts to include them.

Perhaps the most significant recent developments in research on cancer viruses and molecular biology are the very recent findings that demonstrate beyond any reasonable doubt that viral-induced neoplasia is actually a highly specific "virus-directed" phenomenon. Unlike the oncogenesis induced by physical and chemical oncogens (which must be mediated by the cell genome), viral oncogenesis is directed by new genes introduced into the cell by the RNA or DNA genomes of the infecting virus. The entire viral genome may be incorporated and become fully operative within the cell, as occurs frequently with the RNA leukemia and sarcoma viruses of avian and murine species; more often, however, as exemplified by the oncogenic DNA viruses and frequently also by RNA viruses, only part of the total viral genetic information is transcribed in the cell. Since oncogenic transformation of a cell can only occur if the cytolytic activity of these viruses is suppressed, the transcription of only part of the viral genome would favor survival of the cell so that transformation could occur. It is interesting that the avian leukosis and murine leukemia viruses, which often do transmit their entire genome, produce little or no cytolytic activity (as do other myxoviruses) even when virus is produced at high levels in normal or transformed cells.

It is precisely the covert, yet highly specific, behavior of the oncogenic viruses that accounted for their undetected presence in various viral vaccines in the past and now serves as a source of concern for viral vaccines in the process of development. Techniques must be developed for unmasking possible covert leukemia viruses in the tissues and cells of various animal species now being used for the preparation of the newer viral vaccines. Since the RNA leukemia and sarcoma viruses are capable of inducing neoplastic effects in heterologous mammalian species, particularly when given parenterally, and since many of the newer viral vaccines are designed for parenteral injection of children, the need for such techniques is urgent.

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SECTION C. ADENOVIRUSES

DISCUSSION

CHAIRMAN ANDREWES: Thank you very much, Dr. Huebner.

Let us proceed to a discussion of these last two papers. The first discussant is Dr. Robert M. Chanock, Chief of the Respiratory Virus Unit, Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

Dr. Chanock presented a summary of a paper entitled "Immunization against Type 4 Adenovirus by Selective Enteric Infection," which appears in full as an annex to this section on pages 85–93.

CHAIRMAN ANDREWES: The next speaker is Dr. Julius A. Kasel, Medical Virology Section, Laboratory of Clinical Investigations, National Institutes of Health, Bethesda, Maryland.

DR. KASEL: In humans, the degree of neutralizing antibody response elicited by soluble adenoviral antigens is comparable in titer to that following experimental and natural infections. The immunologic nature of serum antibody was studied (a) by comparing the classes of immunoglobulins associated with neutralizing activity after experimental infection or vaccination, and (b) by the ability to protect against virus challenge. These studies were conducted in collaboration with Dr. James R. Lehrich, Dr. Robert H. Waldman, and Dr. John J. Mann.

In Table 1 are shown representative examples of the sequence of appearance of neutralizing antibody in immunoglobulin pools from adult volunteers who received adenovirus Type 4 preparations. These individuals were either inoculated conjunctivally with infectious Type 4 virus or vaccinated intramuscularly with a preparation containing the fiber and hexon soluble antigens. The sera were collected at the indicated intervals and fractionated by gel filtration using Sephadex G–200. After fractionation of each specimen, the fractions were combined to form three pools, containing primarily IgM, IgA, or IgG, respectively. As is shown in the table, in the subject given infectious virus, antibody activity appeared within three weeks in all three immunoglobulin

TABLE 1. ADENOVIRUS TYPE 4 NEUTRALIZING ANTIBODY IN IMMUNOGLOBULIN POOLS

Inoculum	Immunoglobulin	Weeks post-inoculation							
		0	1	2	3	4	10	14	35
Infectious virus	Unfract. serum	0*	0	8	128			64	
(Conjunctival)	IgM	0	0	0	32			8	
(Vol. R. L.)	IgA	0	0	0	4			8	
	IgG	0	0	0	4			32	
Vaccine	Unfract. serum	0	128	1024	512	512	256		64
(Fiber & Hexon antigens)	IgM	0	2	16	<8	<8	0		0
(Vol. L. C.)	IgA	0	32	256	256	128	64		16
	IgG	0	8	128	128	64	64		32

*Titers are expressed as reciprocals of sample dilutions; 0 represents no neutralizing activity in undiluted samples.

pools and persisted for at least 14 weeks. In the vaccinated volunteer, antibody was found in each pool one week after inoculation and persisted for two weeks in the IgM pool and as long as 35 weeks in the IgA and IgG pools.

IgA pools from vaccinated volunteers were absorbed with antiserum to IgG. The results of absorption studies suggest that IgA neutralizing antibody may be formed in response to vaccination with Type 4 soluble antigens. This IgA antibody was present in addition to IgM and IgG antibodies.

The protective effect of vaccination was studied in a virus challenge experiment. The results of a preliminary study are shown in Table 2. Individuals without demonstrable antibody and those who had antibody that had been induced either naturally or by vaccine were given Type 4 virus by a small-particle aerosol; the vaccine group had been immunized six weeks prior to challenge. After the virus challenge, febrile responses and virus shedding were markedly less in the eight men with antibody prior to inoculation than in the four control subjects; the serologic response was similar in all groups. Although the number of subjects was small in the two groups with antibody, it appears that the responses in each group were quite similar.

Since the available data suggest that soluble antigens of adenovirus provide effective immunization against disease, this form of vaccination should be further explored.

CHAIRMAN ANDREWES: Thank you, Dr. Kasel. The next speaker is Dr. John J. Trentin, Head of the Division of Experimental Biology, the Baylor University College of Medicine, Houston, Texas.

DR. TRENTIN: In order to determine the possible adenoviral etiology of human tumors, we have tested approximately 150 human tumors against adenovirus Type 12 tumor-bearing hamster sera containing antibodies against the induced CF tumor antigens.* A significant percentage of the human tumors reacted to such sera but not to normal hamster sera. However, when the adeno-12 hamster tumor antisera were pretested to eliminate those that also reacted against the hamster Fortner Sarcoma antigen, which Sabin has shown to cross-react with some human tissues, the human tumors now failed to react with the adeno-12 tumor antisera.

We believe that these data neither prove nor disprove that adenoviruses play a role in human oncogenesis. It is possible that human adenoviruses are more oncogenic in laboratory animals than in man. Except for one report of *in vitro* transformation of human cells by human adenovirus Type 12,† all adenovirus oncogenesis and *in vitro* transformation that has been demonstrated to date has involved the adenoviruses of one species and the cells of another species, with little or no cytopathic effect and incomplete virus replication. Conversely, the simian, avian, bovine, and canine adenoviruses, some of each of which are oncogenic in hamster cells, are perhaps more likely than human adenoviruses to give little or no cytopathic effect, incomplete virus replication, and oncogenesis in human cells. On the chance that adenoviruses, whether

* McCormick, K. J., Van Hoosier, G. L., Jr., and Trentin, J. J. Attempts to Find Human Adenovirus Type 12 Tumor Antigens in Human Tumors. (In preparation.)

† Sultanian, I. V., and Freeman, G. "Enhanced Growth of Human Embryonic Cells Infected with Adenovirus 12." *Science* **154**: 665–667, 1966.

TABLE 2. INFECTION RESPONSES OF ADULT MALE VOLUNTEERS FOLLOWING AEROSOL INOCULATION WITH ADENOVIRUS TYPE 4

No. of volunteers	Pre-challenge antibody status		No. of men with febrile illness (≥37.6° C)	Per cent of positive specimens		No. of men with post-inoc. Ab titer (≥4-fold increase)
	Origin	Titer (geo. mean)		Throat	Rectum	
4	None	<2*	4	18	55	4
5	Naturally induced	10.5	0	0	3	4
3	Vaccine induced	10.0	0	3	10	3

*Reciprocal of serum dilution.

of man or of animals, may be oncogenic in man, we have studied adenovirus tumor immunization.

Immunity to *transplantation* of isologous tumors induced by adenovirus Type 12 may be produced in adult mice by live adenovirus Type 12;* by irradiated isologous adeno-12 mouse tumor cells; by nonirradiated heterologous adeno-12 hamster tumor cells; and, to a lesser extent, by nonirradiated heterologous adeno-18 hamster tumor cells.

Such tumor transplantation immunity occurs in the absence of CF antibodies against the induced CF tumor antigens. CF antibodies do appear subsequent to tumor growth in animals that were not tumor-immune. We have been able to passively transfer such tumor transplantation immunity by spleen cells but not by serum of immunized hamsters.†

Perhaps most important, we have been able to passively immunize newborn hamsters against *induction* of tumors by adenovirus 12—by simultaneous injection of rabbit anti adeno-12 serum or by prior immunization of the prospective mother with either live or killed adenovirus Type 12 vaccines. Such passive protection of the newborn against tumor induction extended to second and third litters born well over 200 days after the last maternal immunization.‡ This latter system, we believe, constitutes an excellent model for application to humans for the prevention of tumors that might be induced by adenoviruses in infants—if indeed any are, which remains to be determined.

CHAIRMAN ANDREWES: Thank you, Dr. Trentin. The next discussant will be Dr. H. G. Pereira, Head of the Virus Division, National Institute for Medical Research, Mill Hill, London.

DR. PEREIRA: I will limit myself to a few remarks on a point already mentioned by Drs. Mufson and Kasel: the possible use of purified soluble antigens in immunization against adenoviruses. Cells infected with adenoviruses produce large amounts of these antigens that can be purified by relatively simple methods. The scaling up of these procedures to an industrial level should not present insuperable difficulties. Chemically pure antigens free of detectable host materials and of nucleic acids can be obtained. These antigens are very stable and maintain their immunogenic properties throughout the purification procedures. Their capacity to provoke neutralizing antibody responses in human subjects has been demonstrated by several workers. The exact nature of the most important antigen or antigens in this respect has not been established, but work along these lines is in progress.

The use of chemically pure adenovirus vaccines would overcome the problem of oncogenicity, and even preparations derived from human cell lines such as HeLa or KB might prove acceptable.

CHAIRMAN ANDREWES: Thank you, Dr. Pereira. Our last discussant is Dr. A. A. Selivanov, Department of Virology, Institute of Experimental Medicine, Leningrad, USSR.

DR. SELIVANOV: I should like to report on "Studies on Live Adenoviral Vaccines," coauthored by Prof. A. A. Smorodintsev and myself. Monovalent or combined live attenuated adenovirus Types 3, 4, and 7 vaccines inoculated by nasal spray to the upper respiratory tract of susceptible human volunteers were found to stimulate excellent serologic response followed, with some vaccines, by the development of early toxic febrile reactions and weak to moderately severe respiratory illnesses, including sore-throat symptoms. The clinical reaction rate was progressively higher in children of preschool age. Work with respiratory application of vaccine was discontinued as soon as it had been established that the more attenuated, safer adenovirus vaccines from low-temperature strains and strains adapted to chick-embryo cells cannot induce regular antibody production. Among semi-isolated groups of vaccinees having no contact at all with natural adenovirus infections, the postvaccinal humoral immunity tended to disappear gradually during the two or three years

* Trentin, J. J., and Bryan, E. "Virus-Induced Transplantation Immunity to Human Adenovirus Type 12 Tumors of the Hamster and Mouse." *Proc Soc Exp Biol Med* **121**:1216–1219, 1966.

† McDougall, P. T., Van Hoosier, G. L., Jr., and Trentin, J. J. Interactions between Sensitized Lymphoid Cells and Adeno-12-Induced Tumor Cells. (In preparation.)

‡ Trentin, J. J. *Immunization against Tumors Induced by Human Adenovirus. Viruses Inducing Cancer.* W. J. Burdette, Editor. Salt Lake City, Utah: University of Utah Press, pp. 203–208, 1966.

TABLE 1. VIRUS NEUTRALIZING ANTIBODY TITER IN TWO GROUPS OF VACCINATED VOLUNTEERS (TYPE 4)

Description of group	No. in group	Average titer			No. of persons having VN antibodies in a titer 1:10 and lower at days after vaccination		
		Before vaccination	60 days after	840 days after	Before	60 days	840 days
Volunteers isolated, no contact with adenovirus infections.....	40	1:8	1:58	1 10.3	40	0	34
Volunteers in community, contact with adenovirus infections.....	43	1:7.5	1:60	1:22	43	0	7

following respiratory immunization. Where contact occurs, the serological response was maintained at a moderate level for two years and more after immunization (Table 1).

Since 1960 the oral route has been accepted—live Types 3, 4, and 7 adenovirus vaccines enclosed in enteric-coated dragée-candy—as an effective and convenient method of avoiding contamination of the upper respiratory tract (Table 2). Attenuated viruses grown in guinea pig kidneys or in cultures of chick-embryo cells were used for the preparation of the vaccine. No clinical illness was provoked by the oral-enteric immunization after three inoculations. The attenuated viruses multiplied intensively in the intestinal tract and were excreted in the stools without infecting the upper respiratory tract or conjunctiva or being transmitted to contacts.

Small children who cannot swallow the enteric-coated bonbon received the same polyvalent vaccine in the form of a liquid containing original nonheated bovine serum of high inhibitory

TABLE 2. EFFECT OF TYPE 4 ADENOVIRUS VACCINE ADMINISTERED PERORALLY IN DIFFERENT FORMS

Description of vaccine form	No. of persons vaccinated	No. of persons with fourfold or greater antibody increase	Average antibody titer after vaccination	No. of persons showing reaction
Enteric-coated capsule.....	115	80	1:44	0
Enteric-coated bonbon.....	260	205	1:45.5	0
Fluid with inhibitors.....	25	15	1:40	2*

*Slight catarrhal symptoms.

potency against adenoviruses. In such a mixture, prepared 30 minutes before vaccination, the adenoviruses are biologically neutralized and cannot infect the respiratory tract in the process of peroral vaccination. However, in the intestinal tract the serum inhibitors are destroyed by enzymes and the viruses are reactivated to the original level.

In most of the adults and children who were given three doses of monovalent or trivalent vaccine, satisfactory antibody responses—similar to those previously resulting from respiratory vaccination—were obtained. The persons thus immunized were well protected against experimental vaccinal infection and also against natural infection by homologous types of adenoviruses.

Our present Types 3, 4, and 7 vaccine strains were attenuated during prolonged passage through the primary monolayers of guinea pig kidney at low temperature. As preliminary analysis has demonstrated, none of these strains, including Type 7, induced tumor formation in newborn Syrian hamsters.

We may therefore consider our live adenovirus vaccine administered by oral-enteric means to be safe, effective, and very convenient for mass application.

ANNEX TO SECTION C. ADENOVIRUSES

IMMUNIZATION AGAINST TYPE 4 ADENOVIRUS BY SELECTIVE ENTERIC INFECTION

ROBERT M. CHANOCK, R. R. GUTEKUNST, W. LUDWIG, W. P. EDMONDSON, B. F. GUNDELFINGER, AND J. A. KASEL *

DR. CHANOCK *(presenting the paper)*: Adenovirus infection produces its most dramatic impact in semiclosed military recruit populations, where Type 4, and to a lesser extent Types 3, 7, 14, and 21 viruses are common causes of epidemic respiratory tract disease (29). Less dramatic, but probably more important in terms of total morbidity, is the role of adenovirus infection in acute respiratory tract disease of infancy and childhood. In this age group Types 1, 2, 3, 5, and 7 viruses are those most often associated with illness (29, 30).

Although effective vaccines are needed for the eight adenoviruses that are significant causes of respiratory tract disease—Types 1, 2, 3, 4, 5, 7, 14, and 21—we have restricted our efforts to the development and evaluation of a Type 4 virus vaccine. This course was chosen because Type 4 virus epidemics in military-recruit populations offer the ideal setting in which to evaluate vaccine efficacy. In such populations Type 4 virus is often the major cause of respiratory tract disease (16, 27). Thus, vaccine efficacy can be determined from the reduction of crude respiratory tract disease incidence as well as from the reduction of specific adenovirus-associated illness.

In the studies to be described it was not our intention to develop a comprehensive adenovirus vaccine. Rather, we were interested in exploring a new technique, using Type 4 virus as our model, with the expectation that if the method was successful it could be applied to certain other adenovirus types.

PAST DIFFICULTIES

In the past, vaccines were prepared from adenoviruses grown in monkey kidney tissue culture and then inactivated with formalin. The first experimental inactived vaccines, which contained Types 3, 4, and 7 viruses, were extremely effective in preventing adenovirus disease (2, 13). However, subsequent production lots of similar vaccines exhibited variable potency; in some instances very little protection was conferred (27).

In addition a number of problems have arisen regarding the safety of inactivated adenovirus vaccines prepared in monkey kidney culture. First, such vaccines were commonly found to be contaminated with SV_{40} virus, an oncogenic virus of simian origin (7, 28). After the virus strains in the vaccine had been freed of this contamination by treatment with antiserum, another cause for concern became evident. Though treatment with SV_{40} antiserum was effective in eliminating SV_{40} virus, such treatment did not affect another virus contaminant—defective SV_{40} adenovirus hybrid particles in which a portion of the SV_{40} genome was coated with adenovirus capsid (15, 20, 25). Such defective hybrids constituted a definite hazard, since they possessed the oncogenic potential of SV_{40} virus. Attempts to eliminate these hybrids from

* Drs. Chanock, Ludwig, and Edmondson (Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Maryland); Drs. Gutekunst and Edmondson (Naval Medical Field Research Laboratory, Camp Lejeune, North Carolina); Dr. Edmondson (also, E.I.S. Officer, Communicable Disease Center, assigned to the Laboratory of Infectious Diseases, N.I.H.); and Dr. Kasel (Laboratory of Clinical Investigation, National Institutes of Health, Bethesda, Maryland).

adenoviruses propagated in monkey kidney culture failed because the defective particles provided a helper function for adenovirus and without them the adenovirus was unable to replicate in simian tissue culture (26).

BACKGROUND

Faced with the various difficulties just described, we considered it worthwhile to investigate an alternate method of adenovirus immunization. This method was based on the observation that human adenoviruses exhibit a marked predilection for multiplication in the lower intestinal tract (1, 23). Except for a rare infection that leads to intussusception during infancy, intestinal infection with adenovirus is not associated with obvious symptoms or signs of disease (9, 24). Since the usual disease manifestations of adenovirus infection occur in the upper alimentary and respiratory tracts we reasoned that it might be possible to produce a silent immunizing infection by bypassing these areas and directly infecting the lower intestinal tract. This was attempted by administering adenovirus in an enteric-coated capsule that did not release virus until it had passed beyond the stomach.

The initial studies were performed in volunteers who were given Type 4 or Type 7 virus or both enclosed in an enteric-coated capsule (6). These viruses were isolated and grown in human embryonic kidney tissue culture. We found that infection could be initiated by introduction of enteric-coated virus into the lower intestinal tract. Such infection remained localized to the lower intestinal tract and did not spread to the upper alimentary or upper respiratory tract. Intestinal infection was asymptomatic; however, volunteers infected via the intestinal route developed moderately high levels of neutralizing antibody. Interference was not observed when Type 4 and 7 viruses were given simultaneously to the same individual. Finally, infection did not spread from volunteers enterically infected with Type 7 virus to susceptible contacts despite prolonged close association.

After the completion of our preliminary studies, Type 7 adenovirus was found to be weakly oncogenic in newborn hamsters (10). For this reason we discontinued our work with this serotype. All subsequent studies were performed with Type 4 virus strains, which by all available test procedures appear to lack oncogenic potential in the newborn hamster (4).

TYPE 4 VIRUS VACCINE PREPARED IN HUMAN DIPLOID TISSUE CULTURE

Diploid tissue culture. The initial studies of enteric infection were performed with viruses grown in human embryonic kidney tissue culture. Since this type of tissue is not suitable for large-scale vaccine production, we sought another host system for the growth of candidate vaccine strains. The Wistar Institute human diploid fibroblasts were chosen since these cells (a) retain a normal karyotype for as many as 40 cell divisions, (b) are free of demonstrable microbial contaminants, and (c) can provide an extremely large yield of diploid cells at the twentieth to thirtieth passages from low-passage seed stock stored in the frozen state (12).

Virus strain. Strain CL 68578 was recovered by Dr. H. H. Bloom from the throat-swab specimen of a Marine recruit with acute febrile respiratory tract disease. The virus was recovered in Wistar Institute #26 (WI-26) diploid cultures and was propagated thereafter only in these cells or in Wistar Institute #38 (WI-38) cultures. Experimental lots of vaccine were prepared at Wyeth Laboratories, Radnor, Pennsylvania, from virus in its eleventh diploid culture passage (Table 1).

Safety tests. Extensive safety tests performed at Wyeth Laboratories failed to reveal the presence of adventitious microbial agents. Similarly, extensive tests for oncogenicity of the Type 4 vaccine strain in newborn hamsters were negative (Table 2). Newborn hamsters were injected with the highest concentration of crude infected tissue culture fluid or highly purified virus which did not kill them. A total of 147 injected hamsters survived 500 days and 108 animals survived 600 days. Thirty-two hamsters thymectomized at birth and then injected with $10^{7.2}$ TCD_{50} of the vaccine virus have survived 600 days without evidence of tumor formation. Only one hamster developed a tumor, and this was detected on the 468th day after injection. The tumor did not appear to be adenovirus-induced, since it developed at a site distant from the site of virus injection, its histologic appear-

TABLE 1. BIOPHYSICAL AND BIOLOGICAL PROPERTIES OF TYPE 4 ADENOVIRUS VACCINE STRAIN

Property	Type 4 vaccine strain	Other Type 4 strains	Group A adenoviruses (12, 18, 31)	Group B adenoviruses (3, 7, 14, 16, 21)	Group C adenoviruses (1, 2, 4, 5, 6, etc.)
Hemagglutination*	Group 3	Group 3	Group 4	Group 1	Group 2 or 3
Guanine-cytosine ratio of DNA†	58–59%	58–59%	48–49%	48–54%	57–60%
Oncogenicity in newborn hamsters	Neg.	Neg.	High	Low	Neg.

*Rosen's hemagglutination grouping (22).
†Data of Dr. M. Green (19).

ance was unlike that of adenovirus induced tumors, and, finally, we were unable to detect adenovirus T antigen in the tumor or its transplants; nor were we able to detect T antibody in tumored hamsters (4, 14).

Inflammatory lesions developed near the site of injection when a high concentration of Type 4 virus ($10^{7.3}$ TCD_{50}) was inoculated into the brain and spinal cord of monkeys. These lesions involved the meninges, ependyma, choroid plexus, and neurons. Demyelinization did not occur. The inflammatory lesions were produced by the virus, as is evident from the fact that they were not observed when a Type 4 virus-antiserum mixture was injected. Since the natural history of Type 4 adenovirus infection does not include central nervous system involvement, it was felt that the response of monkeys to direct central nervous system inoculation did not necessarily reflect a neurogenic potential of the vaccine virus for man. Similar inflammatory lesions had been observed previously with a Type 7 virus strain. In this instance a more detailed study of the Type 7 virus was performed, and it appeared that the virus produced its inflammatory response without undergoing multiplication (21).

Properties of vaccine strain. As has been said, the Type 4 vaccine strain lacked oncogenic potential in newborn hamsters. This strain exhibited a group 3 hemagglutination pattern, i.e., partial agglutination of rat erythrocytes (22). Its DNA contained a high proportion of guanine-cytosine—58 to 59 per cent (19). These properties indicated that the Type 4 vaccine strain was identical to other Type 4 viruses that had been studied in a similar fashion (Table 1). Furthermore, the Type 4 vaccine strain and the other Type 4 strains that had been characterized were found to belong in Huebner's group C (14). Adenoviruses in group C exhibit group 2 or group 3 hemagglutination patterns, have a high guanine-cytosine ratio in their DNA, and lack oncogenicity for newborn hamsters. Group A adenoviruses, which include the highly oncogenic types 12, 18, and 31, and group B adenoviruses, which include the weakly oncogenic Types 3, 7, 14, 16, and 21, are clearly distinct from group C adenoviruses in hemagglutination properties and DNA composition (14). Thus, the properties of the vaccine strain were characteristic of adenoviruses that lack oncogenic potential in the most sensitive host system known—the newborn hamster.

Response of volunteers to conjunctival inoculation. The behavior of the Type 4 vaccine strain during its early passages in diploid cultures suggested that a variant had been selected during growth in these cells. Initially the virus required 12 to 14 days to produce a complete cytopathic effect in diploid cultures. During subsequent passages the interval decreased to four days (4).

We were interested in determining whether the variant that grew well in diploid cultures exhibited an alteration in virulence for man. For this purpose we tested the response of volunteers to conjunctival inoculation with the vaccine virus (eleventh diploid culture passage). This route of inoculation was chosen because previous studies had shown that Type 4, Type 5, or Type 7 adenovirus induced objective signs of disease—conjunctivitis, pharyngitis, fever—in a high proportion of volunteers thus challenged (3, 5). As Table 3 shows, the six men who received $10^{8.5}$ to $10^{5.5}$ TCD_{50} of the vaccine virus developed only a mild conjunctivitis. Neither fever nor pharyngitis was noted. The conjunctivitis index scores shown in Table 3 represent low values

TABLE 2. TEST FOR ONCOGENICITY OF TYPE 4 ADENOVIRUS VACCINE STRAIN IN NEWBORN HAMSTERS

Passage of virus in tissue culture	Material tested	Inoculation		No. of hamsters surviving				No. with tumor
		Site	Titer (Log_{10})	500 days	600 days	700 days	800 days	
Human diploid fibroblast (WI 26 or 38) passage 11	Tissue culture fluid	IC	6.7 TCD_{50}*	14	14	8	1	0
		IP	6.9	16	16	10	3	0
		SQ	7.2	10	10	8	4	0
		SQ	7.2	32 (thymectomized at birth)	0.T.†			0
Diploid passage 11 KB passage 2	Purified virus§	SQ	6.2 TCD_{50}*	12	12	9	9	1 (Day 468)‡
			6.5	9	9	9	1	0
			6.9	9	9	6	6	0
			7.2	10	10	5	5	0
			6.3 PFU ‖	7	4	4	4	0
			7.0	8	4	4	4	0
			7.5	10	10	O.T.		0
			8.3	10	10	O.T.		0
Total				147	108	63	37	—

*Determined in human embryonic kidney tissue culture.
†On test.
‡No evidence of adenovirus etiology. Tumor developed at a location distant from the site of inoculation; histological appearance of tumor unlike that of adenovirus-induced tumors; extract of tumor and of transplanted tumor did not contain detectable T antigen; finally serum of tumored hamster and of hamsters bearing transplanted tumors did not contain complement fixing antibody for the T antigen of Type 4 or Type 7 adenovirus.
§Virus purified and concentrated by two equilibrium centrifugations in rubidum chloride.
‖ Determined in KB tissue culture dishes.

when compared to the response elicited by other adenovirus serotypes, such as Type 26 and 27, which are not known to cause respiratory tract illness under natural conditions (17). It would appear that the Type 4 vaccine strain had lost some of its virulence for man.

Response of volunteers to enteric administration of virus. The vaccine strain was administered in an enteric-coated capsule to 40 antibody-negative volunteers (neutralizing antibody titer of less than 1:4). Their response resembled that observed previously with the Type 4

TABLE 3. RESPONSE OF VOLUNTEERS TO CONJUNCTIVAL INOCULATION OF TYPE 4 VACCINE STRAIN

Inoculum TCD_{50}-Log_{10}	Number of men (neut. antib. <1:2)				Conjunctivitis-index of severity†	
	Inoc.	Infected*	Conjunctivitis	Temp. >37.5° C or pharyngitis	Total score‡	Maximum daily score
5.5	2	2	2	0	13, 13	2, 2
4.5	2	2	2	0	13, 11	3, 2
3.5	2	2	2	0	13, 10	2, 2

*Type 4 virus recovered from inoculated eye and from throat and/or rectum.
†Grade 1 = Palpebral edema; grade 2 = palpebral and bulbar edema; grade 3 = periorbital edema and/or preauricular adenopathy; grade 4 = edema sufficient to occlude palpebral fissure and/or severe eye pain.
‡Sum of daily scores.

and Type 7 virus strains grown in human embryonic kidney tissue. The enteric-coated Type 4 virus produced a selective infection of the lower intestinal tract that was silent and was associated with the development of moderately high levels of neutralizing antibody (4). Virus did not spread to the respiratory tract of the inoculated individuals or to susceptible cohorts in close personal contact with them. The Type 4 vaccine virus was not detected in serum during the 10-day interval that preceded the development of neutralizing antibody.

Results of field trials with Type 4 vaccine virus. Encouraged by the response of volunteers to the enteric Type 4 virus vaccine, we next evaluated this material for its protective effect against naturally occurring adenovirus disease. The first field trial was initiated in 1964 at Parris Island, South Carolina, where naturally occurring adenovirus infection was uncommon (8). The enteric vaccine was given to 134 Marine recruits, while 146 men from the same training company were fed placebo capsules. A selective intestinal infection, which was silent and was associated with the development of moderately high levels of neutralizing antibody, occurred in the recruits given Type 4 virus. No signs or symptoms referable to the central nervous system developed in any of the vaccinees. The immune response was relatively rapid; serum neutralizing antibody was first detected by the tenth day after infection. The virus recovery data and serologic findings for a random group of 52 vaccinees and 52 placebo men, who were studied in a longitudinal fashion, are shown in Table 4. Type 4 virus was not recovered from the oropharynx of vaccinees nor did infection spread to susceptible contacts who were in close association with the vaccinees for three weeks.

The failure of the Type 4 vaccine strain to spread to the oropharynx or to susceptible contacts was confirmed during a subsequent study at Parris Island and during a study at Fort Ord in 1965 (11), as is also shown in Table 4. Despite the rigors of recruit training, enterically administered Type 4 adenovirus appeared to remain localized to the lower intestinal tract. Furthermore, enteric infection with the Type 4 vaccine strain appeared to be essentially noncommunicable among recruits.

A specific protective effect of the Type 4 virus enteric vaccine was demonstrated when 253 of the recruits from the first field trial at Parris Island were transferred to Camp Lejeune, where Type 4 adenovirus infection was epidemic. The transfer took place three weeks after the men had received the vaccine and at a time when virus shedding from the intestinal tract had ceased (8). Thirty-two recruits in the placebo group were ultimately hospitalized for acute

TABLE 4. PATTERN OF SHEDDING OF TYPE 4 VACCINE VIRUS AND COMMUNICABILITY OF INFECTION

Location and time	Material given	No. of men		No. from whom virus recovered		No. developing fourfold or greater rise in neutralizing antibody
		Without detectable neut. antib. (<1:4)	Total	Throat swab	Anal swab	
Parris Island 1964	Type 4 virus (10^6TCD_{50})	37	52	0†	43†	43
	Placebo*	36	52	0†	0†	0
Parris Island 1965	Type 4 virus ($<10^1$–$10^{6.5}TCD_{50}$)	119	119	0†	84†	84
	Placebo*	24	24	0†	0†	0
Fort Ord 1965	Type 4 virus (10^6TCD_{50})	—	102	0‡	85‡	—
	Placebo*	—	100	0‡	0‡	—

*Placebo controls were in close personal contact with vaccinees in same platoon.
†Tested three times a week for four weeks (8, 11).
‡Tested once a week for four weeks (data of Chin and Lennette, unpublished studies, 1966).

TABLE 5. PROTECTIVE EFFECT OF ENTERIC TYPE 4 ADENOVIRUS VACCINE IN MILITARY RECRUITS

Location	Time	Vaccine administration		No. recruits		Hospital admissions for acute febrile respiratory disease		Adenovirus assoc. acute febrile respiratory disease		Prevalent adenovirus serotype
		Time	Distribution	Vaccine	Placebo	No. or rate in placebo group	Reduction in vaccine group	No. or rate in placebo group	Reduction in vaccine group	
Parris Island, S. C.	1964	3 wks. before adeno. epidemic	50% each platoon.	125	128	40	85%	32	100%	Type 4 (only)
	1965	"	"	339	337	—	—	55	100%	"
	1966	"	"	6,883	6,972	—	—	73	75%	"
Great Lakes, Ill.	1965	4 days after start of training, i.e., after exposure to adeno. epidemic.	50% each company.	386	386	360/1000/9wk.	46%	69/1000/9wk.	69%	Type 4
	1966	Mass immunization of men in 1st 3 wks. of training followed by vaccination on arrival.	85% of population, 10% received placebo	23,015	2,629	119/1000/9wk.	49%	101/1000/9wk.	59%	Type 4, some type 7
Fort Dix, N.J.	1965	On arrival at base	50% each company.	607	638	26.2/1000/wk.	67%	22.5/1000/wk.	96%	Type 4
	1966	"	100% one brigade, 100% placebo in other brigade	3,181	3,164	45.6/1000/wk.	47%	—	—	Type 4
				1,342	1,175	37/1000/wk.	15%	—	—	Type 7 in vaccinees, Type 4 in placebo group

febrile respiratory tract illness associated with Type 4 adenovirus, whereas none of the vaccinees had such an illness (Table 5). This finding indicated that prior enteric Type 4 virus infection was highly effective in preventing acute respiratory tract disease caused by adenovirus of this type (8).

A second field trial performed at Parris Island in 1965 yielded results identical to those of the first study. The Type 4 enteric vaccine provided complete protection against the subsequent challenge of naturally occurring adenovirus infection at Camp Lejeune (Table 5) and there was no evidence that the vaccine produced an untoward effect (11).

A third field trial was performed at Parris Island in 1966. Again there was no evidence that the vaccine produced an untoward effect. In the 1966 study, which involved 6,883 vaccinees and 6,972 controls, the enteric vaccine was estimated to have provided 75 per cent protection against adenovirus disease—somewhat less than that seen during the first two field trials. The basis for this difference is not understood. In each of the trials at Parris Island the vaccine was given three weeks prior to the transfer of the recruits to Camp Lejeune. In addition, Type 4 virus was the only adenovirus recovered during the 1966 adenovirus epidemic at Camp Lejeune.

In 1965 a field trial was performed at Great Lakes, Illinois, in which 50 per cent of the recruits in one company were given the enteric Type 4 virus vaccine four days after the start of training (18). This ecologic setting differed from that of Parris Island in that adenovirus disease was epidemic at the time the vaccine was administered. In fact, the peak of adenovirus illness occurred during the second week of training, whereas at Parris Island adenovirus infection was uncommon. Thus, at Great Lakes the vaccine was in direct competition with rapidly spreading natural adenovirus infection. Despite this competition, the vaccine produced a 46 per cent reduction in hospital admissions for acute febrile respiratory tract disease and a 69 per cent reduction in adenovirus-associated illness (Table 5).

A second field trial was carried out at Great Lakes during 1966. Initially every recruit in the first three weeks of training was given vaccine, and then all incoming recruits received vaccine except for 50 per cent of the men in every fifth company. Over-all, Dr. Peckinpaugh and his staff at Great Lakes observed that the vaccine was associated with a 56 per cent reduction in hospital admissions for acute febrile respiratory tract illness. In neither field trial at Great Lakes was an untoward effect of the vaccine observed. None of the vaccinees developed either central nervous system disease or hepatitis.

The Type 4 vaccine has also been studied at Fort Dix, where adenovirus infection occurs commonly and constitutes the major cause of acute respiratory tract disease. In the first field trial Drs. Rose and Buescher and their associates found that the vaccine was 67 per cent effective in reducing all acute respiratory tract disease requiring hospitalization and 96 per cent effective in preventing adenovirus-associated disease.

In a second field trial at Fort Dix, in 1966, all the recruits in one training brigade were given vaccine over a four-day period. Subsequently, all incoming recruits to this brigade received vaccine at the start of training. At the time of the mass immunization the recruits in another brigade were given placebos, and these were administered subsequently to all incoming recruits to this brigade. As is shown in Table 5, the vaccine provided protection against acute respiratory tract disease during the first two thirds of the study, when Type 4 virus was the prevalent adenovirus in both the immunized and placebo brigades. However, during the last third of the field trial the illness rate in the immunized brigade approached that of the placebo brigade. This dramatic change was associated with a change in the ecology of adenovirus infection in the immunized brigade: Type 7 virus replaced Type 4 virus as the prevalent adenovirus responsible for the major share of acute respiratory tract illness. Perhaps the supression of naturally occurring Type 4 virus infection had created an ecologic vacuum into which Type 7 virus expanded. The emergence of Type 7 adenovirus as the major respiratory tract pathogen after the suppression of Type 4 virus infection by the enteric Type 4 vaccine was not observed at either Parris Island/Camp Lejeune or Great Lakes, although Type 7 virus was detected intermittently at the latter location during the second field trial.

THE FUTURE OF ENTERIC ADENOVIRUS IMMUNIZATION

Thus far approximately 35,000 young adult males have received the enteric Type 4 virus vaccine with no evidence of untoward effect or indication that the vaccine was unsafe. The results of the various field trials demonstrated that the vaccine provided protection against naturally occurring Type 4 virus disease. For these reasons we feel justified in recommending that field trials of the enteric Type 4 virus vaccine in military-recruit populations be expanded.

Only one potential difficulty mars the generally hopeful prognosis for the enteric vaccine. This is the possibility that the vaccine may upset the ecologic balance of adenovirus infection in military populations so that Type 7 virus replaces Type 4 as the major respiratory tract pathogen. Such a shift in adenovirus ecology occurred in one out of three immunized populations during the 1966 field trials. If the vaccine is to be used successfully in the future it will be necessary to understand the conditions that affect a shift in adenovirus ecology and then to prevent such a shift from occurring. If this cannot be accomplished a safe, effective Type 7 virus vaccine would suffice. Currently we are evaluating various techniques for the concentration and purification of Type 7 virus capsid subunits for use in an inactivated vaccine.

The future of enteric adenovirus immunization extends beyond its application to problems of military medicine. A need exists for effective immunization against Types 1, 2, and 5 adenovirus infections during infancy and childhood. Efforts should be made to evaluate the safety and efficacy of enteric immunization with these adenoviruses, since they lack oncogenic potential in hamsters and belong to Huebner's group C. At present it would be unwise to consider the enteric route of immunization for pathogenic adenoviruses that exhibit oncogenic activity in newborn hamsters. However, it is possible that this property of Type 3 and Type 7 adenoviruses has no relevance to human oncology. If so, enteric immunization might then be used to prevent infection by these adenoviruses.

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SECTION D. RHINOVIRUSES

CLASSIFICATION OF RHINOVIRUSES *

HERBERT A. WENNER †

Hixon Memorial Laboratory, Department of Pediatrics,
University of Kansas School of Medicine, Kansas City, Kansas, U.S.A.

DR. WENNER (*presenting the paper*): Probably rhinoviruses were propagated in cultures of human embryonic lung cells during the early studies of the Common Cold Research Unit at Salisbury, England (1). With the reawakened interest in cells cultured *in vitro*, cytopathic agents were detected in human respiratory infections. Among the first of these were strains 2060 (2, 3) and JH (4), which were recovered from human beings experiencing minor respiratory infection. These viruses and their relatives were found using renal cells of monkeys. Shortly thereafter additional strains were found using human embryonic cell cultures. A major development in recognizing the presence of rhinoviruses was the use of embryonic human cells maintained in acid medium and incubated at 33°C on a roller apparatus (5). Shortly afterwards human diploid cells (6) became available; these cells maintained in a proper milieu have been remarkably adept at detecting many viruses, including those associated with the common cold.

By 1961 many cytopathic agents had been recovered from infants, children, and adults during respiratory illnesses. Some of these have been identified (e.g. the parainfluenza group, respiratory syncytial virus). Others, however, particularly those associated in adults with the common cold syndrome, have stood apart. Most rhinoviruses were recovered in cultures of human cells (the H strains), and a few in cultures of monkey kidney cells (the M strains) (5-7). Some of the "new" strains apparently require a more selective biological milieu, such as is found in organ cultures prepared from respiratory epithelium derived from human embryos (8).

BIOPHYSICAL PROPERTIES

Although extensive studies using biophysical procedures have been limited, largely because of the low infectivity titers, relative labilities, and specialized growth requirements of rhinoviruses, still a number of the biophysical properties of rhinoviruses have been defined (9–14). They are small—approximately 18–23 mμ in diameter. In several respects they resemble human enteroviruses: they are the same size and they are ether-stabile; in infected cells they form discrete clusters of spherical particles arrayed in a crystalline pattern (9); they contain ribonucleic acid cores; they display an identical symmetrical structure of the virion (11, 13). They differ from the enteroviruses with respect to primary growth characteristics in cultured cells (5), relative but selective inhibition by 2-(α-hydroxybenzyl)-benzimidazole (14), varying heat stability in the presence of magnesium ions (10), and, possibly, structure of the nucleic acid (11–13). Moreover, they differ completely from human enteroviruses in that they are acid-labile (10, 15), and relatively heat-stabile at 50°C (10); they are not known to produce neuromuscular disease, except for one report of meningo-encephalitis (16), in mice and primate

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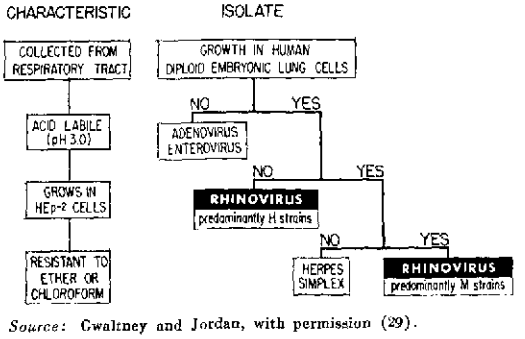
species; and their human habitat appears to be in the nose and throat and they are rarely found in the feces.

On the basis of these properties, rhinoviruses have been placed among the picornaviruses (17). They are separated from human enteroviruses (18) by reason of their relative heat stability, acid lability, and occasional cytopathogenic effect in selected cell cultures (Fig. 1). The single criterion for separating rhinoviruses from enteroviruses has been the extraordinary acid sensitivity of rhinoviruses (the acid-lability test).

SEROLOGICAL PROPERTIES

The early epidemiologic studies of the common cold indicated the existence of multiple serotypes. Six distinct serotypes were identified at the Salisbury Unit (19). Shortly thereafter Ketler *et al.* (15) described 37 rhinoviruses, representing 20 serotypes. Johnson and Rosen (20) contributed five more viruses to the growing list. In rapid succession additional reports came from the National Institutes of Health (21–23), the Merck Institute for Therapeutic Research (15, 24), and several universities—Tulane (25), Chicago (26–28), Virginia (29, 30), and Baylor (31). Investigators in the laboratories of the States of California and Wisconsin (32) also recovered rhinoviruses. By 1964 the large number of known agents having the properties of rhinoviruses (18) became a matter of concern. Advancement of knowledge toward an understanding of the common cold depended on identity of serotypes and the interrelationships between these and other rhinoviruses. The shortage, or indeed the total lack, of satisfactory type-specific animal sera has been a major obstacle to the delineation of serotypes. The Salisbury (19) and Merck (15, 24, 33) studies were unique in providing serologic evidence of numerous serotypes. By 1964, 37 proposed types had been reported—9 M strains and 28 H strains. At that time many more strains were known, and since then a great many more have accumulated. Sorting and classifying became mandatory.



Source: Gwaltney and Jordan, with permission (29).

Fig. 1. Schema for preliminary identification of rhinoviruses.

Efforts to resolve the problem of developing strain-specific antisera were made by the Vaccine Development Board and by the Research Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases (NIAID). Advisory and research personnel concerned with these agencies, collaborating with the Common Cold Research Unit in Salisbury, drew up guidelines and implemented production of antisera for rhinoviruses. Antisera for a wide variety of rhinoviruses were prepared in rabbits, guinea pigs, goats, cattle, and monkeys. As far as possible, the rhinoviruses and antisera were shared. Out of this collaborative program developed a numerical classification schema based on the serological specificities of rhinoviruses. Many people were involved in these labors; however, a large share of the definitive work leading to numerical designation of serotypes was done by Drs. Dorothy Hamre, Vincent Hamparian and A. Z. Kapikian (for others contributing strains see the legend to Table 1).

On 26 June 1966, serological data on 68 rhinoviruses were reviewed by participants in the WHO/NIAID Rhinovirus Collaborative Nomenclature Program. In the sorting, 12 strains were found to be of similar identity. Strain B632 was related but not identical to ECHO-28 and accordingly was designated a relative thereof. Finally, 56 strains were selected as prototypes. Selection of strains as prototypes was based on the sequence of published data relating to them or, if they were not yet described, the chronological order in which they were received at the Ohio State University reference laboratory. A listing of the rhinovirus prototypes with eponyms appears in Table 1. Some 50 of those in the list have been described in published papers (see legend to Table 1).

Published data on rhinoviruses are surfeit

TABLE 1. RHINOVIRUSES: STRAINS DESIGNATED AS PROTOTYPE

Name	Source laboratory	Name	Source laboratory
ECHO 28/2060	NMR #4; Tulane Univ.	NIH 11757	National Inst. Health
B632 (M)	Common Cold Research Unit*	" 33342	" " "
HGP (M)	" " " "	" 363	" " "
FEB	" " " "	" 1200	" " "
16/60	" " " "	" 209 (M)	" " "
Norman	" " " "	" 1794	" " "
Thompson	" " " "	" 56110 (M)	" " "
Coryzavirus type 11	Merck Inst. Ther. Research	" 56882	" " "
" " 12	" " " "	" 58750	" " "
" " 13	" " " "	" 71560 (M)	" " "
" " 14	" " " "	" 1505	" " "
" " 15	" " " "	" 8213	" " "
" " 16	" " " "	Chicago 137-3	University of Chicago
" " 17	" " " "	" 140-F (M)	" " "
" " 18	" " " "	" 151-1	" " "
" " 19	" " " "	" 164-A	" " "
" " 21	" " " "	" 324-H	" " "
" " 22	" " " "	" 363 (M)	" " "
" " 24	" " " "	Charlottesville 79	University of Virginia
" " 25	" " " "	Baylor 1	Baylor University
" " 26	" " " "	" 2	" "
" " 27	" " " "	" 3 (M)	" "
" " 28	" " " "	A2 #58†	Tulane "
" " 29	" " " "	FO-1-3772†	Calif. State Dept. Health
" " 30	" " " "	FO-1-3774†	" " " "
NIH 353	National Inst. Health	FO-1-3928†	" " " "
" 1059	" " "	FO-1-4081†	" " " "
" 1734	" " "	WISC 315 E†	Wisconsin " " "

Modified from data presented at the Rhinovirus Workshop held in Bethesda, Maryland on 22 June 1966. The prototypes are arrayed according to laboratory of origin, not by assigned number. The numerical schema will be reported by the rhinovirus collaborators, to whom I am indebted for the information given above.

For data pertaining to these strains see reference (2) for echovirus Type 28, (19) for Salisbury strains, (15) for coryzaviruses, (20-23), for NIH strains, (26) for Chicago strains, (29, 30) for Charlottesville strain, and (31) for Baylor strains.

NMR = Naval Medical Research Unit No. 4. (M) = M strains; others except as indicated = H strains. FO = Fort Ord.

*Common Cold Research Unit, Salisbury, England.

†No data on M or H character.

with cryptic prefixes—letter-numbers, number-letters, or simply numerical designates—but out of this chaotic situation there is emerging an orderly classification based on serospecificity. Among the existing rhinoviruses there are serotypes closely related to the chosen prototypes. Already a number of these have been recognized (Table 2). In addition, among the 30 or more rhinoviruses still being sorted by Hamparian and Conant (32) there are others that are serologically identical.

PROBLEMS OF CLASSIFICATION

The early efforts at classification were hampered by a multiplicity of cross-reactions between serotypes. In general, rhinovirus antisera prepared in goats and cattle have had consistently low levels of neutralizing activity to heterologous rhinoviruses. However, similar cross-reactivity has not been encountered so often with the antisera of guinea pigs. Some of these reactions have been described by Fenters *et al.* (34). Several cross-reactions encountered with bovine sera are listed in Table 3, which shows cross-reactions between two related rhinoviruses, 2060 and B632 (upper panel), and unilateral or bilateral crosses between six unrelated serum-virus pairs (lower panel). Although these data might suggest that the latter rhinoviruses share minor antigens, additional tests with antisera

TABLE 2. RELATION OF SOME OTHER STRAINS TO PROTOTYPE RHINOVIRUSES (BY RECIPROCAL SERUM NEUTRALIZATION)

Rhinovirus prototype	Related strain(s)	Rhinovirus prototype	Related strain(s)
B632	K779	NIH 56110	Chicago 137–F
Coryzavirus type 22	Chicago 203–F	" 56882	" 248–A
" " 24	NIH 100319		" 04394
" " 25	Chicago 147–H	" 58750	Wisconsin 258–E E2 #33
" " 26	K 2218 NIH 55216	Charlottesville 79	Chicago 201–3C
" " 27	Chicago 127–1	Baylor 1	" 037211 E2 #46
" " 29	" 113–E	" 2	Coryzavirus type 50
" " 30	" 179–E		Charlottesville 202
" " 38	Charlottesville 82	" 3	Coryzavirus type 46 Charlottesville 310
" " 39	Chicago 182–E		
" " 45	" 313–G	FO–1–4081	Coryzavirus type 45 NIH 19143 Chicago 313–G
NIH 209	" H–00052	" 1–3772	Coryzavirus type 34 NIH 16413
" 353	Coryzavirus type 23		
" 1794	Chicago 184–E	" 1–3774	Coryzavirus type 44

See also Table 1. Prefix designates K and E2 from Tulane University.
Source: Data presented by the Rhinovirus Collaborative Study Group. Most of the data have been published (23, 28).

TABLE 3. CROSS-REACTIVITY OF SEVERAL SERUM-VIRUS PAIRS (SERUM NEUTRALIZATION)

PANEL A

Between 2060 and B632

Rhinovirus		Antisera (bovine)		
Strain	Serotype	2060	JH	B632
2060	1A	3072	128	16
JH	1A	2048	192	8
B632	1B	6	12	384

PANEL B

Between some others as yet thought type-specific

Rhinovirus		Antisera (bovine)			
Strain	Serotype	FEB	33342	1059	164-A
FEB	3	1024	24	8	48
NIH 33342	17	48	1536	—	—
" 1059	14	—	16	512	96
Chic 164–A	35	*	8	16	768

*Toxic sera.
Source: Fenters, Gillum, Holper, and Marquis (34).

obtained from other animal species have not been reciprocal.

Data such as those obtained by Fenters *et al.* (34) may have several interpretations. For example, it may be that large domestic animals, even those without prior immunologic experience, develop heterotypic antibodies more readily than guinea pigs, rabbits, or monkeys. Conceivably these animals may have had prior stimulation through infection with rhinovirus serotypes. Although there are few published findings to support such a concept, attention is directed to that fact that the inoculation of heterologous viruses into cattle has occasionally resulted in striking antibody response to serologically unrelated rhinoviruses to which the animal had been previously exposed (34). Such neutralizing activity was not found in preinoculation sera. Another interpretation relates to the development of nonspecific viral inhibitors, a phenomenon we have occasionally encountered in monkeys and rabbits inoculated with human and simian enteroviruses. Hamparian and Conant (32) considered that nonspecific inhibitors might account for these reactions. Sera giving nonspecific responses were treated by dialysis or by repeated absorptions with concentrated HeLa cell suspensions or human liver powder. The nonspecific serum inhibitors were removed almost totally by liver powder, partially by HeLa cells, and hardly at all by dialysis. Specific neutralizing antibodies were left intact after absorption (Table 4). The tentative conclusion with respect to bovine antisera is that the cross-reactive nonspecific inhibitors are protein in nature—possibly globulins—engendered to some antigen other than the rhinovirus virion.

TABLE 4. ABSORPTION OF NONSPECIFIC INHIBITORS FROM RHINOVIRUS ANTISERA

Rhinovirus		Antisera (bovine)				$TCID_{50}$
No.	Strain	Chv 82		B632		
		Before	*After*	*Before*	*After*	
?	Chv 82	96	96	—	—	100
1B	B632	—	—	80	80	100
12	CV 16	36	<12	15	<10	30
28	" 29	64	"	30	"	100
38	Chv 79	18	"	40	"	30
50	A2 #58	36	"	30	"	30

? = Unassigned serotype.
Source: Hamparian and Conant (32). These sera were absorbed with human liver powder.

Several interesting relationships have been described in a study by Tylor-Robinson and Tyrrell (19). Rhinovirus B632 was consistently neutralized by hyperimmune antiserum prepared in monkeys against coxsackievirus A7 (negative preinoculation serum). The 2060 strains (ECHO-28), close relatives of B632, were unaffected by the same sera. Moreover, B632 was not neutralized by A7 antisera prepared in cockerels. In general the immediate serologic response was type-specific (33).

Cross-reactions between unrelated serotypes have occasionally been encountered in sera obtained from human volunteers (35, 36). The circumstances in human beings have been no less complex than in animals. This is particularly true with respect to anamnestic recall, and it also applies to the intervention of subclinical infection from a heterotype.

At the present time serum-neutralization tests are the best available means for measuring antibodies and for differentiating rhinovirus serotypes. The main problems encountered in tests with human sera have been low levels of antibody and breakthrough when virus input in the conventional serum-dilution-endpoint method exceeds $\sim$30 $TCID_{50}$. British workers have used a microplaque technique that measures the kinetics of virus neutralization (37). Comparative studies of sera containing usual levels of neutralizing antibody suggest that either method may be used when the amount of virus in the conventional test is adequately controlled with respect to the virus-challenge dose. Available data indicate that M strains, as contrasted with H strains, generally yield more virus in culture, produce higher levels of antibody, and are more easily worked with in neutralization tests.

There are other problems. One relates to the best measure of serum-neutralizing antibodies and selective sensitivities of cell-culture assay systems. Data from our laboratory illustrate the varying sensitivity of the cell-culture assay systems. Antisera from monkeys inoculated with rhinovirus obtained from KB cells engendered high levels of type-specific antibody when determinations were made in the KB cell line. These high antibody levels were reproducible and they

were type-specific. On the other hand, the antibody endpoints obtained from some of these antisera in WI-26 or WI-38 cells were 5 to 20 times lower. These results were reproducible without notable variation from one laboratory to another (Table 5).

While the problem of breakthrough in the conventional test has been noted infrequently with animal sera, variabilities with different cultural systems require continuing scrutiny. To date workers in this field have found little variation in serum-dilution endpoint, even with somewhat larger virus inputs. Dose-response relationships have not been fully described. Nevertheless, the data obtained using the conventional test have provided the basis for rhinovirus typing. Recently Gwaltney (38) reported a microtiter system for measuring neutralizing antibodies and for differentiating types. An intersecting sera schema like the one developed for enteroviruses may be useful in typing rhinoviruses (39).

Serum plaque-reduction may be more sensitive than the conventional method for revealing antibodies. A major obstacle to the use of plaque-reduction methods is the paucity of data available on plaque-formation by rhinoviruses. We have added several more rhinoviruses to the list of strains reported to produce plaques (21, 40, 41), but far more work needs to be done. The availability of a workable plaque-reduction test would provide a sensitive method for analyzing strain differences among rhinoviruses.

Fortunately many of the problems discussed above do not seem insurmountable. Antisera of sufficient quality for recognizing serotypes can be prepared. Indeed, there are excellent prospects that well-standardized reference sera without heterologous cross-reactivity, or with very low levels thereof, can be prepared in animals, particularly in guinea pigs, using purified virus stocks. Much remains to be learned, however, with respect to optimal antigenic mass, the varying effect of adjuvants, and heterologous overlap during hyperimmunization. At this time the preference is for specific sera of sufficient potency for revealing strain relationships. Undoubtedly interrelationships like those found for strains 2060 and B632 will be encountered again. No doubt subtypes exist among other serotypes.

Summary

Recent collaborative efforts have brought order to the burgeoning groups of rhinoviruses. To date, 56 of 100 or more existing strains have been established as distinct serotypes; some others will soon receive numbers. Information regarding subtypes such as 2060 and B632 are only beginning to appear; intratypic variations, such as those known among human enteroviruses, can be expected. At the present time even less is known about the sharing of minor antigens between types. On the basis of present knowledge the rhinoviruses appear to have the same unique specificity recognized for other picornaviruses (42).

TABLE 5. VARIATIONS OF SERUM DILUTION ENDPOINTS WITH TWO CELL CULTURE SYSTEMS

Rhinovirus		Homologous SDE's by serum neutralization (monkey antisera)					
		WI cells			KB cells		
No.	Prototype strain	No. of tests	SDE	$TCID_{50}$	No. of tests	SDE	$TCID_{50}$
13	353	6	~60	~150	6	500	~50
14	1059	6	~3200	~100	6	10,000	~100
15	1734	6	~100	~80	5	1250	~50
16	11757	6	~120	~200	3	6000	~300
17	33342	6	~2500	~300	5	5000	~200

Numerical entries are geometric mean values, expressed as reciprocal of serum dilution endpoint (SDE). Some liberties have been taken in rounding out the numerical entries. To illustrate the range of values obtained in individual tests, the figures for Type 16—neither the best nor the worst—may be taken as an example. For WI cells the SDE range is 64–420 and the $TCID_{50}$ is 32–350; for KB cells the SDE range is 4096–8000 and the $TCID_{50}$ is 100–500.

Source: Unpublished data obtained at the University of Kansas, NIH, and the Virus and Rickettsial Laboratory, University of California.

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SECTION D. RHINOVIRUSES

TESTS OF RHINOVIRUS VACCINES IN MAN

D. A. J. TYRRELL

Common Cold Research Unit, Salisbury,
Wilts, England

DR. TYRRELL (*presenting the paper*): Rhinoviruses are now known to be one of the main identifiable causes of common colds and similar diseases. It has been shown during the past few years that vaccines can be made with rhinoviruses and that such vaccines can prevent colds produced by these organisms. However, we are still far from having vaccines that can noticeably reduce the number of common colds and similar illnesses in the general population. In the next few minutes I shall attempt to indicate the present state of knowledge and the points on which additional information is required.

The Cultivation of Rhinoviruses

Some rhinoviruses (the M strains) can be grown in tube- or bottle-cultures of trypsin-dispersed monkey kidney cells. The techniques are not basically different from those used for polioviruses, but it is necessary to incubate them at a temperature of about 33°C, to control the pH at about 7, and usually to roll the culture vessels (16). Many more rhinoviruses (H strains) can be grown only in human embryo cells—either primary kidney or semicontinuous diploid fibroblast cell strains. These cells also support the M strains. The M and H rhinoviruses belong to numerous serotypes. Fifty-five of them have now been designated as prototype strains and a numbering system has been internationally agreed upon (Kapikian *et al.*, in preparation). There is also a significant number of rhinoviruses that can be recovered from clinical specimens only by the use of organ cultures of human nasal or tracheal epithelium (15). Certain of these viruses can subsequently be cultivated in human diploid fibroblast cultures of suitably sensitive cell strains. Some, however, have so far been propagated only in organ cultures, probably because they can multiply only in highly differentiated and specialized cells (7).

Preparation of Vaccines

Monkey kidney cell cultures have been used as the substrate for the production of antigens of M strains and human diploid cell strains have been used for both H and M strains (12, 11, 5). Although high yields of viruses can be obtained in organ cultures, the tissue is too scarce to be used for vaccine production. Nevertheless, by using diploid fibroblast strains it is possible to prepare pools of infectious virus from a high proportion of rhinoviruses.

Some of the first batches of rhinovirus vaccines were made by inactivating tissue culture fluids with formalin. When administered by intramuscular injection these vaccines induced antibody responses. It was shown that live virus given intramuscularly produced no better response and that an attenuated live virus given intranasally only produced an antibody response when it produced a cold (5). Virus given by mouth produced neither symptoms nor antibody response, possibly because the acid-labile virus particles were destroyed by gastric juices; even if the virus had reached the lower alimentary tract, where the related enteroviruses multiply, it might not have been able to reproduce because the temperature there was over 33°C. Attempts have nevertheless been made to induce antibodies with a virus strain enclosed in enteric-coated

capsules. One published attempt was unsuccessful (8) and another unpublished attempt had only limited success (Draper, unpublished) (Table 1). Further exploration of the possibilities of using larger doses of virus and adapting the selected strain to grow freely at 37°C might be worth while.

Antigenic Potency

Only in the case of inactivated virus vaccine is there any quantitative information known on the amount of virus required to induce antibody formation. In general one ml of undiluted tissue culture fluid has been administered to volunteers. The fluids have been harvested when an extensive cytopathic effect occurred in the cell sheet, and the infectivity titer of fluids before formalin treatment has usually been 10^5 TCD_{50}/ml or greater. Purified and concentrated antigens have not yet been tested in man. It is possible to adsorb viruses onto aluminium phosphate, retaining their antigenicity, and then to elute them in a somewhat purified and concentrated state (3). They may also be purified and concentrated on a fairly large scale by zonal ultracentrifugation. Some such procedure would probably be necessary in order to combine a useful number of antigens into one injection of polyvalent vaccine. Little is known about the stability of formalin-inactivated rhinovirus vaccines, but they seem to be quite stable on storage at 4°C. However, we have noted a slight decline in the capacity of Type 1A vaccine to induce antibody in man after storage for a year or so (13), and recently it has been found that a vaccine against the H strain DC declined in potency after storage for about six months (Stones and Ramsbottom, unpublished). It would obviously be desirable to enhance the antigenic potency of rhinovirus vaccines by the use of adjuvants, but preliminary studies have shown that incomplete Freund-type oil adjuvant does not improve the response of young adults to an M strain vaccine (13).

The Antibody Response

The antibody response to rhinovirus vaccines has usually been assessed first in animals and then in human adults. The results in man have at times been better than those in animals, and this may be because frequent exposure of human adults to natural infection with related serotypes of rhinoviruses may have "primed" the antibody-producing system to some extent. It is not yet known whether similar antibody responses would also result from the injection of vaccine into young children without antibody. The titers obtained in adults may be as high as or higher than those resulting from natural infection. Good antibody responses have been obtained to vaccines made with H rhinoviruses grown in diploid cells as well as with M rhinoviruses grown in

TABLE 1. ANTIBODY PRODUCTION FOLLOWING ADMINISTRATION OF VARIOUS DOSES OF VIRUS BY DIFFERENT ROUTES TO ADULT VOLUNTEERS (SELECTED RESULTS)

Reference	Serotype and tissue type	Dose TCD_{50}	Active or inactive	Route of administration	No. of subjects	Percentage showing fourfold or greater antibody response
Doggett *et al.*	2 M	10^4	A	By mouth	11 adults	0
"	2 M	$10^{4.4}$	A	Nasal	5 adults	40*
Draper	2 M	10^4	A	By mouth, enteric-coated capsules	9 adults	22
Mascoli *et al.*	32 M	$10^{2.5}$	A	"	9 children	0
"	44 H	10^3	A	"	9 children	0
Doggett *et al.*	2 M	$10^{5.2}$	A	Intramuscular injection	7 adults	100
"	2 M	$10^{4.6}$	A	" "	5 adults	80
"	2 M	$10^{3.6}$	A	" "	7 adults	43
"	2 M	—	I	Two intramuscular injections	40	
Mufson *et al.*	1 M	—	I	" " "	29 adults	79

*Infected volunteers developed colds.

monkey kidney and diploid cells (Stones and Ramsbottom, unpublished). Considering that antibody responses are substantially less frequent after infection with H rhinoviruses than with M rhinoviruses, this indicates a reasonable basic technology for producing antigens. An increase in the antibody titer of nasal secretion as well as of serum has been found after vaccination. This is an indication that antibody induced by vaccination can reach the most susceptible region of the mucous membrane—the one by which the virus probably enters the body in natural infection (1, 2). It is therefore reasonable to expect a protective effect against experimental and natural infections.

Protection by Vaccination

To test monovalent rhinovirus vaccines in small-scale field trials is probably not worth while, since colds may be due to many different serotypes of rhinoviruses and also to other viruses. However, since the disease is so mild it is ethical to administer vaccine to volunteers and then to challenge them by the intranasal administration of a live virus. Such experiments have been done using formalin-inactivated virus. Some of the results are summarized in Table 2. In one study (11) volunteers who had been vaccinated against Type 1A were challenged with a large dose of a living virus that had been passed in tissue culture. Although they shed less virus than the unvaccinated controls, they still developed colds. That is to say, there was no protection against infection with an unrelated rhinovirus. In another trial a group of volunteers challenged with a small dose of rhinovirus Type 2 after vaccination with the same serotype of virus was found to be protected against both infection and disease, whereas another group challenged with rhinovirus Type 1A was not protected (13). It has been shown repeatedly that the resistance of volunteers may be correlated with the possession of significant titers of serum neutralizing antibody against the challenging virus, whether the antibody is induced naturally or by vaccination (11, 2, etc.). It may be concluded from these experiments that vaccines are likely to protect against challenge with homologous virus.

TABLE 2. ATTEMPTS TO PROTECT VOLUNTEERS BY INTRAMUSCULAR VACCINATION AGAINST EXPERIMENTAL INTRANASAL INFECTION WITH RHINOVIRUSES

Serotype of vaccine	Serotype of challenge virus	No. inoculated	Response of volunteers	
			No. with colds	No. infected virus isolated
2.....	1A	13	6	11
2.....	2	28	1	5
None.....	2	23	11	14
1A.....	1A	22	11*	19

*All 11 subjects in this experiment were initially free of antibody and only one cold occurred in the nine volunteers who had antibody titers of 1:32 or greater after vaccination.

Serological evidence shows that the neutralizing antibody produced after natural infection and vaccination with Type 1A induces heterologous antibody against 1B (5). However, these two viruses are unusually closely related serologically (10, 14). Consequently, vaccination with any one serotype would not be expected to protect against more than one or two per cent of colds occurring in the general population. In one small study, never reported in full, it was stated that vaccination with Type 1A vaccine gave protection during an epidemic due to the same serotype in a closed community (12). However, a large-scale field trial of Type 1A rhinovirus vaccine combined with other components was made in several populations of young adults and it seemed that the rhinovirus component produced a reduction of only about 15 per cent in the rate of respiratory disease (9). This is a strange result. It is possible that the vaccine boosted antibody to a number of heterologous viruses. Mogabgab bears out this hypothesis with the results of cross tests using the complement-fixation method, but other work does not support the idea, and natural infections with rhinoviruses seem to be followed by a rather specific antibody response. On the other hand, it would not really be surprising if administration of a potent monovalent vaccine to an adult induced antibody that neutralized some of the other rhinoviruses, probably at least 100, to which he might have been exposed.

We have found that a complement-fixing antigen prepared from HGP virus reacts with the sera of young children that do not neutralize the

same serotype of rhinovirus. This complement-fixing antibody may be produced in response to infection with other viruses—probably other strains of rhinoviruses (Tyrrell *et al.*, unpublished). The antigen may represent the "empty" virus particles that can be separated by ultracentrifugation from crude virus preparations and that react with experimental sera less specifically than "full" particles do (4). This antigen may thus be equivalent to the C antigen of poliovirus and to similar antigens found in preparations of other enteroviruses. In our experience, the antigen is of no diagnostic value, except possibly in first infections with rhinoviruses.

Possible Field Use of Vaccines

As far as I know, rhinovirus vaccines are not at the moment being used to control colds, even on an experimental basis. Nevertheless, we do know how to make antigenically active vaccines, and we know that they can prevent infection and illness induced by the same serotype of virus. There is more to be learned about this subject and in particular about how to make a polyvalent vaccine containing at least a half-dozen strains.

On the other hand we know but little about the detailed epidemiology of colds. In some areas of England a fairly high proportion of isolates has been typed over a number of years using a limited number of sera (Pereira, unpublished), whereas in Glasgow the majority of viruses isolated do not belong to the presently recognized serotypes (Scott, unpublished). Since almost all of the M rhinoviruses isolated in Britain over the past five years belong to known serotypes (Stott, Pereira, and Higgins, unpublished), we might try to make a polyvalent vaccine with a reasonable chance of protecting against all colds produced by M rhinoviruses. However, M rhinoviruses cause a rather small proportion of colds and we do not know how many serotypes of H rhinoviruses might be needed as well, nor do we know whether the viruses isolated in organ cultures, which cause a significant proportion of colds, will turn out to be different serotypes from those we have met already. It is therefore important to discover, through laboratory and epidemiological study, the basic pattern of the distribution of strains. It seems clear that some serotypes are widespread, that a given type does not stay long in any one geographical area, and that several may circulate at once (6). We do not know whether there is a tendency for certain viruses to frequent particular parts of the world. If this should turn out to be the case, we might consider producing different vaccines for different areas. We do not know either whether there is a large but fairly fixed range of different serotypes constantly in circulation or whether new types are continually appearing. Rhinovirus variants can apparently be selected in the laboratory by passage in the presence of guanidine or immune serum (Doggett *et al.*, in preparation), and it is therefore possible that a similar process may occur when viruses are spreading in a partially immune population.

Conclusion

There is obviously a long road to tread before rhinovirus vaccines can be widely used. Some say they never will be. Of one thing I am certain, however: ultimately successful measures for the control of colds will have to maintain immunity against rhinoviruses. The modicum of antibody that we now carry is undoubtedly a valuable safeguard against frequent and troublesome infections. Although this level may be difficult to raise, it certainly should not be allowed to decline.

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SECTION D. RHINOVIRUSES

PROBLEMS AND APPROACHES TO CONTROL OF RHINOVIRUS INFECTIONS *

WILLIAM S. JORDAN, JR.

University of Virginia
Charlottesville, Virginia, U.S.A.

DR. JORDAN (*presenting the paper*): Investigators searching for *the* common cold virus have been defeated by success. Instead of one virus, or a few, that might be used for immunization, a great number have been identified in recent years, and the prospect of a centavalent vaccine looms as a virologist's nightmare on the acute respiratory disease horizon. This ironic turn of events is exemplified particularly by the case of rhinoviruses.

Rhinoviruses are at present the most important known cause of the common cold in young adults, having been associated with 10 to 30 per cent of respiratory illnesses in schoolboys (20), university students (13, 15, 16, 30), military recruits (2), and industrial populations (15, 21, Gwaltney *et al.*, in press). Isolation rates in children, however, have seldom exceeded 5 per cent (2, 15, 21, 25)—a puzzling difference, that will be considered in some detail later.

In the 10 years since rhinovirus Type 1 was first isolated, all the conditions believed necessary for the development of an effective cold vaccine have been satisfied (22): rhinoviruses can be propagated readily in the laboratory; their etiologic relationship to the common cold has been confirmed in volunteer subjects (4, 5, 27, 35, 38); it has been shown that immunity follows infection, with resistance being directly related to antibody level (5, 13, 14, 19, 21, 27, 35); and experimental vaccines have provided varying degrees of protection (26, 31, 35). But despite these achievements, the immediate prospects for the prevention of rhinovirus infections by immunization are slim indeed. Data from field studies indicate that there are at least 100 rhinovirus serotypes, and possibly more than that (13–16, 28–30). These types are antigenically distinct and induce highly specific antibody responses in man (4, 14, 21, 22, 35). Such antigenic multiplicity and immunologic specificity must be responsible in large part for the frequent recurrence of common colds, and they certainly present great obstacles to the preparation of a protective polyvalent rhinovirus vaccine.

Nevertheless, there might be hope for such a vaccine within the capabilities of present technology if it could be shown that a limited number of types, even 10 or 20, were responsible for the majority of rhinovirus illnesses. The data so far available on this point are not encouraging. In longitudinal studies of three to four years' duration in Charlottesville, Virginia (Gwaltney *et al.*, in press), Chicago (16), and Philadelphia (15), the number of different types identified has been 48, 50, and 53, respectively; the number of types represented by single isolates has been 17, 8, and 27, respectively. In these and other areas rhinovirus types have shown a random distribution with respect to time. Occasionally focal epidemics have been caused by only a few

* Studies in the author's laboratory referred to in this paper were conducted under the sponsorship of the Commission on Acute Respiratory Diseases, Armed Forces Epidemiological Board, and were supported in part by the Office of the Surgeon General, Department of the Army, and in part by the Vaccine Development Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

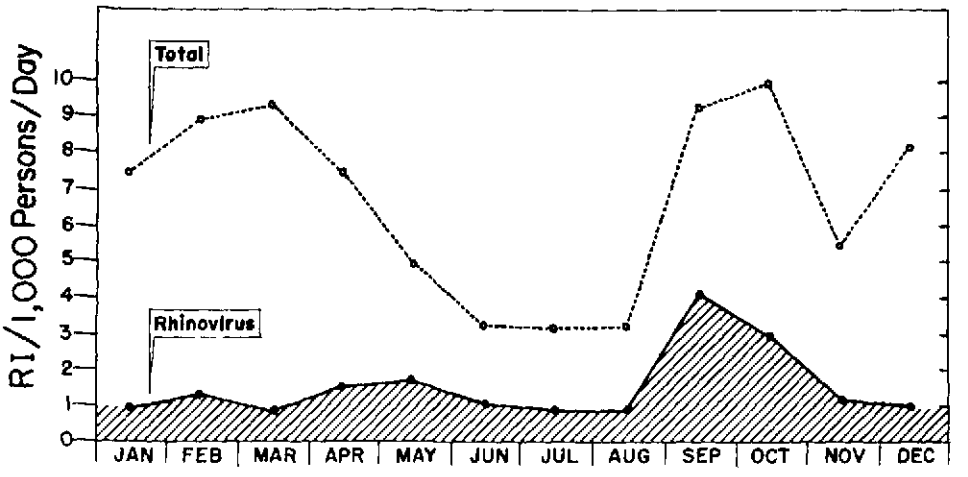
types, but most often multiple types have circulated simultaneously and then disappeared, to be replaced by other types in successive seasons. As a result, even the most commonly occurring rhinovirus serotype will account for only a small portion of all rhinovirus infection (28). It is not known how many different rhinovirus antigens can be compressed into an inactivated vaccine, but the number of known types has probably already exceeded this limit. Many isolates have not yet been typed because they are not neutralized by available antisera, so the data cited actually understate the problem.

In the face of this dilemma, we may ask whether live virus vaccines have anything to offer. Might it be possible to administer single, attenuated types seriatim by mouth, as for polioviruses or adenovirus Type 4, or intranasally, as has been tried for influenza? With good antigens and a lot of public education, an immunization program entitled "Rhinovirus of the Month" could be instituted. Given 100 serotypes and starting at the age of six, a well-dosed child would be protected by the age of 16, provided immunity lasts for 10 years. Antibody has been shown to persist for over a year after immunization (35) and for as long as seven to eight years after natural infection (37), but the duration of type specific immunity remains to be determined. The duration of immunity may vary with the type, and there is evidence that antibody to H strains persists less well than antibody to M strains (37). More data are needed on this point. Unlike other picornaviruses, rhinoviruses are not excreted in the feces (2), and viruses fed in milk (9) or in enteric-coated capsules (24) have failed to provoke antibody responses. It has not yet been shown that prolonged laboratory passage can attenuate a strain of rhinovirus so that it will induce antibody without illness when given intranasally (9). I believe this approach should be pursued, for reasons I shall give later.

While we debate whether the frequency and severity of rhinovirus illnesses justify such measures, and while we wait for the dense, type-specific antigens (7) of many of these viruses to be packed into a reasonable volume and enhanced with an adjuvant more effective than those tested to date (17, 35), we must in the meantime continue to carry out epidemiologic studies directed toward clarifying the behaviour of rhinoviruses. From such studies may come information to guide us in the formulation of vaccines and to suggest other approaches to control.

As tedious as it will be, serotype identification and classification must continue if the full scope of the problem is to be defined. This effort has been aided by the WHO Collaborative Program, and it will be greatly facilitated when adequate quantities of typing sera are provided by the Vaccine Development and Reference Reagents Programs of the National Institutes of Health. How many types are there? What are the contemporary immunologic deficiencies of various populations? Is there any consistency to the cycling of serotypes? Why did Type 1 virtually disappear for several years after its simultaneous prevalence in the United States and Great Britain? More attention must be paid to both host and environment. What are the factors affecting host resistance? Is there such a thing as meterological activation of latent infection? What factors facilitate transmission?

It is now clear that seasonal factors lead to fluctuation in the incidence of rhinovirus infections (16, 20, 29, 39). Although December and January were peak months during one 14-month study of a military population (29), a consistent pattern of prominent peaks in September and October with low periods from January through March has been observed in several civilian populations in the United States (16) and Great Britain (20, 39). During a three-year surveillance of a group of young adult insurance company office workers in Charlottesville (Gwaltney *et al.*, in press) there was a regular annual peak of illness in September and early October associated with a high rhinovirus isolation rate. Data for the three years were pooled to obtain the curves in Figure 1. Rhinoviruses were isolated from 23 per cent of all the illnesses sampled, and rhinovirus infections occurred during all months of the year. Because an epidemic of rhinovirus infections initiated the respiratory disease season each year, and rhinovirus isolation rates were lower at other times, 40 per cent of all rhinovirus illnesses occurred during the early fall. Since the etiology of most of the nonrhinovirus illness was not defined, the figure illustrates the unhappy fact, documented by

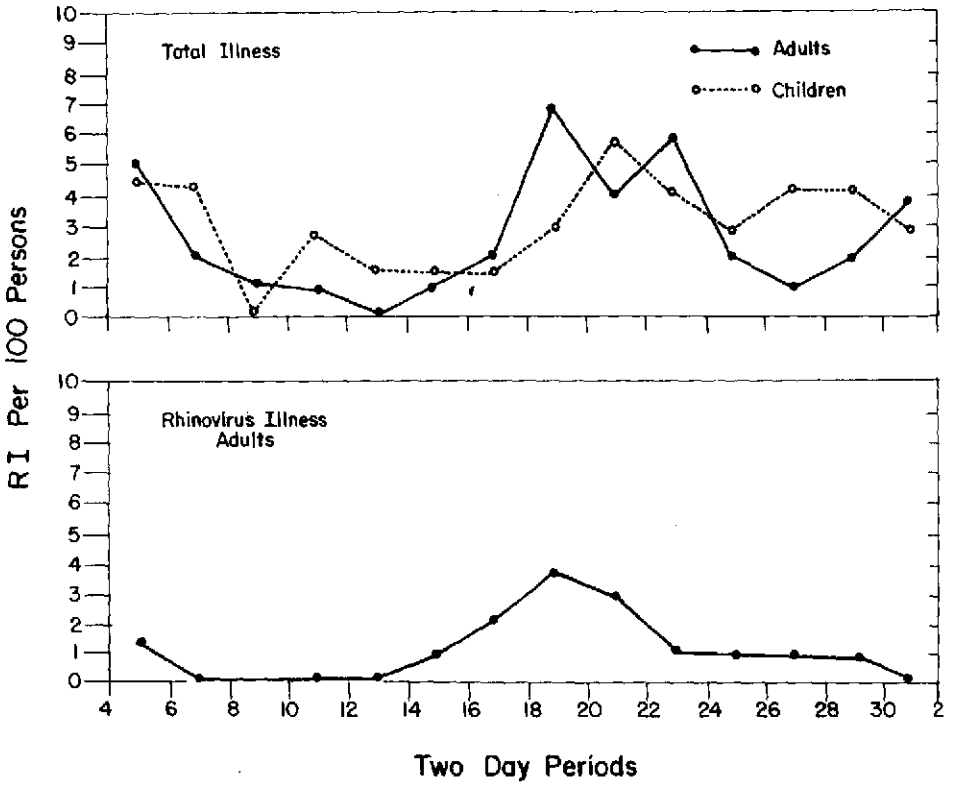


The curves above, which represent combined data for a three-year period, depict the seasonal variation in the occurrence of respiratory illness and of rhinovirus infections in an industrial population of young adult office workers. Rhinoviruses were isolated from 23.3 per cent of the illnesses sampled. The rhinovirus illness rate was derived by applying the isolation rate for a given interval to the total respiratory illness rate.

Fig. 1. Seasonal occurrence of respiratory illness—Combined data, March 1963-March 1966.

many other studies, that the agents responsible for nearly half of all acute respiratory illnesses are yet to be identified.

A number of things happen in the fall. People return from vacation; the weather changes; children go back to school. Since school-age children are said to be greater spreaders of respiratory infections than adults (1, 8), my associates and I undertook a short-term study of 50 families of the Charlottesville insurance company employees in the fall of 1965 (J. O. Hendley *et al.*). There were 68 respiratory illnesses in 37 of the 50 families during the four-week period that included the peak of rhinovirus infections in all employees. Sixty-four (94 per cent) of the family illnesses were sampled. The rhinovirus isolation rate for adults was 42 per cent. Much to our surprise, none of the specimens from ill children were positive, although their illness rate was just as high (Fig. 2). The illness rate was highest in children not attending school, and the rate increase in schoolchildren as in all children, lagged slightly behind that in adults. Whether the failure to isolate rhinoviruses from children at this time was due to our decision not to take nasal specimens from the young children or to other factors is not known. The data are consistent with the low isolation rates reported for children by others (2, 15, 21, 25) and with the observation that antibodies to rhinoviruses currently prevalent in adults are infrequent in children (34, 36). Certainly, here in a setting in which to seek confirmation of a role for interferon or for depression of relative susceptibility through some nonspecific blockage of cell invasion by viruses. We hope to obtain clearer answers from a more extensive family study conducted in the fall of 1966, in which observations were begun sooner and continued longer. Preliminary results for the first six weeks indicate that agents resembling rhinoviruses have been isolated with equal frequency from children and adults with illness. This time nasal specimens were collected from all children. However, these specimens yielded only a few more isolates than concomitant pharyngeal swabs, and the difference between the two years remains unexplained. Of particular interest is the fact that a mass testing of all members of the families in August, one to two weeks before the respiratory illness rate began to increase, yielded rhinoviruses from 7.6 per cent of asymptomatic children, as against 2.4 per cent of asymptomatic adults. It is too early to tell whether enough other agents were isolated from ill children to support the suggestion that interference by other viruses later suppressed rhinovirus illness in these children.



The curves above show the occurrence of illness in 50 families (100 adults, 53 schoolchildren, and 16 preschool children) during a four-week period beginning at the opening of school. Thirty-eight illnesses occurred in adults, 30 in children. Both nasal and pharyngeal swabs were collected from adults and older children; a pharyngeal swab only was collected from 23 children under 10 years of age. No rhinoviruses were isolated from children.

Fig. 2. Occurrence of respiratory illness in families, September 1965.

With regard to nonspecific resistance, very interesting data have been derived from both epidemiological and volunteer studies. A transient period of resistance has been noted following respiratory illness in adults (3, 22, 23, 32), but not in children between the ages of 5 and 14 (3). During a study of illnesses in families, Dingle and his associates (8) sought evidence for such a period by studying secondary attack rates. Children had slightly reduced secondary attacks through the 22nd day; rates for adults were slightly reduced for a period of 10 to 69 days. In this study the specific etiologies were not known, and persistent susceptibility following a recent infection, rather than resistance, was the impressive feature.

Investigators at the National Institutes of Health (5) noted that volunteers rechallenged with the same rhinovirus after one month, when induced homologous antibody titers were relatively low, exhibited complete resistance to illness and almost complete absence of virus shedding. In another series of experiments (12), resistance to homologous virus was demonstrated at two weeks, when no serum antibody was detectable; after this same interval no illness was induced in a group of volunteers inoculated with a serologically unrelated virus. Some degree of resistance to heterologous reinoculation was still present after five weeks, but it was gone by 16 weeks. The nature of this resistance mechanism is unknown. It is not due to type-specific nasal antibody, and perhaps not to interferon. The phenomenon certainly warrants further study, particularly since prospects for a vaccine are so gloomy. If only such resistance could be induced by infectious, but attenuated, intranasal rhinoviruses, it might be possible to provide non-

specific protection while one serotype per month was being sniffed to gain specific immunity. The nose appears to be not only the most susceptible route of rhinovirus infection but also the principal site of viral multiplication. There is increasing evidence that nasal antibody is important in resistance to respiratory infection, including that due to rhinoviruses. Perhaps this is another argument for attenuated nasal vaccines.

Two other major approaches to prevention remain: interference with the spread of rhinoviruses through environmental control, and antiviral chemoprophylaxis. What scant information is available indicates that rhinoviruses spread rather poorly (19, 30), and I detect little enthusiasm for further trials of such measures as ultraviolet irradiation (10, 11, 40) or the control of dust (6, 40) or humidity (33), which have failed in the past. Perhaps when we know more about how rhinoviruses spread and initiate infection new approaches to environmental control will become obvious. As for antiviral chemoprophylaxis (18), the availability of safe drugs that would block the attachment or replication of rhinoviruses in the respiratory tract might well make all other approaches obsolete. Such drugs are being sought in a number of laboratories. Should any compound show promise in volunteer experiments, early fall would seem to be the logical time for field trials.

It seems appropriate to conclude this discussion of prevention with a word about treatment. The mechanism by which rhinoviruses produce tissue damage and symptoms is unknown. If this mechanism were understood, perhaps it would be possible to devise therapeutic agents more useful than the ineffective antihistamines and popular cold remedies.

In summary, the battle against rhinovirus illnesses and other respiratory infections has just been joined. The multiplicity of rhinovirus serotypes and the existence of agents yet to be identified present a formidable challenge to the prevention of the common cold by immunization. Apart from chemoprophylaxis, "the best hope," our chairman Dr. Andrewes has said, "of preventing not only the common cold but all these respiratory infections may lie in understanding their natural history" (1).

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SECTION D. RHINOVIRUSES

DISCUSSION

CHAIRMAN ANDREWES: The first of the discussants is Dr. William J. Mogabgab, Department of Medicine, Tulane University, New Orleans, Louisiana.

DR. MOGABGAB: It is generally recognized that a limited number of strains within most families of infectious agents are responsible, by virtue of properties known as virulence or antigenic patterns, for most infections or illnesses, at least within a variable period of time and in certain kinds of populations.

Table 1 shows that a limited number of rhinovirus types were recovered from two groups of young adults. Although such results are dependent on sensitivity of the isolation techniques, the data from serologic studies shown in Table 2 reveal that a majority of the colds in university students were associated with neutralizing antibody responses to six of these types. A similar study of young military personnel in an adjoining state (Table 3) demonstrated that the same six types—1A, 1B, 2, 29, 30, and 31—accounted for almost a half of the common colds. The Type 2 recovered from the university group was actually a variant of HGP and probably should be designated as 2B.

Analysis of the neutralizing antibody levels (Table 4) demonstrated that half of the university students were susceptible to infection with these types, since most infections occurred in the absence of detectable antibody. Table 5 reports similar observations in young military personnel. The individuals in both groups were from a variety of areas in the United States.

These findings suggest that an inactivated vaccine containing a limited number of antigenic types might have a considerable effect in pre-

TABLE 1. RHINOVIRUSES RECOVERED FROM UNIVERSITY STUDENTS WITH UPPER RESPIRATORY ILLNESSES, NEW ORLEANS, LOUISIANA

Year	No. of specimens	Rhinovirus types										Totals
		1A (GL2060)	1B (K779)	2 (K2211)	15 (1734)	25 (K2218)	29 (179E)	30 (106F)	31 (140F)	38 (CH79)	45 (E246)	
		No. of isolates										
1962–63	190	0	2	2	0	0	0	0	0	0	0	4
1963–64	292	2	1	0	0	4	4	0	1	0	1	13
1964–65	372	4	0	0	1	1	3	5	0	1	0	15
1965–66	376	2	0	26	0	2	0	1	21	0	0	52
Totals	1230	8	3	28	1	7	7	6	22	1	1	84
		*In Trainees, Keesler Air Force Base, Mississippi**										
1962–63	414		6	3		1	6	1	1			20

*Also one T32 and T43.
In WI-26, WI-38, or MK-2 cultures.

TABLE 2. SEROLOGIC INCIDENCE OF RHINOVIRUS INFECTIONS IN UNIVERSITY STUDENTS AS DETERMINED BY NEUTRALIZING ANTIBODY RESPONSES, TULANE UNIVERSITY, NEW ORLEANS, LOUISIANA, 1958-1966

Illness		Type 1A (GL2060)	Type 1B (K779)	Type 2 (K2211)	Type 29 (UofC, 179E)	Type 30 (UofC, 106F)	Type 31 (UofC, 140F)	Type 45 (E2-46)
Mild respiratory illnesses	+/total	6/66	28/505	38/455	17/440	3/14	18/99	2/11
	%+	9.0	5.5	8.4	3.9	21.4	18.2	18.2
All other respiratory illnesses	+/total	0/19	8/263	13/280	7/277	6/24	3/24	0/3
	%+	0.0	3.0	4.6	2.5	25.0	12.5	0.0
All respiratory illnesses	+/total	6/85	36/768	51/735	24/717	9/38	21/123	2/14
	%+	7.1	4.7	6.9	3.3	23.7	17.1	14.2

TABLE 3. SEROLOGIC INCIDENCE OF RHINOVIRUS INFECTIONS IN AIRMEN AS DETERMINED BY NEUTRALIZING ANTIBODY RESPONSES, KEESLER AIR FORCE BASE TRAINEES, 1958-1963

Illness		Type 1A (GL2060)	Type 1B (K779)	Type 2 (K2211)	Type 25 (K2218)	Type 29 (UofC, 179E)	Type 30 (UofC, 106F)	Type 31 (UofC, 140F)	Candidate (K2305)
Afebrile upper respiratory illnesses (colds)	+/total	9/100	68/475	13/310	2/18	7/169	2/36	1/17	0/24
	%+	9.0	14.3	4.2	11.1	4.1	5.6	5.9	0.0
All other respiratory illnesses	+/total	14/51	11/303	7/255	0/12	3/160	2/27	0/20	0/18
	%+	27.5	3.6	2.7	0.0	1.9	7.4	0.0	0.0
All respiratory illnesses	+/total	23/151	79/778	20/565	2/30	10/329	4/63	1/37	0/42
	%+	15.2	10.2	3.5	6.7	3.0	6.3	2.7	0.0

TABLE 4. DISTRIBUTION OF RHINOVIRUS NEUTRALIZING ANTIBODY TITERS IN UNIVERSITY STUDENTS WITH RESPIRATORY ILLNESSES, TULANE UNIVERSITY CAMPUS, 1958-1966

Virus		Percentage with reciprocal neutralizing titers							Total illnesses
		<4	4	8	16	32	64	>64	
Type 1A	ab↑A	83		17					6
(GL2060)	C		17	50			17	17	6
1958–1965	no ab↑	46	13	13	9	2	6	11	79
Type 1B	ab↑A	78		17	5				36
(K779)	C			47	20	17	8	8	
1958–1966	no ab↑	37	9	18	16	7	7	6	732
Type 2	ab↑A	80	6	12	2				51
(K2211)	C			25	18	18	18	21	
1958–1966	no ab↑	50	13	13	11	5	5	3	684
Type 25	ab↑A	100							4
(K2218)	C			50		25	25		
1963–1966	no ab↑								0
Type 29	ab↑A	58	13	25	4				24
(UofC, 179E)	C			33	46	8	4	8	
1962–1966	no ab↑	65	9	14	8	2	1	1	693
Type 30	ab↑A	100							9
(UofC,106F)	C			22	33	11	22	11	
1963–1964	no ab↑	38	14	14	25	3	3	3	29
Type 31	ab↑A	71	19		10				21
(UofC,140F)	C			24	10	28	24	14	
1965–1966	no ab↑	53	19	8	10		7	3	102
Type 45	ab↑A	100							2
(E246)	C			50	50				
1963–1964	no ab↑	75	8	8		8			12

ab↑ = Neutralizing antibody increment of fourfold or greater.
A = Acute. C = Convalescent.

TABLE 5. DISTRIBUTION OF RHINOVIRUS NEUTRALIZING ANTIBODY TITERS IN AIRMEN WITH RESPIRATORY ILLNESSES, KEESLER AIR FORCE BASE, 1958-1963

Virus		Percentage with reciprocal neutralizing titers							Total illnesses
		<4	4	8	16	32	64	>64	
Type 1A	ab ↑ A	70	12	9	9				23
(G12060)	C			22	39	22	4	13	
1958–1963	no ab ↑	59	3	11	13	5	5	4	127
Type 1B	ab ↑ A	76	13	10	1				79
(K779)	C			34	34	18	9	5	
1958–1963	no ab ↑	41	10	19	14	6	4	6	699
Type 2	ab ↑ A	85	5	5	5				20
(K2211)	C			50	20	10	20		
1958–1963	no ab ↑	55	12	14	11	4	3	1	545
Type 25	ab ↑ A	100							2
(K2218)	C				50		50		
1962–1963	no ab ↑	68	11	11	7		3		28
Type 29	ab ↑ A	100							10
(UofC), 179E)	C			10	20	10	40	2	
1962–1963	no ab ↑	61	13	13	10	1	1	1	319
Type 30	ab ↑ A	100							4
(UofC, 106F	C				75		25		
1962–1963	no ab ↑	51	5	6	14	2	7	15	59
Type 31	ab ↑ A	100							1
(UofC, 140F)	C					100			
1962–1963	no ab ↑	50	14	6	22	6		2	36
Candidate	ab ↑ A								0
(K2305)	C								
1962–1963	no ab ↑	79	5	5	9		2		42

ab ↑ = Neutralizing antibody increment of fourfold or greater.
A = Acute. C = Convalescent.

TABLE 6. PROTECTIVE EFFECTS OF RHINOVIRUS TYPE 1A VACCINES

Year	Populations	Vaccines*	No. of subjects	Observation period (weeks)	Respiratory disease (rate/1000/week†)		Relative reduction in rate (%)
1958–59	University A	Placebo	55	16	22.7	(A)	26
		Rhinovirus, T1A	59		16.9		
	University B	Placebo	86	28	17.4	(B)	19
		Rhinovirus, T1A	86		14.1		
1958–59	Industrial	Influenza	72	14	43.6	(C)	23
		Influenza, Rhinovirus, T1A	72		33.6		
1959–60	Industrial	Influenza	125	26	24.8	(D)	13
		Influenza, Rhinovirus, T1A	125		22.1		
1960–61	Industrial	Influenza	500	20	5.3	(E)	17
		Influenza, Rhinovirus, T1A	500		4.4		
1961–62	Industrial	Influenza	335	22	7.5	(F)	33
		Multivalent	335		5.0		
1962–63	Military	Placebo	1430	11	11.8	(G)	20
		Rhinovirus, T1A	1427		9.4		

*Rhinovirus, T1A vaccine was prepared from virus grown in monkey kidney cultures. Influenza was standard polyvalent vaccine. Multivalent vaccine included influenza Types A2, B1, parainfluenza Type 1, and rhinovirus T1A, all propagated in monkey kidney cultures. Two injections of 1 ml subcutaneously, separated by intervals of 3 to 8 weeks, were given. About 50% of each group received only one dose.

†(A) and (B) rates were determined from cases appearing at the campus dispensary. (C) and (D) included all upper respiratory illnesses that occured in each group except febrile illnesses (influenza-like) in (D). (E) and (F) included illnesses that caused absence from work. (G) were trainees at Keesler Air Force Base.

venting the common cold. The use of just one of these types (1A) in a vaccine was found to be capable of reducing rates of afebrile respiratory illnesses (Table 6).

Although these were preliminary experiments in small groups, the results were uniformly consistent. It is noteworthy that viruses antigenically related to that in the vaccine were not recovered in the last study. Nevertheless, they still might have been present in the population and these results could have been due to heterotypic antibody responses. Thus, there appears to be reasonable basis for a positive approach to immunization against many common colds.

CHAIRMAN ANDREWES: Thank you, Dr. Mogabgab. I will call next on Dr. Dorothy M. Hamre, Department of Medicine, University of Chicago, Chicago, Illinois.

DR. HAMRE: There now seems to be little doubt that the reason for repeated acute respiratory infections is the large number of viruses, particularly the large number of serologic types of rhinoviruses that can cause these infections. All of the evidence presently available indicates that there is no cross immunity among the 55 serologic types of rhinoviruses characterized to date.

Observations on medical students enrolled for four years in our study of acute respiratory illnesses support this apparent specificity of immunity to rhinovirus serotypes. Over the four-year period 425 specimens were obtained during the acute phase of respiratory illness from 55 medical students. On the basis of virus isolation and/or fourfold-or-greater rise in antibody titer, 42 per cent of the illnesses could be specifically diagnosed and 29.5 per cent were rhinovirus infections. Thus, 125 rhinoviruses were isolated from acute specimens. From these same students eight rhinoviruses were isolated from control specimens. At least 41 serotypes are represented among the 133 rhinoviruses recovered from these students. Not all of these rhinoviruses have been typed.

TABLE 1. RHINOVIRUSES ISOLATED FROM 55 STUDENTS OVER 4-YEAR PERIOD

No. of viruses	No. of students	Percentage
0	8	14
1	6	12
2	18	33
3	12	22
4	6	12
5	1	2
6	3	5
7	1	2

TABLE 2. VIRUS ISOLATION FROM TWO STUDENTS

Student 0452		Student 0483	
Date	Virus	Date	Virus
10/23/61	R53	5/21/63	UR*
1/23/62	R16	10/15/63	R43
4/16/62	R54	5/11/64	Neg.
4/15/63	433D	6/8/64	UR
1/13/64	R45†	9/21/64	UR
5/11/64	Neg.	1/4/65	UR
12/9/64	Neg.	6/7/65	UR

*Untyped rhinovirus.
†Not ill.

These students averaged a total of seven common colds during their four years as medical students, with a range from 2 to 15. Eighty-six per cent of the students yielded specimens positive for rhinoviruses. The number of isolations from each student ranged from 1 to 7 (Table 1). In no case was the same serologic type isolated twice from different illnesses from the same student (see examples in Table 2).

Our study of acute respiratory illnesses among medical students has been in progress for seven years.* Over this period we have isolated strains belonging to 41 of the 55 characterized rhinovirus types and strains of 23 apparently new types. Antigenic variation such as that occurring among influenza Type A viruses is probably not common among rhinoviruses, but variants related to but not identical with known types are being isolated in increasing numbers as the volume of rhinovirus serotyping increases. Each year there has been an increase in rhinovirus isolations during the fall and spring season. During these periods of high prevalence many serologic types are isolated, but it has not been possible to detect any pattern of periodicity for any rhinovirus type.

Prevention of common colds by means of the usual killed virus vaccine does not appear encouraging. The use of live attenuated strains of rhinoviruses certainly merits investigation. Further consideration should also be given to environmental or host factors that affect the spread of these viruses.

CHAIRMAN ANDREWES: Thank you, Dr. Hamre. We have a few minutes for free discussion if there are any here who would like to take part.

DR. MELNICK: Dr. Huebner referred to potential problems in regard to foreign oncogenic determinants being present in adenoviruses, particularly the papovavirus SV_{40} tumor determinant. I would like to mention some recent studies by Dr. Butel and her colleagues at Baylor that bear on the attempts to adapt candidate vaccine viruses to monkey kidney cultures in the absence of the SV_{40} determinants.

One of the monkey-kidney-adapted adenoviruses was recently subjected to close study. Even though the virus does not have SV_{40} tumor determinants, the virus stock was found to be made up of a mixture of two particles—true adenoviruses and defective adenoviruses that will not grow unless an ordinary adenovirus is present. This was demonstrated by showing that infection required two-hit kinetics.

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Transcapsidation procedures have made it possible to transfer the genome from this defective virion to another adenovirus so that the genome governing monkey adaptation is now covered by an adenovirus capsid of another type. This is similar to the situation with the SV_{40} adenovirus hybrid. We do not know where this new genetic material comes from, but we do know that it carries with it the determinant for growth in monkey kidney cultures. There is no evidence that it is derived from SV_{40}.

Those who are interested in developing adenovirus vaccines, particularly live vaccines, by use of monkey-kidney-adapted virus will have to consider whether such adapted viruses carry genetic material in addition to the adenovirus genome.

DR. FOX: I would like to speak of both adenoviruses and rhinoviruses, referring to a continuing study of families that we conducted for four and one half years in New York.

It has been a real problem to determine whether or not adenoviruses cause disease. Dr. Mufson, I believe, indicated that some 5 per cent of respiratory illnesses studied were attributed to adenoviruses. Another way to approach the subject is to look at adenovirus infections that are picked up without reference to illness and, from these, to make an estimate of the proportion of infections that are temporally related to illness. In our experience it turned out to be about 45 per cent. This is not to say that all of these illnesses were necessarily due to the adenoviruses that were infecting these individuals, but it gives an idea of the upper limit of illness potential. From this, we need to subtract a proportion attributable to background illness, which I do not know how to estimate.

Also notable is the high proportion (over 60 per cent) of adenovirus associated illness manifesting fever.

On the rhinovirus front I did want to say that in New York the fall peak of rhinoviruses was not observed. Our peak was in the winter and spring. Also, we found children infected just as frequently as adults. Finally, we found that about 30 per cent of the infections occurred in the absence of illness.

DR. TYRRELL: I suppose that children are said to be less frequently infected with rhinoviruses than adults because they also suffer from respiratory infections due to a lot of other viruses. Therefore, if you calculate the ratio of those infected with rhinoviruses to the total number of illnesses, then the ratio appears to be smaller in children than adults. If you use the number of rhinovirus infections per year, the frequency would probably be the same in both children and adults. I believe Dr. Fox was referring to the latter figure just now.

SECTION E. MYCOPLASMA VACCINES

CLASSIFICATION OF MYCOPLASMA OF MAN

D. G. FF EDWARD

Wellcome Research Laboratories, Beckenham,
Kent, England

DR. EDWARD (*presenting the paper*): The first isolation of a mycoplasma from man was made nearly twenty years ago by Dienes and Edsall (6). Subsequent reports by Dienes and co-workers showed that mycoplasmas could frequently be isolated from both male and female genital tracts. These reports were confirmed by other investigators, who were content only with reporting the isolation of pleuropneumonia-like organisms, the strains not being examined further. In some cases the method of identification was not such as to distinguish between mycoplasmas and bacteria in the L-phase (9).

In 1951 Morton and colleagues (18) isolated mycoplasmas from the mouth by aerobic cultivation. Subsequent observations by Dienes and Madoff (7) suggested that the buccal strains differed from the genital ones.

Work in my laboratory with animal strains of mycoplasma showed that modifications of standard bacteriological techniques permitted quite large numbers of strains to be studied in detail. Moreover, strains could be grouped according to biological and serological properties and assigned to species. Using these methods, 91 strains isolated from the human genital tract were examined. All the strains except one were found to belong to a single species, first termed "Human Type 1" (21) but later, as part of a comprehensive attempt at nomenclature for the mycoplasmatales, named *Mycoplasma hominis* Type 1 (10). Serological examination was aided by the coincidental discovery that antisera produced a specific inhibition of growth. Isolations of *M. hominis* were made both from patients with urethritis and from healthy controls, thus discounting any etiological role of this species in nongonococcal urethritis.

Three strains received from other laboratories as human genital isolates—Campo being the prototype—differed in their serological and other properties and were classified as *M. hominis* Type 2. Subsequently Lemcke (17) showed that these strains belonged to *M. arthritidis*, a species pathogenic for rats. In view of this finding, the Type 2 serotype of *M. hominis* has been withdrawn (11) and the Type 1 strains may be called simply *M. hominis*. There remains the problem of the real origin of the few Campo-like strains allegedly isolated from the urethra in the United States. If they did not come from the urethra, they could have resulted from laboratory misadventures during their long subcultivation. It is noteworthy that no Campo-like strain has been found among the many genital isolates made in Europe or among a group of 100 subjects examined in Canada.

Mycoplasmas requiring anaerobic cultivation were isolated from cases of ulcerative balanitis by Ruiter and Wentholt (26). They were assigned to a second species, *M. fermentans* (10). Subsequent studies have shown this species to be represented among the inhabitants of the urethra and vagina.

Nicol and Edward (21), using anaerobic cultivation, isolated mycoplasmas from the saliva. These organisms differed from the genital strains and were assigned to a third species, *M. salivarium* (10).

Chanock, Hayflick, and Barile (3) isolated a mycoplasma from tissue cultures infected with Eaton agent—a cause of primary atypical pneumonia; it was named *M. pneumoniae* (4). The pathogenic role of this organism in causing respiratory infection is now firmly established, as is its identity with Eaton agent. The demonstration that a mycoplasma was definitely pathogenic for man produced the current interest in the mycoplasmas among medical microbiologists. And the need to identify this species in the sputum and throat led to the recognition of other species resident in the oropharynx, presumably as commensals. In independent and concurrent investigations by two groups, newly recognized isolates were named *M. pharyngis* (5) and *M. orale* (32), respectively. Since these strains admittedly belong to the same species, an opinion as to the correct name has been sought from the Judicial Commission on Bacteriological Nomenclature. A second group of isolates has been provisionally named *M. orale* Type 2 (33). *M. hominis* itself has been isolated from the oropharynx, and in volunteer studies its introduction into the throat has caused exudative pharyngitis and tonsillitis (19). A single strain, "Navel," unique in its properties (17), was isolated from a skin lesion affecting the umbilicus (27). It has not been classified further.

The "T strains" represent a subgroup of mycoplasma, differing in the small size of their colonies (T for tiny) and in their ability to metabolize urea (23). They were first recognized by Shepard (28), who isolated them from the genital tract but at first was unable to subculture them. Unfortunately, for some time his work was discredited because an early attempt at subcultivation in chick tissue culture led to contamination with an avian mycoplasma (29). Successful serial subculture was obtained by Ford (13), and there is now no doubt that the "T strains" belong among the mycoplasmatales. Serological studies have shown that there are at least six serotypes (23). Nomenclature and further classification is premature until more is known about their properties and relationship to large colony mycoplasmas. Their role in genitourinary infection is also uncertain. Mycoplasmas of the "T strain" type may be more widespread, for their isolation has already been reported from cattle (Taylor-Robinson, to be published).

These species and strains, together with the regions of the body from where they have been isolated and comments on their pathogenicity, are listed in Table 1.

The classification of *Mycoplasma* was originally based on biological properties and findings from a limited number of serological tests. It has now been confirmed by other serological tests and by the use of the newly developed nucleic acid homology technique (25). Species

TABLE 1. HUMAN MYCOPLASMAS

Species (or strain)	Location	Pathogenicity
M. pneumoniae	Respiratory tract	Pneumonia
M. hominis	Genital tract Oropharynx	Low grade pathogen (abscesses, etc.) (Not cause of nongonococcal urethritis)
M. fermentans	Genital tract (leukemia, etc., by tissue culture)	Unknown
M. salivarium	Oropharynx	Commensal
M. orale *M. pharyngis*	Oropharynx Bone marrow in leukemia	Commensal Unknown relationship to leukemia
M. orale Type 2	Oropharynx	Commensal
Strain "Navel"	Skin lesion	Unknown
"T strains" (6 serotypes)	Genital tract	Unknown, including relationship to nongonococcal urethritis

differ antigenically, although the presence of common antigens is revealed by some techniques. Moreover, strains within one species can differ quite markedly. There is a general problem in mycoplasma classification as to how one should define species and serotype. In practice, growth-inhibition tests, including the recently developed metabolic inhibition tests (24, 34), are the most useful serological methods for identification. The latter method is also applicable to the study of the "T strains" (23) and, because of its sensitivity, can be used to study antibody response in human sera.

Biological properties are most useful in distinguishing *M. pneumoniae*, which ferments glucose and is unique among the human species in producing a potent hemolysin for guinea pig erythrocytes, believed to be peroxide (30); in causing hemadsorption; and in reducing tetrazolium (Table 2). It is also less sensitive to inhibition by methylene blue, a property that facilitates its selective isolation (15). The abilities of certain species to ferment glucose, of others to metabolize arginine with the formation of alkali, and of "T strains" to metabolize urea have recently been used for the development of metabolic inhibition tests (23, 24, 34). The property of forming "film and spots" was found by Edward (8) to be useful in differentiating species.

The foregoing covers all the "human" mycoplasmas known to date. In the last few years there have been a number of reports of the isolation of "animal" mycoplasmas from human tissues, usually after preliminary passage in tissue culture. There has been a tendency to assume that mycoplasmas are always host-specific. This is perhaps dangerous and should not itself be used to discredit as laboratory contaminations all reports of the isolation from man of mycoplasma species normally found in animals. It is true that many of these reports should be looked at with caution for other reasons, particularly because the isolations were made from tissue cultures, which are notoriously liable to contamination. But the question of host-specificity of mycoplasmas should be approached with an open mind, especially as it is already known not to be absolute among the animal mycoplasmas.

I will now list the animal mycoplasmas whose isolation has been reported from man. The Negroni agent, isolated in England from cases of human leukemia by passage in human fetal tissue cultures, has been identified as *M. pulmonis*, a pneumonia-causing pathogen for mice and rats (12, 16). Strains isolated from leukemia by another U.S. group by means of tissue culture have now also been identified as *M. pulmonis* (16), although at first they had been named *M. mergenhagen* because they differed from other human species. This is not the occasion to discuss the etiological role of mycoplasmas in leukemia, but to avoid any suggestion that *M. pulmonis* in particular is associated with leukemia I would like to point out that *M. fermentans* has also been isolated by means

TABLE 2. BIOLOGICAL PROPERTIES OF HUMAN MYCOPLASMAS

Species (or strain)	Colonial morphology	Growth		Glucose fermentation	Hemolysis	"Film and spots"	Tetrazolium reduction	Splitting of Arginine	Inhibition by 0.002% methylene blue
		Aerobic	Anaerobic						
M. pneumoniae	Granular	+ slow	+ slow	+	+ (β)	−	+	−	−
M. hominis	"Fried egg"	+	+	−	− or ±	−	−	+	+
M. fermentans	"Fried egg"	Tr	+	+	− or ±	+	−	+	+
M. salivarium	"Fried egg"	Tr	+	−	− or ±	− or +	−	+	+
M. orale Type 1	"Fried egg"	±	+	−	±	...	−	+	+
M. orale Type 2	"Fried egg"	±	+	−	±	...	...	+	...
"T strains"	Tiny	−*	+	−	...	−	...	−†	...

*Grows aerobically in fluid media.
†Metabolizes urea.
... No observations available.

of tissue culture (20) and that the few successful isolations from bone marrow directly on to cell-free media were of *M. orale* (1, 14).

Butler and Leach (2) described an agent that appeared suddenly in an HEp-2 cell line during maintenance. This agent, called GDL, has been subsequently identified as *M. hyorhinis*, a pathogen for pigs (22, 31). There have been a number of reports of the isolation of GDL-like organisms from tissue cultures of human tumors (16). Bartholomew (to be published), using preliminary passage in tissue culture, isolated mycoplasmas from rheumatoid arthritis and lupus erythematosis; these were also identified as *M. hyorhinis*. An isolate similarly obtained through tissue culture from Reiter's syndrome was found to be Campo-like. Three isolations of Campo-like strains were made directly in cell-free media by Jannson and Wager (to be published), two of them from the joints of rheumatoid arthritis patients. As stated previously, Campo-like strains belong to the species *M. arthritidis*. Earlier reports of the presence of this species in the genital tract should be regarded with suspicion.

These reports are summarized in Table 3. *M. pulmonis*, a pathogen for rats and mice, *M. hyorhinis*, a pathogen for pigs, and *M. arthritidis*, a cause of polyarthritis in rats, have been isolated from human material after preliminary passage in tissue culture. There is one recent report of the isolation of *M. arthritidis* directly in cell-free media. These isolations may have resulted from contamination or other technical errors. Only future work will show whether mycoplasmas pathogenic for animals do in fact inhabit human tissues and to what extent they are significant as a cause of disease.

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TABLE 3. REPORTED ISOLATIONS OF ANIMAL MYCOPLASMAS FROM MAN

Species	Disease	Method of isolation	Habitat in animal	Pathogenicity
M. pulmonis	Leukemia	After T.C. passage	Respiratory tract, rats and mice	Pneumonia
M. hyorhinis	Tumors, arthritis, etc.	After T.C. passage	Respiratory tract, pigs	Sometimes arthritis
M. arthritidis Campo-like	Reiter's syndrome	After T.C. passage	Rats	Polyarthritis
M. hominis Type 2′	Rheumatoid arthritis	Directly on cell-free media		

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SECTION E. MYCOPLASMA VACCINES

FIELD EVALUATION OF KILLED *M. PNEUMONIAE* VACCINE*

WILLIAM J. MOGABGAB

Department of Medicine

Tulane University School of Medicine, New Orleans, Louisiana, U.S.A.

DR. MOGABGAB (*presenting the paper*): The amount of illness, especially pneumonia, caused by *Mycoplasma pneumoniae* in young airmen at Keesler Air Force Base, Mississippi, provided a need for as well as an opportunity to evaluate means of prevention (1-3). Because of the ready growth of this organism in media suitable for vaccine preparation for human administration, an inactivated vaccine was the most direct approach. This type of vaccine had been shown to be well tolerated in man and it was capable of evoking an antibody response (1, 4, 5). Antigenicity as well as protective effects of an inactivated *M. pneumoniae* vaccine administered to young military personnel are described in this paper.

MATERIALS AND METHODS

Population. Young airmen 18 to 21 years of age in training at Keesler Air Force Base on the Mississippi Gulf coast for periods of 6 to 12 months were housed in dormitories, usually three or four men in a room. These men had completed basic training during a period of a few weeks at another base and were now engaged in technical and classroom work. Medical facilities, including outpatient clinics, were mostly concentrated in the hospital. Data for illness rate determinations and for special case studies are derived from the men who appeared voluntarily for medical care. Specimens for laboratory study were obtained each week from 15 to 25 individuals who had respiratory disease of four days' or less duration. These people served as a sample of the illness spectrum for each period. The clinical features of the cases were tabulated by a nurse who had been trained for this purpose. The following classification system was used: (a) URI, afebrile—common-cold-like with temperature $< 101°F$; (b) URI, febrile—influenza-like with temperature $> 101°F$; (c) pharyngitis with dysphagia; (d) laryngitis tracheitis, or bronchitis, and (e) pneumonia. The diagnosis of pneumonia depended on the demonstration of definitive infiltrates in chest roentgenograms (10). As controls, persons with a variety of other conditions—allergies, gastroenteritis, chronic cough, sinusitis, otitis media, and no obvious disease—were included.

Most of the trainees arriving at Keesler Air Force Base between March 1964 and June 1966 participated in the vaccine trials. Men were omitted at intervals only when the vaccine supply was exhausted. Immunization was usually performed during the first few days of reception on the base. Vaccine and placebo were administered on the basis of the last digit of the service number.

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Data processing by the Division of Medical Computing Sciences, Tulane University School of Medicine.

Vaccine and administration. Inactivated alum-adsorbed *Mycoplasma pneumoniae* vaccine was prepared by the Merck Institute for Therapeutic Research (5). The organism was propagated in a serum-free artificial liquid medium, concentrated and refined, inactivated with formalin, and incorporated into alum adjuvant. Vaccine and placebo were administered intramuscularly to alternate individuals in a dose of 1.0 ml. After October 1965 a second injection was given one month later to some of the men. All lots of vaccine had previously been shown to be antigenic in animals (5). Pre- and post-vaccine blood specimens were obtained from a sample of the men inoculated with each lot to determine antibody response. In addition to the men reporting to the hospital with possible vaccine reactions, 15 individuals reported to the immunization clinic until the fourth day for checking of local or systemic reactions.

Data processing. Names, service numbers, date of immunization, and other information on each vaccinee was recorded on tape at the Tulane Biomedical Computer Center. Data on change in status of the individual or departure from the base were also noted. In addition, information on all subsequent visits to the hospital for any type of respiratory disease, diagnoses, and days hospitalized were included for analysis.

Recovery of M. pneumoniae. Recovery was made from throat swabs and pharyngeal washings. Throat swabs were streaked on 0.002 per cent methylene blue agar plates, and pharyngeal washings with Eagle's solution were inoculated into diphasic PPLO broth cultures containing 20 per cent horse serum, Fleischmann's 20–40 yeast extract, 1 per cent glucose, penicillin, thallium acetate, and amphotericin (6–8). Identification was accomplished by subculture after 10 days' incubation to PPLO-agar plates followed by overlay with 10 per cent sheep's blood agar for observation for beta-hemolysis. Reduction of 2-, 3-5-triphenyltetrazolium chloride (TTC) was also used (4).

Antibody determinations. Antigen for complement fixation was prepared by growth of *Mycoplasma pneumoniae* strain FH in PPLO broth for 16 days, which was then treated with phenol or treated by boiling (9). In testing, two units of antigen and two exact units of complement were used with fixation overnight at 4°C. Neutralizing antibody was measured by colony reduction with sera that had been inactivated at 56°C for 30 minutes. Approximately 100 colonies per plate were used as the challenge dose, with one hour at 37°C as combination time for the agent with antibody prior to plating. Convalescent sera reduced the colony counts tenfold or more. Subsequently, neutralizing antibody was determined by mixing 10 infectious doses of *M. pneumoniae* with twofold dilutions of inactivated serum in 1.0 ml of PPLO broth (unheated horse serum). Growth of the agent in each culture was then determined by reduction of tetrazolium, and titers were calculated on the basis of initial dilution of serum-inhibiting growth (9).

Antigenicity of vaccine. Blood samples were obtained at the time of vaccination and three weeks thereafter; they were also taken three weeks after the second injection, when given. Antibody response was determined by complement fixation and by neutralization through inhibition of tetrazolium reduction. In some instances neutralizing antibody was determined by colony reduction. In all cases serum was inactivated at 56°C for 30 minutes, but the PPLO medium contained 20 per cent unheated horse serum.

RESULTS

Antibody responses following vaccination. The amount and distribution of complement-fixing and neutralizing antibody responses from successive lots of *M. pneumoniae* vaccine are shown in Table 1. Neutralization was a more sensitive index of antibody increments than complement fixation. Antibody determination using tetrazolium-reduction inhibition (TRI) provided quantitative results that were not obtained from the colony reduction procedure. Variations in potency of different lots of vaccine were not appreciable, but an increase in the size of the dose or a second injection one month later both caused an increase in response. In order to preclude the possibility of inhibition by nonspecific substances, no serum supplements, fresh or heated, were used in these antibody determinations.

To predict the degree of efficacy of *M. pneumoniae* vaccine, the distribution of initial anti-

TABLE 1. ANTIBODY RESPONSE TO INACTIVATED MYCOPLASMA PNEUMONIAE VACCINE IN YOUNG AIRMEN, KEESLER AIR FORCE BASE, MISSISSIPPI, 1964-1966

Dose			CF antibody increment							Colony reduction		TRI neutralizing antibody increment								
			Totals	0	2x	4x	8x	16x	32x	64x	Totals	No.	Totals	0	2x	4x	8x	16x	32x	64x	>64x
1.0 ml	No.	V	268	108	48	69	25	10	6	2	210	131	268	73	4	50	38	36	28	7	32
		P	257	240	11	3	2	1			197	9	257	243	4	3	3	1	3		
	%	V		40	18	26	10	4	2	1	62			27	2	19	14	13	10	3	12
		P		93	4	1	1	1			5			95	2	1	1	1	1		
1.0 ml x 2 (1 mo. apart)	No.	V	60	23	21	10	4	1	1				58	12	2	6	7	11	8	3	9
		P	45	32	8	3	2						45	38	3	3	1				
	%	V		38	35	17	7	2	2					21	3	10	12	19	14	5	16
		P		71	18	7	4							84	7	7	2				

TRI = Tetrazolium reduction inhibition; V = Vaccine; P = Placebo.

body titers in individuals with subsequent increments in convalescent sera was compared to the distribution in persons with none. Complement-fixation and tetrazolium-reduction inhibition were used for determination of amount of antibody. Low titers were probably protective, since most of the men with antibody increases had lower initial titers than those who did not demonstrate a rise (Table 2).

Reactions to vaccination. Reactions to administration of vaccine consisted of tenderness to palpation of the injected site in about a fifth of

TABLE 2. DISTRIBUTION OF MYCOPLASMA PNEUMONIAE ANTIBODY TITERS IN AIRMEN WITH PNEUMONIA, KEESLER AIR FORCE BASE, MISSISSIPPI, 1965–1966

By complement-fixation (no. with antibody titer)

		<4	4	8	16	32	64	128	256	>256	Total
With antibody rise	No.	79	10	23	8	11	1				132
	%	59.8	7.6	17.4	6.1	8.3	0.8				
No antibody increment	No.	51	16	32	29	22	18	8	2	20	198
	%	25.8	8.1	16.2	14.6	11.1	9.1	4.0	1.0	10.1	

By TRI neutralization (no. with antibody titer)

		<4	5.7	11.3	>16	Total
With antibody rise	No.	39		2		41
	%	95.1		4.9		
No antibody increment	No.	52	11	12	93	168
	%	30.9	6.5	6.5	56.4	

TABLE 3. COMPARISON OF VARIOUS DIAGNOSTIC PROCEDURES FOR MYCOPLASMA PNEUMONIAE INFECTION IN YOUNG AIRMEN WITH RESPIRATORY DISEASE, KESSLER AIR FORCE BASE, MISSISSIPPI, 1959-1966

Diagnostic procedure	With antibody response by						With isolation	
	Complement-fixation		Colony reduction		TRI neutralization			
	+/No.	%	+/No.	%	+/No.	%	+/No.	%
Complement fixation	536/3249	16.5	28/55	50.9	37/49	75.5	149/245	60.8
Colony reduction	28/60	46.7	58/396	14.6			23/59	39.0
TRI neutralization	37/112	33.0			49/251	19.5	22/61	36.1
Isolation	149/328	45.4	23/44	52.3	20/48	41.7	277/2122	13.1

Sera were inactivated at 56°C for 30 minutes for all procedures.
TRI = Tetrazolium-reduction inhibition.
Number positive/total determinations is underlined for each procedure.

the men examined on the following day. Immediate pain was not observed. Considerable variation in different vaccine lots was noted. Systemic reactions were rare and could not be clearly associated with the vaccine.

Evaluation of diagnostic procedures for M. pneumoniae *infections.* The results obtained from various techniques for antibody titer determinations and from isolation of the organism are shown in Table 3. Complement fixation appeared most sensitive in determining serologic response to infection. Certainly it was the most practical technique. Nevertheless, any of the procedures used were successful for detecting from a half to two thirds of the infections. Another indeterminate factor in laboratory diagnosis was the time of onset of infection. Many of the individuals had had some symptoms of an upper respiratory illness for one to three weeks prior to onset of the acute sickness under study. Another problem was antibiotic administration, especially in the case of erythromycin, which caused growth-inhibition serum titers of 1:64 to 1:256 in the TRI neutralization test. Antibiotic administration undoubtedly affected recovery of the organism as well (10).

Table 4 affords an analysis of complement fixation and isolation as determinants of infection by *M. pneumoniae.* A crude antigen consisting of broth cultures treated with phenol was

TABLE 4. SEROLOGY AND RECOVERY OF MYCOPLASMA PNEUMONIAE AS DETERMINANTS OF INFECTION, KEESLER AIR FORCE BASE, MISSISSIPPI, SEPTEMBER 1959-JUNE 1966

Clinical categories	Diagnostic procedures				
	Complement fixation		Isolation		Complement fixation or isolation
	All	With isolation positive	All	With CF positive	
	No. positive/No. cases				
URI outpatients	110/1862	28/62	90/1230	28/72	106/1169
URI hospitalized	39/508	8/16	25/261	8/15	23/252
Total percentage positive	6.3	46.2	7.7	41.3	9.1
Pneumonia	387/879	113/250	162/631	113/158	292/623
Percentage positive	44.0	45.2	25.7	71.5	46.9
Totals	536/3249	149/328	277/2122	149/245	421/2044
Percentage positive	16.5	45.4	13.1	60.8	20.6

TABLE 5. REDUCTION IN MYCOPLASMA PNEUMONIAE INFECTIONS IN VACCINATED AIRMEN WITH RESPIRATORY ILLNESSES, KEESLER AIR FORCE BASE, MISSISSIPPI, MARCH 1964-JUNE 1966

Clinical classification	Serology			Isolation		
	Vaccine	Placebo	Percentage of reduction	Vaccine	Placebo	Percentage of reduction
	No. of cases			No. of cases		
URI, afebrile	9	8	0.0	11	14	21.4
URI, febrile	1	7	85.7	1	5	80.0
Pharyngitis	0	1	100.0	1	1	0.0
Bronchitis	0	2	100.0	0	1	100.0
All URI	10	18	44.5	13	21	38.1
Pneumonia	46	72	36.1	22	33	33.4

*Onset of illness was more than a month after vaccination with a single dose of 1.0 ml.
†By complement fixation.

used. Probably a purified complement-fixing antigen with less anticomplementary activity, as described by Kenny *et al.*, would have resulted in greater sensitivity (11). Fragility of the organism and requirements for growth lowered the sensitivity of isolation as a diagnostic procedure.

Determination of vaccine effectiveness in airmen with laboratory evidence of infection by M. pneumoniae. Results of laboratory studies on airmen with respiratory disease are shown in Table 5. Reduction in incidence of pneumonia, although quite appreciable at 36 per cent, probably could have been greater, since cases documented by antibody response were found after vaccination. Conversely, there were more individuals without serologic evidence of infection in the placebo group than in the vaccinated group. This might have been predictable, since the serologic diagnosis methods have not been sufficiently sensitive. Also, many individuals were first seen one to two weeks after onset of illness, when antibody titers had already risen. In fact, reduction in the incidence of serology-negative illnesses provided an index of the number of infections that were not detected by these procedures.

Similar results were obtained from analysis based on recovery of the organism (Table 5). Thus, the protective effect of the vaccine was demonstrable with two different laboratory diag-

TABLE 6. EFFECT OF MYCOPLASMA PNEUMONIAE VACCINE IN YOUNG AIRMEN, KEESLER AIR FORCE BASE, MISSISSIPPI, MARCH 1964-JUNE 1966 *

Clinical class†	Vaccine	Placebo	Vaccine	Placebo	Percentage of reduction in rate
	No. of cases		Rate/1000		
URI, afebrile	704	680	65.2	65.4	0.0
URI, febrile	156	159	14.4	15.3	1.9
Pharyngitis	439	481	40.7	46.2	8.7‡
Bronchitis	45	68	4.2	6.6	33.8‡
All URI	1344	1388	124.5	133.4	3.2‡
Pneumonia	85	149	7.9	14.3	42.9‡

*Vaccine: 10,797; placebo: 10,402. Single injection, 1.0 ml intramuscularly.
†Illnesses that occurred more than six months after vaccination were excluded.
‡Significant at the 95 percent confidence level.

nostic procedures for infection by *M. pneumoniae*, but with both procedures vaccine failures were observed.

Protective effect of Mycoplasma pneumoniae *vaccine as determined by rates of upper-respiratory illnesses and pneumonia in vaccinated and placebo groups.* Occurrences of respiratory diseases according to clinical classification are shown in Table 6. Except for bronchitis and, to a lesser degree, pharyngitis, there was little reduction in incidence of upper respiratory illnesses. However, the protective effect of the vaccine on pneumonia rates was quite evident, as shown by the marked decrease among the vaccinated airmen.

Since these results were based on clinical diagnoses (including chest roentgenograms), but without specific laboratory evidence of *M. pneumoniae* infection, they may fail to represent the full effectiveness of the vaccine. Nevertheless, this was not demonstrable by analysis of the illnesses that received complete laboratory study. Such results suggest that most of the pneumonia was caused by *M. pneumoniae* and that the vaccine did not produce immunity in all instances.

DISCUSSION

In the absence of sufficient knowledge of the biology of *M. pneumoniae*, an inactivated vaccine was a more practical approach to prophylaxis than the attenuated live vaccines or antibiotic suppression. Obviously these and other means of prevention must also be investigated. On the other hand, inactivated vaccine can be prepared in sterile medium and without living cells or animal serum for growth of the organism, thus avoiding many vexing problems of production. In addition, the scarcity of local tenderness, febrile reactions, or other toxic effects from administration of inactive vaccine provided another incentive for its use. The fact that most of the pneumonias in young airmen in training were caused by *Mycoplasma pneumoniae* indicated a need for definitive action.

Determination of the antigenic potency of this vaccine for man presented problems, since there was no means of measuring antigenic content directly and the fraction that stimulated protective antibody was not known. Complement fixation proved to be the most practical approach for rapid evaluation of the potency of successive lots of vaccine even though it underestimated the number of responders and the amount of antibody stimulated. Determination of neutralizing antibody by colony-reduction did not supply quantitative information, but tetrazolium-reduction inhibition did provide these data and was a much more sensitive means of detecting responders. This method was also suitable for deriving an index of the amount of antibody that could be considered protective.

Whether or not the protective effect of inactive *Mycoplasma pneumoniae* vaccine can be increased by concentration of the antigen has not yet been determined. Antibody response was enhanced by this procedure, but sufficient increase in potency to prevent most of the infections was not achieved. It did not seem likely that more concentrated preparations would cause serious local or systemic toxic reactions. Although a booster dose appeared to be a reasonable solution, the percentage of responders did not increase markedly. However, the number of men who received a second injection was inadequate for evaluation of an index of protection. The administrative problems of a two-dose program in a military population with an intensive training schedule make this method less desirable and probably account for the small number of men who received both injections.

Summary

An inactivated *Mycoplasma pneumoniae* vaccine was administered parenterally to young airmen in training. Over 21,000 men received alternately vaccine or placebo during the period from March 1964 to April 1966. Local or systemic reactions to the vaccine were inconsequential. Almost three fourths of the men demonstrated a neutralizing antibody response following a single injection of vaccine. A second dose did not increase the level appreciably. Fewer responders were shown by complement-fixing antibody.

Serology and isolation of the organism showed, respectively, a 36 and a 33 per cent reduction in the number of men with pneumonia. However, the over-all lowering of the pneumonia rate was 43 per cent. This discrepancy was explainable on

the basis of lack of sensitivity of the diagnostic procedures and by failure to detect many of the illnesses until the first or second week after onset.

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SECTION E. MYCOPLASMA VACCINES

MYCOPLASMA PNEUMONIAE INFECTION— PROSPECTS FOR LIVE AND INACTIVATED VACCINES

R. M. Chanock, C. B. Smith, W. T. Friedewald, R. Gutekunst, P. Steinberg, S. Fuld, K. E. Jensen, L. B. Senterfit, and B. Prescott *

DR. CHANOCK (*presenting the paper*): *Mycoplasma pneumoniae* is an important lower respiratory tract pathogen of man (1, 2, 7, 10). It is a common cause of bronchitis and/or pneumonia, particularly in the 5-to-30 year old age group (2, 7). The need for an effective vaccine is quite clear: morbidity has been estimated at one pneumonia illness per 1,000 persons per year in the civilian population (1), and this figure probably represents an underestimate of considerable magnitude. In addition, there are several high risk populations, such as military recruits and college students, in whom morbidity may exceed that of the general population by as much as twentyfold (3). Finally, an effective vaccine is needed because of the often prolonged interval before illness caused by *M. pneumoniae* is identified correctly and therapy can be instituted.

PROPAGATION OF ORGANISMS FOR VACCINE PRODUCTION

In the initial studies in which *M. pneumoniae* was identified as a mycoplasma, a complex medium was employed for growth of the organism (4). It contained a high concentration of horse serum (20 per cent), yeast extract (2.5 per cent), and an infusion of beef heart (4). Although useful for experimental and serodiagnostic studies, such a medium was clearly not suitable for the preparation of either a live or an inactivated vaccine because of the danger of sensitization, especially to horse serum antigens. Two different solutions to this problem have recently been developed.

First, it was found that the organism grew to moderately high titer when a chloroform extract of egg yolk was substituted for horse serum and a chemically defined tissue culture medium was substituted for beef heart infusion (11). In this manner, sensitizing components of the original medium were replaced by materials safe for topical or parenteral administration. The egg yolk extract medium was used for the production of several lots of experimental inactivated vaccines that were tested in 11,693 young adults with no evidence of untoward effect (9).

Second, it has been found that under proper conditions *M. pneumoniae* can be adapted to grow on a glass surface as a confluent layer of mycoplasmas (22). Figure 1 shows a large bottle in which *M. pneumoniae* has grown as a confluent layer. Figure 2 illustrates the dynamics of mycoplasma growth on a glass surface. With an inoculum of $10^{9.5}$ colony forming units (CFU), maximum growth of viable organisms is attained by the fourth to fifth day in horse serum-yeast extract-beef heart infusion medium. During subsequent incubation the number of viable organisms decreases, but the total mass of antigen (measured by complement fixation) increases for another three to four days. The organisms that have grown on the glass adhere

* Drs. Chanock, Smith, Friedewald, and Prescott (Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Maryland); Drs. Gutekunst, Steinberg, and Fuld (Naval Medical Field Research Laboratory, Camp Lejeune, North Carolina); and Drs. Jensen and Senterfit (The Chas. Pfizer Company, Inc., Terre Haute, Indiana).

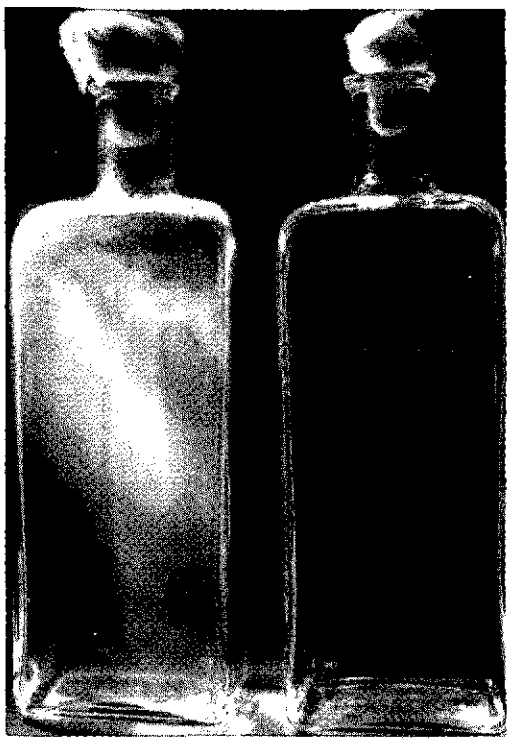

Fig. 1. *Mycoplasma pneumoniae* colonies attached to glass surface of Povitsky bottle. Left—Layer of *Mycoplasma pneumoniae* on glass surface, culture fluids decanted. Right—Uninoculated broth control, culture fluids decanted. Both surfaces stained by Dienes method.

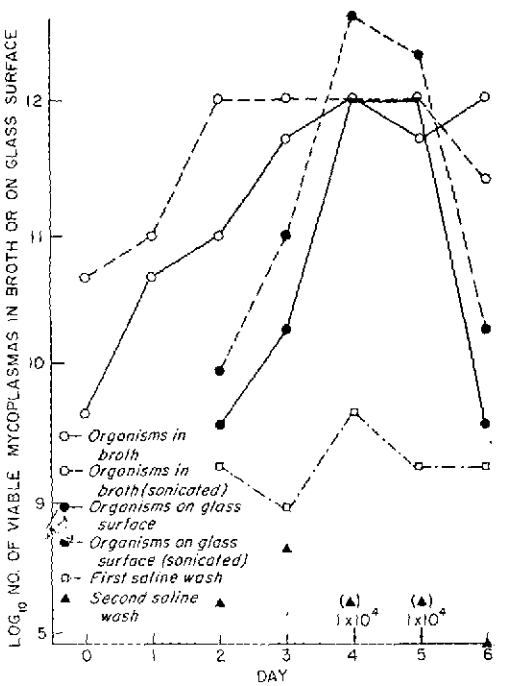

Fig. 2. Growth curve of *Mycoplasma pneumoniae* cultured in Povitsky bottles.

tenaciously to the surface of the bottle and resist removal by multiple washings with saline or balanced salt solution (Fig. 2). Divalent cations do not appear to be involved in the attachment process, since versene does not remove the organisms from the glass surface. However, they can be removed either by scraping or by treatment with 0.25 per cent trypsin solution.

Large quantities of organisms can be produced by the glass surface technique. For example, approximately 287 mg of wet weight of organisms can be grown routinely in a Povitsky bottle that has 540 cm^2 of glass surface available for mycoplasma growth. Organisms on glass can be washed free of detectable traces of growth medium constituents and then suspended in buffered saline to the desired concentration. This method has been used for the production of experimental vaccine lots in which the residual serum concentration was reduced to 4×10^{-11} by multiple washings of the glass-surface-grown mycoplasma sheet. Using a sensitive guinea pig anaphylaxis technique, we have been unable to detect the presence of serum in such preparations (17).

CORRELATION OF GROWTH-INHIBITING ANTIBODY WITH RESISTANCE TO *M. PNEUMONIAE* INFECTION AND ILLNESS

Before vaccines could be evaluated for immunogenicity it was necessary to determine which antibodies to *M. pneumoniae* correlated with resistance to infection and illness. In a series of volunteer studies involving 155 men we observed a significant correlation between growth-inhibiting antibody (measured by the tetrazolium-reduction inhibition technique) and resistance to febrile illness caused by *M. pneumoniae* (Table 1). This correlation was evident for illness produced by organisms grown in tissue culture and by those grown in mycoplasma broth medium. Immunofluorescent antibody also exhibited a significant correlation with resistance, whereas indirect hemagglutination antibody did not (23).

TABLE 1. PROTECTIVE EFFECT OF GROWTH-INHIBITING ANTIBODY AGAINST EXPERIMENTALLY INDUCED M. PNEUMONIAE ILLNESS

Challenge strain (10^6 CFU)	Pre-existing growth-inhibiting antibody*	No. of men	No. who developed febrile respiratory tract disease		
			With pneumonia	Without pneumonia	Total
PI 898 monkey kidney culture passage 2	No	25	3	10	13
	Yes	27	0	0	0
FH broth passage 9 or PI 1428 broth passage 4	No	14	0	2	2
	Yes	9	0	0	0
FH broth passage 12 or 55 or PI 1428 broth passage 2, 25 or 27	No†	45	3	14	17
	Yes†	35	0	3	3
Total	No	84	6	26	32
	Yes	71	0	3	3

*Presence of growth-inhibiting antibody in undiluted serum.
†Tests performed with 5 per cent inactivated guinea pig serum in medium; other tests performed without this additive.

Two recent epidemiologic studies of *M. pneumoniae* infection in a population of military recruits indicated that growth-inhibiting antibody was associated with resistance to naturally occurring infection (3). Serum was obtained from 227 men prior to their exposure to the organism, and the presence and level of growth-inhibiting antibody were determined (Table 2).

In the first study infection occurred two to three times more often among recruits who lacked antibody than among those who possessed it. However, approximately one fourth of the men with pre-existing antibody became infected, which suggests that reinfection was not uncommon in this population. Thus the protective effect of antibody was only relative, not absolute.

In the second study shown in Table 2 10 men had high levels of pre-existing antibody—1:64 or greater. These men apparently escaped infection, since we were unable to detect an antibody rise or to recover the organism from oropharyngeal swab specimens collected at weekly intervals.

TABLE 2. M. PNEUMONIAE INFECTION AND ASSOCIATED PNEUMONIA IN SEVEN PLATOONS OF MARINE RECRUITS STUDIED DURING MAY-OCTOBER 1965 AT PARRIS ISLAND, S.C. AND CAMP LEJEUNE, N.C.

Study	Interval	Growth-inhibiting antibody at start of training	No. of recruits	Infection detected by rise in growth-inhibiting antibody		No. *M. pneum.* associated pneumonias
				No. of men	Percentage	
1	May–Sept. (14 wks.)	No*	45 (49%)	21	58†	2
		Yes	47	10	23‡	0
		Total	92	31	41	2
2	July–Oct. (14 wks.)	No*	41 (30%)	33	80	2
		Yes	94	22	23	0
		Total	135	55	41	2

*Antibody not detected in undiluted serum.
†Forty-five recruits studied during first nine weeks of training; thereafter only 26 men studied; indicated percentage equals combined percentage of both phases of study.
‡Similar to †; 47 recruits studied during first nine weeks and 31 studied during subsequent five weeks of training.

Complete resistance in this setting was impressive, for the men with high antibody levels were exposed to a challenge that successfully infected 80 per cent of the seronegative recruits. These findings suggest that highly immunogenic vaccines might well be capable of eradicating *M. pneumoniae* from populations with a high incidence of infection.

Growth-inhibiting antibody was also associated with resistance to illness caused by *M. pneumoniae*. During a 14-week period of surveillance, 4 of the 86 recruits who lacked antibody developed a pneumonia associated with *M. pneumoniae* infection, whereas such illness was not detected in the group of 141 men with pre-existing growth-inhibiting antibody (Table 2).

Growth-inhibiting antibody may not be solely responsible for resistance to infection and illness. Possibly another immune mechanism, which develops parallel to growth-inhibiting antibody, may be more important. In any case, growth-inhibiting antibody is a useful barometer of host resistance to *M. pneumoniae* illness, and for this reason we have chosen to use it to evaluate the immunogenicity of experimental vaccines.

INFECTIVITY AND VIRULENCE OF TWO STRAINS OF *M. PNEUMONIAE* AT DIFFERENT PASSAGE LEVELS IN MYCOPLASMA GROWTH MEDIUM

Previous studies in volunteers indicated that serial passage of *Mycoplasma pneumoniae* in broth medium was associated with a decrease in the virulence and infectivity of this organism in man (5). Hopes were raised that an attenuated vaccine could be developed using organisms propagated for a certain number of passages in broth medium. Unfortunately, subsequent studies have not justified our original optimism, but this approach to immunoprophylaxis is certainly far from exhausted.

A summary of recent experience with organisms propagated in broth medium is shown in Table 3. In these studies each of the volunteers lacked detectable growth-inhibiting antibody prior to administration of *M. pneumoniae*. After serial passage in broth medium both the FH and PI-1428 strains exhibited decreased infectivity in man (19). Early passage material of both strains infected all the volunteers tested, whereas only some of the volunteers were infected with the 55th passage of the FH strain or the 27th passage of the PI-1428 strain. Although the infectivity of the high passage FH and PI-1428 suspensions was reduced, five of the volunteers infected with these materials developed respiratory tract illness, in two instances associated with fever. From this we conclude that virulence and infectivity were not completely dissociated during serial passage of the organism in broth medium. For this reason, the high-passage suspensions that were evaluated did not appear suitable for use as immunizing agents.

Continued attempts to select attenuated variants of *M. pneumoniae* appear indicated in the

TABLE 3. RESPONSE OF VOLUNTEERS WHO LACKED DETECTABLE GROWTH-INHIBITING ANTIBODY TO INFECTION WITH TWO STRAINS OF M. PNEUMONIAE

Strain of *M. pneumoniae**	Passage Level in broth or agar medium	Number of antibody-negative men†				
		Challenged	Infected‡	Illness		
				Total	Febrile	Pneumonia
FH	12	9	9 (100%)	3	1	0
	55	8	3 (38%)	3	1	0
	2	22	22 (100%)	17	11	3
PI–1428	25	13	13 (100%)	7	5	0
	27	6	4 (66%)	2	1	0

*Inoculum contained approximately 10^6 colony forming units (CFU).
†Growth-inhibiting antibody not detectable in undiluted serum.
‡As determined by fourfold or greater rise in growth-inhibiting antibody 3-4 weeks following challenge.

light of recent findings with *M. mycoides*, the agent of bovine pleuropneumonia, and *M. mycoides var. capi*, a cause of pneumonia in goats. Single colonies were picked from avirulent and virulent strains of these organisms. The progeny of these single colonies were then tested for virulence in the appropriate susceptible host (13). A virulent strain of *M. mycoides* was found to be a mixed population containing avirulent as well as virulent organisms (13). Similarly, an attenuated strain of *M. mycoides* was shown to be a mixed population containing both avirulent organisms and organisms of moderate virulence. Perhaps high-passage suspensions of *M. pneumoniae* are heterogeneous with respect to virulence. For this reason it would be worthwhile to continue the search for an avirulent strain of *M. pneumoniae* by testing the progeny of single colonies derived from a high passage broth grown suspension of this organism.

INACTIVATED VACCINES

Preparation and immunogenicity. At present the development of an acceptable live attenuated vaccine does not appear imminent. In contrast, the prospects for a safe, highly immunogenic, inactivated vaccine are excellent. During the past three years inactivated vaccines have been prepared by two different techniques. The first vaccines were prepared from organisms grown in broth containing a chemically defined solution of amino acids and vitamins (Eagle's medium or Leibovitz's medium) enriched with a chloroform extract of egg yolk. After maximal growth in this medium the organisms were inactivated by formalin and then concentrated by centrifugation (11). More recently, vaccines have been prepared from organisms grown on glass using a medium containing calf serum, yeast extract, and a chemically defined solution of amino acids and vitamins. After mycoplasma growth on the glass surface reached a maximal level, the organisms were washed free of broth medium constituents. Concentration was achieved by scraping the organisms from the glass and suspending them in a small volume of buffered saline. The concentrated purified suspension was then inactivated with formalin (18).

The relative potencies of vaccine lots grown in egg yolk extract broth and on a glass surface are shown in Table 4. In rabbits, the egg yolk extract vaccine lots were comparable in potency to the first glass-surface grown vaccines (lots 799 and 808). Apparently the latter preparations had been harvested too early, for when a longer period of incubation was employed (for example, see lot 821 in Table 4) immunogenicity for rabbits was greatly increased. A preparation with such increased immunogenicity could be diluted 1:500 and still stimulate the development of growth-inhibiting antibodies in all inoculated rabbits. Indeed, rabbits injected with undiluted vaccine prepared in this manner developed a mean antibody titer of 1:2048 or greater.

Potency tests were also performed in hamsters, which were injected with varying dilutions of vaccine and later challenged with 10^3 CFU of *M. pneumoniae*. Failure of the challenge suspension to grow in the hamster lung was interpreted as evidence of resistance induced by the vaccine. In hamsters the glass-surface-grown vaccines were more potent than the egg yolk extract vaccines (Table 4). The glass-surface-grown vaccines induced resistance to pulmonary multiplication of *M. pneumoniae* in 65 to 96 per cent of hamsters injected with a 1:125 dilution of vaccine.

When the vaccines shown in Table 4 were tested for immunogenicity in man an anomalous result was observed. The egg yolk extract vaccine lots were more potent in man than the glass-surface-grown vaccines, despite the latter vaccines' higher concentration of organisms and their greater immunogenicity in rabbits and hamsters. The explanation of this difference in immunogenicity for man and animals is not clear. Conceivably, the egg yolk extract provides an adjuvant effect in man but not in rabbits or hamsters. The composition of the growth medium is known to influence the lipid composition of the membrane of *M. laidlawii* (16). Another possibility is that the egg yolk extract favors the production of a more highly antigenic lipoprotein membrane than does the medium used for glass-surface propagation of *M. pneumoniae*. These two possibilities are currently under investigation.

Alum appeared to enhance the immunogenicity of a glass surface grown vaccine that was initially of low potency (Table 4). Similar reports of an adjuvant effect of alum have been described by others (12).

TABLE 4. EVALUATION OF M. PNEUMONIAE VACCINES IN LABORATORY ANIMALS AND MEN

Vaccine			Immunogenicity in laboratory animals				Immunogenicity in antibody-free volunteers†	
			Rabbits			Hamsters		
Type	Lot No.	Titer of *M. pneumoniae* before formalin inactivation CFU/ml*	Highest dilution of vaccine that stimulated growth-inhib. antib. in all animals	Arithmetic mean titer of growth-inhib. antibody 4 weeks after injection of vaccine		Percentage resistant to *M. pneumoniae* challenge 15 days after injection with 1:125 dilution vaccine	No. tested	No. who developed growth-inhibiting antibody
				Undiluted	1:125			
Egg yolk broth	40	1×10^6	1:5	128	2	20	28	17 (61%)
	295	1×10^6	1:25	272	6	35	12	7 (58%)
Glass surface grown organism	799	5×10^8	1:5	50	—	75	14	5 (36%)
	808	5×10^8	1:5	66	2	65	17	3 (18%)
	821	5×10^{10}	1:500	>2048	290	93	13	2 (15%)
	821 plus alum	5×10^{10}	1:500	>2048	544	86	12	7 (58%)

*CFU = Colony forming units/ml.
†Growth-inhibiting antibody not detectable in undiluted serum.

Protection against experimental disease. Earlier in this presentation we indicated that naturally induced growth-inhibiting antibody correlated with resistance to *M. pneumoniae* infection and illness. To determine whether vaccine-induced antibody exhibited a similar correlation with resistance, 19 adult volunteers were given two 0.5 ml injections of lot 40 egg yolk extract vaccine (20). This vaccine was inactivated with formalin and the organisms were concentrated by centrifugation. Each of the 19 volunteers lacked detectable growth-inhibiting antibody prior to injection of the vaccine. Ten men responded to the vaccine by developing detectable levels of growth-inhibiting antibody (Table 5). When challenged with 10^6 CFU of a virulent sus-

TABLE 5. PROTECTIVE EFFECT OF GROWTH-INHIBITING ANTIBODY INDUCED BY INACTIVATED M. PNEUMONIAE VACCINE

Material injected	Antibody detectable 6 wks. after injection, i.e., at the time of challenge*	No. of men	No. who became ill following challenge with 10^6 CFU of PI–1428 pass. 2 *M. pneumoniae*			
			Total	Pneumonia	Febrile respiratory disease	Afebrile tracheobronchitis or systemic symptoms§
Inactivated vaccine	Yes	10	1†	0	1	0
	No	9	7	3	7‡	0
None or placebo	No	13	10†	0	4‡	6

*Each man lacked detectable growth-inhibiting antibody prior to administration of vaccine or placebo.
†Significant difference $P < .004$ (Fisher exact test).
‡Significant difference $P < .05$ (Fisher exact test).
§Malaise and/or headache.

pension of *M. pneumoniae,* only one of the 10 men who had developed vaccine-induced antibody became ill. This man developed a brief febrile illness. In contrast, 10 of 13 antibody-negative men who did not receive vaccine became ill. Although the numbers involved were small, the difference in illness rates between the men who responded to the vaccine and the control volunteers was statistically significant ($P < .004$). This difference suggested that vaccine induced growth-inhibiting antibody was associated with significant but not complete protection against experimentally produced illness.

In contrast to the protective effect described above, an anomalous effect was observed in volunteers who did not develop detectable antibody after the injection of vaccine (Table 5). Experimental infection in this group led to more severe illness than that observed in the control volunteers. Although the vaccine did not stimulate the development of detectable growth-inhibiting antibodies in the former group, it appeared to affect their reactivity to infection. A similar paradoxical effect of vaccines of low potency has been observed by other workers (6, 8). The present experience suggests that caution should be exercised in evaluating inactivated vaccines of low potency in man. Since the paradoxical enhancement effect was observed in those volunteers who failed to develop antibody, it is probable that such an effect will not be seen when vaccines of greater potency are tested.

Protection against naturally occurring disease. The protective efficacy of inactivated egg yolk extract vaccine was evaluated at the Parris Island Marine Recruit Training Center in South Carolina. Vaccine was administered to 11,693 young men, while 11,813 were given a placebo. During the field trial four different vaccine lots of similar potency were used. Untoward reactions were not noted (9).

For the past seven years *M. pneumoniae* infection has been both endemic and widespread at Parris Island (3). During this period the organism was responsible for 38 per cent of X-ray-confirmed pneumonia illnesses, the average incidence being 1.5 per 1,000 men per month (3). In such a setting the development of vaccine-induced resistance faced obvious competition from naturally occurring infection. Despite this handicap a protective effect of the egg yolk extract vaccine was observed (Table 6). There was a 24 per cent reduction in crude pneumonia incidence: 55 pneumonias occurred in the vaccine group, whereas 72 occurred in the placebo group. More striking was the reduction in X-ray-confirmed *M. pneumoniae* pneumonia. We observed a 46 per cent reduction in such pneumonia: 13 cases were identified in the vaccine group and 24 in the placebo group. This difference was statistically significant ($P = .05$).

Although a definite protective effect was observed in the field trial, it is clear that the egg yolk extract vaccine was not of sufficient potency to recommend its widespread use. Vaccines of greater potency are needed if effective protection is to be provided for most susceptible individuals. The importance of the field trial lies in the finding that an inactivated vaccine was capable of inducing resistance to naturally occurring *M. pneumonia* illness.

Future of inactivated vaccines. A number of approaches to the development of a more potent inactivated vaccine are currently under study. The possible adjuvant effect of egg yolk extract for *M. pneumoniae* and the role of lipid nutrients in the antigenicity of the organism are both under investigation. In addition, glass-surface-grown suspensions of the organism, which were of low immunogenicity in volunteers, are being further concentrated and precipitated with alum in an attempt to increase their antigenicity for man. Success in any one of these areas could lead to the rapid development of an acceptable and potent immunogen for the prevention of *M. pneumoniae* illness.

For the next few years *M. pneumoniae* vac-

TABLE 6. PROTECTIVE EFFECT OF INACTIVATED EGG YOLK EXTRACT M. PNEUMONIAE VACCINE, PARRIS ISLAND, SOUTH CAROLINA, 1965-1966

Group	No. of recruits	No. of pneumonia illnesses (diagnosis confirmed by X-ray findings)		No. of *M. pneumoniae* pneumonia illnesses*	
Vaccine	11,693	55	24% reduction	13	46% reduction†
Placebo	11,813	72		24	

*Recovery of *M. pneumoniae* at the onset of illness and/or a fourfold or greater rise in CF or growth-inhibiting antibody during convalescence.
†Significant difference P = .05.

cines will probably be prepared from whole organism suspensions grown either on a glass surface or in egg yolk extract broth. However, the future of inactivated vaccines may well lie in the use of highly purified antigens derived from the organism. Recent studies have shown that the major serologically reactive constituent of *M. pneumoniae* is a low molecular-weight phospholipid (14, 15, 21). This material represents only 1.5 per cent of the dry weight of the organism, yet it accounts for almost all of the complement-fixing activity of *M. pneumoniae*. Although not capable of stimulating the development of antibody in animals, the active phospholipid combines with and blocks the activity of both indirect hemagglutinating antibody and growth-inhibiting antibody. In this sense the phospholipid material is a hapten that requires a protein carrier to be immunogenic.

The active phospholipid is soluble in acetone or chloroform-methanol and can be extracted from the oragnism with either of these lipid solvents. Subsequent purification and concentration of the hapten can then be achieved, without loss of activity, by silicic acid column chromatography followed by thin-layer chromatography (Table 7). During the various purification procedures complement-fixing activity cannot be dissociated from growth-inhibiting antibody blocking activity. The phospholipid hapten, which has an approximate molecular weight of 1.000 to 2.000, contains glycerol, phosphate and two fatty acids one of which contains 16 carbon atoms, while the other contains 18 carbon atoms. Thus the hapten resembles lecithin in structure but differs from it in lacking choline (14).

We are currently attempting to couple the purified phospholipid hapten to a purified protein acceptable for parenteral injection. In this way, it may ultimately be possible to prepare a highly purified vaccine for the prevention of *M. pneumoniae* illness.

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TABLE 7. RECOVERY OF CF ACTIVITY AND GROWTH-INHIBITING ANTIBODY BLOCKING ACTIVITY FOLLOWING SILICIC ACID COLUMN CHROMATOGRAPHY OF M. PNEUMONIAE LIPID EXTRACT

Material		Eluting fluid	Activity of indicated fraction			
			CF-units per mg		Growth-inhibiting antibody blocking activity per mg*	
Fraction	1	Benzene-hexane (6:94)	<0.4		<0.4	
	2	" " (1:4)	"		"	
	3	" " (6:4)	<1.6		"	
	4	Benzene	<3.2		"	
	5	CHC_3-Benzene (3:1)	"		0.4	
	6	CHC_3	<16		<2.0	
	7	CHC_3-CH_3OH (20:1)	<51		1.6	(0.4%)
	8	" " (9:1)	6553	(65%)	306	(85%)
	9	" " (4:1)	3277	(32%)	38	(11%)
	10	" " (1:1)	26	(0.3%)	<0.4	
	11	" " (1:20)	205	(2%)	5	(1.4%)
	12	Ether	26	(0.3%)	9	(2.5%)
Total		—	10,087		360	

*One unit of inhibitory activity = highest dilution of fraction capable of inhibiting 8–16 units of growth-inhibiting antibody.

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SECTION F. COMBINED RESPIRATORY VIRUS VACCINES

DEVELOPMENT AND FIELD EVALUATION OF COMBINED POLYVALENT RESPIRATORY VIRUS VACCINES

M. R. Hilleman, R. E. Weibel, A. F. Woodhour, J. Stokes, Jr., C. C. Mascoli, M. B. Leagus, A. A. Tytell, and P. P. Vella *

DR. HILLEMAN (*presenting the paper*): A rational approach to immunologic control of the acute respiratory infections of man necessitates the discovery of the agents responsible and the assessment of the importance of each of them in relation to the amount and severity of illness they cause. Spatial and economic considerations impose severe restrictions on the number of agents that can be included in a vaccine and make it imperative that only the important ones be used.

During 1962, on the basis of a combination of laboratory study and survey of the literature relating to persons in open populations, we prepared an estimate of the relative importance of each of the various known respiratory disease agents that would be referable to natural occurrence of illness in the community (7, 12, 16). There is nothing in the newer developments to alter the 1962 appraisal significantly, and it is reproduced in slightly modified form in Figure 1. The estimates are presented in the figure according to age group and clinical circumstance. In general, illnesses among the outpatients were mild and confined to the upper respiratory tract, while those among hospital patients were more severe and involved the lower respiratory tract.

It is quite clear that respiratory syncytial (RS), adenovirus, parainfluenza, influenza, and *Mycoplasma pneumoniae* infections contribute prominently to respiratory illnesses in childhood and that rhinovirus, influenza, and mycoplasma infections are of prime importance in adults. With this information at hand, we set about developing vaccines against the respiratory syncytial, parainfluenza, influenza, and mycoplasma agents. Work on adenoviruses has been minimized owing to the known carcinogenic quality of a large number of adenovirus serotypes (10). The rhinoviruses comprise at least 54 distinct serotypes (4, 13, 23) and offer little hope at this time for practicable control by killed vaccines. Live virus vaccines have also shown little promise to date. Live rhinovirus Types 32 and 44 fed in enteric-coated capsules by our group (24) to seronegative volunteers failed to demon-

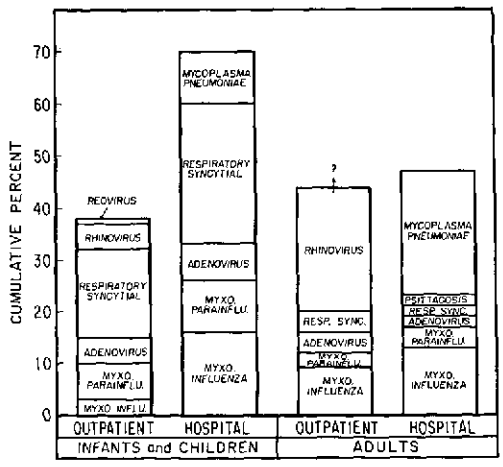


Fig. 1. Estimate of relative importance of respiratory disease agents, according to age and severity (modified from Hilleman *et al.* (16)).

* From the Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania, and the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia.

strate antibody response against the homologous serotypes.

The multiplicity of important agents in acute respiratory illnesses necessitates the effective combination of vaccines into polyvalent formulations. Though competition and immunologic exclusion might attend single dose administration of two or more antigens (1, 2), this has not been a serious problem to date with mixed viral antigens such as polyvalent influenza or poliovirus vaccines or influenza-adenovirus combination. In spite of the promise of polyvalent vaccines, few attempts have been made to prepare and evaluate such vaccines except for the formulations just mentioned (3, 5, 17–20, 25, 27, 29, 41).

The past several years' studies in our laboratories (6, 8, 11, 12, 14, 21, 22, 26, 31, 33–35, 40) have been devoted in large measure to the development of polyvalent killed virus vaccines using highly purified and concentrated viral antigens that were precisely quantified. The vaccines in alum formulations proved completely safe, and the antibody responses in animals and in man were of such magnitude as to give considerable promise. Accordingly, large-scale studies were initiated during 1964 among children in the Havertown-Springfield suburb of Philadelphia for the purpose of evaluating antibody responses to the vaccines and of measuring protective efficacy against the natural disease.

STUDY 50: TRIAL OF HEPTAVALENT VACCINE IN 1964–1965

A controlled study of a mixed heptavalent vaccine containing RS-parainfluenza 1, 2, 3-*Mycoplasma pneumoniae*-influenza A and B agents was carried out by our group (34) during the respiratory disease season of 1964–1965. The subjects were 407 children three to five years of age in kindergarten and nursery schools, who were alternately selected to receive vaccine or to serve as controls. The children in the vaccinated group were given three doses of vaccine one month apart and were bled prior to vaccination and one month after the third dose. They were observed twice weekly for respiratory illness for 19 weeks during January through May of 1965, and specimens for virus or mycoplasma isolation were taken from the children who were ill with respiratory disease. Further detail is presented elsewhere (34).

The vaccinated and control groups had a similar serologic status for each agent prior to vaccination, which indicated their comparability. Figure 2 summarizes the serologic responses to the individual components of the vaccine based on comparison of the prevaccination antibody titer and the titer following the third dose of vaccine. Parainfluenza and influenza antibodies were assayed by the hemagglutination-inhibition (HI) method, and those against RS virus and *Mycoplasma pneumoniae* were measured by the serum-neutralization procedure. The responses to the parainfluenza and influenza viruses were excellent while those against the RS and mycoplasma agents were less.

The same findings are presented in simplified form in Figure 3, which shows the seroconversion rate for each vaccine component. The heptavalent vaccine stimulated homologous antibody in 94 per cent or more of the children initially seronegative to parainfluenza and influenza viruses, in 79 per cent of those seronegative to *Mycoplasma pneumoniae*, and in 33 per cent of those without antibody to respiratory syncytial virus. The geometric mean titers were lowest against the RS and mycoplasma components.

The antibody response was less striking in persons who displayed antibody initially: 49 to 89 per cent of the children who were initially seropositive to the parainfluenza or influenza viruses showed a fourfold or greater increase in antibody after vaccination; 6 per cent responded similarly to RS virus; and 19 per cent to the mycoplasma component (Fig. 4). The lower rate of response in children who had antibody initially compared with initial seronegatives was of no great concern since the human subjects without antibody were those most vulnerable to severe infection and consequently in greatest need of protection by vaccine.

The protective efficacy of the heptavalent vaccine was evaluated by comparing the cumulative attack rates, by week, in the vaccinated and control groups (Fig. 5). For analysis, the mild afebrile or weakly febrile (100°F or less, oral) were separated from the severe cases, all of which had a fever of above 100°F. The figure shows that there was a reduction in total respiratory illness, in mild illness, and in severe illness as a result of vaccination. The reduction was

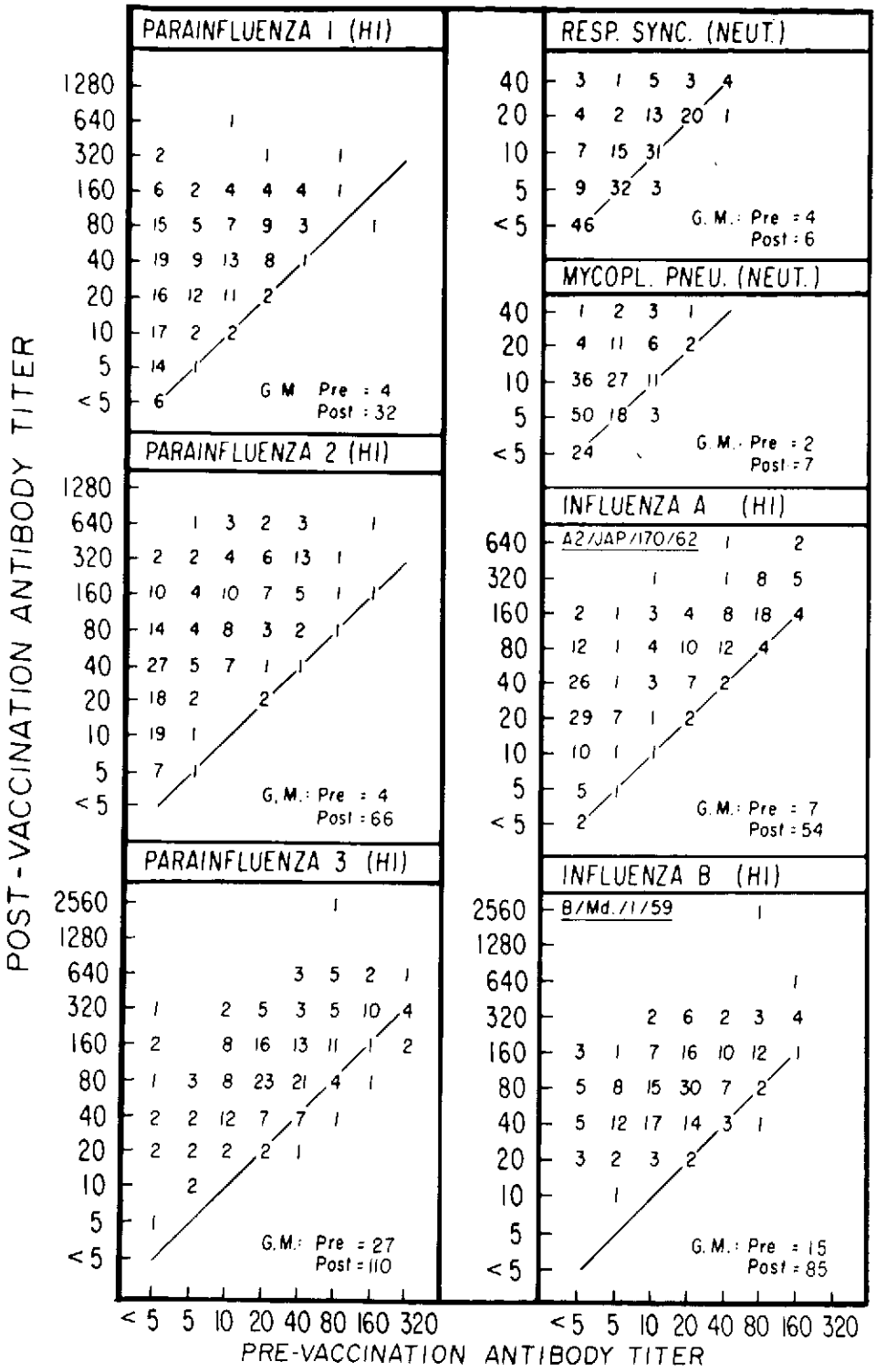
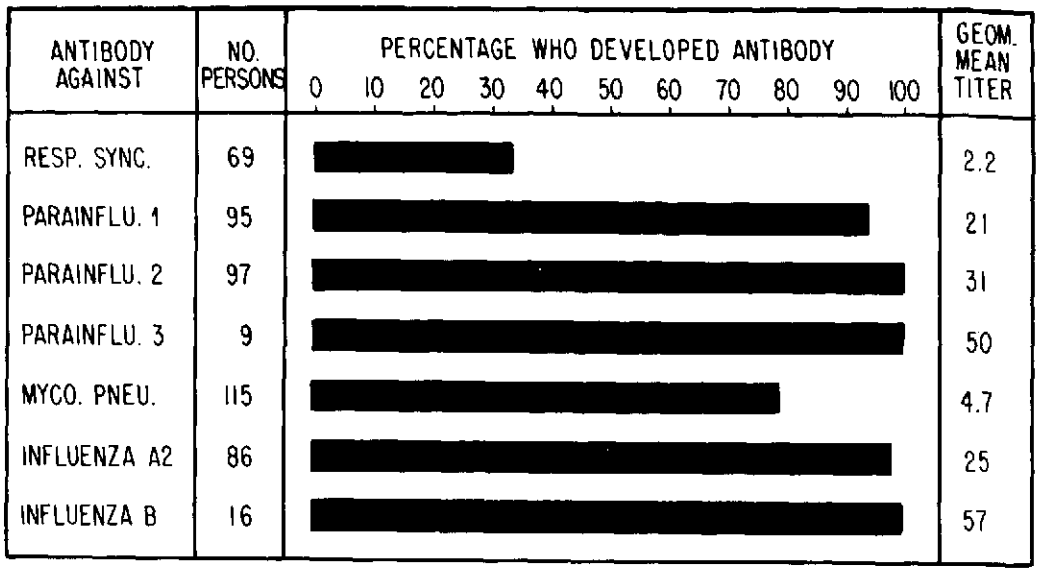
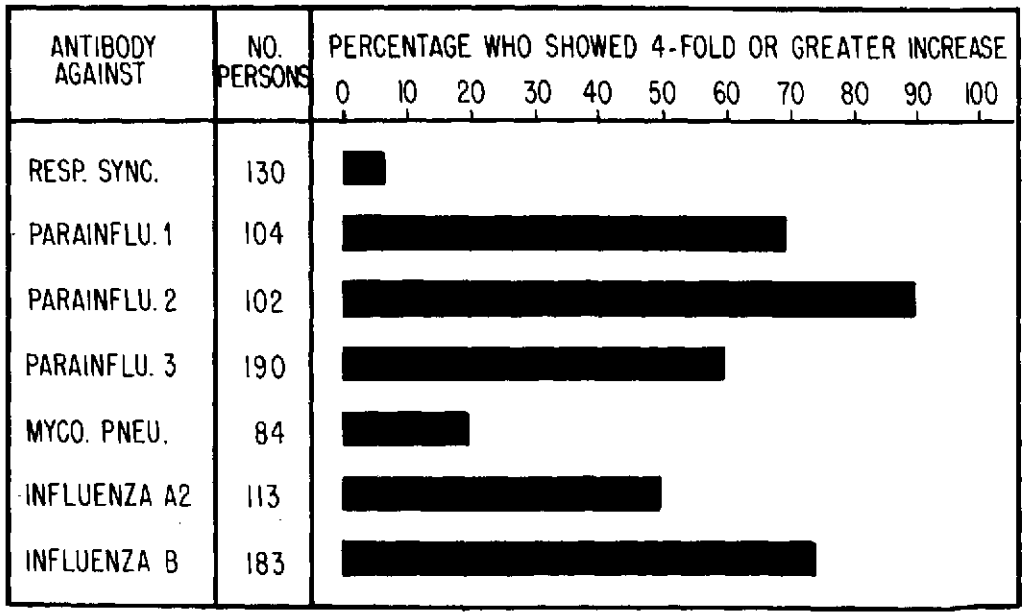


Fig. 2. Antibody responses against homologous agents among children who received three doses of heptavalent alum-precipitated RS-parainfluenza-mycoplasma-influenza vaccine in 1964 1965 trial (Study 50).



Respiratory syncytial and *M. pneumoniae* titers were by neutralization and others were by hemagglutination-inhibition.

Fig. 3. Seroconversion rates among initially seronegative preschool children given three doses of heptavalent respiratory virus vaccine in 1964–1965 trial (Study 50).



Respiratory syncytial and *M. pneumoniae* titers were by neutralization and others were by hemagglutination-inhibition in 1964–1965 trial (Study 50).

Fig. 4. Fourfold or greater antibody increases among initially seropositive preschool children given three doses of heptavalent respiratory virus vaccine in 1964–1965 trial (Study 50).

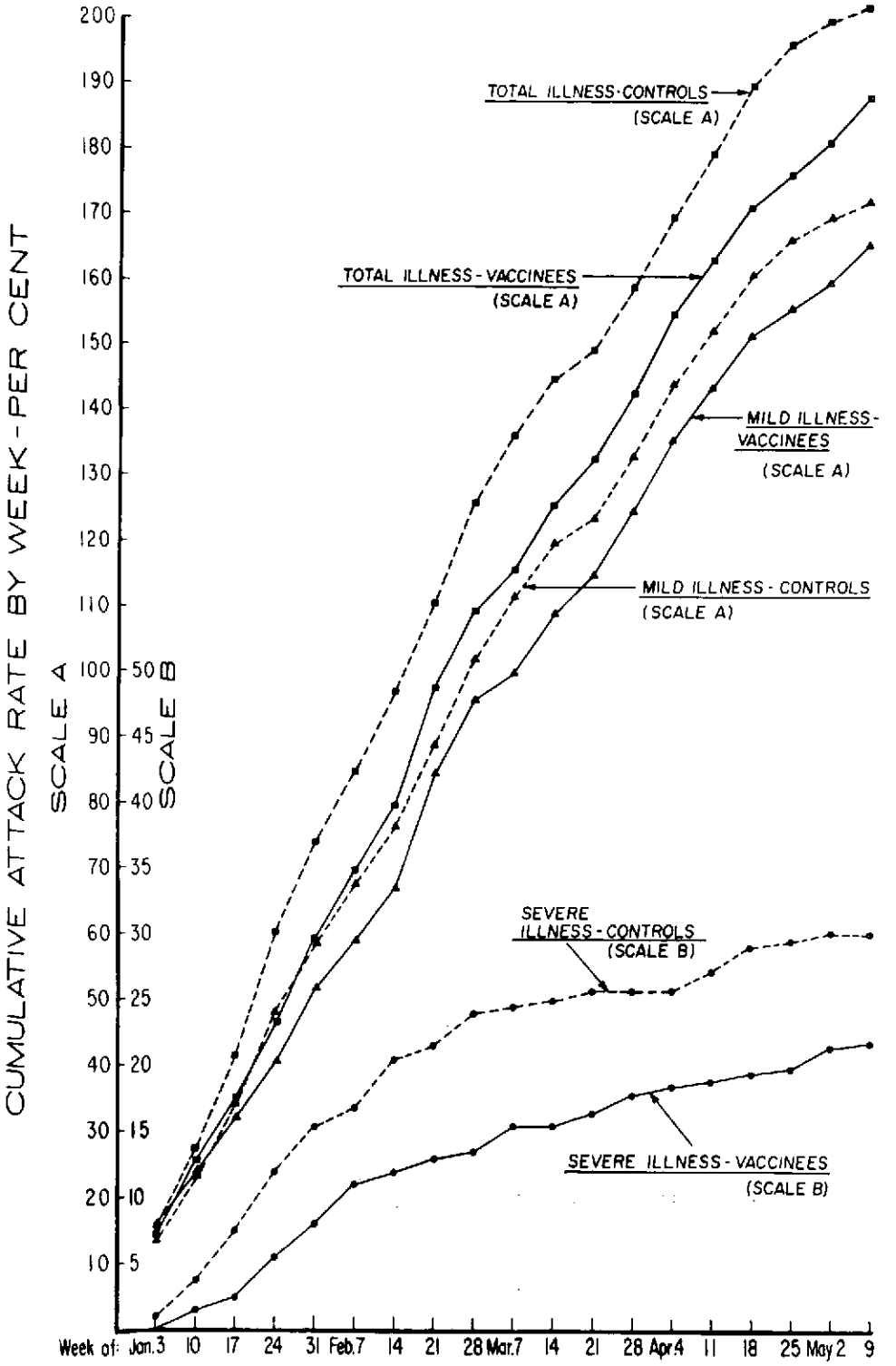


Fig. 5. Cumulative attack rates for total, severe, or mild respiratory illnesses, according to time, among all children vaccinated with heptavalent respiratory syncytial-parainfluenza-mycoplasma-influenza vaccine or held as unvaccinated controls, in 1964–1965 trial (Study 50).

greatest in the cases with febrile disease. This was expected, since the agents represented in the vaccine are those most likely to cause severe febrile disease.

The reduction in respiratory illnesses resulting from vaccination occurred during the first 10 weeks of observation, the period when the respiratory disease rates were the highest. No effect was apparent during the subsequent nine weeks, when the over-all rates were low. A summary evaluation of vaccine efficacy is presented in Table 1, in terms of attack rates for total, severe, or mild illness for the entire period and for the first 10 weeks of study. The protective effect was mainly against severe illness, and this was of the order of 36 per cent for the first 10 weeks of study and 28 per cent for the entire period. Both these values are significant statistically. The 15 per cent reduction in all respiratory illness shown during the first 10 weeks was of borderline significance. The finding of the protective efficacy of the vaccine was substantiated in further analyses in which the vaccinated and the control groups were compared for average occurrence of respiratory illness per child and average illness severity score assigned to the cases.

The rates for isolation of viruses and mycoplasma from the cases were too low to permit an estimate of agent-specific attack rates of illness. However, the rate for recovery of *Mycoplasma pneumoniae* from vaccinees compared with controls was in the ratio of 1:8, which indicates that the mycoplasma component of the vaccine was effective. The marked absence of influenza A_2 in the Philadelphia area during the period of study, the occurrence of influenza B only during the period of observation (22) and the serologic response data, together with the agent isolation results, supported the judgment that the vaccine efficacy was due mainly to the parainfluenza and mycoplasma components. The findings in the study were viewed as highly promising and as indicative that substantial protection against acute respiratory illnesses can be achieved by prophylactic immunization.

TABLE 1. EVALUATION OF EFFICACY OF THE ALUM-PRECIPITATED HEPTAVALENT RS-PARAINFLUENZA-MYCOPLASMA-INFLUENZA VACCINE IN 1964-1965 TRIAL (STUDY 50)

Time period	Illness	Ratio Attack rates vacc./ control	Difference (efficacy)	Significance (P)
Total period	Total	1/1.08	−7%	None
	Severe	1/1.38	−28%	0.05
	Mild	1/1.04	−4%	None
First 10 weeks	Total	1/1.18	−15%	0.05-0.10
	Severe	1/1.57	−36%	<0.025
	Mild	1/1.12	−10%	None

Respiratory illness rate/100 observations.

STUDY 70: TRIAL OF MONOVALENT AND TRIVALENT VACCINES IN 1965–1966

After the promising trials of heptavalent vaccine in 1964–1965, it was decided to carry out tests of monovalent RS, monovalent *Mycoplasma pneumoniae*, and trivalent parainfluenza 1, 2, 3 vaccines in nursery and kindergarten classrooms in the Havertown-Springfield community (35). Simplified formulations were used in order to facilitate identification of the active agent or agents in the vaccines which were responsible for protection. The vaccines were prepared in essentially the same manner as the heptavalent preparation except that the content of RS viral antigen in the RS vaccine was markedly increased and the influenza virus antigens were not used. Further, only two doses of vaccine were given in the 1965–1966 trial, in contrast to three doses employed in the 1964–1965 tests of heptavalent vaccine.

The subjects were 750 children three to five years of age in 45 kindergarten and nursery school classrooms in the Havertown-Springfield area. The children were divided at random in each classroom into four groups to receive one of the three vaccines or to serve as unvaccinated controls. The children in the vaccinated groups were given the two doses of vaccine one month apart during October and November 1965. Blood samples for serologic testing were taken prior to vaccination and again one month after the second dose. The children were observed twice weekly for respiratory illness for 20 weeks during January through May of 1966. Specimens for virus or mycoplasma isolation were taken from the children who were ill with respiratory disease. Details of the data on the trial are published elsewhere (35).

The vaccinated and the control groups had a similar serologic status for each agent prior to

vaccination, which indicates the comparability of the groups. Figure 6 summarizes the homologous antibody responses in the children to the vaccines. Parainfluenza antibody was measured by the HI technique, and antibody against the RS virus and *Mycoplasma pneumoniae* was assayed by the serum-neutralization method. The antibody responses to the parainfluenza viruses were excellent and those to the RS virus were considerably greater than had been obtained earlier with the heptavalent vaccine. This was due to the use of a greater amount of antigen in the RS vaccine. The response to mycoplasma was somewhat less than to the other agents; this appears to be related more to an insensitivity of the means for detecting neutralizing antibody than to the response itself, since the vaccine is highly protective against the natural disease.

The findings in Figure 6 are shown in simplified form in Figure 7. Ninety-five to 96 per cent of the initially seronegative children developed antibody against the parainfluenza viruses after only two doses of vaccine. Seventy-four per cent showed a serologic response to RS virus and 60 per cent to the mycoplasma.

The percentages of persons who displayed

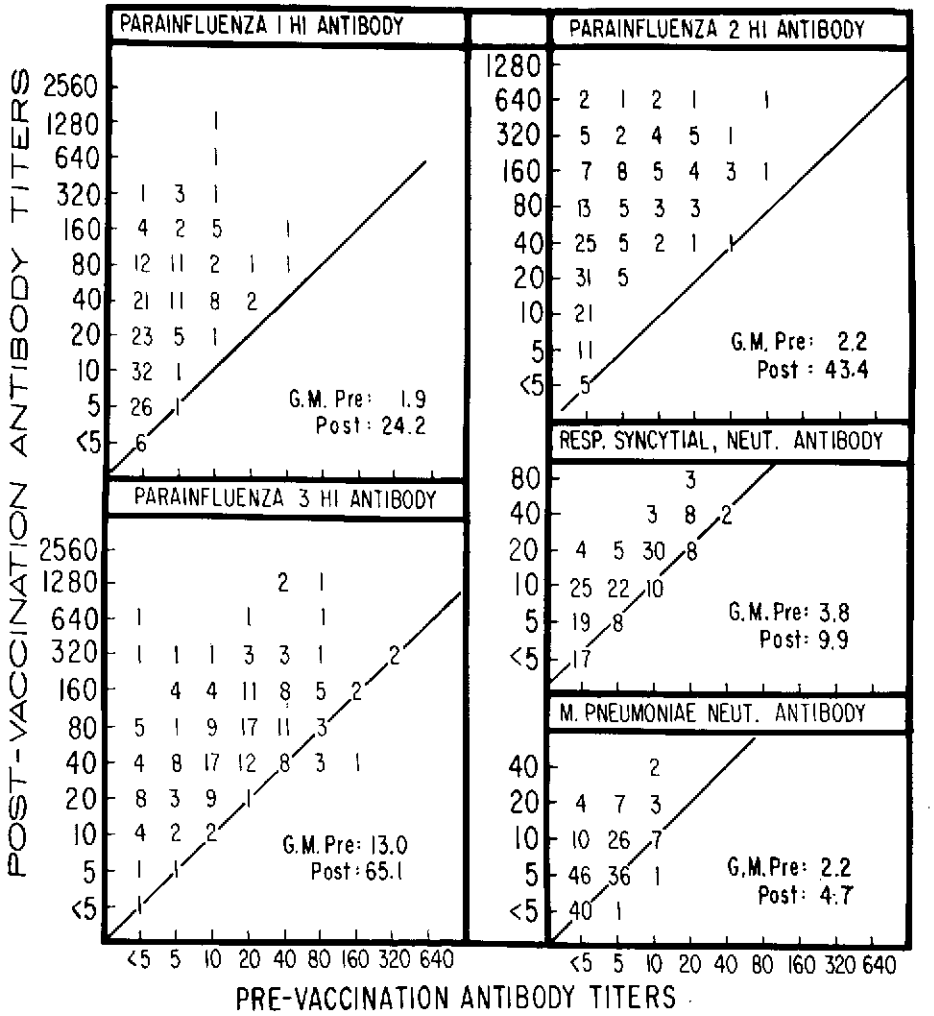
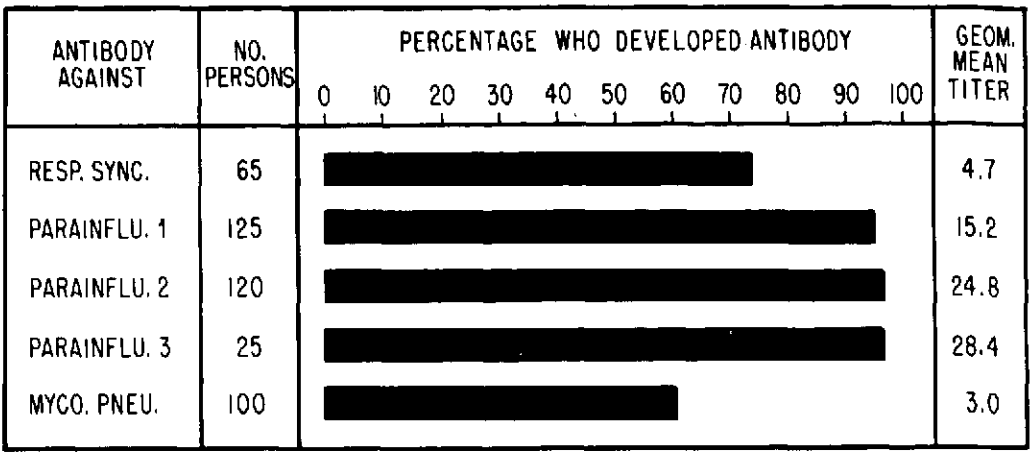


Fig. 6. Homologous antibody responses against respiratory syncytial or parainfluenza Types 1, 2, 3 viruses or *M. pneumoniae* in children who received two doses of killed alum-precipitated RS, mycoplasma, or parainfluenza 1, 2, 3 vaccine in 1965–1966 trial (Study 70).

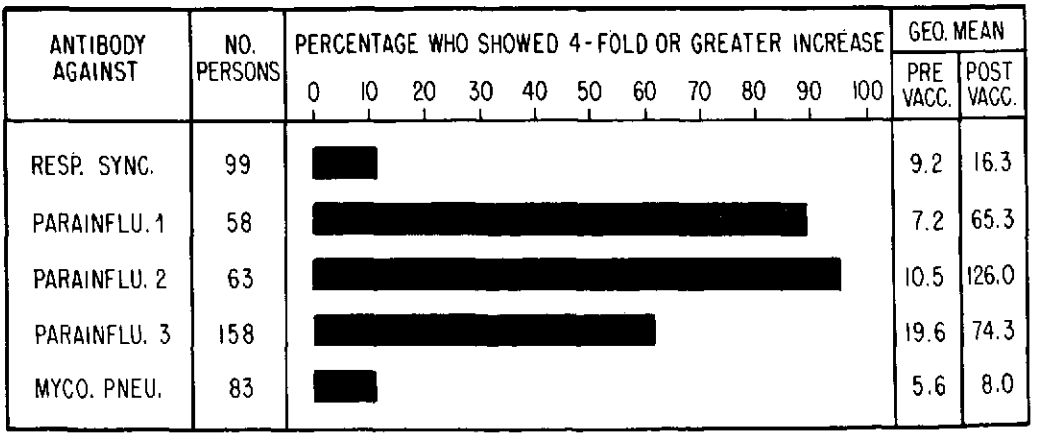


Respiratory syncytial, and *M. pneumoniae* titers were by neutralization and parainfluenza by hemagglutination-inhibition.

Fig. 7. Seroconversion rates among initially seronegative preschool children given two doses of killed alum-precipitated RS, *Mycoplasma pneumoniae*, or parainfluenza 1, 2, 3 vaccine in 1965–1966 trial (Study 70).

antibody initially and who showed a fourfold or greater increase in antibody after two doses of vaccine are shown in Figure 8. Striking booster responses were observed for the parainfluenza agents, ranging from 61 to 95 per cent. The fourfold or greater rises in titer against RS and mycoplasma were each 11 per cent, and this was consistent with the findings in the study with heptavalent vaccine.

The protective efficacy of the vaccines was evaluated by comparing the cumulative attack rates, by week, for each vaccine group with those of the controls. The findings are shown in Figure 9. The rate for respiratory illness during 1965–1966 was only about one third as high as in the preceding year, when the heptavalent vaccine was tested. Hence, the rates are very low. The analyses were made in terms of total, severe, and mild disease, as for the heptavalent vaccine study. There was a striking decrease in illness among children who received *Mycoplasma pneumoniae* vaccine as compared



Respiratory syncytial and *M. pneumoniae* titers were by neutralization and others were by hemagglutination-inhibition (Study 70).

Fig. 8. Fourfold or greater antibody increases among initially seropositive preschool children given two doses of killed alum-precipitated RS, *M. pneumoniae*, or parainfluenza 1, 2, 3 vaccine (Study 70).

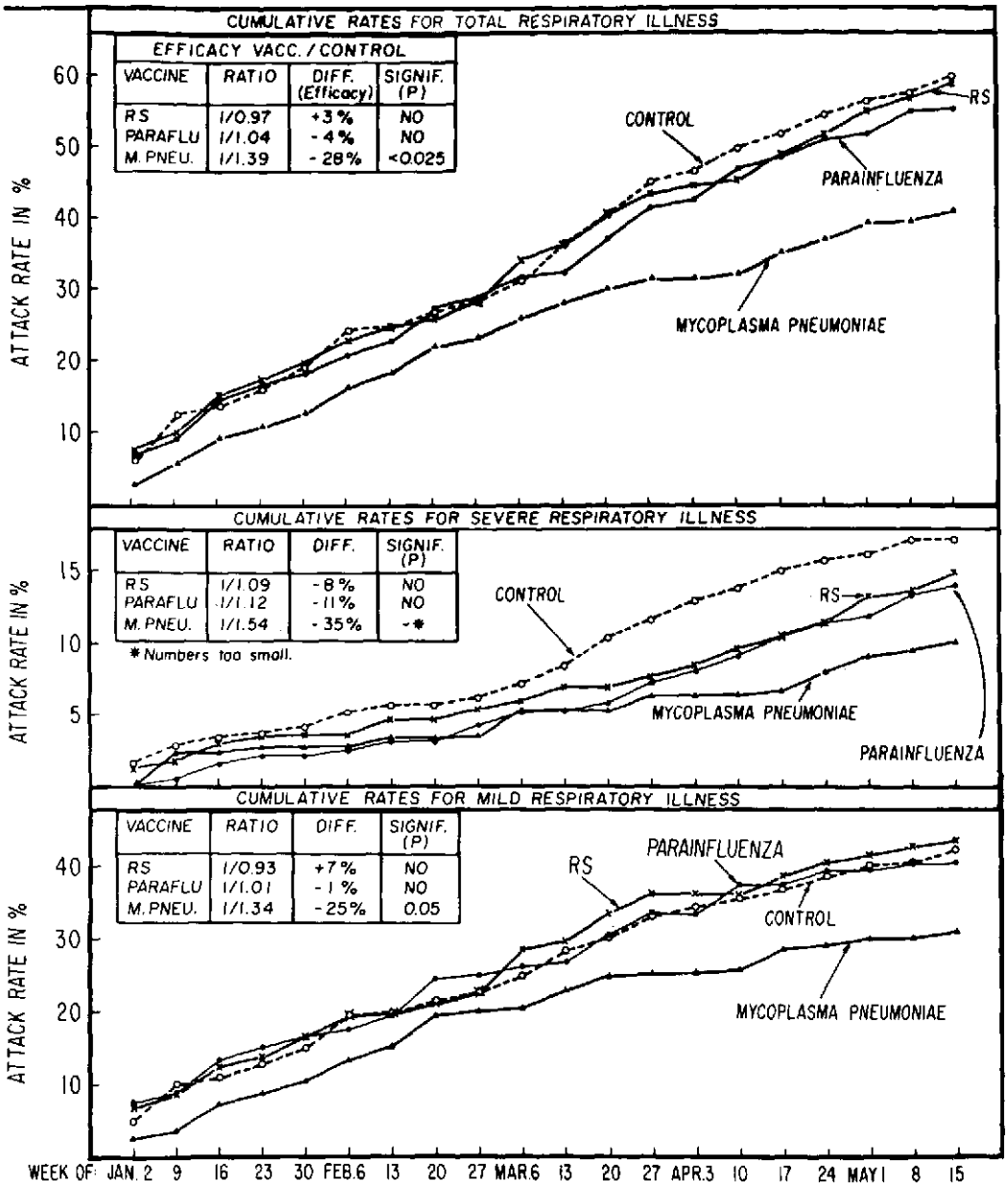


Fig. 9. Cumulative attack rates for total, severe, or mild respiratory illnesses, according to time, among all children vaccinated with killed alum-precipitated respiratory syncytial, parainfluenza types 1, 2, and 3 mixed, or mycoplasma vaccine, or held as unvaccinated controls in 1965–1966 trial (Study 70).

with the controls, and this difference was statistically significant when total respiratory disease and mild respiratory disease rates were observed. As might be expected, the greatest reduction, 35 per cent, was observed for severe illness. The numbers, however, were too small to permit statistical confirmation. The efficacy demonstrated for the mycoplasma vaccine was consistent with the findings obtained in the 1964–1965 heptavalent vaccine study and with the very substantial reduction in lower respiratory tract illness demonstrated in large-scale field tests of the same vac-

cine at Keesler Air Force Base by Dr. Mogabgab (28). There was also an 8 per cent reduction in severe disease among children who received RS vaccine and of 11 per cent among recipients of parainfluenza vaccine. Though consistent in pattern of development, these figures were not confirmed by statistical analysis. The rates for recovery of specific agents from the cases for diagnostic purposes were so low as to preclude any attempt at refining the data in terms of agent-specific attack rates.

ANTIBODY RESPONSES IN INFANTS

It was apparent from the studies that though excellent antibody responses against these agents may be obtained in nursery and kindergarten children, the attack rates for diseases caused by these agents are apparently low and the efficacy of the vaccines might be better shown in children of younger age. It was important, therefore, to establish whether very young children respond serologically to the vaccine (21).

Table 2 summarizes the antibody responses in young infants who were given two doses of the heptavalent vaccine described above for Study 50. It should be noted that only two, rather than three, doses were given and that all children were initially seronegative to all agents except influenza B and parainfluenza 3 in one child (5 months). It may be seen that, considering that the only two doses of vaccine were given, the infants responded remarkably well to all the agents except RS virus. Only two of the eight children responded to RS virus, but this was after only two doses of a RS vaccine that was known not to contain sufficient antigen.

IMMUNOLOGIC ADJUVANTS

Because of the extreme multiplicity of kinds of antigens worthy of inclusion in killed vaccines, means must be developed for minimizing the volume per dose, the required number of doses, and the cost. The best hope for a practicable solution lies in a safe and effective immunologic adjuvant. Adjuvants should facilitate the achievement of a more durable and a higher level of immunity employing a smaller antigenic mass in fewer doses than would be attainable if the corresponding aqueous material were employed.

Two years ago, my colleagues and I described the development and clinical testing of a new adjuvant called adjuvant 65 (8, 15, 30, 32, 36, 38, 39). This consisted of an aqueous vaccine in peanut-oil emulsion employing Arlacel A (mannide monooleate) as emulsifier and aluminum monostearate as stabilizer. The adjuvant works very well with a variety of viral antigens, but has been applied most extensively to influenza virus vaccine. More recently it has been used with a highly purified influenza virus vaccine newly developed in our laboratories (36, 38). The virus

TABLE 2. INDIVIDUAL HOMOLOGOUS ANTIBODY RESPONSES AMONG INFANTS RECEIVING TWO DOSES OF HEPTAVALENT RS-PARAINFLUENZA-MYCOPLASMA-INFLUENZA VACCINE IN 1964-1965 TRIAL (STUDY 50) (ALL WERE INITIALLY SERONEGATIVE UNLESS OTHERWISE SHOWN)

Age	Child No.	Reciprocal of antibody titer against							
		RS (Post)	Paraflu. 1 (Post)	Paraflu. 2 (Post)	Paraflu. 3 (Post)	Myco. pneu. (Post)	Influenza A (Post)	Influenza B	
								Pre	Post
5 mos.	482	<5	40	10	80*	10	80	20	20
7 mos.	326	<5	10	160	10	<5	20	80	80
8 mos.	299	<5	<5	40	160	<5	80	40	160
9 mos.	118	<5	5	20	10	10	20	10	80
11 mos.	308	<5	20	80	640	<5	20	10	160
1 yr.	99	<5	<5	20	10	5	80	20	40
1 yr.	189	40	5	20	40	5	10	10	40
1 yr.	442	20	10	80	80	10	80	20	160

*Prevaccination titer was 1:80.

in the vaccine is purified by combined physical and chemical procedures. In this work, only contemporary influenza A and B virus strains were used, since strains of an earlier vintage have offered no significant contribution to the efficacy of the vaccines. Figure 10 illustrates representative findings in tests in which persons were given one or two doses of the adjuvant with the purified vaccine in adjuvant as compared with purified aqueous or ordinary Sharples vaccine. The purified aqueous and Sharples aqueous vaccines performed roughly the same. The very large titer increase and sustained high antibody level attending use of the adjuvant are clearly shown. Elsewhere in this program (37), it will be reported that the enhancement of antibody response attending the use of adjuvant 65 is retained for at least three years.

No significant adverse local or systemic effect has been noted in short- or long-term studies in about 15,000 persons to date, including tests for induction of immediate and delayed hypersensitivity. Short-term and long-term toxicity tests in a variety of animals, with particular attention to local and systemic pathology, autoimmune disease, hypersensitivity, cancer and

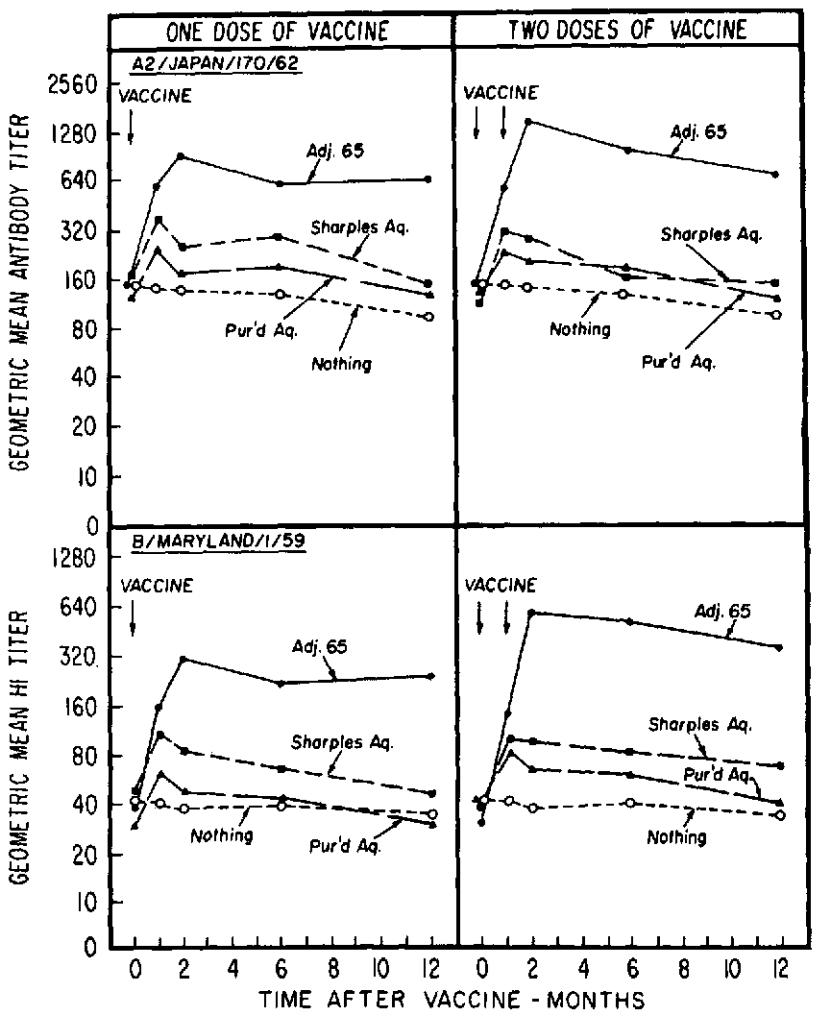


Fig. 10. Study 55. Antibody response in adult human subjects according to time after one or two injections of adjuvant 65, purified aqueous, or Sharples aqueous bivalent influenza virus vaccine given at zero (one dose) or at zero and one month (two doses).

teratogenicity, have been carried out with no significant adverse effects to date. All the components of the adjuvant enjoy the precedent of long-term use in materials employed in man. The general composition of the adjuvant components is monitored by gas chromatographic analysis, and they are tested for freedom from polynuclear aromatic hydrocarbons that might be carcinogenic. Presence of the hepatocarcinogenic aflatoxin in the peanut oil is excluded. All three components of the emulsified adjuvant break down after intramuscular injection and are metabolized, as is shown by radioactive tracer analysis.

Summary

I think it is clear that the substantial progress that has been made in virus discovery now permits a definition of specific etiology in the majority of respiratory disease cases and delineates which viruses are sufficiently important to be worth including in a mixed polyvalent vaccine. The large body of experiences with experimental vaccines reported here indicate that a sizable portion of the respiratory disease spectrum can be controlled by vaccines if a large enough antigenic mass is employed and if these vaccines can be used in highly polyvalent preparations. The findings in the present studies indicate that the parainfluenza vaccines have achieved a degree of immunologic performance equal to that of influenza virus vaccines. The *Mycoplasma pneumoniae* vaccine is highly protective, as was shown in the present studies and in the extensive tests carried out by Dr. Mogabgab at Keesler Air Force Base. Respiratory syncytial virus is also immunogenic, but a greater antigenic mass must be employed in future vaccines to achieve maximal efficacy. It is evident from the present studies that future vaccines should be evaluated in a group of children younger than the three-to-five-year-olds, in which the attack rates would be higher for the agents under consideration for vaccine use. The development of the highly effective and safe immunologic adjuvant 65 offers promise of providing the extra boost, both in antibody level and in duration of antibody elevation, that should bring these vaccines into practicable routine use.

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SECTION E. MYCOPLASMA VACCINES AND SECTION F. COMBINED RESPIRATORY VIRUS VACCINES

DISCUSSION

CHAIRMAN ANDREWES: We will now proceed with the discussion of both Sections E. and F. The first discussant is Dr. L. Hayflick, of the Wistar Institute, Philadelphia, Pennsylvania.

DR. HAYFLICK: Perhaps some of the apparent difficulties encountered in the development of a really effective vaccine against *Mycoplasma pneumoniae* infection can be traced to the tacit belief that these microorganisms behave like viruses or bacteria. This is, perhaps, exemplified by their inclusion here at this Conference. I believe that greater progress can be made in the field of "mycoplasmology" if less time is spent attempting to fit bacteriological and virological concepts into this uniquely shaped field, which requires its own special techniques.

The mycoplasmas have limited and, in many ways, unique synthetic capabilities, with the biochemistry of the progeny mycoplasmas often dependent upon the nature of the culture medium. Examples of this are known to those of us who have isolated mycoplasmas from clinical materials only to find that subcultivation of the isolate is frequently impossible or requires a long period of adaptation. I am reminded of the time when I first grew the Eaton Agent on the then new media formula that I had devised.* Mycoplasmas readily appeared on the plates that were inoculated with the tissue culture passaged material, but it took six months of hard work before I was able to make the first successful subcultivation. Subcultivations are as easy to make now, as are the subcultivations of ordinary bacteria. It is probable that the antigenic composition of mycoplasmas is partly influenced by the kind of substrate upon which they are grown. This may be particularly important in the case of *Mycoplasma pneumoniae*, whose growth is more dependent upon the presence of whole protein in the medium than is the growth of other mycoplasmas.* We might benefit from the fund of knowledge on this point accumulated in the development of vaccines against *M. mycoides*, the etiological agent of contagious bovine pleuropneumonia. Thus the massive yields of glass-grown mycoplasmas or the serum-free growth described in this session may in themselves be relatively unimportant and may partly account for the less-than-hoped-for results. It is quite probable that the quantitative aspects of the *M. pneumoniae* antigenic mass are less important than the qualitative properties presumably influenced by the medium components.

Moreover, other routes of vaccine administration should be explored. There is good reason to believe that the intranasal administration of live, attenuated *M. pneumoniae* might be a good approach. Investigation of living attenuated *M. pneumoniae* administered parenterally or to the intestinal tract by enteric-coated capsules should also be pursued.

There is also the possibility of the existence of localized or tissue-fixed antibody to *M. pneumoniae*, especially since the organism is found to be associated predominantly with the surface of the bronchial epithelium and, unlike viruses, does not require cell penetration for replication. If this is so, other approaches to vaccination become apparent.

* Chanock, R. M., Hayflick, L., and Barile, M. F. "Growth on Artificial Medium of an Agent Associated with Atypical Pneumonia and Its Identification as a PPLO." *Proc Nat Acad Sci (USA)* **48**:41–49, 1962; and Hayflick, L. "Tissue Cultures and Mycoplasmas." *Texas Rep Biol Med* **23**, Suppl 1:285–303, 1965.

* Hayflick, L. *op cit.*, and Hayflick, L., and Chanock, R. M. "Mycoplasma Species of Man." *Bact Rev* **29**:185–221, 1965.

In any case, I am sure that we have not heard the last from the mycoplasmas and their association with human disease. If findings in domestic and laboratory animals can properly be extrapolated to man, then it is likely that some day evidence may be forthcoming to link the mycoplasmas and the recently recognized T strains to some kinds of arthritis, rheumatic conditions, and nongonococcal urethritis in man. I expect that we may be only at the threshold of vaccines against infections caused by the mycoplasmas, and that by "cutting our teeth" on *M. pneumoniae* vaccines we will be acquiring the techniques for production of other mycoplasma and T-strain vaccines of the future.

CHAIRMAN ANDREWES: The next discussant is Dr. B. P. Marmion, Department of Microbiology, Monash University Medical School, Melbourne, Australia.

DR. MARMION: A case seems to have been made by the speakers for the effectiveness of inactivated whole cell vaccine from *M. pneumoniae* in reducing the incidence of lower respiratory tract illness in the populations studied.

It may well be that inactivated whole cell vaccine will prove to be sufficiently immunogenic and nontoxic to be an adequate vaccine without further refinement. However, it is possible, as Dr. Chanock has foreshadowed in his discussion of the phospholipid fraction of the organism, that chemical extracts of *M. pneumoniae* may provide a refined vaccine with the essential antigenic determinants free of other superfluous cell components. In this context, I should like to summarize briefly the findings of a group of us in Melbourne—Drs. Plackett, Lemcke, and Shaw and myself—on the analysis of the immunochemical composition of *M. pneumoniae*.

1. Serologically reactive fractions can be extracted from *M. pneumoniae* with chloroform-methanol (2:1 v/v) by the Folch method as applied by Kenny and Grayston, by the Dafaalla B method (warm ethanol with acetone precipitation in the cold), or by a warm aqueous phenol method resembling that of Westphal. If these techniques are used in sequence, material is still extracted by each, so that they probably extract different substances or different fragments of the same larger complex.

2. These extracts react with human convalescent or rabbit hyperimmune sera by various techniques. We have used mainly complement fixation and gel diffusion.

3. The chloroform-methanol extract shows the most potent reactivity with homologous human or rabbit antisera. Extracts by the Dafaalla B or the phenol method show more heterologous reactions.

4. Material extracted by the Dafaalla B method has not been characterized. The phenol extract contains about 14 per cent galactose and traces of glucose and mannose; its serological activity is reduced by treatment with periodate, but more investigation is required to find out (a) whether the galactose-containing portion has actually been synthesized by the organism and (b) whether the serological reactivity resides in it or in some other component of the phenol extract.

5. Examination of the major reactive fraction, the chloroform-methanol (2:1 v/v) extract, shows that it has the following properties:

a. It is heat- and alkali-stable.

b. Treatment with pronase and lipase (15 hours) does not alter reactivity.

c. Treatment with phospholipase C does not alter the reactivity of the CM extract, but does substantially reduce the reactivity of a phospholipid, the Difco cardiolipin antigen, tested with a WR positive serum.

d. The serological reactivity of CM extract is substantially reduced by treatment with periodate and by carbohydrase. In preliminary experiments the WR reactivity of Kolmer cardiolipin was not reduced by periodate treatment.

e. The whole CM 2:1 extract from *M. pneumoniae* reacts with WR + sera from patients with syphilis, and the WR antibody (or reagin) that cross-reacts may be adsorbed from these sera with crude beef heart WR antigen. However, some serologically reactive subfractions of the CM extract do not react with WR positive sera; that is, the WR reactive component is only a part of the whole extract. The human and rabbit anti-*M. pneumoniae* sera we use react only feebly with Kolmer cardiolipin CF antigen and not at all with phosphatidyl glycerol, synthetic phosphatidyl serine or phosphatidyl ethanolamine.

f. Lecithin and phosphatidyl ethanolamine both have an auxiliary effect on the CF reac-

tivity of certain of the subfractions of the CM extract. This aspect is of some importance in the testing of subfractions of the lipids, particularly neutral lipids.

g. The whole (2:1 v/v) CM extract is only feebly antigenic in rabbits and guinea pigs,* but appears to block the antibody for growth inhibition that is present in the rabbit antisera we are using.

h. Fractionation of the whole CM extract by methods that will separate acidic lipids or phospholipids on the one hand and neutral lipids on the other reveals that serological reactivity is associated with components in *both* of these general classes. The CF reactive neutral lipid fractions do not contain phosphorus in detectable amounts. The reactivity of both acidic and neutral lipid fractions is periodate sensitive. They react as different entities in gel diffusion. The reaction of the serologically active neutral lipid fraction is not inhibited in gel-diffusion systems by cardiolipin. If extracts are made from organisms whose lipids are labeled with C^{14} or tritium, labeled components appear in both the serologically reactive acidic and the neutral lipid fractions, which suggests that both fractions contain components that have been made by the organism.

i. Analysis of serologically reactive acidic and neutral lipid fractions by thin layer chromatography on silica gel indicates that there are a number of serologically reactive subfractions within each group. Some difficulty has been experienced in obtaining homogeneous subfractions and more work is required before weight can be placed on the results of chemical analysis of materials eluted from the various spots on the TLC plates.

We would advocate some caution in concluding that the chemical nature of the antigenic determinants of *M. pneumoniae* has been established. Whether those haptens, once identified, can be made effective antigens in a vaccine is of course a separate problem.

CHAIRMAN ANDREWES: The last discussant is Dr. Keith E. Jensen, Medical Research Laboratories, Chas. Pfizer and Company, Inc., Groton, Connecticut.

DR. JENSEN: The objectives described by Dr. Hilleman form part of that ideal concept, the combination of biologics, about which many have dreamed. A course of injections to provide children with resistance to a spectrum of acute respiratory diseases has been almost technically feasible, almost within our grasp, since 1962. Four years ago, when we demonstrated that children would produce antibodies in response to vaccine complexes of diphtheria-pertussis-tetanus, influenza, and parainfluenza, there seemed grounds for hope that a highly useful biologic could soon be developed. Why, then, is it not yet available? What has retarded rapid progress toward this goal?

Two considerations stand out. First, there were medical questions concerning the epidemiological importance of parainfluenza: Was widespread use of such a vaccine warranted? Some investigators would argue that such proof is still lacking. Secondly, there was the fear that avian leukosis viruses (common contaminants of egg fluids) might be oncogenic in man; this led to the conclusion that new vaccines, even when inactivated with formaldehyde, must be from leukosis-free eggs. We then elected to explore whether harvests from infected monkey kidney cultures would be sufficiently rich in parainfluenza antigens to be a practical means of vaccine manufacture and so avoid the egg problem. After vigorous efforts to improve yields from the tissue cultures and demonstrations that even when highly concentrated by centrifugation such harvests had only marginal antigenic potency, it must be concluded that the egg is a better source.

As for other components of a new combined vaccine, we now sense that it may be some time before a highly potent, safe, and practical vaccine with inactivated respiratory syncytial virus will be developed, since conventional approaches have fallen short. The danger of sensitizing with a weakly antigenic product, and so doing the child a disservice, may deter further exploration with monovalent or polyvalent materials. As Dr. Chanock described earlier today, the same phenomenon has been seen with *M. pneumoniae* vaccine. This indicated that complete departures from conventional methods of vaccine produc-

* Kenny, G. E., and Grayston, J. T. "Eaton Pleuropneumonia-like Organism (*Mycoplasma pneumoniae*) Complement-Fixing Antigen: Extraction with Organic Solvents." *J Immunol* **95**:19–25, 1965.

tion and/or administration may be necessary to achieve the desired effects. An oily adjuvant, although it enhances response to some kinds of antigens (e.g., influenzal), has not appeared efficacious in our hands with other viral antigens such as the RS virus.

I should also like to comment briefly on *M. pneumoniae* infections in families, particularly in children. It is now well recorded by Dr. Grayston and his colleagues in Seattle that school-age children have infections with this agent that result in pneumonia, bronchitis, and other disease of the respiratory tract. Early in 1965, an epidemic was followed in 89 families living in Terre Haute, Indiana, where suspected index cases were seen in a pediatric clinic. Among the patients who were later shown to belong to families infected with *M. pneumoniae*, 51 were less than five years old and 46 were five to eight years old. Antibody increases and/or positive throat cultures were demonstrated in 19 cases (37 per cent) in the younger group and in 25 patients (54 per cent) in the five-to-eight group. As others have also noted, for unknown reasons boys were more frequently infected than girls—in fact, 13 out of 26 boys (50 per cent) between less than one year and four years old presented illness associated with evidence of infection with tetracycline. Although all index cases received the drug, alternate families were given placebo, and infection-illness rates were compared with those found in families in which all members were given therapeutic doses of the drug for a week. The laboratory results indicated that the infection rate was only slightly lowered by treatment (48 per cent with placebo, 39 per cent with drug), but the incidence of infection-associated illness was significantly higher in placebo families (14 out of 58, or 69 per cent) than in treated families (14 out of 47, or 30 per cent). We concluded that—though most of the disease is mild—this agent can be of importance in a pediatric practice and that family epidemics might be controlled by tetracyclines pending the development of safe, effective vaccines.

SESSION III

ENTEROVIRUSES

Tuesday, 8 November 1966, at 8:30 a.m.

CHAIRMAN
DR. JOHN R. SEAL

RAPPORTEUR
DR. HERBERT A. WENNER

Section A.

Poliomyelitis

Presentation of Papers by:
Dr. Sven Gard
Dr. Albert B. Sabin
Dr. Herald R. Cox
Dr. D. Ikić
Dr. M. P. Chumakov
Dr. W. Chas. Cockburn

Discussants:
Dr. George W. A. Dick
Dr. Andrew J. Rhodes
Dr. Jacobus D. Verlinde
Dr. Manuel Ramos Alvarez

Section B.

Coxsackie and Echoviruses

Presentation of Paper by:
Dr. Joseph L. Melnick

Discussants:
Dr. Marina K. Voroshilova
Dr. Norman R. Grist
Dr. Dorothy M. Horstmann
Dr. Manuel Ramos Alvarez

SECTION A. POLIOMYELITIS

INACTIVATED POLIOMYELITIS VACCINE PRESENT AND FUTURE

SVEN GARD

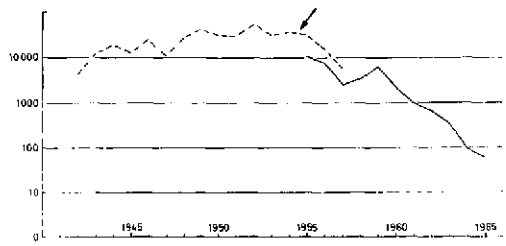
Department of Virology, Karolinska Institute
Stockholm, Sweden

DR. GARD (*presenting the paper*): There seems to be a widespread notion that inactivated poliovirus vaccine may have a certain protective value but that by itself it cannot effect complete control of the disease or, more particularly, of the circulation of the virus in the community. In a few countries where only inactivated vaccine has been used, however, both the disease and the virus have virtually disappeared. Proponents of the live vaccine have suggested, as an explanation of this fact, that mass application of live vaccine in neighboring areas could have shielded against an influx of wild virus strains. In a country thus protected, they contend, the native wild virus would eventually disappear, presumably after exhaustion of the pool of susceptible intermediates functioning as links in a continuous chain of infections.

A mechanism of this kind could undoubtedly account for the cessation of virus circulation in very small, truly isolated communities, like the Eskimo villages in northern Alaska. But the thought that it might operate in a region as large as Norway, Sweden, and Finland combined, and account for the virtual extinction of infection in the course of five years, seems slightly fantastic. These three countries constitute a continuous land area of 428,000 square miles, equalling that of Spain, France, and Great Britain taken together, or more than 12 per cent of the continental U.S.A. They have a population of 16 million, with approximately 250,000 annual births. Moreover, their people have intense contact with all parts of the world (each year, for instance, about a million Swedes spend some time abroad—a large number in Mediterranean countries where the probability of exposure to polioviruses is considerable).

The proposed explanation is not only theoretically unlikely but also disregards existing evidence obtained experimentally and otherwise. In this paper I intend, by summarizing the experience gained in Sweden, to show that complete control of poliomyelitis, including virus dissemination, can be achieved by the use of inactivated vaccine alone and to point out the necessary conditions.

It has been reported from several countries, among them the USSR, Czechoslovakia, and Hungary, that mass vaccination with inactivated virus failed to prevent the reappearance of epidemic poliomyelitis with practically unchanged attack rates. In the United States a certain drop in morbidity rates was observed in 1956 and 1957, which was attributed to the widespread use of Salk vaccine (10). If, however, the earlier history of the disease in this country is taken into consideration, it is questionable whether this reduction carries much significance (Fig. 1). In the late 1940's and early 1950's the United States had had a period of high poliomyelitis activity, and experience has shown that such peaks are often followed by a number of years of comparative quietude. At all events, the attack rates recorded in the years 1956–1960 deviate from those of the high-activity period by only one order of magnitude or less, and no downward trend is evident. Not until 1960 or 1961 was a substantial reduction in at-



(· · · ·) paralytic cases (——)
Arrow indicates initiation of mass immunization with Salk vaccine
Source: Communicable Disease Center, Atlanta, Georgia (10).

Fig. 1. Poliomyelitis in the USA, 1942–1965, total number of cases reported.

tack rates initiated, and by this time live vaccine was being widely used.

This point is further illustrated by the excerpts from the U.S. Public Health Service surveillance reports summarized in Table 1. Without precise knowledge of vaccination coverage by geographic regions, ages, and social groups, an evaluation of the protective value of the vaccine is not feasible. However, the high rate of vaccine failures is conspicuous, as it represents consistently almost one third of the total number of verified paralytic cases. The obvious conclusion is that the vaccines used could not have been very efficient.

VACCINE POTENCY

It goes without saying that efficacy is a function of the antigenic potency of the vaccine. In this respect the vaccines released in the 1950's did not come up to standard. In a field trial in 1955 (7) the relationship between vaccine potency, determined in guinea pig tests, and serologic conversion rates in prevaccination triple-negative children was studied. The results indicated that a conversion rate of 90 per cent after primary immunization with two 1 ml doses of vaccine would require a Type 1 extinction limit value of approximately 2.1 log, and a 99 per cent conversion rate, 2.5 log (Fig. 2).

Table 2 shows Type 1 extinction limits of 20 randomly selected lots of vaccine released in various countries in the period 1955–1958 and those of the first 21 lots produced by the Swedish National Bacteriological Laboratory (5). None of the commercial vaccines had a potency value above 1.5, and some were hardly more potent than so much colored water. The best of them could not be expected to induce measurable antibody responses in more than 50 per cent of the vaccinees. Besides, with such marginal antigen doses a considerable proportion of demonstrable serologic conversions would probably represent pure IgM responses, evoking only a short-lived immunological memory and offering little prophylactic benefit.

The unsatisfactory quality of these vaccines was due at least in part to the rigid precautions and the stringent technical regulations imposed on vaccine manufacture after the Cutter incident in April 1955. These precautions no doubt improved the safety of the vaccine, but they did so at the expense of its potency. The end result was a general loss of faith in this type of product.

Apart from the complex kinetics of chemical

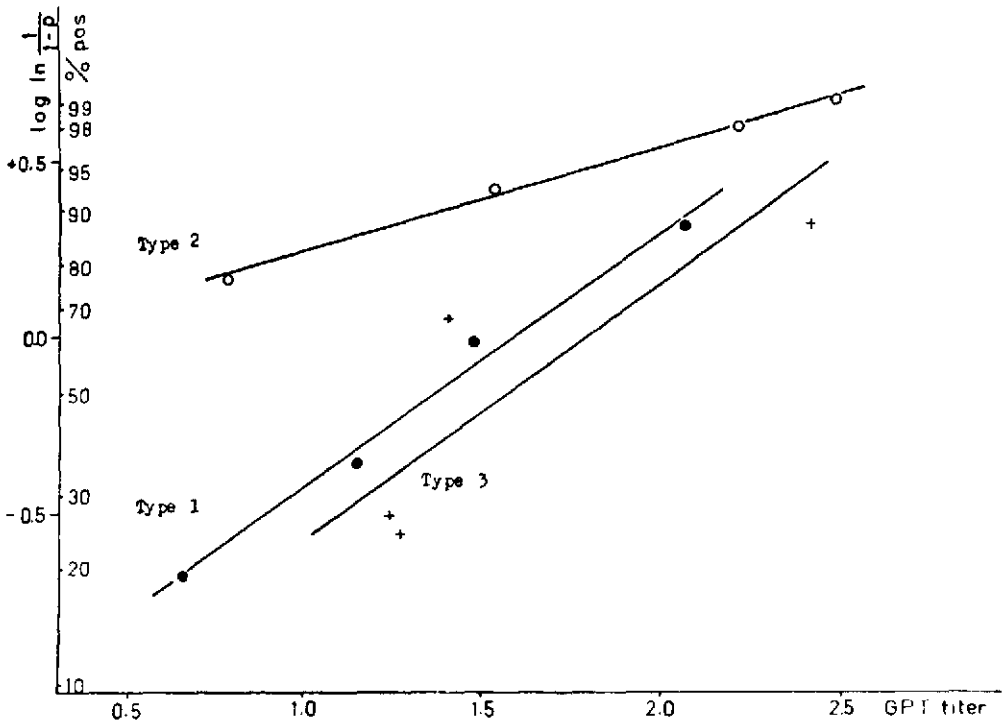
TABLE 1. PARALYTIC CASES OF POLIOMYELITIS, UNITED STATES, 1956–1963

	Total No. of cases	Percentage of total cases having received Salk vaccine (No. of doses of vaccine)				
		0	1	2	3	4 +
1956	7,911					
1957	2,499					
1958	3,697					
1959	6,289	48.8	6.5	7.2	11.5	2.5
1960	2,525	57.6	8.6	10.0	16.4	7.4
1961	829	57.1	7.3	9.5	15.5	10.6
1962	425	63.9	6.9	8.3	10.5	10.4
1963	328	63.3	7.9	6.3	13.0	9.5

TABLE 2. DISTRIBUTION OF POTENCY VALUES OF VACCINES RELEASED IN 1955–1958

Extinction limit	Type 1	
	a	b
≤0.00	2	
0.01–0.50	3	
0.51–1.00	11	2
1.01–1.50	4	9
1.51–2.00		6
2.01–2.50		4
≥2.51		
Mean	0.67	1.62

a = Commercial vaccines.
b = Vaccines prepared by the Swedish National Bacteriological Laboratory.

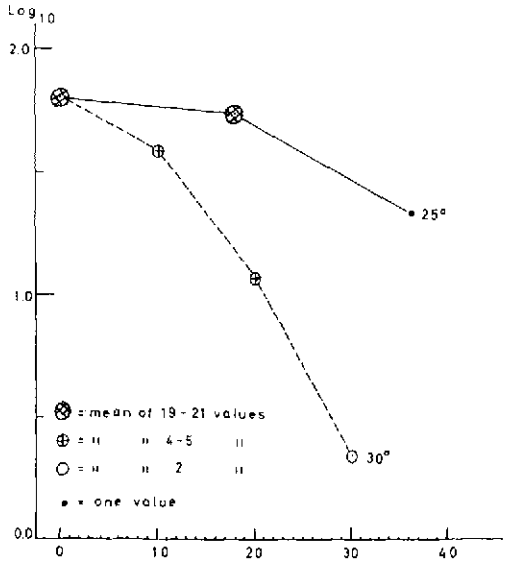


Logit-transformed conversion rates plotted against guinea pig extinction limit titers.
Source: Gard, *et al.* (7).

Fig. 2. Vaccine potency and serologic conversion in man.

virus inactivation (4), the main difficulty encountered in the production of an inactivated poliovirus vaccine has been the fact that most inactivating agents, including formaldehyde, seem to transform the native N into H-antigen, which cannot induce the production of neutralizing protective antibody. This reaction, however, is temperature dependent (Fig. 3)—and apparently more so than inactivation of infectivity. Thus, by lowering the temperature of inactivation the margin between safety and maintained potency can be widened. The Swedish vaccine was originally inactivated at 25°C for eight weeks, but more reproducible results have since been obtained by inactivation at 30°C with a holding time of four weeks, with no appreciable impairment of the potency.

Another point of great importance is the choice of virus strains. We found in early experiments (8) that the immunizing capacity in guinea pigs of Mahoney strain Type 1 was inferior to that of a certain wild strain of moderate virulence, as was Leon Type 3 to the Sauckett strain, and so

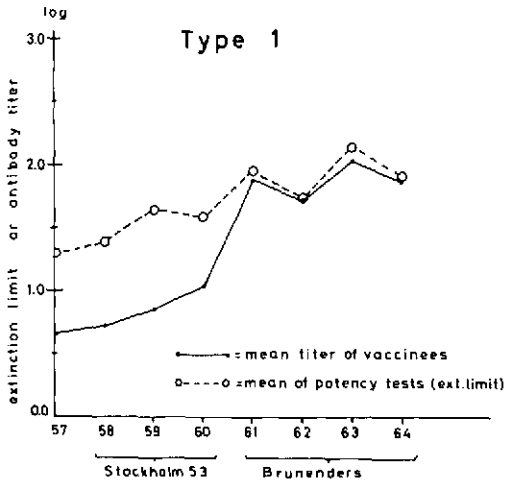


Guinea pig extinction limit titer values after varying holding times. Average values.
Source: Böttinger, M., *et al.* (3).

Fig. 3. Formol-inactivation of N-antigen at 25°C and 30°C.

we naturally chose the latter strains of each type for our vaccine. When in 1961 the Type 1 strain originally used was exchanged for the attenuated Brunenders strain, it was observed, much to our surprise, that the antigenicity of the Type 1 component of the vaccine was significantly higher for man than for guinea pigs. It had previously been well known that poliovirus Type 2 was relatively much more antigenic in man than in guinea pigs. Apparently the human organism is capable of recognizing intratypic qualitative differences that find no expression in immunization tests with guinea pigs. Thus, even though potency tests performed in experimental animals give reliable results when the same strains of virus are being used, they may be misleading when different strains are being compared.

Figure 4 shows comparisons between Type 1 potency values of the vaccines used in Sweden and the average serum titers obtained in prevaccination triple-negative children after primary immunization with two doses of the corresponding vaccines. The high correlation between the two is obvious, as is the effect of the introduction in 1961 of the Brunenders strain. As is shown, the potency of the vaccines has gradually improved over the years. No distinct changes have been made in production methods that could account for this trend. I believe, however, that the phenomenon is not uncommon—that as familiarity with a certain procedure increases, the results tend to improve. The present product seems to fulfill reasonable requirements for potency.



Guinea pig extinction limit titers and average serum titers expressed in log units. Brunenders strain introduced in 1961. *Source:* Böttiger, M. (2).

Fig. 4. Vaccine potency and average titer values after primary immunization.

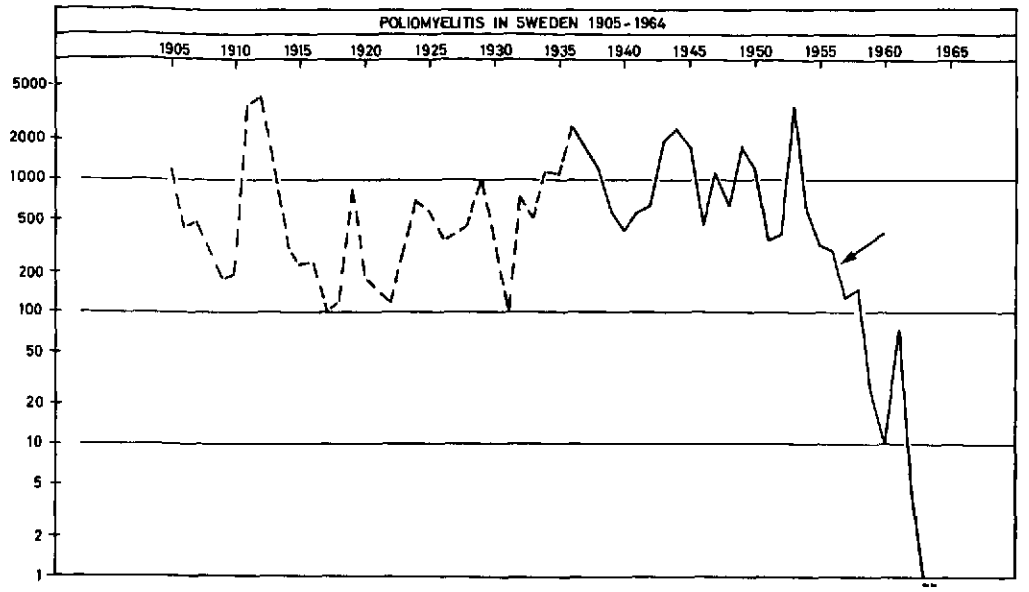
SAFETY

Mass vaccination in Sweden has always been restricted to the off season, from February or March to May, in order to reduce to a minimum the chance appearance of unassociated poliomyelitis in the vaccinees. This precaution has paid off. Among 5.4 million vaccinees who have received between 15 and 20 million inoculations there has not been a single reported case of neurological complications within 30 days after inoculation that could be even remotely associated with the vaccine.

Extraneous viruses have not presented any serious problems. Since February 1958 only cynomolgus monkeys have been used for preparation of tissue cultures. SV_{40} virus has not been encountered on any occasion. Pools of monkey serum or gamma globulin kept in storage, sera from guinea pig potency tests, sera from vaccinees, and leftovers of old vaccine lots have been examined. So far there is nothing to indicate that any Swedish vaccine has contained SV_{40} virus, live or dead.

PERFORMANCE IN THE FIELD

The results of mass application of the vaccine have been spectacular. In the course of five years the morbidity rate decreased from a 20-year annual average of 1,132 cases to virtually zero. Figure 5 shows reported cases of poliomyelitis in Sweden since 1905, when notification was first made compulsory. Up until 1935 paralytic and nonparalytic cases were reported together, but since 1936 paralytic cases have been reported separately. I should like to emphasize that prior to vaccination the disease had been continuously present in the country for more than 55 years. Its activity had varied somewhat from one year to the next, but there had been no really quiet periods and the fluctuations had been small when measured in terms of orders of magnitude. Therefore, the sharp decline in morbidity rates immediately following



Total number - - - - -
Paralytic cases only ——.
Arrow indicates initiation of mass vaccination.

Fig. 5. Reported cases of poliomyelitis in Sweden annually since 1905.

the introduction of mass vaccination carries a distinct significance.

The steady downward trend was interrupted in 1961 by a local outbreak of 55 cases in Gothenburg. Since detailed vaccination records were available, the protective effect of the vaccine could in this instance be calculated to at least 98 per cent (1).

I should like to point out that the effect of the vaccination became quite apparent at a time when live vaccine had not yet been applied anywhere on such a scale as to have any possible effect on the morbidity rate in Sweden.

Figure 6 gives further details on the relationship between vaccination and morbidity rates. In 1957 a total of 717,000 children in the top-risk age group—4 to 11 years—received their primary inoculations. As a result, this age group was virtually eliminated from the morbidity distribution pattern for that year, whereas in nonvaccinated age groups morbidity rates were the same as in previous years.

In 1959, by which time the group between 2 and 26 years had been covered and a total of 2.1 million persons (out of a population of 7.7 million) had been vaccinated, a marked decline in attack rates among nonvaccinated individuals became apparent, and the trend was further accentuated in the following years. This indicated a diminished rate of exposure, which was also reflected in a decreasing rate of virus isolations in routine examinations of stools from patients in infectious disease wards.

The probable explanation for this unexpected development was found in experiments in which prevaccinated individuals were fed live attenuated virus (6), and the rate and duration of

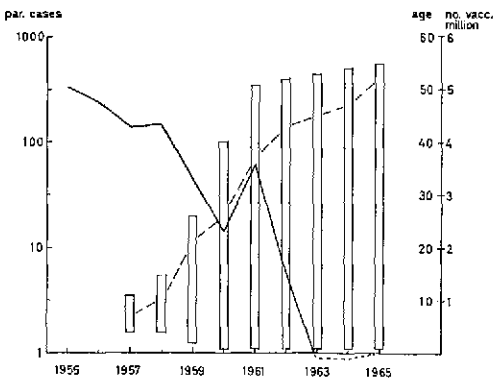
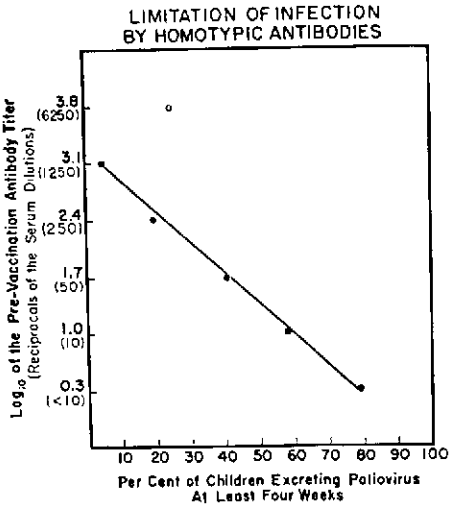


Fig. 6. Paralytic poliomyelitis (log scale) (——), number of vaccinated persons (- - - -), and age groups vaccinated (open bars).

virus excretion were observed to be inversely correlated to the serum titer at the time of feeding. At very high titers the alimentary tract might be completely insusceptible to infection with even large doses of virus (Fig. 7). These observations, which have since been repeatedly confirmed, indicate that, contrary to what was originally believed, circulating antibodies might measurably affect the susceptibility of the mucous membranes of the alimentary tract to implantation of the virus. A sufficiently extensive immunization with a sufficiently potent inactivated vaccine would therefore provide an effective brake on the circulation of the virus in the community.

Vaccine failures have been remarkably few. A total of five paralytic cases have been reported in persons who had received at least two doses of early vaccines. Four of these cases were relatively mild; in only one case was residual severe paralysis recorded.

As of now 5.4 million persons, or 71 per cent of the total Swedish population, have received at least two inoculations of vaccine. Coverage below age 55 is approximately 85 per cent and below age 20 at least 95 per cent. This is apparently sufficient to keep the domestic situation under control, but it is still unsatisfactory in that it does not completely exclude the possibility that persons visiting regions abroad where the risk of exposure still exists might contract the disease and/or import the virus into the country.



Source: Pagano *et al.* (9).

Fig. 7. Percentage of children excreting virus for at least four weeks as a function of serum titer at time of feeding.

In 1963 no case of poliomyelitis was reported, but Type 3 virus was found on one occasion in sewage in Gothenburg.

In 1964 one Type 2 strain was isolated from a child from Tanzania. The family had been residing in Stockholm for some time and had repeatedly received visits from African students.

In 1965 an unvaccinated Swedish journalist contracted paralytic poliomyelitis after a week in Baghdad. She returned home and infected her unvaccinated infant daughter but not her adequately vaccinated son. No further spread of the infection could be traced.

In 1966 a nurse, who had worked with poliomyelitis patients for more than 20 years and who had not cared to be vaccinated, contracted paralytic poliomyelitis during a vacation in the Canary Islands. No secondary spread of virus was observed.

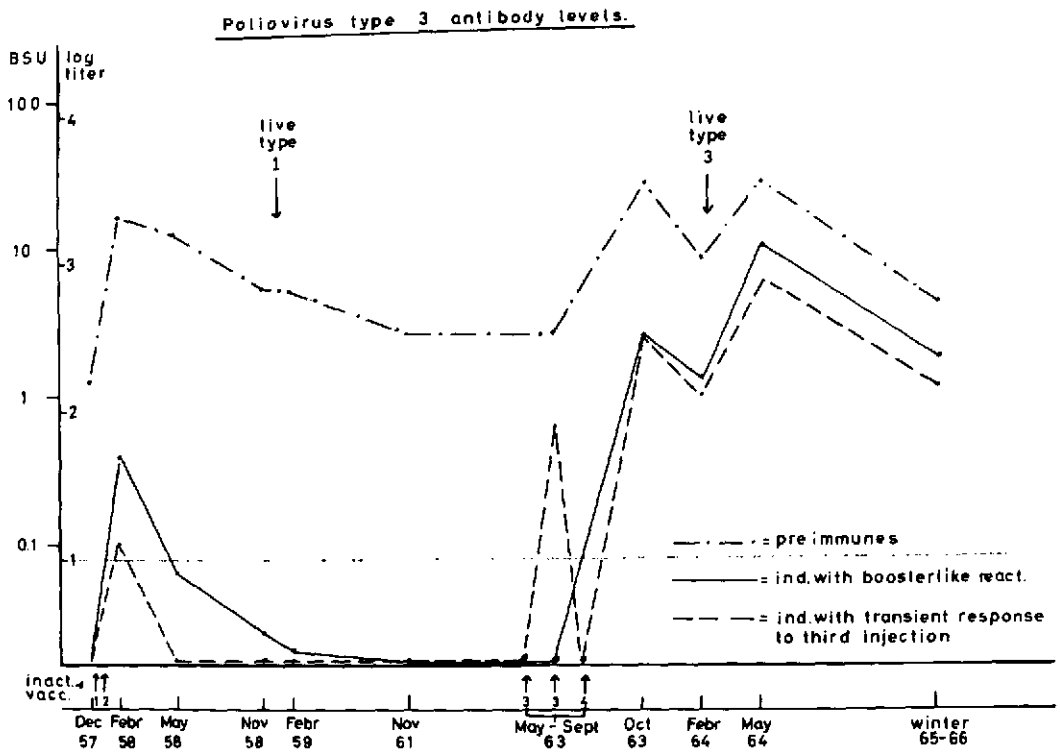
Finally, this year one child in Malmö developed a mild paralytic disease and a sibling developed a nonparalytic infection. Neither of them had been vaccinated. The father runs a restaurant in which many immigrant workers had been employed as dishwashers. The source of infection could not be definitely established. An extensive survey of the whole residential area failed to unveil any further poliovirus infections.

With the exceptions mentioned, no strains of poliovirus have been recovered from between 5,000 and 10,000 stool samples annually examined during the last four years.

At present the threat of virus imported from abroad can apparently be met only by intensified vaccination propaganda.

DURATION OF IMMUNITY

The problems of safety and efficacy seem to have been satisfactorily solved. However, the equally important question of duration of immunity has not yet been answered once and for all. I should like to open the discussion of this point by referring to results obtained in the course of a study (2) on the use of live attenuated Type 3 virus (Fig. 8). In 1957 all members of a group of 20 volunteer families were given two inoculations of the comparatively weak trivalent vaccine then available. Those with preexisting natural immunity responded with clear-cut booster effects and their serologic titers have since stayed at a high level. The previously non-immunes could be divided into two groups. In group "a," a minority of about one fourth,



Source: Böttiger, M. (2).

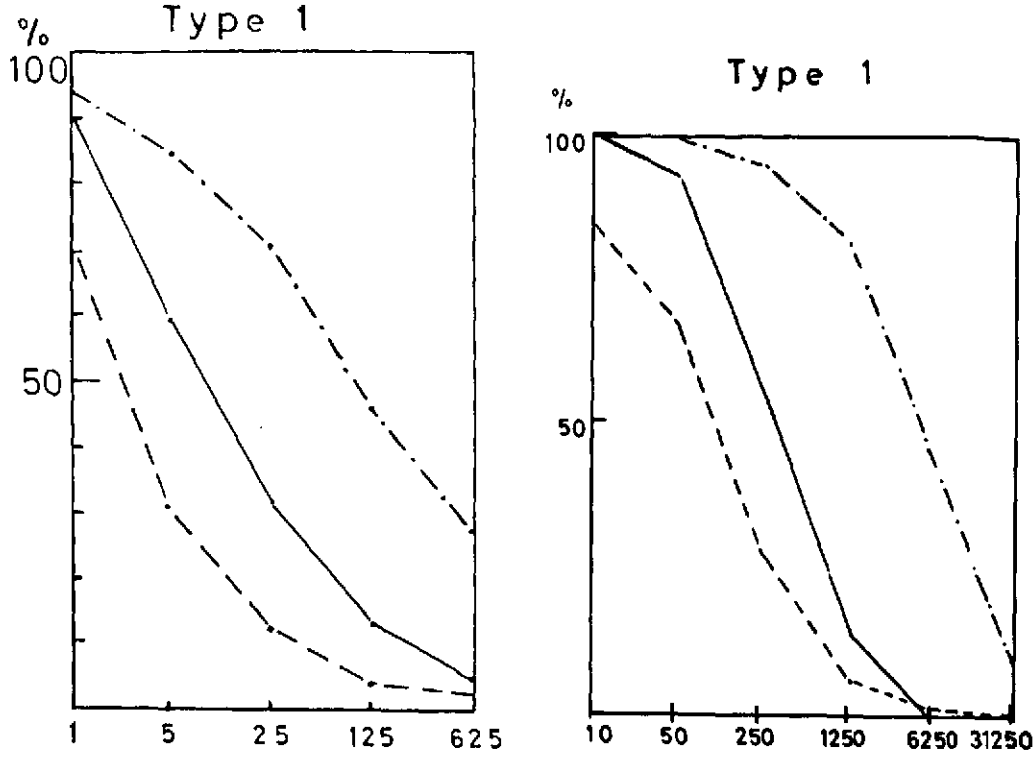
Fig. 8. Type 3 antibody levels after primary immunization with inactivated vaccine and effect of a booster injection five and one half years later.

titers were low, responses were transient, and antibodies were no longer demonstrable one year later. In group "b," the three-quarter majority, primary titers were higher and, after a decline during the first years, serum antibodies remained demonstrable at a low but constant titer level for at least five and one half years.

In 1963 all subjects were given a booster inoculation with a potent vaccine. On this occasion group "b" gave a typical secondary response with very high titers, which were not significantly enhanced after a subsequent feeding of live virus. Group "a," on the other hand, gave a primary response; only after a second inoculation six months later were typical booster effects obtained.

We have interpreted this observation as an indication that the early vaccine, although sufficiently potent to produce IgM responses in all vaccinees, did not have enough antigen content to induce IgG antibody formation in more than 75 per cent of the subjects. Without IgG responses no lasting immunological memory will be evoked and later inoculations will not produce booster effects. It is not sufficient to obtain demonstrable serologic conversion; the antigen dose must be large enough to produce IgG responses.

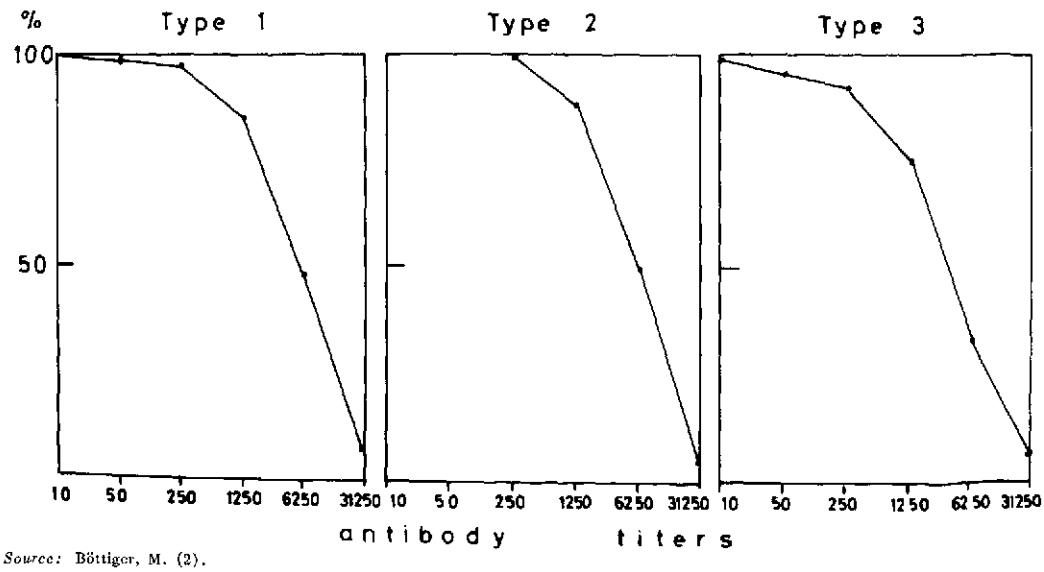
Present evidence thus indicates that the early vaccines did not come up to the potency standard that should be required. Follow-up studies of groups of prevaccination triple-negatives have also shown that up to 25 per cent of those vaccinated in 1957 and 1958 had no demonstrable antibodies four to five years after the third dose. We have therefore recommended a fourth inoculation five years after the primary immunization. This routine will be relatively easily maintained in the future when children receive their first immunization at the health centers and their second booster injection in the first grade of school. The question is whether this will be any longer necessary after the considerable improvement in the quality of the vaccine.



Source: Böttiger, M., *et al.* (3).

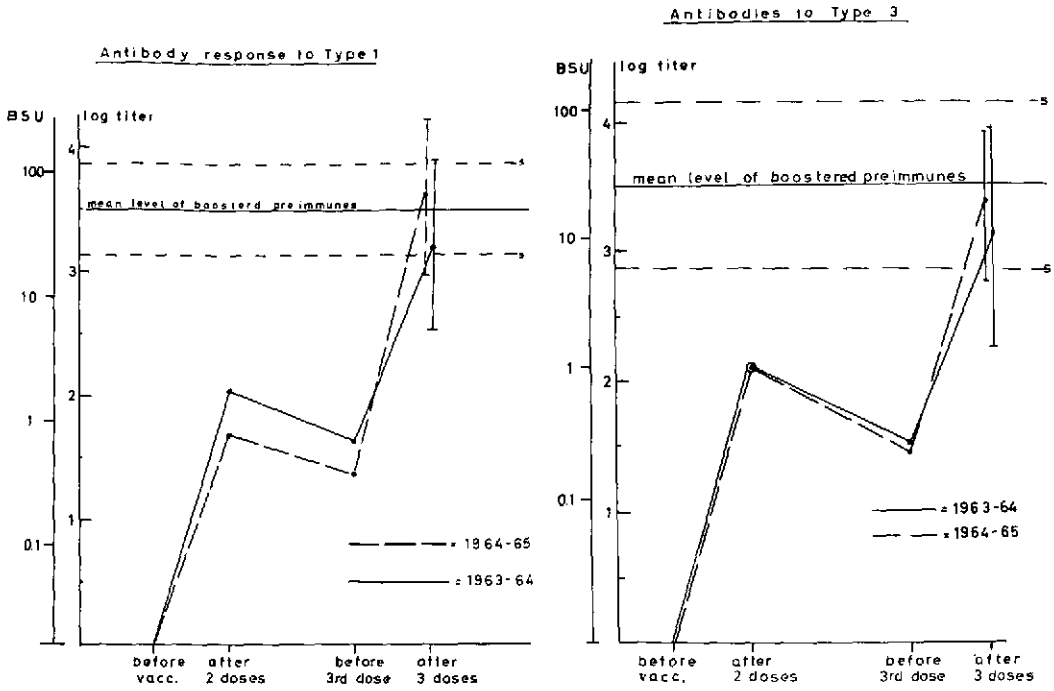
Figs. 9 and 10. Immunity profile of prevaccination triple-negative children two weeks after primary immunization (——) and before (- - - -) and after booster injection (— — —), 1957-1958 and 1963-1964.

"Immunologic profiles" after vaccination with 2 doses in 63 and 1 in 64



Source: Böttiger, M. (2).

Fig. 11. Immunity profiles against Types 1, 2, and 3 after completed immunization with present vaccines.



Types 1 and 3. Broken lines indicate 95 per cent confidence limits of mean titer of hyperimmunized pre-immune adults.
Source: Böttiger, M. (2).

Figs. 12 and 13. Titer levels in hyperimmunized pre-immunes and in prevaccination triple-negative children after primary immunization and before and after booster injection.

Figures 9 and 10 show the results obtained with the 1957–1958 and 1963–1964 vaccines, respectively. The increase in median Type 1 titers from 125 to 6,250 is a measure of the improvement in potency. Figure 11 shows the uniform results now obtained with all three types of virus. Finally, Figures 12 and 13 compare vaccination effects with reactions in natural immunes. A naturally pre-immune person reacts to an inoculation of inactivated vaccine with a typical secondary response. The resulting high titers cannot be significantly enhanced by further hyperimmunization with either inactivated or live virus—they represent a sort of maximum response. The figures show that titer levels reached after completed immunization fall within this maximum response range. Continued follow-up studies on consecutive groups of vaccines will eventually provide an answer to the question of duration of immunity. There seems to be reason to believe that the immunity conferred by the present potent vaccines will prove to be as long-lasting as that following infection with live virus.

THE FUTURE

As far as Sweden is concerned, reasons for any major changes in vaccination policy are lacking. Two questions have been raised, however. Pediatricians are anxious to change the vaccination schedule so that all necessary injections will be given in the first months of life. Since we may expect in the future to deal with an infant population having uniformly very high titers of maternal antibodies, to meet this request would most probably require the production of vaccines of substantially higher potency values than those now available. This can hardly be achieved without an expensive concentration procedure. To be sure, at present about 75 per cent of the original N-antigen activity is lost in the inactivation process. Since this loss seems to be built into the inactivation technique, it probably cannot be avoided. Concentration of the virus is technically feasible, and practicable methods have already been developed, but the cost of the product thus obtained is at present prohibitive. One possibility for lowering the

cost would be to use easily cultivable permanent cell strains, or possibly cell-lines giving good virus yields, for the tissue cultures.

The other question concerns the application of multipurpose combined vaccines. It has already been shown by several groups that inactivated trivalent poliovirus vaccine can be incorporated into triple-vaccine or diphtheria-tetanus toxoid preparations without impairment of the immunogenic activity of any of the components. Other inactivated vaccines, for instance those against measles and RS virus, can also probably be added to such a vaccine cocktail. From an administrative and organizational point of view such a procedure would greatly simplify routine immunization of children against the growing number of infectious agents that seem to call for prophylactic measures. This appears to be one of the major advantages of inactivated vaccines over live ones.

The greater the number of components in a combined vaccine, the greater will be the need for purification of the various antigens. The development of practicable purification methods should therefore be encouraged. Purification would serve two purposes: it would remove substances causing unwanted side reactions and, by eliminating nonessential antigens, it would reduce immunologic competition to a minimum —a requirement that will assume greater urgency as the number of essential antigen components increases.

Summary

Mass immunization with inactivated poliovirus vaccine has been shown to be a highly effective method for control of both the paralytic disease and the circulation of virus in the community. However, this effect can be obtained only if (a) the vaccine is sufficiently potent to induce the formation of IgG antibodies in practically 100 per cent of the vaccinees, and (b) a sufficiently large proportion of the epidemiologically significant age groups is immunized.

Vaccine potency depends on the choice of vaccine strains as well as on the method of inactivation. Tests in animals may not always provide reliable guidance in strain selection. The question of the duration of immunity induced by inactivated vaccine is not yet definitely settled, but the prospects for achieving a long-lasting immunity appear to be good.

Tasks for the future include the production of purified multivalent vaccines and possibly the preparation of concentrated products for the immunization of infants in the first months of life.

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SECTION A. POLIOMYELITIS

POLIOMYELITIS: ACCOMPLISHMENTS OF LIVE VIRUS VACCINE

ALBERT B. SABIN

The Children's Hospital Research Foundation
University of Cincinnati College of Medicine, Cincinnati, Ohio, U.S.A.

DR. SABIN (*presenting the paper*): Six years have now passed since the mass use of live attenuated oral poliovirus vaccine was initiated as a public health measure in many parts of the world, and each year since 1960 additional countries have conducted mass vaccination campaigns with this type of vaccine. Although I know of no accurate statistics, the estimate that altogether more than 350 million people have received oral poliovirus vaccine is probably fairly reliable. This figure includes populations of most of the countries in Europe and North America and many countries or regions in South America, Africa, Asia, and Oceania.

The vaccine has not been uniformly administered in all parts of the world or even necessarily in different parts of a single large country. The variations involved patterns of mass use such as initial mass campaigns carried out over short periods of time with varying degrees of coverage of the most susceptible age groups versus routine administration over long periods of time, dosage schedules using monovalent or polyvalent vaccines or both, repetition of immunization, extent of ongoing immunization of the new generations of children, exclusive use of oral vaccine, or continued use of both oral and inactivated virus vaccine, and so on. Despite the great number of variables in the use of the oral vaccine and the frequent inadequate follow-up and analysis of data in a number of countries, some tentative conclusions may nevertheless be drawn concerning the requirements for eradication or control of the disease and the naturally occurring polioviruses under different social, hygienic, and climatic conditions.

The objective in the mass use of oral poliovirus vaccine is not only to protect a large number of vaccinated individuals against the paralytic disease, which can also be achieved to a large extent by inactivated virus vaccine, but also to break the chain of transmission of the virulent polioviruses so that the disease can be more effectively controlled or entirely eliminated from large population groups when only a certain proportion of the susceptible population has been vaccinated—a proportion that *a priori* can be expected to vary in areas with high and low prevalence of polioviruses. The prediction, based on early experimental field studies in different parts of the world (13), that initial mass immunization campaigns with oral vaccine carried out rapidly and covering 70 to 80 per cent of the susceptible child population would quickly effect such a break in the chain of transmission, which could then be maintained by adequate ongoing immunization of the new generations of children and of other vaccinated newcomers, seems already to have been fulfilled in several areas of the world that have temperate climates and good health services. Judgment regarding the complete disappearance of the disease is possible only where reports of clinically diagnosed cases of poliomyelitis-like paralysis or weakness are thoroughly investigated by competent critical clinicians and virologists to eliminate those cases that are clinically not like poliomyelitis and virologically not related to infection with a poliovirus.

Results in countries with good health services. Czechoslovakia, with a population of 14 mil-

lion, was one of the first countries to have a national mass oral poliovirus vaccine campaign with adequate subsequent vaccination of the new generations of children and to conduct extensive, ongoing systematic clinical and virologic studies (14). The first nationwide campaign was carried out in the spring of 1960 in the face of a mounting outbreak of poliomyelitis. It was designed to reach children from 2 months to 14 years of age. The schedule involved an initial administration of Type 1 vaccine followed by a mixture of Types 2 and 3 in 1960 and a repetition of the same schedule in the spring of 1961 for the children vaccinated in 1960 and those born since then. The coverage was reported as being at least 90 per cent. Since 1961 the new generations of children have been vaccinated in the spring the same way and revaccinated the following year. The outbreak in 1960 was rapidly halted, and from July 1960 to the present time there has not been a single confirmed case of paralytic poliomyelitis of local origin. Moreover, annual studies on thousands of stool specimens from young, healthy children failed to detect any naturally occurring polioviruses. This seems to be a good example of the predicted eradication of both the disease and the naturally occurring polioviruses from a country that previously had a constant, though variable, annual prevalence of paralytic poliomyelitis, including also the three years following the mass use of inactivated virus vaccine.

It would appear that comparable or nearly similar results may have been achieved in other central, eastern, and southern European countries using somewhat different but not too dissimilar vaccination schedules. In the USSR, where almost 90 per cent of the population under 21 years of age had received all 3 types of the vaccine by the middle of 1960, subsequent vaccination involved not only the oncoming generations of children but also the annual revaccination of almost all children up to eight years of age. On the basis of clinical and virologic studies of limited samples from various parts of the USSR, Professor M. P. Chumakov has expressed the opinion that paralytic disease of poliovirus etiology has been completely or almost completely eliminated in most of the Soviet Union, but he points out at the same time that because of the country's vast and varied climate and population and almost inaccessible districts there remain localized areas of inadequate vaccination and surveillance.

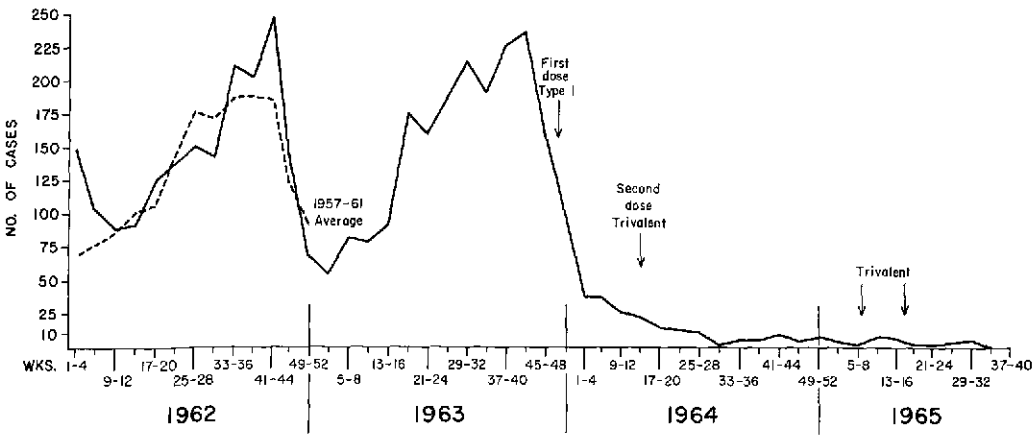
Belgium, with a population of about 9.3 million, provides an interesting example of the effects of a mass campaign with oral vaccine that reached only about 60 per cent of the children between 3 months and 15 years of age and about 35 to 40 per cent of the adults up to age 40. Table 1 shows the number of cases reported annually from 1950 to 1957, when no vaccine was used, from 1958 to 1962 when inactivated vaccine was used; and after the mass campaign with monovalent oral vaccines, which began 17 March 1963. Oral vaccine has been given on a voluntary basis in 1964, 1965, and 1966, but a large proportion of the children have remained unvaccinated. The effect of oral vaccine has been rapid, and despite the relatively low coverage, the disease has been practically eliminated. During the five years of comparably extensive use of inactivated vaccine there was still a total of 958 cases—a definite reduction from the 2,731 cases reported in the preceding five years when no vaccine was used (2, 3, 6). The extraordinary effect of the oral vaccine in Belgium with such a large proportion of the susceptible population still unvaccinated is most likely the result of a break in the chain of transmission of the naturally occurring polioviruses in a country with a relatively low prevalence of such viruses prior to vaccination.

Spain and Italy, which have larger and more diverse populations and climates, provide interesting examples of different kinds of mass campaigns with oral vaccine that have had rapid and marked effects but have failed to eliminate certain foci of continued paralytic activity of the naturally occurring polioviruses—foci with a higher prevaccination prevalence of polioviruses and a lower vaccine coverage. The data for Spain (11, 15), which has a population of about 31 million, are shown in Table 2 and Figure 1. The number of cases increased from an average of about 1,100 per annum during the period 1950–1954 to an average of about 1,700 per annum during 1957–1961. Despite continued use of inactivated vaccine, although not on an extensive scale, the annual number of cases continued to increase: there were 1,853 in 1962 and 1,959 in 1963. An extraordinarily well-organized free mass campaign with oral vaccine was carried out in December 1963, during

TABLE 1. POLIOMYELITIS IN BELGIUM (1950-1966)

Vaccine used	Year		No. reported
None	1950		86
	1951		118
	1952		897
	1953		184
	1954		198
	1955	2,731	979
	1956		1,038
	1957		332
Inactivated	1958		162
	1959		142
1.5 million children	1960	958	301
(6/12–15 yrs.) in 1958	1961		203
and then continued	1962		150
Oral	1963		16 *PRE* + 22 *POST*
	1964		1?
Mass campaign	1965		1?
begun 17–III–63	1966 (9		2 (+3?)
3 million (3/12–40 yrs.)	months)		

From data of the Ministry of Public Health. The official report of the Ministry shows two cases for 1964, but both of these had their onset in 1963. The one case recorded with a question mark next to 1964 refers to a rapidly fatal case of quadriplegia with respiratory difficulty that was not reported as poliomyelitis and was not sufficiently investigated to permit a diagnosis. The question marks next to the cases in 1965 and 1966 indicate doubt that polioviruses were responsible for the disease.



Source: F. Pérez Callardo *et al., Revista de Sanidad e Higiene Pública* **39**:537-561, 1965.

Fig. 1. Poliomyelitis in Spain.

TABLE 2. POLIOMYELITIS IN SPAIN (1950-1966)

Period	No. cases/annum
1950–1954	1,103 (average)
1957–1961	1,711 (average)
1962	1,853
1963	1,959
1964—First 24 wks	152 – 70% confirmed
—Last 28 wks	51 – 25% "
1965	70 – 44% "
1966—First 6 mos	160 – 50% "

which Type 1 vaccine was administered to 4.3 million children between 2 months and 7 years of age, or 95 per cent of Spain's population in this most susceptible age group. Three months later, in April 1964, 4.6 million children of the same age group—99 per cent of the total—received a dose of trivalent vaccine. The effect of this campaign was immediate. First, cases due to the Type 1 virus practically disappeared, and after the second campaign with trivalent vaccine a dramatic total reduction took place. This reduction was even more marked than the number of reported cases would indicate, since most of the small number of reported cases either were clinically not like poliomyelitis or failed to yield polioviruses. During the first half of 1965 the children born since the previous campaign in 1964 received two doses of trivalent vaccine: 584,000 received the first dose and 554,000 received the second. A further drop in total number of reported and confirmed cases occurred in 1965. It was followed, however, by a slight rise in 1966, mostly in the southern provinces. Of the 31 confirmed cases in 1965, 18 were unvaccinated and 12 had received only one dose; the vaccination status of one was unknown. Among the 79 virologically confirmed cases in 1966, 55 were unvaccinated, 15 had received one dose, and only 4 had received two doses. These data indicate that the vaccine used in Spain is protective but that dissemination of paralytic strains of polioviruses has not been eliminated in some areas of the country, particularly in the southern provinces.

Italy, which has a total population of about 51 million and climatic and social conditions that differ widely between the northern and southern regions of the country, provides still other lessons of general interest regarding the effects of the mass use of oral vaccine (4, 8, 9). Some of the significant data for Italy are shown in Figure 2. After six years of extensive use of inactivated vaccine Italy continued to have large numbers of cases of paralytic poliomyelitis. A very well organized nationwide free mass campaign with the three monovalent oral vaccines during the spring of 1964 had an immediate effect. A dose of trivalent vaccine was then offered to the same persons in November 1964. The vaccine was offered to persons between the ages of 3 months and 20 years, with high priority given to those under 6 years of age. By May 1965 the full course of three monovalent doses and one trivalent dose had been given to 80 to 90 per cent of the children under 6 years in most of the northern provinces and to about 50 to 60 per cent in most of the southern provinces. An energetic government organization continued to vaccinate the new generations of children as well as others who were missed in the original campaigns. Although there is a continuing drop in the number of reported cases—and the reported number is not based on persistence of paralysis or clinical or virologic confirmation—small numbers of cases still occur, mainly in the southern areas with a higher prevaccination prevalence of polioviruses and with a lower vaccine coverage. Almost all the reported cases are now in unvaccinated or incompletely vaccinated children. Thus, the problem is not so much the ineffectiveness of the vaccine due to interference by other enteroviruses but rather the failure to effect sufficient interference with the chain of transmission in areas of high poliovirus prevalence and insufficient vaccine coverage. A coverage of 50 to 60 per cent that appeared to be sufficient in Belgium is not sufficient for the southern parts of Italy.

The events in the United States, in my opinion, provide another example of how mass vaccination of a portion of the population with oral vaccine has cut down the general dissemination of polioviruses to such an extent that the disease has been practically eliminated among most of the approximately 200 million people—many of whom, including a large number of young children, unfortunately remain unvaccinated. Some of the pertinent data for the United States, derived from the official morbidity and mortality

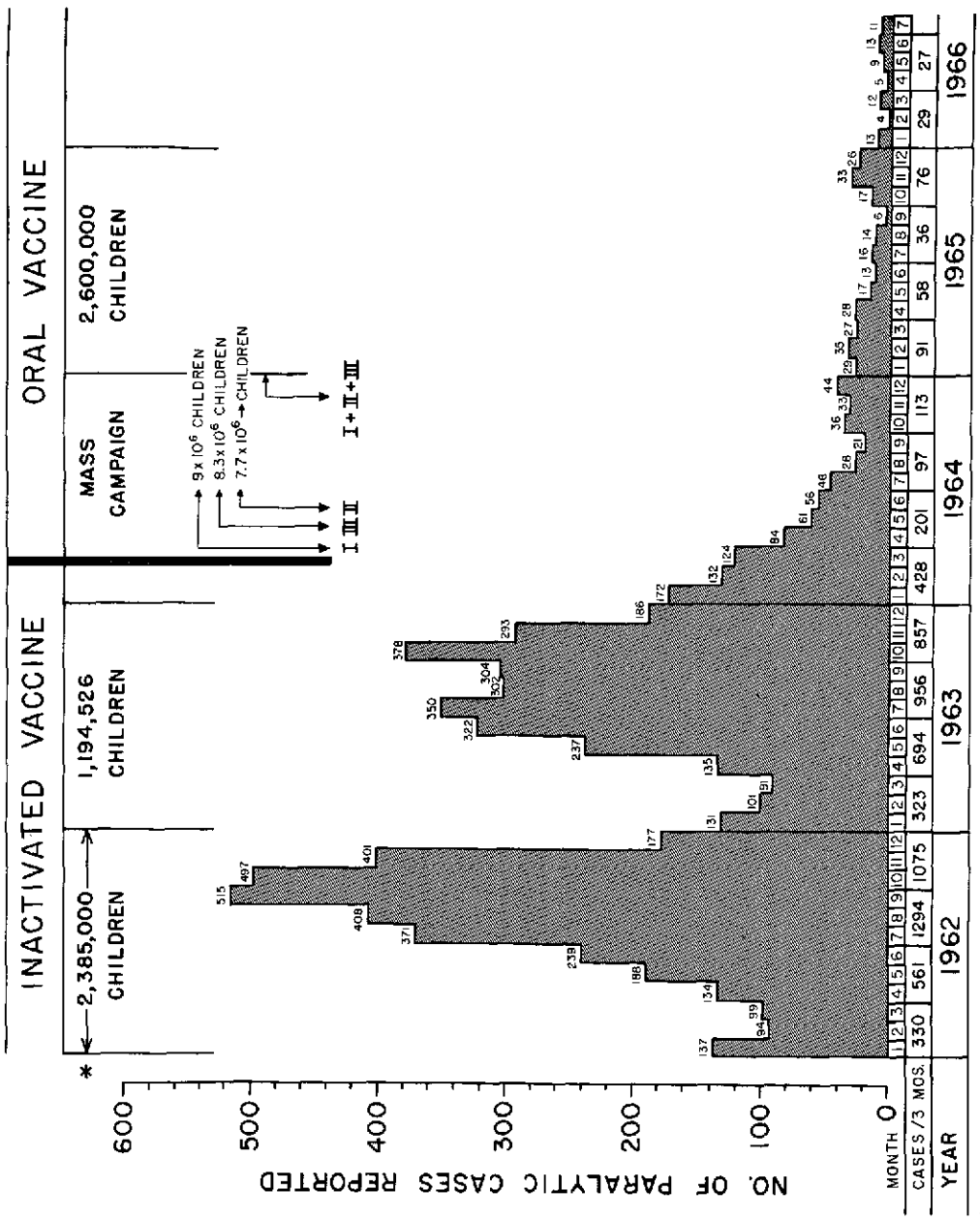


Fig. 2. Paralytic poliomyelitis in Italy, 1962-1966.

reports of the U.S. Public Health Service, are shown in Table 3. Despite the considerable diminution in the number of cases following the extensive use of inactivated vaccine from 1955 on, thousands of paralytic cases continued to occur annually, and regional epidemics broke out each summer. In 1961 and 1962 oral vaccine was used to abort incipient epidemics, and from the end of 1962 to 1964 about 100 million persons of all ages received oral vaccine in exceptionally well-organized community campaigns under the direction of local medical societies. In 1964 for the first time there was no increased summer prevalence and no outbreak requiring emergency mass vaccination. A total of 91 cases were reported, 11 of them in Texas, and there is reason to believe that many of the 91 cases were not caused by poliovirus. The downward trend continued in 1965, Texas contributing 18 out of the 61 reported cases. During the first 9 months of 1966, 53 of the 66 reported cases occurred in Texas counties with a population of only 1.9 million, and some of those reported in the other states could also be traced to Texas. The Texas cases have occurred predominantly among unvaccinated Mexican-Americans along the Mexico border at points where there have been outbreaks of poliomyelitis. A nationwide immunization survey conducted in the United States in September 1964 indicated that in the 1-to-4-year age group 67 per cent had received one dose of oral vaccine (presumably mostly only Type 1) and 47 per cent had three doses. Since these survey data represent an average for the entire country, and since many communities have had no mass campaigns with oral vaccine, the extraordinarily low incidence of poliomyelitis achieved throughout the country (except among unvaccinated children along the Mexican border) suggests that important breaks in the chain of transmission of the polioviruses in the massively vaccinated regions have also greatly reduced dissemination of naturally occurring polioviruses in the entire country.

Most probably, some of the small European countries that use only inactivated vaccine and recently have had practically no poliomyelitis—for example Finland, where a recent serologic survey (7) showed that about 60 per cent of the children under three years of age have no demonstrable antibody—are also to a large extent protected by the massive use of oral vaccine elsewhere in Europe.

In Asia, Japan provides an example of another large country with highly developed health services in which governmentally directed mass use of oral vaccine during the past five years has almost completely eliminated the disease (12). Israel has demonstrated that good health services can overcome the special problems posed by subtropical countries—namely, year-round high level of dissemination of polioviruses and other enteric viruses coupled with a high incidence of infection during the first year of life. Poliomyelitis has been practically eliminated from Israel since the first antiepidemic mass campaign with oral vaccine in 1961 has been followed by an excellent ongoing program of oral vaccination of infants during the first year of life. This achievement has taken place despite

TABLE 3. POLIOMYELITIS IN THE U.S.A. (1958-1966)

Vaccine used	Year	Paralytic cases*
Inactivated	1958	3,201
(since 1955)	1959	5,472
	1960	2,218
Oral—Epidemic	1961	829
" —Epidemic + some community	1962	691
" —Community + epidemic	1963	336
" —Community	1964	91 (Texas–11)
" —Ongoing	1965	61 (Texas–18)
" —Ongoing	1966 (9 mos.)	66 (Texas–53)†

*Only cases with residual paralysis two months after onset.
† The 53 cases occurred in 22 counties with a population of 1.9 million.

the fact that the polioviruses are still active in several Arab communities within its borders in which it has not been possible to vaccinate many of the children (5, 12).

Results in countries with poorly developed health services. Mass campaigns with oral vaccine have been carried out in many subtropical and tropical countries of South America, Africa, and Asia where the health services, like the countries they belong to, are in various stages of development. Dramatic decreases in numbers of cases have occurred within a few weeks after mass campaigns carried out during nonepidemic periods. However, even when mass campaigns reached as much as 90 per cent of the susceptible age groups, as in the Brazilian states of Guanabara and São Paulo, the chain of transmission of polioviruses was only partly interrupted. Insufficient coverage in localized regions, the continuous immigration of unvaccinated children from other areas with high prevalence of polioviruses, and failure to vaccinate more than a small proportion of the children born after the mass campaigns have all contributed to the continued occurrence of large numbers of cases, predominantly among unvaccinated infants (12).

Severe epidemics of poliomyelitis, which still occur in economically less developed countries with poorly developed health services, will continue to require good antiepidemic vaccination programs with oral vaccine. Recent experience in Ceylon (1), Guyana * (10), and other places has shown how limited is the beneficial effect of antiepidemic mass vaccination when it is begun late and when trivalent vaccine is administered slowly over a period of many weeks instead of a few days. It seems to me that it would be very helpful if the Regional Offices of the World Health Organization could be prepared to assist such countries in emergency antiepidemic campaigns in the following ways:

1. Arranging for local health authorities to give prompt notification of an unexpected increase in cases.
2. Arranging for *rapid* isolation and typing of viruses so that the appropriate monovalent vaccine can be used.
3. Making prior arrangements for storage of emergency stocks of vaccine that can be made available rapidly and without cost to needy countries.
4. Sending consultants familiar with the tactics and logistics of organizing a rapid mass campaign.

Conclusion. The experience of the past six years has shown that oral poliovirus vaccine coupled with highly developed, dedicated health services can eliminate poliomyelitis either nearly or completely. Elimination can be effected more easily in regions with relatively low prevaccination dissemination of polioviruses, but with greater effort it can still be achieved in regions with high poliovirus prevalence. In economically less developed countries with inadequate health services single mass campaigns are of limited transitory value when only a small proportion of infants born thereafter are immunized during the first six months of life.

* Formerly British Guiana.

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SECTION A. POLIOMYELITIS

DURATION OF IMMUNITY FOLLOWING LIVE POLIOVIRUS VACCINE

HERALD R. COX

Viral and Rickettsial Research, Lederle Laboratories
American Cyanamid Company, Pearl River, New York, U.S.A.

DR. COX (*presenting the paper*): It is now generally accepted that live oral poliovirus vaccines confer an excellent degree of protection against poliomyelitis, but there is still comparatively little definitive knowledge concerning the duration of protection. Today an attempt will be made to summarize the information that is available at present.

Let me qualify my report on two points. First, in the studies to be discussed none of the investigators were able to rule out the possibility that naturally occurring wild strains of poliovirus had appeared in the vaccinated population during the years following the original vaccinations. This fact must be kept in mind when statements are made that poliovirus antibodies persisted for certain periods of time, since wild strains are known to give booster effects in antibody titers just as can be demonstrated by feeding additional doses of poliovirus vaccines. Second, in view of the time limitations, no attempt will be made to describe the test procedures used for assays of antibody.

Encouraging results were reported by Koprowski and his associates (11–14), with the attenuated strains that were first used in man. Neutralizing antibodies were found to persist for at least eight years in seven children who were fed the rodent-adapted TN Type 2 strain of poliovirus (16). In 1958, eight years after immunization, sera were tested and the results were compared with those obtained on sera taken from the same individuals in 1955 or 1956. The 1958 sera showed Type 2 antibody titers* ranging from 16 to 256, with a median of 64. On the average, the eight-year sera were only a single twofold dilution less active in antibody content than sera taken six years after vaccination, and only about eightfold less active than sera collected one or two months after vaccination. All of the seven children who received the SM N-90 Type 1 chick-embryo-adapted strain when tested four and one half years after vaccination showed antibody titers ranging from 16 to 512, with a median of 128. The antibody titers at four and one half years after vaccination were only about twofold less than the titers in blood taken from the same individuals two and one half years after vaccination. Of 13 infants vaccinated at five days to six months of age, 12 retained homotypic antibodies one to two years after feeding of the SM Type 1, the CHAT Type 1, the TN Type 2, or the Fox Type 3 attenuated strains. The only child who failed to show demonstrable antibody had been fed the Fox Type 3 virus. The results in general supported the concept that significant seroimmunity after live poliovirus immunization is of long duration.

Sabin reported in 1959 (18) that five triple-negative children who received the three currently used (Sabin) vaccine strains at three-week intervals early in 1957 and six adults who likewise received one or more vaccine strains early in the same year showed no significant changes in their antibody titers when tested two years later.

Horstmann and her associates (9) used three different dosage schedules of Sabin poliovirus strains to immunize 351 preschool children in the city of New Haven, Connecticut, in 1960,

* Titers are expressed throughout as the reciprocal of the dilutions.

The children on schedule A received monovalent vaccines against Types 1, 3, and 2, each given separately and in that order at monthly intervals, and adjusted so that all doses were 0.2 ml in volume and contained 100,000 tissue culture infective doses ($TCID_{50}$) of virus. Those on schedule B first received a monovalent Type 1 vaccine, followed six weeks later by a bivalent Types 2 and 3 vaccine. All contained 100,000 $TCID_{50}$ of each virus type. Those on schedule C received trivalent vaccine in two doses, six weeks apart, each containing one million $TCID_{50}$ of each virus type. No significant differences were found in the results obtained from the three different administration schedules. One year after vaccination 63 of the children were bled again and their antibody levels were compared with titers determined six weeks after vaccination. No attempt was made to separate the children according to vaccination schedule. At the end of the one-year period 90, 97, and 64 per cent of the children showed antibody titers of 16 or greater for Types 1, 2, and 3, respectively. The comparative figures for six weeks after immunization were 95, 98, and 88 per cent. The geometric mean antibody titers decreased from 478 to 173 for Type 1, from 766 to 402 for Type 2, and from 278 to 48 for Type 3. The authors stated however, that when the blood specimens that gave negative or low-level results were retested using a more sensitive, longer incubation test the percentages of children with antibodies 16 or greater were 95 for Type 1, 100 for Type 2, and 95 for Type 3.

Buser and Schär reported in 1961 (1) on the persistence of antibodies in children living in the city of Bern, Switzerland, who had received Koprowski's CHAT Type 1 and Fox Type 3 strains (15). The children were immunized with monovalent vaccine preparations, receiving 10^6 $TCID_{50}$ of Type 1, and 10^5 $TCID_{50}$ of Type 3. The Type 3 vaccine was given at least four weeks after Type 1. Of 20 children tested shortly after vaccination with Type 1 poliovirus in 1958, all showed antibody titers of 5 or greater and 18 showed titers of 50 or greater. Two years later all of the same children still had antibody titers of 5 or greater, and 19 of the 20 showed antibody titers of 50 or greater. Similarly, of 20 children tested shortly after vaccination with Type 3 poliovirus in 1958, 17 showed antibody titers of 5 or greater, and 16 had titers of 50 or greater. Tested again one and one half years later, these same children gave essentially the same results: 17 of the 20 showed titers of 5 or greater, and 16 had titers of 50 or greater.

In 1962 Zacek and his associates (20) reported on the persistence of neutralizing antibodies in children in Czechoslovakia who had been vaccinated about a year previously with monovalent vaccines furnished by Dr. Sabin and which represented aliquots of the original 1956 lots. The vaccines were given separately at monthly intervals in that order: Type 1, Type 3, and Type 2. The dosage of each type contained 100,000 $TCID_{50}$. Tests were run on 257 sera from March 1959 bleedings (one to three months after vaccination) and on 261 sera from March 1960 bleedings (13 to 15 months after vaccination). With the 1959 sera the percentages of children who showed neutralizing antibody titers of 16 or greater were 95, 96, and 89 for Types 1, 2, and 3, respectively, whereas one year later the percentages were 83, 89, and 58, respectively. During the one-year period the geometric mean antibody titers decreased from 172 to 48 for Type 1, from 334 to 84 for Type 2, and from 94 to 15 for Type 3.

Dobrowolska (7) has reported on studies carried out in Poland during 1958 and 1959 with Koprowski's strains. No data are given concerning the amount of virus in the doses used for immunization, nor are details given on how the serological tests were carried out, but it is stated that "serological examination showed a high rate of conversion for both Types 1 and 3." At the end of 1961 and the beginning of 1962 a second study was done to determine the duration of antibodies in children who showed no detectable antibodies before immunization. No quantitative data are given, but the conversion rate to poliovirus Type 1 was stated as ranging from 87 to 100 per cent at the end of a two-month period, and from 86 to 100 per cent one to two years later. The conversion rate for Type 3 ranged from 66 to 100 per cent two months after immunization, and from 81 to 100 per cent one to three years later.

In 1963, Buser, Schär, and Fleury (2) reported the results of a study in Switzerland on 102 children vaccinated five years previously with Koprowski's CHAT Type 1 strain. Only children who were originally triple-negative were included, although 15 per cent of them apparently possessed maternal antibodies at the time the study was initiated. Of the 102 children, 47

had also participated in a controlled study with the W-Fox Type 3 strain four and one half years earlier. In 1958 the Type 1 virus was fed in a dosage of 10^6 $TCID_{50}$, and six months later the Type 3 strain was fed in a dosage of $10^{5.3}$ $TCID_{50}$.

The authors state that no serum samples were available from 1958 to use for comparative titrations in 1963, but all titrations were performed in the same laboratory by the same technician using the same procedures as in 1958. Of the 102 children tested for Type 1 antibodies in 1958, all showed antibody titers greater than 5, and 92 per cent showed greater than 50. For the five-year period, 100 per cent of the children had Type 1 antibody levels greater than 5 and 81 per cent had titers greater than 50. Of the 47 children tested for Type 3 antibodies in 1958, all showed antibody titers greater than 5 and 98 per cent showed titers greater than 50. For the five-year period, 98 per cent of the 47 children showed Type 3 antibody levels greater than 5 and 62 per cent had titers greater than 50.

In 1965 Just and Ritzel (10) published their results concerning students in Basel, Switzerland, who were immunized against Type 1 poliovirus in the spring of 1960 by being fed 10^6 $TCID_{50}$ of Koprowski's CHAT strain and then immunized against Types 2 and 3 two years later by being fed Sabin strains in a dosage not stated. Four years later 119 of them were bled again and their sera were checked for persistence of neutralizing antibodies. No significant differences were found among the antibody titers for the three types of polioviruses. The average titers were 83 for Type 1, 58 for Type 2, and 35 for Type 3. The geometric means for the three types were calculated at 73 for Type 1, 55 for Type 2, and 33 for Type 3. In only six serum samples were no demonstrable neutralizing antibodies present. Four had none against Type 3, one lacked antibodies to Type 2, and one specimen was negative for both Types 1 and 3. The authors stated in their summary that they did not consider revaccination on a broad scale to be necessary in Basel at that time.

Thus far we have not been able to obtain a copy of the Proceedings of the X Symposium of the European Association against Poliomyelitis and Allied Diseases held in Warsaw in 1964. However, Just and Ritzel (10) report that most workers whose findings were presented at that meeting agreed that neutralizing antibodies persist in relatively good titers for at least four years after oral immunization with a living poliovirus vaccine.

For example, oral vaccination against poliomyelitis was carried out in Poland for the first time in 1959–1960. Apparently there have been no mass vaccination programs since then. In 1963, neutralizing titers were determined on about 6,000 subjects, and were found in 80 to 90 per cent of them. Although quantitative data are not available, the Polish investigators concluded that effective immunization persisted for at least four years (17, as cited in 10).

Mass immunization against poliomyelitis was undertaken in Czechoslovakia in 1960, with revaccination in 1961. In tests run two and one half years later neutralizing antibodies were found in nearly 100 per cent of those examined (21, as cited in 10).

Voroshilova and her associates (19, as cited in 10) reported studies carried out in Moscow in 1963 to determine the persistence of neutralizing antibodies in children and adults who had been immunized with oral poliovirus vaccine for the first time in 1959. Neutralizing antibodies with a titer of 8 or greater were found against all three types in 81 to 93 per cent of the children.

In Switzerland, DuPan and Wiesmann (8, as cited in 10) tested 100 schoolchildren two years after oral immunization. Neutralizing antibodies were still present for Type 1 in 92 children, for Type 2 in 93 children, and for Type 3 in 81 children. The authors concluded that sufficient immunity was still present two years after the original immunization to make revaccination unnecessary.

In 1966 Cabasso and his associates (5) reported on the status of poliovirus antibody for 91 children living in the Hillsborough County, Florida, area.

These children, triple-negative at the time of vaccination, had been immunized three years previously by the feeding of two doses, eight weeks apart, of trivalent vaccines prepared from the Sabin strains. The concentration of virus per dose ranged from $10^{5.4}$ to $10^{5.9}$ for Type 1 (mean $10^{5.7}$), $10^{4.8}$ to $10^{5.2}$ for Type 2 (mean $10^{4.94}$), and $10^{5.7}$ to $10^{5.9}$ for Type 3 (mean $10^{5.8}$). Previous studies had been made on these children one and two years after immunization, since they

represented a group selected from about 200,000 individuals immunized in 1962 (3, 4).

Of the 91 children tested, antibody titers of 8 or greater were found for Type 1 in 98 per cent, for Type 2 in 100 per cent, and for Type 3 in 97 per cent. At the level of 16 or greater the figures were 81 per cent for Type 1, 100 per cent for Type 2, and 95 per cent for Type 3. The geometric means of the titers were 50 for Type 1, 330 for Type 2, and 110 for Type 3.

This past April serum specimens were obtained from 71 children in the same group previously reported on by Cabasso *et al.* (5, 3, 4). These sera represented the fourth year after immunization. In a personal communication Dr. John S. Neill, Director of the Hillsborough County Health Department, Tampa, Florida, stated that of the 71 children, 33 had definitely received a booster dose of trivalent oral poliovirus vaccine between May 1965 and February 1966, one had probably received a booster, and 37 had apparently not received a booster. The child who probably received a booster dose of vaccine was included with the 33 children definitely stated to have received a booster dose. Table 1 shows the percentage of the 71 children with neutralizing antibody titers of 16 or greater at one month, one year, two years, three years, and four years. The beneficial effect of feeding a booster dose of vaccine at the end of the third year may be seen in the fourth-year column. The Type 1 antibody titers in particular were increased. Table 2 shows the geometric means of the neutralizing antibody titers of the 71 children calculated at one month, one year, two years, three years, and four years. Here again, the beneficial effects of feeding a subsequent dose of poliovirus vaccine at the end of the third year are seen in the fourth year-column, which shows increases in the geometric mean antibody titers for all three types of polioviruses.

Summary

It is apparent that the various studies reviewed here support the concept that significant seroimmunity of relatively long duration occurs after the feeding of living poliovirus vaccines, either as monovalent, bivalent, or trivalent preparations. This finding is believed to be valid, even though booster effects due to the presence of naturally occurring wild strains of poliovirus undoubtedly took place in many, if not all, of the studies. A booster effect, almost certainly due to wild strains of poliovirus, was observed in the Hillsborough County, Florida, vaccinated population during the second year after immunization (4). Evidence of this effect appears in the second year data presented in Table 2. The geometric mean antibody titers are approximately the same for Type 1 in the one and two-year columns, whereas the titers for Types 2 and 3 are actually higher in the second year. This is contrary to the nor-

TABLE 1. PERCENTAGES OF 71 CHILDREN WITH NEUTRALIZING ANTIBODY TITERS OF 16 OR GREATER AT 1 MONTH AND AT 1, 2, 3, AND 4 YEARS

Virus type	Percentage with antibody titers 16 or greater at				
	1 month	1 year	2 years	3 years	4 years
1	99	92	92	83	94*/84†
2	100	100	100	100	100/97
3	100	99	96	96	97/97

*Refers to 33 children who definitely received a third year booster dose of trivalent oral poliovirus vaccine and one child who probably received such a booster.
†Refers to 37 children who did not receive a vaccine booster.

TABLE 2. GEOMETRIC MEANS OF NEUTRALIZING ANTIBODY TITERS OF 71 CHILDREN WITHOUT POLIOVIRUS ANTIBODY WHEN VACCINATED

Virus type	Geometric mean titer* at				
	1 month	1 year	2 years	3 years	4 years
1	198	76	74	56	102†/50‡
2	793	419	434	343	462/246
3	481	165	198	132	170/123

*Titers expressed as reciprocal of the dilutions.
†Refers to 33 children who definitely received a booster dose of trivalent oral poliovirus vaccine at the end of the third year and one child who probably received such a booster.
‡Refers to 37 children who did not receive a vaccine booster.

mal expectation of generally lower values as time passes.

We must agree that until polioviruses are eliminated no unconfined human population can be protected from natural exposure to wild strains.

Finally, it is extremely difficult to state with certainty the time at which it would be advisable to give a booster dose of vaccine. In the Hillsborough County study, a booster dose of oral trivalent poliovirus vaccine given at the end of the third year significantly increased antibody levels. If I were asked to make a recommendation, I would say that booster doses should be given at least every fourth year to keep antibody titers at a generally high level.*

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* Since this paper was written, I have learned that the Swiss Health Authorities have recommended the administration of a booster dose of live attenuated trivalent polio vaccine every three years (22).

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SECTION A. POLIOMYELITIS

POLIOVACCINES PREPARED IN HUMAN DIPLOID CELLS

D. Ikić

Institute of Immunology
Zagreb, Yugoslavia

Dr. Ikić (*presenting the paper*): For the preparation of virus vaccines in Yugoslavia we are currently using primary tissues approved for this purpose in accordance with all national and international requirements.

The disadvantages of primary cells are well known. The problem of the safety of substrates for the preparation of vaccines has become even more complex since the discovery that only one part of the oncogenic virus, the genome, can be incorporated into another viral virion and thus lead to the formation of a hybrid possessing oncogenic properties. We must therefore be alert to contamination not only by the oncogenic infective virus itself but also by its genome (9, 22).

In an effort to bypass the shortcomings of primary cells, human diploid cells have become the object of increasing interest as a new method for preparing virus vaccines. There is a general need for a substrate that can be more thoroughly tested, standardized, and certified—one that can be used for the preparation of many various vaccines.

Live oral poliovaccine has been prepared in the United States and Yugoslavia using human diploid cells as a substrate (6, 10). In the United States, Switzerland, Sweden, and Yugoslavia nearly 200,000 individuals have been vaccinated and revaccinated with monovalent and trivalent poliovaccine (1, 6, 7, 11, 14, 16–19).

Human diploid cells have also been used in the United States in the preparation of the following vaccines: adenovirus Type 4, which has been administered in the form of enteric capsules to more than 50,000 persons (2, 4, M.J. Rosenbaum, unpublished observations); rabies, which has given very satisfactory results (23); rhinovirus, which is still in the experimental stage (15); and rubella, which has been administered to a group of 31 children (21).

In Yugoslavia and the USSR a measles vaccine has been developed using human diploid cell strains. A group of 100 children in the USSR were inoculated with it subcutaneously (3), and several thousand children in Yugoslavia were similarly vaccinated. The Russians have also prepared a vaccine against tick-borne encephalitis in human diploid strains (O.G. Andzaparidze, personal communication).

In my country attempts are being made to develop smallpox and influenza vaccines using this substrate (12).

I shall now describe in detail our experience in Yugoslavia with live oral poliovaccine prepared in the HDC WI-38 strain.

Preparation of Live Oral Polio Vaccine in HDCS (WI-38)

Koprowski's strains of Types 1, 2, and 3 live oral polio vaccines were used. Human diploid cell strain WI-38 was used as a substrate. The vaccine was prepared and controlled according to guidelines suggested for the control of HDCS for vaccine production and according to the Requirements for the Production of Koprowski Strains of Attenuated Poliovirus Vaccine (25, 26).

The titers of attenuated poliovirus prepared in HDCS are very nearly the same as the titers of virus prepared in monkey kidney tissue culture.

Tests for neurovirulence in monkeys and tests for genetic stability after the first human passage (LTE marker, T marker, Bovine marker) showed no significant difference between the vaccine prepared in HDC and that prepared in MKTC. This was also true of the immunizing potency, i.e., the conversion rate of antibodies.

Further details on all these investigations are to be found in my publications (10, 11, 13).

Plan of the Study

Since 1964 a total of 183,600 children have been vaccinated and revaccinated with live oral polio vaccine. In addition, between April and June 1963, 11,007 children were vaccinated with WM-3 poliovirus vaccine prepared in HDCS or MKTC substrates. The vaccine prepared in diploid cells was marked with the letters B and C, and that prepared in monkey kidney tissues with letters A and D. Using the Latin Square according to the statistical tables of Fisher and Yates (5), all children were allocated at random into four groups: A, B, C, and D. The test did not include a placebo group. All children covered by the study had been vaccinated against poliomyelitis in 1962 or 1963 with the vaccine prepared in MKTC.

Designed as it was, the test could only determine whether HDCS used in the preparation of virus vaccines can, during the follow-up period, cause diseases attributable to an agent contained in HDCS and not in MKTC. Particular attention was paid to infectious hepatitis, the virus of which was suspected of being present in HDCS. The duration of the follow-up period was the same in all groups. The random allocation of vaccinated children into four groups served to attenuate other factors beyond the vaccine itself that might affect the results of vaccination. The vaccination program was developed in graduated stages, beginning with a small group. In 1964, after some necessary data had been collected, vaccination and revaccination with HDCS vaccine began on a larger scale.

Similarity of the Vaccinated Groups

Information was kept on the socioeconomic standing of the children's parents, the urban-rural distribution of the vaccinated population, age and sex distribution, incidence of infectious diseases, percentage of children covered by vaccinations other than polio, frequency of children's visits to medical institutions, contacts between pediatricians and children, and the results of follow-up visits by visiting nurses. No difference was found among the A, B, C, and D groups on the basis of any of these criteria. Thus, the random sampling had fulfilled its purpose.

All children in the study were covered by the social insurance program and were under the control of the corresponding medical centers. Each child had a file in the medical center it belonged to, and the centers from time to time organized routine controls and examinations of the children in their areas. Histories of diseases were kept in the centers' records. The data presented in this report have been taken from such records. All children in the study had approximately the same number of routine examinations. If a child fell ill during the follow-up period more than once, each diagnosis was taken into account. The diseases have been grouped according to the WHO classification. The intervals of follow-up—0 to 3 months, 3 to six months, 6 to 12 months, 12 to 24 months, and over 24 months—were adequate to reveal any differences among the groups with respect to diseases that could have shorter or longer incubation after vaccination. Analysis and statistical evaluations showed that there was no significant difference among the groups with respect to either the number of diagnoses made by the physicians or the length of follow-up period.

Infectious hepatitis in vaccinees. According to the reports of the clinicians supervising the trial, 13 cases of infectious hepatitis were recorded among the vaccinated children during the entire follow-up period (Table 1). The diagnoses were made on the basis of anamnestic data, clinical findings (hepatomegaly, jaundice), and laboratory findings (liver function tests). Epidemiological inquiry suggested contact or infection as plausible sources of the disease in 9 of 13 cases. The cases of infectious hepatitis were distributed by groups as follows: 6 cases appeared in Group A, 5 in Group B, 1 in Group C, and 1 in Group D. Seven of the 13 cases of hepatitis appeared in Groups A and D and 6 in Groups B and C. The lack of difference between Groups A+D and B+C strongly supports the

TABLE 1. CASES OF INFECTIOUS HEPATITIS REPORTED DURING THE TWO-YEAR OBSERVATION PERIOD, YUGOSLAVIA, 1964–1966

Initials, age, and sex of patient	Group code	Interval between vaccination and onset of illness (days)	History		Form of disease	
			Contact	Injec.	Icteric	Unicteric
Š.T.,12,F	A	2			+	
P.B.,12,F	A	161			+	
P.B.,9,F	A	179	+	+	+	
M.J.12,M	A	165	+			+
O.Ž.,9.M	A	170			+	
L.E.10,F	A	225	+		+	
Z.M.10,M	D	176	+		+	
T.Ž.,14,F	B	104	+	+	+	
H.B.,10,M	B	185	+			+
V.T.,10,M	B	195	+			+
Š.S.,14,M	B	182				+
M.S.,10,M	B	196	+		+	
S.Ž.,10,M	C	207	+			+

belief that the WI-38 strain of HDC is free of the virus of infectious hepatitis.

Number and percentage of affected children. Statistical analysis of the number and percentage of children affected by diseases during the follow-up intervals showed no difference between the groups vaccinated with HDCS and those vaccinated with MKTC vaccines.

Number and percentage of diagnoses grouped according to the International Classification of WHO. The diseases recorded during the follow-up intervals were statistically analyzed by groups, according to the International Classification of Diseases of WHO (24). Again, there was no significant difference between the HDCS and MKTC groups.

Data on other Children Vaccinated and Revaccinated with Oral Polio Vaccine Prepared in HDC System

Since 1964 polio vaccine prepared in HDC has been used for both primary vaccination and revaccination. The number of children in both categories has steadily increased. To date 25,800 children have received Type 1, 17,400 children have received Type 2, and 23,100 children have received Type 3 in primary vaccination. In addition, 117,300 children have been revaccinated with trivalent poliovaccine. Thus, the total number of children covered by primary vaccination and by revaccination is 183,600. A comparison of the children vaccinated with live oral polio vaccine prepared in HDCS and with the same vaccine prepared in MKTC revealed no significant difference indicating that human diploid cells harbor viruses or other agents pathogenic for man.

DISCUSSION

More than 250,000 people throughout the world have received vaccines prepared in human diploid cells. The results obtained thus far in the field show without question that human diploid cells (WI-38 strain) are free of the virus of infectious hepatitis and other agents that might have caused diseases during the follow-up periods.

Nothing that would be disadvantageous or that would speak against the introduction of HDCS system has been noticed so far in any of the field trials, nor has any adventitious virus been revealed in intensive laboratory investigations.

Minimum requirements have been established for HDCS. They are stricter than the requirements for primary cell cultures, and, indeed, primary cell cultures cannot always meet these standards (20).

Studies of the characteristics and markers of HDCS have helped in clearly defining and differentiating this system from cell lines (H. Eagle, personal communication).

In view of all this, it is necessary for us to reconsider the substrates we have at our disposal for the preparation of virus vaccines.

Nobody wants to challenge seriously the use of primary cells in the preparation of virus vaccines, but we do think that this method should be defined more precisely so that a primary tissue culture can represent a more uniform population that is capable of meeting some of the criteria laid down for human diploid cell strains.

In any case, we must try to develop a substrate for vaccine production that is more uniform and offers greater possibility for being standardized. In this respect, human diploid cells have an ad-

vantage over primary cell cultures. Our ultimate goal is to have standardized and certified cells for virus growth, a standardized growth and maintenance medium, a standardized and certified seed virus, and a handling procedure that would exclude the possibility of contamination of cells.

We have now accumulated a lot of experience with HDCS, both in the laboratory and in the field. This method offers great possibilities. We do not see anything that would speak against licensing this substrate for at least those vaccines that are administered orally. Our experience here is indeed sufficient.

As a matter of fact, in active prophylaxis, in medicine, and in everyday life we regularly apply procedures that from the point of view of safety are far less tried than the use of human diploid cells. But in these cases we are less critical because the procedures have become habit.

In order to better characterize and standardize substrates for the preparation of vaccines, it would be useful in the future to carry out further studies on markers that distinguish human diploid cells from cell lines, to collect data on the comparative testing of HDCS and primary cells as substrates for the preparation of virus vaccines, both in the laboratory and in field studies, and to further standardize tissue culture media (8), including calf serum or a substitute therefor.

The use of well-characterized substrates for virus vaccine production is as justified and necessary as the use of a well-characterized seed virus.

Summary

All laboratory examinations carried out so far showed that the human diploid cell strains (HDCS) are free of adventitious viruses.

All three types of poliovirus propagated in HDCS yield titers nearly as high as those obtained with monkey kidney tissue culture (MKTC) preparations.

Propagation in human diploid cells does not affect the genetic stability of any of the three types of poliovirus, and the conversion rate of antibodies after primary vaccination with all three types of live oral poliovaccine prepared in human diploid cells corresponds to the results obtained with vaccine prepared in MKTC.

Live oral poliovaccine prepared in HDCS was applied in Yugoslavia for the first time in 1963. It was used for both primary vaccination and revaccination. So far, more than 180,000 children have been vaccinated and revaccinated. Groups of children who received either the vaccine prepared in HDCS or that prepared in MKTC were studied over a two-year period, and clinical diagnoses were collected and compared. The findings show no significant difference between these groups, indicating that HDCS harbors viruses pathogenic for man.

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SECTION A. POLIOMYELITIS

THE PRESENT STATUS OF POLIOVIRUS IMMUNIZATION IN THE USSR

M.P. CHUMAKOV and M.K. VOROSHILOVA

Institute of Poliomyelitis and Viral Encephalitides
USSR Academy of Medical Sciences, Moscow, USSR

DR. CHUMAKOV (*presenting the paper*): Because of the possibility of pandemic spread and increased frequency of severe epidemics, poliomyelitis constitutes, in the fullest sense, an international medical problem. The medical, personal, familial, and social consequences of paralytic poliomyelitis are tremendous, and their significance is not inferior to that of more common infectious diseases—influenza, hepatitis, and the like.

The history of poliomyelitis has shown that not a single country, whether great or small, rich or poor, whatever its level of public health, in the end is safe from the epidemic rise and spread of poliomyelitis. Numerous examples may be found in all parts of the world. In the Soviet Union the incidence of poliomyelitis was high during the period from 1954 to 1960, and it reached its peak in 1958.

Since poliomyelitis has a large number of subclinical forms and obscure sources of infection undetectable by common clinical and laboratory methods of investigation, the incidence of this disease can be halted only by immunizing the entire susceptible population starting at the age of two to three months.

It is for this reason that such great attention is given to the problem of developing and applying specific prophylactic measures against the disease.

In the last 10 years scientists and practicing medical workers have been collaborating fruitfully within the framework of the World Health Organization and international agreements on problems of virology, including the control of poliomyelitis. The most important result of this collaboration has been the widespread introduction into public health practice of live poliovirus vaccine from strains attenuated by Dr. Sabin and the consequent eradication of epidemic manifestations of poliomyelitis in a number of countries. The results of this work in our country have been published in several reports. Although it would be premature to speak of the achievement of complete eradication of poliomyelitis in some countries, the fact remains that a relative well-being with regard to poliomyelitis now exists in those countries where the widest mass immunization against poliomyelitis has been carried out, particularly with live poliovirus vaccine or with a combination of live and killed vaccine.

During the period 1957–1960 over 12 million children in the USSR received multiple injections of Salk inactivated poliomyelitis vaccine (Soviet production). However, such a small-scale vaccination program was clearly insufficient for our country, particularly at a time when the epidemic incidence of poliomyelitis was continuing to increase in a number of regions. Moreover, we soon came to the conclusion that immunization with Salk inactivated vaccine, which reduced the number of poliomyelitis cases among vaccinees three or fourfold, was not stopping the circulation of the causative virus among the child population fast enough. Although the inactivated vaccine created seroimmunity, it did not produce adequate immunity against the invasion of poliomyelitis virus in the alimentary tract, nor did it always prevent multiplication of poliovirus in the intestinal tract in vaccinated partially immune children. Because of this, poliovirus was not completely eliminated from

child communities and it continued to circulate in the chain of asymptomatic cases until a new outbreak. Thus, even widespread use of Salk inactivated vaccine does not wipe out the potential foci of poliomyelitis. In the United States, where over 100 million persons received Salk vaccine between 1954 and 1962, outbreaks of poliomyelitis occurred not infrequently during this period and several hundred strains of poliomyelitis virus were isolated from patients annually. Similar situations occurred in Israel (1958–1959), in Hungary, and in some other countries, despite great efforts spent in giving multiple (3–4) injections of Salk vaccine.

In our country poliovirus vaccine strains selected by Dr. Sabin have been studied carefully and tested under various conditions since 1956. In 1958 a large batch of live poliovirus vaccine from Sabin strains (SLV) was prepared at our Institute and in 1959 it was used for oral immunization of over 15 million persons ranging in age from 2 months to 20 years, and in some areas (Estonian SSR) to 60 years. We are proud that the use of Sabin vaccine strains for the mass immunization of millions of people had its "start in life" in our country. The technology of large-scale production and control of this vaccine was also first developed in our country, since before our work in 1958 only about 30 liters of Sabin vaccine had been prepared. In 1958–1959 we had already prepared over 30 million doses, and in the past eight years we have produced more than 40,000 liters of this vaccine for primary oral immunization and revaccination of over 200 million persons in the USSR and 30 other countries.

Thanks to international collaboration within the framework of WHO and the active assistance of Dr. Sabin, the problems of standardizing the manufacturing technology and of effecting biologic control of live poliovirus vaccine were successfully solved. We have now achieved extremely high reproducibility and consistency in the production of large lots of poliovirus vaccine that meet the high requirements of international standards.

When in 1961, in connection with the adoption of new international requirements for oral live poliovirus vaccine (developed with due regard for the experience of our Institute), the need arose to purify Sabin seed vaccine strains from latent SV_{40} virus and to find monkeys not contaminated with this virus, we successfully accomplished these tasks also, without any impairment of the genetic stability of attenuated poliovirus strains. Our seed viruses of Types 1, 2, and 3, purified from SV_{40} according to our own original technique, were thoroughly tested in 1962–1963 at the Division of Biologics Standards of the National Institutes of Health, in Bethesda, Maryland, thanks to the kind cooperation of Dr. Murray, and also at Dr. Melnick's laboratory in Houston, with quite satisfactory results. During the past five years we have been using these purified lots of Sabin seed viruses exclusively.

For the production of kidney tissue cultures we use kidneys from African green monkeys that have been previously quarantined for six weeks and that have been demonstrated to be serologically negative for SV_{40} infection. The kidney tissue cultures are further checked by the immunofluorescence method for the absence of contamination with SV_{40} and other spontaneous simian viruses. To increase the thermal stability of the final preparations of live poliovirus vaccine we use 1 M $MgCl_2$, according to the well-known finding of Wallis and Melnick.

In the past eight years over 115 million persons from two months of age on up have been immunized with Sabin live vaccine in the USSR. Every year vaccinations against poliomyelitis are given to more than 4.5 million persons, including primary vaccinations of newborn babies reaching two months of age and revaccinations of children up to three years of age.

The most convenient form of live poliomyelitis vaccine has proved to be dragee-candy containing one vaccination dose each of vaccine poliovirus Types 1, 2, and 3. The candied vaccine is used most widely for immunization of children over one year of age and adults. In addition, we often use 2–4 drops of the liquid trivalent vaccine administered in a lump of sugar.

For one vaccination dose we use about 500,000 tissue culture units of poliovirus Type 1, 200,000 units of Type 2, and 300,000 units of Type 3—all Sabin vaccine strains.

We are currently studying in torrid subtropical areas of our country the possibility of increasing the effectiveness of live poliovirus vaccine implantation in the intestinal tract of vaccinated children to counteract the interference

by nonpoliomyelitis enteroviruses widely circulating in these areas. The problem is that such an undertaking calls for a considerable increase in the amount of poliovirus Type 1 administered. We think, however, that we can achieve good implantation of the vaccine and at the same time avoid a considerable increase of vaccine virus concentration by repeating the oral vaccinations every four months for all children under three years of age using the usual doses of live vaccine.

We believe that revaccination with Sabin oral live vaccine is absolutely necessary, even though many authors disagreed with us. In the first place, possible interference by enteroviruses can prevent effective multiplication of vaccine strains in a number of cases; in the second place, repeated vaccination is necessary to achieve favorable results, since in some effectively vaccinated children the level of antibody gradually declines. Since after campaigns of mass immunizations with Sabin live vaccine latent immunization has been reduced sharply and local strains of wild poliovirus have been eliminated almost completely, the artificial "boosting" of collective immunity by repeated use of oral poliovirus vaccine becomes necessary. With the inexpensive vaccine now available it is quite easy to regularly revaccinate children up to three years of age, and this is what we are doing in our country.

In carrying out mass vaccinations against poliomyelitis in our country we have watched over the safety, implantation, and serologic activity of the live vaccine produced by our Institute on a regular and continuing basis. Owing to this work, which has been done by several dozens of virological laboratories in the country over a number of years, solid evidence of the complete safety and reactogenicity, as well as the high immunologic activity, of Sabin oral live poliovirus vaccine has been accumulated. During all these years, despite the tremendous scale of live vaccination in the USSR, there has not been a single accident or complication as a result of vaccination with SLV. Even in an accidental increase of 500 or 1,000 times a single dose there was no untoward reaction in primarily vaccinated children. We have not observed any valid association with vaccine poliovirus in the few rare cases of paralytic disease occurring within the first 30 days after vaccination. Of course, in cases where the occurrence of poliomyelitis disease coincides with the vaccination period it is sometimes very difficult to establish beyond a doubt the lack of causal association with vaccine poliovirus. However, by comparing all the data obtained in investigations of a number of similar cases over several years we have definite indications that these cases were not causally associated with vaccination.

The analysis of cases of paralytic diseases clinically similar to poliomyelitis occurring at the time of vaccination campaigns gives no ground for the frequently voiced opinions of some authors regarding the alleged selectively high pathogenicity of Sabin poliovirus Type 3 vaccine. Our data show that such few cases as were observed were coincidental not only with Type 3 vaccination, but also with Types 1 and 2 immunization. We regard all these cases either as coincidental infection with wild strains circulating at the time of vaccination or as casual detection of extraneous clinically similar involvements of the nervous system.

The fact alone that poliovirus Type 3 has been isolated from the feces of diseased vaccinated children must not be regarded as decisive evidence of causal association, since Type 3 vaccine poliovirus, as compared to the other types of vaccine poliovirus, is known to be excreted frequently and massively by children who have been repeatedly vaccinated. In some cases we observed excretion of a mixture of wild and vaccine Type 3 poliovirus or a mixture of wild Types 1 and 2 viruses with vaccine Type 3 virus or nonpoliomyelitis enteroviruses. To put all suspicions to rest, and to completely clear up the question of a possible mixed infection with wild and vaccine strains of poliovirus, we reisolate the poliovirus in every case where a paralytic disease coincides with a vaccination campaign. Successful accomplishment of this task will require further improvement of the methods for isolation and identification of poliovirus in cases of mixed infection or in cases where the true causative agent of the disease is masked by the intensive excretion of vaccine poliovirus.

Figures 1–6 and Table 1 show data on the epidemiological effectiveness of mass oral vaccinations with Sabin live vaccine in the USSR. Since 1962–1963 seasonal peaks have been eliminated and there has been a general marked decline in the incidence of paralytic poliomye-

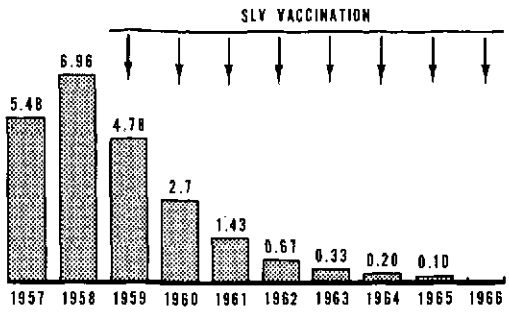

Fig. 1. Paralytic poliomyelitis in the USSR (1957–1966), annual average per 100,000 population.

litis, including complete disappearance of cases in some of the republics and large regions of the USSR. This decline has been taking place steadily and gradually for several years, and it is possible that a large portion of the cases reported over the period were not really poliomyelitis, since reporting was based on clinical evidence only. Selective investigation of such cases by laboratory, serologic, and virologic methods usually permits us to exclude the diagnosis of poliomyelitis.

We feel that the problem of the etiology of poliomyelitis-like paralytic diseases not causally associated with poliovirus Types 1, 2, and 3 requires further detailed investigation. It is necessary to specify the role of certain nonpoliomyclitis enteroviruses that may be frequently isolated from paralytic patients with serological confirmation of infection. In some cases we have been able to demonstrate the etiologic role of

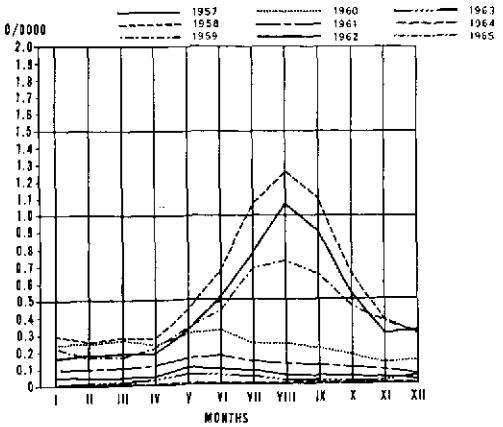

Fig. 2. Paralytic poliomyelitis in the USSR.

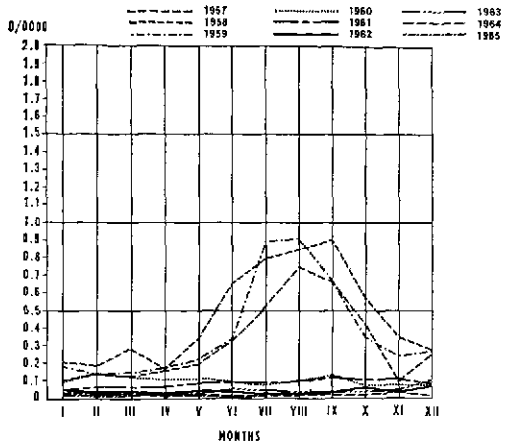

Fig. 3. Paralytic poliomyelitis in 13 republics of the USSR (excluding RSFSR and Ukrainian SSR).

AB-IV-Coxsackievirus A7, though it should be recognized that most of the other cases were not associated either with this or with polioviruses. The group of poliomyelitis-like diseases etiologically unassociated with polioviruses may possibly take in diseases of the CNS of variable origin, including noninfectious syndromes.

Primary oral immunization and revaccination with SLV produce rapid development of specific antibodies in the vaccinees. Figures 7–9 compare the results of serological examination of children before and after mass vaccination campaigns. Of special interest are the results of surveys in 1966 in Moscow, Minsk, and several other large towns in the USSR. These data were obtained from surveys of large groups of children and adolescents. For example, in Moscow among 472 children and adolescents 96.2

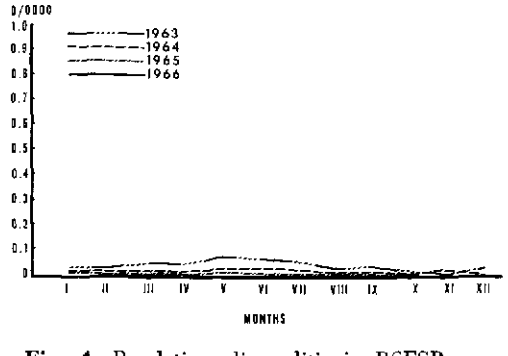

Fig. 4. Paralytic poliomyelitis in RSFSR per 100,000 population.

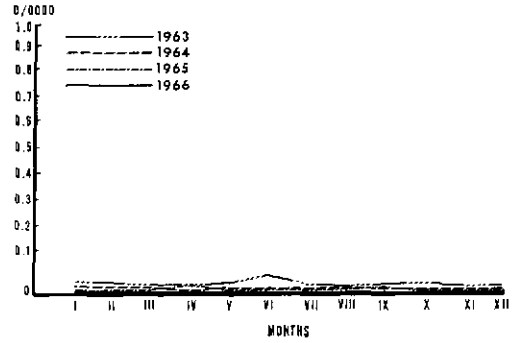

Fig. 5. Paralytic poliomyelitis in Ukrainian SSR per 100,000 population.

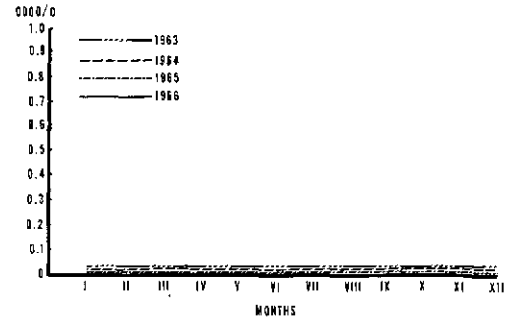

Fig. 6. Paralytic poliomyelitis in Estonian SSR per 100,000 population.

per cent had antibody for all three types of polioviruses, whereas only 2.6 per cent had no antibody for Type 1, 0.8 per cent had none for Type 2, and 1.5 per cent had none for Type 3. The geometric mean antibody titers were 1:84, 1:158, and 1:69, respectively. These data are for sera tested in dilutions of 1:4 and higher. A noticeable reduction in the number of positive sera is observed with dilutions of 1:8 and higher or 1:32 and higher. These data point up the need for continued revaccination of younger age groups if antibody levels are to be kept at 1:32 and higher.

In 1962–1963 the vaccination schedule in the USSR was temporarily changed: vaccinations were started at 12 months of age or over and were given only once a year. This resulted in a rapid accumulation of completely susceptible children in the one-to-two-year age group and increased the circulation of wild polioviruses among the population. After return to the original vaccination schedule, according to which

TABLE 1. PARALYTIC POLIOMYELITIS IN THE SOVIET REPUBLICS (MONTHLY AVERAGE PER 100,000 POPULATION)

Republic	Population in 1964 (millions)	Year			
		1963	1964	1965	1966
RSFSR	126.0	0.0366	0.0158	0.0070	0.0130
UkSSR	45.0	0.0090	0.0020	0.0008	0.0006
KazSSR	11.0	0.0291	0.0283	0.0038	—
UzbSSR	9.2	0.0191	0.0216	0.0033	0.0041
BSSR	8.6	0.0333	0.0158	0.0041	0.0001
GSSR	4.4	0.0033	—	0.0066	0.0033
AzSSR	4.2	0.0350	0.2033	0.0733	0.0425
MolSSR	3.2	0.0025	0.0008	0.0012	—
LitSSR	2.9	0.0008	—	0.0002	—
KirSSR	2.4	0.0166	0.0166	0.0083	—
LatSSR	2.2	—	—	—	—
TadSSR	2.2	0.1466	0.0275	0.0366	0.0333
ArmSSR	2.0	0.0625	0.0625	0.0291	0.0083
TurSSR	1.7	0.2591	0.0044	0.1291	0.1050
EstSSR	1.3	—	—	—	—
USSR	226.4	0.0250	0.0160		

— No cases.

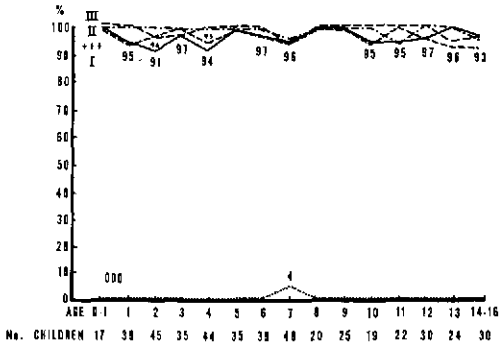

Fig. 7. Comparison of results of serological examination of children before and after mass vaccination campaigns—Moscow, 1966, 472 children $\geqslant$ 1:4.

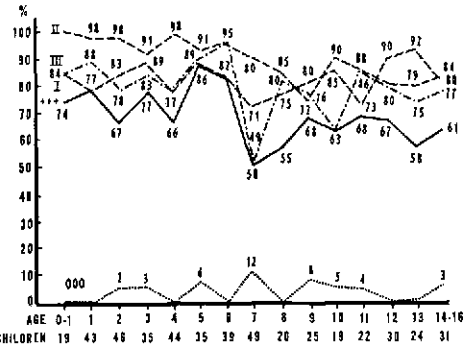

Fig. 9. Comparison of results of serological examination of children before and after mass vaccination campaigns—Moscow, 1966, 481 children $\geqslant$ 1:32.

primary immunization was started at the age of two months, the situation improved rapidly. At the present time we are giving three vaccinations at four-month intervals to all children from the age of two months to three years. In addition, schoolchildren are revaccinated in the first grade and again in the eight or ninth grade. It is quite possible that for the great majority of children and adolescents revaccination is not absolutely necessary; however, even occasional cases of declining or lacking immunity in vaccinees may be eliminated with this schedule of periodic vaccination.

Systematic surveillance of healthy children for carrier state or polioviruses and other enteroviruses performed after mass vaccinations with SLV has revealed a very sharp reduction in the number of poliovirus isolations from stools. It would be wrong to claim, however, that the circulation of wild poliovirus strains has ceased completely. In 1964–1965 wild poliovirus strains, mostly of Types 1 or 2, were isolated in some southern republics of the USSR. Small outbreaks of true poliomyelitis (up to 20 cases) have been observed in occasional areas. It should be emphasized that these cases of poliomyelitis were always connected with breaks in the regular vaccination of younger age groups. The only cause found for increased incidence was insufficient attention to vaccination on the part of local public health workers who had prematurely excluded poliomyelitis from the list of important public health problems.

Our work is not done. We must continue to watch over the circulation of polioviruses and other enteroviruses and we must not relax our efforts to create specific immunity to poliomyelitis in growing children.

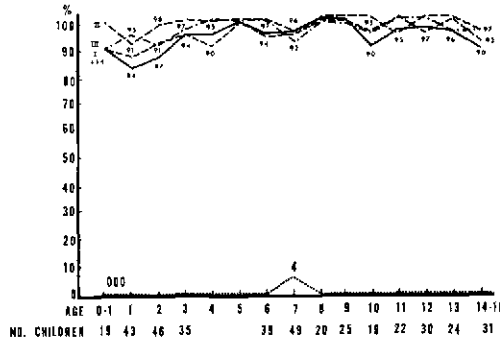

Fig. 8. Comparison of results of serological examination of children before and after mass vaccination campaign—Moscow, 1966, 481 children $\geqslant$ 1:8.

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SECTION A. POLIOMYELITIS

THE STATE OF POLIOMYELITIS IN THE WORLD

S. G. DROZDOV and W. CHAS. COCKBURN

Virus Diseases, Division of Communicable Diseases, World Health Organization
Geneva, Switzerland

DR. COCKBURN (*presenting the paper*): In the decade and a half since poliomyelitis virus vaccine was first used, the decline in the incidence of the disease in well-vaccinated communities has been so dramatic that some people are inclined to assume it no longer merits much attention.

The purpose of this report is to look at the present state of poliomyelitis in the world as a whole and to show that there are still serious problems, potential and actual, to be faced now or in the near future.

The statistical information has been derived mainly from WHO publications, including the *World Health Statistics Annual* (previously entitled *Annual Epidemiological and Vital Statistics*), the *Epidemiological and Vital Statistics Reports*, and the *Reports on the World Health Situation*. Other information has been taken from publications by particular authors. The figures on incidence of the disease are of course not accurate, and the limitations imposed by the inaccuracies have always to be borne in mind. The information does, however, permit general trends to be defined.

For the purposes of this review the world has been divided into the following regions: (1) Europe; (2) United States of America, Canada, Australia, and New Zealand; (3) islands in Oceania; and (4) tropical and semitropical countries.

Poliomyelitis in Europe

Table 1 shows the number of cases in 21 European countries in 1964 and the average of the annual figures for 1951–1955.

Except in the five countries listed at the end of the table (France, Greece, Italy, Portugal, and Spain), the incidence of disease in 1964 was very low. Many countries had fewer than 10 cases and only three had more than 20. Information from the USSR could not be readily fitted into the table, but in that country a very great decline occurred—from an average annual rate of 6.4 per 100,000 in 1956–1958 to about 0.1 per 100,000 in 1964. Altogether, in the 21 countries listed in the table there has been an 11-fold increase. In the 16 countries in the first part of the table, however, there has been an 84-fold decrease, whereas in the five countries in the second part of the list there is only a three-fold decrease. In four of these five countries adequate vaccination campaigns have been started only recently. (Portugal, as far as is known, has not yet initiated a program of this type.) The campaigns are already having an effect on the incidence of the disease. Recently available data for 1965 show several marked declines from the figures of the previous year. The incidence has dropped in France from 533 to 290, in Italy from 919 to 262, and in Spain from 196 to 64. Data are not yet available for Greece. The number of reported cases in Portugal rose slightly—from 225 to 280.

Most of the countries experiencing substantial decreases have used live vaccine, usually containing Sabin strains, and a high proportion of the susceptible population has been vaccinated. In Finland, the Netherlands, Norway, and Sweden, killed vaccine has been used exclusively or mainly and has given results as good as those in countries using live vaccine.

TABLE 1. POLIOMYELITIS IN EUROPEAN COUNTRIES, 1964 AND 1951–1955

Country	No. of cases, 1964	Average annual no. of cases, 1951–1955
Austria	6	607
Belgium	2	475
Czechoslovakia	0	1,081
Denmark	2	1,614
Finland	4	372
German F.R.	62	3,702
Hungary	3	583
Iceland	0	193
Ireland Rep.	17	121
Netherlands	16	599
Norway	15	979
Poland	14	2,226
Sweden	1	1,526
Switzerland	6	956
United Kingdom	65	4,381
Yugoslavia	21	361
Total	234	19,746
Ratio		1 :84
France	533	1,672
Greece	179	367
Italy	919	3,335
Portugal	225	112
Spain	196	1,004
Total	2,052	6,490
Ratio		1 :3
Grand total	2,286	26,236
Ratio		1 :11

TABLE 2. POLIOMYELITIS IN THE UNITED STATES OF AMERICA, CANADA, AUSTRALIA, AND NEW ZEALAND, 1964 AND 1951–1955

Country	Cases in 1964	Average annual no. of cases, 1951–1955	Decrease ratio
United States	121	37,864	1 :313
Canada	21	3,922	1 :187
Australia	23	2,187	1 :95
New Zealand	1	405	1 :405
Total	166	44,378	1 :267

Poliomyelitis in the United States of America, Canada, Australia, and New Zealand

The number of cases reported in these four countries in 1964 and the average of the annual figures for 1951–1955 are shown in Table 2. In all the countries except Australia killed vaccine was used in the early phases and live vaccine was used mainly or exclusively in the later phases. In Australia killed vaccine was used exclusively until 1964 (6). The decrease from 1951–1955 to 1964 ranges from 95-fold in Australia to over 400-fold in New Zealand.

It may be stated with confidence that in the areas so far considered, if the countries with good programs continue to carry them on effectively and if the few countries that have not yet done so set up effective programs in the near future, poliomyelitis will soon cease to be a disease of public health importance.

Data on the effects of immunization on the prevalence of the natural poliomyelitis viruses are available from only a few countries. In the Estonian SSR in 1959–1962 (5), it was shown that wild viruses practically disappeared from the population in little more than a year after the introduction of live poliomyelitis virus vaccination. Similar evidence was obtained by Dömök and his colleagues (4) in Hungary. Gard (11) and Böttiger and her colleagues (1) reported that in Sweden natural poliomyelitis viruses were practically eliminated as a result of systematic campaigns with inactivated vaccine.

More information on the behavior of the natural poliomyelitis viruses is necessary, however, for the proper development of immunization programs in the future. Fortunately, in Europe there is an opportunity to compare over a period of time the experience of countries that have used mainly killed vaccines with those that have used mainly or exclusively live vaccines. WHO is seeking to sponsor a collaborative study of this subject in suitable countries.

Poliomyelitis in the Islands of Oceania

In the islands of Oceania poliomyelitis morbidity shows great variations from year to year, particularly in the smaller islands. Annual morbidity rates between 1950 and 1964 varied from

0.0 to 397 per 100,000. In the larger islands—Fiji and Papua and New Guinea—cases tend to occur each year and the incidence is less variable; it ranged from 0.1 to 8.1 per 100,000 population between 1950 and 1964. In the islands on which vaccine campaigns have been carried out —for example, Cook Islands, Fiji, Western Samoa—the incidence of disease has fallen rapidly. It seems reasonable to assume that adequate vaccination of populations in these islands would be as successful as it has been in Europe, North America, Australia, and New Zealand.

Poliomyelitis in Tropical and Semitropical Countries

The state of affairs in most of the tropical and subtropical countries contrasts with the generally successful control of the disease in the parts of the world already discussed. The information available from many of these countries is less accurate and comprehensive than the data from the countries already dealt with. It may be safely assumed that the real incidence of cases and deaths is higher and not lower than the figures indicate.

Geographical Distribution of Poliomyelitis Morbidity

Tables 3, 4, and 5 present information on the incidence of poliomyelitis in 26 countries in Africa, 22 countries in Central and South America, and 15 countries in Asia. These countries represent, respectively, about 80 per cent of the total African population, about 60 per cent of the Central and South American population, and about 20 percent of the Asian population. The tables give the average annual number of cases for 1951–1955 and 1960–1964 and the actual number of cases for 1964. The average annual number of cases in 1951–1955 is compared with the average number for 1960–1964—a procedure slightly different from that followed in Tables 1 and 2. This form of presentation is intended to provide more accurate comparisons in countries where reporting practices may have varied from year to year.

TABLE 3-a. POLIOMYELITIS MORBIDITY IN AFRICAN COUNTRIES EXPERIENCING INCREASED INCIDENCE, 1964 AND ANNUAL AVERAGES FOR 1951–1955 AND 1960–1964

Country	Cases in 1964	Average annual no. of cases, 1960–1964	Average annual no. of cases, 1951–1955	Ratio 1960–1964/ 1951–1955
Nigeria	465	235	4	58.8 :1
Libya	581	186	26	7.2 :1
Algeria	172 [a]	142 [b]	33	4.3 :1
Malawi	28 [c]	56 [d]	16	3.5 :1
Ethiopia	32	34	10	3.4 :1
Tunisia	6 [e]	76 [f]	23	3.3 :1
Upper Volta	96	49	18	2.7 :1
Kenya	430	618	270	2.3 :1
Tanganyika*	303	266	121	2.2 :1
Uganda	120	248	119	2.1 :1
Zambia	84 [c]	77 [f]	39	2.0 :1
Madagascar	18	18 [g]	10	1.8 :1
Gabon	2	24	15 [h]	1.6 :1
La Réunion	10	11	7	1.6 :1
Zanzibar & Pemba*	1	8	5	1.6 :1
Mozambique	52	43	39	1.1 :1
Total	2,400	2,091	755	2.8 :1

*Republic of Tanzania since 6 May 1964.
[a] Data for 1961. [b] Data for 1960–1961. [c] Data for 1962. [d] Data for 1960–1962.
[e] Data for 1963. [f] Data for 1960–1963. [g] Data for 1961–1964. [h] Data for 1952–1954.

TABLE 3-b. POLIOMYELITIS MORBIDITY IN AFRICAN COUNTRIES EXPERIENCING DECREASED INCIDENCE, 1964 AND ANNUAL AVERAGES FOR 1951–1955 AND 1960–1964

Country	Cases in 1964	Average annual no. of cases, 1960–1964	Average annual no. of cases, 1951–1955	Ratio 1960–1964/ 1951–1955
Mauritius	3 [a]	1 [b]	80	1:80.0
Senegal	35	40	100 [c]	1 : 2.5
Ghana	28	25	59 [d]	1 : 2.4
Angola	81	138	314	1 : 2.3
Congo (Kinshasa)	524	421	925	1 : 2.2
Southern Rhodesia	59	71	127	1 : 1.8
U.A.R.	417	288	475	1 : 1.6
South Africa	107	398	616	1 : 1.5
Cameroon	28	14	16	1 : 1.1
Ivory Coast	50 [a]	23 [b]	26	1 : 1.1
Total	1,332	1,419	2,738	1 : 1.9
Total for all countries listed in tables 3a and 3b	3,732	3,520	3,493	1 : 1.0

[a] Data for 1963. [b] Data for 1960–1963. [c] Data for 1952–1955. [d] Data for 1951–1954.

The information shows that poliomyelitis is common in tropical and semitropical areas. Comparison of the data for 1951–1955 with those for 1960–1964 shows twofold or greater increases in 11 of the 26 African countries (Table 3a), in 5 of the 22 Central and South American countries (Table 4a), and in 7 of the 15 Asian countries (Table 5a). Twofold or greater decreases were observed in 5 African, 6 Latin American, and 2 Asian countries (Tables 3b, 4b, and 5b). Of the countries experiencing twofold or greater increases in the average annual

TABLE 4-a. POLIOMYELITIS MORBIDITY IN CENTRAL AND SOUTH AMERICAN COUNTRIES EXPERIENCING INCREASED INCIDENCE, 1964 AND ANNUAL AVERAGES FOR 1951–1955 AND 1960–1964

Country or area	Cases in 1964	Average annual no. of cases, 1960–1964	Average annual no. of cases, 1951–1955	Ratio 1960–1964/ 1951–1955
Dominican Republic	19 [a]	90 [b]	— [c]	
Guyana*	—	99	3	33.0:1
Peru	607	561	107	5.2:1
Colombia	622	401	103	3.9:1
Ecuador	84	87	43	2.0:1
Guatemala	76	130	71	1.8:1
Martinique	2	4 [d]	3	1.3:1
Nicaragua	47 [a]	109 [e]	82 [f]	1.3:1
Venezuela	271	332	286	1.2:1
El Salvador	20 [a]	52 [b]	49	1.1:1
Total	1,748	1,865	747	2.5:1

*Formerly British Guiana.
[a] Paralytic cases only.
[b] For 1963 and 1964 only paralytic cases are recorded.
[c] For 1951–1953; for 1954–1955 there are no available data.
[d] Excluding 1961.
[e] For 1961 and 1964 only paralytic cases are recorded.
[f] For 1954 only paralytic cases are registered.

TABLE 4-b. POLIOMYELITIS MORBIDITY IN CENTRAL AND SOUTH AMERICAN COUNTRIES EXPERIENCING DECREASED INCIDENCE, 1964 AND ANNUAL AVERAGES FOR 1951–1955 AND 1960–1964

Country or area	Cases in 1964	Average annual no. of cases, 1960–1964	Average annual no. of cases, 1951–1955	Ratio 1960–1964/ 1951–1955
British Honduras	—	0.2 [a]	4	1:20.0
Costa Rica	10	36	297 [b]	1: 8.2
Uruguay	2 [a]	29 [d]	158	1: 5.4
Trinidad and Tobago	4	8 [e]	42	1: 5.2
Jamaica	57	49	168	1: 3.4
Mexico	391	645 [f]	1,365	1: 2.1
Puerto Rico	—	106	146	1: 1.4
Honduras	38	29	40 [g]	1: 1.4
Argentina	521	967 [f]	1,071	1: 1.1
Chile	394	429	468	1: 1.1
Paraguay	60	47 [h]	54 [i]	1: 1.1
Cuba	1	144	144	1: 1.0
Total	1,478	2,489	3,953	1: 1.6
Total for all countries and areas listed in Tables 4-a and 4-b	3,226	4,354	4,700	1: 1.0

[a] One case for 1960–1964; data for British Honduras are not included in total calculation.
[b] Excluding 1958. [c] Data for 1963. [d] Data for 1960. [e] Excluding 1963.
[f] For 1960 and 1961 only paralytic cases are registered. [g] Data for 1951 and 1955.
[h] For 1961 only paralytic cases are registered. [i] Data for 1954–1955.

TABLE 5-a. POLIOMYELITIS MORBIDITY IN ASIAN COUNTRIES EXPERIENCING INCREASED INCIDENCE, 1964 AND ANNUAL AVERAGES FOR 1951–1955 AND 1960–1964

Country or area	Cases in 1964	Average annual no. of cases, 1960–1964	Average annual no. of cases, 1951–1955	Ratio 1960–1964/ 1951–1955
Turkey	244	642 [a]	43 [b]	14.9:1
Lebanon	230	247	34	7.3:1
Hong Kong	37	157	34	4.6:1
Macau	8	9	2	4.5:1
Ceylon	197	539	196	2.8:1
Iraq	226	175	74	2.4:1
Jordan	70	92	46	2.0:1
Philippines	357	402	295	1.4:1
Singapore	18	71	55	1.3:1
Taiwan	508 [c]	537 [d]	478 [e]	1.1:1
Total	1,895	2,871	1,257	2.3:1

[a] For 1960 only paralytic cases are registered.
[b] For 1953 and 1954 only paralytic cases are registered.
[c] Provisional data for 1961.
[d] Data for 1960–1961.
[e] Data for 1955.

number of cases, the total rose from 679 to 1,987 in the 11 African countries; from 256 to 1,238 in the 5 Latin American countries; and from 429 to 1,861 in the 7 Asian countries—altogether a rise of from 1,364 to 5,086. Clearly, there is a very substantial change in the situation.

Only a few of the tropical and semitropical countries have used killed vaccine to any great extent. The results, though apparently good in Israel and Jamaica, were equivocal in the others.

Live vaccine has been used in a larger number of countries, but in many cases the programs have been limited to a narrow age group and unfortunately have often been allowed to lapse after one or two annual campaigns.

Only a few countries have used live vaccine on a large scale—in Africa, the Republic of the Congo (Kinshasa, formerly Leopoldville), Mauritius, South Africa, Tunisia, and the United Arab Republic; in South and Central America, Cuba, Jamaica, Mexico, and Puerto Rico; in Asia, Ceylon, Hong Kong, Israel, Japan, and Singapore. The results, as the tables show, have been generally satisfactory, but not in all

TABLE 5-b. POLIOMYELITIS MORBIDITY IN ASIAN COUNTRIES EXPERIENCING DECREASED INCIDENCE, 1964 AND ANNUAL AVERAGES FOR 1951–1955 AND 1960–1964

Country	Cases in 1964	Average annual no. of cases, 1960–1964	Average annual no. of cases, 1951–1955	Ratio 1960–1964/ 1951–1955
Israel	21	57 [a]	704	1 :12.4
Cambodia	3	7	29	1 : 4.1
Indonesia	7	67	129	1 : 1.9
Japan	98	1,714	2,414	1 : 1.4
Malaysia	175	111	124 [b]	1 : 1.1
Total	304	1,956	3,400	1 : 1.7
Total for all countries and areas listed in Tables 5-a and 5-b	2,199	4,827	4,657	1.0 :1

[a] For 1960 and 1961 only paralytic cases are registered.
[b] For 1955 only paralytic cases are registered.

countries. At this stage it is difficult to know whether the apparent lack of success in those with less favorable results is due to the short intervals that have elapsed since the program was begun or to some other factors.

Despite the deficiencies in the information available, it is justifiable to conclude that poliomyelitis has greatly increased in the tropics and semitropics in the last few years and that under the present circumstances it will probably continue to do so.

Age Distribution of Poliomyelitis in Tropical and Semitropical Countries

Information on the ages of poliomyelitis patients is not readily available for many countries in tropical and semitropical areas. Those for which it was available in 1955–1957 and 1958–1961 are shown in Table 6. The data may not be representative of Africa, Latin America, and Asia as a whole, but they provide two observations of interest: in most of the countries two thirds to three quarters of the reported cases occurred in the 0–4 years age group, and in most countries the proportion of cases in this age group was greater in 1958–1961 than it was in 1955–1957. Poliomyelitis in tropical countries remains, therefore, a disease of young children; it is still the classical infantile paralysis. The increased proportion of cases in the 0–4 years age group is probably explained by the declining infant mortality in these areas and the consequent population rise in this age group.

Infant Mortality and Incidence of Poliomyelitis

Payne (21) has pointed out that there is a broad correlation between a declining infant mortality ratio and a rising incidence of reported cases of poliomyelitis, and Paul (20) has said, "A general supposition . . . is that in countries where the infant mortality falls below 75 per 1,000 the poliomyelitis case rate will be worth watching." Comparative figures are shown for 25 countries in Asia and South and Central America in Table 7. In 1951–1955, 14 of the 25 countries had infant mortality rates of 75 or more. By 1960–1964 only 4 countries remained in this category. Of the 10 countries which fell below the 75 per 1,000 level in the period, 7 reported an increasing number of poliomyelitis cases. And the 3 that reported fewer cases in 1960–1964 than in 1951–1955 have had vaccination programs of greater or lesser intensity.

There is no reason to suppose, however, that the countries in which the infant mortality rate was below 75 in both periods and which have not had extensive vaccination campaigns are at either greater or lesser risk than those in which the rate has fallen below 75 recently. Perhaps the most interesting feature of the table is that

TABLE 6. PERCENTAGE OF POLIOMYELITIS PATIENTS IN THE 0–4 YEAR AGE GROUP IN CERTAIN COUNTRIES OF AFRICA, SOUTH AND CENTRAL AMERICA, AND ASIA

Country or area	Percentage of patients in the 0–4 yr. age group	
	1955–1957	1958–1961
AFRICA		
Angola	54	83
Mozambique	70	70
S. Rhodesia	65	71
South Africa	61	70
AMERICAS		
Argentina	75	80
Chile	83	85
Colombia	65	74
Costa Rica	81	84
Jamaica	48	60
Mexico	88	95
Nicaragua	91	94
Paraguay	61	85
Peru	88	94
Puerto Rico	71	85
Venezuela	86	82
ASIA		
Hong Kong	64	86
Israel	88	77
Japan	79	83
Jordan	96	94
Lebanon	92	94
Philippines	82	72
Taiwan	96	97
Turkey	64	66

in every country listed the infant mortality rate has declined, usually to a considerable extent, between the periods studied.

Levels of Antibody

Further information on the usual age of infection with poliomyelitis virus is obtained from serological surveys. Data were examined for studies between 1958 and 1963 in 30 tropical and semitropical countries. They showed, as is well known, that 70 to 80 per cent of children have antibody by the time they are five years old. Such general assessments, however, may be misleading. Fox, Gelfand, and others (8, 9, 12, 13) have demonstrated the cyclic process by which one type of poliovirus tends to be replaced after a certain time by another. Cabasso and his colleagues (2) have shown that immunity to the different types may be very variable (Table 8). They estimated in studies in six Latin American countries that from 10 to 48 per cent of the subjects might not have antibody to poliomyelitis virus Type 1, from 6 to 61 per cent to Type 2, and from 1 to 31 per cent to Type 3. Although Type 1 is probably the main cause of clinical disease in tropical and semitropical countries, outbreaks due to Types 2 and 3 are not uncommon (7, 23).

Table 9 shows a recent example of the distribution of poliomyelitis virus antibodies in a tropical urban population (Ibadan), based on data supplied by the Poliomyelitis Commission of the Nigerian Western Region Ministry of Health (19).

We have concentrated attention on reports tending to show that antibody levels may be lower in children in warm climates than is generally believed because we consider that much more study of this subject is now required. Many of the early surveys were carried out on groups of children chosen, often of necessity, without too much attention to their being fully representative of the whole child population. The influence of urbanization and other environmental changes on antibody levels also requires further study.

The Efficacy of Live Poliomyelitis Vaccines in Hot Climates

Studies in temperate climates have shown that the live poliomyelitis virus vaccines multiply satisfactorily in the gut of those to whom they are administered regardless of whether the preparations are monovalent, bivalent, or trivalent. In early studies children without antibody were shown to respond very well to monovalent vaccines. Similar results were found later with trivalent vaccines when at least two and preferably three doses were administered not less than four to six weeks apart.

Sabin and his co-workers (24) made a special trial with trivalent vaccine in Toluca, Mexico, where a large proportion of the children

TABLE 7. RELATIONSHIP BETWEEN THE INCIDENCE OF POLIOMYELITIS AND THE INFANT MORTALITY RATES (DEATHS PER 1,000 LIVE BIRTHS)

Country or area	Infant mortality rates		Number of cases of poliomyelitis	
	1951–1955	1960–1964	1951–1955	1960–1964
ASIA				
Israel	36.2	26.6	704	57
Japan	48.0	28.6	2,414	1,714
Malaya	86.2	64.3	124	111
Ceylon	75.6	54.4	196	539
Hong Kong	69.2	39.2	34	157
Jordan	80.9	54.5	46	92
Philippines	96.4	71.1	295	402
Singapore	63.5	32.8	55	71
Taiwan	33.4	70.0	478	537
AMERICAS				
Argentina	63.8	61.0	1,071	967
Chile	131.3	123.0	468	429
Costa Rica	82.2	68.2	297	36
Honduras	62.0	42.2	146	106
Jamaica	69.8	48.8	168	49
Mexico	89.7	71.4	1,365	645
Puerto Rico	62.0	42.2	146	106
Uruguay	50.9	46.3	158	29
Colombia	109.8	93.0	103	401
Ecuador	115.4	96.4	43	87
El Salvador	80.8	72.6	49	52
Guatemala	99.3	89.3	71	130
Guyana	76.4	52.6	3	99
Nicaragua	75.3	63.2	82	109
Peru	98.3	58.5	107	561
Venezuela	73.4	50.5	286	332

were carrying enteroviruses belonging to a variety of species. Ten weeks after one dose of trivalent vaccine the conversion rates among the whole group of negative children (single, double, and triple negative) were 68 per cent for Type 1, 82 per cent for Type 2, and 43 per cent for Type 3. After a second dose the conversion rates were 96 per cent for Types 1 and 2, and 72 per cent for Type 3 (Table 10).

Khozinski and his colleagues (15) showed that children without immunity in Tashkent, where the ambient temperature is high, responded well after one feeding of trivalent vaccine; 92 per cent developed antibody to Type 1, 85 per cent to Type 2, and 70 per cent to Type 3.

The investigations in Tashkent and Toluca and other similar studies have provided an apparently sound basis for recommending trivalent vaccine in at least two and preferably three doses for children in the tropics, and, as is shown in Table 10, there have been a number of reports of satisfactory results. Other campaigns, however, have given results that, measured by serological conversion, were poor. In Singapore (16) the seroconversion rate to Type 1 after two doses of trivalent vaccine was only 50 per

TABLE 8. ESTIMATED SERONEGATIVE PERSONS AMONG POPULATIONS LATER FED MONOVALENT OR TRIVALENT ORAL POLIOVIRUS VACCINE *

Country	No. of persons fed†	Estimated number and percentage seronegative							
		Type 1		Type 2		Type 3		Triple-negative	
		No.	%	No.	%	No.	%	No.	%
Colombia	154,000	31,600	20.5	53,100	34.5	55,400	36.0	17,700	11.5
Costa Rica	270,000	98,500	36.5	43,200	16.0	50,000	18.7	28,700	11.0
Cuba	7,800	780	10.0	440	5.7	1,100	14.0	78	1.0
Nicaragua	76,500	24,100	31.5	10,700	14.0	23,700	31.0	3,300	4.3
Peru	200	90	47.5	120	61.0	130	64.0	60	31.0
Uruguay	325,000	90,000	27.5	56,850	17.5	117,000	36.0	16,250	5.0
Total	833,500	245,070	29.4	164,410	19.7	247,330	29.7	67,088	8.0

*Source: Cabasso, V. J., Jungherr, E. L., Moyer, A. W., Roca-Garcia, M., and Cox, H. R. "Oral Poliomyelitis Vaccine, Lederle—Thirteen Years of Laboratory and Field Investigation." *New Eng J Med* 263:1321–1330, 1960.
†Mostly children 6 months to 10 years of age.

cent. In South Africa (25) a similarly low conversion rate to Type 1 (51 per cent) was obtained after one feeding of trivalent vaccine, but the results improved considerably after a second dose. In Brazil only 33 per cent of the children in a study developed antibody to Type 1 after two doses of trivalent vaccine, and in Nigeria only 27 per cent developed antibody to Type 1 under a schedule of one dose of Type 2 monovalent vaccine followed by one dose of trivalent vaccine. In another small trial with Type 1 poliovaccine in Ibadan, Nigeria, the results were also unsatisfactory (18).

TABLE 9. AGE DISTRIBUTION OF CHILDREN WITH POLIOMYELITIS VIRUS ANTIBODY BEFORE VACCINATION, IBADAN, NIGERIA

Age group (months)		Poliovirus Type 1	Poliovirus Type 2	Poliovirus Type 3
5–7	No. tested	21	21	20
	% immune	4.8	0	15.0
8–11	No. tested	62	64	63
	% immune	14.5	3.1	26.9
12–15	No. tested	68	66	66
	% immune	29.4	12.1	48.5
16–19	No. tested	69	67	69
	% immune	50.7	13.4	47.8
20–23	No. tested	20	20	20
	% immune	45.0	5.0	50.0
24 +	No. tested	50	52	50
	% immune	68.0	48.0	68.0
Total	No. tested	290	290	288
	% immune	37.2	15.5	44.8

Source: Nigeria, Western Region Ministry of Health Poliomyelitis Commission. "Poliomyelitis Vaccination in Ibadan, Nigeria, during 1964 with Oral Vaccine (Sabin Strains). A Report." *Bull WHO* 34:865–876, 1966.

We have drawn attention mainly to the seroconversion rates obtained with Type 1 vaccine, since this type remains the most important. The conversions to Types 2 and 3 are shown in the table but are not discussed in the text.

Some of the unsatisfactory results may have been due to the use of vaccines that were of poor potency on manufacture or that lost potency for one reason or another before they were fed to the children. Other organizational failures may sometimes have been responsible. However, the general belief is that the main reason for failure is the presence of other enteroviruses in the gut, and recent studies (e.g., Melnick and associates, personal communication) confirm that sometimes 80 or 90 per cent of children in the tropics may be carrying enteroviruses and that often two or three different types may be present simultaneously. Sabin and his coworkers (24) considered that the good results in Toluca were due to the mass campaign, in which a very high proportion of susceptible children were fed simultaneously, thus providing for a period an effective substitute for wild enteroviruses, partly by the direct feeding of the vaccine and partly by the spread of vaccine

TABLE 10. SEROCONVERSION RATES AFTER VACCINATION WITH LIVE POLIOVIRUS VACCINES IN TROPICAL AND SEMITROPICAL AREAS

Country or area	Population	Type of vaccine and scheme of vaccination	Seroconversion			Bibliography ref. No.
			Type 1	Type 2	Type 3	
1) USSR*	Single-, double- and triple-negative children	One dose of trivalent Sabin vaccine	66.7	88.6	75.0	3
	Single-, double- and triple-negative children	Two doses of trivalent Sabin vaccine	76.5	85.7	89.0	
2) Bolivia	Children 6 months to 8 years of age	Two doses of trivalent Sabin vaccine	86.9	100.0	96.2	14
3) Brazil	Single-, double- and triple-seronegative children	Two doses of trivalent Sabin vaccine	33	94†	64	Milovanovic, 1965 personal communication
4) Costa Rica	Children 0–13 years of age	One dose of trivalent Lederle vaccine	87	43	91	22
		Two doses of trivalent Lederle vaccine	97	69	100	
5) Hong Kong	Children 6 months to 5 years of age	Two doses of trivalent Sabin vaccine	66	98.5	95	10
6) Mexico (Toluca)	Single-, double, and triple-negative children	One dose of trivalent Sabin vaccine	68	82	43	24
		Two doses of trivalent Sabin vaccine	96	96	72	
7) Nigeria	Children 5 months to 3 years of age	Type 2 monovalent vaccine followed by trivalent vaccine (Sabin strains)	26.9	64.5	47.1	19
8) Singapore	Triple-negative children 6 to 11 months of age	Two doses of trivalent Sabin vaccine	50	98.5	58.8	16
9) South Africa	Seronegative children under 16 years of age	One dose of trivalent Sabin vaccine	51.4	87.3	85.4	25
		Two doses of trivalent Sabin vaccine‡	93	98	99	
		Three doses of trivalent Sabin vaccine‡	100	100	100	
10) Surinam	Children 6 months to 5 years of age	One to three doses of trivalent Sabin vaccine	85	90	66	17
11) USSR (Tashkent, Uzbek SSR)	Seronegative children, 0 to 15 years of age	One dose of trivalent Sabin vaccine	91.8	83.7	69.5	15

*Data given as examples of high seroconversion rates received in temperate climate countries.
†The author's opinion is that the high level of seroconversion might be strengthened by naturally occurring circulation of wild poliomyelitis virus Type 2.
‡Second and third doses of vaccine were given to failures after first and second feeding. Total seroconversion is shown.

strains to contacts. This may well have been the case in Toluca, but a mass campaign is not always practicable. In fact, there is considerable objection to such campaigns because they do not provide satisfactory means of regularly immunizing the children born into the community each year. Only a scheme that fits into routine immunization programs will meet long-term requirements. Therefore, if interference by other enteroviruses is the main reason for the poor results obtained in places such as Nigeria and Brazil, means must be sought for making vaccines effective when used routinely for immunization in tropical areas.

Discussion

There is a great contrast between poliomyelitis in temperate climates and in the tropical and semitropical belt. In the temperate areas vaccination has reduced the former high incidence almost to vanishing point. In many tropical and semitropical countries the incidence of the disease is increasing progressively. This is shown by the increased number of reported cases (though the limitations of this means of assessing the real incidence of the disease have to be kept in mind) and is supported by information on antibody levels and on the behavior of infant mortality rates.

Though the incidence has not yet reached alarming levels in all the tropical countries, large outbreaks have occurred recently in some of them. The general incidence is likely to continue to increase in the near future, and further large outbreaks may be expected to occur.

It is not suggested that all countries with increasing rates should immediately undertake vaccination campaigns, but the time has come for national health authorities to give careful consideration to the question of how to deal with this potentially dangerous situation.

In this connection, the World Health Organization plans to assist in two directions. Arrangements are being discussed with the Director of the WHO International Reference Center for Enteroviruses and collaborators in tropical countries for a study of the efficacy of live vaccines in the tropical belt. Consideration will be given to the administration of higher doses than are now usually employed and also to the administration of lower doses at more frequent intervals. The inhibiting influence of breast milk on the multiplication of vaccine strains in the gut will also be studied further. Arrangements are also being made to have 1.5 million doses of vaccine readily available for use in emergencies and to provide, mainly through the WHO Reference Centers for Enteroviruses, but also through other suitable laboratories, for the rapid dispatch of staff and equipment to assist national workers in the event of epidemics.

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SECTION A. POLIOMYELITIS

DISCUSSION

CHAIRMAN SEAL: We now come to the discussion of Section A, on poliomyelitis. Our first discussant this morning is Dr. George W. A. Dick, Professor of Microbiology at the Bland-Sutton Institute, Middlesex Hospital, London, England.

DR. DICK: At one time it was claimed that interference with the spread of wild virus in a community was a unique attribute of oral polio vaccine, but it is now clear to almost everyone that this is not so—not only from the studies of Sven Gard and others in Europe but also from the earlier use of Salk vaccine in North America and elsewhere.

Individuals immunized with potent inactivated polio vaccine excrete less virus than nonimmunized people, and this reduction is sufficient to prevent the normal circulation of wild poliovirus in a well immunized community.

As with most things we buy, the best is usually the most expensive. Potent inactivated polio vaccine is no exception, but I wonder if costs might not be greatly reduced if we could use continuous or semicontinuous cell lines for virus production. I see little fundamental objection to this method for the production of *inactivated* virus vaccine. Indeed, with our present knowledge, if we were now to embark on the production of virus vaccines for the first time I believe we might well opt for semicontinuous or continuous cell lines, rather than primary monkey kidney cells, as the lesser of two evils.

Some years ago I visualized inactivated polio vaccine, in various combinations with other antigens, as the ideal vaccine for developed countries, and oral polio vaccine, because of its lower cost and in some circumstances easier administration, as the ideal for the developing countries. Now I am not so sure.

I think it has now become obvious to most of us that whirlwind mass campaigns with oral vaccine are of little value unless they are followed up by well-organized continuing programs. It may be that the best policy for some developing countries is to build up polio immunization gradually over the years, integrating it with other vaccine programs, rather than launching a country-wide mass campaign. A continuous breeze is certainly better than a whirlwind followed by a calm.

It is important not to become so mesmerized by the concept of total eradication that an undue proportion of the available human and material resources of a developing country are devoted to one particular project. There are situations in which a reduction of 70 per cent in the number of cases of poliomyelitis might be achieved without undue strain on resources, whereas the effort required for eradication might be too great to be justified. The fact that we cannot always achieve total eradication should not deter us from making a more modest start.

It is now obvious that oral vaccine is relatively inefficient in some of the developing countries because of the difficulties in establishing regular gut infection in infants and young children. There seems no way of getting round this problem except by increasing the dose of virus, which would add to the cost and possibly reduce the margin of safety.

In many countries polio vaccine will be but one of the vaccines needed in a continuing immunization program. Were it not for the present problem of high cost, I would say that combined vaccines containing potent inactivated poliovirus antigens are best.

CHAIRMAN SEAL: Our next discussant is Dr. Andrew J. Rhodes, Professor and Head of the Department of Microbiology, School of Hygiene, University of Toronto, Toronto, Canada.

DR. RHODES: Canada's experience in the field of vaccination against poliomyelitis goes back to the original Francis trial of inactivated vaccine in 1954. Inactivated vaccines were introduced into public health programs in 1955 and are widely used to this day. In most parts of Canada almost every child must by now have received several shots of inactivated vaccine.

In 1960 and 1961 trials of attenuated (Sabin) vaccine were carried out and in 1962 the product was widely used. From 1963 on, the attenuated vaccine has continued to be used on a wide scale, more recently mainly in children.

In Canada we regard the Salk and Sabin vaccines not as alternative immunizing agents but rather as complementary media. Each has its advantages and its disadvantages. Both are well within the financial grasp of Canadian public health authorities.

Like many other countries, Canada had suffered for a long time from epidemic poliomyelitis, and some outbreaks had had a very high attack rate. In 1953 the attack rate was 260 paralytic cases per million population (over 3,000 cases).

The incidence declined markedly with the widespread use of Salk vaccine in 1956, 1957, and 1958. In 1961 the incidence was only 5 cases per million.

Sabin vaccine began to be widely used in 1962, and this may well have given poliovirus its *coup de grace*. In 1965 we had only two cases in the country, and to date this year only one—interestingly enough, an unvaccinated adult male who had immigrated about a year earlier.

I should like to discuss some of the reasons for this truly spectacular advance in preventive medicine and for the change in the ecology of poliovirus. I shall deal mainly with public health aspects. Some of the reasons are inherent in the nature of the products themselves and others are related to the methods of administering the products to the susceptible population.

Both vaccines are highly effective preventives. They are easy to administer and they cause few adverse reactions. They provide durable resistance and probably break the cycle of transmission. Of great importance is the fact that they are highly acceptable to the public and to the health professions. Furthermore, they can be meshed into regular immunization programs with a minimum of disturbance of ongoing schedules.

Our practice in Canada may be divided into epidemic control procedures and regular elective immunization programs.

In regard to epidemic use, the appearance of even one or two cases of paralytic illness in a Canadian community justifies a vigorous attack. All epidemiological contacts are given trivalent Sabin vaccine. If cases have occurred outside this group, then a more general campaign is recommended.

With reference to the elective use of polio vaccines, we have developed three alternative schedules, each starting at about three months of age. They are all based on the long-standing use of combined antigens in Canada. Combined antigens in which Salk vaccine is included were introduced by Connaught Medical Research Laboratories of the University of Toronto in 1959. More recently the polio component has been purified. The three alternative schedules are as follows:

1. Basic immunity is provided by giving three injections of DPT polio (quadruple) antigen at 3, 4, and 5 months of age. This immunity is stimulated by quadruple antigen given at 18 months and again at school entry.

2. Basic immunity is provided by giving the same course of quadruple antigen, but this is followed by trivalent Sabin vaccine at 6, 8, and 18 months and again at school entry.

3. DPT antigen is used for immunizing at 3, 4, and 5 months and Sabin is given at 3, 5, and 8 months. A booster of Sabin vaccine is given at 18 months and at school entry. This is the procedure preferred in the Province of Quebec. For adults, unless there is any urgency or particular risk of exposure, we prefer to give Sabin vaccine only after a preliminary course of Salk vaccine.

The program outlined for children also includes smallpox vaccine between 5 and 18 months of age and measles killed or live attenuated vaccine (Table 1). Connaught Laboratories are currently testing a five-way product—DPT-polio-measles.

As to the future, we anticipate that all newborns and immigrant children will continue to be immunized against poliomyelitis on a regular basis as they are now. The current need to boost immunity developed by killed vaccine with

TABLE 1. ELECTIVE IMMUNIZATION SCHEDULES RECOMMENDED FOR USE IN CANADA (NATIONAL ADVISORY COMMITTEE ON IMMUNIZING AGENTS)

Age or grade of subject (Approximate)	Combined antigens			Smallpox vaccine	Poliomyelitis vaccines			Alternative measles vaccines		
	Diphtheria toxoid	Pertussis vaccine	Tetanus toxoid		Sabin only	Salk and Sabin	Salk only	Killed and live	Live	Live, for older children*
Months 3	x	x	x		Sabin	in D P T P	in D P T P	Killed†		
4	x	x	x		Sabin	in D P T P	in D P T P	Killed†		
5	x	x	x	x‡	Sabin	in D P T P	in D P T P	Killed†		
6						Sabin				
7										
8					Sabin	Sabin				
9									Live, further attenuated	
10										
11										
12								Live		
18	x	x	x		Sabin	Sabin	in D P T P			
School entry (Grade 1)	x	x	x	x	Sabin	Sabin	in D P T P			Live, further attenuated
Mid school (Grade 5)	x	—	x	x§	Sabin	Sabin	in D T P			
School leaving (Grade 10)	x	—	x	x§	Sabin	Sabin	in D T P			

*For susceptible older children, i.e., those who have not previously had measles or been immunized against measles.
†Provisional until measles combined antigen available.
‡ Primary smallpox vaccination may be done at any age between 5 and 18 months, *but two live vaccines should not be given at the same session.*
§Optional.

attenuated polio vaccine may eventually cease to exist in many parts of the country.

Finally, I should like to stress that the successful reduction of poliomyelitis in Canada to near vanishing point is the result of teamwork between the two Canadian manufacturers (Connaught Medical Research Laboratories and the University of Montreal Institute of Microbiology), the Federal Laboratory of Hygiene in Ottawa, the federal and provincial health departments, numerous local medical officers of health, and a cooperative general public.

CHAIRMAN SEAL: Our next discussant is Dr. Jacobus D. Verlinde, Laboratory of Medical Microbiology, University of Leiden, Leiden, Netherlands.

Dr. Verlinde presented a summary of a paper entitled "Virological Surveillance in Vaccinated Children's Populations," which appears in full as an annex to this section on pages 218–222.

CHAIRMAN SEAL: Our last formal discussant in Section A is Dr. Manuel Ramos Alvarez, Children's Hospital, Mexico City.

DR. RAMOS ALVAREZ: The use of Sabin's live poliovirus vaccine in some tropical and subtropical areas has so far failed to provide results in the control of poliomyelitis comparable to those achieved in some countries with temperate climates. One of the reasons for this failure, as was pointed out earlier in the session, has been insufficient use of the vaccine and irregular immunization of the constantly emerging new generations of children. Other factors may also play a role; for example, the presence of a number of enteroviruses in the bowel of children at the time of vaccination can interfere with the intestinal multiplication of the vaccine strains and consequently with the development of immunity. This interference may be responsible for a certain proportion of the vaccine failures observed in these areas. The high incidence of proved or suspected enterovirus infection among the low socioeconomic groups in many Latin American countries makes it highly desirable to obtain precise information concerning the behavior of the vaccine in such populations.

Table 1 shows that the administration of $10^{5.5}$ TCD_{50} of any of the monovalent vaccines in the presence of an enteric viral infection failed to protect an important proportion of a group of vaccinated children, as measured by the development of homotypic antibodies (41 per cent in those fed Types 1 and 2 viruses and 50 per cent in those fed Type 3). The results of tests with single doses of monovalent and trivalent vaccines fed to children under similar conditions are shown in Table 2. The over-all antibody response for Types 1 and 2 viruses in children fed the trivalent vaccine compares favorably with that obtained with the respective monovalent preparation; however, the response for Type 3 virus was of a somewhat lower magnitude. In order to overcome these deficiencies in antibody formation, the feeding of several doses of vaccine is indicated. Table 3 shows the complete antibody conversion rates after the administration of three consecutive doses of trivalent vaccine at intervals of seven to eight weeks. The total conversion rates to triple-immune were 45, 74, and 83 per cent after the first, second, and third doses, respectively. Table 4 shows the results obtained with monovalent vaccines when fed serially at four-week intervals (Types 1, 3, and 2). Four weeks after the last type was fed, only 46

TABLE 1. INTERFERENCE OF NON-POLIO (NP) ENTEROVIRUSES PRESENT AT TIME OF VACCINATION UPON INTESTINAL MULTIPLICATION OF VACCINE STRAINS AND ANTIBODY FORMATION (DOSE FED: $10^{5.5}$ TCD_{50} OF EACH TYPE)

Type of vaccine	NP virus isolations at time of feeding	No. fed	Percentage with		Total percentage for each type		
			Virus excretion	Antibody conversion*	No. fed	Virus excretion	Antibody conversion
1	Positive	64	45	59	103	58	73
	Negative	39	79	95			
2	Positive	44	52	59	87	69	77
	Negative	43	86	95			
3	Positive	62	42	50	91	56	65
	Negative	29	86	96			

*Four to five weeks after vaccination.

TABLE 2. POLIOVIRUS ANTIBODY CONVERSION RATES AFTER A SINGLE DOSE OF MONOVALENT OR TRIVALENT VACCINE IN CHILDREN WITH AND WITHOUT NONPOLIO (NP) ENTEROVIRUS INFECTION AT TIME OF FEEDING

Antibody type	Nonpolio enterovirus at time of feeding	Seroconversion rates				Total seroconversion rates			
		Monovalent vaccine		Trivalent vaccine*		Monovalent vaccine		Trivalent vaccine	
		No. tested	Percentage converted	No. tested	Percentage converted	No. tested	Percentage converted	No. tested	Percentage converted
1	Positive	64	59	45	69	103	73	114	77
	Negative	39	95	69	83				
2	Positive	44	59	52	69	87	77	116	84
	Negative	43	95	64	97				
3	Positive	62	50	40	50	91	65	127	50
	Negative	29	96	87	49				

Dose Fed: Monovalent Vaccine: $10^{5.5}$ TCD_{50} of each type
Trivalent Vaccine: Type 1—$10^{5.7}$ TCD_{50}; Type 2—$10^{5.0}$–$10^{5.8}$ TCD_{50}; and Type 3—$10^{5.4}$ TCD_{50}.
*Seven to eight weeks after feeding vaccine.

per cent of the subjects had converted to triple-positive.

The evidence obtained in this study indicates the need for the administration during non-epidemic periods of three or four properly spaced consecutive doses of trivalent vaccine if control of poliomyelitis is to be attempted in heavily infected areas.

TABLE 3. COMPLETE SEROCONVERSION RATES TO TRIPLE-IMMUNE IN CHILDREN FED THREE CONSECUTIVE DOSES OF TRIVALENT VACCINE AT INTERVALS OF SEVEN TO EIGHT WEEKS

Seronegative to type(s)	No. of subjects fed	Percentage of conversion to triple-positive after indicated dose		
		First	Second	Third
1	6	83	100	
2	11	82	91	100
3	17	53	81	87
1+2	14	71	93	100
1+3	15	60	85	92
2+3	25	48	76	86
1+2+3	81	28	62	74
All	169	45	74	83

TABLE 4. COMPLETE SEROCONVERSION RATES TO TRIPLE-IMMUNE IN CHILDREN FED ONE DOSE OF EACH OF THE MONOVALENT VACCINES AT INTERVALS OF THREE TO FOUR WEEKS *

Seronegative to type(s)	No. of subjects fed	Percentage of conversion to triple-positive four weeks after feeding last type of vaccine
1	21	67
2	29	62
3	18	55
1+2	12	42
1+3	9	33
2+3	15	27
1+2+3	23	17
All	127	46

*$10^{5.5}$ TCD_{50} each of Types 1, 3, and 2, administered in that order.

CHAIRMAN SEAL: We have two short discussions to add to the program at this point. For the first of these I would like to call on Dr. Voroshilova of the USSR.

DR. VOROSHILOVA: Under the guidance of the WHO Virus Diseases Unit, headed by Dr. Cockburn, the Regional Reference Center for Enteroviruses in Moscow participated in a study of vaccination with live Sabin vaccine under conditions in tropical and subtropical areas.

Difficulties of vaccination under such conditions were encountered as early as 1959, and the problems were particularly severe during the vaccination campaigns in the central Asian regions.

We have since learned that certain changes should be made in the vaccine dosages: for Type 1 the dosage should be increased to 500,000 units; for Type 2, to 200,000; and for Type 3, to 300,000.

To date we have carried out serologic studies in a number of subtropical regions of the USSR and also abroad, particularly in Burma. Figure 1 shows the results of serologic investigations conducted on 418 Burmese children. These data are interesting because they show that the most susceptible population in countries such as Burma are children up to three years of age. Thus, vaccination campaigns should be concentrated much more heavily on this group than on older children.

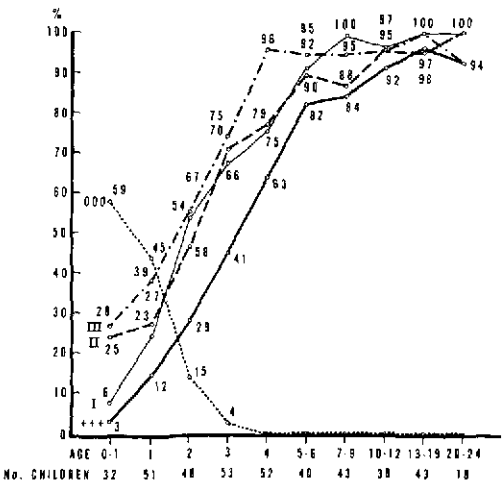

Fig. 1. Serologic investigations conducted on 418 children, $\leqslant 1{:}4$ (Burma, 1966).

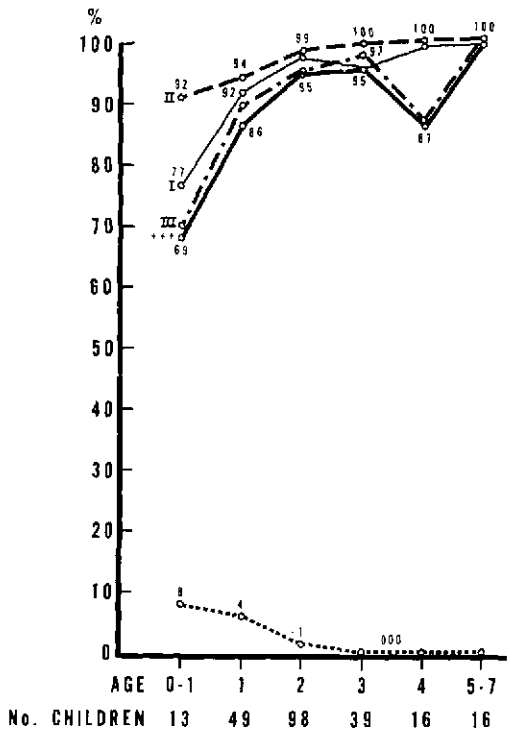

Fig. 2. Results of serologic survey, 213 children, $\leqslant 1{:}4$ (Ashkhabad, 1966).

Figure 2 shows the results of a serological survey conducted in Ashkhabad in the Central Republic. The sera were taken at random. This study demonstrated that the existing system of vaccination and revaccination of children up to three years of age has led to good results. Nearly 100 per cent of the children over one year of age and approximately 70 per cent of those up to one year had developed antibodies to each of the three types of polioviruses.

CHAIRMAN SEAL: I would like now to call on Dr. Karel Zácek, Institute of Epidemiology and Microbiology, Prague, Czechoslovakia, who will comment on the experience of six years of surveillance in that country.

DR. ZÁCEK: As Dr. Sabin has already mentioned, nationwide vaccination and revaccination campaigns with Sabin's oral poliomyelitis vaccine were carried out in Czechoslovakia in 1960 and 1961. They covered more than 90 per cent of the total child population from two months to

15 years of age. Since then, newly born children have been vaccinated each spring.

As a part of a complete surveillance program, regular virological surveys for the presence and circulation of enteric viruses have been carried out since 1960, for which purpose several thousand stool specimens are collected among the healthy population in September and October every year. These samples are collected mainly from children's institutions, nurseries, and kindergartens in all parts of the country. Usually not more than one stool specimen is taken from any one child.

Polioviruses were recovered in 0.12 per cent of over 2,500 specimens collected in 1960, in 0.03 per cent of about 3,200 specimens taken in 1961, and in 0.04 per cent of about 2,500 specimens tested in 1962. However, not a single poliovirus was isolated from more than 5,000 stool specimens collected and tested during the following three years—1963, 1964, and 1965.

CHAIRMAN SEAL: Since time permits, we will proceed with a general discussion. Perhaps Dr. Gard would care to make additional comments.

DR. GARD: On the matter of the protection afforded by neighboring countries whose populations have been vaccinated with live vaccine, I would like to point out that the effects of mass use of inactivated vaccine in Sweden were observed at a time when live poliovirus had not been applied anywhere else on such a scale as to have had any possible effect on developments in our country. So I think our experience does show, as Dr. Dick pointed out, that inactivated vaccine alone can achieve the same effect as was previously attributed only to live vaccine.

DR. FOX: I think the crucial point that Dr. Gard was speaking of, and that has concerned many of us here, is the effectiveness of inactivated vaccine in reducing the occurrence of infection, as distinguished from disease. I dare say our observations in Louisiana some years back have been pretty much at the root of some of the beliefs that inactivated vaccine is not very effective in this respect. I would like to say that I am quite impressed with the data that Dr. Gard has presented.

There was a suggestion of evidence in our own experience in Louisiana that inactivated vaccine did at least slightly abbreviate the period of excretion, even though we could not demonstrate any reduction in the extent of intrahousehold spread of virus. I think the key to the problem is almost certainly the potency of the vaccine used, and I am prepared to believe that with a sufficiently potent vaccine, as apparently they had in Sweden, a significant reduction in the spread of wild polioviruses can probably be achieved.

DR. PLOTKIN: With regard to the problem of oral polio vaccination in underdeveloped countries, I would like to point out that another effect may be involved—namely protein deficiency. Dr. Katz and I have found that protein-starved children have deficient antibody response to oral poliovaccine.

Also, I would urge that further attention be given to the vaccination of children around two months of age. Only in this age group would there be any hope at all of children being relatively free of enterovirus excretion. The intensity of enterovirus carriage in older children in the tropics really has to be seen to be believed, and it is going to be very difficult to overcome. On the other hand, our data on newborn children show very little enteroviral carriage. We may find that somewhere along the age spectrum there is a point at which enteroviral carriage is relatively low.

DR. STOJKOVIC: Since 1960 we have been using Sabin oral vaccine in Yugoslavia. I would like to add some of our experiences to the report of Dr. Zácek.

We have been studying the carrier rate of poliovirus after vaccination in healthy young children. In 1963 we found only three polioviruses with T-minus or T-plus-minus characteristics in a total of 2,000 stool specimens; in 1964 we found only one poliovirus in approximately 2,000 stool specimens; and in 1965 we found no poliovirus at all in 1,700 stool specimens, although we detected other enteric viruses in 5 per cent.

Thus, the results obtained in Yugoslavia were similar to those noted by Dr. Zácek.

DR. JACKSON: I wonder if Professor Chumakov would explain further the differentiation of the cases of polio occurring in the vaccinated

people and the differentiation of vaccine-induced and natural infections. Can we now assume that in both children and adults there is no risk of vaccine-induced cases?

DR. CHUMAKOV: On the basis of epidemiological and laboratory data obtained from the study of suspected cases, we may assert that no connection exists between the occurrence of paralytic poliomyelitis and vaccination with live Sabin vaccine.

Despite the large scale of antipoliomyelitis vaccination campaigns in the USSR, no cases of paralysis produced by the Sabin vaccine have been recorded. Some cases of paralysis reported during the mass vaccination period were found after histopathologic and other tests to be mere coincidence. Other cases that had been diagnosed as poliomyelitis in adults were found after such tests to have no connection with the disease, the original diagnosis having been incorrect. In other cases any connection of the paralysis with antipolio live virus vaccination could be excluded when fecal isolations showed a mixture of wild poliovirus of one type and a vaccine poliovirus of another type—or of a vaccine poliovirus of a type other than the one to which the patient had developed an increased level of antibody. There were a few isolated cases on which complete data could not be obtained. I mentioned these in my report earlier this morning.

On the basis of eight years of mass vaccination in the USSR and on the basis of a detailed comparison of all survey results, it has been found that in most of the cases occurring one to two months after vaccination there was no conclusive proof of poliomyelitis produced by vaccination with live poliovaccine.

ANNEX TO SECTION A. POLIOMYELITIS

VIROLOGICAL SURVEILLANCE IN VACCINATED CHILDREN'S POPULATIONS *

J. D. VERLINDE

Laboratory for Medical Microbiology, State University
Leiden, The Netherlands

DR. VERLINDE (*presenting the paper*): Since 1957 killed poliovirus vaccine has been used in annual poliomyelitis immunization programs in the Netherlands. On an average, 80 to 90 per cent of the child population up to at least 14 years of age has been vaccinated. In a few limited areas, however, religious objections have impeded the vaccination campaign.

Oral vaccine was used in a few small-scale trials several years ago (3, 4) and more recently in some children's homes (5) and in limited areas having a low vaccination status at the time of an outbreak. All three types were administered in the field trials, but only Type 1 was given on the other occasions, since it had been found to circulate in the population at risk.

During the 30-year period prior to the introduction of killed vaccine, epidemics of poliomyelitis occurred every four or five years, and the disease continued to be prevalent, although at a lower level, during the interepidemic years. The effect of vaccination is demonstrated in Figure 1, which shows the average annual incidence of poliomyelitis over a series of four-year periods before and after the introduction of vaccination.

Although there can be no doubt that the high vaccination status of the child population is responsible for the sharply decreased incidence of poliomyelitis, we have become interested in the dissemination of poliovirus under present conditions. A group in our laboratory, under the direction of Dr. J. B. Wilterdink, has been working on this problem for several years. At the same time, two of my associates, first Dr. D. Metselaar and later Dr. R. A. de Haas, studied in the Central Public Health Laboratory, Paramaribo, Surinam, the dissemination of polioviruses in the local population before, during, and after an outbreak of poliomyelitis (2). This outbreak was successfully controlled by trivalent oral poliovirus vaccine (6).

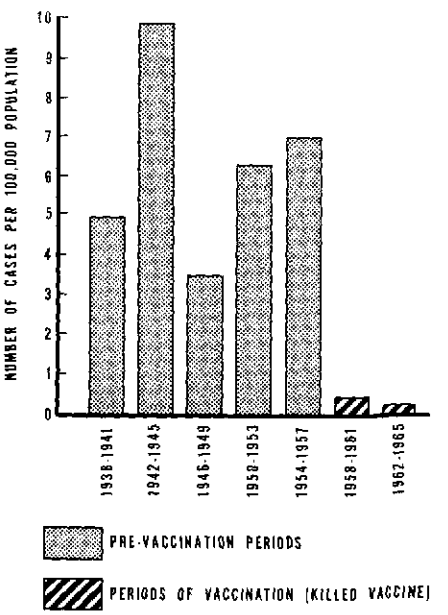

Fig. 1. Average annual incidence of poliomyelitis per 100,000 population in four-year periods.

* Aided with grants from the Princess Beatrix Fund, the Organization for Health Research T.N.O., and the Netherlands Foundation for the Advancement of Tropical Research (WOTRO).

It is evident from virological studies of patients and from surveys of children's homes and nursery schools in the Netherlands that the relative frequency of poliovirus isolations has decreased during the last eight years, although not to the same extent as has the incidence of clinical poliomyelitis. A continuing and fluctuating dissemination of polioviruses in the child population is still observed (Figure 2).

An analysis of the poliovirus carrier rates has been made in several studies, two of which will be briefly reported here.

In the fall of 1963 a limited outbreak of poliomyelitis Type 1 (6 paralytic and 4 nonparalytic cases) on the island of Tholen in the southwestern part of the country provided an opportunity to compare the poliovirus carrier rates in nonvaccinated children with rates in children who had been immunized with killed vaccine. Stool samples were collected prior to the administration of Type 1 oral vaccine. Data from the poorly vaccinated communities on this island (25 per cent of the children had received a complete course of killed vaccine), were compared with those on nursery school children in the city of The Hague (75 per cent had received a complete course of killed vaccine), where not a single case of poliomyelitis had been observed during the period in question. The age groups in the two areas were not comparable, except for the 4-to-6 year group, which was present in both.

The poliovirus carrier rates were determined at one single occasion. A relatively low rate was determined in The Hague (nonepidemic conditions) and a high one on the island of Tholen (epidemic conditions) (Table 1). The proportion of carrier rates was exactly one and one half times higher in the nonvaccinated than in the vaccinated children in both areas, however. Since studies carried out by others have also shown the same proportions of intestinal carrier rates among vaccinated and nonvaccinated contact children during a Type 1 outbreak, a certain regularity of occurrence may be presumed. Marine and associates (1) observed this difference only in children with a relatively high antibody level and not before the third to fourth week after onset of the disease in the index children. The frequency of fecal excretion during the first three weeks was the same in both vaccinated and nonvaccinated individuals.

A second study, a longitudinal one, included a monthly virological survey of approximately 200 children of the 1-to-4 age group in five children's homes in different regions of the country and, in addition, virological examination of sewage samples collected twice monthly from some 25

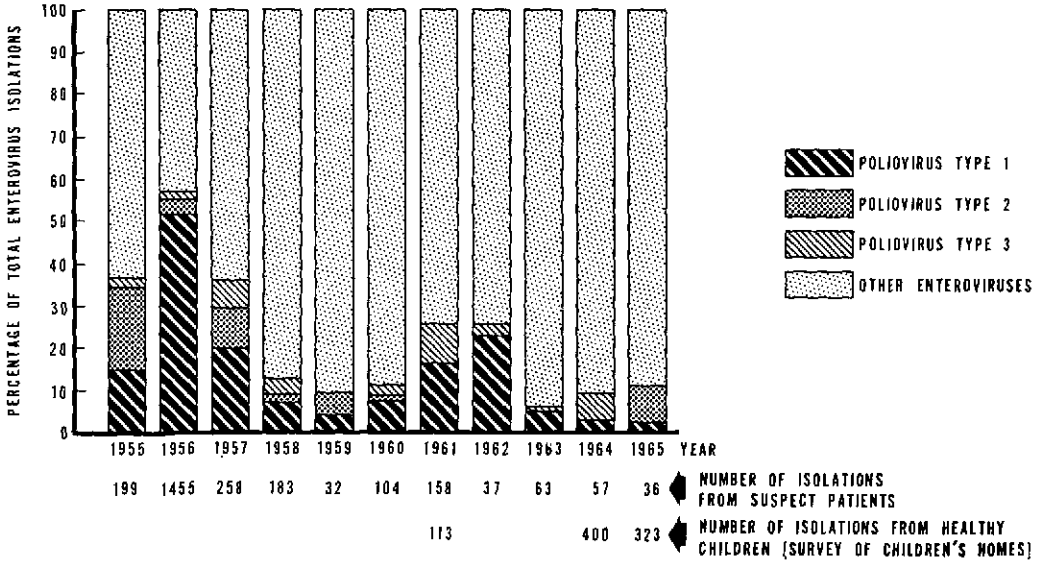

Fig. 2. Relative frequency of polioviruses isolated from patients suspected of enteroviral infection and from healthy children.

TABLE 1. CARRIERS OF POLIOVIRUS AMONG CHILDREN IN THE 4-TO-6 YEAR AGE GROUP

Conditions in groups		Nonepidemic	Epidemic
Types of poliovirus		Types 1 and 3	Type 1
Total number of children examined		482	48
Completely vaccinated (killed vaccine)	Total	404	19
	Percentage	84%	40%
	Carriers	19	6
	Percentage	4.7%	31.6%
Not vaccinated or not completely vaccinated	Total	78	29
	Percentage	16%	60%
	Carriers	5	14
	Percentage	6.4%	48.3%

sewage plants throughout the country (7). Figures 3 and 4 show that the level of dissemination of Types 1 and 3 was relatively low in 1964 and extremely low in 1965. To our surprise, Type 2, which had not been isolated at all during the four preceding years, suddenly appeared in two children's homes and in the nearby sewage plants in an eastern and southern area of the country close to the German and Belgian frontiers. Within five months approximately 35 per cent of the children in both homes were found to be carriers of poliovirus Type 2, some of them for more than 60 days. Almost all children had received a full course of killed vaccine, the intervals between the last injection and the isolation of Type 2 virus ranging from two months to four years. This is particularly striking since Type 2 is considered to be the most potent antigen in killed vaccine.

Four arbitrarily chosen strains of the Type 2 virus failed to exhibit paralytic activity in cynomolgus monkeys following intracerebral and intraspinal inoculation of amounts of virus ranging from 100 to 1,000,000 TCD_{50}. Slight poliomyelitic lesions, however, were found in the lumbar cord region of monkeys that had been inoculated with the higher concentrations of virus. Hence the virus has to be regarded as a highly attenuated one.

The origin of the Type 2 virus remains obscure. The possibility of its having slipped in from across the border has to be taken in account, since trivalent oral vaccine was administered in 1965 on a large scale in both of the adjoining countries. But if this vaccine is the source of the Type 2 virus, it is puzzling why only Type 2 was recovered and not Types 1 and 3.

Continued examination of sewage in 1966 revealed that Type 2 has been disseminated throughout almost the entire country, since it has been isolated occasionally from approximately 25 per cent of the sewage plants examined. This pattern resembles the dissemination of a naturally occurring strain.

It may be concluded from these studies that one has to be continually alert to the occurrence of alternating periods with lower and higher levels of dissemination of polioviruses, even in regions with a high vaccination status, and not only among nonvaccinated individuals but also among those who have received a complete course of killed vaccine.

The vaccination status of the Surinam children is poor. From the end of September 1962, when a virological survey was started, to January

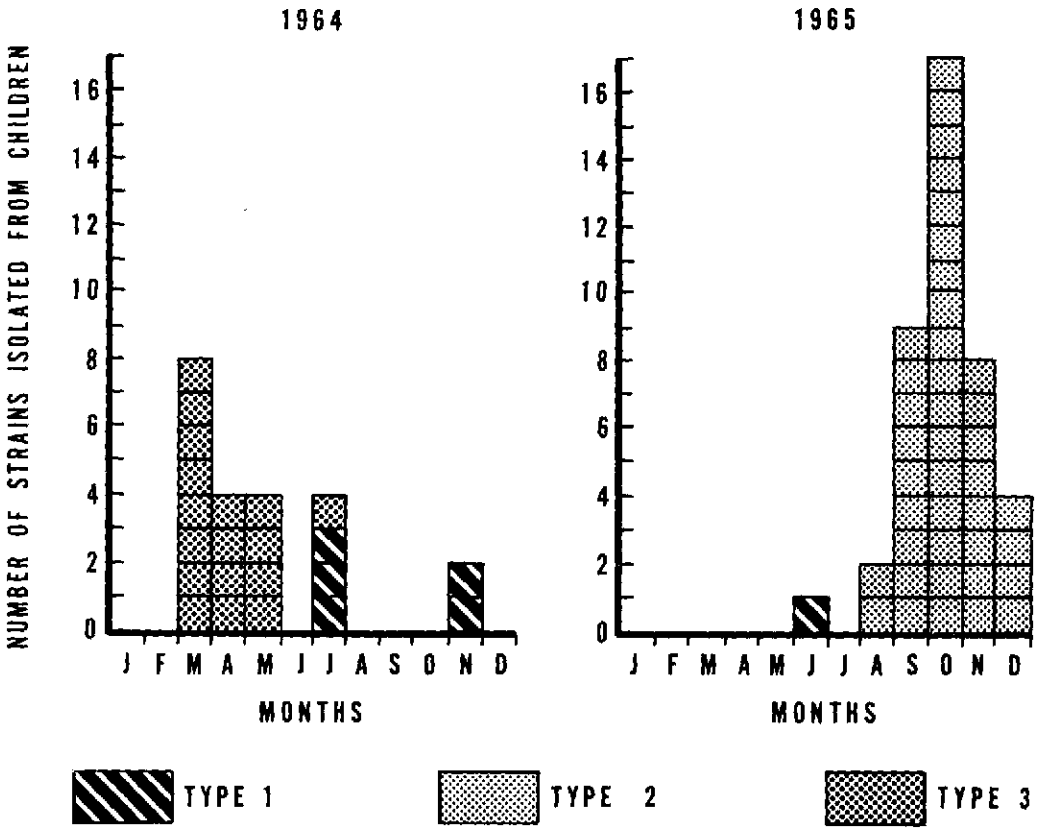

Fig. 3. Distribution of polioviruses in children's homes, 1964 and 1965.

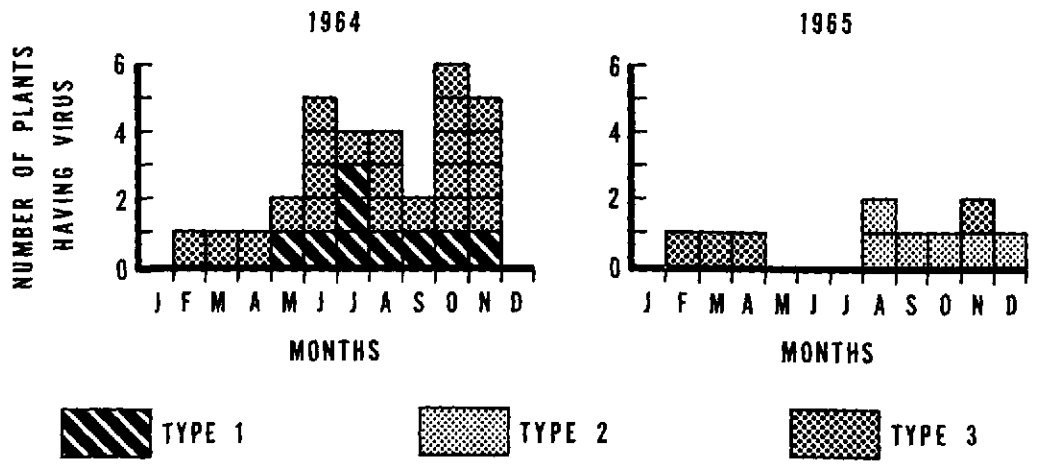

Fig. 4. Distribution of polioviruses in sewage plants, 1964 and 1965.

1963, the poliovirus carrier rate increased from almost zero to 30 per cent. An outbreak of clinical poliomyelitis began in early January 1963. Due to local circumstances, the administration of trivalent oral vaccine was not started before several cases of poliomyelitis Type 1 had been reported. Roughly 75 per cent of the children up to 14 years of age received the first dose of oral vaccine, and 65 per cent received the second dose. The number of reported cases dropped rapidly, and after the administration of the second dose only one case was reported.

The seroconversion rates were lower than in temperate climates—85, 90, and 66 per cent for Types 1, 2, and 3, respectively. This is probably due to high levels of dissemination of other enteroviruses.

The vaccination status of children born after the outbreak is still poor, and recent virological surveys indicate the formation of a new reservoir of susceptible individuals.

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SECTION B. COXSACKIE AND ECHOVIRUSES

IMPORTANCE AND PROSPECTS FOR CONTROL OF COXSACKIEVIRUS AND ECHOVIRUS INFECTIONS *

JOSEPH L. MELNICK

WHO International Reference Center for Enteroviruses
Department of Virology and Epidemiology
Baylor University College of Medicine, Houston, Texas, U.S.A.

DR. MELNICK (*presenting the paper*): The types of illnesses caused by the enteroviruses are numerous, depending upon the target organ attacked. These illnesses have been recently reviewed in the fourth edition of *Viral and Rickettsial Infections of Man* (1, 2) and are listed in Tables 1 and 2 for purposes of reference. The most common illness caused by the Coxsackie and echoviruses that is serious enough to require hospitalization is aseptic meningitis, which often occurs in epidemic form.

Despite the introduction and widespread use of poliovaccines, it is important to note their failure to influence the incidence of aseptic meningitis in countries where poliomyelitis has been virtually eliminated. In the United States, for example, there have been over 2,000 reported cases of aseptic meningitis in the past four years (3), whereas paralytic poliomyelitis has shown a marked decline over the same period (Fig. 1). These figures would suggest that poliovirus does not *currently* cause a significant number of cases of aseptic meningitis.

Since 1962 state public health laboratories have reported isolations of enteroviruses to the Communicable Disease Center as a part of its continuing surveillance program. The nonpolio enterovirus isolations by type and year are summarized in Table 3 (3). The most frequently isolated virus varies from year to year, particularly among the group B Coxsackieviruses, but echovirus 9 has been consistently prevalent during the four-year period shown. In 1965 focal outbreaks due to specific types occurred in widely scattered parts of the country. These outbreaks were caused by echoviruses 6 and 9 and Coxsackieviruses B2 and B5.

A similar experience has also been noted in other parts of the world. In Sweden, where polio epidemics were brought to a halt by vaccination, aseptic meningitis epidemics have continued (4), as is shown in Table 4. The epidemic types shifted from echo 6 in 1954, to echo 6 and Coxsackie A9 in 1955, to echo 9 in 1957, and to Coxsackie B5 in 1961.

Enterovirus meningitis is usually a benign disease and almost all patients recover.

Some people have expressed concern that with the elimination of paralytic poliomyelitis other enteroviruses would become more virulent. Experience seems to indicate, however, that the nonpolio enteroviruses are no more virulent today than they were before the control of poliomyelitis. This is illustrated by data in Table 5 obtained in California during the period 1956–1960, when paralytic poliomyelitis was being brought under control by vaccination. In this study the number of Coxsackie and echoviruses associated with acute paralytic disease remained constant throughout the period (5).

The nonpolio enteroviruses have not been related to epidemics of paralytic disease, except for Coxsackie A7, which has caused outbreaks in the Soviet Union (6) and in Scotland (7).

* Work described from the author's laboratory has been aided in part by a grant, AI 05382, from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

TABLE 1. THE CLINICAL SYNDROMES CAUSED BY COXSACKIEVIRUSES *

Group	Syndrome or clinical feature	Types
A (23 types)	Herpangina	2, 4, 5, 6, 8, 10
	Aseptic meningitis	2, 4, 7, 9, 10
	Paralysis (infrequently)	7, 9
	Exanthem	4, 9, 16
	Common cold	21, 24
	Hepatitis	4, 9
	Pneumonitis of infants	9, 16
	Undifferentiated febrile illness	All types mentioned above
B (6 types)	Pleurodynia	1 to 5
	Aseptic meningitis	1 to 6
	Paralysis (infrequently)	2 to 5
	Severe systemic infection in infants, meningoencephalitis, and myocarditis	1 to 5
	Pericarditis, myocarditis	1 to 5
	Undifferentiated febrile illnesses	1 to 6
	Rash	5
	Hepatitis	5
	Pneumonia	5

*Source: Dalldorf, G., and Melnick, J. L. "Coxsackie Viruses." In *Viral and Rickettsial Infections of Man*, pp. 474–512, 1965.

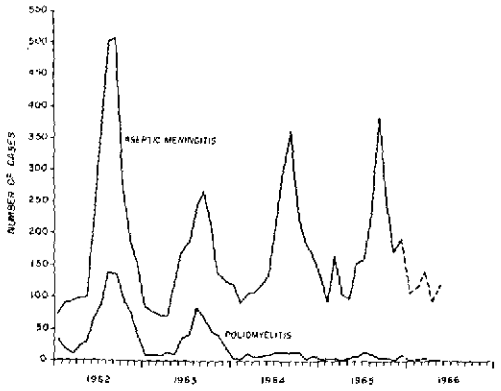
TABLE 2. CLINICAL ASSOCIATIONS OF ECHOVIRUSES *

Syndrome or clinical feature	Echovirus types†
Aseptic meningitis	
Sporadic cases	1, *2*, 3, *4*, *5*, *6*, 7, *9*, *11*, 12, 13, *14*, *15*, *16*, *17*, *18*, *19*, *20*, 21, 22, *23*, 25, *30*, *31*, 32
Outbreaks	*4*, *6*, *9*, *11*, 16, *30*
Paralytic disease	1, *2*, *4*, *6*, 7, 9, *11*, *16*, 18, *30*
Encephalitis	2, 3, 4, 6, 7, *9*, 11, 14, 18, *19*
Ataxia	*9*
Guillain-Barré syndrome	*6*, 22
Exanthematous disease generally maculopapular)	
Sporadic cases	1, 2, 3, *4*, 5, 6, 7, *9*, *11*, 14, *16*, *18*, 19
Outbreaks	*2*, *4*, *9*, *11*, *16*
Enanthems (with and without exanthems)	6, *9*, *16*
Epidemic myalgia	1, 6, 9
Pericarditis	1, 9, 19
Myocarditis	6, 9
Diarrheal disease	
Sporadic cases	1, 2, 5, 6, 7, 9, 11, 12, 13, 14, 15, 18, 19, 21, 22, 23, 24
Outbreaks	*11*, 14, 18
Respiratory-enteric disease	1, 11, 19, 20
Lymphadenopathy	2, 4, 9, 16, 20
Hepatic disturbances	4, 9
Respiratory syndromes	1, 3, 6, 11, 19, 20
Ocular disturbances	1, 4, 6, 9, 16, 20

**Source:* Melnick, J. L. "Echoviruses." In *Viral and Rickettsial Infections of Man*, pp. 514–544, 1965.
†Figures in italics indicate isolations from blood or cerebrospinal fluid or other parenteral sources as well as from feces or pharynx.

Sporadic cases due to this virus have been reported from Japan, the United States, Scandinavia, and Switzerland. Table 6 lists the data from Scotland, where Coxsackievirus A7 was isolated from 54 patients, mostly during outbreaks in 1959 and 1963. Grist (7) noted that only 10 (or 19%) were paralyzed, as compared with 70 per cent of the patients from whom poliovirus was isolated during the same period. Paralysis was less severe than that due to poliovirus and cleared up more rapidly and completely. Only one case was fatal.

Epidemiological evidence that Coxsackie A7 virus was the cause of paralysis was obtained in the 1959 outbreak, when tests of feces from 123 patients during the peak of the outbreak showed Coxsackie A7 virus in 5 of 6 paralytic cases, 18 of 57 nonparalytic meningitis cases, and only 1 of 60 cases of other diseases. Further similarity to poliovirus was shown by the ability of representative strains to cause paralytic disease in monkeys, giving a histological picture in certain respects like that of poliomyelitis, as originally reported for this virus by Voroshilova and Chumakov (6).



Source: Communicable Disease Center. Reported Incidence of Notifiable Diseases in the U.S.--Annual Supplement to the Morbidity and Mortality Weekly Report.

Fig. 1. Reported cases of aseptic meningitis and paralytic poliomyelitis in the United States, 1962-1966 (by month).

Global reporting of enterovirus isolations. In recent years the WHO Virus Diseases Unit has initiated a program to receive, collate, and distribute quarterly reports of virus isolations from WHO Reference Centers for Enteric and Respiratory Virus Diseases and from a number of collaborating national laboratories (8). In 1965, 30 laboratories reported regularly, but information from over 120 is included for this year because several of the reporting laboratories collected information from other parts of their respective countries. This program is already providing useful data and will undoubtedly prove to be an ever-increasing source of valuable epidemiological information, particularly as the number of reporting laboratories continues to increase.

The relative frequency of the main enterovirus

TABLE 3. NONPOLIO ENTEROVIRUS ISOLATIONS IN THE UNITED STATES, 1962–1965 *

Year	No. of states reporting enteroviruses of any type	Echovirus types					Coxsackievirus types							Total
		4	9	11	14	Other	A9	B1	B2	B3	B4	B5	Other	
1962	34	54	115	23	16	75	15	15	57	119	7	32	100	628
1963	28	72	104	32	27	120	91	137	28	11	44	6	65	737
1964	23	34	50	18	7	112	49	33	52	0	66	5	41	467
1965	23	1	171	14	13	135	8	6	59	3	22	38	16	486

Source: Communicable Disease Center. *Morbidity and Mortality Weekly Report* 15:194, 1966.
*This table does not include enterovirus isolations made by viral diagnostic laboratories outside the state health department.

TABLE 4. ENTEROVIRUSES RECOVERED BY TISSUE CULTURE METHODS FROM PATIENTS OF THE HOSPITAL FOR INFECTIOUS DISEASES IN STOCKHOLM, 1953–1961 *

Year	Polioviruses			Echoviruses								Coxsackieviruses							Not identified
	1	2	3	1	5	6	7	9	11	15	16	A7	A9	B1	B2	B3	B4	B5	
1953	392	1				1	1	6									2		4
1954	6	3	11		2	38	1					2					3		7
1955	6	32	37			19		3			1		17				2		17
1956	3	1	23			3		10					1						22
1957	8	2	3					201						2	2		2		7
1958	43	2				1		3	9				1			2	9	4	12
1959	9									1						8	3		4
1960	1			1					2						1	1	1	2	8
1961								12					8	1	2		3	58	10
Totals: 1953–1961	468	41	74	1	2	62	2	235	11	1	1	2	27	3	5	11	25	64	91

**Source*: Svedmyr, A. "Variation of Dominant Types of Enteroviruses during a Sequence of Years." *Arch Ges Virusforsch* 13:167–168, 1963.

TABLE 5. SPECIFIC VIRUSES ASSOCIATED WITH CASES OF CLINICAL PARALYTIC POLIOMYELITIS, CALIFORNIA, 1956–1960 *

Type of virus	Number of patients, by year					
	Total	1956	1957	1958	1959	1960
Cases studied	706	272	103	102	138	91
Poliovirus	437					
Type 1	360	156	19	44	83	58
Type 2	5	2	1	2	0	0
Type 3	72	22	19	15	12	4
Coxsackievirus	31					
Type A9	1	0	1	0	0	0
Type A16	1	0	0	0	1	0
Type B2	8	2	1	2	2	1
Type B3	2	1	0	1	0	0
Type B4	7	1	5	0	0	1
Type B5	11	4	2	4	0	1
Type B6	1	0	0	0	0	1
Echovirus	22					
Type 4	7	5	1	0	0	1
Type 6	4	3	0	0	1	0
Type 9	2	0	0	2	0	0
Type 11	1	0	0	0	0	1
Type 13	1	0	0	0	1	0
Type 14	3	1	1	1	0	0
Untyped	4	2	0	0	1	1
Mumps	13	10	2	1	0	0
Herpes simplex	6	0	4	0	1	1
St. Louis encephalitis	1	1	0	0	0	0
Total:						
Nonpolioviruses						
No. of patients	73	30	17	11	7	8
Per cent	10%	11%	17%	11%	5%	9%

*Source: Lennette, E. H., Magoffin, R. L., and Knouf, E. G. "Viral Central Nervous System Disease. An Etiologic Study Conducted at the Los Angeles County General Hospital," *JAMA* 179: 687–695, 1962.

types isolated in 1965 is shown in Table 7. The large number of Coxsackie B5 isolates is striking, particularly when the 1,563 isolates in 1965 are compared with only 318 reported in 1964. In 1965 it was possible to note the movement of Coxsackie B5 in Europe: the United Kingdom reached its peak of isolations during the third quarter and Finland only in the fourth quarter.

TABLE 6. COXSACKIEVIRUS A7 ISOLATIONS *

Year	Paralytic cases	Nonparalytic cases	Total
1956	1	0	1
1959	7 (1 fatal)	30	37
1961	0	1	1
1963	2	13	15
Total	10	44	54

*Source: Grist, N. R. "Coxsackie A7 Virus and Paralytic Poliomyelitis." *Anglo German Med Rev* 3: 108–111, 1965.

During 1965 all the group B Coxsackieviruses were commonly isolated, as were A4, A9, and A16. Among the echoviruses, Types 4, 6, 7, 9, and 14 were particularly prominent. Polioviruses continued to be isolated from patients with CNS disease, indicating that the vaccine had not been adequately or properly used.

The proportion of the different virus groups causing CNS disease is shown in Table 8. The most frequent of the Coxsackieviruses were B5, A9, B2, B3, and of the echoviruses, Types 6, 4, 9, and 14. The relative role of Coxsackie and

TABLE 7. 1965 DATA FROM WHO VIRUS DISEASES UNIT *
(BASED ON INFORMATION FROM 120 LABORATORIES)

Virus	No. of isolates	Virus	No. of isolates	Virus	No. of isolates
Coxsackie A4	44	Echo 1	73	Polio 1	551
A9	149	4	154	2	182
A16	57	6	536	3	248
B1	104	7	123		
B2	131	9	210	Reo	97
B3	214	11	86		
B4	146	12	89		
B5	1,563	14	124		
B6	60	27	35		
Others	249	Others	255		
Total	2,717	Total	1,685		

*Source: Data supplied to WHO Virus Diseases Unit, Geneva.

echoviruses in causing virus infections of the CNS is shown by age groups in Figure 2. That enteroviruses may play a role in respiratory illness, although admittedly a lesser one, was shown by a similar analysis of virus infections in which Coxsackieviruses were isolated in appreciable numbers from young children with respiratory illness.

Prospects for control by vaccination. In the above discussion I have emphasized enteroviral meningitis, for such outbreaks have been most regularly documented. However, members of the group also caused other illnesses, as may be seen from the lists in the first two tables. It would certainly be desirable to eliminate enteroviral myocarditis of the newborn and also pleurodynia, if this could be accomplished.

If we had a safe, potent nonpolio enterovirus vaccine, how could it be tested for efficacy in the field? This question is not easy to answer.

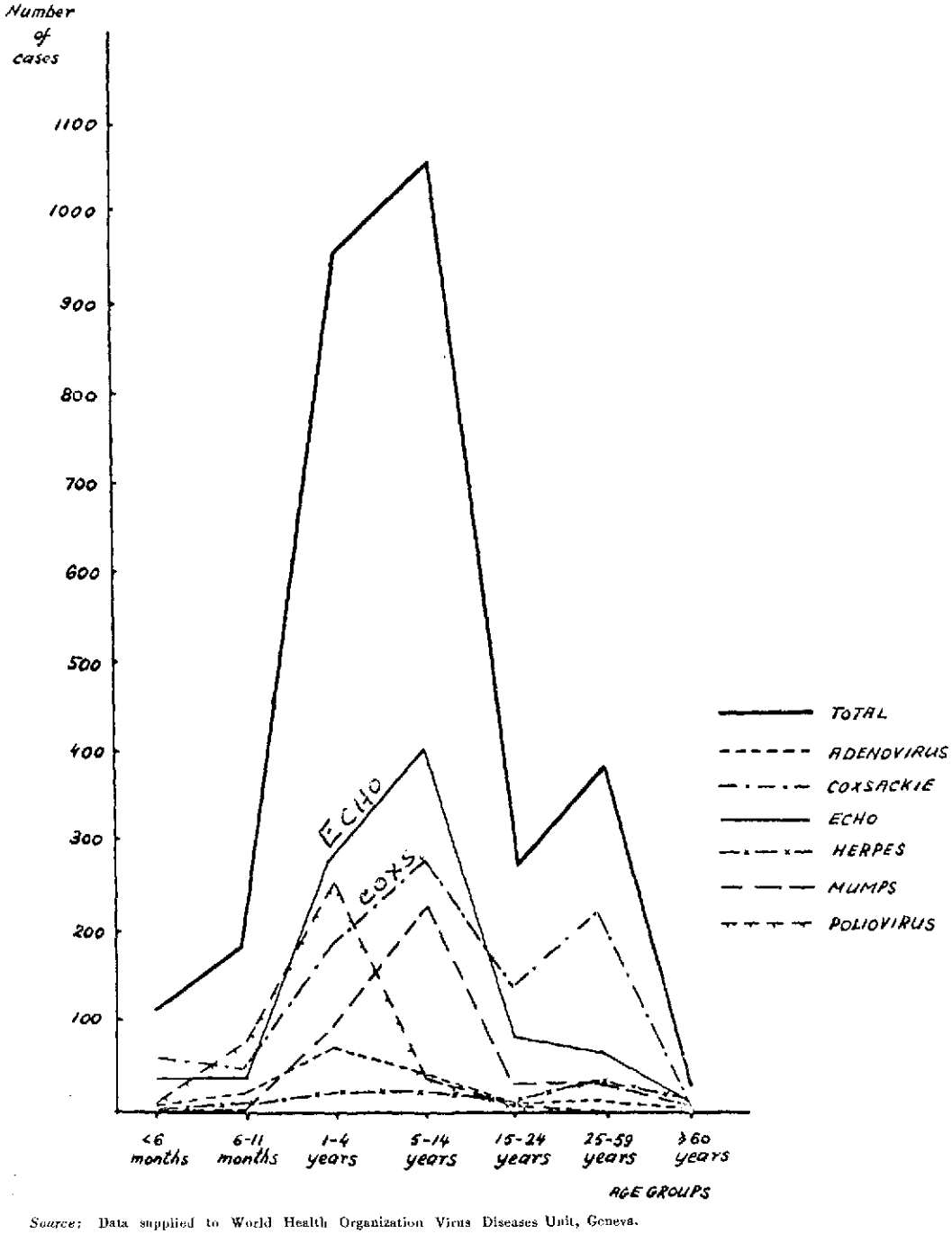
In regions like the United States it would be necessary to vaccinate high proportions of susceptibles in large areas and to compare enterovirus disease with equally large control populations. Thus, for example, we might vaccinate all children under 15 years of age in Connecticut and Rhode Island and compare them with the same age groups in the neighboring states of Massachusetts and New York. To make a statistically valid study, the vaccine would have to be administered a sufficient number of times to ensure maintenance of seroimmunity of all susceptibles, including those born into the study, over a period of at least 10 years. Are the diseases currently produced by the enteroviruses worth the great cost and effort that would be involved? I doubt it.

Could one obtain an answer in another way? In the course of our studies on the etiology of infant diarrhea, we deliberately selected Karachi, an area of high incidence, for a field study (9). As in our continuing studies in Houston, we could find no significant difference in virus isolation rates between the diarrheal patients and the control group. In Karachi 78 per cent of 842 children studied were excreting an enteric virus. When we examined the isolates for dif-

TABLE 8. PERCENTAGE DISTRIBUTION OF VIRUSES ISOLATED FROM PATIENTS WITH ACUTE CNS DISEASE *

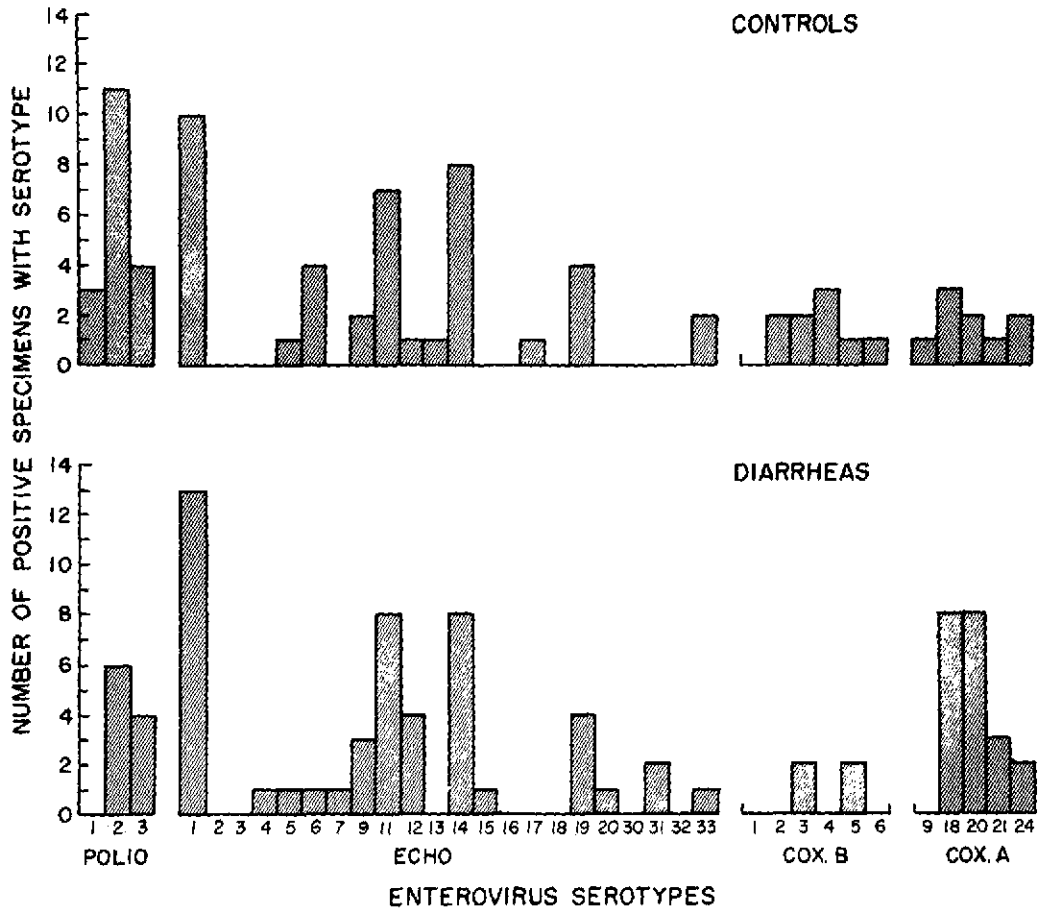
Viruses	1963	1964	1965
Coxsackie†	29	26	32
Echo‡	22	34	29
Polio	26	11	13
Mumps	17	19	12
Adeno	2	4	5
Herpes	2	3	3
Other	2	3	6

*Source: Data supplied to WHO Virus Diseases Unit, Geneva.
†Most frequent types of Coxsackie viruses, in descending order: B5, A9, B2, and B3.
‡Most frequent types of echoviruses, in descending order: 6, 4, 9, 14.



Source: Data supplied to World Health Organization Virus Diseases Unit, Geneva.

Fig. 2. Virus infections of the CNS, by age groups, 1965.

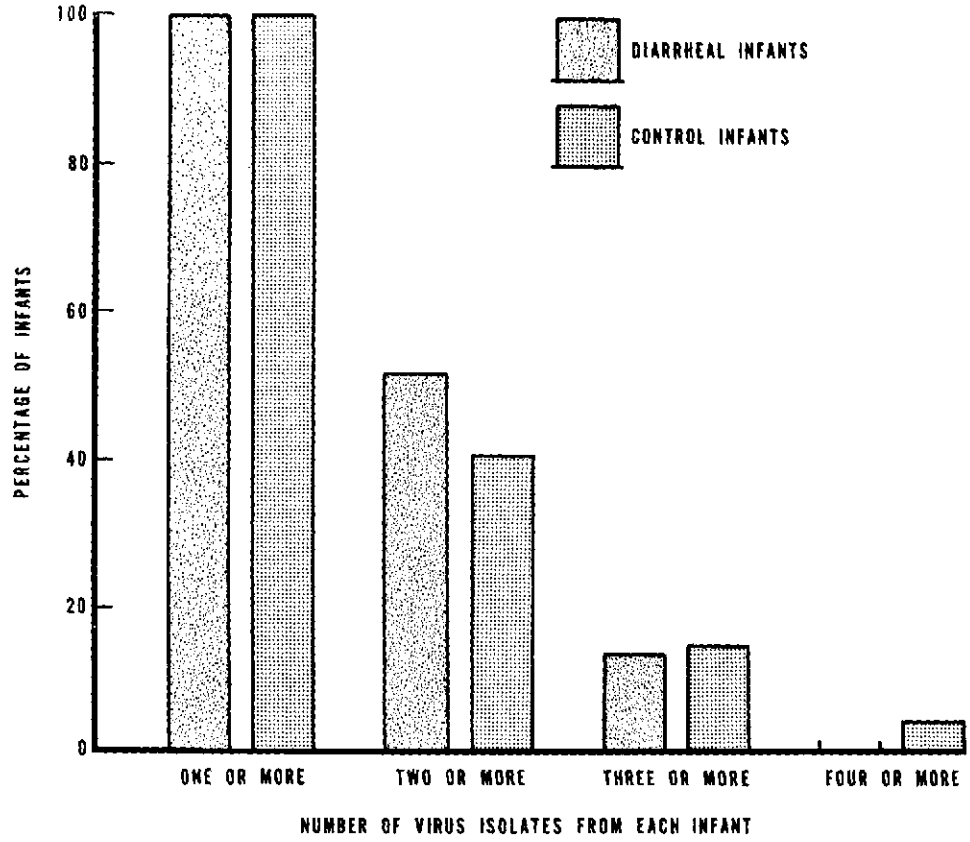


Source: Parks, W. P., Melnick, J. L., Queiroga, L. T., and Ali Khan, H. "Studies of Infantile Diarrhea in Karachi, Pakistan. I. Collection, Virus Isolation and Typing of Viruses." *Amer J. Epidem* 84:383-395, 1965.

Fig. 3. Comparison of the number of enterovirus serotypes identified in diarrheal infants and matched controls in Karachi, Pakistan, April-July 1964.

ferent enteroviral serotypes (Fig. 3), there was a slightly increased recovery of Coxsackievirus A strains, and also of adenoviruses, in the diarrheal children. Even more striking was the observation that many of these children were excreting two, three, or even four viruses at the same time (Fig. 4). There was no difference in multiple virus excretion between patients and controls (10).

The reason for showing these data at this time is that they indicate that some populations are different from those in New England and are heavily infected with enteroviruses. The use of an enteroviral vaccine in such an area under controlled conditions might offer a rapid solution to the question of the effectiveness of the vaccine. For example, if a vaccine containing the group B Coxsackieviruses were given to 1,000 children aged 3 to 12 months, the effect on the health of these children, as well as on their viral flora, could be followed to determine whether the effect of the vaccine became visible. However, among such a poor population, the frequency of bacterial infections and the deplorable state of nutrition probably have a far greater effect on their state of health than infection with enteroviruses. Nevertheless, studies could be readily set up and an answer quickly obtained on whether or not the prevalent viral flora can be influenced by vaccination—or perhaps by chemotherapeutic agents when they become available.



Note: Rectal swabs from diarrheal and control infants who yielded single viruses were subsequently tested to determine whether the infants might have been harboring additional viruses. This was done by carrying out subsequent isolation attempts in the presence of type-specific antiserum against the first virus isolated. The percentages of this group whose swabs yielded two, three, or four viruses are shown (10).

Fig. 4. Percentages of diarrheal and control infants harboring two, three, and four viruses at the same time, Karachi, Pakistan, 1964.

Even though it appears unnecessary to vaccinate large groups against enterovirus infections, which are usually mild, it may be wise to have vaccine available for use under certain conditions, as when outbreaks of myocarditis occur among newborn infants. Also, methods for preparing potent enteroviral vaccines should be available in case more virulent strains should suddenly make their appearance.

New work on enterovirus vaccines. Because formalin, which is customarily used for inactivating viral vaccines, reacts with and denatures not only viral nucleic acid but also the antigenic protein of the virus, methods for preparing enteroviral vaccines using procedures other than formalin inactivation have been explored.

One new method developed by Wallis and myself depends on growth of the virus in cells containing small amounts of vital dye, such as neutral red. Virus grows well in such cells, but the progeny virus is now extremely photosensitive and can be completely inactivated by brief exposure to visible light. Such inactivated virus has proved to be virtually as antigenic as live virus in stimulating the production of neutralizing antibodies.

The application of photosensitizers to vaccine preparation has not been seriously considered

previously because of the undesirable presence of heterotricyclic dyes in the final vaccine. With advances in methodology (11), totally photosensitive virus harvests can be prepared in the absence of free dye. Brief exposure of such virions to white light results in the direct inactivation of their internal nucleic acid cores. The surface antigens should not be deleteriously affected, and the method should yield killed-virus vaccine of high potency.

The last part of this report is concerned with the antigenic potency of photosensitized vaccines as compared to formalin-killed vaccines.

Echovirus Type 7 was used as a model for determining optimal conditions required for producing photosensitive virus. Echoviruses are normally photoresistant, but photosensitive stock was readily prepared by three passages through dye-treated cultures. The third-passage virus yielded a harvest that contained $10^{7.5}$ PFU/ml, but when an undiluted sample of the harvest was exposed to light for 15 minutes no detectable infectivity remained. The optimal conditions of passage were determined to be the following: pretreatment of cells with 1:40,000 neutral red for 2 hours, removal of free dye, addition of 1 PFU of virus per cell, and harvest 24 hours later.

Since all free dye is removed before the addition of virus seed, it was necessary to determine how long the pretreated cells, once infected, would continue to yield totally photosensitive virus. As shown in Table 9, it was found that treated cells could be infected up to three days after dye treatment and still yield totally photosensitive virus.

TABLE 9. PRODUCTION OF PHOTOSENSITIVE ECHOVIRUS 7 FROM CELLS INOCULATED WITH VIRUS AT VARIOUS INTERVALS AFTER TWO-HOUR PRETREATMENT WITH NEUTRAL RED *

Time interval between dye treatment of cells and virus inoculation†	Log PFU/ml Dark	Log PFU/ml Light
None (Inoculated immediately)	7.5	0.0
24 hrs.	7.3	0.0
48 hrs.	7.7	0.0
72 hrs.	7.2	0.0
96 hrs.	7.5	2.5
120 hrs.	7.1	4.1

*Source: Wallis and Melnick, unpublished data.
†Virus harvested 24 hours after inoculation.

Photosensitive virus was prepared for three representative enteroviruses: echovirus 7, Coxsackievirus A9, and Coxsackievirus B3. They were exposed to white light for 15 minutes and tested in rabbits for the capacity to induce neutralizing antibodies. For purposes of comparison, formalinized vaccines were prepared from the same strains but from photoresistant virus grown in dye-free cultures. The results of the vaccine infectivity tests and of the neutralization tests of the sera from the inoculated rabbits are shown for echovirus 7 in Table 10. By the 30th day, the rabbits immunized with live virus responded with a mean antibody titer of 1,070; those injected with formalinized vaccines yielded a mean titer of 90, and those receiving the photoinactivated vaccines developed a mean titer of 640. Thus the photoinactivated vaccine gave a sevenfold greater antibody response than the formalinized preparation. Similar findings were obtained with photosensitized Coxsackievirus B3 and A9 vaccines, and our preliminary data indicate that the method can be applied to other virus groups.

As I come to the end of this discussion, it is obvious that I have not mentioned the possible development of attenuated virus strains as potential vaccines. At first glance this procedure might seem easy, since many of the enteroviruses are naturally of low virulence for man; however, with no knowledge of how laboratory markers correlate with human virulence, field testing of such live viruses as potential vaccines would be an almost impossible undertaking at this time.

In concluding, we may say that the methodology of preparing enterovirus vaccines has progressed to the point where potent killed virus vaccines for any known type can be made. More difficult to answer are the questions of how to test their efficacy in the field and whether vaccines are warranted for virus illnesses that are usually of a relatively benign nature. Even with the Coxsackieviruses in group B, and Coxsackie A7, which can cause serious and even fatal illness, the incidence of serious illness is so low that widespread vaccination cannot even be considered.

It must be emphasized that with poliomyelitis rapidly being brought under control other enterovirus infections have not become more virulent in nature. Nevertheless, public health virologists must remain alert to the possibility of a mutation

TABLE 10. ECHOVIRUS TYPE 7 VACCINES *

Virus treatment	Log PFU/ml											
	Live virus vaccine			Formalinized vaccine				Photoinactivated vaccine				
Untreated (dark)	7.6 (V) [b]			—				7.4				
Exposed to light 15 min	7.5			—				0.0 (V)†				
5 days at 37° C	4.2			0.0 (V)†				—				
Bleedings (days)	**Serum titers**											
	Rabbit No.			Rabbit No.				Rabbit No.				
	1	2	3	5	6	7	8	9	10	11	12	
0	0	0	0	0	0	0	0	0	0	0	0	
10th	10	640	160	0	0	0	0	160	40	10	40	
20th	160	1,280	320	0	80	0	0	320	320	160	320	
30th	320	2,560	320	40	160	80	80	1,280	640	320	320	
Mean titers (30th day)	1,070			90				640				

*Source: Wallis and Melnick, unpublished data.
†(V) indicates "vaccine" used to inoculate rabbits listed in corresponding columns of lower part of table.

toward increased virulence in one or more of the common enteroviruses. If this should occur, then the most advanced methodology available for vaccine production should be quickly brought into use.

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SECTION B. COXSACKIE AND ECHOVIRUSES

DISCUSSION

CHAIRMAN SEAL: The first of our scheduled discussants is Dr. Marina K. Voroshilova, of the Institute of Poliomyelitis and Viral Encephalitides, USSR Academy of Medical Sciences, Moscow.

DR. VOROSHILOVA: The existence of numerous immunologic types of enteroviruses capable of producing epidemic outbreaks of aseptic meningitis, Bornholm disease (epidemic myalgia or pleurodynia), and other enterovirus infections makes the prophylaxis of these infections by means of immunization with specific inactivated or live vaccines practically impossible. Nor can the problem be solved by prophylaxis with gamma globulin, since many lots do not contain sufficiently high antibody titers to control the most important enteroviruses.

Considerable advantages appear to be found in our suggestion to introduce prophylaxis based on the interfering effect of some enteroviruses not pathogenic for man on the causative agents of the diseases in question.

For this purpose, in our studies at the Institute of Poliomyelitis and Viral Encephalitides we selected highly active strains of echoviruses 1, 7, 8, 12, and 20, which had been isolated from healthy children in mass surveys of children's institutions. Nearly all the children who were infected with these viruses remained healthy throughout the observation period. The initial isolation of the viruses had been performed in rhesus monkey kidney cell cultures, and all the strains were free of SV_{40}. Two to five liters of the stock virus were prepared. After careful control tests in tissue cultures and in animals, including monkeys, echovirus 1 strain was used for oral vaccination of seronegative children in five special boarding schools. Initially four children were vaccinated, and then in the course of several trials 51 more children between the ages of two and three were added to the group. All the children examined, including those who before immunization had been carriers of wild enteroviruses, showed implantation of echovirus 1 and developed antibody to it with no clinical symptoms.

To bring epidemic outbreaks of enterovirus infections to a rapid halt, we consider it advisable to use oral vaccines of the most active strains of several immunologic types capable of stopping the circulation of wild enteroviruses.

In addition, we believe it expedient to develop an oral vaccine against Coxsackie A7 virus since it produces poliomyelitis-like diseases, including paralytic and even fatal types.

In passages at low temperature, variants were obtained that caused no disease in monkeys when inoculated by the intracerebral and intraspinal routes. Studies of these variants are now in progress.

TABLE 1. CLINICAL FORMS OF 108 CASES CONNECTED WITH VIRUS TYPE AB-IV—COXSACKIE A-7

Clinical forms		No. of cases
Spinal		
1. With remaining symptoms	27	36
2. Without remaining symptoms	9	
Bulbo-spinal		
1. Fatal cases	2	3
2. With remaining symptoms	1	
Pontine		6
Meningo-encephalitis		3
Nonparalytic poliomyelitis or aseptic meningitis (mild cases of meningo-encephalitis)		59
Fever disease		1
Total		108

CHAIRMAN SEAL: Our next discussant is Dr. Norman R. Grist, Professor of Infectious Diseases, University of Clasgow, Scotland.

DR. GRIST: I share Dr. Melnick's doubt whether the public health importance of nonpolio enteroviruses is sufficient to require general prophylactic immunization. Most enterovirus infections are minor or symptomless, and the possibility of serious cumulative damage from a series of such infections remains speculative. Our experience during the past decade resembles that of others in that paralysis has been a rare and sporadic consequence of infection with echoviruses or Coxsackieviruses other than Type A7.* Outbreaks of Coxsackievirus A7 infection with paralysis have been reported from several countries but to date have been too small and infrequent to necessitate general prophylactic immunization, though this situation might change in the future.†

Perhaps the heart, more often than the nervous system, may be seriously affected by Coxsackieviruses. Of over 800 group B (mostly Type 5) infections reported in Britain last year approximately 5 per cent had cardiac disease,‡ although effective virological investigations are probably performed far less often in cardiac than in neuroparalytic illnesses. The special danger of outbreaks of systemic infection in newborn infants was mentioned by Dr. Melnick. Such outbreaks have been infrequent (I have encountered none), but specific prophylactic measures for use in these circumstances would be most valuable.

Since the greatest risks from these viruses come from periodic outbreaks or epidemics of one or other of their numerous serotypes, I feel that specific prophylaxis could very practicably be directed against threatening outbreaks. This would require a rapid and flexible action, conceivably along such lines as the following:

1. Interference or ecologic displacement by administration of living avirulent enterovirus (not necessarily of the same antigenic type).
2. Rapid immunization by noninfectious vaccine prepared from the appropriate virus by some preorganized method such as that described by Dr. Melnick.
3. Chemotherapy—not yet available, but ideal for such situations as neonatal outbreaks in maternity units.

CHAIRMAN SEAL: Our next discussant is Dr. Dorothy M. Horstmann, Professor of Epidemiology and Pediatrics, Yale University School of Medicine, New Haven, Connecticut.

DR. HORSTMANN: As Dr. Melnick has indicated, attempts to control the extensive spectrum of disease due to Coxsackie and echoviruses do not seem to be either practical or desirable. Most of the infections are unapparent, and those that are presented as aseptic meningitis, pleurodynia, or a variety of other syndromes are mild and self-limited. It is true that paralytic disease resembling poliomyelitis has been caused by certain Coxsackie A and B viruses and a few echoviruses, but, excluding neonatal encephalomyocarditis (which is a generalized disease), not many more than 12 or 15 fatal cases of CNS disease due to these agents have been reported in the world literature. The behavior of the enteroviruses is unpredictable, however, and it would seem advisable to have at least a prototype vaccine available in case one of the now known—or unknown—Coxsackie or echoviruses should suddenly appear in epidemic form, inducing severe disease in man. Such pattern of behavior is not an unfamiliar event; this is essentially the case of the polioviruses beginning in the latter part of the nineteenth century.

In the past few years, with the advent of effective vaccination against poliomyelitis, there has been some speculation whether other enteroviruses would become more virulent as a result and "take over" the role of the departing polioviruses as causes of severe CNS and other disease. After more than five years' experience, the answer seems to be a clear-cut no. The presence or absence of wild polioviruses has had little impact on the behavior of other members of the

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* Grist, N.R., and Bell, B. J. "Paralysis Due to Enteroviruses Other than Polioviruses." In *Proceedings of the IX Symposium of the European Association against Poliomyelitis and Allied Diseases* (Brussels), pp. 366–271, 1963.

Grist, N. R., and Bell, B. J. "Enteroviruses Isolated in Western Scotland, 1960–1964." *Health Bull* (Edinburgh), 1967 (in press).

† Grist, N. R. "Coxsackie A7 Virus and Paralytic Poliomyelitis." *Anglo German Med Rev* 3:108–111. 1965.

‡ Grist, N. R. "Viral Cardiomyopathy." In *Symposium—Disorders of the Heart and Circulation.* Publication No. 31 of the Royal College of Physicians of Edinburgh, pp. 117–128, 1966.

large enterovirus family. This may be associated with the fact that in communities where oral poliovirus vaccines are used on a continuing basis there is a certain amount of circulation of those attenuated poliovirus strains which have, to a certain extent, replaced the wild viruses.

We have conducted long-term follow-up studies of an urban population of some 30,000 in which community-wide vaccination of 86 per cent of the children in 1961 has been followed by regular immunization of newborn babies in the first few months of life. Weekly examination of sewage in this community, from a sewer line serving 20,000 persons, has revealed the regular presence of polioviruses throughout the year. These have had, for the most part, the same *in vitro* markers as the vaccine strains. Of the 49 sewage samples tested in 1965, 48 contained at least one poliovirus type and many had all three types. In addition, as many as half the specimens collected during the summer months contained other enteroviruses—Coxsackie and echoviruses. Even so, at no time was there evidence of unusual disease incidence in the population.

Further direct evidence that the vaccine strains were circulating among susceptible children in this community was revealed by the isolation of polioviruses from prevaccination rectal swabs of 8 per cent of the children attending the Well Child Conference in 1965. Coxsackie and echoviruses were also recovered from some of the children during the summer months (July-October).

Although there has been no evidence of increased virulence of Coxsackie and echoviruses, it is still possible that any one of these viruses might undergo a change and present a serious disease threat. We have recently experienced an outbreak of aseptic meningitis and mild febrile illness in New Haven due to an agent that had rarely been recovered before and had not been known to be associated previously with an epidemic of infection or disease. There was considerable difficulty in identifying the virus, but, with the help of Dr. Lennette's laboratory, it was eventually found to be an echo 11 [1]. Fortunately, this agent proved to be only mildly pathogenic for man. Had it been highly virulent, however, a considerable time would have been required before its nature was known, and an effective vaccine could have been prepared. If ever a highly pathogenic agent should appear on the scene, there will be a need for means of immunization. The more experience we have had with various techniques for vaccine development, such as the one described by Dr. Melnick, the better will be our position in terms of the rapid production of an effective immunizing agent.

CHAIRMAN SEAL: Our last discussant this morning is Dr. Manuel Ramos Alvarez, Head of the Virus Laboratory, Children's Hospital, Mexico City, who was also a discussant in Section A.

DR. RAMOS ALVAREZ: Although certain types of Coxsackie and echoviruses have been isolated from the central nervous system in fatal cases of paralysis, their role in the vast majority of the nonpolio paralytic diseases is still undefined. I shall attempt here to summarize briefly some of the virological studies carried out on many of the patients admitted to the Children's Hospital of Mexico City during the last few years with paralysis as the main clinical manifestation.

Viral and serological tests in properly collected specimens showed a high incidence of nonpolio enterovirus infection among children with paralytic disease. The highest incidence of isolation of these nonpolioviruses (Coxsackie and echoviruses) was found among children between two and five years of age (Table 1). The patients were arbitrarily divided into two groups

TABLE 1. ISOLATION OF POLIOMYELITIS AND OTHER ENTEROVIRUSES FROM RECTAL SWABS OF CHILDREN WITH FLACCID PARALYSIS AS THE MAIN CLINICAL MANIFESTATION *

Age group (years)	Number of cases tested	Percentage of children with virus indicated		Total percentage of isolation
		Polioviruses	Nonpolioviruses	
<1	51	92	8	100
1	63	78	17	95
2	33	42	42	84
3–5	44	14	59	73
6–12	27	7	33	40
All	218	54	29	83

*Specimens for test obtained one to 10 days after onset of paralysis and within the first 24 hours after admission to the hospital.

TABLE 2. INCIDENCE OF POLIOVIRUSES AND NONPOLIOVIRUSES (NP) AMONG CHILDREN WITH FEBRILE AND AFEBRILE PARALYTIC DISEASES

Age group (years)	Febrile disease				Afebrile disease			
	No. tested	Percentage of children with virus indicated			No. tested	Percentage of children with virus indicated		
		Polio-virus	NP	Total isolation		Polio-virus	NP	Total isolation
<1	51	92	8	100				
1	54	91	7	98	9	0	78	78
2	18	78	17	95	15	0	73	73
3–5	14	43	43	86	30	0	60	60
6–12	7	28	14	42	20	0	40	40
All	144	82	12	94	74	0	62	62

according to presence or absence of fever at the time paralysis appeared (Table 2). Among children with febrile paralysis, the over-all viral isolation recovery rate was 84 per cent for polioviruses and only 12 per cent for nonpolioviruses; in children with afebrile paralysis, polioviruses were not recovered at all, whereas nonpolioviruses (Coxsackie and echoviruses) were isolated in 62 per cent of the cases. Table 3 shows the frequency of polio and nonpolioviruses among children with paresis only.

Serological tests for all three types of polioviruses in acute and convalescent sera of patients from whom a Coxsackie or an echovirus was recovered ruled out simultaneous infection with these viruses. Similar tests with the nonpolioviruses recovered from rectal swabs of these children showed a significant antibody rise during convalescence in most of the patients tested, thus indicating that infection with these agents and clinical symptoms were concurrent. Clinical follow-up on some of the patients with nonpolio paralytic diseases showed in most instances a complete clinical recovery three months to a year after discharge from the hospital, although in some cases there were severe or slight sequelae two years later.

Routine spinal fluid examinations in the nonpolio paralytic cases showed abnormal findings in 50 per cent of the patients tested (Table 4). The mortality among the nonpolio paralytic cases was 25 per cent. The mortality for paralytic poliomyelitis was 12 per cent (Table 5). In seven out of eleven fatal nonpolio paralytic cases viral isolation carried out before death (2 to 3 days after onset of paralysis in four cases, and 7 days after onset of paralysis in three cases) were positive for nonpolioviruses. The time elapsed between onset of paralysis and death was 6 to 7 days in six cases, 9 days in two

TABLE 3. ISOLATION OF POLIOMYELITIS AND OTHER ENTERIC VIRUSES (NP) FROM RECTAL SWABS OF CHILDREN WITH PARESIS

Age group (years)	No. tested	No. of children with viruses indicated		Total isolation
		Poliovirus	NP	
1–4	6	2	4	6
5–12	11	0	5	5
All	17	2	9	11

TABLE 4. SPINAL FLUID ABNORMALITIES WITHIN SEVEN DAYS AFTER ONSET OF PARALYSIS OR PARESIS IN CHILDREN WITH NONPOLIOVIRUS INFECTION

Group	Number	Percentage showing changes indicated		
		None	Proteins increased cells within normal limits	Cells increased
With paralysis	38	50	29	21
With paresis	15	67	7	26

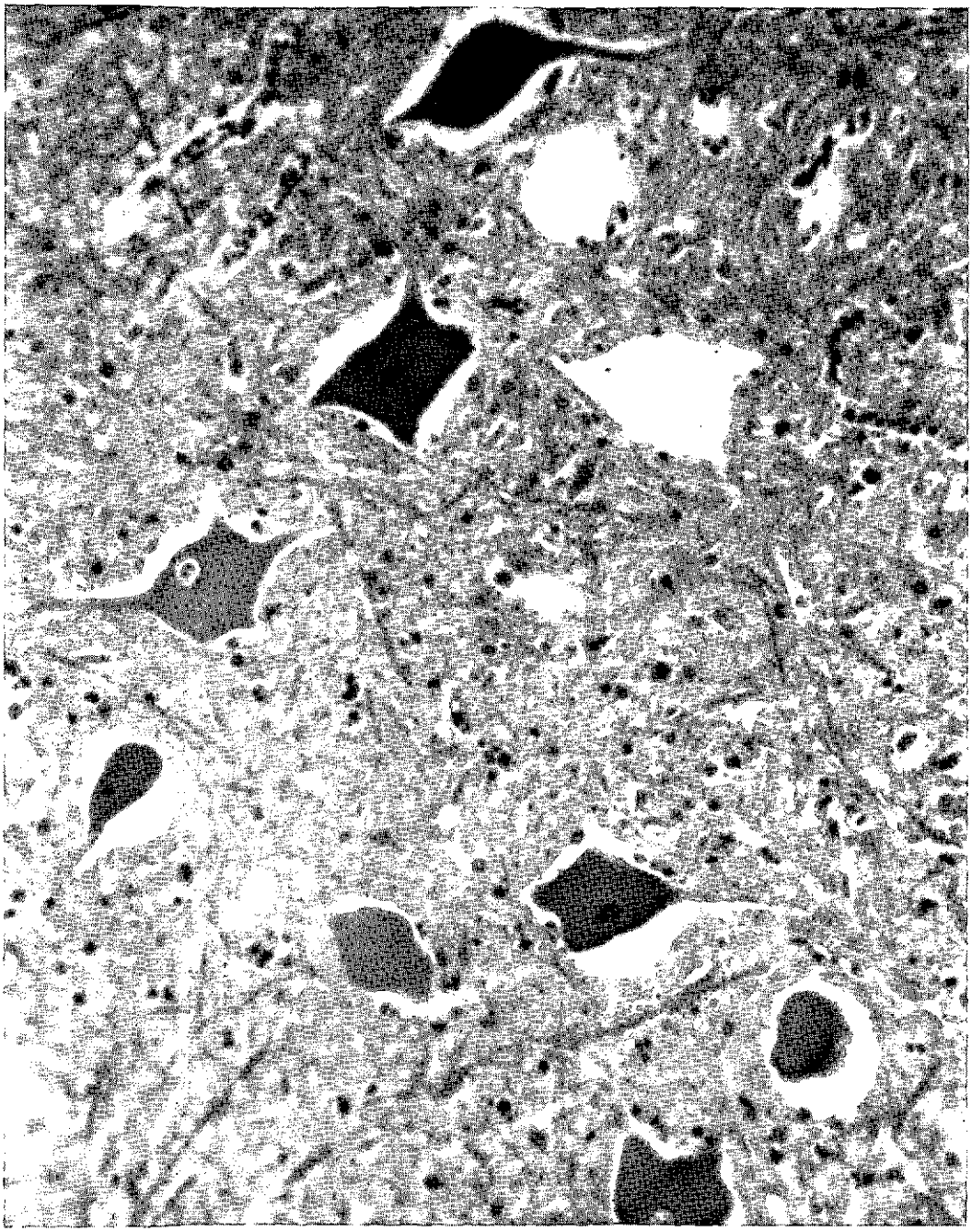

Fig. 1. Neuronal degeneration in the anterior horn of the lumbar cord, H&E, 10X.

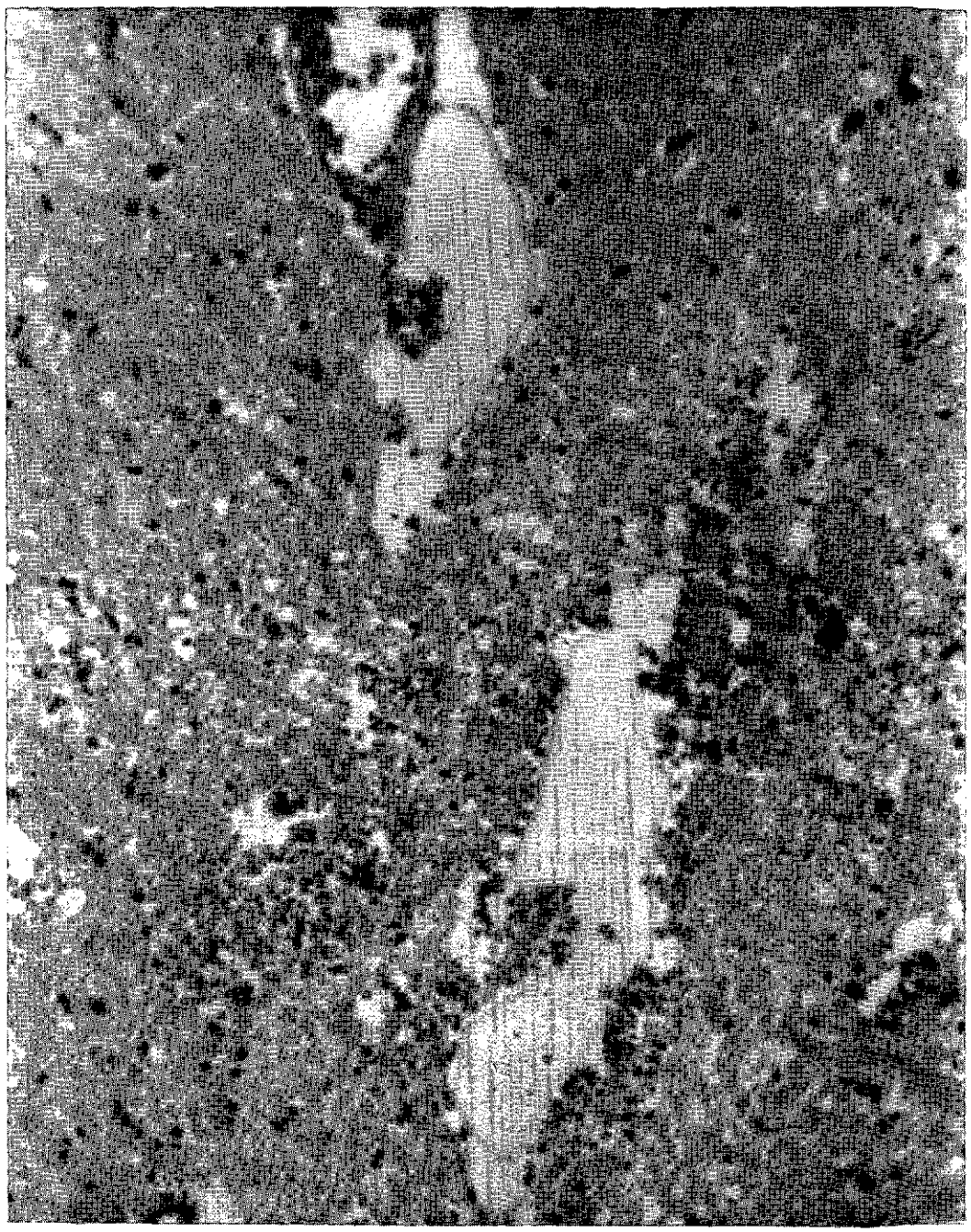

Fig. 2. Hemorrhagic lesions observed in the medullae, H&E, 10X.

TABLE 5. COMPARATIVE MORTALITY OF PARALYTIC DISEASES AMONG CHILDREN WITH POLIOVIRUS AND CHILDREN WITH NONPOLIOVIRUS (NP) INFECTION

Group	No. of cases	Mortality	
		No.	Percentage
Poliovirus	120	15	12
NP*	71	18	25

*Included eight patients with paresis.

cases, and 13 days in three cases. Repeated attempts to isolate virus from the central nervous system in seven patients were unsuccessful, although it should be pointed out that these studies were conducted 8 to 15 hours after death.

The main histological findings were moderate to severe neuronal degeneration with multiple areas of hemorrhage and marked perineuronal edema at different levels of the central nervous system (Figs. 1 and 2). In three cases, one of them with severe neuronal changes, there were significant lesions in the roots, similar to those described in acute infectious polyneuritis. In addition to the neuronal changes, in three cases there was glial mobilization and in two cases there were some areas with perivascular cuffing. Two cases also showed focal areas of meningeal infiltration. Nonspecific myocarditis was observed in two cases and hepatitis in three.

Although the significance of the nonpolio Coxsackie and echoviruses encountered in these

TABLE 6. NONPOLIOVIRUSES ISOLATED FROM RECTAL SWABS OF CHILDREN WITH PARALYSIS AND CHILDREN WITH PARESIS

Group	Echo		Coxsackie		Unclassified
	Type	No. of strains	Type	No. of strains	
Paralysis	4	2	A9	1	35
	6	1	A18	2	
	21	1	A20	1	
	Total	4	B2	2	
			B5	2	
			Untyped	18	
			Total	25	
Paresis	20	1	A9	1	6

paralytic diseases is difficult to evaluate, especially since no one virus in particular predominates (Table 6), certain facts should be emphasized. Nonpolio paralytic diseases have represented an important proportion of the total number of cases of paralysis admitted to the Children's Hospital over the past few years. There has been a high incidence of virus isolations (Coxsackie and echoviruses) from the stool of these patients. Serological tests in properly collected serum specimens have indicated that these nonpoliovirus infections and the clinical symptoms were concurrent, which suggests that an etiological association could exist at least in some patients.

SESSION IV

ARBOVIRUSES AND HERPESVIRUS GROUP VIRUSES

Tuesday, 8 November 1966, at 2:00 p.m.

CHAIRMAN
DR. A. S. PARODI

RAPPORTEUR
DR. ANTHONY M.-M. PAYNE

Section A

Arboviruses

Presentation of Papers by:
Dr. J. Casals
Dr. W. McD. Hammon
Dr. Ronald B. MacKenzie

Section B

Herpesvirus Group Viruses

Presentation of Papers by:
Dr. Robert N. Hull
Dr. Thomas H. Weller

Discussants (Sections A. and B.):
Col. Edward L. Buescher
Dr. Karl M. Johnson
Dr. Charles L. Wisseman, Jr.
Dr. Donald R. E. MacLeod
Col. William D. Tigertt
Dr. T. F. McNair Scott

SECTION A. ARBOVIRUSES

CLASSIFICATION OF ARBOVIRUSES AND DELINEATION OF CLINICALLY IMPORTANT TYPES

JORDI CASALS

Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut
and The Rockefeller Foundation, New York, U. S. A.

DR. CASALS (*presenting the paper*): On examining the program of this Conference it can be seen that none of the sets or families of viruses except the arboviruses and hepatitis is introduced by a speaker who has been given the task of outlining their classification or present knowledge of their etiology. In all other instances the respective sessions begin outright with reports on the subject matter of this Conference—on vaccination.

While, understandably, the entire session on viral hepatitis is dedicated to a review of the present knowledge of its etiology, there seems to be less of a reason, at least on the surface, for setting aside one third of the time allotted to the arboviruses for what, in essence, consists of reporting on classification and definitions. However, that reason becomes clearer when it is realized, as it soon is on examining the extent of knowledge about the epidemiology and basic properties of the arboviruses, that there are large lacunae in this knowledge. It may then be assumed that it is the function of this report to point out these gaps in our knowledge; and also to point out that, in fact, there are some misunderstandings as to what constitutes an arbovirus, deriving on the one hand from a misconception of the definition and on the other from lack of the proof required by the definition.

In this paper, therefore, an attempt will be made first to examine the properties or conditions needed for including a virus among the arboviruses—or, to put it in other words, to define the arboviruses. Secondly, once an agent is included in this set, certain criteria are followed for its subsequent classification; these criteria will be described next.

Recently there have been two conferences dealing, in part, precisely with questions of classification of arboviruses. One was during the Ninth International Congress for Microbiology (Moscow, July 1966) and the second was a meeting of a Scientific Group on Arboviruses and Human Disease called by WHO (Geneva, September 1966). To the persons in the audience who participated in one or both of these meetings, much that will be said here will be repetitive. Furthermore, some of the basic concepts expressed here will find expression also in the report of the Scientific Group just mentioned (in preparation); except in questions of detail, these concepts can be said to represent the compromise view of several persons who gave the matter a great deal of thought.

DEFINITION

The term arbovirus is an abbreviation for "arthropod-borne animal virus," by which is meant a virus that in nature is transmitted between vertebrates by hematophagous arthropods; the virus multiplies in the tissues of both arthropods and vertebrates. In the former, the virus does not produce observable illness, or lesions; in the vertebrate it causes lesions, illness and a period of viremia during which an arthropod feeding on the vertebrate acquires the virus. This multiplies in the body of the arthro-

pod, which can transmit it by bite to another susceptible vertebrate only after a period of time, called the extrinsic incubation, has elapsed. It is this biological transmission requiring an extrinsic incubation in the arthropod, the close epidemiological association between arthropod vector and vertebrate host and the existence of a reservoir—either the arthropod itself or a vertebrate in which the virus causes hardly any disease manifestations other than viremia—that defines an arthropod-borne animal virus.

Although biological transmission is basic in the definition, it must not be concluded that an arbovirus cannot be transmitted in nature by other means, such as droplet infection, contamination of food, or insects acting as flying needles; there are examples of transmission of disease to man under such circumstances. It is, however, known or assumed that for all arboviruses there is a natural basic epidemiological cycle of the type described.

CRITERIA FOR INCLUSION AMONG THE ARBOVIRUSES

In a strict sense, a virus should be considered arthropod-borne only when the basic definition is fulfilled, either by observation of the undisturbed natural cycle or by its experimental reproduction under conditions closely simulating events in nature. This criterion is difficult to meet and impractical to apply; it can be asserted that it has only been satisfied in the case of three or four arboviruses. Since there are at present well over 200 arboviruses, it is easy to realize how far we are from establishing the definition generally.

This complete but elusive criterion not having been met, others have been accepted, with various degrees of authority, for the inclusion of a virus in the set; it is generally agreed, however, that while provisional inclusion may be based on less than convincing evidence, final acceptance of a virus as arthropod-borne must be based on proof that it multiplies in an arthropod and that the latter can transmit it by bite after a long enough period of time to rule out mechanical transmission.

The criteria for the inclusion of a virus among the arboviruses are of two types: properties related to the natural transmission cycle, and properties not so related.

Properties Related to the Natural Transmission Cycle

There are gradations in the completeness with which the natural cycle of the different arboviruses are known and, consequently, in the weight given this knowledge for the classification of a virus.

1. *Observation in nature and complete reproduction of a continuous natural cycle.* Viruses for which this knowledge is available—yellow fever, dengue, sandfly fever—constitute classic examples of epidemiological investigation accomplished even before the agents involved were identified as viruses; because in the particular ecological circumstances that prompted the reported studies, disease in man was an essential feature, these examples stand out sharply. It must be realized, however, that present knowledge of the epidemiology of arboviruses shows that the involvement of man is generally unimportant in the perpetuation of these viruses in nature.

2. *Epidemiological observations and reproduction of a cycle with a minimum of experimental disturbance.* The examples mentioned above are apt to remain for a long time the only ones in that category; fulfillment of the criterion is highly impractical, even if man as the natural host is left out. But demonstration of the arthropod-borne nature of a virus can be and has been accomplished by using a criterion, or a number of them, that are considered at present to be just as decisive. The arthropod-borne nature of several agents has been established on the basis of overwhelming epidemiological and other ecological evidence showing association between virus, host, and vector; elements of this evidence are repeated isolation of the virus from a given arthropod species, disappearance of the active virus from an area with abatement of the arthropod or with seasonal reduction in numbers of arthropods, and close association between arthropods and natural reservoirs or amplifier hosts. In addition, there has been, with these viruses, experimental reproduction of a transmission cycle—it need not be the one or the only one operating in nature—under conditions in which the artificiality is reduced to a minimum.

The association of *Culex tarsalis* with Western equine and St. Louis encephalitis in the western

part of the United States, of *Culex tritaeniorhynchus* with Japanese encephalitis, of *Ixodes persulcatus* with Russian tick-borne, Far Eastern encephalitis, and of *Dermacentor andersoni* with Colorado tick fever are examples of this type of epidemiological observations. In addition, with these and a few other viruses the observation and experimental reproduction of a cycle has been completed, with the single exception that the cycle may be discontinuous—the same individual vertebrate or arthropod may not have been used in the reproduction of the cycle—or the first infected link in the chain may have been infected with syringe and needle, rather than in nature (10, 20, 22).

3. *Reproduction, complete or partial, of a transmission cycle under experimental and artificial conditions.* The usual procedure consists in infecting a host by inoculation of a viral suspension and feeding an arthropod on the host in subsequent days; after a sufficient number of days have elapsed to allow for virus multiplication in the arthropod and to rule out mechanical transmission, the arthropod is allowed to feed on a new, susceptible host; transmission of the virus indicated by disease, viremia, or the development of antibodies is an indication of successful completion of the cycle. The artificial conditions in this system are, usually, infection of the first host in the chain by inoculation with syringe and needle, the use of an unnatural host (mouse, hamster, or chick), and the use of an arthropod species that may have no epidemiological significance in nature.

In other instances the cycle is initiated by artificially infecting the arthropod by inoculation or allowing it to feed on a mixture containing virus in the form of infected tissue suspension and normal blood; then transmission to a vertebrate host is attempted.

At times the cycle is not completed and proof of infection of either the arthropod or the vertebrate is derived from inoculation and titration of suspensions of tissues from vector or host in susceptible experimental systems.

4. *Serial propagation by experimental inoculation of hematophagous arthropods.* Serial propagation of a virus by experimental inoculation of blood-sucking arthropods with infected tissues of the arthropods, preferably salivary glands, is accepted as evidence that a virus can use arthropods in a natural transmission cycle. Viruses belonging in other families may have been isolated occasionally from hematophagous arthropods in nature; however, evidence that multiplication in series occurs is either missing or not well established. A true arbovirus may, on the other hand, fail to propagate under these conditions owing to the use of an unsatisfactory arthropod.

5. *Circumstances of isolation.* These circumstances refer to some of the ecological or epidemiological factors included in paragraph 2, the main difference being that under the present heading are included agents for which the information is fragmentary and incomplete. A virus is provisionally considered to be arthropod-borne when it has been repeatedly isolated from wild-caught mosquitoes or other arthropods; or from the blood of vertebrates (mammals, birds), particularly in the course of epidemics or epizootics that from their course and other circumstances appear to be arthropod-borne rather than due to other sources; or from the blood or tissues from sentinel animals so exposed that arthropods are the likely source of contact.

Properties Unrelated to the Natural Transmission Cycle

A number of arboviruses defined on grounds described in the preceding section have been found to have certain physical, chemical, or morphological properties in common; therefore, by means of *a posteriori* reasoning, the presence of these properties in a virus leads to entertaining the possibility that the virus may be arthropod-borne. Some of them are basic properties of the virion (see next section); others are simple observations on pathogenicity for experimental animals or data of a serological order. Since these properties are not limited to the arboviruses, the fact that a virus possesses them is no certain indication that it is arthropod-borne.

1. *Serological relationships.* The existence of a serological relationship with an established arbovirus has been considered sufficient evidence for the inclusion of a virus in this set. In some cases there is no other evidence, either because it has not been investigated or, if it has, the virus has failed to propagate in the arthropod used. Examples of the first are Dakar bat virus, Negishi, Ossa, Bujaru, Chagres, and Ber-

tioga; and of the second, Modoc, bat salivary gland virus (Rio Bravo), and Pacui. It is legitimate to inquire whether there may exist arboviruses defined only serologically that are not arthropod-borne in the strict sense.

2. *Effect of ethyl ether and sodium deoxycholate.* This effect has been investigated with the majority of arboviruses. In an analysis (4) involving about 160 to 170 arboviruses, 120 have been reported as being inactivated by these chemicals; between 35 and 40 that had not been tested or for which no report was available belong among the grouped arboviruses, and 6 similarly untested agents were in the ungrouped category. Five viruses—or antigenic groups—were reported either as not being inactivated or as being inactivated to a very low degree; this relative resistance sets them apart from the rest of the arboviruses. The viruses are African horse sickness; bluetongue of sheep; epizootic hemorrhagic disease of deer, New Jersey type; Corriparta; and Nodamura.

3. *Nucleic acid.* In the analysis mentioned, reports were available on 20 or 21 arboviruses; in all cases RNA was found. At present, however, this is not a procedure routinely applied to candidate arboviruses.

4. *Susceptibility of laboratory animals.* With rare exceptions, such as dengue Type 3 in the early passages, arboviruses can be propagated serially with great ease in newborn mice by the intracerebral route of inoculation. While the question of early selection of viral populations of different pathogenicity must be borne in mind, it is a good working guideline to assume that a virus that does not propagate easily in newborn mice is not an arbovirus.

PROPERTIES OF THE ARBOVIRUSES

A full consideration of the properties of the virion of the arboviruses is not contemplated at this time; suffice it to say that an analysis of the known data (WHO report, in preparation) clearly shows that there are few of these agents for which the type of nucleic acid, symmetry, and presence or absence of an envelope are known. From the estimated size and the observed shape of a few of these agents, it is suspected or anticipated that a large proportion of the arboviruses may fall into a class having cubic symmetry and an envelope. However, exceptions are already known; the viruses in the vesicular stomatitis group have been described as having helical symmetry; and other agents show such low susceptibility to the action of ether and sodium deoxycholate (see above) that the absence of a lipid-containing envelope can be strongly suspected.

It seems reasonable to conclude, at this time, that on the basis of the properties of the virion the arboviruses constitute a heterogeneous collection and that they will pertain to more than one taxon in a universal system of classification (25).

CLASSIFICATION

Arboviruses can be classified according to various criteria, depending on the purpose to be served. A system of classification based on antigenic relationships detected by serological tests is in general use (2); according to this system, all related viruses constitute an antigenic group. In addition to grouped viruses there are a number still ungrouped.

Table 1 lists the antigenic groups now known, some of which are still somewhat ill defined; also given are the number of viruses in each group and a few representative names. The number of serotypes in some of the groups is still an unsettled question because of the diversity of the criteria followed in establishing a serotype. In addition, certain groups are listed—African horse sickness and bluetongue of sheep—that in other contexts are considered to be each a single virus with serological subtypes.

It is hardly necessary to point out that a table of this kind is not static; changes are anticipated with time in the number of groups, either because new ones are created or old ones may emerge; and, of course, additional viruses are continually being reported.

PROBLEMS INHERENT IN THE SEROLOGICAL CLASSIFICATION OF ARBOVIRUSES

The basic observation for the antigenic classification of arboviruses is the detection of cross-reactions between agents, with the understanding that quantitative expression of this overlap is necessary. In the majority of cases there is no difficulty in interpreting the experimental results; in some instances, however, a

TABLE 1. ANTIGENIC CLASSIFICATION OF ARBOVIRUSES

Group	Reference	Viruses in group: Number*	Viruses in group: Sample of names
A	Casals and Brown, 1954 (5)	20–22	EEE, WEE, Chikungunya
B	Casals and Brown, 1954 (5)	37–39	RSSE, JBE, yellow fever, dengue
C	Casals and Whitman, 1961 (7)	12	Apeu, Caraparú, Oribóca
African horse sickness	Theiler, 1908 (24)	9	No names, letters or numbers
Anopheles A	Spence *et al.*, in prep.	2	Anopheles A, Lukuni
Anopheles B	Sousa Lopez *et al.*, in prep.	2	Anopheles B, Boracea
Bakau	Elisberg *et al.*, 1960†	2	Bakau, Ketapang
Bluetongue of sheep	Howell, 1960 (15)	12	No names, letters or numbers
Bunyamwera	Casals and Whitman, 1960 (6)	11–15	Bunyamwera, Ilesha, Wyeomyia
Bwamba	Kokernot *et al.*, 1957 (16)	2	Bwamba, Pongola
California	Whitman and Shope, 1962 (27)	5–11	California, Tahyna, Melão
Capím	Shope, 1960†	4	Capím, Bush-bush, Guajará
Changuinola	Bruckner and Bensabath, 1963†	4	Changuinola, Irituia
EHD of deer	Shope *et al.*, 1960 (23)	2	New Jersey, South Dakota
Guama	Whitman and Casals, 1961 (26)	4	Guama, Catú, Mojú
Kaisodi	Pavri *et al.*, in preparation	2	Kaisodi, TP 94
Kemerovo	Libikova *et al.*, 1965 (17)	4–6	Kemerovo, Tribec, Hughes
Koongol	Doherty *et al.*, 1963 (11)	2	Koongol, Wongal
Nyando	EAVRI, Report, 1965 (13)	2	Nyando, Eretmapodites 147
Phlebotomus fever	Causey and Shope, 1965 (8)	11	Neopolitan, Sicilian, Chagres
Piry	Shope, 1966†	2	Piry, I 653514
Quaranfil	Clifford *et al.*, in prep.	2	Quaranfil, Johnston
Simbú	Casals, 1957 (2)	7	Simbú, Oropouche, Manzanilla
Tacaribe	Mettler *et al.*, 1963 (18)	4	Tacaribe, Junín, Machupo
Timbó	Causey *et al.*, 1966 (9)	2	Timbó, Chaco
Turlock	Work, 1962†	2	Turlock, Umbre
Vesicular stomatitis	Myers and Hanson, 1962 (19)	3	New Jersey, Indiana, Cocal
Ungrouped	Numerous	50+	Rift Valley fever, Colorado Tick fever.

*From data available at the WHO International Reference Center for Arthropod-borne Viruses, Yale University, and in the literature.
†Unpublished personal communications.

decision can be extremely difficult. There are two main areas in which confusion is likely: (1) relationships between antigenic groups, and (2) the definition of a serotype.

Relationships between Antigenic Groups

By definition this situation should be ruled out; it has, however, occurred that once certain antigenic groups were established, one or more viruses of a group showed serological cross-reactions with one or more viruses of another group, the overlaps being generally at a very low titer and not with all standard tests. This type of relationship was first noted between the Bunyamwera and California groups (27); between groups C, Guama and Capím (Shope, personal communication, 1963); and between Bunyamwera, California, Bwamba, Simbú, and possibly Koongol (3). It is anticipated that, in time, accord will be reached concerning the designation of the hierarchic groupings, either by creating a new designation for these loosely connected groups, such as overgroup or supergroup, or by regarding the connected groups as one large group and the present groups included in it as subgroups. For the present, and for practical reasons, it seems best to maintain the groups as separate and acknowledge the existtence of intergroup relationships.

Definition of Serotype

It is well established that arboviruses have distinct antigenic variants; this fact may result in difficult decisions when it is a question of

either establishing a new serotype or including a new isolate within an existing serotype. No criterion for decision can be given or suggested for extreme cases except that which seems to prevail in other areas of taxonomy: that in the long run experience and general acceptance will decide. In the meantime, it is strongly to be urged that in reporting work with arboviruses a clear statement be made of the strains used. Furthermore, in conducting serological surveys it is advisable to use the particular strains prevalent in the area; and for preparation of vaccines, strains should be selected with due consideration to their antigenic coverage, other circumstances being equal.

CLINICALLY IMPORTANT TYPES OF ARBOVIRUSES

A little over one fourth of the described arboviruses are known to induce human disease; some have caused large epidemics, others small outbreaks, still others sporadic cases or laboratory infections.

Human infections are often subclinical or inapparent and detectable only by the subsequent development of antibodies; when the disease is manifest its clinical type depends on the predominant localization and multiplication of the virus in certain organs, tissues, or systems. In the course of an outbreak it is generally noted that all gradations may occur between the asymptomatic, subclinical infection and the type of fully developed disease considered characteristic of the virus in question; in addition, the fact that different viruses can cause a similar syndrome renders an etiological diagnosis on clinical grounds extremely difficult and at best tentative.

A systematization of the clinical manifestations of infection by arboviruses is not easy; several forms can nevertheless be discerned at the "typical" level, which are not necessarily the most generally observed. The following syndromes are associated with arbovirus infections: encephalitis; hepatitis with hemorrhagic manifestations; dengue- or grippe-type illness, with myalgias, arthralgias and rash; hemorrhagic fevers; meningo-encephalitis, often with diphasic course; and general systemic disease with no localizations. No exhaustive review of the viruses causing the different types is intended here; the idea is rather to mention the principal agents that have been associated with different syndromes, particularly agents that have caused outbreaks of fair magnitude or frequency in preference to viruses responsible for sporadic or rare cases.

Encephalitis is caused by Eastern, Western, and Venezuelan equine encephalitis virus, group A; Japanese, Murray Valley, St. Louis and tick-borne (Far Eastern subtype, mainly), group B; and California encephalitis virus, California encephalitis group (CE). Meningo-encephalitis, usually mild and often with a diphasic course is associated with infection with tick-borne virus of group B (Central European subtype) and also with Kemerovo, a tick-borne virus of the Kemerovo group.

Dengue- or grippe-type illnesses with joint and muscle pains, also rashes of various aspects, in diverse association, have been caused by infections with chikungunya, O'nyong-nyong, and Ross River, group A; dengue Types 1, 2, 3, and 4 and West Nile, group B; and, usually with less prominent arthralgia, by Neapolitan and Sicilian sandfly fever viruses, phlebotomus group, and Colorado tick fever, an ungrouped virus.

Hepatitis with hemorrhagic manifestations of the intestinal tract is the typical form of severe yellow fever.

Hemorrhagic fevers are caused by viruses belonging in different groups or ungrouped. It is of interest to note that some of the viruses responsible cause, in other ecological conditions, rather mild and generally nonfatal disease. Chikungunya has been incriminated in a small proportion of cases of hemorrhagic fever of Southeast Asia, usually mild, though there seems to be evidence that it has also caused fatal cases. The immense majority of these fevers in Southeast Asia (Bangkok, Philippines, Singapore) and India are due to infection with dengue viruses 1 to 4; the circumstances that have promoted the pathogenic activity of these agents is unknown. Other group B viruses that induce hemorrhagic fevers are Omsk hemorrhagic fever and Kyasanur Forest disease viruses. Crimean hemorrhagic fever is due to an ungrouped tick-borne agent; work with this agent is, however, curtailed because of the lack of a good laboratory system. There are other hemorrhagic fevers, Argentinian and Bolivian, due to viruses belong-

ing in the Tacaribe group and designated Junín and Machupo respectively.

Generally a systemic type of illness, with only fever, malaise, loss of appetite, short duration, and complete recovery as the full manifestation of infection, has been associated with viruses of various groups: Mayaro, group A; most of group C human pathogens; Guarôa, in the Bunyamwera group; Bwamba, in the Bwamba group; and Oropouche, in the Simbú group.

A few final remarks will illustrate certain aspects of recent, or relatively recent, activity in human populations of some of the listed viral types.

O'nyong-nyong virus was unknown until 1959, when an epidemic broke out in Uganda and spread to Kenya, Tanganyika, and Nyasaland; during a period of little more than two years it was estimated to have affected about two million persons in East Africa (12). This epidemic stopped short of being a real calamity because no lethality was associated with it; the event, however, illustrates the possibilities for community disruption inherent in an arbovirus.

Venezuelan equine encephalitis had been shown to be responsible for a small human outbreak in 1952 in Colombia and for a number of laboratory infections. All these cases were mild or relatively so, with no encephalitic involvement nor lethal outcome; two earlier fatal cases, in Trinidad in 1943, had been linked with this agent, but the causal sequence was not clear on subsequent examination. Beginning in 1962 a series of major outbreaks occurred in Colombia and Venezuela extending over a period of a year or two; the cases were estimated in the thousands, with perhaps 10 to 15 per cent diagnosed as encephalitis, and a not inconsiderable number of them were fatal. Another feature of this epidemic was the frequency with which the virus was isolated from nasopharyngeal washings; in a series of patients examined by Briceño-Rossi (1), 11 per cent who had circulating virus also yielded virus from the throat. For this reason and also on the basis of epidemiologic considerations, it was concluded that droplet transmission had been common.

The discovery of the causal association between the dengue viruses and hemorrhagic fevers of Southeast Asia has been one of the outstanding occurrences of recent years. Interest in this syndrome began with the isolation of dengue strains in the Philippines in 1956, and was considerably aroused by the epidemic in Bangkok in 1958; hemorrhagic fevers of this type have since been noted in other countries in the area with, more recently, an invasion of India. Since 1958, in Thailand, about 28,000 hospitalized cases have been reported, with 1,800 deaths, almost 50 per cent of the cases having occurred since 1964 (14); in India a rough estimate for 1963 was about 100,000 cases, of which 500 were hospitalized and 200 died (21). The high mortality associated with the syndrome in children makes this one of the truly serious types of arbovirus infection; and the fact that it is caused by viruses that in the past had not been suspected of lethal capacity for man is disquieting.

Among other epidemics due to group B viruses that are worthy of mention as illustrating the constant threat presented by arboviruses is the recent one caused by SLE virus in Texas, in which nearly 250 confirmed cases were observed, with 22 deaths, from the middle of July to the end of September 1966. And it may also be well to remember here that yellow fever is not a thing of the past; even though an effective vaccine exists, it is not always available at the right time and place. Outbreaks have occurred since 1959 or 1960 in various countries—Ethiopia, Portuguese Guinea, Senegal, and Argentina.

Infection with California virus with resulting clinical encephalitis is a new development in the increasing pathogenic potential of arboviruses; while this is still a minor public health problem in the United States, 59 serologically confirmed or presumptive cases were reported in 1965 in states in the eastern half of the country. In another area of the world, western Siberia, a new arbovirus infection, caused by Kemerovo virus, was discovered four years ago; its potential threat is still undetermined.

Oropouche virus infection is still another example of an epidemic that can develop, reach its peak, and vanish in a period of three to five weeks. In the city and suburbs of Belém, Brazil, in 1961, over 7,000 cases were reported; no fatalities or severe cases of crippling illness were reported. Prior to the Belém outbreak only one human infection with this virus had been described.

Types of hemorrhagic fevers different from those caused by group B viruses have stimulated attention in recent years. Argentinian hemorrhagic fever, caused by Junín virus, has been known since the middle fifties, but the isolation of the etiological agent in 1958 promoted studies on the disease; its importance as a public health problem is of concern to the zone affected, owing to the not inconsiderable annual number of cases—from 300 to over 1,000, with a mortality that in various outbreaks or districts has varied from 3 to 15 per cent—and to the clear indications that the virus has been spreading to or has been uncovered in new areas. A somewhat similar disease, more severe in its mortality rate, is the Bolivian type of hemorrhagic fever caused by Machupo virus, described in an accompanying paper (see pp. 260–265); in the communities affected this was a truly devastating disease. Mention of these two types of hemorrhagic fevers could not be omitted; however, they still present a taxonomic problem. There is no clear evidence that the responsible viruses are arthropod-borne, even in a limited sense; while Junín virus has been isolated from mites on a few occasions, the epidemiological significance of this fact has not been appraised. In the case of Machupo virus, transmission of the disease to man is best explained by mechanisms other than by arthropod bite; whether a natural cycle exists involving arthropods is not known.

This survey of arboviruses that are, or may be, clinically important has had to be superficial because of time limitations; it is hoped, however, that it may have served to stress some of the problems presented by the arboviruses and their present and potential threat to public health.

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SECTION A. ARBOVIRUSES

PRESENT AND FUTURE OF KILLED AND LIVE ARBOVIRUS VACCINES

WILLIAM MCD. HAMMON

Department of Epidemiology and Microbiology
Graduate School of Public Health, University of Pittsburgh
Pittsburgh, Pennsylvania, U.S.A.

DR. HAMMON (*presenting the paper*): From the standpoint of the epidemiologist and the manufacturer, the problems concerning arbovirus vaccines for man are distinctly different from those discussed in prior sessions of this Conference. These previously discussed respiratory and enteric disease agents are almost completely world-wide in distribution, are generally transmitted rather directly from man to man, and have been carried by him wherever he has gone and apparently successfully established there. They are present in tropical, temperate, and even arctic areas and in urban and rural environments. Man is the reservoir and at some time in life, frequently at many times, he is exposed to almost every one of them, if not all. By contrast, arboviruses are usually zoonoses, each dependent for maintenance on one or a few specific arthropod vectors and lower vertebrate hosts. Man usually does not serve as a means of transport and is only a temporary, dead-end host. The vectors are frequently sharply localized. The temperature requirement for viral multiplication in the vector is relatively rigid and sharply limited seasonally in much of the world. The lower-vertebrate host may also be geographically limited and seasonally inactive as a result of migration, estivation, or hibernation. Vector and lower vertebrate together usually constitute the reservoir. The result is a very spotty geographical distribution, seasonal and sporadic occurrence, and at times complete disappearance. Each arbovirus has a distribution pattern differing from the other, though with frequent overlapping. Many viruses in the tropics are never encountered elsewhere. Some large populations spend a lifetime without exposure to a single arbovirus and others are exposed to perhaps 50 or more during childhood. The countries that are most highly developed tend to have the smallest problem, possibly because the population has refused to tolerate the vectors, and certainly they are the best able to minimize the problem by controlling the vectors adequately. Where the problems are most numerous and severe, less is and can be done about it. Fortunately, vaccine in many instances can be considered a secondary line of defense, since arbovirus diseases are susceptible to control by killing one or more links in the reservoir, the reservoir being nonhuman. This usually consists in vector control. These are some of the fundamental differences between the vaccine problem for respiratory or enteric viruses, and that for arboviruses.

As a rule, these differences have the following results in respect to arbovirus vaccines: (1) interest is usually locally restricted for any one vaccine, (2) profit from development and sale is unlikely, (3) application is impractical in most instances because of sporadic and unpredictable epidemic occurrence, (4) morbidity rates are low even during epidemics, and (5) indication for use is frequently limited to laboratory and other research personnel. All this applies generally, with certain outstanding exceptions such as yellow fever, dengue, Japanese B encephalitis, and the tick-borne encephalitides

of Europe and Asia. For most of these, vaccines of some degree of effectiveness are already in use.

To look at the problem in another way, however, there are over two hundred known viruses believed to be arthropod-borne, and almost half are known to produce human disease, though many on the basis of only one or two recognized cases. This, however, probably greatly underestimates the potential, since most of these agents have only been looked for or found in their little ecological niche or reservoir within the last few years and the human population of the area has not been studied. Thus, the size of the human disease problem is completely unknown and will not be known for many years.

Of all these viruses affecting man there is only one for which we currently have an approved and available *live* virus vaccine for man—yellow fever. We have *inactivated* virus vaccines, similarly approved for human use and available, for two viruses—Japanese B encephalitis (JBE) and Russian spring-summer (RSSE) or tick-borne encephalitis (TBE) virus. Obviously the need is far greater than this, and a number of others are in development and a few used in limited numbers of human volunteers. Some of these newer vaccines will be briefly described. First, however, let us consider what use would be made of vaccines in the United States if they were now available for some of our well-recognized serious arbovirus problems.

Eastern equine (EEE), Western equine (WEE), St. Louis (SLE), and California encephalitis (CE) viruses produce epidemics in the United States that result occasionally in serious morbidity and in fatalities. This year SLE virus has produced two major urban epidemics in Texas, and Ohio has again had its share of CE type cases. As an example, SLE virus will be considered.

A year ago, one of my associates and recent graduate students, after completing some work on a JBE vaccine in cell culture, took a strain of St. Louis virus that had been cloned from a Tampa, Florida, isolate (17); grew it readily in primary tissue culture to a high titer; filtered it; inactivated it with formalin; observed its rate of inactivation to be a first order type of reaction until it was no longer detectable in small quantities; prolonged inactivation for three times this period, as Salk had proposed for poliovirus; then tested its potency as a vaccine in mice (2) in the same manner as is required by the Government's minimum standard for JBE vaccine (19). It was a more potent product than any Japanese B encephalitis vaccine so far made by us or others. In all probability this could be made quickly on a commercial scale and would pass all reasonable safety tests. Let us therefore assume that a safe and effective inactivated cell culture vaccine for St. Louis virus could be made available. Is a commercial producer likely to become interested? Will the city health departments of St. Louis, Tampa, St. Petersburg, Houston, Dallas, Corpus Christi, Kansas City, and others, where large serious epidemics have occurred, order it for use in 1967 or 1968? I believe not, but I might be in error. If this vaccine produces the expected type of response, like that of inactivated JBE, RSSE, poliovirus, and many others, the recommended regime will be three, possibly four doses, the first two given a month apart and the third preferably at a longer interval. The following year a booster should be given and possibly a booster on an annual basis for at least a few years. The interval between injections can be shortened in the face of an epidemic, but immunization will be less effective, and by the time the community is adequately immunized the period of risk may have largely or even completely passed.

St. Petersburg and Tampa had a three-year experience of epidemic prevalence and now for at least three years no virus has been found in birds, mosquitoes, or man. Houston may not be struck again for many years. The virus was recognized as present in several parts of Texas in studies we made in the early 1940's (8, 10), but Houston appears to have remained free for many years until just recently. In the Yakima Valley of Washington and in the southern San Joaquin Valley of California, St. Louis encephalitis occurred endemically and in seasonal epidemics that we studied for a number of years beginning in 1940. Mosquito control took care of the problem, accidentally in one area (22) and purposely in the other (21), and vaccine is not now needed. One can hardly recommend an effort to produce a human vaccine of the inactivated type for mass, routine use for any of our currently recognized United States epidemic-producing arboviruses. Such vaccines,

however, are very badly needed for certain laboratory workers at high risk.

A live virus vaccine, on the other hand, provided it gave many years of protection and the protection began almost immediately after administration, would be an entirely different matter and might find great usefulness in epidemic and interepidemic situations. However, none of these developed for North America would find a market in Europe, Asia, Africa, or Australia because other viruses, not these, present problems there. The SLE example is paralleled by many other situations in the world.

As to immediate, recognized problems for which vaccine might well be used, if we exclude biological warfare, groups of laboratory and research workers, and mass military exposure (major problems nonetheless), we must rank high such diseases as the dengues and their related hemorrhagic fevers of Southeast Asia, chikungunya, tick-borne encephalitis of Europe and Asia, Japanese B encephalitis, Venezuelan encephalitis, and a few others. These all have relatively widespread distribution and have caused major epidemic problems. Sandfly fever is omitted since *Phlebotomus* can usually be so readily controlled with DDT. Before individual consideration of these, some generalization is possible.

An inactivated mouse brain vaccine, usually treated with formalin, can be readily produced for most arboviruses, and vaccines of this kind have been used successfully in almost every laboratory for the immunization of experimental animals. Several have been used for man, principally for JBE and RSSE. The dangers of brain vaccines have long been recognized, and every effort is being made to replace them with primary cell culture harvests; except for rabies, a brain source of other vaccine viruses is relatively unique. Group A arboviruses and yellow fever virus vaccines have been produced in whole chick embryos, usually as crude, heavy suspensions. Sensitization and the presence of other viruses related to malignancy are currently deterrents to their use. Thus, the accent is on cell cultures. These also have their drawbacks, which will undoubtedly be discussed in detail by others. The relative uniqueness of the arbovirus problem has to do with the fact that no cell culture system will produce most arboviruses in the desired titer, and most of the cell lines that do for one or more viruses have not been previously used as sources of human vaccine. Thus, to meet most licensing requirements very extensive human volunteer studies will be needed; possibly 100,000 or more persons must be injected with each new type of tissue or cells employed, for a determination of the safety of the tissue source alone.

The next general problem is that of live virus vaccines. As was pointed out earlier, live virus vaccines are usually assumed to protect longer and more quickly, and only one dose is required; for example, yellow fever and smallpox, as against JBE and RSSE in the inactivated group. All must be attenuated if given by inoculation, since this is the normal route of disease producing infection.

Attenuation is usually no longer a difficult task with the many types of cell cultures available, plaque picking for clones, and the recognition and utilization of certain markers. The unanswered question is what type and degree of attenuation are needed for man and how can this be determined in the laboratory without risk to human volunteers at any stage. This we cannot answer. For the greater number of arboviruses there is no laboratory animal model that can be used to determine whether peripheral inoculation (as occurs with an arthropod bite) of a selected clone will or will not produce the clinical syndrome normally induced in man. In most instances only the intracerebral route of inoculation will produce disease in a laboratory animal, and even that may be only in a newborn mouse. Failure to produce encephalitis in a newborn mouse can hardly serve as a means of selecting an attenuated strain of dengue virus for vaccine to protect against either the classical human febrile disease or the severe hemorrhagic fever syndrome. Lower primates do not become ill by any route of injection with virulent dengue virus, yet after attenuation (as determined in human volunteers) this changed virus may produce encephalitis in the monkey (25). In fact, the virus population used was selected by serial mouse brain passage. A strain of JBE virus that we recently "attenuated" in cell culture became greatly attenuated for the central nervous system of the mouse, hamster, burro, monkey, and chimpanzee but then would not produce any infection or antibody in man (9).

These examples illustrate the dilemma of what constitutes a satisfactory clone of attenuated virus. But this is only a small part of the problem. What if a suitably attenuated virus does give immunity but also produces viremia, and this then permits infection in an arthropod vector? Will reversion to virulence occur on serial passage? Will such a potential of the virus delay licensing and use? Suppose that after extensive testing, two well-recognized but colonized mosquito vectors do not become infective after feeding on 10 or 20 vaccinated volunteers at the height of viremia. How about wild mosquitoes of several other genera and species in other parts of the world which have not even been tested for virulent virus? Where does one stop in guaranteeing safety to the communities where vaccinees may be sent or where persons are vaccinated locally because of a limited laboratory exposure to an exotic agent? What if yellow fever vaccine could not have been released until all treetop monkey-feeding mosquitoes of the jungles of South America and Africa had had to be tested on human volunteers for serial passage to determine the clone susceptibility?

And so one can continue to present the multiple problems of live virus vaccines for highly fatal or crippling disease. However, recent law in the United States has become extremely specific and limiting on the use of human volunteers to the point where, with all previously recognized difficulties, it is obvious that only a very unusual type of scientist with special personal characteristics of aggressiveness, persistence, and many other qualities will, or should, with knowledge of what lies before him, undertake to develop and test a live virus vaccine for any arbovirus that produces a dangerous disease in man. The difficulties are greatest when the disease in question is one of great risk, but the need in such a case is correspondingly greatest.

In place of the many years of careful development and progressive human-volunteer testing required for a live virus vaccine, most inactivated vaccines are relatively easily and quickly developed up to the point of field testing for effectiveness. In general, today's requirements for a cell culture vaccine are a safe cell culture system free from recognized adventitious agents, low final foreign protein content, filtration prior to and possibly again during the inactivation, repeated monitoring of the rate of inactivation during the production of each lot, final safety testing for any residual free virus by inoculation of many susceptible animals and cell systems with probably at least two liters of every batch produced, and suitable stabilization for prolonged storage. As with any vaccine, there should be a high antigen content so as to require a minimal number of injections of modest amounts. Neutralizing antibody response in volunteers should be readily detectable and remain at such a level for upwards of a year at least. A booster should result in an increased titer and duration of response. After this comes field testing somewhere in the world in a population at adequate risk to a predictable high incidence of disease, preferably with placebo controls and an effective clinical and laboratory follow-up for adequate evaluation. Such a test may well require vaccination of a million or more persons and a period of several years for a clear-cut answer. Challenge of volunteers, as with respiratory viruses, is no more acceptable than it would have been with poliovirus or yellow fever virus, unless a mild disease like dengue is under consideration. Such testing, adequately performed, will be in most instances more difficult than the "Francis test" of inactivated poliovirus vaccine (6), because of the limited geographic areas involved and the inadequacy of medical services, transportation, and communication in many of the involved communities, plus the presence of many more diseases to consider in differential diagnosis.

INDIVIDUAL VACCINES

Yellow fever vaccine, the first arbovirus vaccine, is by now quite well known. The so-called French or neurotropic type resulted from serial mouse brain passage and produces serious encephalitis in children with sufficient frequency to result in recommendations that it not be used in children under ten years of age. Even older ages are not entirely exempt from severe disease. It has been given mixed with vaccinia by scarification. The 17D strain selected after many chick embryo cell culture passages is also neurotropic in mice. It has produced encephalitis in children, almost entirely in infants, but with much less frequency than the mouse adapted

virus. Its big safety hurdle involved hepatitis from human serum used as a stabilizer. This led to a major series of tragedies, but the risk is now eliminated by excluding human serum. The accepted protection period from a single injection is recognized to be at least ten years.

Russian spring summer encephalitis virus vaccine was used in millions of persons as a crude formalin inactivated mouse brain suspension. Its effectiveness has not been challenged, but reports differ as to the morbidity resulting from its administration. Apparently allergic demyelinizing encephalomyelitis from the CNS tissue and active infectious encephalitis from incomplete inactivation have both presented problems. More recently a chick embryo primary cell culture suspension, inactivated with formalin in the presence of the cell suspension and the final product after clarification and filtration adsorbed on aluminum hydroxide, has been used extensively, routinely, on humans. Early reports are that it is safe and effective in producing antibodies when given in a series of four doses over a period of four to six months; and annual booster doses are recommended for at least three years (3).

So far as importance with respect to vaccine needs is concerned, Japanese B encephalitis poses probably the greatest or next to the greatest current challenge. Crude mouse brain inactivated with formalin has been licensed for use by the United States and Japan, and chick embryo similarly in the United States. Use in adults by Americans after World War II was considered inadequately effective though never put to a controlled field trial. A field trial in children using the American product was reported as having produced protection (29). Recently workers at the Japanese National Institute of Health have developed several more purified mouse brain vaccines claimed to be adequately free from CNS tissue antigens (15). One or more of these is in routine use in children in certain areas of Japan and a placebo field trial conducted among children in Taiwan has been reported as showing good protection (13).

This terminates the report on arbovirus vaccines currently approved or licensed for routine mass usage in man.

Vaccines licensed and prepared for horses in the United States for Western and Eastern equine encephalomyelitis have received considerable human application. These are chick embryo suspensions that have been inactivated. They have successfully produced antibody in man, and their use by laboratory workers appears to have reduced the incidence of laboratory-acquired disease and fatalities. Because of hindrances imposed by recent United States Government regulations, this human use and apparent protection is no longer available. This also applies to a few other vaccines never produced in adequate quantities or used in sufficient numbers of persons to merit manufacturing and licensing for man by a licensed producer of viral vaccines.

Mention was made earlier of attempts in our laboratory to produce an attenuated JBE primary hamster kidney cell live virus vaccine. It was tested in terminal cancer patients for oncolytic and immunizing potential (9). It was too noninfectious. Then it was inactivated with formalin, since infectious titers in cell culture approaching 10^{10} TCD_{50}/ml were available, and this has been shown to produce an excellent antibody response in adults after two or three doses (5). Since it was harmless in volunteers before inactivation, the amounts needed for safety testing after inactivation were not prohibitive for the production of a trial lot in a modest university laboratory.

Venezuelan equine encephalitis (VEE) virus has been recognized for many years as one requiring a vaccine carrying a high priority. The first vaccine was prepared in the usual manner by inactivation of a virulent strain. Inactivation was determined by mouse inoculation. When it was finally used in a large number of persons at one time it was recognized as having produced considerable disease by infection (26). Man was much more susceptible than the most susceptible laboratory animal recognized. When inactivated enough to render it noninfectious for man it was also nonimmunizing.

A strong U.S. Army group undertook the attenuation of VEE in primary guinea pig heart cells and finally a promising attenuated strain was established (1). This has received rather extensive human trial in large numbers of exposed laboratory and field workers (16, and personal communication with Lieutenant Colonel R. W. McKinney) and during an epidemic in Venezuela. Mild febrile disease following

vaccination is not uncommon, and an occasional aseptic meningitis has been reported, but the attenuated clinical picture, as with measles, is much more acceptable than the regular disease and the routine use of this vaccine in persons at high risk is apparently quite justifiable. Virus can be isolated from the throats of vaccinees, but actual transmission to other intimate associates, though searched for, has not been demonstrated. A low-grade viremia occurs in many persons, but *Aedes triseriatus*, an effective vector of wild virus, has failed to become infected or infective and requires higher titers than those apparently available before it can serve as a vector.

Vaccine development for dengue is also clearly indicated but has received inadequate attention. There are at least four rather distinct immunologic types of dengue, plus antigenic variants of these (11). At least four types have been demonstrated to be present in two large cities, and three types have been shown elsewhere. Limited early work with inactivated virus has given no encouragement to the development of an unconcentrated inactivated preparation. This, however, may not be the final answer. If a polyvalent vaccine is to be made available it appears likely that it may have to be inactivated, which presents a serious challenge.

Live attenuated dengue virus was first prepared by Sabin and Schlesinger from relatively low passage mouse brains (25). Further passage tended to lead to neurotropic virulence in monkeys. A modified dengue disease with rash was produced in volunteers which gave a few months' heterologous protection to experimental challenge and a more enduring homologous response. Further selection of strains by Wisseman and his associates led to a more modified Type 1 (31), which was recently used in a field test in Puerto Rico (Dr. C. L. Wisseman, Jr., personal communication) during an outbreak caused by a strain of dengue closely related to Type 3 (23). A significant degree of protection was reported. Sabin reported interference between Types 1 and 2 given together (25). It would appear that in Southeast Asia and India polyvalent vaccine is needed. On the other hand, Halstead and Russell present arguments to the effect that the serious hemorrhagic fever syndrome of the Southeast Asian dengue type occurs only after one or more dengue or other group B arbovirus experiences, resulting in rapid 7S antibody production early in the infection that leads to the serious disease manifestation (7, 24). They postulate that previous sensitization is an essential factor in etiology. If this should prove to be the case—and I have reservations—dengue vaccination of either monovalent or polyvalent type in areas where multiple types are present would be contraindicated, since it might lead to a more serious disease syndrome after exposure to the next virus of a different type.

I know of only three other arbovirus vaccines for which there are published reports of human trials: Rift Valley fever, Colorado tick fever, and Langat virus. Rift Valley fever vaccine also is a cell culture formalin-inactivated product producing a good antibody response after three injections (20). Both primary monkey and hamster kidney have been used. Colorado tick fever virus vaccine was produced in suckling mouse brains; the virus was then absorbed on calcium phosphate and eluted by sodium phosphate, then inactivated with formalin. Two or three injections in man produced significant antibody levels for a short period of time (28). An attenuated Langat virus clone grown in chick embryo fibroblasts was tested in human volunteers in the USSR as a possible live virus vaccine against TBE. No clinical symptoms or viremia were detected, but neutralizing antibodies were produced (27). In addition to these, I have been informed by Dr. T. R. Rao (personal communication) that an inactivated vaccine for Kyasanur Forest disease made from cell culture is undergoing human trial in India. This vaccine development is proceeding at the Haffkine Institute.

A very large number of reports have appeared on many other attenuated, partially attenuated, and inactivated arbovirus vaccines tested only in laboratory or domestic animals. These are too numerous to review here.

An approach of great interest for world travelers, airline crews, certain military personnel, and laboratory personnel, and one that may give rise to a vaccine with a wider market than most others, is the development of a polyvalent vaccine or combination of vaccines, live or inactivated or in a mixed series, providing broad coverage for large groups of antigenically related viruses. Casals has shown this to be

quite reasonably applicable to a number of group A agents (2), and we and many others have examined the more difficult problem in group B (2, 12, 14, 18, 30). Combinations of live attenuated and certain inactivated agents in series may well be the answer as soon as several attenuated strains within the same group become available. There is considerable animal experimental work to support the usefulness of this concept.

In summary, it may be stated that epidemiological considerations place arbovirus vaccines in a unique category. Attenuated vaccines, though they appear to be the most desired, need not be expected in great numbers in the near future because of inherent dangers and difficulties. Inactivated vaccines from primary cell culture sources for many viruses can be quickly developed and produced, but most have limited or no use in public health programs, though for laboratory personnel and other special high-risk groups they are of great importance. To meet the need for these latter groups will require heavy subsidy, for such vaccines will not be developed and marketed for profit. Most of them are unlikely ever to be proved effective in man in adequate field trials. Except for a disease like dengue, challenge inoculation cannot be employed to demonstrate effectiveness. Thus, effectiveness may be predicated entirely on results in animals and on the production of specific antibody in man.

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SECTION A. ARBOVIRUSES

POSSIBILITIES FOR CONTROL OF HEMORRHAGIC FEVERS IN LATIN AMERICA

R. B. MACKENZIE, M. L. KUNS, and P. A. WEBB*

DR. MACKENZIE (*presenting the paper*): A clinical syndrome known as hemorrhagic fever has been recognized in two South American republics—since before 1953 in Argentina and since 1959 in Bolivia (1, 2). The clinical diseases of the two countries are similar, characterized by headache and fever with leukopenia, often followed by gastrointestinal bleeding, severe hypotension, and central nervous system manifestations. Junín virus has been shown to be the etiologic agent of Argentine hemorrhagic fever (AHF), while its counterpart in Bolivia (BHF) is caused by Machupo virus (3, 4, 5); the two agents share a common complement-fixing antigen not only with each other but with two other viruses, Tacaribe and Amaparí. Tacaribe virus, for which the group has been named (6, 7), was isolated from frugivorous bats in Trinidad in 1956 (8). Amaparí was first isolated in 1964 in Brazil, from rodents of the genera *Neacomys* and *Oryzomys* and from laelaptid mites combed from the same *Oryzomys* (9). No evidence of human infection due to Tacaribe or Amaparí viruses has yet been demonstrated. Figure 1 shows the great geographic distances that separate the source localities of the four known members of the Tacaribe group.

From 1958 through 1965, more than 6,000 cases were reported as occurring in Argentina. A significant majority of the cases were males over the age of 15 years, primarily migratory agricultural harvest workers who became ill during the autumn months of April, May, and June. While population data in the context of AHF epidemiology are not readily available, it appears that the attack rates have been much higher among the migrant workers who manually harvest corn and live in temporary dwellings adjacent to the fields than among permanent residents of the epidemic areas. The basic epidemiologic pattern of AHF seems to have changed little since its first description.

A similar preponderance of adult male cases, with seasonal variation, was noted in Bolivia through 1962 (2); however, the age-sex dis-

Fig. 1. Locations of source localities of Tacaribe group viruses in South America.

* Dr. Mackenzie (The Rockefeller Foundation, Bogotá, Colombia); Drs. Kuns and Webb (Middle America Research Unit, Balboa Heights, Canal Zone).

tribution shifted to a general one in 1963, when an epidemic struck San Joaquín, a town of 2,500 in the Department of Beni (10, 11). From 1959 through 1964, more than 1,100 cases occurred in the two Bolivian locations seen in Figure 2.

Subsequent to 1962, serological studies were used in estimating total Bolivian case numbers (10, 11); clinical diagnoses were used in compiling the Argentine estimates. Case fatality rates ranged between 15 and 30 per cent in Bolivia and were generally less than 10 per cent in Argentina. Serological studies in Bolivia have failed to demonstrate the occurrence of subclinical infection.

Evidence of movement of epidemic foci, or "fronts," has been sought in Bolivia. It appears that BHF struck in two distinct areas more or less simultaneously; they were situated about 70 miles apart in Bolivia's tropical grasslands. The human population appears to have been completely susceptible to the disease. A disastrous outbreak among 600 inhabitants of a 50-square mile area called Orobayaya was terminated in 1962 when the inhabitants fled to a small town 20 miles away; the disease did not follow them (2, 11). The second Bolivian area, that of San Joaquín, was closely observed over an 18-month period in 1963 and 1964. A peripheral advance of no more than 10 miles in each of two directions occurred. Meanwhile, the epidemic was seen to progress slowly, like a slow-burning grass fire, within San Joaquín itself; it required more than a year to affect the entire town, which has an area of about one quarter of

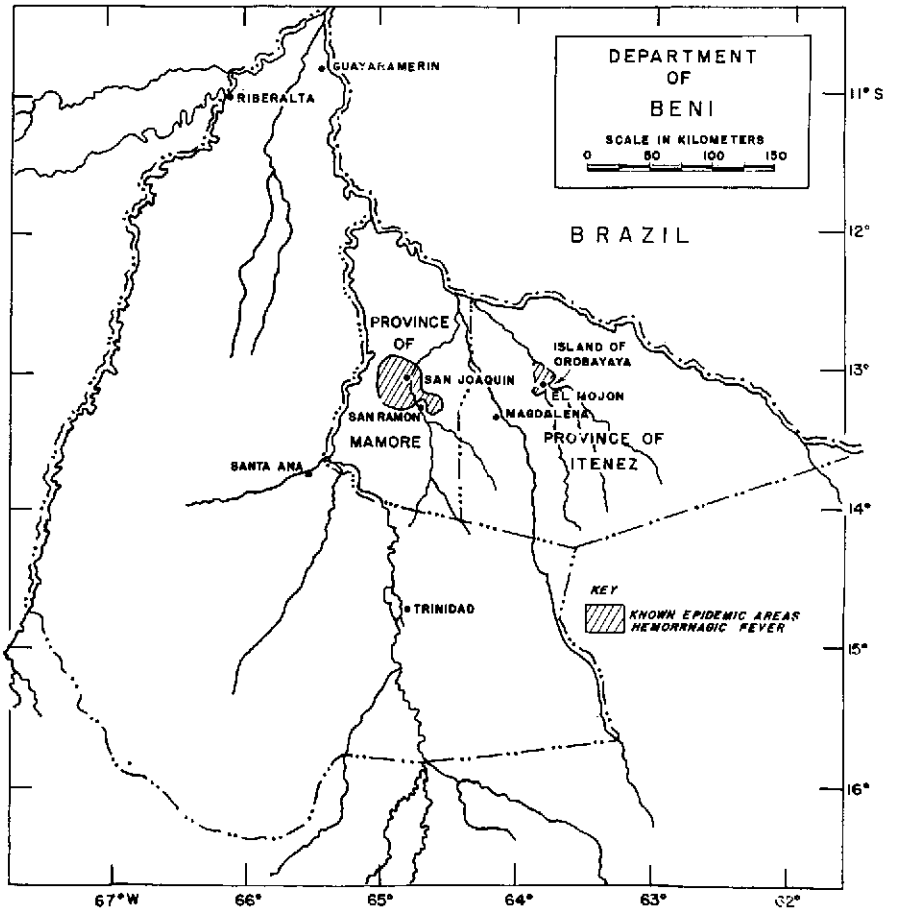

Fig. 2. Map of Northeastern Bolivia showing known epidemic hemorrhagic fever sites through 1965.

a square mile (10, 11). Hemorrhagic fever in Argentina also appears to be spreading slowly from the original focus.

Thus, South American hemorrhagic fevers have been rural, focal, intense, and persistent, without evidence of rapid frontal advance. The only documented foray of a hemorrhagic fever virus into a town—San Joaquín, Bolivia—produced a severe human epidemic; otherwise the viruses have been restricted to completely rural settings.

Every indication points to the paradomestic rodent *Calomys callosus* as the reservoir host of Machupo virus in Bolivia. *Calomys* were numerous in and around the houses of San Joaquín in 1963 and 1964 (12). Asymptomatic infection with chronic viruria was experimentally induced in laboratory reared *Calomys* (13); *Calomys* caught in houses producing hemorrhagic fever cases were found to harbor virus (14), while the same species taken from unaffected houses were negative. Meanwhile, thousands of arthropods taken at the apparent height of virus transmission in San Joaquín were not productive of virus.

The fields near which AHF cases were encountered were heavily populated with wild rodents, chiefly *Calomys laucha, Akodon arenicola, Oryzomys flavescens* and *Mus musculus*. Junín virus has been recovered not only from *Calomys*, but also from *Akodon* and *Mus* (15, 16). It has also been recovered, unlike the virus in Bolivia, from laelaptid mites (17). Though human-to-human transmission has been documented for both Junín and Machupo viruses, it has not been significant in epidemic maintenance.

Thus, in the case of BHF, evidence supports a hypothesis of direct transmission of virus from chronically infected rodents to humans, possibly by contamination of food. In the case of AHF, some findings would suggest a similar method of transmission, but virus has been reported from a broader spectrum of rodents and from arthropods; no full evaluation has been made of their relative epidemiologic significance. But, most important, hemorrhagic fever is *rodent associated* in both Bolivia and Argentina; Machupo and Junín viruses appear to be primarily parasites of rodents and are probably not disseminated by either winged arthropods or birds. The subsequent discussion of possibilities for effective control of human disease draws heavily on these assumptions.

SPECIFIC APPROACHES TO DISEASE PREVENTION

Ecological

A. *Rodent control.* In certain instances, the limitation of human contact with infected rodents would seem to be a feasible method of hemorrhagic fever control. Such was the case in San Joaquín, Bolivia, where one variety of rodent, *C. callosus*, predominated, lived in close contact with humans, and was subject to little effective competition or predation. These conditions made it possible to limit control measures to the confines of the town itself. A three-pronged attack was made, consisting of trapping, poisoning, and a community-wide clean-up campaign designed to destroy nesting sites and limit food sources for *Calomys*. These measures not only reduced the number of rodents but effectively halted the epidemic. Follow-up consisted of continued trapping and removal of protective cover, together with the importation of cats, the population of which had become depleted after the application of insecticide for malaria control was initiated in 1959 (11).

Though the immediate results of the rodent control program were spectacular, residents of San Joaquín, observers and investigators alike, were concerned that hemorrhagic fever cases would again recur. The townsfolk were informed that sporadic cases could be expected. An intensive surveillance program, which had been in existence and functioning efficiently for several months, was continued; it consisted not only of registering and obtaining paired sera from all hospitalized patients, but also of weekly house-to-house canvass in a search for mild illness; acute and convalescent sera were similarly obtained from such persons. Meanwhile, a regular program of rotational rodent trapping was begun. Using 15 traps per night per house, each house was subjected to an average of 90 trap-nights per month. Complete records were maintained of all captures, and skins and skulls were saved for specific identification. The control program work was done by part-time workers, all residents of San Joaquín. During the 24 months of postepidemic follow-up, a total of 24

nonfatal cases were confirmed serologically; it is probable that one additional fatal case occurred. The cases that occurred during that period were largely concentrated in the northwest corner of the town; their distribution in relation to the rates of *Calomys* captured per house per year are shown in Figure 3. The rodent capture rate in the northwest corner was double that of the other parts of San Joaquín, implying that persistent foci of *Calomys* did remain in San Joaquín and that the continuance of cases, at a low level of incidence, was related to their presence.

Though AHF is also rodent-associated, it may be that multiple or nonparadomestic species are involved, and an arthropod vector as well. If this is so, disease prevention by way of trapping and poisoning rodents might be impractical

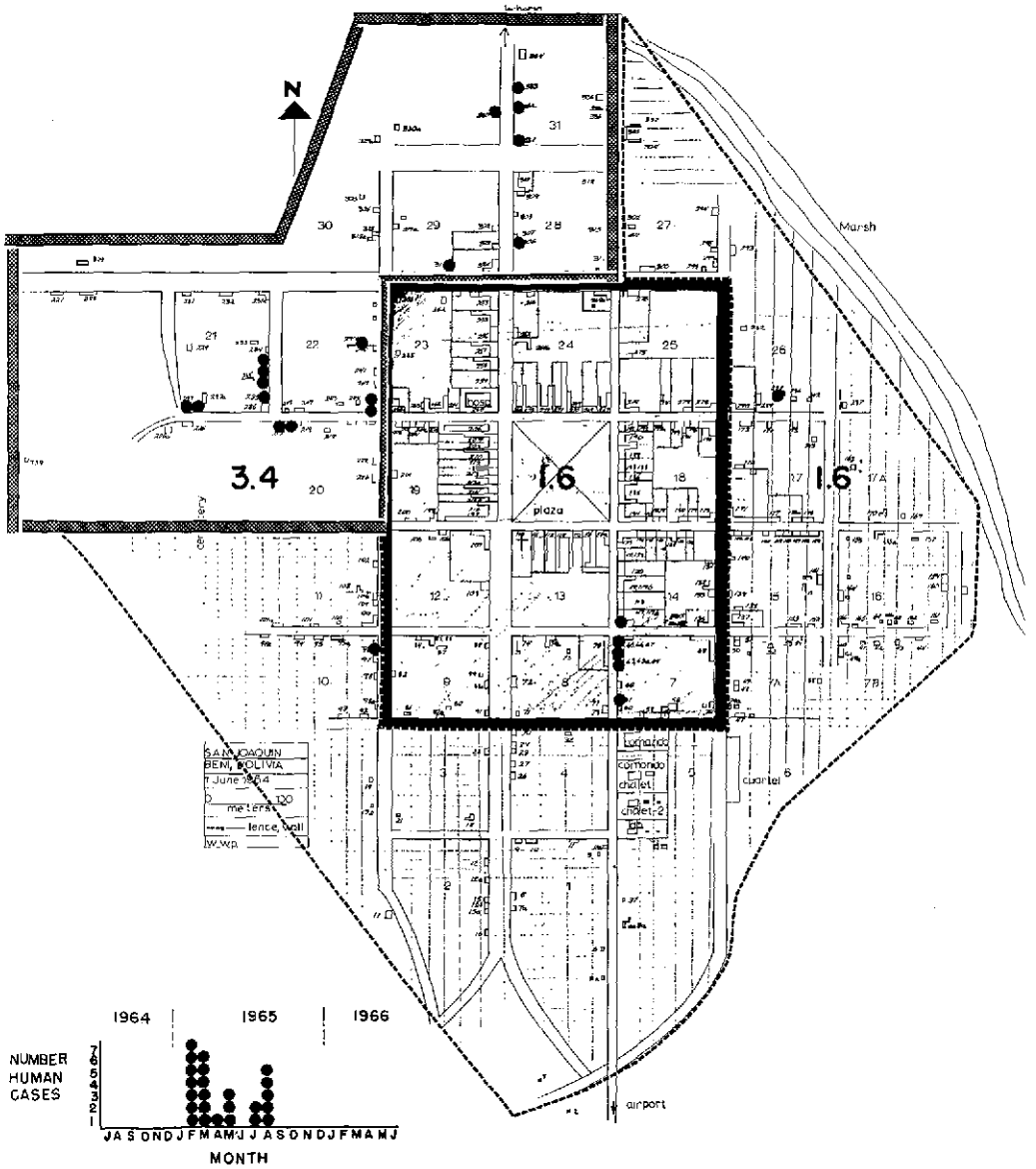

Fig. 3. Confirmed BHF cases, San Joaquín, July 1964-December 1965, with rodent captures per-house per-year shown for three areas.

or ineffective. The introduction of stronger competitors or predators that do not have the ability to serve as reservoirs themselves might be considered. It was observed in Bolivia that certain river-bank towns were populated with *Mus* and *Rattus* apparently to the exclusion of *Calomys*. Though not without danger, such a method of biological control might sometimes deserve consideration. After confirmation that cats are not susceptible to Machupo virus infection, they were reintroduced into San Joaquín to supplement trapping. Though their effect on a *Calomys* population has not been measured, it is possible that the intimacy of the rodents' contact with humans is decreased by the presence of cats.

B. *Modification of human activity.* As a supplement to or substitute for rodent control, a modification of human practices of crop harvesting, food storage, and ordinary hygiene might well diminish the frequency of human infection. The incidence of hemorrhagic fever in Argentina is said to have been reduced in certain areas where mechanical corn pickers have been introduced. Attack rates in Argentina and Bolivia appear to be highest among members of the lowest socioeconomic groups, among whom hygiene receives the least attention.

Immunological

Postinfection immunity to Machupo virus appears to endure for at least three years and may well be permanent; a similar response might be expected after Junín virus infections. A major step forward has been made by Parodi *et al.*, who have successfully inactivated Junín virus by photosensitization in the presence of neutral red (20). Not only have they been able to protect guinea pigs against challenge, but administration to two persons who had experienced natural infections more than a year previously resulted in an increase in complement-fixing antibody without causing symptoms of infection. In guinea pigs, nonlethal Tacaribe virus has been shown to protect against lethal doses of Junín virus (18, 19). Whether such protection is humoral or is due to chronic infection with viral interference is not clear. No observations have been made that affirm or deny the existence of cross-protection between Junín and Machupo virus among humans. Nor is anything known of the behavior of Amapari or Tacaribe viruses in humans.

Gamma globulin prepared from pooled convalescent plasma was prophylactically administered to high-risk investigators working in Bolivia. Careful evaluation was not possible, and some impressions as to its beneficial effect are subjective. Further evaluation is well warranted, since such a rapid and safe, though temporary, protective measure would be most valuable.

Discussion and Summary

South American hemorrhagic fever viruses most certainly appear to be rodent-associated; and any current effort to *prevent* human infection should be directed at interrupting the transmission of virus, directly or indirectly, from rodents to humans. It is unlikely that any single means of rodent control would be effective in all situations.

Several approaches to rodent control are possible and methods need to be tailored to each local situation, but only after a determination of what the host reservoir is and how the virus is transmitted. Differences in human activities can be most striking among South American communities. In Bolivia, for example, the populations that are at risk live in areas in which there are no roads and travel is chiefly by oxcart trail or riverboat. Thus, most residents of northeastern Bolivia never leave the province in which they are born, and there is little population movement over distances greater than 50 miles. In contrast, the epidemic area of Argentina is laced with highways and railroads; a great deal of farm work is done by migratory labor, and it is likely that large numbers of new susceptibles are fed into the AHF areas each year.

The development of a vaccine to Machupo virus would be of great value, not only to Bolivians but also to laboratory workers outside of Bolivia. But it seems that a most acute need is for the protection of Argentine agricultural workers. Until we know the degree of cross-protection in humans between the viruses of South American hemorrhagic fevers, one can only speculate as to the ultimate value of any single vaccine. Meanwhile, it seems that hemorrhagic fever control in South America can only be effected through the medium of limiting human exposure.

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SECTION B. HERPESVIRUS GROUP VIRUSES

VACCINATION AGAINST HERPESVIRUS INFECTIONS

R. N. HULL AND F. B. PECK, JR.

Lilly Research Laboratories
Indianapolis, Indiana, U.S.A.

DR. HULL (*presenting the paper*): Viruses belonging to the herpes group are known to infect or to be carried by various animal species. The virus from each species is a serologically distinct entity, although some cross-reactions are seen, especially among those recovered from primates. The type and severity of disease produced by these viruses in their natural host varies, but in some instances they are most significant as etiologic agents when transmitted to a foreign host. The ecology of herpesviruses is a very interesting subject in itself, but is beyond the scope of this presentation.

HERPESVIRUS SIMIAE

In our efforts to prevent or to modify the diseases produced by these viruses, we have studied two of the primate viruses, herpes simplex from man and B virus from Asiatic monkeys, and also pseudorabies, which is important in domestic animals. These studies, beginning first with B virus, have been in progress for over 10 years. After many failures, a method was finally devised that yielded an inactivated B virus vaccine that stimulated antibody in experimental animals and afforded protection against direct challenge with large doses of virulent virus. These findings have previously been described (3) and will not be reviewed in detail. The vaccine produced fairly high antibody titers in guinea pigs, in the range of 1:32 to 1:512, but response in the rabbit was considerably less, with titers on the order of 1:4 to 1:16. It was demonstrated, however, that antibody levels of 1:4 or greater afforded a solid immunity in rabbits against challenge with several thousand infective doses of live virus. All efforts, including prior hyperimmunization with herpes simplex virus, failed to enhance the antibody response in rabbits. It was noted also that antibody produced in rabbits was short-lived and generally dropped to undetectable levels within three to four months after immunization unless booster doses were given. In the light of these observations, it was rather surprising to find that the antibody assayed one week following primary immunization was of the 7S variety.

The preliminary evaluation of B virus vaccine in man was reported in a second publication (4). In these studies a total of 129 persons were immunized at various times and by several different immunization regimes. The antibody levels for both B virus and herpes simplex were determined prior to immunization, and B virus antibody titers were measured after vaccine administration. Although some information was obtained that suggested that one route of inoculation might be preferable to another, the over-all results are compiled and presented in the top portion of Table 1. As can be seen, 18.5 per cent of the group were found to be negative for herpes simplex antibody, and 50.3 per cent were negative for B virus antibody prior to vaccination. (All the individuals negative for herpes simplex antibody were also negative for B virus antibody; in addition, 41, or 58 per cent, of those found positive for herpes simplex antibody were negative for B virus antibody. These data are not included in the table.) Thus, 50.3 per cent of the total were negative for B virus antibody before immunization, and this was re-

TABLE 1. IMMUNIZATION WITH B VIRUS VACCINE: ANTIBODY STATUS

Antibody type	<1:4 No.	<1:4 Per cent	>1:4 No.	>1:4 Per cent
Before and after primary immunization (129 persons)				
Herpes simplex prior to vaccine	24	18.5	105	81.5
B virus prior to vaccine	65	50.3	64	49.7
B virus post vaccine	46	35.6	83	64.4
After numerous booster doses (253 persons, including those above)				
Herpes simplex	64*	25.3	189†	74.6
B virus	49	19.3	204	80.6

*<1:8
†>1:8

duced to 35.6 per cent after primary immunization. The response, or seroconversion, was essentially limited to individuals who were negative for B virus antibody but positive for herpes simplex antibody. Only 2 of the 24 individuals negative for herpes simplex antibody responded to the B virus vaccine.

The lower portion of Table 1 contains data from another study, which will be discussed shortly. Since they supplement or extend these observations, it seems pertinent to introduce them, in part, at this point. Most of the 129 persons for whom data are presented, plus an additional number for a total of 253, have continued to receive booster doses at three to six-month intervals over a period of several years. All participants in the program were bled just before and seven days after their most recent booster dose, and both sera were assayed for B virus antibody. The sera were screened also for antibody to herpes simplex. It is seen that after these booster doses the percentage of individuals negative for B virus antibody was further reduced to 19.3 per cent.

In a limited study reported previously, it appeared that small intradermal doses of vaccine were equal if not superior to 1.0 ml doses given by the subcutaneous or intramuscular route. It has been a general finding in both man and experimental animals that frequent small doses of this vaccine produce better antibody response than do massive doses or concentrated preparations. In man, as in rabbits, antibody titers obtained after primary immunization were of short duration, and within six months most of the individuals who had responded to primary immunization lost their titers. In view of these findings the immunization schedule was revised as follows:

Primary immunization: 0.1 ml I.D. in each forearm, at days 0, 7, 14, and 42
First booster series: 0.1 ml I.D. in each forearm at three-month intervals for one year
Maintenance dose: 0.1 ml I.D. in each forearm at six-month intervals

Many of the people included in the original studies described above and in previous reports have continued on such a schedule for approximately six years. Except for an occasional spot-check of a few individuals, no further serological studies were undertaken until recently.

During the first six months of this year the antibody status of all persons receiving the vaccine (a total of 253) was studied before and after their six-month booster dose. Many have now received as many as 20 doses. The results of this study are shown in Table 2. It appeared that at this stage in the program about 75 per cent of the people had remained positive for B virus antibody during the six-month period preceding the booster dose, and that the booster did little to enhance titers, as the mean titer increased only from 1:7.6 to 1:8.8. Sixteen individuals, however, showed seroconversion from titers of <1:4 to 1:4 or greater. This finding was the only

TABLE 2. ANTIBODY STATUS OF 253 PERSONS BEFORE AND ONE WEEK AFTER A SIX-MONTH BOOSTER DOSE OF B VIRUS VACCINE

Antibody level*	No. of persons Before booster	No. of persons After booster	Per cent after booster
<1:4	65 (25%)	49	19.3
1:4	32	38	15.0
1:8	44	48	19.0
1:16	41	44	17.4
1:32	48	54	21.3
>1:32	23	20	8.0

*Mean titer: 1:7.6 before booster dose; 1:8.8 afterward.

evidence that supported the continuation of the six-month booster dose in this group of people. It was pleasing to find that titers in general were higher than those obtained after primary immunization.

It was both surprising and disturbing, however, to note that 26 of 64 persons negative for herpes simplex antibody (<1:8) responded to the booster dose. This was in direct contrast to previous observations made at the time of primary immunization. In an earlier study a group of 10 herpes-negative persons, who had remained negative to both B virus and herpes simplex antibody after a full course of B virus vaccine, had been given an additional four doses of herpes simplex antigen, and only one responded. It was later determined, however, that this individual was subject to recurrent infections with herpes simplex, while none of the others had any history of such infection. These findings led us to the hypothesis that these adults negative for herpes simplex antibody might have been a unique group composed of individuals with an immune tolerance to this virus. If so, they might also be considered resistant to herpesvirus infection. This theory was challenged, however, when the last known human infection with B virus occurred in an individual apparently negative for herpes simplex antibody. If there is any remaining truth to the hypothesis, it will have to allow for a small subgroup, within the herpes-negative group, of individuals who have been sensitized to herpesvirus or are subject to recurrent herpes infection but do not have the usual elevated antibody titers to herpes simplex virus. Such people apparently have the capacity to respond to these antigens. There is much yet to be learned about the immunology of herpesvirus infection.

HERPESVIRUS SUIS

Inactivated pseudorabies virus vaccine was prepared by essentially the same procedure as that developed for B virus vaccine, except that the virus was grown in a continuous rabbit kidney cell strain, LLC-RK$_1$ (2). The vaccine was antigenic for both rabbits and mice and afforded protection to the latter against severe challenge with live virus. After these studies in laboratory animals a limited investigation was undertaken in the immunization of cattle. Five of eight six-month-old calves were given multiple 2.0 ml doses, and antibody levels were determined six weeks after the last dose. At that time all eight animals were challenged with 10^7 $TCID_{50}$ of the vaccine strain. Two weeks later the survivors plus an additional two control animals were challenged again with a field isolate of pseudorabies virus. These studies and the results obtained are summarized in Table 3.

Antibody titers determined six weeks after

TABLE 3. IMMUNIZATION OF CALVES WITH INACTIVATED PSEUDORABIES VIRUS VACCINE AND THE EFFECT AGAINST DIRECT CHALLENGE

Calf No.	Antibody levels		Challenge results	
	Before immunization	Six weeks after vaccination	First test at six weeks*	Second test at eight weeks†
907	<1:4	1:32	Survived	Survived
916	<1:4	1:64	Survived	Survived
923	<1:4	1:32	Survived	Died
925	<1:4	1:64	Survived	Survived
927	<1:4	1:32	Survived	Survived
451‡	<1:4	<1:4	Died	—
911‡	<1:4	<1:4	Died	—
924‡	<1:4	1:4	Survived	Survived
523‡	<1:4	—	—	Died
524‡	<1:4	—	—	Died

*Challenged with vaccine strain.
†Challenged with field isolate.
‡Unvaccinated controls.

immunization and prior to direct challenge ranged from 1:32 to 1:64 in the vaccinated animals. One control animal developed a titer of 1:4. All five of the vaccinated animals resisted the first challenge dose, as did also the one control animal with a titer of 1:4. Four of the five vaccinated calves also resisted the second challenge with the field isolate, while both of the controls inoculated with this virus succumbed to the infection (1).

HERPESVIRUS HOMINIS

Shortly after the first potent inactivated B virus vaccine was obtained we undertook similar studies with herpes simplex virus. This virus, also grown in primary rabbit kidney cultures, produced good yields on the order of $10^{6.5}$ to $10^{8.0}$ $TCID_{50}$/0.5 ml, with an average of $10^{7.3}$. It was filtered and inactivated in the same manner as has previously been described for B virus. It stimulated antibody in guinea pigs to levels of 1:16 to 1:1024, with an average of about 1:256. Rabbits responded with lower titers, in the range of 1:4 to 1:32. The vaccine strain, identified as Mayo 1814, was lethal for rabbits by the intraperitoneal route or by inoculation of the scarified cornea, and was also highly virulent for adult mice by intracerebral inoculation. The virus produced dermal lesions in guinea pigs after inoculation into this tissue but did not cause generalized infection and death.

Protection tests were performed in these species in which vaccinated animals were compared to nonimmune controls. In a typical experiment twenty 12- to 15-gram mice were given two 0.5 ml intraperitoneal doses of herpes simplex antigen and, along with a similar number of uninoculated controls, were challenged with 600 LD_{50} of live virus. Eighty per cent of the vaccinated animals survived this challenge, which was 100 per cent fatal in the control group. In other tests with rabbits, the challenge dose was administered by inoculation of the scarified cornea of the right eye with virulent virus and the left eye with spent medium from uninoculated cultures. Immunization was accomplished by intraperitoneal inoculation of the herpes simplex antigen. Upon challenge, all animals developed lesions in the right eye only, but the vaccinated rabbits all survived the infection. Nearly all the unvaccinated animals developed encephalitis and died. Thus, in these primary herpetic infections of rabbits, the antigen prevented encephalitis and death, but not the local eye infection. As was mentioned previously, the vaccine strain produced skin lesions, but not encephalitis in guinea pigs. In this species the herpes simplex antigen readily protected against the development of dermal lesions in challenged animals. The vaccine strain was compared with numerous heterologous strains, including fresh isolates, both by cross-serum-neutralization tests and by cross-protection tests in mice, without evidence of any notable antigenic differences.

Early studies on the ability of the antigen to stimulate a neutralizing antibody response in humans were disappointing. In a human potency test, in which the dosage schedule was similar to that used in the guinea pig potency test, only one individual who was negative for herpes antibody demonstrated an increase in titer, and this individual had an attack of herpes simplex infection during the study period. Of the remaining nine subjects who were initially positive for herpes simplex antibody, none demonstrated a significant rise in titer (Table 4).

Subsequently, we have also found that subjects suffering from recurrent herpes simplex infection also fail to respond to the antigen (Table 5). For example, of a group of 25 individuals treated with 10 doses of antigen, none developed a fourfold rise in titer. Only 11 of the 25 subjects demonstrated a twofold rise, which we feel is of doubtful significance. In addition to the lack of neutralizing antibody

TABLE 4. HERPES SIMPLEX ANTIGEN: NEUTRALIZING ANTIBODY RESPONSE IN HUMAN POTENCY TEST

Subject	Before antigen	1 week after 4th dose
1 through 9	<4	<4
10	<4	32
11	32	32
12–16	64	64
17–19	64	128

Dosage: 1.0 ml subcutaneous at 0, 7, 14, 28 days.
Bleeding: Days 0 and 35.

TABLE 5. NEUTRALIZING ANTIBODY RESPONSE TO HERPES SIMPLEX ANTIGEN IN INDIVIDUALS WITH RECURRENT HERPES SIMPLEX INFECTION

Subject	Before treatment	After treatment
1	8	8
2–4	32	32
5–6	32	64
7–8	64	64
9–10	64	128
11–14	128	128
15–19	128	256
20–22	256	256
23–24	256	512
25	512	512

Dosage: 1.0 ml weekly for 10 weeks.

response, complement-fixing antibodies also fail to rise after use of the antigen or recurrent disease. These findings are in agreement with the results of Lepine *et al.* (6), who used an antigen similar to ours in the treatment of recurrent herpes simplex infection.

Evaluation of the possible clinical effectiveness of the antigen was undertaken with considerable skepticism, because of the inherent clinical variability of this disease and the lack of a suitable serologic assay system. The observed failure of antibody to rise after recurrences of the natural disease, coupled with the almost complete ineffectiveness of multiple doses of antigen to produce a serologic response in humans, was and continues to be a serious deterrent to evaluation of this antigen. The well-recognized facts that recurrent herpes simplex infection in humans may be altered by physical, chemical, and psychic stimuli and that irregular periods of remission and exacerbation without known triggering mechanisms may occur also hamper effectiveness studies. However, with the encouragement and cooperation of the late Dr. Frank R. Heilman of the Mayo Clinic and Dr. G. John Buddingh of Louisiana State University, clinical studies were begun early in 1959 and have continued with the active cooperation of many dermatologists, ophthalmologists, and internists throughout the United States.

The basis for the method of use of the antigen rests on the following observations. Although recurrent herpes simplex infection exhibits a wide variety of clinical manifestations, the lesions themselves have a common histological characteristic: early in the development of the lesion there is a striking similarity to the histologic picture seen in acute hypersensitivity states such as urticaria. Marked edema of the corium occurs, with accompanying dilation of blood vessels and perivascular infiltration of leukocytes and mast cells. As in urticaria, the edema is out of proportion to the interstitial leukocytic infiltration. In addition, the development of cutaneous tuberculin-like hypersensitivity following infection with herpesvirus also lends credence to the possibility that herpes simplex virus has allergenic properties that may be operating in the individual with recurrent herpes simplex infection. Consequently, a classic "desensitization" approach was proposed as a possible method for controlling the disease in a manner similar to that used for the control of other allergic states.

Clinical studies were undertaken in patients suffering from recurrent herpes simplex infection. They were treated much as in other desensitization programs—a primary series of frequently spaced doses of antigen followed by periodic reinforcing doses in subjects whose clinical state warranted maintenance therapy for continued remission of their disease. A series of at least ten 1.0 ml subcutaneous doses of antigen was arbitrarily selected as the primary course of injections. If improvement occurred, further maintenance doses of 1.0 ml were recommended for the patients who required them.

It has been extremely difficult to obtain a satisfactory group of patients that is large enough to be studied on a double-blind basis utilizing herpes simplex antigen and a placebo. However, we were fortunate in being able to sponsor two studies by independent investigators (Table 6). Both groups consisted of patients suffering from severe and frequently recurring disease. Both had the same treatment plan, but the time of the effectiveness evaluation differed.

In the first study (W. B. Mullin, unpublished data), 12 subjects received a series of ten 1.0 ml subcutaneous injections of the antigen, and 11 subjects received an identical series of injections of placebo consisting of nutrient medium 199. Ten of the 12 patients receiving the antigen had significantly fewer attacks from the time of initial injection of antigen to six months after the last injection. Only 2 of the 11 sub-

TABLE 6. EFFECT OF HERPES SIMPLEX ANTIGEN ON RECURRENCE RATE

	Number of patients		
	Total	Improved	Unchanged
Study A* Antigen	12	10	2
Placebo	11	2	9
Study B† Antigen	23	16	7
Placebo	29	22	7

*Period of evaluation: From initial injection to six months after last injection.
†Period of evaluation: From six months after last injection to 36 months after last injection.

jects receiving the placebo had a significant decrease in their attack rate.

In the second study (5), however, no difference in effectiveness was demonstrated when the rate of recurrence of lesions was determined during a period from 6 months to 36 months after the last injection of antigen. Comparison of these two studies indicated that the antigen appeared to be temporarily effective in decreasing the rate of recurrence of lesions but that patients tended to revert to their pretreatment recurrence rate after the use of the antigen was discontinued. These findings are analogous to what is seen in patients undergoing desensitization therapy for allergic disorders. In addition, the normal variability of spontaneous remission over a period of months undoubtedly contributed to some of the improvement noted in Study B, in which a decrease in recurrence rate was found in both placebo and antigen groups.

We have also accumulated data from the clinical use of the antigen in a variety of patients with herpes simplex infection. Records of 399 subjects treated with the antigen have thus far been analyzed. A general review of their pretreatment status appears in Table 7.

Since we have been primarily interested in and have selected individuals with a long history of recurrences, half of the patients in this group have had their recurrent attacks for more than eight years. A very important factor in analyzing these data is the frequency of recurrent attacks. Over 30 per cent of the patients had a history of at least 11 episodes of recurrence per year. Sixty-nine per cent of the cases gave a history of facial lesions, 13 per cent were treated because of active herpetic keratitis, 9 per cent received therapy for genital herpes, and 14 per cent for aphthous stomatitis, although the latter is not generally considered to be due to herpesvirus. Fourteen per cent of the patients also had a history of multiple sites—face and eye, face and mouth, and so on.

TABLE 7. HERPES SIMPLEX ANTIGEN: PATIENT ANALYSIS

Total subjects	399 (45% male—55% female)	
Age range	76% 3rd—6th decade	
Past history of recurrences:		
<1 year	10%	1–3 years 20%
4–8 years	19%	>8 years 50%
Frequency of recurrent lesions:	Attacks per year	Distribution
	<3	20%
	3–6	35%
	7–10	13%
	≥11	32%
Site of lesions:	Face	69%
	Eye	13%
	Genital	9%
	Aphthous stomatitis	14%
	Other	6%
	Multiple sites	14%

In analyzing the results of therapy, the patients were classified according to whether they had received (a) a primary series of at least ten weekly 1.0 ml subcutaneous injections of antigen or (b) the primary series as defined above plus a series of periodic booster doses, usually at four- to eight-week intervals. The decision whether or not a patient improved after therapy was based on a comparison of the post-treatment and pretreatment recurrence rates. A period no less than twice the usual period of normal remission prior to treatment was chosen as the minimum period of observation. For example, a patient with recurrences every eight weeks prior to therapy was considered to be improved if no recurrences occurred for at least 16 weeks during and after therapy.

The primary series of injections was given to 153 subjects (Table 8). Of these, 111 were classified as improved, 38 remained unchanged, and 4 were classified as becoming worse during treatment. Out of 133 subjects who received both the primary series of injections and periodic booster doses, 119 were considered to be improved and 14 showed no change from the pretreatment status.

These results, however, must be viewed in the light of the known variability of recurrence of herpes simplex infections, especially in subjects who have relatively few recurrences per year or who have variable remission histories. Therefore, in order to help rule out the possibility of chance, we have analyzed the data from a highly selected group of patients in the series (Table 9). Thirty-five subjects were found who had suffered from recurrent herpes simplex infection of the face for more than eight years, who had recurrences of one to two weeks' duration at least 11 times a year, whose lesions recurred at regular intervals and were either stationary or increasing in severity, and who had had no periods of relative quiescence of the disease for at least three years prior to therapy with herpes simplex antigen. In addition, their disease had not been affected by previous forms of therapy such as smallpox immunization, X-ray, and antimetabolites. This group of subjects represents, we believe, the most severe and most refractory type of patient, and hence the type most likely not to benefit from nonspecific therapy.

TABLE 8. GENERAL EVALUATION OF EFFECTIVENESS OF HERPES SIMPLEX ANTIGEN

Series	Total*	Improved	Unchanged	Worse
Primary only	153	111	38	4
Primary plus booster doses	133	119	14	—

*Does not include 18 subjects lost to follow-up or 95 subjects receiving less than primary series of injections.

TABLE 9. EFFECTIVENESS OF HERPES SIMPLEX ANTIGEN IN SELECTED CASES OF SEVERE RECURRENT FACIAL LESIONS

Series	Total	Improved	Ineffective
Primary	18	13	5
Primary plus booster doses	17	15	2
Complete remissions		10	
Partial remissions		18	
Ineffective		7	

Eighteen of these patients received a primary series of at least 10 injections of antigen. Seventeen others received the primary series plus periodic booster doses. On the basis of the criterion for effectiveness outlined above, 28 subjects experienced either complete or partial remission of their disease and 7 failed to respond. We feel that these results are significant because in this particular group of patients all previous attempts at therapy had failed and the recurrence rate had been regular in spite of repeated attempts to control the disease either psychologically or otherwise. Many of these patients have now been followed for three to six years. In the majority, the improvement has been maintained only so long as periodic maintenance doses of antigen were administered. It seems unlikely that the pronounced clinical improvement that occurred in the group would have happened by chance.

We have also analyzed data obtained from the use of the antigen in patients suffering from recurrent active herpetic keratitis. Herpes simplex virus is probably the most important current cause of keratitis. Treatment of this condition has been uniformly poor except in cases of superficial ulceration amenable to therapy by

iododeoxyuridine. Keratitis is an especially severe problem in individuals with facial herpes, since repeated seeding of the cornea with herpes simplex virus from facial or other lesions may occur. The use of the antigen in these patients was undertaken with great caution because of the possibility that the series of injections might stimulate activity. Treatment was instituted in these cases by the initial injection of 0.1 ml of a 1:10 dilution of antigen followed by gradually increasing doses until the usual 1.0 ml subcutaneous dose was achieved. Thereafter, the usual primary series of injections were given followed by periodic maintenance doses.

Of the 47 subjects in this series (Table 10), all had typical histories of active herpetic keratitis that had not responded to previous therapy with antimetabolites or other classic therapeutic measures. None had clinical evidence of decreasing severity of their lesions. Ten patients in the series were not followed long enough to determine whether their lesions were affected by the use of herpes simplex antigen. Of the 37 subjects who were, 19 had recurrent herpes simplex lesions elsewhere in addition to their herpetic keratitis. Four subjects were included who had been suffering from herpetic keratitis for less than one year. In each instance, however, the lesions were continuous and progressive in character. Eight subjects had been suffering from herpetic keratitis for periods of one to three years, and 25 had had their disease for more than three years.

TABLE 10. EFFECTIVENESS OF HERPES SIMPLEX ANTIGEN IN ACTIVE HERPETIC KERATITIS

Total subjects		47
Lost to follow-up		10
Total analyzed		37
Multiple sites of infection		19
Duration of infection	<12 months	4
	1–3 years	8
	≥4 years	25
Total patients with active disease with duration of	≥1 year	33
Results: Complete remission		14
Partial remission		16
No effect		3

Of the 33 subjects with active disease of one year's duration or more, 14 have experienced a complete remission and 16 have shown a marked decrease in the number and frequency of their attacks. Three continued to experience their usual episodes of recurrence in spite of therapy with the antigen. Of the 4 patients whose disease was of less than 12 months' duration, 2 experienced complete remission and 2 were classified as partial remissions. While it is true that some of the keratitis patients might have experienced complete or partial remission without therapy, we feel that the clinical improvement in 90 per cent coinciding with the onset of therapy is a much higher success rate than would have occurred by chance.

The results of therapy in these patients with facial or corneal herpes simplex infection are most encouraging. While we do not know whether the original premise that the lesions of herpes simplex infection are allergic in nature is a correct one, the antigen seems to have been effective. It is most logical to assume that this is due to some humoral factor engendered by the use of the antigen. This factor, if present, is not measurable by standard laboratory procedures for determining the antigenic response of the human to injection of the antigen. It was with a great deal of interest, therefore, that we learned of the studies performed by Tokumaru (7).

If, as Tokumaru has described, patients with recurrent herpes simplex infection are deficient in γ A immunoglobulin synthesis, it could be hypothesized that herpes simplex antigen stimulates the formation of γ A immunoglobulins. These immunoglobulins, with their high avidity for herpes simplex virus, might neutralize or so alter the virus that it is no longer capable of stimulating the tissue reaction that ultimately produces the pathologic lesion typical of recurrent herpes simplex infection. Furthermore, the finding of a deficiency of γ A antibody synthesis offers a new avenue of approach in determining possible serologic evidence of the effectiveness of herpes simplex antigen. Obviously, the findings of Tokumaru must also be considered in connection with the treatment of many allergic disorders by the injection of specific allergens.

The extensive laboratory tests performed on B virus vaccine to insure its safety were described previously (4). Twenty consecutive lots

were produced and tested without a single incidence of inactivation failure. Eight of these were used in clinical studies. The herpes simplex antigen was similarly tested; however, the volumes tested were smaller and more nearly approximated those required for other licensed products. A total of 11 lots that passed all the laboratory tests was employed in the clinical studies described above. One lot, which was not used, was found to contain residual live virus. It was determined, however, that the lot had inadvertently been treated with 1 in 4,000 final concentration of formalin rather than with the prescribed 1 in 400. The small amount of virus that escaped the action of formalin in this lot was readily detected by the rabbit kidney tissue culture safety test on two different occasions. As has been said, both the B virus vaccine and the herpes simplex antigen were prepared from virus propagated in kidney tissue cultures prepared from three-week-old domestic rabbits. Over the past 10 years we have used thousands of these cultures in our research, development, and control laboratories in the pursuit of this program; we have yet to isolate the first adventitious agent from rabbit kidney tissue cultures.

As a result of the clinical studies of both B virus vaccine and herpes simplex antigen, we have been able to collect a considerable amount of data on the clinical safety of material prepared from primary rabbit kidney tissue culture. Consequently, we have reviewed the data for evidence of systemic or local reactions following the use of vaccine prepared in this manner.

A total of 652 subjects has received an aggregate of more than 10,000 doses of B virus vaccine or herpes simplex antigen. The most common finding in the herpes simplex group has been erythema, sometimes accompanied by induration and mild tenderness, which has occurred in approximately 20 per cent of the subjects. Two individuals refused to continue therapy because of this. Another subject developed a sterile abscess after the second injection of the antigen. Local reactions occurred after each injection of antigen in only four subjects. In addition, we have reports of two patients with a previous history of generalized erythema multiforme accompanying bouts of recurrent herpes simplex infection. The injection of herpes simplex antigen in these two patients resulted in a local erythema multiforme lesion at the site of injection.

Constitutional reactions were mild and consisted mainly of headache, malaise, and a feeling of weakness or light-headedness. Eight subjects exhibited these symptoms. Another developed a punctate rash after the first injection of antigen. (This patient was highly allergic to a variety of substances.) One additional subject developed chills after each dose of antigen, but continued therapy. None of the subjects with eye lesions developed sudden or unexpected flare-ups coincident with injection of the antigen, although one did experience a period of reactivation of his keratitis during therapy. In the B virus group intradermal dosage produced local erythema and occasional pruritis, usually within 24 hours of injection. No systemic reactions occurred.

There was no evidence of a direct correlation between the number of injections of antigen administered and the development of local or systemic reactions. No increase in reaction incidence could be found in patients having more than 10 injections compared to those having fewer. From these data it is evident that these antigens, prepared in rabbit kidney tissue culture systems, have not been associated with severe or untoward side effects.

Acknowledgment

We very gratefully acknowledge the assistance of many of our associates in the pursuit of these studies. Personnel in the Biological Research Laboratories assisted in the preparation and laboratory evaluation of B virus and pseudorabies virus vaccine and of herpes simplex antigen, and performed serological tests in support of clinical studies. The Biological Development Laboratories produced the more recent clinical trial lots of herpes simplex antigen, and the Biological Control Laboratories performed the necessary tests for safety, purity, and potency on all clinical trial lots. The assistance and cooperation given by the Industrial Medicine Department in the clinical evaluation of B virus vaccine were paramount to its success and completion.

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SECTION B. HERPESVIRUS GROUP VIRUSES

PROSPECTS FOR IMMUNIZATION AGAINST VARICELLA AND CYTOMEGALOVIRUS INFECTIONS

THOMAS H. WELLER

Department of Tropical Public Health and
Center for Prevention of Infectious Diseases
Harvard School of Public Health, Boston, Massachusetts, U.S.A.

DR. WELLER (*presenting the paper*): Varicella-zoster or V-Z virus (51, 60) and the human cytomegaloviruses or CMV (38, 42, 56, 57) were isolated some 10 years ago. Morphologically, biologically, and biochemically, the V-Z and CMV viruses possess affinities to the herpesvirus group and, like herpes, are ubiquitous in the human population. As I discuss the V-Z and CMV viruses, I shall not review the literature of the past decade, but rather shall refer to selected observations relating to the problem of immunization.*

Primary contact with V-Z virus induces varicella, and it is assumed that the syndrome of herpes zoster follows the activation of a latent V-Z virus infection, although the manner in which V-Z virus persists in the quiescent state and then becomes active is obscure. Our knowledge of the consequences of primary or secondary contact with the CMV viruses is imperfect. Congenital infections with CMV may produce widespread visceral and nervous system involvement (55), and in this respect CMV viruses rival rubella virus in producing congenital damage. Infection acquired after birth often occurs without overt disease (37). On the other hand, recent work indicates that postnatal contact may result in hepatitis (17, 47) or in a syndrome manifested by persistent fever and hepatosplenomegaly associated with mononucleosis-like changes in the peripheral blood (2, 23). In older subjects a cytomegaloviral pneumonia or enteritis may dominate the clinical picture; whether such an episode represents a primary response on the part of the human host, is the result of reinfection, or reflects activation of a pre-existing viral process remains unknown. I shall defer comment on the social significance of these agents.

Consideration of Selected Characteristics of the V-Z and CMV Viruses

The morphologic similarity of the V-Z and CMV viruses and their affinity to the herpes group has been documented repeatedly (3). Melnick and his co-workers (31) have placed the V-Z and CMV viruses in herpes subgroup B on the basis of an avid cell association; however, as is noted below, this attribute is perhaps a cultural artifact and not a unique *in vitro* behavioral characteristic. In this presentation, it is appropriate to emphasize biologic and epidemiologic dissimilarities between the V-Z and the CMV agents, accepting that our knowledge is incomplete and that today's assumptions may be proved false. Immunologic and epidemiologic evidence suggests that there is but one antigenic type of V-Z virus; in most individuals a single attack of varicella is the rule. For the human cytomegaloviruses, in contrast, a con-

* For general reviews of the subject of V-Z virus, reference may be made to Downie (11) and Weller (52, 53); for reviews of the CMV agents, to Medearis (30), Weller and Rowe (58), Weller (54), and Hanshaw (15).

siderable degree of antigenic heterogeneity exists among the members of the group (56). In substantiation of this view, Medearis (30) found great variation in the neutralizing antibody patterns of five congenitally infected infants, and of their mothers, as assayed with three strains of virus. Working with acquired infection in older children, Hanshaw and his co-workers (17) studied 25 patients with viruria; 21 had neutralizing antibodies for the Davis strain, 12 for strain AD 169, and 4 were negative for Davis and AD 169 antibodies, which suggests the existence of yet other antigenically different strains. Again, in the significant report from Finland by Kääriäinen and his co-workers (20; Kääriäinen, personal communication, 1966), the development of complement-fixing antibody in high titer was observed in one patient, yet the serum did not neutralize two heterologous strains of CMV. These observations suggest that we err in referring to *the* human cytomegalovirus and should instead speak of the human cytomegaloviruses. A number of questions are thus raised. Does natural infection with one antigenic variant protect against infection with other variants? Do the variants differ in pathogenic potential? Such fundamental questions remain to be answered.

Let us consider the phenomenon of "latency" as it relates to human infections with V-Z and CMV viruses. Varicella manifests some peculiar epidemiologic and biologic characteristics. We accept varicella as a highly contagious clinical entity. Virus, however, can be recovered consistently only from the fresh cutaneous lesion. Attempts to demonstrate virus in nasopharyngeal secretions are usually unsuccessful (12, 32), which may or may not be due to insensitivity of the tissue culture indicator system, as suggested by Gold. The significant feature is the rapidity with which virus disappears from view within the host—morphologically, biologically, and epidemiologically—to remain hidden for years or decades and then to reappear with increasing frequency as time goes by. Hope-Simpson (18) has re-emphasized the relationship between zoster attack rates and advancing age in the general population; he estimates that half of the people reaching age 85 will have experienced an attack of zoster. Such a unique host-parasite relationship with long periods of latency obviously poses obstacles to the evaluation of a supposedly "attenuated" V-Z virus as an immunizing material.

The CMV host-parasite relationship is a somewhat different pattern, in which the state of viral latency may be more apparent than real and containment of the infectious process is achieved slowly and more or less imperfectly. After either congenital or postnatal CMV infection, viruria or viral excretion in the saliva may persist for months or years; in one of our patients viruria was demonstrated over a 52-month period (55). With the passage of time, the amount of virus excreted gradually declines. Viruria is rarely demonstrated in apparently normal adults. Yet the recent reports by Kääriäinen (19, 20) suggest that an inapparent cytomegaloviremia may be common in adults; of a group of five seronegative open-heart surgical patients who underwent extracorporeal circulation in which separate pools of fresh heparinized blood were employed, all subsequently developed high titers of CMV complement-fixing antibody. Four showed no clinical evidence of CMV infection, but the fifth developed a prolonged fever, with a mononucleosis, and was shown to be a CMV excreter. That virus was transmitted by the fresh blood, which was used two to four hours after collection, was further suggested by studies on a control group. The controls included 14 patients subjected to heart surgery and 21 additional cases, all of whom had received stored citrated blood. In the patients given stored blood no CMV antibody response occurred.

A high cytomegalovirus carrier rate in the adult population is suggested by this important series of observations. Whether the adult carrier state reflects a smoldering primary infection or is the result of reinfection with one or more antigenic varieties is unknown. Kääriäinen (20) also observed that in some patients who already had low levels of pre-existing cytomegalovirus complement-fixing antibody prior to open-heart surgery, high titers developed after extracorporeal circulation in which fresh blood was employed. This observation may indicate that new heterotypic infections had been induced in the recipients.

The so-called avid cell-virus relationship observed *in vitro* with the V-Z and CMV agents, with the attendant difficulties in the performance of neutralization tests, has led to widespread use of the complement-fixation (CF) reaction.

Comment on the specificity and nature of the complement-fixing antibody response is pertinent. The production of active V-Z complement-fixing antigens has been simplified by the use of infected cell extracts; various cell lines of human origin (6, 39, 48) or of monkey origin (49) are satisfactory. Our observation (59) that CF antibodies decline within a few months after an attack of varicella, often becoming undetectable, has been amply confirmed (6, 13, 50). The potential usefulness of the CF test is further circumscribed by the recent demonstration that V-Z virus and herpes simplex virus apparently share minor antigens. Kapsenberg (22) observed that 12 of 49 patients with herpes infection developed fourfold or greater rises in V-Z antibody titers, and Svedmyr (48) demonstrated V-Z antibody rises in 9 of 14 virologically proven cases of herpes simplex infection. Comparable results were obtained by Ross *et al.* (36), who further noted that certain patients with varicella developed increased herpes CF titers. The limitations of the CF test are becoming obvious.

Present concepts on the epidemiology of cytomegaloviruses are based in large part on findings obtained with the CF reaction, originally introduced by Rowe and his co-workers (38). Application of the CF test, for example, indicates that 81 per cent of the adults in Washington (38), 54 per cent in London (46), and over 60 per cent in Stockholm (7) possess antibodies. Further, in institutions or boarding schools where intimate contact facilitates spread of the cytomegaloviruses, high rates are demonstrable in childhood (46, 55). Except in early infancy, when titers may be low (30, 55), the results of CF tests have shown a good correlation with the occurrence of virologically proven infection. Medearis (30) demonstrated that the CMV antigens involved in the CF reaction show broad strain reactivity; tests done with antigens prepared from 14 strains yielded essentially identical results. While the CF reaction is useful in the investigation of disease, caution is indicated in the interpretation of survey data obtained with this technique. We possess scant information on the persistence of CF antibody in man after a primary infection at levels associated with possible activation of a latent infection, or on those resulting from the repeated contact with homologous or heterologous strains that must occur with these ubiquitous agents. Accurate information will emerge only with recognition of the problems posed by antigenic heterogeneity and through the widespread application of neutralization tests employing homologous and heterologous strains. Present difficulties in the correlation of CF antibody survey data (which may reflect a relatively recent contact with any of the antigenic variants in the group of cytomegaloviruses) with data obtained by the performance of neutralization tests with a single strain (which may reflect the long-term as well as the current experience with but one antigenic member of a family of viruses) are illustrated by data such as those obtained by Carlström (7).

The Need for Vaccines

We turn now to the social significance of the V-Z and CMV agents, with particular reference to new information bearing on the practical indications for the development of vaccines. It is appropriate to consider four categories of significance: (1) congenital damage that may impose a burden on society for the lifetime of the affected individual; (2) the problems of morbidity and mortality associated with infections naturally acquired after birth; (3) new problems associated with iatrogenic disease; and (4) indirect problems posed to the health of the public by the confusions of differential diagnosis.

There is no evidence that maternal varicella produces congenital malformations or is related to prematurity (28, 41). Congenital infection with the cytomegaloviruses, on the other hand, has since 1962 (55) been recognized as responsible for mental retardation associated with microcephaly, various other central nervous system abnormalities, and hepatitis with hepatosplenomegaly. Confirmatory evidence relating the CMV to mental deficiency continues to accumulate (16, 30, 45). Overt congenital infections are commonly seen in pediatric centers, but prevalence data are lacking. Inapparent infection may also produce significant brain damage (15). In a survey of 100 apparently healthy newborns carried out by Stern in London (personal communication, 1966), three excreters of CMV were detected; one remains apparently normal at three months of age, a second developed hepatosplenomegaly, and the third has by three months developed microcephaly and mental retardation.

Clearly, congenital CMV infections are of great social significance.

The age distribution of varicella deserves comment, particularly as varicella pneumonia occurs predominantly in adults and may be fatal. In temperate regions varicella is classically considered a benign disease of childhood. It seems peculiar that varicella pneumonia, which is not uncommon, was first recognized as recently as 1942. The question may be raised whether this change reflects improved diagnostic standards, the unmasking by antibiotics of a condition previously concealed by secondary bacterial pulmonary infections, or a gradual modification of host-parasite relationships. Nonfatal varicella pneumonia may result in diffuse nodular pulmonary calcifications, as was first established in New Zealand by Mackay and Cairney in 1960 (26), and numerous cases have now been observed in Australia (1, 24). These observations, plus the demonstration by Bocles *et al* (5) that in varicella pneumonia abnormalities in alveolar capillary gas exchange persist after clinical recovery, add to the significance of nonfatal pulmonary involvement.

The status of varicella in tropical areas is not well defined. From personal observation it would appear that contact with V-Z virus in the tropics is often delayed until adulthood, although few data are available on this point. Two reports from Ceylon substantiate this view (10, 29). When adequate statistics become available, varicella may be recognized as a more serious problem in tropical than in temperate areas. Herpes zoster, of course, is age-dependent and will increase as life expectancy is extended.

As was noted earlier, the significance of acquired CMV infection is being elucidated; the prevalence of the recently described syndromes of CMV hepatitis and of CMV mononucleosis is now unknown.

Iatrogenic V-Z or CMV disease is an important complication of the use of steroids, immunosuppressives, or roentgen therapy. That certain patients on steroid therapy may die if infected with V-Z virus was first emphasized 10 years ago (14). Likewise, a fulminant varicella or cytomegalovirus infection may occur in patients under therapy for leukemia or other malignant processes. Zoster has long been associated with radiation or arsenic therapy. The increasing use of organ transplantation and of immunosuppressive agents has produced new problems. Zoster developed as an annoying complication following renal transplantation in 8 per cent of the patients in one series (34). Much more serious is evidence that an overwhelming cytomegalovirus infection may contribute to or be responsible for a fatal outcome in transplantation cases. The study of Kanich and Craighead (21) is illustrative. Evidence of active CMV infection was found at autopsy in 8 of 25 (32 per cent) renal homotransplant recipients who had received immunosuppressive drug treatment.

Mention may again be made of transfusion induced cytomegalovirus mononucleosis (19). Thus, iatrogenic V-Z CMV infections are rapidly assuming great significance.

Varicella is also of public health import, when confused with variola. This year variola minor appeared in England in February; the disease was reported as varicella until April, by which time 22 cases had occurred (25). Similarly, the newly recognized calcified miliary lesions of varicella pneumonia will pose problems in the interpretation of roentgenographic surveys.

Certain Problems to be Considered in the Production of Experimental Vaccines

The idea that the V-Z and CMV agents are avidly cell-associated *in vitro* is misleading—a point of practical import. While V-Z virus is cell-related under most conditions of propagation, the virus-cell association is not an inherent characteristic, as was demonstrated by Caunt (8, 9), who recovered cell-free infectious virus after propagation in cultures of human thyroid tissues. Although we obtained growth of V-Z virus in cultures of rabbit testicular tissue (60), little use has been made of nonprimate tissues. Therefore, the recent reports of Söltz-Szöts (43, 44) describing the propagation of V-Z virus in cultures of embryonic guinea-pig tissues, with the appearance of infectious virus in high titer in the fluid phase, come as a surprise and need confirmation.

The human cytomegaloviruses present a problem of cell specificity but not of strict cell avidity. At the moment CMV are routinely propagated in cultures of human fibroblasts, although human myometrial cells also suffice. After serial

propagation—sometimes necessarily prolonged—infectious virus usually appears in the culture medium in significant amounts. Search for other cell systems should continue. At present, human diploid fibroblast lines appear to be the sole candidate cells for possible vaccine production.

Definitive studies of the antigenic constitution of V-Z and CMV viruses have awaited the availability of suitable reference sera and the subsequent analysis of strains by sensitive plaque reduction or comparable procedures. Recently such a test for CMV and the preparation of reference sera in monkeys has been described (33). A V-Z antiserum that is active in the CF and direct fluorescent antibody reactions has also been prepared in monkeys (40). Yet the use of monkey reference antisera must be carefully controlled. Monkeys harbor as yet unclassified cytomegaloviruses (4, 27), some of which share common CF antigens with a human CMV strain. Our knowledge of the possible occurrence of simian V-Z counterparts is nonexistent, and we lack definitive data on the susceptibility of higher primates to human V-Z virus. That the monkey is not completely refractory to human V-Z virus was demonstrated by Rivers 40 years ago (35). Until we are able to work with monkeys known to be virginal with respect to past contact with antigens present in the human CMV or V-Z agents, the results obtained with monkey reference antisera should be interpreted conservatively.

Man is the only species now available as a test subject for experimental CMV or V-Z immunizing materials. At present, under no circumstances would the trial of live virus materials in man appear justified. A search for an alternative test model, possibly in the form of a lower primate with an artificially modified defense mechanism, is indicated. In the interim, exploration of the nature of the response of man to inactive virus might be initiated. A logical starting point would be exploration of the possibility that stimulating doses of killed V-Z virus might boost pre-existing antibody titers in elderly people and might thus minimize the misery so commonly the consequence of herpes zoster.

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SECTIONS A. AND B.

ARBOVIRUSES AND HERPESVIRUS GROUP VIRUSES

DISCUSSION

CHAIRMAN PARODI: We will now proceed with the discussion of both Sections A and B. Our first discussant is Colonel Edward L. Buescher, Walter Reed Army Institute of Research, Washington, D. C.

COL. BUESCHER: Vector-transmitted virus diseases, unlike those transmitted to man by direct or indirect contact, have complicated life cycles for their etiologic agents involving arthropods, lower-animal hosts, with man more frequently than not involved more as an accident than as an essential part of the natural transmission cycle. Infection of each of the natural hosts is characterized by systemic invasion from the basic portal of entry with or without ultimate localization in a target organ. These two general characteristics are the bases for concepts of the control of arthropod-borne virus infections: remove man from the effective natural transmission cycle either by protecting him from exposure to arthropod bite or direct contact with virus shedding hosts or by rendering him immune by immunization.

Both approaches have been attempted in the last 30 years to control those diseases that in temperate zones have a seasonal epidemic occurrence. Indeed, it was the need to understand their natural history that prompted the extended investigations of the tick-borne viruses in the Soviet Union; of Western equine, Eastern equine, and St. Louis encephalitis in the United States; of Japanese encephalitis in Japan; and of Murray Valley encephalitis in Australia. As the data emerged it became clear that the problems presented by ecological control varied for each virus, and often for the same virus in different geographic areas. Thus approaches to the control of tick-borne disease are different from those for mosquito-borne infections, and since mosquito behavior varies with species, the control of disease transmitted by several vectors either by the same or by different means requires methods specific for the vector species. These considerations are even more complex in tropical areas where infections appear to be endemic and transmission cycles more complex.

Rendering susceptible humans immune appears on the surface to be the simpler approach and the one sharing the larger number of common problems. The reviews of Drs. Casals and Hammon emphasized the multiple speciation of the agents. This multiple speciation, however, does not pose the problem presented by the rhinoviruses, because seldom do more than 10 or 15 agents appear to be active in a given area and rarely are all of them important causes of human disease. The problem of vaccines, then, is a technological one, which by virtue of the specific geographic distribution of the viruses themselves lends itself to a unique experience in multiple simultaneous development. The biological vagaries of the viruses notwithstanding, it seems clear that technical advances in vaccine development for any arbovirus will be significant and applicable to others that are important causes of human disease.

CHAIRMAN PARODI: Thank you, Col. Buescher. The next discussant is Dr. Karl M. Johnson from the Middle America Research Unit, Balboa Heights, Canal Zone.

DR. JOHNSON: Dr. Mackenzie emphasized the value of rodent-control measures in the prevention of South American hemorrhagic fever, particularly in Bolivia. This is entirely proper; I know of no other example of a virus disease

affecting man in which knowledge concerning the malady was effectively applied so rapidly after the etiologic agent was first recognized. Although much more epidemiological information is needed, there are some indications that the Argentine disease may not be as readily controlled by such methods. Therefore, I should like to make four points concerning future prospects for the development of safe, successful vaccines for Tacaribe-group agents, particularly Junín and Machupo viruses.

The first is that it is nearly inconceivable that such vaccines will come from private industry, for reasons already discussed.

The second is that any attempt to solve the problem must cope with the fact that Machupo, and probably fresh isolates of Junín, viruses are highly hazardous organisms. Machupo virus has already killed two persons in laboratories under circumstances that suggest aerosol transmission. There is no doubt that in terms of engineering it will be costly to combat this sobering situation in order that effective work may proceed.

The third point is that we must know much more about the properties of these viruses before even pilot vaccine experiments can be attempted. Unfortunately, I do not believe that we can use the facts that are now being uncovered about certain arboviruses. For although Tacaribe agents contain RNA and have lipid coats, no details of their morphology are known. I further believe that when pictures *are* made, these viruses will turn out to be larger than, and in some ways different from, arboviruses such as yellow fever, Sindbis, or Venezuelan equine encephalitis.

The final consideration is that there are truly major obstacles ahead in terms of safety testing for candidate vaccines. In the case of inactivated preparations, what host can we use to test for residual infectivity? For live vaccines, we face the excruciating hurdle of deciding whether virus attenuation for any other host implies a safe margin for man.

This somewhat gloomy forecast deserves a codicil. Considerable experience in several laboratories during the past few years indicates that Tacaribe virus itself may be studied without undue hazard to personnel. It can be and is being used to learn more about this entire virus group. Another promising lead is the discovery that the Central American marmoset, *Saguinus geoffroyi*, is a clinically sensitive host for Machupo virus, with an LD_{50} comparable to the infant hamster. Since it is possible to inoculate relatively large amounts of virus, marmosets may prove useful in tests for residual infectivity; they also may be important hosts for preliminary assessment of virus attenuation. Finally, there does exist a most important resource—small groups of scientists who have seen these ravaging diseases and who remain dedicated to their elimination from human medicine.

CHAIRMAN PARODI: The next discussant will be Dr. Charles L. Wisseman, Jr., Professor and Head, Department of Microbiology, University of Maryland School of Medicine, Baltimore, Maryland.

DR. WISSEMAN: I should like to report very briefly on the current status of a live attenuated Type 1 dengue vaccine. Through manipulation in suckling mice, which included serial terminal dilution passages, the partially attenuated Type 1 dengue strain of Sabin was considerably modified. After characterization in laboratory animals, this new strain was first tested in small groups of human volunteers at doses of 10^4 and 10^5 suckling mouse i.c. LD_{50}. Aside from minimally detectable regional lymphadenopathy, no clinical reaction was discernable. However, neutralizing antibodies began to appear in two weeks and were present in all subjects after three weeks. A larger field trial in airborne troops, done in collaboration with the Department of Virus Diseases of the Walter Reed Army Institute of Research and involving about 300 men, revealed the following:

1. Reactions to the vaccine were minimal. No man lost time from duty because of it.

2. A mild rash, often unnoticed by the subject, occurred in about 12 per cent of the vaccinees. It caused no difficulties.

3. Titration of the vaccine in man indicated that a small dose (10–100 suckling mouse i.c. LD_{50}) would induce antibody formation.

4. Vaccinated subjects resisted challenge with unmodified Type 1 dengue virus 60 days after vaccination.

A limited field trial in young adult males in Puerto Rico during the 1963 dengue epidemic, involving approximately 600 vaccinated and 600 control subjects, revealed that: (1) the vaccine

was well tolerated under field conditions; and (2) it reduced the attack rate of the heterologous Puerto Rican dengue type to about half of that in the controls, beginning about three weeks after vaccination. Thus, it appeared to confer a significant degree of heterologous immunity.

Since the vaccine consists of a suspension of infected suckling mouse brain, we are currently involved in an attempt to discover whether passenger murine viruses are present and, if they are, to eliminate them.

CHAIRMAN PARODI: We now have Dr. Donald R. E. MacLeod, Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

DR. MACLEOD: In a previous report * the preparation and trial in animals of a formalin-inactivated B virus vaccine was described. Though some degree of protection was obtained, the potency was low and freedom from residual live virus could not be assured. We came to the conclusion that with so virulent a virus, unless special methods for testing for residual virus could be developed, we could not prepare a vaccine for human use. Safety precautions in handling monkeys had been greatly increased; furthermore, we had evidence of the protective effect of immune monkey antiserum in animals. We therefore abandoned the attempt to prepare a vaccine and continued studies of the use of immune serum for passive prophylaxis.

* MacLeod, D. R. E., Shimada, F. T., and Walcroft, M. J. "Experimental Immunization against B Virus." *Ann NY Acad Sci* **85**:980–989, 1960.

We were able to report, in 1960, that rabbits could be protected against intracutaneous inoculation of B virus by infiltration of the site with immune monkey gamma globulin. This serum concentrate was prepared by precipitation with ammonium sulphate. We have prepared several lots and, since 1960, have kept a small stock for use in humans after possible exposure to B virus.

Experiments have been made in rabbits on the protective effect of immune globulin given at different times after infection and inoculated by different routes. The results of three representative tests are given in Table 1. Rabbits were inoculated intradermally with 200 $TCID_{50}$ B virus contained in 0.2 ml; then, after various intervals, immune monkey serum concentrate was inoculated into the same site or into the muscles of the same or of the opposite limb. The volume injected locally was 0.75 ml, which is about the volume required to infiltrate a small skin wound in a man. The volume injected into the same limb (0.2 ml) is approximately equivalent to 5 ml in a man, an amount which can be injected into the arm. The volume injected into the opposite limb (0.8 ml) is approximately equivalent to 20 ml in a man, a volume that can be given into the gluteal muscles. The rabbits were observed for 42 days. Animals dying during this period were examined *post mortem*. The brain and cord of one rabbit that died in each group of three were examined microscopically.

Local infiltration with the immune globulin six hours after infection protected all rabbits. Local injection of serum 24 hours after infection

TABLE 1. PASSIVE PROTECTION OF RABBITS AGAINST B VIRUS BY IMMUNE MONKEY GAMMA GLOBULIN GIVEN AFTER INFECTION

Challenge B virus intracut.* ($TCID_{50}$)	Interval, virus to gamma globulin (hours) No. of rabbits died. Observ. period-42 days				
	1/2 hr.	2 hrs.	6 hrs.	24 hrs.	Controls
	G.G. locally. Intracut. 0.25 ml. Subcut. 0.5 ml.				
200	N.T.	0/3	0/3†	1/3	3/3
	G.G. same limb. Intramusc.‡ 0.2 ml.				
200	1/3	1/3	1/3	2/3	3/3
	G.G. opposite limb. Intramusc.‡ 0.8 ml.				
200	3/3	3/3	2/3	3/3	3/3

*Posterior aspect of left leg.
†One of three rabbits injected died of pneumonia after 30 days. No evidence of B virus infection.
‡Hamstring muscles.

protected two of three rabbits. Antiserum injected into the muscles of the same limb protected some of the animals, when given 6 hours after infection and possibly even when given after an interval of 24 hours. Inoculation of serum into the opposite limb, however, gave no protection.

From the results of these and other experiments in rabbits we have considered the following procedures justified for prophylaxis after possible exposure to B virus. The wound site is infiltrated with approximately 0.75 ml of immune monkey globulin as soon as possible, and 5 ml is injected into the muscles of the same limb. If this is not possible because of the site of the wound, as for example the eye, or if virus fluid is swallowed, 20 ml of the serum is injected into the gluteal muscles. This may be of some value, even though experiments in rabbits have provided no evidence that it would be effective.

Immune monkey gamma globulin has been injected into seven persons (Table 2). No reactions have occurred. The probability of exposure to B virus was too low to draw any conclusions regarding the prophylactic effect of the serum. Two individuals received two doses of 5 to 15 ml and one subject has been given eleven doses—nine small (0.1 to 0.4 ml) and two large (15 ml). Passive antibody to B virus was detected 1 to 28 days after the first large dose,

TABLE 2. INOCULATION OF HUMANS WITH MONKEY GAMMA GLOBULIN

No. of persons	No. of doses	Volume injected (ml)	Reactions
7	1 or more	5 to 20	None
2	2	5 to 15	None
1	11	0.1 to 15	None

which followed five small doses, but not after the second injection of 15 ml (Table 3). The reason for this is not known. It may be due to the development of antibody to the monkey gamma globulin, after repeated doses, or to variability in the test system or in the potency of the immune globulin used.

CHAIRMAN PARODI: Thank you, Dr. MacLeod. Our next discussant is Colonel William D. Tigertt, Director, Walter Reed Army Institute of Research, Washington, D. C.

COL. TIGERTT: I should first like to ask you to appreciate my predicament; being No. 5 in a panel of six discussants sometimes restricts what one can say.

I should like to refer briefly to problems of administration of attenuated arbovirus vaccines to man—and to one in particular that I suspect we shall all hear much more about in the future.

Dr. Hammon indicated that in his opinion millions of people should be involved in any appropriate field trial. I think it is imperative

TABLE 3. RESULTS OF 11 INOCULATIONS OF MONKEY GAMMA GLOBULIN IN ONE SUBJECT

Date	Volume (ml)	Route	Remarks
			No antibody to B virus or herpes simplex virus
17 Dec. 1958	0.1	Intracutaneous	
18 Dec. 1958	0.2	Subcutaneous	
19 Dec. 1958	1.0	Intramuscular	
5 Jan. 1959	0.1	Intracutaneous	
11 Feb. 1959	0.1	Intracutaneous	
	0.3	Subcutaneous	
18 Feb. 1959	15	Intramuscular	Antibody to B virus and herpes simplex virus detected 1 to 28 days after inoculation
12 July 1960	0.2	Intracutaneous	
7 Apr. 1961	0.1	Intracutaneous	
	0.3	Subcutaneous	
July 1963	0.1	Intracutaneous	
	0.2	Subcutaneous	
19 July 1966	0.1	Intracutaneous	
	0.2	Subcutaneous	
30 Sept. 1966	15	Intramuscular	No antibody to B virus or herpes simplex virus detected 2 to 28 days after inoculation

to consider what might occur after the administration of any product to so many people. I am particularly concerned at the moment with the small fraction of such individuals who have some derangement in their immunologic response mechanisms.

It has been known for years that individuals with Hodgkin's disease and related problems very frequently suffer severe illnesses when exposed to several viruses. This is known to be true of some of the attenuated vaccine viruses now available for study or in actual use. Similarly, and perhaps less obviously, I think that all of us would eliminate from any such field trial individuals with a fully expressed (Louis) bar picture. Yet the information that is now becoming available, through neurologists, indicates that this particular hereditary expression of illness very frequently is not a full-blown picture and that here individuals with quite modified levels of gamma globulins might be included in such a study. Finally, there have been repeated reports that individuals with a history of recurrent furunculosis are poor subjects to include in such a study. I offer no answer to these problems; I am not sure that the answer is available at present. But I do think the area in question deserves very serious investigation in the future.

CHAIRMAN PARODI: Our last formal discussant is Dr. T. F. McNair Scott, Professor of Pediatrics, University of Pennsylvania, Philadelphia, Pennsylvania.

DR. SCOTT: Drs. Hull and Peck have presented procedures for the prophylaxis of the three important infections of the herpes group of viruses:

1. *B virus (Herpesvirus simii).* It is clear from their data that B virus vaccine is effective in producing antibodies in man that can be recalled by repeated inoculations. This procedure would seem to be adequate for monkey handlers. Perhaps it should also be recommended for a group of persons not usually thought of as monkey handlers—that is, those engaged in using these animals for psychological and other tests, since as a rule they are not oriented to thinking of the dangers of this virus infection.

2. *Virus of pseudorabies (Herpesvirus suis).* This is not a hazard to human health but is an economic hazard. It is relatively uncommon in the United States, and the authors indicate the potential value of a formalin-killed vaccine. In Eastern Europe, where the disease is a major problem and where inactivated vaccines had been tried unsuccessfully for years,* live attenuated vaccines have now been developed and successfully tested in the field † using a variant described in 1957.‡

3. *Virus of herpes simplex (Herpesvirus hominis).* Prophylaxis is required (a) to prevent the primary infection in certain individuals who are at risk of death, and (b) to prevent recurrences of herpes in individuals in whom the recurrences occur in the eye, with a serious risk of corneal scarring and blindness, or are distressingly frequent.

(a) Neonates often die as the result of a primary herpetic infection. No active immunization of them is feasible, and so passive immunization must be considered when clinical herpetic infection is recognized in a woman at the time of or shortly before delivery. The only source of antibodies is commercial gamma globulin, which should be administered to the infant immediately after birth. In most lots of commercial gamma globulin there are 1,200 to 2,000 units of neutralizing antibody per ml; 5 ml to an average newborn should therefore provide a protective circulating level of about 20 units per ml.

The reported failures of this procedure may be due to the fact that commercial gamma globulin consists entirely or almost so of the Gamma G fraction of immunoglobulin. Tokumaru § recently observed that maternal serum completely neutralized a given dose of herpes virus in one hour even when diluted 1:16, but the cord blood, which did not contain Gamma A fraction or the maternal serum from which Gamma A

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* Manninger, R. and Mócsy, J. *Traité des maladies internes des animaux domestiques.* Paris: Vigot Frères, pp. 457–464, 1959.

† Bartha, A. "Attempts at Attenuating the Virulence of Aujeszky's Disease Virus." *Magy Allatorv Lap* **16**:42 45, 1961.

Barócasai, G., and Tanezer, D. "Experiences in active Immunization against Aujeszky's Disease." *Magy Allatorv Lap* **17**:350–351, 1962.

Škoda, R., Brauner, I., Sádecký, E., and Somogyiová, J. "Immunization against Aujeszky's Disease with Live Vaccine. II. Immunization of Pigs under Laboratory Conditions." *Act Virol (Praha)* **8**:123–134, 1964.

‡ Tokumaru, T. "Pseudorabies Virus in Tissue Culture. Differentiation of Two Distinct Strains of Virus by Cytopathogenic Pattern Induced." *Proc Soc Exp Biol Med* **96**:55–60, 1957.

§ "A Possible Role of γ A Immunoglobulin in Herpes Simplex Infection in Man." *J Immun* **97**: 248–259, 1966.

had been removed, was unable to neutralize the same dose of virus completely, even when undiluted. The provision of an injectable preparation of Gamma A might be a useful addition to this prophylactic procedure.

(b) The prophylaxis of recurrences is a much more difficult problem. The authors have indicated that their vaccine has been effective through the mechanism of desensitization. Similar results with inactivated vaccines have been reported by others,* but these are difficult to interpret because of the suggestibility of the subjects and there are hazards to this procedure. For example, one individual without previous history of clinical herpes developed an attack of genital herpes after inoculation; another had a flare-up of acute keratitis.† Erythema multiforme has also been induced by this procedure. The authors indicate that they have not encountered similar difficulties.

Passive immunization may be possible for those with recurrent ocular manifestations. Individuals liable to recurrent herpes have been shown to belong to a population of poor Gamma A producers.‡ The stress that always precedes a recurrence may reduce the already low Gamma A below a critically protective level, thus allowing multiplication of the reactivated virus in the avascular cornea. The provision of added Gamma A through the routine use of eye drops by those at serious risk might be explored.

CHAIRMAN PARODI: Thank you, Dr. Scott. To start our free discussion, I recognize Dr. Blaškovič.

DR. BLAŠKOVIČ: I should like to make reference first to arboviruses. It is a well-established fact that domestic milk-giving animals play a role in the epidemiology of tick-borne encephalitis (TE) in European countries. These animals, after being bitten by a virophorous tick, develop viremia and subsequently excrete the virus with the milk. Goats are of extreme importance as a natural focus of tick-borne encephalitis. Their infectious raw milk serves as a source of human infection, and the viremic animals infect blood-sucking uninfected ticks.

In 1959 and 1960 at the Institute of Virology, Czechoslovak Academy of Sciences, Bratislava, an immunization program involving domestic animals—goats, sheep, and young cattle—was carried out using both formalin-inactivated and virulent viruses. The immune response after one immunization dose was favorable to the live virulent vaccine. The vaccination procedure was suggested to elicit an immune state and to prevent further viremia and virus excretion with the milk. The immune animals were no longer a source of infection either for man or for uninfected ticks.§

It was clear, however, that an immunization program cannot be undertaken with live virulent vaccine. Mayer of our laboratory developed a clone of TE virus designated Hy-HK$_{28}$/2″ from a virulent prototype strain Hypr. This clone, after numerous passages in human amniotic and hamster kidney cells differed in many genetic markers from the parental virus; it multiplied only in intracerebrally infected mice (Table 1). Its virulence in intrathalamically inoculated *M. mulata* monkeys decreased at least 7 logs when compared with a virulent TE virus strain.‖ This clone did not induce viremia in subcutaneously inoculated goats, and no virus excretion by milk occurred in either laboratory or field experiments. A marked resistance developed against the challenge in the highly virulent virus (Table 2). The clone is also immunogenic in sheep and cattle. Its practical use for immunizing domestic animals (goats, cattle, sheep) is being considered.

With reference to the matter of herpesvirus group viruses, I should like to comment briefly on the paper presented by Dr. Hull and to support the comments of Dr. Scott.

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* Lépine, P., de Rudder, J., Maurin, J., and Henocq, E. "Essac de thérapeutique de l'herpès récidevant par un vaccin préparé en culture cellulaire et inactivé par les rayons ultra-violets. I. Préparation du vaccin et essacs d'immunisation sur l'animal." *Sem Hop Paris* **40**:1471–1473, 1964.

Henocq, E., de Rudder, J., Maurin, J. and Lépine, P. "Essac de thérapeutique de l'herpès récidevant par un vaccin préparé en culture cellulaire et inactivé par les rayons ultra-violets. II Essais cliniques." *Sem Hop Paris* **40**:1474–1480, 1964.

† Chapin, H. B., Wong, S., and Reapsome, J. "The Value of Tissue Culture Vaccine in the Prophylaxis of Recurrent Attacks of Herpetic Keratitis." *Amer J Ophthal* **54**:255–265, 1962.

‡ Tokumaru, *op. cit.*

§ Blaškovič, D. "Note on the Problem of the Prevention of Tick-Borne Encephalitis." *J Hyg Epidem (Praha)* **3**:132 137, 1959; and Blaškovič, D. Edit. The Importance of Deliberate Immunization of Domestic Animals in a Natural Focus of Tick-Borne Encephalitis. Publishing House of the Slovak Academy of Sciences, Bratislava, 118 pp. (in Slovak with English and Russian summary), 1962.

‖ Mayer, V. "A Mutant of Tick-Borne Encephalitis (TE) Virus with Lost Neurovirulence for Monkeys." *Acta Virol (Praha)* **10**:573, 1966.

TABLE 1. CHARACTERISTICS OF TICK-BORNE ENCEPHALITIS VIRUS CLONES DIFFERING IN VIRULENCE FOR MACACCA MULATTA MONKEYS

Parental virus strain	"Hypr"	
Clone	P III-E	Hy-HK$_{28}$"2"
Characteristics	$ic^+ sc^+ s^+ t^+ e^+ u^r$	$ic^+ sc\ s\ t\ c\ u^s$
Neurovirulence in LD_{50}/ml	$\geq 3.16 \times 10^7$ N^+	$\leq 0.3 \times 10$ N

ic^+: Virulence for 6–8 g mice after intracerebral inoculation.
sc^+, sc: Ditto after subcutaneous inoculation.
s^+, s: Plaque diameter of clones in chick embryo cell cultures.
t^+, t: Degree of thermoresistance at 50° C.
e^+, e: Elution from hydroxylapatite columns by various Na_2HPO_4 molarities.
u^r, u^s: Denaturation of infectivity and haemagglutinating activity by 2 M urea at 35° C.
N^+, N: Degree of virulence for *M.mulatta* monkeys after intrathalamic inoculation.

A large-size plaque variant (diameter >3 mm) of pseudorabies virus, Bucharest strain, was continuously passaged in our laboratory on chick embryo cell tissue cultures, undergoing there 620 passages. The clone of this variant was found to be sufficiently attenuated in its virulence for pigs to be worthy of field trials. After good results in these trials, a commercial attenuated pseudorabies virus vaccine has been manufactured in Czechoslovakia since 1963. It is used in large piggeries with very satisfactory results. Since the virus did not completely lose its virulence for cattle, they are not vaccinated with the vaccine.

Pseudorabies infection in pigs is considered a serious veterinary health problem in the countries of the Danube Valley. However, according to some reports, some other countries are also concerned about this problem.

TABLE 2. TICK-BORNE ENCEPHALITIS VIRUS IN BLOOD AND MILK OF GOATS AFTER SUBCUTANEOUS ADMINISTRATION OF THE VIRULENT P III-E CLONE OR AFTER THE BITE OF VIROPHORIC IXODES RICINUS TICKS

Nonimmunized goats

Animal no.	Infections dosis	Day after infection 3	4	5	6	7	8	9	10	11	12
1		●	0	0	0	●	0	0	0	0	0
		+	0	0	0	+	0	0	0	0	0
2	10^6	●	0	0	●	●	0	0	0	0	0
	LD_{50}	+	0	0	+	+	0	0	0	0	0
3		0	●	0	●	0	0	0	0	0	0
		0	+	0	0	0	0	+	0	0	0
4		0	●	●	●	0	0	0	0	0	0
		0	+	+	+	0	0	0	0	0	0
5		●	●	●	●	0	0	0	0	0	0
	ticks*	−	−	−	−	−	−	−	−	−	−
6		●	●	●	0	0	0	0	●	0	0
		−	−	−	−	−	−	−	−	−	−

Goats, challenged 3 months after immunization with 10^6 LD_{50} of the attenuated Hy-HK$_{28}$ "2" clone (one shot)

Animal no.	Challenge dosis	Day after challenge 3	4	5	6	7	8	9	10	11	13
7		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
8	10^6	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
9		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
10	10^9	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
11		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
12	ticks*	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0

●: virus in blood
+: virus in milk
0: no virus recovered
−: non lactating goat
*: 8–9 ticks per goat

DR. KAUFMAN: As one primarily interested in the blinding problem of recurrent herpes, I was fascinated by Dr. Hull's paper. I think it is important to bring to the attention of the audience some of the confusion in this general area.

Therapies such as repeated injections of polio vaccine, of rabies vaccine, or of smallpox vaccine, substances such as bee venom, and a variety of nostrums have all been claimed to be capable of preventing recurrent herpes. Most of this evidence has been based on results with patients who had had many recurrences, which appeared to decrease in severity or intensity. Such evidence is at best enormously difficult to evaluate, especially in view of the great psychological component involved. Even if we do not question the adequacy of the double-blind control (which is indeed open to question) the numbers are too small to be of any real significance.

It is important to remember that a beautiful study by Carroll in Boston and workers in Pennsylvania and in New York indicates that, of patients who have previously had recurrent attacks of ocular herpes simplex, some 67 per cent of those who receive no additional treatment will not have another attack within the next two years.

The need for adequate controls in evaluating this type of vaccine treatment is great, and the evaluation itself is extremely difficult.

To my knowledge, none of the other uncontrolled vaccine studies has shown improvement at a rate any higher than that of untreated patients.

DR. UNGAR: I should like to ask Dr. Hull two questions. First, what type of vaccine did he use—an autogenous vaccine in each instance, or a stock vaccine? Second—in connection with the opinion he expressed that the treatment probably results in desensitization and his remark that some of the findings seen in the skin biopsy resemble tuberculin reaction—did he see this reaction after the primary immunization or in patients given booster doses?

I wholehcartedly support the view of the previous speaker. I have had a chance to follow some of herpes keratitis trials, and at the end someone always said: "I wish we had a few hundred more controls to see what the spontaneous remissions are."

DR. MELNICK: My question is also addressed to Dr. Hull. We have heard repeatedly over the last year or so about the work he has been doing with B virus vaccine, and certainly the results he presented today are as good as, or better than, any that I have seen to date.

I want to ask him about the availability of this vaccine. How can it be made available to monkey handlers and other laboratory workers who are at risk working with this dangerous virus?

DR. HULL: First, the question about what strain of herpesvirus was used and whether it was an autogenous strain from the patient or a general laboratory strain.

The strain used throughout these studies is known as the Mayo 1814 strain and was isolated originally by Dr. Heilman at Mayo from a patient with recurrent herpes. This has been compared with many other strains, and we see no appreciable antigenic differences between it and fresh isolates and other laboratory strains.

As to Dr. Melnick's question concerning the availability of the B virus vaccine, this is a question we anticipated. The matter is beyond the realm of science, actually; it is the concern of management and legal people. All I can say at this time is that a license application has been filed with the Division of Biologics Standards and that thus far we have not felt we could release the vaccine outside of our own laboratories until this license was obtained.

I should like to pass the question about skin reactions on to my co-author, Dr. Peck.

DR. PECK: The hypothesis that the pathologic lesion we know as herpes simplex might be the result of tissue hypersensitivity is based on the rather striking similarity of the histologic picture of these lesions as they develop to those seen in allergic states such as urticaria and possibly even the tuberculin reaction. Histologically these and the lesion of developing herpes simplex show perivascular cuffing with mononuclear cells, very slight if any intracellular infiltration of leukocytes, and edema all out of proportion to the amount of inflammatory response seen. This was pointed out many years ago by Unna and remains true today.

I believe Dr. Kaufman mentioned the difficulties on double-blind studies. No one will agree

with him more than I. It took over two years just to obtain at one center the number of patients with what we hoped was severe enough herpes to get some kind of answer. It has been our experience that most dermatologists and ophthalmologists who hold teaching positions at various universities or are in private practice will only have one or two patients with severe recalcitrant lesions in their practice at any one time. This makes it almost impossible to conduct double-blind studies.

Furthermore, I have not yet found an ophthalmologist—and we have many of them cooperating with us—who is willing to give placebo to a patient with active herpetic keratitis that has not responded to anything else and is steadily getting worse. They want to try to prevent blindness if they can.

Variability of response to therapy is a problem that we must face in the evaluation of this antigen. It is the reason that we picked the most highly selected group of recalcitrant patients with facial lesions who have had no periods of remission for at least 36 months before they received their series of doses of antigen. They had previously had such treatments as smallpox vaccine, poliomyelitis vaccine, X-ray, even surgical procedures to try to remove the skin and subcutaneous tissue from these infection sites—all to no effect, and with no periods of remission.

This is why we feel that we may possibly be on the right track. Tokumaru's work is extremely important, I think, and we hope that the study we now have under way will give us some serologic evidence of the effectiveness of this vaccine. We have had none so far.

DR. DEINHARDT: Without wishing to add to the anxiety about herpes B virus of Asiatic monkeys, I should like to mention that South American nonhuman primates have their own simian herpesvirus, which was isolated simultaneously by Dr. Melnick's group and by us several years ago. Perhaps fortunately, we do not know what this virus, marmoset or platyrrhine herpesvirus, will do in man, and I have no intention of trying to find out.

The only saving grace is that animals preimmunized with herpes simplex of man will be protected against disease if challenged with the South American primate herpesvirus. With the permission of Dr. Hull, I can state that there even seem to be two antigenically different herpesviruses of South American monkeys.

This new group of herpesvirus is dormant in a variety of South American monkey species, and in handling these monkeys one should be aware of it.

SESSION V

EXANTHEMS AND MUMPS

Wednesday, 9 November 1966, at 8:30 a.m.

CHAIRMAN

DR. O. V. BAROYAN

RAPPORTEUR

DR. W. CHAS. COCKBURN

Section A.

Rubeola

Presentation of Papers by:

Dr. John F. Enders
Dr. Erling C. Norrby
Dr. René Labusquière
Dr. Conrado Ristori
Dr. V. M. Bolotovsky
Dr. Frank T. Perkins
Dr. Harry M. Meyer, Jr.

Discussants:

Dr. W. Chas. Cockburn
Dr. Samuel L. Katz
Dr. Saul Krugman
Dr. M. V. Milovanović
Dr. M. Matumoto
Dr. François Kalabus
Dr. Anton J. Schwarz

Section B.

Rubella

Presentation of Papers by:

Dr. John L. Sever
Dr. J. Corbett McDonald

SECTION A. RUBEOLA

PRESENT STATUS OF LIVE RUBEOLA VACCINES IN THE UNITED STATES

JOHN F. ENDERS and SAMUEL L. KATZ *

DR. ENDERS (*presenting the paper*): To assess comprehensively the present status of live rubeola vaccine is not feasible within our time limit. We shall therefore confine ourselves in the main to a consideration of certain aspects of the experience with mass vaccine in the United States (1, 2), supplementing this with other relevant data. In this way we hope to contribute to the broader evaluation of the status of these vaccines that this session will produce.

As a beginning it may be useful to recapitulate once again the essential facts regarding the development and properties of the three live measles vaccines that have so far been employed in this undertaking. The development of measles vaccine was preceded by the demonstration that the virus could be isolated or cultivated *in vitro* in appropriate cell systems and that under those conditions its virulence for man was eventually attenuated.

Of the attenuated measles vaccines to be considered, the first was described in 1958 and is now usually referred to as the Edmonston B attenuated live measles vaccine (3). It was developed in our laboratory after the demonstration that measles virus could be isolated and propagated in human and simian cell cultures. After adaptation to chick cell systems it was found that the so-called Edmonston strain induced a modified infection in monkeys and in susceptible children. This infection is characterized principally by the absence of demonstrable viremia and virus excretion, lack of transmissibility to susceptible contacts, and absence or modification of the characteristic signs and symptoms of measles. The first lots of vaccine prepared and tested were designated Edmonston A to distinguish them from those subsequently referred to as Edmonston B, which consisted of the same virus that had been subjected to several additional passages in chick cell systems. In March 1963, Edmonston B vaccine was licensed for general distribution in the United States.

Two years later, in February 1965, the further attenuated live measles vaccine developed by Schwarz and first described in 1961 (4) was licensed for distribution. This preparation was derived from Edmonston A virus that Schwarz had subjected to an additional prolonged series of passages in chick embryo cell systems. During these passages the reactogenicity of the virus became further reduced. The further-attenuated virus (FAV), like Edmonston B vaccine, consists of virus propagated in chick embryo cell cultures.

A third live vaccine, also licensed in early 1965, consists of chick embryo-adapted Edmonston virus grown in dog kidney cell cultures.

We shall comment here on the principal features of the experience gained so far in large scale vaccination with two of these products: Edmonston B vaccine administered with or without gamma globulin, and the further-attenuated vaccine of Schwarz given without gamma globulin. The dog kidney vaccine, which is distributed only with the recommendation that it be administered with gamma globulin, until very recently has not been widely employed.

Quantity of Vaccine Distributed

Any inference about the effects of mass vaccination must, of course, be based on the num-

* From the Research Division of Infectious Diseases, The Children's Medical Center; The Children's Cancer Foundation and the Departments of Bacteriology and Immunology and of Pediatrics, Harvard Medical School, Boston, Massachusetts.

ber of persons vaccinated. The quantities of vaccine distributed by the manufacturers provide a basis for estimating this number. According to the Communicable Disease Center (CDC) of the U.S. Public Health Service, approximately 15 million vaccine doses were distributed in the United States during the three-year period from March 1963 through March 1966. Of this total it is estimated that about 7 million doses were released during the first 24 months and about 7.9 million during the last 14 months.

As a further basis for evaluation we have independently attempted to estimate the proportional distribution of Edmonston B and FAV. In making this attempt we are indebted to certain manufacturers of these products for their cooperation. Although our estimates based on incomplete information are extremely crude, they suggest that during the three-year period 8 to 9 million children received Edmonston B and 3 to 4 million received FAV. It would also be desirable to know the amounts of Edmonston B vaccine that have been administered with and without gamma globulin. Again, unfortunately, exact figures are not available. However, with the help of estimates supplied through the kindness of certain of the producers of vaccine we may state with some confidence that in 1963 and 1964, when Edmonston B was the only vaccine available, almost all of it that was distributed was used with gamma globulin, as was a high proportion of that distributed in 1965 and 1966. A smaller quantity of Edmonston B vaccine, estimated at about 600,000 doses, was also released for use without gamma globulin during 1965 and 1966.

SAFETY

Incidence of Complications

In confirmation of the low incidence of serious complications recorded in earlier field trials in this country and abroad, the numbers of reported severe illnesses or deaths associated closely in time with administration of either of the vaccines is extremely small in comparison to the number of persons inoculated. It has been possible to collect only 30 examples of unusual sequelae associated closely in time with vaccine administration.

Approximately half of these consisted of central nervous system manifestations: encephalitis, aseptic meningitis, cerebellar ataxia, loss of motor function, or seizure disorders. Four cases of thrombocytopenic purpura have followed use of vaccine, and three infants underwent exacerbation of atopic eczema. There have been three cases of tuberculous meningitis, one of them fatal. Other fatal cases that have been temporarily associated with the immunization procedure are one case of sudden death, one child who succumbed with exfoliative dermatitis, one case of fulminant hepatitis, one of acute rhabdomyolysis with myoglobinuria, and one of acute hemolytic anemia with renal cortical necrosis. Prior to the licensing of the vaccine, a patient with leukemia developed giant cell pneumonia after receiving live attenuated measles virus. We are also aware of an infant with the "Swiss" type of agammaglobulinemia who developed a similar pneumonia after measles vaccination.

It is not possible to give an accurate figure on the incidence of febrile seizures among children with elevated temperatures in the post-vaccination period, but this has apparently been rare enough or of insufficient concern to warrant consistent reporting. Only 12 such cases were reported during the early period, during which more than 4.5 million doses of Edmonston B were initially distributed. It is noteworthy that this low incidence of convulsive disorders contrasts with figures reported in studies conducted elsewhere. No entirely satisfactory explanation for these discrepancies has as yet been presented.

It should be emphasized that, except possibly with the leukemia cases, there is no direct evidence that the vaccine virus was etiologically involved in any of the cases just summarized. Moreover, there is no indication from the available data that one type of vaccine was more often associated with these complications than another.

However, the temporal association of vaccination with tuberculous meningitis, although extremely rare, again raises the question of the vaccine's possibly having an activating effect on the tuberculous process—a question that has already been considered by certain investigators. At present, obviously, no final answer can be given. But *a priori* it appears unlikely that the vaccine has this effect, not only because of the rarity of the association, but also because of the

lack of evidence suggesting that vaccination acts as a potentiating agent in tuberculosis, although such evidence has been sought in two studies on the effect of live measles vaccine in tuberculous children under treatment. Clearly, however, additional clinical investigations and epidemiological analysis on this point are desirable. Until such studies are completed there seem to be no substantial grounds for requiring tuberculin testing before vaccination in community immunization programs. For, as the Public Health Service Advisory Committee on Immunization Practice has lately emphasized (6), the risk from natural measles often far outweighs the theoretical hazard of possible exacerbation of undiagnosed tuberculosis.

Lack of Evidence for Extraneous Viruses in Vaccine

In considering the safety of any live virus vaccine, the possibility that unrecognized agents may be unwittingly included must always be kept in mind. The very low incidence of reported complications suggests that extraneous viruses capable of causing clinical signs of acute infection have not been present in the live vaccines. As to the possible presence of oncogenic agents or others capable of inducing occult chronic infections, the data, of course, are silent, since the period of observation has been relatively short. The requirements for the manufacture and safety-testing of live measles vaccine were devised, however, to eliminate the inclusion of such agents insofar as is possible by means of available techniques.

The possibility that measles virus itself may possess oncogenic potential was suggested by the increased incidence in chromosome breakage in leukocytes observed during natural measles and to a less degree after measles vaccination. So far as we are aware, there is no indication from observations made either in the present mass vaccinations or in earlier trials that live measles vaccine is oncogenic. We may note that recent experimental data likewise give no support to this hypothesis; Hilleman and his associates (7) found measles virus to be nononcogenic in hamsters, and Dr. George Diamondopoulos in our laboratory, in a study of hamster embryonic cells chronically infected with this agent during a period of over two years, obtained no evidence of enhanced oncogenic transformation attributable to the virus.

Reactions to Live Vaccine after Immunization with Inactivated Virus

For the sake of completeness, mention should be made at this point of recent reports, as yet largely unpublished, of unusual reactions that have followed the inoculation of attenuated vaccines in subjects who had previously received inactivated measles vaccine. Since Dr. Katz will later discuss them in detail, we shall only remark here that no such reactions in previously unvaccinated recipients have been reported during mass immunization (8).

Acceptance of Live Measles Vaccines

General acceptance of a vaccine depends largely on two factors, which tend to be inversely related: (1) the gravity of the disease against which protection is sought, and (2) the danger and discomfort of vaccination. Trials of experimental lots of Edmonston B vaccine showed that it induced fever of 103°F (39.5°C) or higher in approximately 30 per cent of the recipients and a modified rash in approximately 50 per cent. Although in many instances these reactions were not accompanied by malaise or disability, they were regarded by many investigators as too severe to be freely acceptable by parents and physicians as a prophylactic against a disease that is often predominantly benign. Concern over possible harmful effects of the febrile reactions was also expressed. Accordingly, means to reduce the reactogenicity were sought, and were soon found in the concomitant administration of gamma globulin or in further attenuation of the virus. Both procedures reduced the incidence of marked febrile reactions to Edmonston B vaccine by about 15 to 20 per cent and the occurrence of rash by about 35 to 40 per cent. With the manufacture of Edmonston B vaccine on a large scale and under carefully controlled conditions, and with its use in larger trials, it became apparent that the reactogenicity was of an order compatible with its release for general distribution without gamma globulin. On the basis of the estimates of the number of doses distributed, it appears that such Edmonston B vaccine, even when administered without gamma globulin, has been acceptable to many persons charged with

the responsibility of inoculation. A preference for the less reactogenic material, however, is also clearly indicated.

EFFECTIVENESS

In past trials the effectiveness of live measles virus vaccines has been assayed by determining (1) their capacity to induce and maintain a durable antibody response in susceptible persons, and (2) the degree of protection afforded upon subsequent intimate exposure to naturally occurring cases of the disease.

Antibody Response

The results of early trials of the vaccines showed that they stimulated specific antibody formation within two to three weeks after inoculation in 90 per cent of susceptibles as measured by neutralization, complement-fixation, or hemagglutination-inhibition techniques. The maximum mean levels of antibody attained with Edmonston B vaccine without gamma globulin closely approximate those induced by natural measles. When the vaccine is given with gamma globulin this level is slightly lower. After the administration of further attenuated vaccine, the average peak titer is significantly lower, although the number of vaccinees responding is of a high order.

The persistence of antibodies over varying periods has been documented in several studies. Thus, as an example, we may refer to a small group of children whom we originally inoculated with Edmonston A vaccine. In a continuing study we have determined that the level of neutralizing antibodies in each of 10 of these children has remained essentially unchanged throughout the eight-year period. In larger groups of originally seronegative children who received Edmonston B vaccine alone or with gamma globulin or further attenuated virus hemagglutinin-inhibiting antibody has been detected by Krugman and his associates in every child who was studied four to six years after vaccination. Since it has long been recognized fom the results of passive measles immunization that even a very low concentration of antibody may confer protection, such findings give much reason to expect that vaccination with attenuated live measles virus will confer durable and solid resistance comparable to that resulting from the naturally acquired disease.

Lately, however, it has been questioned whether the antibody responses induced by Edmonston B and by further attenuated vaccine will prove equally durable, not only because of the differences in the average maximal response but also because the subsequent decline in titer of HI antibody as measured by the Rosen technique is more pronounced and continues at a more rapid rate following administration of further attenuated vaccine (10). Indeed, at the end of two years no antibody was detected by this method in a significant proportion of sera. However, when the more sensitive HI test of Norrby was applied to the same sera, or when neutralization tests were performed, antibodies were found in all or nearly all cases four years after vaccination, as Dr. Krugman's data indicate (9). The titers of antibody often tended, however, to be lower than those found in comparable sera of Edmonston B vaccinees.

The possibility therefore remains, though rendered more remote by these recent observations, that complete extinction of antibody formation may occur sooner in certain persons vaccinated with the further attenuated material, which theoretically might be accompanied by reversion to a state of susceptibility. If adequate records are kept on a sufficient number of vaccinees as mass immunization is continued with both vaccines, it may eventually be possible to arrive at a final answer to this important question.

Protective Effectiveness of the Vaccines

In confirmation of the expectation of prophylactic effectiveness afforded by studies of antibody response, it soon became apparent in most trials that for periods of one to two years these vaccines are highly effective in protecting against measles under conditions of known intimate exposure. We must note, however, that in a recent large study of FAV in England (11), which Dr. Perkins will presumably discuss, the protection afforded during the first six months was only about 85 per cent.

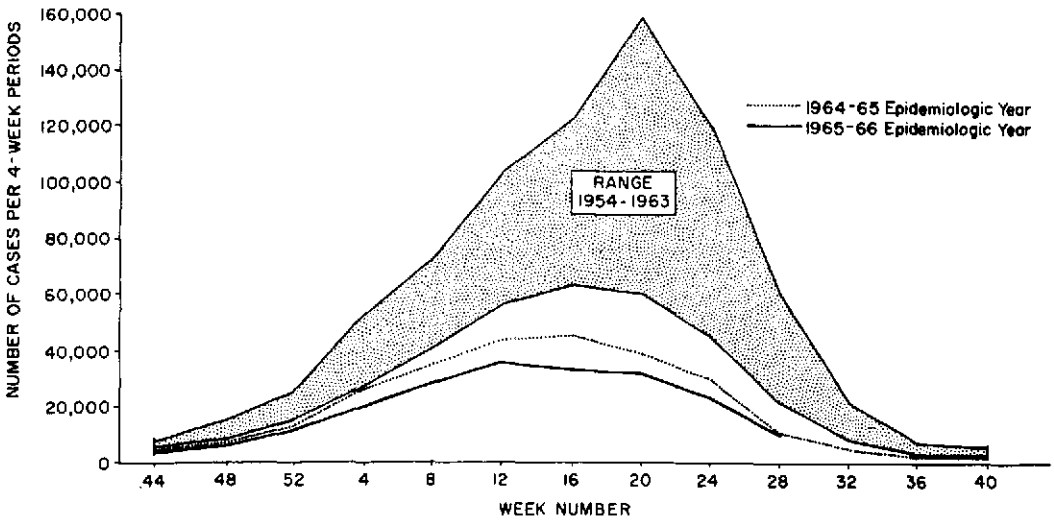
There are few precise data obtained under exacting conditions showing unequivocally that a high level of protection is maintained over longer intervals. In a few instances, however, in which

regular follow-up studies have been done, it has been demonstrated that a solid state of resistance persists for at least four years. In support of this statement we can cite as an example our experience with the original group of institutionalized children vaccinated in 1958 (12). Six of these were exposed to measles 4⅔ years later. None developed the disease, whereas two unvaccinated children originally included as controls became infected. In another trial of Edmonston B vaccine, conducted during a long-term family study at the Children's Hospital Medical Center in Boston, vaccinated children have been carefully followed up to the present time. Among 39 of these children, 31 were exposed to measles in the family at intervals ranging from four months to four years after vaccination (13). All proved to be resistant. In approximately 300 normal home-dwelling children vaccinated with FAV who have been closely studied, Krugman has recorded no cases of measles over a period of three years. In other comparable groups of children who received Edmonston B alone or with gamma globulin the same investigator has not encountered a case of measles during periods of four and five years after vaccination.

Effectiveness of Vaccine in Mass Immunization

On the basis of data on vaccine effectiveness of the sort we have attempted to illustrate by the foregoing examples, it seemed justifiable to expect a prompt and marked reduction in the annual incidence of measles if the vaccination of young children in the susceptible age groups were instituted on a sufficiently large scale throughout the country. Moreover, the durable resistance conferred by a single inoculation suggested that if most of each year's new crop of susceptible children were vaccinated, measles might be eliminated from the community within a reasonable time.

The results of the mass immunization recently summarized by the Communicable Disease Center lend support to the first of these expectations (1, 2). Although only an estimated two fifths of the total susceptible child population had been vaccinated by the end of March 1966, the measles case incidence during the first 26 weeks of that year was the lowest recorded in 20 years. The incidence for 1965 was also below that for any of the preceding 10 years as shown in Figure 1. Although other factors may also have been involved in this abrupt decline in the incidence of measles in the



Source: "Measles Surveillance." Report No. 1 (1 August 1966), U. S. Public Health Service, Communicable Disease Center, Atlanta, Georgia.

Fig. 1. Measles cumulated by four-week periods, United States—Epidemiologic years 1964-1965 and 1965-1966, compared with 10-year period, 1954-1963.

United States, it is difficult to escape the conclusion that vaccination played a major role. We can anticipate that the continuation and expansion of immunization will soon provide data for a more confident judgment of the effectiveness of live virus vaccines in the control of measles in large populations.

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(12) See Ref. 3, Paper II.

(13) See Ref. 3, Paper VII.

SECTION A. RUBEOLA

PRESENT STATUS OF KILLED MEASLES VACCINE*

ERLING C. NORRBY

Department of Virus Research, Karolinska Institute,
School of Medicine, Stockholm, Sweden

DR. NORRBY *(presenting the paper)*: Research on killed measles vaccines, as compared to live measles vaccines, has been considerably less intensive. The most obvious reason for this is that live vaccines of well-documented effectiveness are now available.

In addition, doubts concerning the possibility of establishing long-lasting and complete protection with a killed vaccine alone have also probably deterred research efforts in this area. However, some recent results have made the validity of this preconceived opinion somewhat questionable (53, 55).

The present review will give a description of different types of killed vaccines reported in the literature and discuss the influence of various general parameters on the state of immunity established and on reaction patterns occurring in connection with exposure to live virus. Finally, some general suggestions on directions for the future development of killed measles vaccines will be proposed.

Types of Killed Measles Vaccines

Two main types of killed measles vaccines can be distinguished: inactivated "whole" virus vaccine, and products containing purified antigen(s) derived from the virus envelope.

The first vaccines of the "whole" virus type gave disappointing results (17, 25), but the product subsequently developed by Warren and co-workers yielded more encouraging results (63, 65). It was prepared by propagating the Edmonston A strain of measles virus in cynomolgus monkey kidney tissue cultures. Infectivity of harvested material was eliminated by treatment with formalin, after which the killed antigen was concentrated and partially purified by adsorption onto alum. This vaccine has been available for general use in the United States for several years. A similar product derived from chick-embryo grown virus has also recently been released for general use (30, 57, 58).

The second type of killed measles vaccine contains purified hemagglutinin (HA) (52–54). So far, this product has only been prepared in minor batches for use in small-scale field trials. Treatment with Tween-80 and ether has been used as a means to disrupt envelope structures (41). Purification of envelope antigen(s) has been obtained by physicochemical techniques. Attempts to develop techniques applicable on a large scale for the preparation of purified HA have met with some difficulty.

Methods for the preparation of purified antigen(s) from envelope structures should preferably be based on knowledge of the immunobiological architecture of this component. However, the present information on myxoviruses is far from complete. It is known that the virus envelope is derived from a modified cell membrane and that consequently the normal cellular components and the virus antigens form integrated parts. The lipid part of the membrane appears to be taken in a ready-made form from the host cells. Hoyle (27) was the first to show that ether extraction could be used as a means of disrupting the influenza virus envelope. Later studies have shown that this

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method of lipid extraction gives rather large fragments (36). A more effective disintegration was obtained using deoxycholate and, in particular, dodecyl sulphate. However, treatment with the latter agent led to a loss of certain biological activities concomitant with conversion into small particle products (67). It was also shown that extraction of Newcastle disease virus material with ether—even in combination with a detergent (26), which improves the disintegrating effect—caused the appearance of fragments still containing host cell components, including the lipids (11). In order to eliminate all these, a methanol, or occasionally methanol-ethanol, extraction was needed. The product thus obtained could not be brought into aqueous solution, although in the suspended form it still retained its immunogenic activity. Similarly, it was found that the lipid components of the influenza virus envelope could be effectively extracted with methanol-chloroform (12). This treatment gave a precipitate of virus material that had lost the major part of the biological activities originally present. However, almost all activities were recovered when the precipitate was dissolved in 67 per cent glacial acetic acid. Under these conditions the activities were carried by small molecular weight components (sedimentation coefficient less than 4S). Elimination of glacial acetic acid by dialysis against buffered saline of pH 7.2 led to an aggregation of the small components into products that were very heterogeneous with regard to their sedimentation characteristics.

These data on means of fractionation are of considerable importance in considering possible ways of producing a measles vaccine product containing purified antigen. Measles virus in most respects exhibits biological characteristics that justify its classification as a large myxovirus (43). In keeping with these characteristics, it was found that a disruption of envelope structures could be obtained by treatment with Tween-80 and ether (41, 42, 44) and even more effectively with deoxycholate (46). A first step in a purification procedure would preferably be ultracentrifugation of harvested material in its crude form at a moderately high speed, which would lead to recovery of most of the viral antigens in the precipitate (42, 44). Further treatment could then, in principle, proceed along two different lines. One would be to apply the disintegration techniques described above, extracting almost all lipids and host cells components. The product obtained might be used as is or, if necessary, purified further. This alternative, however, might be somewhat complicated because of the product's insolubility in aqueous media. The second possibility would be to accept the presence of nonviral components remaining in the products when a milder disintegration technique, Tween-ether or deoxycholate treatment, is used. Further purification would then be aimed at eliminating nonenvelope components, e.g., the virus-specific nucleoprotein antigen. Presumably some kind of ion exchange chromatography technique would be most apt to use for this purpose. An example of a situation in which a general procedure of the latter kind was chosen is the technique described for preparation of an influenza vaccine containing purified HA (9). This vaccine was prepared by Tween-ether treatment of the material and elimination of nucleoprotein antigen by precipitation with lanthanum acetate.

Factors Affecting Immunity

Age of recipients. The aim of vaccination against measles must naturally be to provide protection against the disease as soon as passive immunity due to maternal antibodies has vanished—at the age of about six months. With live vaccine, the absence of antibodies is an absolute prerequisite for vaccination. With killed vaccine, however, maternal antibodies exert only a modifying effect, the magnitude of which depends on the relative proportions of concentration of circulating antibodies and of antigen content in the vaccine applied. Attempts to immunize children under six months in connection with DPT vaccination have in fact been undertaken (1, 32, 35). With the vaccine used for these immunizations only very poor antibody responses were obtained. Nonetheless, subsequent administration of moderately attenuated live virus vaccine resulted in almost symptomless infections and elicited vigorous anamnestic antibody responses.

Dose of antigen. The result of primary immunization with a killed vaccine depends to a great extent on the antigenic potency of the preparation used. The amount of antigen present in the product can be quantified by antigenic

extinction limit tests in guinea pigs (65). An increase in the potency of preparations up to a certain value is paralleled by an increase in the geometric mean titers of the vaccinees (58, 65). As the potency values 90 beyond this point the serological responses asymptotically approach those seen after natural measles (52, 57), which is to be considered the maximum antibody response to a primary immunization. Very little information is available on the importance of the dose of antigen used for booster injections. In one field trial it was found that two vaccines of different antigenic potency when used for primary immunization evoked similar antibody titers (52) but elicited markedly different anamnestic responses (53). This may imply that the range of responses to different amounts of antigen is wider for secondary than for primary immunization. However, to draw such a conclusion from these results alone is somewhat uncertain, since only the low-potency preparation was absorbed onto an adjuvant.

Adjuvant. The presence of adjuvant in the form of aluminum phosphate increases the immunizing effectiveness of the products used for primary immunization with regard both to actual titers recorded (63) and to the degree of sensitization obtained (54; Norrby *et al.*, to be published). However, adjuvant-free antigen preparations appear to be equally effective in eliciting anamnestic antibody responses (53, 54).

Immunization schedule. In most field trials undertaken so far the immunizing effect of three monthly doses has been studied (8, 16, 18, 19, 21, 31, 38, 49, 52, 69). This antigenic stimulus is to be regarded as a protracted primary immunization, the effectiveness of which depends on the dose of antigen present in the preparation. The effects of a fourth injection administered more than six months after the primary immunization have mainly been noted for live vaccine products (1, 5, 16, 18, 19, 21, 24, 31, 35, 37). Comparatively few studies have been undertaken to analyze the boostering effect of killed vaccine (33, 53, 54, 59). It is a well-established fact that in most cases after primary immunization with killed vaccine no symptoms occur upon subsequent injection of live measles vaccine. However, under such conditions there appears to be a slight risk of getting a somewhat lower percentage of takes of the live vaccine (19) and also, under certain circumstances, a less satisfactory persistence of antibody titers (6). A booster injection with killed vaccine elicited vigorous antibody responses in all trials.

Clinical Reactions Connected with Administration of Killed Vaccine or Killed Followed by Live Vaccine

As a rule, the injection of killed measles vaccines gives very few general clinical reactions. A local infiltration, which may sometimes develop into a sterile abscess, is occasionally seen after the administration of an adjuvant-containing vaccine. On one occasion a booster injection of alum-containing vaccine caused what presumably was a generalized allergic reaction (53). This points to the need for future caution. A local reaction at the place of injection of live vaccine in children preimmunized with killed vaccine has also been reported (13).

Experiences of Antibody Responses to Immunization with Killed Measles Vaccines Alone

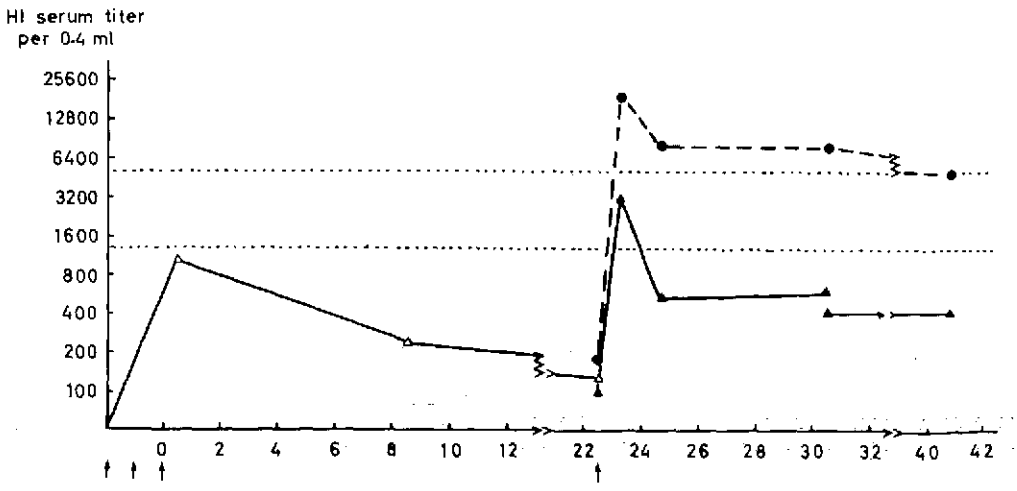
Three monthly doses of either a "whole" virus, alum-containing, formalin-inactivated vaccine (1, 5, 16, 18, 19, 21, 24, 31, 35, 37) or a product of purified HA not containing adjuvant (52) gave conversion rates of from 90 to 100 per cent. The antibody titers reached were about 25 to 50 per cent of those appearing after natural measles. The relative concentrations of various antibodies measured by different techniques appeared to be similar in the two cases. Several controversial statements have been made concerning the persistence of antibodies, presumably due to a variation in the sensitivity of the different serological techniques applied. By the use of a sensitive hemagglutination-inhibition (HI) technique (41, 45) it was found that antibody titers declined 6- to 12-fold during the 8 to 11 months after vaccination (51, 53, 54). After this, stabilization occurred and virtually no change in titers took place over a long period of time.

Completion of an immunization schedule consisting of primary injections of killed vaccine and a booster injection of the same kind of vaccine causes impressive booster responses. In one trial, children who had received three

monthly doses of a "whole" virus, formalin-killed vaccine as their primary immunization were given a booster injection of either the same product or purified HA 22 months later (52). The increases in antibody titers were 35- and 100-fold, respectively, in the two groups (Fig. 1). Injection of the product containing purified HA in most cases even resulted in the appearance of antibodies in absolute titers higher than those seen after natural measles. In another field trial (54) a booster injection of a moderately potent product of purified HA caused a 20-fold and an 80-fold increase in antibody titers in children who had received purified HA and formalin-killed vaccine, respectively, as their primary immunization (Fig. 2). The antibody decrease after the booster injection followed the same kinetic pattern as after primary immunization. However, there was a tendency for the antibody titers to stabilize at a relatively higher level. During a period of 18 months after a booster injection the reduction was 4- to 8-fold (53; Norrby *et al.*, to be published). A somewhat more rapid fall-off in titers was recorded in the group of children who had received purified HA both for primary and secondary immunization (Fig. 2; Norrby *et al.*, to be published). Similar results from studies of formalin-killed vaccine have also been reported by others (33). However, less encouraging results—i.e., a more rapid decrease in titers after a booster injection—have been described in one publication (59).

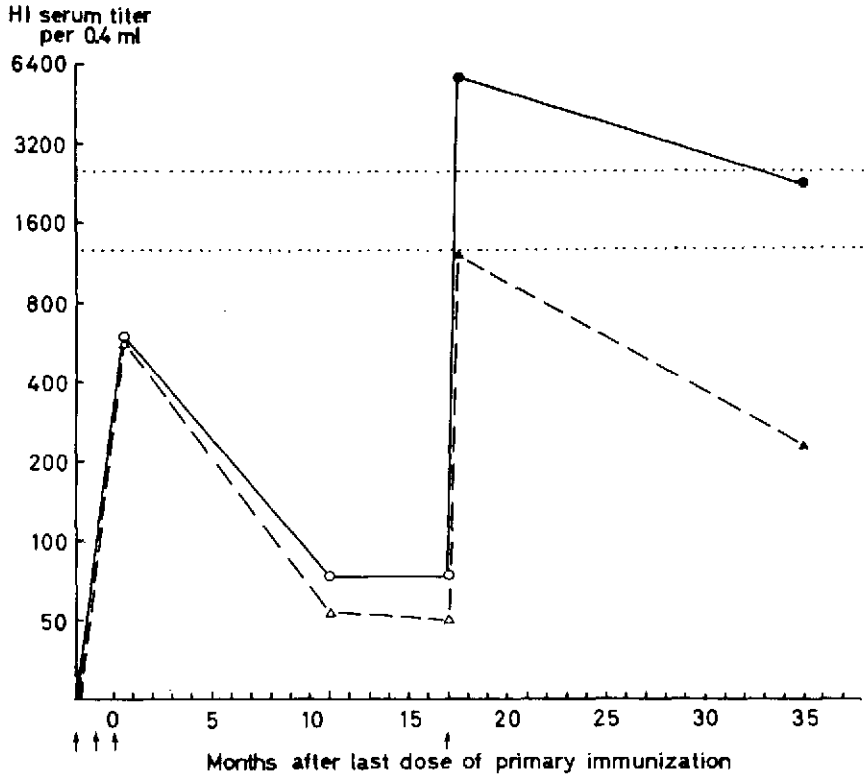
Characteristic Features of Immunity and Clinical Protection Conferred by a Natural Measles Virus Infection as Compared to Vaccination with Killed Antigen

The immunity acquired through a natural measles virus infection is stable and extends throughout life even in the absence of re-exposure (4, 56). The relative importance of various defense mechanisms with respect to this state of immunity has not as yet been clearly elucidated. That circulating antibodies alone are highly protective can be inferred from the prophylactic effect of the administration of gamma globulin (29, 62). However, experiments with monkeys have shown that reinfection and local multiplication of virus in the respiratory tract (14), and even viremia (64), can occur in the presence of circulating antibody. The latter situation may presumably be explained by the fact that the virus can be harbored in the formed elements of blood, which are known to support its multiplication (3).



Note: The four injections are indicated by arrows. The same type of vaccine (▲—▲) or a product containing purified HA (●---●) was given as booster injection. The finely dotted lines ..... the range *within which* 90 per cent of the HI titers of early measles convalescent sera fell.

Fig. 1. Variation in geometric mean HI titers in a group of children immunized with three monthly doses of "whole" virus, formalin-inactivated vaccine followed by a fourth injection 22 to 23 months later.



Note: Three monthly doses (arrows) of either formalin-killed, "whole" virus vaccine (○—○) or a high-potency product containing purified HA (△···△) were given as a primary immunization. Seventeen months later a booster was given of a moderately potent product of purified HA. Finely dotted lines indicate the range of HI titers of early measles convalescent sera.

Fig. 2. The variation in geometric mean HI titers in children immunized with killed measles vaccine.

There has been discussion of the possible importance of cell-bound immunity for the protection against measles seen after recovery from the disease in cases of agammaglobulinemia (28). It is possible, however, that many of the patients in question were suffering from hypoglobulinemia rather than agammaglobulinemia. The finding that immunization with killed vaccine in a number of situations of this kind conferred protection (39) also points in this direction. It should be mentioned that attempts to demonstrate delayed hypersensitivity by skin tests in individuals immune to measles have so far failed (15).

The degree of protection against natural measles after three monthly doses of killed vaccine is about 90 to 95 per cent during the first year after vaccination and about 70 to 75 per cent during the second year (18, 22, 31, 33, 38, 51–54, 59, 70). Even so, a considerable number of mild or symptomless infections, as judged from booster responses, have been reported. Since antibodies can be detected with sensitive techniques for two years after primary immunization, we must assume that reinfections can occur in spite of these antibodies. This has been directly demonstrated in some cases (51, 54). Even an HI serum titer of 80 prior to exposure did not provide complete protection. This titer value should be compared to the HI serum titer value, which theoretically should be obtained after administration of a dose of gamma globulin giving complete protection. The value obtained in such a calculation is only about 6 (54). The important conclusion to be drawn from these facts is that there must be a *qualitative* difference between the state of immunity conferred by circulating antibodies derived from a regular measles infection and that conferred by vaccination with killed vaccines. The reason for

this difference must at present remain a matter of conjecture. Difference in avidity of antibodies could play some, probably minor, part. A possible absence of essential antigen(s) in the killed vaccines might be another plausible explanation. However, no clear-cut difference has been found between the relative concentration of different antibodies, measurable with the various serological techniques available, after a regular measles infection and after immunization with killed vaccine. It should be mentioned, though, that a somewhat lower ratio between HI and neutralizing antibodies in preparations of gamma globulin as compared to late convalescent sera has been noticed (15; Norrby, unpublished). No atypical features were found regarding the kinetics of the appearance of IgM and IgG antibodies after immunization with killed vaccine (54, 61). It might be of interest to complete this set of investigations with a quantitative estimation of IgA antibodies, since these are known to be the most essential ones for protection in secretions of the respiratory tract (2).

A booster injection with killed vaccine was found to evoke potent anamnestic responses (33, 53, 54). This made it feasible to analyze the extent to which the antibodies' qualitative inferiority, in terms of clinical protection, could be compensated for by their quantitative abundance. Table 1 presents some results obtained in one of the small field trials undertaken by the author and his co-workers (55). The trial, which was described earlier in this report, involved children given a primary immunization with three monthly doses of a "whole" virus, formalin-killed vaccine and then 22 months later a booster injection of either the same product or purified HA. Because of the latter circumstances, the data on clinical and serological reactions to exposure to natural measles during the period 8 to 18 months after the booster injection are not strictly comparable. However, taking the serum titers at their face value, it can be concluded that children with high HI antibody titers—160 to 640—could in some instances be reinfected. Only in one single case were any clinical reactions observed, although impressive rises in antibody titers were recorded. In children with still

TABLE 1. CLINICAL AND SEROLOGICAL REACTIONS IN 12 CHILDREN EXPOSED TO CLEAR-CUT CASES OF MEASLES 8 TO 18 MONTHS AFTER BOOSTER INJECTION

Child No.	Type of vaccine given as booster	Time for exposure; months after booster	Clinical reaction	8 months post-booster serum titers		18 months post-booster serum titers	
				HI	CF	HI	CF
3	FK	14	0	160	<20	20,480	640
6		15	0	640	20	10,240	320
19		15	0	160	<20	10,240	640
22		13	*	640	20	128,000	1280
39		14	0	1280	40	640	20
46		?	—	1280	40	10,240	320
62		14	0	160	20	80	20
7	TE	16	0	20,480	1280	20,480	640
20		13	0	20,480	640	10,240	320
21		14	0	20,480	640	10,240	320
34		12	0	10,240	320	2560	320
48		13	0	10,240	640	5120	320

*Case history appears in source.
FK = formalin-killed, "whole" virus vaccine.
TE = Tween-ether treated product containing purified HA.
Source: Norrby, E., Lagercrantz, R., and Gard, S. "Measles Vaccination. VI. Serological and Clinical Follow-Up Analysis 19 Months After a Booster Injection." *Acta Paediat Scand* 55:457–462, 1966. Reproduced by permission.

higher H1 serum titers—10,000 to 20,000—neither a clinical nor a serological reaction was detectable.

In many children who had acquired partial protective immunity by vaccination with killed virus, exposure to measles resulted in modified symptoms and, occasionally, atypical complications (33, 51, 53–55, 59). Most often there were only mild symptoms from the respiratory tract, a moderate fever of short duration, and either a faint rash or none at all. Occasionally the rash was atypically distributed, appearing only in the peripheral parts of the limbs. Unexpected complications in the form of pneumonia have been noticed in some vaccinees with good antibody titers. Reactions of this type were seen in 5 children out of 125 exposed to natural measles in one study (59) and in 2 out of 38 exposed in other studies (53, 55). These pulmonary complications often displayed a considerable degree of severity. They generally started to develop a few days after the first catarrhal symptoms had been noticed and lasted for three to seven days. The etiology of these pneumonias is not known. Possible alternative explanations are either a superinfection with a separate microorganism or a localized reaction between measles antigen and antibody. The latter situation would then be analogous to the "virus pneumonia syndrome" that can occur in variola-immune individuals.

General Conclusions and Remarks on the Present Status and Future of Killed Measles Vaccine

Some of the results discussed above clearly demonstrate that the possibilities of providing long-lasting and protective immunity by the use of killed measles vaccine alone are much more favorable than had previously been thought. However, many factors, some of which have not yet been carefully analyzed, are of importance for the state of immunity established. At our present state of knowledge the optimal procedure for immunization appears to be the following:

Primary immunization should be effected with an adjuvant-containing product. The antigen content of the vaccine should be adjusted to represent the smallest amount giving maximal or near-maximal antibody response. Theoretically, this dose of antigen could be given as one injection, although two might possibly be needed. About six months or so after the primary immunization a booster injection should be given. This should be of high antigenic potency. The addition of adjuvant is presumably not needed.

Though this procedure appears simple, it involves a number of problems that have not yet been completely analyzed. One is the choice of adjuvant, a problem that is not peculiar to measles immunization but affects vaccinations in general. Another important problem is the kind of killed product to be used. The formalin-killed "whole" virus vaccine available today can provide good protection, but it is not satisfactory for use in its present form as the sole product for immunization. Many arguments speak for a further development of a vaccine containing purified HA (20, 48). Accumulated evidence, mainly of an indirect nature, indicates that HA isolated from the nucleocapsid of the virus and the major part of the lipids of the envelope stimulates the production of protective antibodies as effectively as crude virus material does. The absence of lipids might be an advantage, since they are believed by some to play a role in hyperergic reactions causing measles encephalitis (34). The elimination of the nucleoprotein can also be of potential value. At least a nucleic-acid-free preparation would more or less guarantee the absence of extraneous infectious agents. Furthermore, the immunizing effect of purified HA might be partially enhanced by the elimination of nonessential antigens. This effect was recently demonstrated in comparative studies of "whole" virus, formalin-killed influenza vaccine and a product containing purified HA (23). One practical advantage that might also be mentioned is that purified HA, unlike whole virus products, can be sterilized by filtration (63).

It should be stressed that the development of a vaccine product containing purified HA is still in the experimental stage. Large-scale production has posed problems. In particular, our lack of knowledge concerning immuno-biological features of the envelope of myxoviruses has been a handicap. All studies on this subject should be encouraged.

Several general problems connected with the use of killed vaccine also warrant further in-

vestigation. Why is it that the immunity produced by killed vaccine is qualitatively inferior to that acquired after a natural measles infection? The answer to this question depends partly on the availability of further information concerning naturally acquired life-long immunity. Another basic problem pertains to the quality of clinical protection. The pulmonary complications seen in some vaccinees with good titers of circulating antibodies call for further investigation. Should this reaction be caused by a local antigen-antibody reaction, it will constitute a serious argument against the use of killed vaccine alone for immunization.

The factor of overwhelming importance for the future fate of killed measles vaccines is, of course, the attitude prevailing toward live measles vaccines. The matter boils down to one question: Is there really a need for any killed vaccine today, when the further attenuated virus strains available seem to cause almost no clinical reaction and result in an immunity equivalent to that caused by natural measles? The answer to this question is naturally related to what should be required of a vaccine to be used universally in a community. To say that it should provide a protection comparable to that conferred by natural measles, that it should be harmless to the individual, and that it should be practical to apply is simply to speak truisms. The further attenuated live vaccine certainly fulfills the first and the last of these requirements. Whether it is completely harmless might be debated, although few precise data on this point are available at present. We know that in a considerable number of cases attenuated virus has caused the appearance of chromosomal breakages in white blood cells (40). Although the significance of this finding is difficult to evaluate, it probably is justifiable to deduce that the appearance of such gross changes in the vital structures of cells may well imply the occurrence of invisible disturbances of unknown potential danger. Against this background it would seem advisable to avoid a massive virus reproduction in the organism. The more general advantage of eliminating a trauma that must of necessity leave some traces in the organism should also be mentioned. Hypothetically, one could imagine the establishment of a carrier state of measles virus in the organism similar to what can be obtained *in vitro* (47, 60). Such an *in vitro* carrier state has been found to affect the chromosomal structures of the cells (Nichols *et al.*, to be published).

How then does the inactivated vaccine satisfy the general requirements stated above? As regards protection, present evidence indicates that a long-lasting immunity can be obtained. This requires the inconvenience of repeated injections of antigen, which is in contrast to the formerly predominant idea that their number could probably be rather small. If the antigen is incorporated into a polyvalent vaccine its application will not add any extra burden to the vaccination calendar. It has been shown that formalin-killed, "whole" virus vaccine in combination with polio vaccine (7, 66) and purified HA combined with a polio-DPT vaccine product (10) are effective for immunization against measles. The potential danger for the individual when a purified and well-defined antigen product is used for immunization must be considered minimal.

In conclusion, it should be stressed that the type of population to be immunized would have a deciding influence on the vaccine policy applied. In a developing country in which people live under primitive conditions and measles infections and their sequelae are responsible for 10 to 20 per cent of the mortality in young children, the primary aim of vaccination should be to reduce this mortality rate. In this case a further attenuated live virus vaccine should be used, provided, first, that for practical and financial reasons similar results cannot be achieved by the use of an inactivated vaccine, and, second, that the general physical condition of the vaccinees will allow them to resist the mild virus infection. In a highly developed country the problems associated with vaccination are somewhat different. The motivation for vaccination is not so acute; the aim is rather to eliminate a traumatic disease and its dangerous sequelae, such as encephalitis. Under these circumstances it would seem advisable to analyze the possibility of conferring lifelong protection by the use of inactivated vaccine alone.

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SECTION A. RUBEOLA

MASS RUBEOLA IMMUNIZATION IN AFRICA

RENÉ LABUSQUIÈRE

Organization for Coordination and Cooperation in the Control of Major Endemic Diseases for the Countries of Central Africa
Yaoundé, Federal Republic of Cameroon

DR. LABUSQUIÈRE *(presenting the paper):* Although accurate medical statistics are largely unavailable in Africa, it is evident that measles is responsible for widespread ravages and could account for approximately 50 per cent of the deaths among children between one and four years of age (2). Excessive measles-associated mortality and morbidity may be due to many diverse factors—malnutrition, associated infections, and a way of life that imposes early massive contagion, to mention three. Climate may also play an important role. Although measles is distributed throughout all of Africa, it is particularly severe in certain countries—for example, Upper Volta, Mali, Niger, and Chad. In these places the season of extreme dryness corresponds each year to the measles epidemic period. One has the impression that the malignant course of measles in this area may be related to the superimposed hazard of acute dehydration.

In the past, personnel engaged in the practice of tropical medicine in these areas attempted only supportive treatment rather than the control of the disease, since no effective prophylactic measures were available. When Dr. Enders and his associates isolated and attenuated the measles virus (3), immunization finally appeared as a possible solution to this public health problem.

PRELIMINARY STUDIES

While visiting the National Institutes of Health (NIH) in June 1961, Dr. Paul Lambin, then Minister of Health of Upper Volta, proposed a pilot study in Ouagadougou using the experimental live measles vaccine. Dr. Lambin's initiative led to an association of African, French, and American public health personnel that has evolved in five years into a major international effort to control measles in Africa.

In late 1961 more than 20,000 American children had been safely immunized with the attenuated measles strain, but vaccine-associated fevers and rash were common. It was at that time that the first pilot study was undertaken in Ouagadougou by Dr. Harry M. Meyer, Jr., Dr. Daniel D. Hostetler, Jr., and Mrs. Barbara C. Bernheim of the NIH, in cooperation with the Upper Volta Ministry of Health. The purpose of the study was to determine whether or not the live virus vaccine would evoke excessive reactions when inoculated into susceptible African children, for whom epidemic measles could be particularly severe. By early 1962 the results of this first Upper Volta pilot study (7) had established the safety and efficacy of the attenuated virus, with or without gamma globulin, for African populations.*

In October 1962 a second pilot study demonstrated that measles vaccine could be effectively administered by jet injection, yielding seroconversion rates of 97 to 100 per cent in children eight months of age and older. The study also established the feasibility of jet in-

* An experimental Enders B-level measles vaccine, produced and donated without charge by Merck, Sharp and Dohme was used in Upper Volta for the two pilot studies and for the first mass immunization program.

jection for administering mixtures of measles, smallpox, and yellow fever vaccines (8).

FIRST MASS CAMPAIGN IN UPPER VOLTA (4, 6, 9)

On the basis of the encouraging results of the pilot study, a program of mass immunization against measles was conducted from November 1962 to March 1963. Since over 90 per cent of the population resided in rural areas remote from medical installations, the general organization of the project was entrusted to the Upper Volta Endemic Disease Control Service (Service des Grandes Endémies), of which I was Director.

Methods. The program was carried out by eight vaccination teams, each consisting of two male nurses and a chauffeur. Each team was equipped with a Willys jeep, jet injectors, a portable generator, and a butane-electric refrigerator. Vaccinations were scheduled sequentially in each of the nine medical sectors of Upper Volta. The program was coordinated and supervised by the same NIH group that had previously carried out the pilot studies. In each sector the chief medical officer, the personnel under his supervision, and all available vehicles were put at the disposal of the group for the duration of the campaign in that particular area. Firm instructions were issued to the administrative or political authorities so that the campaign would receive maximum publicity and attract the greatest possible number of children. Wherever possible, a propaganda team was sent in first to inform the village of the vaccination team's arrival and to assemble families for vaccination. In view of the amount of vaccine available for reaching the population at risk, it was decided to direct the campaign chiefly toward all children six months to four years of age. In all, 731,548 persons, or 19.7 per cent of the estimated total population of Upper Volta (3,710,000), were vaccinated.

Reactions. Vaccine reactions can be estimated quite accurately in adequately controlled pilot studies. In a mass campaign, the monitoring of side effects is much less precise, but useful information can still be obtained. Data collected during the Upper Volta campaign were based on the following indicators: (1) expected and actual pediatric mortality and morbidity in the two large hospitals at Bobo-Dioulasso and Ouagadougou; (2) general observations and reports of sector physicians and health personnel in rural dispensaries during the post-vaccination period; (3) evaluation of general public acceptance.

The hospital in Ouagadougou, which serves an area in which 200,000 children were vaccinated during the hot dry season, reported an increase in morbidity in the weeks following vaccination; however, no significant change was noted in mortality. Barbotin and Poulain (1) reported 75 hospital admissions and 10 deaths. Only one of the deaths appeared to be vaccine-associated. The hospital in Bobo-Dioulasso reported a decrease in both morbidity and mortality during the month following vaccination. Reports by sector physicians indicated that about 50 per cent of the vaccinated children experienced febrile reactions, sometimes accompanied by gastroenteritis. Some of the physicians felt that the most severe reactions occurred in children who were in a poor general state of health and were already under treatment for malaria or pulmonary infections. Still, no fatalities were known to occur and the general impression was favorable.

Public acceptance of the vaccination program was best indicated by the large numbers of mothers who throughout the entire program brought their children to be vaccinated.

Results. By January 1963 about 50 per cent of the total pediatric population had been immunized. The immediate result was a marked decrease in the number of measles cases reported during the next three months—the time of year when the incidence of measles normally reaches its peak (Fig. 1).

During the first year after the mass inoculation, the number of reported cases of measles remained relatively low. The total number of cases recorded each month during the period 1960–1964 is shown in Table 1.

In 1966 Sansarricq reported that the incidence of measles had increased to two thirds of the prevaccination level (13). Since very few supplementary vaccinations were administered (24,676 in 1963, 33,752 in 1964, and 26,738 in 1965), this sudden rise would indicate that during the first two years reasonably good control is main-

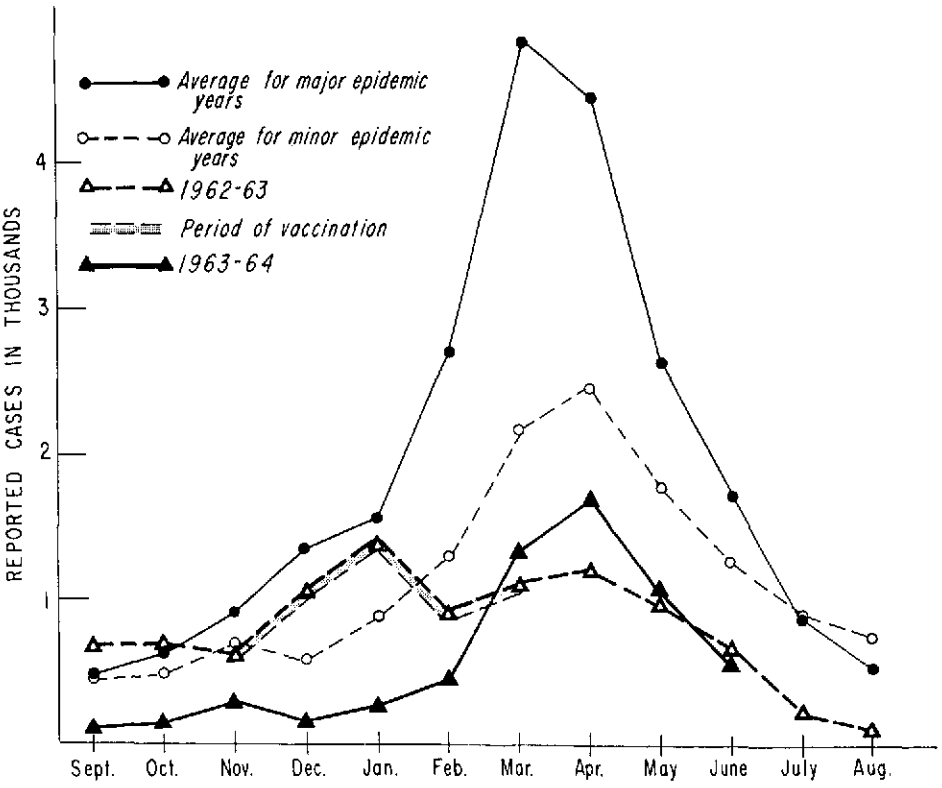

Fig. 1. Effect of vaccination on reported measles cases in Upper Volta.

TABLE 1. NUMBER OF MEASLES CASES REPORTED MONTHLY IN UPPER VOLTA, 1960–1964

Month	1960	1961	1962	1963	1964
January	2,054	557	2,386	1,408	254
February	3,063	1,046	4,360	897	429
March	7,505	1,293	5,268	1,090*	1,334
April	4,932	2,581	6,405	1,188	1,682
May	2,125	1,693	3,466	940	1,053
June	1,625	1,257	1,814	620	521
July	596	863	683	194	184
August	276	771	595	69	—
September	183	1,036	700	95	—
October	260	1,017	710	140	535
November	473	1,304	620†	278	457
December	360	2,478	1,072	160	457

*End of mass immunization program.
†Beginning of mass immunization program.

tained, but that by the third year there is a return to the typical epidemic pattern.

Several cases of natural measles occurred in children who were reported to have been vaccinated. In carefully conducted pilot studies measles vaccination can be almost 100 per cent successful, but in large campaigns somewhat lower seroconversion rates are to be expected, particularly in Africa where the transportation and proper storage of vaccine is often difficult. In addition, the results of limited serologic tests carried out in one of the nine medical sectors pointed to considerable variation in the effectiveness of the vaccinating teams. These differences indicate the need for careful selection of reliable personnel and for thorough training programs.

As a result of the experience in Upper Volta we have made the following recommendations: (1) that supplementary vaccination programs be conducted each year to immunize new children in the population who have reached six months of age; (2) that vaccination preferably be performed between the end of the rainy season and the beginning of the hot, dry season, before the onset of the annual measles epidemic.

EXTENSION OF VACCINATION TO OTHER AFRICAN COUNTRIES

After the mass campaign in Upper Volta, the United States Agency for International Development (AID) provided technical assistance for limited immunization trials in seven other West African countries—Niger, Dahomey, Ivory Coast, Mali, Guinea, Senegal, and Mauritania—through the West African regional health organization, the OCCGE.* Under this program, Dr. Meyer and Mrs. Bernheim trained African nurses from the seven countries and then supervised the nurse-trainees during limited trial campaigns in their countries of origin. Approximately 100,000 children were inoculated and the results confirmed the earlier favorable experiences in Upper Volta (10).

These trials were followed by mass campaigns supported by AID, with the technical assistance of the Communicable Disease Center, Atlanta, Georgia. When the mass campaigns are completed, nearly 3 million additional vaccinations will have been performed, 1 million of them with the Schwarz strain.

The Senegalese medical authorities are of the opinion that the Enders B-level vaccine can cause serious reactions. Rey *et al.* (11) reported blindness and pneumonia among the reactions observed. They also suspected transmission of the vaccine to unvaccinated children. The use of the Schwarz strain in French-speaking West Africa followed a pilot study by the same group in which 182 Senegalese children were vaccinated with this preparation. The results of the study indicated that 98 per cent of the vaccinees developed antibodies after inoculation, that the reactions were extremely mild, and that vaccination as late as three days after exposure to the natural disease was protective (12).

The immunization programs carried out in the ex-French West African countries impressed the French-speaking nations in Central Africa. Cameroon (5) organized an immunization program, and Chad followed suit shortly thereafter. To date over 700,000 inoculations of Enders B-level vaccine produced by Merck, Sharp, and Dohme have been given in the two countries. No deaths have been observed and reactions have not been excessive.

PROSPECTS FOR FURTHER VACCINATION IN AFRICA

Nearly 4 million measles vaccinations have been performed in Africa. The current programs will continue until the end of 1966, at which time a new five-year plan involving United States assistance to 19 countries will go into effect. In the countries of the OCEAC† alone (Cameroon, Chad, Central African Republic, Gabon, and Congo (Brazzaville)), which have a combined population of about 10 million, 3,200,000 antimeasles inoculations are expected to be given. Other vaccinations, of course, are also scheduled. The planning of this program has taken into consideration our present concepts of mass immunization.

In the light of past experience, the OCEAC felt it necessary to plan not only for the next

* L'Organisation de Coordination et de Cooperation pour la Lutte contre les Grandes Endémies, Bobo-Dioulasso, Republic of Upper Volta.

† L'Organisation de Coordination pour la Lutte contre les Endémics en Afrique Centrale, Yaoundé, Federal Republic of Cameroon.

five years but for the years thereafter as well. The campaign in Upper Volta has demonstrated the need for complementary immunization of uninoculated measles-susceptible infants. So far all of the immunization programs have been carried out by specialized teams. The consequent demands for personnel, equipment, and operating funds have compromised the normal activity of the Endemic Disease Control Service and used up a disproportionate share of its meager budget. In addition, the rapid pace at which these teams must work to meet their heavy schedules is often incompatible with a high degree of precision. We have therefore decided to integrate these vaccination campaigns into the routine multiphased activity of the Endemic Disease Control Service.

The national Endemic Disease Control Services are the successors of the first "prospection" team created in 1917 by Colonel Jamot, which then became the Sleeping Sickness Service and later the General Service of Mobile Hygiene and Prophylaxis.

The following guidelines laid down by Jamot remain valid after half a century of experience:

1. In territories where the proportion of medical personnel is low, where distances are great and populations are widely scattered, and where murderous endemic diseases reign, no appreciable results may be expected from fixed medical installations.

2. One of the first tasks is to determine which among the evils to combat should be attacked first, it being well understood that the aim is not to carry out pure research in beautiful institutes but to work at an urgent task: to prevent men from dying.

. . .

. . .

5. Among the possible means of attack . . . we will choose those whose effectiveness has been established and that lend themselves to large-scale application.

. . .

7. The instrument of action shall be a specialized service operating with the aid of mobile teams, which will carry out mass prophylaxis of major endemic diseases in rural areas.

These guidelines are still valid and may well be applied to measles control.

Today each country has its own Endemic Disease Control Service. The services are divided into several sections, each one headed by a chief medical officer. International coordinating organizations have been created—the OCCGE in West Africa and the OCEAC in Central Africa—that enable the national services to participate in unified health plans, notably in the area of vaccinations.

Each Endemic Disease Sector acts on the basis of a specific program, using mobile teams to visit the entire sector according to schedule that varies depending on the country and the circumstances. The completely equipped team visits each small village assembly center and can examine from 400 to 500 persons daily. The Service is responsible for the detection, prevention, and treatment of endemic diseases. One of the primary prophylactic actions of these teams is vaccination; thus measles immunization has its place in the routine activity of the Endemic Disease Control Service.

I shall cite a few figures to indicate the degree of effectiveness of these organizations. At the end of World War II in Central Africa alone there were 50,000 cases of trypanosomiasis, half of them in the Central African Republic; today there are only about 100. There are nearly 700,000 cases of leprosy in the two ex-French federations; in certain countries more than 50 per cent of these cases are apparently cured. More than 100 million smallpox vaccinations have been given; the sporadic cases of the disease that still occur can often be traced to sources outside the ex-federations or to difficulties encountered in the transportation or storage of the vaccine. More than 85 million yellow fever vaccinations had been performed by 1958; although the disease was believed to have disappeared, recent outbreaks in Senegal indicate the need for continuing immunization. Over the next five years 11 million yellow fever vaccinations are planned for the OCEAC alone. These few fragmentary figures indicate the efficiency of the mobile team, or the "French system." There is no doubt that good results could be obtained if this system were applied to the fight against measles.

The apparent disadvantages of integrating measles immunization into the service are: (1) the schedule would be slower, since the teams have other responsibilities; (2) automatic jet-injectors could not be fully utilized; and (3)

each team would have to carry special equipment, such as jet-injectors and refrigerators. The advantages, however, are undeniable: (1) the operation would be more economical, since many procedures could be accomplished at the same time, using the same personnel, vehicles, gasoline, and equipment; (2) the population would be better protected, since the people would receive more complete and thorough surveillance and treatment as well as multiple vaccinations; and (3) the vaccinating personnel would be better supervised by the habitual team leader, thus obviating the necessity of setting up specialized measles immunization teams at the expense of other programs.

We therefore offer the following recommendations for future mass measles immunization programs in the French-speaking African nations: (1) integration of measles immunization into the routine activities of the mobile teams of the Endemic Disease Control Service; (2) provision of all mobile teams with a complete set of the simplest and sturdiest equipment, which would enable them to perform all required vaccinations, as well as their habitual activities; (3) regular visits of these teams to all zones according to a fixed schedule determined by the particular conditions in each country; (4) supplementary measles vaccination during each team visit in rural zones and twice a year in urban areas of all children having reached the age of six months since the previous passage; (5) continuation of regular vaccinations for an unlimited period.

Summary

Following the encouraging results of the first mass measles immunization program in Upper Volta, in which nearly 750,000 children were vaccinated, the African member nations of the OCCGE have performed almost 3 million immunizations and those of the OCEAC nearly 750,000. Beginning in 1967, a five-year plan of assistance by the United States will continue this effort. Through the integration of this measles vaccination program into the activity of the Endemic Disease Control Service, we hope to obtain a progressive decrease in the incidence of measles and consequently a reduction of excessive infant mortality in Africa.

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SECTION A. RUBEOLA

MASS MEASLES IMMUNIZATION IN SOUTH AMERICA

C. RISTORI, J. M. BORGOÑO, R. GREIBER, AND O. AVENDAÑO*

National Health Service and Bacteriological Institute of Chile, Santiago, Chile

DR. RISTORI (*presenting the paper*): Chile was the first country in Latin America to launch a mass vaccination campaign against measles. What led to the program was a high measles mortality rate and the progress made in prevention of the disease since the development of the Enders live attenuated vaccine.

EPIDEMIOLOGICAL CHARACTERISTICS

In Chile, as in other Latin American countries, measles has some special characteristics:

1. An inverse correlation between mortality and children's socioeconomic and nutritional status (2).

2. A high mortality rate, which has shown a tendency to increase in the last 10 years (Table 1).

3. A correlation between the seriousness of the disease and climatic conditions. Mortality is four times higher in the provinces of the cold, moist south than in those of the warm, dry north (2).

4. A higher mortality in younger groups, with an inverse correlation between age and severity (1) (Tables 2, 3, 4).

* In addition to the authors, the Chilean National Health Service team conducting this investigation included the nurses Elsa Heras and Blanca Carrillo.

TABLE 1. RELATIVE IMPORTANCE OF MEASLES AMONG ALL CAUSES OF DEATH AND AMONG DEATHS FROM ACUTE COMMUNICABLE DISEASES, CHILE, 1956–1965

Year	Total No. of deaths	Deaths due to acute communicable diseases	Deaths due to measles		
			Number	Percentage of total deaths	Percentage of deaths caused by communicable diseases
1956	83,744	2,008	453	0.5	22.5
1957	91,073	2,057	677	0.7	32.9
1958	88,611	3,274	1,575	1.8	48.1
1959	93,292	3,140	1,147	1.2	36.5
1960	93,625	4,154	2,116	2.3	50.9
1961	91,551	3,916	1,822	2.0	46.5
1962	94,569	4,462	2,455	2.6	55.0
1963	98,901	4,085	2,243	2.3	54.9
1964	94,111	5,072	3,264	3.5	64.3
1965	91,491	2,943	1,211	1.3	41.1

TABLE 2. DEATHS FROM MEASLES IN CHILE, 1956–1965: DISTRIBUTION BY AGE GROUP

Year	Total No. of deaths	Under 1 year		1–4 years		5–9 years		10 and over	
		No.	%	No.	%	No.	%	No.	%
1956	453	186	41.1	243	53.6	17	3.8	7	1.5
1957	677	243	35.9	405	59.8	20	3.0	9	1.3
1958	1,575	629	39.9	891	56.6	48	3.0	7	0.4
1959	1,147	463	40.4	626	54.6	36	3.1	22	1.9
1960	2,116	895	42.3	1,098	51.9	101	4.8	22	1.0
1961	1,822	805	44.2	919	50.4	67	3.7	31	1.7
1962	2,455	1,091	44.4	1,239	50.5	98	4.0	27	1.1
1963	2,243	968	43.2	1,165	51.9	79	3.5	31	1.4
1964	3,264	1,441	44.1	1,613	49.4	161	4.9	49	1.5
1965	1,211	567	46.8	568	46.9	46	3.8	30	2.5

There are other countries in Latin America where the situation is similar and measles is the most important cause of death among the acute communicable diseases. In some of these, vaccination programs are currently under consideration. A mass campaign has already been initiated in Peru, employing the same vaccine and technique as in Chile. For the time being, these are the only two countries in the Hemisphere that are performing mass vaccination of children.

PRESENT STATE OF THE VACCINATION PROGRAM IN CHILE

The first Chilean trial with the Enders attenuated vaccine was carried out in July 1962 (3, 4). Although the vaccination produced general reactions in approximately 30 per cent of the children, no serious accidents or complications were observed. It was also verified that the addition of gamma globulin did not significantly reduce the intensity and number of these reactions to justify a measure that complicates the procedure, especially in the case of mass vaccination programs.

In view of the excellent immunological and epidemiological results obtained, a program was thereupon initiated to protect most children eight months to three years of age with live vaccine. A preliminary report was submitted in July 1964 to the Seminar of the Children's International Center in Paris (5).

TABLE 3. AGE DISTRIBUTION OF MEASLES DEATHS IN CHILE, 1964–1965

Age (years)	1964		1965	
	No. of deaths	%	No. of deaths	%
−1	1,441	44.1	567	46.8
1	984	30.1	370	30.6
2	350	10.7	122	10.1
3	174	5.3	53	4.4
4	105	3.2	23	1.9
5–9	161	4.9	46	3.8
10–14	24	0.7	16	1.3
Over 14	25	0.8	14	1.2
Total	3,264	100.0	1,211	100.0

TABLE 4. DISTRIBUTION OF DEATHS FROM MEASLES AMONG INFANTS UNDER 1 YEAR, CHILE, 1964–1965

Age (months)	1964		1965	
	No. of deaths	%	No. of deaths	%
Under 6	417	28.9	178	31.4
6	161	11.2	70	12.3
7	165	11.5	62	10.9
8	203	14.1	69	12.2
9	171	11.9	67	11.8
10	160	11.1	60	10.6
11	152	10.5	61	10.8
Unknown	12	0.8	—	—
Total	1,441	100.0	567	100.0

During the second half of 1964 the program was intensified and was extended to other parts of the country. Thus far, more than 620,000 children have been immunized (Table 5).

Vaccination is being conducted in the maternal and child centers all over the country where babies and preschool children are examined monthly. In some poor sectors where the population is dense and the proportion of children high, vaccination is performed along the street or door-to-door, in order to obtain the maximum coverage in the minimum period of time.

The response of the public and pediatricians has been very favorable. Large numbers of mothers bring their children to the maternal and child centers or to any other place where vaccination is being performed. They are instructed to report any severe reactions appearing during the two weeks following vaccination. Fewer than 5 per cent of the total number of vaccinees return for consultation.

VACCINES EMPLOYED

The entire program in Chile thus far has been carried out using the Edmondston B strain of attenuated virus. More than 520,000 children received the chick embryo vaccine manufactured by Merck, Sharp, and Dohme Laboratories, and about 100,000 children the dog kidney vaccine prepared by the Phillips Roxanne Laboratories. Although gamma globulin was not used the reactions observed with the chick embryo vaccine were minor and without clinical consequence. By contrast, severe reactions were frequently observed with dog kidney vaccine.

SEROLOGICAL STUDIES IN CONNECTION WITH THE PROGRAM

Persistence of Antibodies

The antibody level in the blood serum of four groups of children vaccinated one month, one year, two years, and three years earlier is shown in Table 6.* The hemagglutination-inhibition test was used. Up to three years after vaccination the antibody level is still very satisfactory.

Immunological Response of Undernourished Children

Because of the high percentage of undernourished children in Chile, as in most Latin Ameri-

* This investigation was performed by Dr. Maurice R. Hilleman of the Merck Institute for Therapeutic Research.

TABLE 5. NUMBER OF VACCINATED CHILDREN BY HEALTH ZONES, CHILE, 1963–1966

Health zones	1963	1964	1965	1966* (1st semester)	Total	% of vaccinees in age groups under 5 years
I	1,891	6,437	10,409	1,768	20,505	39.3
II	447	6,331	17,632	5,971	30,381	45.1
III	86	3,096	2,031	1,009	6,222	39.2
IV	6,821	37,385	13,718	7,263	65,187	69.1
V	25,561	108,299	61,865	27,880	223,605	59.5
VI	1,123	3,773	35,271	3,019	43,186	67.3
VII	1,281	27,714	14,237	11,762	54,994	63.4
VIII	958	2,644	11,727	1,105	16,434	39.3
IX	4,298	10,152	44,186	12,440	71,076	51.2
X	1,950	6,674	24,466	3,258	36,348	35.8
XI	965	4,000	15,102	4,834	24,901	38.3
XII	859	7,859	15,389	3,474	27,581	55.1
XIII	185	2,737	3,606	774	7,302	75.0
Total	46,425	227,101	269,639	84,557	627,722	54.7

*Preliminary information.

TABLE 6. PERSISTENCE OF SERUM ANTIBODIES AFTER MEASLES VACCINATION, CHILE, 1966

Time after vaccination	No. of serum samples	Antibody titer						
		−4	4	8	16	32	64	128
1 month	19	4	1	—	—	2	3	9
1 year	16	—	2	—	4	2	3	5
2 years	16	—	1	1	4	5	1	4
3 years	18	—	3	—	1	3	4	7

can countries, and the severity of measles in these children, it was considered necessary to study their immunological response to vaccination. The results of complement-fixation tests carried out in 42 children are shown in Table 7: 83.3 per cent demonstrated seroconversion, which is held to be quite satisfactory in view of the frequent failure of the CF reaction to detect initial immunes.

Immunological Response of Infants under Eight Months

A significant number of measles cases in Chile occur in infants under nine months of age, which is considered the lower limit for vaccination. Although, as is well known, the persistence of maternal antibodies reduces the immunity level conferred by vaccination, we decided to determine the percentage of satisfactory responses in infants six to eight months old and the possibility or desirability of lowering the age limit for vaccination.

Table 8 shows antibody levels, using the complement-fixation test, in a group of 30 infants vaccinated at six months of age, in a group of 27 vaccinated at seven months, and in a group of 31 vaccinated at eight months. The

TABLE 7. SEROCONVERSION IN VACCINATED UNDERNOURISHED CHILDREN AGED 8 MONTHS TO 3 YEARS, CHILE, 1964–1965

No. of children	Negative	1:20	1:40	1:80 and over	% of conversion
42	7	12	20	3	83.3

TABLE 8. RESULTS OF COMPLEMENT-FIXATION TEST IN SANTIAGO CHILDREN VACCINATED AT 6, 7, AND 8 MONTHS OF AGE

Age (months)	No. of negative sera	No. of positive sera		Total blood samples	% conversion
		1:20	1:40		
6	13	11	6	30	56.6
7	11	8	8	27	59.3
8	8	13	10	31	74.2

findings argue against the immunization of children under eight months of age, since the seroconversion obtained for the six-month-old children was only 56.6 per cent; for the seven-month-olds, 59.6 per cent; and for the eight-month-olds, 74.1 per cent. Regardless of this finding, the frequency and severity of measles has forced us to start immunization at the age of eight months.

EPIDEMIOLOGICAL EVALUATION

The favorable effect of the immunization program in reducing measles morbidity and mortality became apparent in 1965, when only 1,211 deaths occurred. This was little more than one third the incidence for the previous year.

A clear correlation between reduced mortality and the initiation of the program is observed in Table 9. In the provinces where a high degree of coverage was achieved in 1964, there was an 81.8 per cent reduction in deaths in 1965; in those where the program was launched during the first half of 1965, the decrease was 58.4 per cent; and in those that started the program during the second half of 1965, the decrease was 29.5 per cent.

An outbreak of measles in January-March 1965 in the Province of Magallanes coincided with the initiation of the vaccination program (Table 10). Of 2,141 children vaccinated, only 6 contracted measles and one died. In contrast, of 3,991 unvaccinated children in the same age group there were 511 cases of measles and 26 deaths.

In another communication, we reported on the results of follow-up observation of 2,000 vaccinated children and a control group of the

TABLE 9. DEATHS FROM MEASLES BY PROVINCE ACCORDING TO DATE OF VACCINATION, CHILE, 1964–1965

Date of vaccination	Province	No. of deaths		% reduction
		1964	1965	
1964	Aconcagua	55	2	96.4
	Valparaíso	119	11	90.8
	Santiago	793	137	82.7
	Curicó	37	30	18.9
	Talca	87	13	85.1
	Maule	50	6	88.0
	Linares	81	34	58.0
	Total	1,222	233	81.8
1st SEMESTER 1965	Ñuble	65	109	
	Concepción	342	121	64.6
	Arauco	112	29	74.1
	Bío-Bío	229	36	84.3
	Malleco	137	55	60.0
	Cautín	132	77	41.7
	Valdivia	180	41	77.2
	Osorno	188	45	76.1
	Llanquihue	195	91	53.3
	Magallanes	5	55	
	Total	1,585	659	58.4
2nd SEMESTER 1965	Tarapacá	23	7	69.6
	Antofagasta	17	29	
	Atacama	58	4	93.1
	Coquimbo	204	36	80.9
	O'Higgins	60	77	28.3
	Colchagua	78	35	55.1
	Chiloé	10	111	
	Aysén	7	20	
	Total	457	319	29.5
Total		3,264	1,211	62.9

same size 10 months after vaccination (5). A similar experiment was then undertaken with two groups of children under the same conditions but with a 30-month follow-up period. Table 11 shows the results of this investigation, which confirms the high efficacy of the vaccine. Of 2,011 unvaccinated children, during a total observation period of 34,252 months, 340 cases of measles and 24 deaths occurred. By contrast, among 2,042 vaccinated children, with a total observation period of 26,510 months only 12 cases and one death occurred.

In a further study of the efficacy of the vaccine, a review was made of all cases of measles among children hospitalized in Santiago during 1965. At that time, 43 per cent of the total popu-

TABLE 10. MEASLES EPIDEMIC IN PUNTA ARENAS, CHILE, JANUARY–MARCH 1965

Population (aged 8 months to 5 years)		Morbidity		Mortality	
		No. of cases	Rate per 100,000 population	No. of deaths	Rate per 100,000 population
Vaccinated	2,141	6	280	1	46.7
Unvaccinated	3,991	511	12,803	26	651.4
Total	6,132	517	8,431	27	440.3

lation under five years of age had been vaccinated. Table 12 presents data that are quite conclusive: 256 nonvaccinated children were admitted to hospitals with measles, and 30 died; only five vaccinated children were admitted with measles or its complications, and not one of them died. This means that only 3.8 per 100,000 vaccinated children needed hospitalization, whereas in the unvaccinated population of the same age group, the figure was 135 per 100,000.

COMMENTS

This study confirms that vaccination with Enders live attenuated virus vaccine is highly effective in protecting against measles. Its application in Latin American countries, where the mortality is extremely high, can result in a large saving of lives. The two-year vaccination program in Chile has been very extensive; in 1965 there were about 2,000 fewer deaths than in 1964. The vaccination technique is simple, and the addition of gamma globulin is unnecessary when vaccines causing only slight general reactions are used.

At present we are studying the immunological response and the local and general reactions produced by a combined vaccine against measles and smallpox. For this purpose, 30,000 doses of vaccine were provided by the Merck, Sharp, and Dohme Research Laboratories. We found that the reactions in the first 5,000 children vaccinated were no greater than those observed with each of the vaccines given separately. Jet injection of the vaccine has been readily accepted by the public and the medical profession. If these preliminary observations are confirmed, the simultaneous use of these two vaccines will represent a great advantage because of the simplified method.

Because of the high level of protection conferred by the measles vaccine, it is recommended that every developing country with a high measles mortality rate incorporate mass measles vaccination into its public health programs. We are pleased to see that Peru has already introduced the procedure, and we hope that other Latin American countries will follow the example.

TABLE 11. CASES AND DEATHS FROM MEASLES IN CHILDREN UNDER 6 YEARS OF AGE, CHILE, 1964–1966

Status	No. of children	Months of observation	Morbidity		Mortality	
			No. of cases	Rate per 100,000 population	No. of deaths	Rate per 100,000 population
Unvaccinated	2,011	34,252	340	16,907	24	1,193
Vaccinated	2,042	26,510	12	587	1	49

TABLE 12. MEASLES CASES ADMITTED TO THE HOSPITAL, SANTIAGO, 1965*

Age groups	Unvaccinated		Vaccinated	
	Cases	Deaths	Cases	Deaths
Under 6 months...	35	4	0	0
7–11 months.....	73	5	1	0
1 year.....	87	14	3	0
2 years.....	22	0	1	0
3 years.....	20	3	0	0
4 years.....	10	3	0	0
5 years.....	9	1	0	0
Total.....	256	30	5	0

*Among a population with 43 per cent of children under five vaccinated.

Summary

The results obtained in Chile after three years of vaccination with the Edmonston B strain of Enders attenuated vaccine have been reported. More than 620,000 children from eight months to five years of age—54.7 per cent of the population in that age range—have been vaccinated. The serological studies reveal the persistence of serum antibodies for at least three years; good immunological response in underfed children; and unsatisfactory serological response in infants under nine months of age.

The total number of deaths in 1965 was the lowest registered in the last five years and was 2,053 lower than in 1964.

An epidemic occurred in the Province of Magallanes at the same time that vaccination was being carried out; this permitted demonstration of the high level of efficacy of the vaccine, through a comparison of the measles rates among vaccinated and among unvaccinated children.

A 30-month follow-up of about 2,000 vaccinated and 2,000 unvaccinated children showed 12 cases of measles with one death in the vaccinated group, as against 340 cases with 24 deaths in the control group.

An investigation of children hospitalized in Santiago during 1965 was carried out. At that time 43 per cent of the city's population between eight months and five years of age had been vaccinated; 256 unvaccinated children were hospitalized with measles and there were 30 deaths, whereas only 5 vaccinated children were hospitalized and there were no deaths.

Because of the high level of protection conferred by the vaccine and the fact that it produces only minor reactions, even when gamma globulin is not used, it is recommended that every developing country with a high measles mortality rate incorporate the mass vaccination of children into its health programs.

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SECTION A. RUBEOLA

IMMUNIZATION AGAINST MEASLES IN THE USSR

V. M. BOLOTOVSKY

Central Research Institute of Epidemiology, Ministry of Health
Moscow, USSR

DR. BOLOTOVSKY (*presenting the paper*): The wide spread of measles and its high incidence in children, frequently accompanied by serious complications and in many countries by a high mortality rate, have impelled scientists from various countries to concentrate their efforts on obtaining safe and effective vaccines against this disease. Marked progress was made in this field after 1954, when Enders and his co-workers demonstrated that measles virus could be isolated and cultivated in tissue culture and thus paved the way for the study of methods for attenuating the virus. In 1958 the first vaccinal strains for the production of live vaccines became available. By now a good many data have been accumulated on both live and inactivated measles vaccines.

Valuable advances have recently been made in the USSR in the study and formulation of live measles vaccines. In 1958 A. A. Smorodintsev and his co-workers suggested the Leningrad 4 strain, which was isolated from a measles child, passaged repeatedly in human tissues and chick fibroblasts, and then adapted to the primary monolayer of newborn guinea pig kidney culture. The vaccine produced severe reactions, however, requiring the simultaneous injection of gamma globulin. In 1962 the same authors proposed the Leningrad 16 (L-16) strain isolated directly from a measles patient in a monolayer guinea pig kidney culture. This culture has been employed in the USSR for the serial production of live measles vaccine. Tests have revealed that the clinical reactions are mild and simultaneous administration of gamma globulin is not necessary. The measles vaccine prepared from the L-16 strain is now being used in the USSR to immunize children between the ages of 1 and 8 years.

Meanwhile, as part of the WHO Measles Immunization Program, controlled field trials have been carried out to evaluate different measles vaccines separately and in combination. In these tests coded preparations were used for vaccination, children in all age groups were selected at random for immunization, and placebo was used parallel with vaccines.

The only results taken into account were those registered in children who had been seronegative before vaccination, because the existence of even slight specific immunity inevitably affects the reactivity of the vaccinated organism and can substantially change the quantitative reactogenicity and epidemiological efficacy of the vaccines tested (Table 1). Thus, for exam-

TABLE 1. EXAMINATION DATA OF HI TEST IN INSTITUTIONALIZED CHILDREN WITHOUT MEASLES HISTORY

Age groups	No. of children	Seropositive		Geometric mean titer in log2
		Absolute No.	%	
1–2	85	0		
2–3	189	17	8.9	4.8
3–4	208	42	20.2	4.9
4–5	131	34	25.9	5.4
5–6	42	14	33.3	5.8
6–7	19	8	42.1	6.6
Total	674	115	17.0	4.9

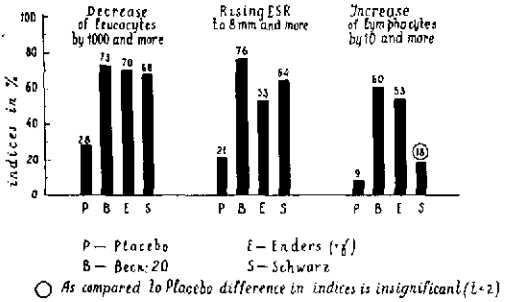

Fig. 1. Blood cell changes in persons given live vaccines against measles (7–10 days).

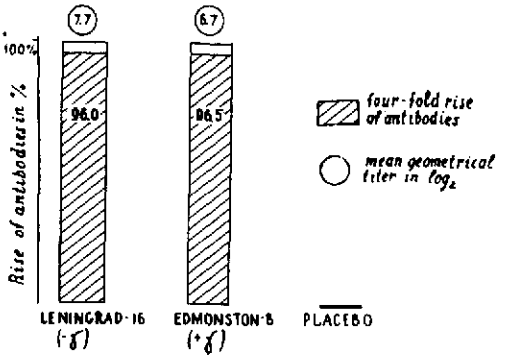

Fig. 3. Immunogenicity of live measles vaccines.

ple, in conducting a controlled test of efficacy, measles antibody with a geometric mean titer of 4.9 $\log_2$ was found in 115 (17 per cent) out of 674 children from one to seven years with no measles history. Time limitations prevent my analyzing here the results of all the comparative studies. Moreover, the results differed slightly from those obtained in similar studies carried out in Canada, Czechoslovakia, Yugoslavia, and other countries. I need only mention the results of the analysis of blood from children inoculated with different live vaccines (Fig. 1). With all the vaccines tested, leukopenia of short duration and acceleration of the erythrocyte-sedimentation rate were recorded, though in varying degree. The changes observed in the leukocyte formula appeared to be correlated with the levels of reactogenicity of the vaccines.

The vaccine prepared from the L-16 strain was studied in two controlled tests. In the first, levels of reactogenicity and immunogenicity of the L-16 and Edmonston B vaccines were compared. The Edmonston B vaccine* was administered with gamma globulin (0.022 ml per 1 kg of body weight) and the L-16 vaccine† without (Fig. 2). The reactogenicity of the two vaccines proved similar both in level of temperature reactions and in extent of rash (Fig. 3). In the hemagglutination-inhibition test, the immunogenicity proved to be identical as well.

In the second controlled test the L-16‡ and Schwarz§ vaccines were compared. Their reactogenicity was the same with regard to levels of temperature reactions, catarrhal manifestations, and rash (Fig. 4). The study of immunogenicity of the vaccines by the hemagglutination-inhibition reaction (Table 2) showed 100 per

* Merck, Sharp and Dohme, U.S.A.
† Pasteur Institute, Leningrad.
‡ Institute of Virus Preparations, Moscow.
§ Glaxo, United Kingdom.

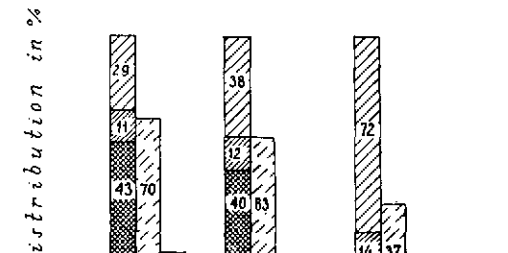

Fig. 2. Reactogenicity of live measles vaccines.

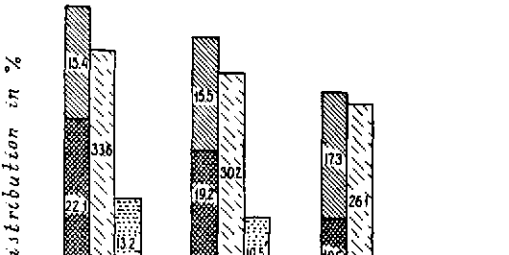

Fig. 4. Reactogenicity of live measles L-16 and Schwarz vaccines.

TABLE 2. HI DATA IN SERA OF CHILDREN VACCINATED WITH LIVE MEASLES VACCINES

Preparation		Smorodintsev		Schwarz		Placebo	
		Absolute No.	%	Absolute No.	%	Absolute No.	%
No. of sera		79	100.0	78	100.0	75	100.0
Including seronegative sera		36	45.6	36	46.2	34	45.3
Finite titer of paired sera	<1:4					34	100.0
	1:4	1	2.8				
	1:8	1	2.8	1	2.8		
	1:16	4	11.1	3	8.3		
	1:32	3	8.3	6	16.7		
	1:64	9	25.0	18	50.0		
	1:128	13	36.1	8	22.2		
	1:256	5	13.9				
	1:512						
Total		36	100.0	36	100.0	34	100.0
Geometric mean titer in $\log_2$		6.1		5.8			
Geometric mean titer		1:69		1:56			

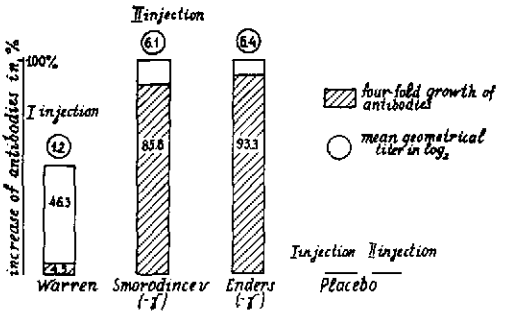

Fig. 5. Immunogenicity of combined scheme of vaccination against measles.

cent seroconversion with practically equal geometric mean titers. Fourfold and higher antibody increases in the second sera (Table 3) occurred in 83.3 per cent with the L-16 vaccine and in 88.9 per cent with the Schwarz.

A study of reactogenicity, immunogenicity, and epidemiological efficacy was also made for a vaccination scheme using Pfizer (U.S.A.) killed measles vaccine. First the killed vaccine was administered, and then 28 to 30 days later an injection of live vaccine (L-16 and Edmonston B without gamma globulin) was given. It was demonstrated that combined vaccination was practically areactogenic (the number of reactions in the experimental group of children was the same as in the group twice inoculated with placebo).

As to the immunogenicity of this method, it was shown that the seroconversion rate after the administration of the killed vaccine was 46.3 per cent and that after subsequent administration of live vaccine all the children showed an increase in antibody titer (Fig. 5). Table 4 presents the measles morbidity rate registered during the first year of observation.

Combined immunization would take care of a considerable number of cases in which live measles vaccines are contraindicated. This is very important because of the great need for protection of children who suffer from certain diseases or who are simply undernourished. However, not enough knowledge has yet been accumulated on the duration of the postvaccinal immunity under such a vaccination scheme; we

TABLE 3. HI RESULTS ON SERA OF CHILDREN VACCINATED WITH LIVE MEASLES VACCINES

Preparation	Smorodintsev		Schwarz		Placebo	
	Absolute No.	%	Absolute No.	%	Absolute No.	%
Seronegative paired sera...	36	100.0	36	100.0	34	100.0
Increase in antibodies.....	36	100.0	36	100.0		
Titer 1:32 and higher.....	30	83.3	32	88.9		

TABLE 4. EPIDEMIOLOGICAL EFFICACY OF THE COMBINED METHOD OF MEASLES VACCINATION

Preparation	No. of vaccinated children with contacts	Morbidity		Efficacy		Mean mistake	Statistical value			
		No. of cases	Per 1000 persons	K	E(%)		To placebo	To Warren + Smorodintsev (−γ)	To Warren + Enders (−γ)	To Warren
Placebo	196	46	234.7			±3.1		47.3	52.0	9.4
Warren +Smorodintsev (−γ)	171	11	64.3	3.7	72.6	±1.9	47.3		46	22.1
Warren+Enders (−γ)	171	9	52.6	4.5	77.6	±1.7	52.0	4.6		24.6
Warren	61	11	180.3	1.3	23.2	±4.9	9.4	22.1	24.6	

do not yet know whether frequent revaccinations are necessary. Furthermore, the efficacy of the method needs confirmation in larger communities—that is, on a district, city or provincial scale.

Single immunization with the L-16 live vaccine as now practiced in the USSR has proved epidemiologically effective. According to data reported by the Institute of Epidemiology of the Kirghiz Soviet Socialist Republic (Central Asia), the measles morbidity rate among children in three cities of the Republic immunized in 1962 with L-16 vaccine was considerably different from that registered in nonvaccinated groups (Fig. 6). It is noteworthy that, despite

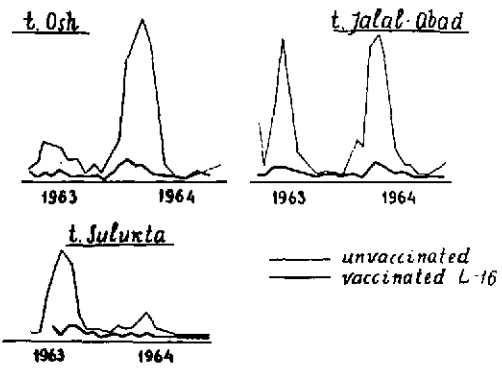

Fig. 6. Morbidity among the children vaccinated with live measles vaccine and unvaccinated ones, 1963–1964.

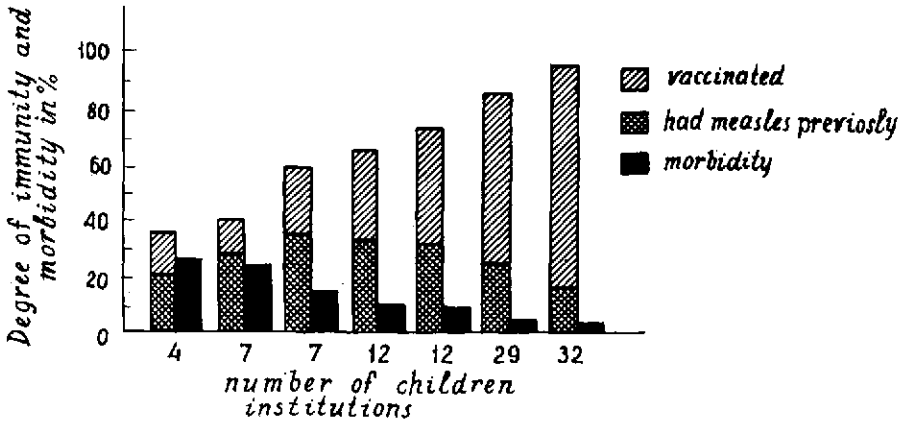

Fig. 7. Measles morbidity in foci with different degrees of immunity.

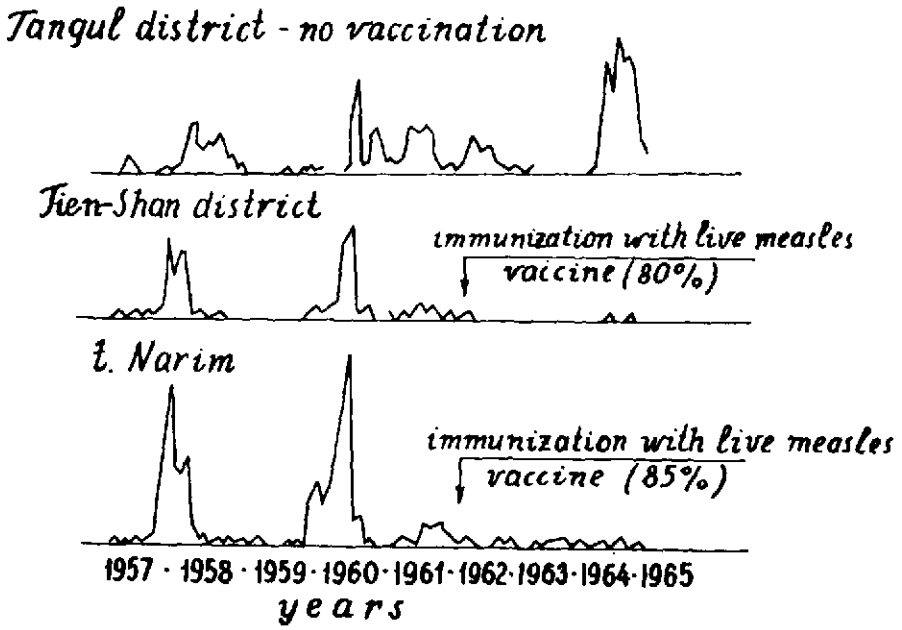

Fig. 8. Influence of active measles immunization on epidemic process.

the lower proportion of immunization, the vaccinated children did not participate in the periodic rises in measles morbidity recorded in those cities, though the scope of vaccination there was rather limited.

It is absolutely clear that for the epidemiology of the disease to be affected a certain level of immunity among susceptible children must be maintained. In this respect the observations made in a number of children's institutions with different levels of immunity are significant (Fig. 7). The consistent results there were entirely corroborated under field conditions in two districts where 80 to 85 per cent of the susceptible children between the ages of one and eight years were immunized against measles (Fig. 8).

More than 4 million children in the USSR have now been immunized with the L-16 vaccine. Further immunization to protect a majority of susceptibles against measles will inevitably reduce the number of cases sharply and may preclude the epidemic spread of this infection.

SECTION A. RUBEOLA

RUBEOLA IMMUNIZATION IN THE UNITED KINGDOM

FRANK T. PERKINS

Division of Immunological Products Control, National Institute for Medical Research (Hampstead Laboratories), London, England

DR. PERKINS (*presenting the paper*): Before reporting on the measles vaccines and schedules used in clinical trials in Great Britain, I should like to discuss the disease and its effect on our community. Figure 1 shows the incidence of measles in England and Wales in the past decades. There is clearly a biennial pattern in which the case incidence rises to about 500,000 and there are always more cases of measles reported than of any other infectious disease. Deaths due to measles, however, have decreased markedly over the period in question, probably due to the use of antibiotics. Still, it is pertinent to ask how serious the disease is today.

In 1963 a survey of complications occurring in children after measles was made by Miller

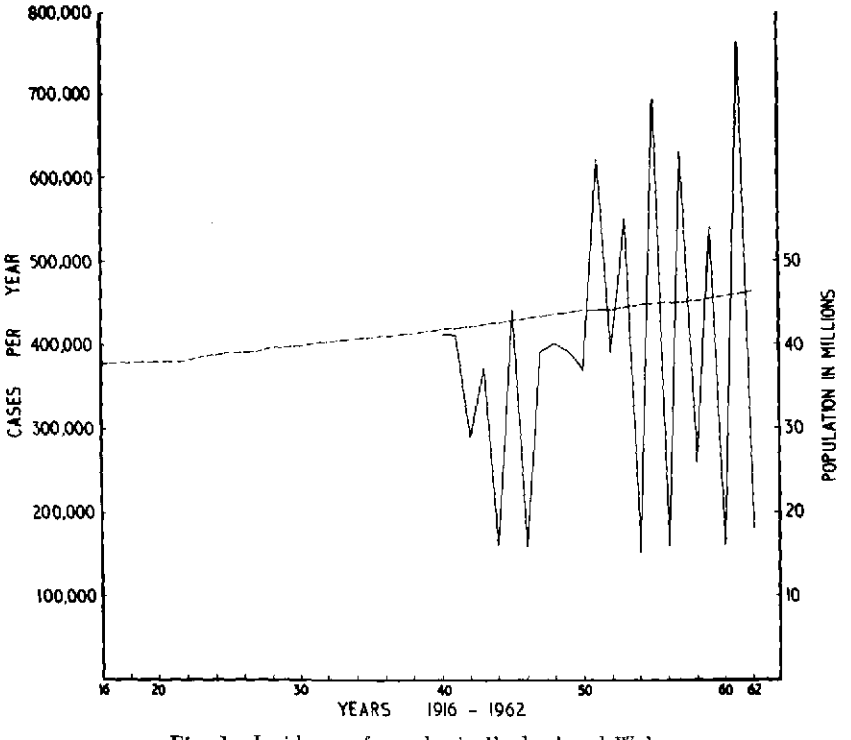

Fig. 1. Incidence of measles in England and Wales.

(3). Undoubtedly, there are limitations to the accuracy of the findings of such a postal survey, but the results surprised many who thought the disease was mild and did not warrant a large investigation. A total of 53,000 cases occurring at all ages up to 20 years were reported on, and about one in every 15 patients suffered from at least one complication. The most numerous were infections of the respiratory tract (38 in every 1,000 cases), and otitis media (25 per 1,000). Neurological disturbances occurred in 4 out of 1,000 cases. There were 610 hospital admissions (11.5 per 1,000) and 12 deaths (0.2 per 1,000). In an epidemic of about 500,000 cases, therefore, we may expect 19,000 cases of respiratory infection, 12,500 cases of otitis media, and 2,000 cases with neurological disturbances, some of which will result in irreversible damage. Clearly there is a need for a safe and potent vaccine.

Since measles was still not regarded by some as a serious disease, we felt it important to study reactions to the vaccine on a small scale before further investigating the protection against the disease.

The first Medical Research Council trial was carried out in January 1964 to determine the clinical reactions and antibody responses to live vaccines produced from further attenuated strains by both Glaxo Laboratories (Schwarz strain) and the Wellcome Foundation (MV/20). The vaccines were either given alone or preceded by a single dose of inactivated vaccine produced by Pfizer, Ltd. The Wellcome MV/20 strain gave a slightly higher incidence of reactions than did the Schwarz strain, but the antibody responses were also higher. Temperature records showed that in many of the children a febrile reaction occurred between the 6th and 9th day after receiving the live vaccine. These occurrences were greatly reduced by a preceding dose of inactivated vaccine. None of the children was seriously ill. As a result of the trial, it was generally agreed that the vaccines were safe even when given alone (Medical Research Council Report, 1965).

Since the information obtained from a trial involving only 300 children is necessarily limited, it was decided to plan a much larger investigation in the fall of 1964. In this second trial attempts were made to assess the frequency and degree of severity of reactions occurring after vaccination and to study the protective effect of the vaccines. The investigation was too large to obtain blood samples from all the subjects, and thus it was decided to take blood samples from children between one and two years old—the age group in which the maternal antibody had been lost and in which there was the highest proportion of nonimmune children. Blood samples were taken from about 150 children, and the antibody responses were compared with those of the previous trial.

Children whose parents registered them for the trial were assigned at random by date of birth to one of three groups to receive (a) killed vaccine (Pfizer) followed one month later by live vaccine (Glaxo); (b) live vaccine (Glaxo) alone; or (c) no vaccine. We decided to use the Schwarz strain only because in our first trial of any size we wanted the least number of reactions. The controls in this trial were not given a placebo vaccine but were promised vaccine later. Of the 36,530 children who took part, 10,625 were given killed and live vaccine, 9,577 were given live vaccine alone, and 16,328 remained unvaccinated. Attempts were made to reach all children, whether vaccinated or not, during the third week after the live vaccine was given and again at three and six months. The analysis of the results up to the six-month follow-up has recently been published (Medical Research Council, 1966), but I would like to mention a few of the salient points here. The continued analysis of the trial has not significantly altered the findings.

The trial was started at the beginning of a measles epidemic. Figure 2 shows the reported incidence of measles per 100,000 population in all areas taking part in the trial. The shaded areas of the horizontal blocks indicate the period during which the inoculations were given.

During the third week after the live vaccine was given, that is, after the most likely time for reactions to occur had passed, 98 per cent of the mothers were reached and asked about the health of the child since vaccination. The results obtained from a representative sample of about 11 per cent of all the children are shown in Table 1. The proportion of children reported as being unwell was 61 per cent among those given live vaccine alone and 54 per cent among

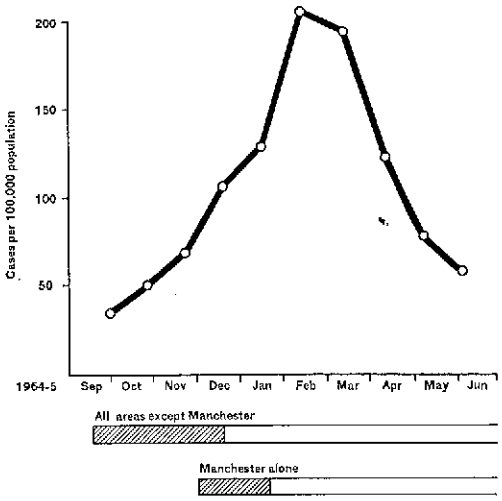

Fig. 2. Reported incidence of measles per 100,000 population in all areas taking part in the trial.

those given killed vaccine first. It is interesting to note that in the sample group 38 per cent of the controls were also reported as being unwell during the three-week period. Loss of appetite, disturbed sleep, malaise, rash, and fever occurred more frequently in the vaccinated children, whereas respiratory symptoms and diarrhea occurred with similar frequency in all groups.

Information about convulsions is given for all trial children in Table 2. All but one of the 30 convulsions were associated with fever, and the time at which they occurred is most interesting. More than half the convulsions after live vaccine alone occurred between day 6 and day 9, that is, when a fever induced by the vaccine was most likely. In contrast, there was no similar concentration of occurrences in the group given killed and live vaccine or in the control group. The table also shows that 20 per cent of the convulsions would not have occurred if children with a family history of fits had been excluded. It was considered that a convulsion following the administration of live vaccine was part of a general febrile reaction that is common in children of this age and is not serious.

In general, it was found that, although a few reactions caused disquiet, the great majority of children either remained well or had only trivial complaints. Furthermore, the proportion of children visited by the doctor or admitted to the hospital was no greater in the vaccinated groups than in the unvaccinated controls.

The protection against measles given by the vaccines was high and the analysis based on confirmed cases is shown in Table 3. The incidence of measles in the group given live vaccine alone was about one sixth, and that in the group given killed and live vaccine about one eighth of that in the unvaccinated group. There was no statistically significant difference in the degree of protection shown by the two schedules; for both it was about 85 per cent over the six-month period of the study. A similar analysis was made of those children in the representative sample who were known to have been in contact with measles at home during the six months after vaccination. The attack rates were 9 per cent in the killed-and-live vaccine group, 6 per cent in the live vaccine group, and 83 per cent in the unvaccinated controls. These results indicate a degree of protection of about 92 per cent.

The incidence of confirmed measles reported

TABLE 1. SYMPTOMS REPORTED AT THE THIRD WEEK FOLLOW-UP IN THE REPRESENTATIVE SAMPLE, EXCLUDING CASES OF MEASLES

Group	No. in sample	Symptoms										Seen by family doctor		Admitted to hospital	
		Children unwell		Off food/ vomiting		Disturbed sleep/malaise		Rash		Fever					
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Killed/live vaccine.....	1,127	608	54	281	25	448	40	154	14	42	4	120	11	4	0.4
Live vaccine...	1,015	620	61	317	31	454	45	190	19	65	6	111	11	2	0.2
Unvaccinated control.....	1,732	665	38	268	15	368	21	22	1	20	1	249	14	7	0.4

TABLE 2. CONVULSIONS DURING THE FIRST THREE WEEKS FOLLOW-UP IN ALL TRIAL CHILDREN

Group	No. of children	Convulsions		No. associated with fever	No. with family history of fits	Day of convulsion after vaccination												
		No.	Rate per 1,000			0	3	4	5	6	7	8	9	10	11	12	13	14–21
Killed/live vaccine.....	10,625	7	0.7	7	1			2				1					1	3
Live vaccine..	9,577	18	1.9	17	3	1				3	3	2	3				1	5*
Unvaccinated control.....	16,328	5	0.3	5	2		1		1					1				2

*One child had an afebrile convulsion and was subsequently found to be epileptic.

during the first month is also shown in Table 3. It is interesting to observe that even at this early stage some protective effect is given by the vaccines, especially in the group that received killed and live vaccine. There were two possible explanations for the earlier protection apparently afforded by the killed-and-live vaccine schedule. One is that the live vaccine may be giving a booster response, the killed vaccine having laid down a basal immunity. The second is that in the live vaccine group some of the early vaccine reactions may have been reported as measles. Some doubt has been expressed about the efficacy of the vaccines because the protection was not 100 per cent. In the small study undertaken to ensure that the vaccines were giving serological conversion it was seen that about 5 per cent of the children vaccinated by each schedule gave no antibody response, and in these children no protection could be expected. Indeed, 100 per cent protection has never been attained by a vaccine in such a large-scale clinical trial; it was not achieved by pertussis, BCG, or poliomyelitis, and why it should have been expected of this vaccine is not clear to us.

TABLE 3. INCIDENCE OF MEASLES DURING THE FIRST SIX MONTHS AFTER VACCINATION IN ALL TRIAL CHILDREN

Group	No. of children	Confirmed cases of measles			
		During the first month		During the entire 6-month period	
		No.	Rate per 1,000	No.	Rate per 1,000
Killed/live vaccine...	10,625	12	1.1	128	12
Live vaccine.	9,577	31	3.2	156	16
Unvaccinated control....	16,328	166	10.2	1,531	94

Doctors were asked to report on the severity of the disease. The results are given in Table 4. The proportion of severe cases was small in all three groups, but in the mild and moderate categories it is evident that vaccination by both schedules substantially modified the attack. An analysis was also made of the nature of complications accompanying the disease with particular reference to bronchitis, pneumonia, otitis media, and convulsions (Table 5). About 25 per cent of the cases involved complications, the incidence being distributed fairly evenly between the three groups. Bronchitis was the most common complication. It is worth noting that so far no case of encephalitis has been reported in any of the trial children.

It is clear that the giving of killed vaccine before live vaccine lowers the incidence of reactions. However, the killed-and-live vaccine schedule used in the United Kingdom requires two injections, and the number of children failing to return for the injection of live vaccine is sufficiently high to cause concern. All the control children in the trial have been offered killed and live vaccine, and of about 7,800 given killed vaccine, about 1,480 (19 per cent) have failed to return for the live vaccine. When the mothers were asked why the children had not

TABLE 4. DEGREE OF SEVERITY OF MEASLES IN CASES CONFIRMED BY A PHYSICIAN

Group	No. of cases	Physician's assessment of degree of severity						Four or more home visits by physician	
		Mild		Moderate		Severe			
		No.	%	No.	%	No.	%	No.	%
Killed/live vaccine	128	99	77	26	20	3	2	6	5
Live vaccine	156	120	77	35	22	1	1	8	5
Unvaccinated control	1,531	728	48	737	48	66	4	149	10

returned, about half of them reported the child as being unwell but did not suggest that this was due to the vaccine. If a killed-and-live vaccine schedule is used, therefore, it is essential that the mother clearly understand that immunity cannot be expected until after the live vaccine has been given.

Live measles vaccine given alone may cause a mild attack of measles with none of the severe complications that sometimes occur after the natural disease. The majority of reactions seen by the doctor are trivial. Whatever the reaction symptom, the natural disease is still far more severe. Even the incidence of convulsions associated with the vaccine (1.9 per 1,000) is far lower than that accompanying the natural disease (7.7 per 1,000). If measles were a disease in which the child had only a small chance of being infected, these vaccine reactions might not be justified. However, few people escape measles, and the complications in some cases are very serious. It would be misleading to report that measles vaccine was being used on a wide scale in the United Kingdom. Some people do not feel that a mild reaction is justified, even for protection against the disease. The vaccine is being used, however, and when we have more data on the duration of protection it may well be used on a wider scale.

None of these studies so far has answered two important questions:

1. How long does the protection last?
2. What proportion of our child population must be vaccinated to break the chain of transmission of the disease and thus prevent the biennial epidemics?

The first question can only be answered by long-term studies, which are already in progress. If there is evidence of waning immunity in our vaccinated children during the expected 1966–1967 epidemic, then we must consider giving reinforcing doses. To give a short-lived immunity and postpone the disease to an age when it may be more inconvenient or dangerous would be unjustifiable.

The second question is quite fascinating and it is one that has not been answered by any study. We are attempting to answer it by vaccinating all children with no history of measles

TABLE 5. SPECIFIC COMPLICATIONS ASSOCIATED WITH CONFIRMED CASES OF MEASLES

Group	Total No. of confirmed cases	Specific complications								Cases admitted to hospital	No. of deaths
		Bronchitis		Pneumonia		Otitis media		Convulsions			
		No.	%	No.	%	No.	%	No.	%		
Killed/live vaccine	128	20	16	0	0	5	4	2	2	0	0
Live vaccine	156	29	19	0	0	2	1	2	1	1	0
Unvaccinated control	1,531	304	20	10	1	57	4	10	1	22	3

in several areas and comparing the incidence of the disease in the areas before and after vaccination. When we have an answer to this question we look forward to reporting our findings.

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SECTION A. RUBEOLA

COMBINED MEASLES-SMALLPOX AND OTHER VACCINES

H. M. MEYER, JR., H. E. HOPPS, B. C. BERNHEIM, AND R. D. DOUGLAS

Division of Biologics Standards, National Institutes of Health
U. S. Public Health Service, Bethesda, Maryland, U.S.A.

DR. MEYER (*presenting the paper*): There has been considerable field experience in the use of combined live virus vaccines and in simultaneous but separate inoculation of various live attenuated agents. By 1947 more than 12 million West Africans had been scarified with a mixture of smallpox and French neurotropic yellow fever vaccines (19). Immunization with mixed or simultaneously inoculated smallpox and 17-D yellow fever vaccines has also been evaluated in several pilot studies (3, 6, 11, 12). In recent years trivalent and bivalent oral poliovirus vaccines have seen extensive use (7, 23). Combinations of live attenuated measles and mumps viruses have been employed in Russia (24). American and African children have been immunized with jet-injected mixed live measles and smallpox vaccines (14, 25), and in Africa a trivalent combination of attenuated measles, smallpox, and 17-D yellow fever viruses has been jet-inoculated into triple-negative children (14, 15). Simultaneous inoculation of vaccinia virus and BCG vaccine has received attention as a possible approach to smallpox and tuberculosis control (10). Simultaneous but separate inoculation was also used in a small study of monovalent oral poliovirus vaccine and live measles vaccine (5).

There are major advantages, but also problems, associated with combined or simultaneous vaccination with living agents. In this paper we shall present data obtained in our earlier studies of combined vaccines and use this information as a background for discussing the subject.

MATERIALS AND METHODS

The materials and methods used in the clinical trial of combined measles, smallpox, and yellow fever vaccines in Ouagadougou, Republic of Upper Volta, have been described in detail previously (14). The 545 children participating in the study were placed in five groups of approximately equal size and given vaccines singly or in combination as follows: (1) measles, (2) smallpox, (3) yellow fever, (4) measles and smallpox, and (5) measles, smallpox, and yellow fever. The majority of the children were less than one year of age, and none had a history of previous measles or of smallpox or yellow fever immunization.

After an initial physical examination, the children were inoculated with 0.5 ml volumes of the appropriate vaccine or mixture of vaccines by means of an automatic jet-injection apparatus. The vaccinees were observed for a three-week period to determine the occurrence of vaccine-related reactions. The following vaccines were employed: (1) an experimental lot of Enders B-level attenuated measles virus vaccine grown in chick embryo tissue cultures (this material was donated by Dr. M. R. Hilleman, Merck Institute for Therapeutic Research, West Point, Pennsylvania); (2) a commercial lot of chick-embryo-type bacteriologically sterile smallpox

vaccine (Lederle Laboratories, Pearl River, New York, Lot 2096-12A); and (3) a commercial lot of 17-D yellow fever vaccine (National Drug Company, Philadelphia, Pennsylvania, Lot 5312). The three vaccines were supplied in the lyophilized state and were maintained under refrigeration until rehydrated and, where appropriate, mixed immediately prior to use. The calculated amounts of the attenuated virus administered to each recipient under these conditions were as follows: measles, 12,000 tissue culture infectious $dose_{50}$ ($TCID_{50}$); smallpox, 1,500,000 $TCID_{50}$; and yellow fever, 6,300,000 mouse-lethal doses (LD_{50}). Sterile blood specimens were collected from children immediately prior to and 18 to 20 days after vaccination. The sera were aseptically separated from the clots, stored, and then shipped in the frozen state to the National Institutes of Health for antibody assay. The antibody assay methods used in testing the sera from vaccinated children included hemagglutination-inhibition (HI) for measles (22) and smallpox (4) and neutralization tests for measles (13), smallpox (1), and yellow fever (9).

RESULTS

Clinical Response to Vaccination

No unusual or unexpectedly severe reaction to vaccination occurred in any of the 545 vaccinated children. Children in groups receiving attenuated measles virus alone or in combination with the other vaccines experienced more fevers than those in the group inoculated with smallpox vaccine alone or with yellow fever vaccine alone (Fig. 1). The percentage of children with temperatures of 39.0°C or higher on any occasion during the 21-day observation period was remarkably constant in the three groups: measles, 20.0 per cent; measles-smallpox, 22.8 per cent; and measles-smallpox-yellow fever, 20.6 per cent.

A maculopapular rash was detected between the ninth and the fifteenth postvaccination days in 45 to 52 per cent of the children in each group receiving measles vaccine. These rashes were usually considerably modified and only rarely resembled the cutaneous manifestations of natural measles.

Smallpox vaccination was followed by the

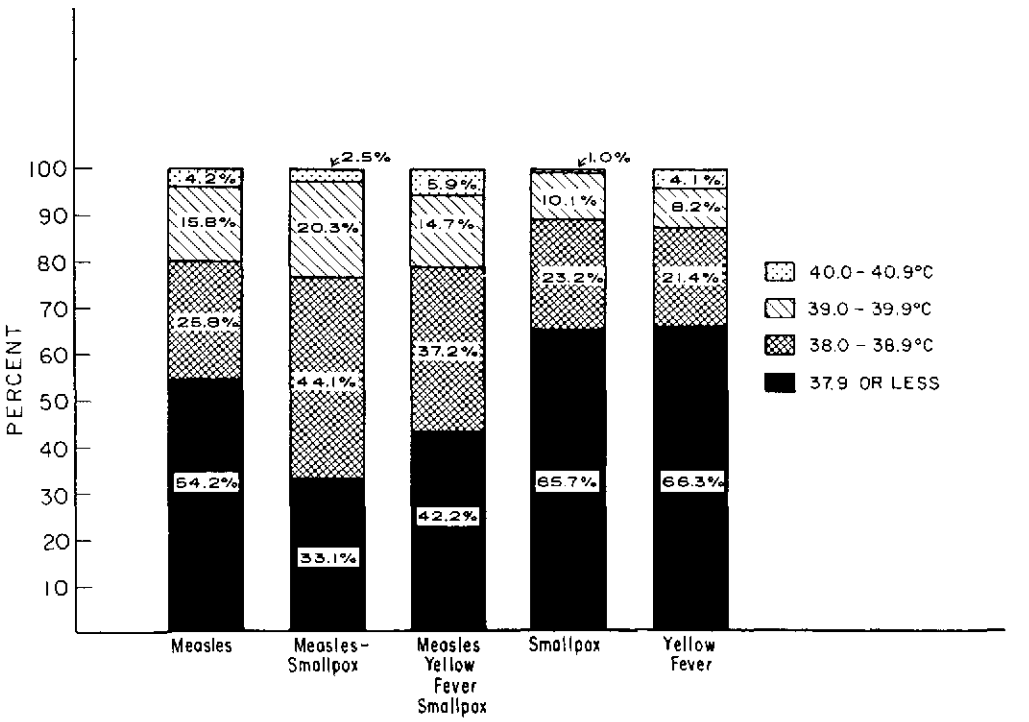

Fig. 1. Maximum temperature during 21-day post vaccination period.

development of the typical primary cutaneous vaccinal reaction in 99 per cent of susceptible children. The addition of measles or measles and yellow fever viruses to the vaccine did not influence the character of the skin lesions.

During the course of surveillance, many vaccinees developed symptoms or signs of infection or parasitic diseases. Common complaints included diarrhea, respiratory illness, otitis media, purulent conjunctivitis, and skin infection. These problems seemed to bear no relation to the type of vaccine administered, since they were about equally distributed in the five groups.

Antibody Response to Vaccine

In the groups receiving measles vaccine, alone or in combination, 97 to 100 per cent developed measles HI antibodies (measles alone, 97 per cent; measles-smallpox, 100 per cent; measles-smallpox-yellow fever, 98 per cent). The data in Figure 2 provide more detailed information regarding the level of measles HI antibody response. There was little difference in the geometric mean titers (GMT) (143, 152, and 182) for the three groups.

Smallpox vaccine evoked an HI antibody response in 99 to 100 per cent of susceptible children inoculated (smallpox alone, 100 per cent; measles-smallpox, 99 per cent); measles-smallpox-yellow fever, 100 per cent). As Figure 3 illustrates, HI antibodies for vaccinia tended to be highest in the group receiving only smallpox vaccine and lowest in the children receiving the mixture of the three vaccines. These differences in antibody titers are statistically significant at the level $P > 0.005$.

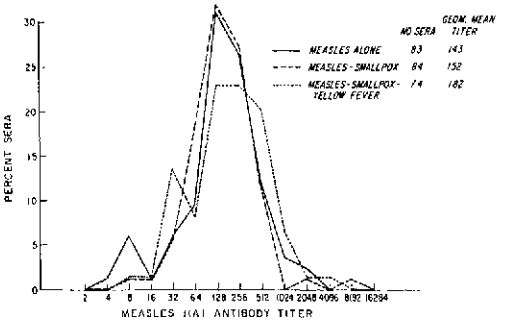

Fig. 2. Measles antibody response of vaccinated children.

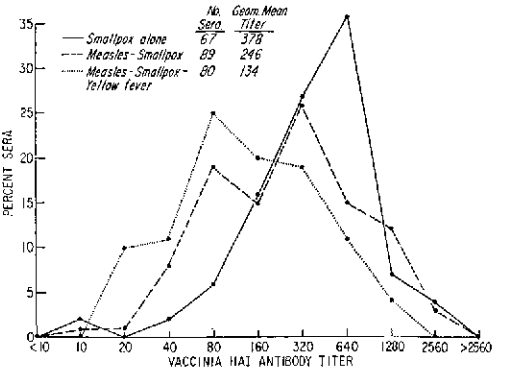

Fig. 3. Smallpox antibody response of vaccinated children.

Yellow fever neutralizing antibodies were assayed by a mouse-protection test. The criteria used for calculating yellow fever seroconversion have been described in an earlier report (14). The results, summarized in Table 1, revealed that 7 per cent of the children inoculated with yellow fever vaccine alone failed to develop neutralizing antibodies while 19 per cent of the trivalent vaccine group remained serologically negative.

Neutralizing antibody tests for measles and vaccinia virus were performed on representative numbers of sera from groups receiving monovalent and polyvalent vaccines.

Table 2 lists the results of measles virus neutralization in comparison to HI determinations on sera from 20 children who had received measles vaccine alone, 9 who had received measles-

TABLE 1. YELLOW FEVER NEUTRALIZING ANTIBODY RESPONSE OF CHILDREN INOCULATED WITH YELLOW FEVER VACCINE ALONE OR IN COMBINATION WITH OTHER VACCINES

Type of vaccine	Yellow fever neutralizing antibody response		
	Positive*	Equivocal*	Negative*
17-D Yellow fever	47/72† (65%)	20/72 (28%)	5/72 (7%)
Measles-smallpox-yellow fever	51/81 (63%)	15/81 (19%)	15/81 (19%)

*See text for method of calculation.
†Numerator denotes number of children with specified antibody response; denominator denotes number of children inoculated.

TABLE 2. MEASLES NEUTRALIZING AND HEMAGGLUTINATION-INHIBITING ANTIBODY RESPONSE OF CHILDREN INOCULATED WITH MEASLES VACCINE ALONE OR IN COMBINATION

Type vaccine	Antibody test	Measles antibody titer									No. vaccinated	Geometric mean titer
		<8	8	16	32	64	128	256	512	1024 or>		
Measles	Neut.		1	3	4	10	2				20	43.7
	HI		1		1	2	10	6				119.4
Measles-smallpox	Neut.				4	3	2				9	54.5
	HI					1	4	2	2			187.4
Measles-smallpox-yellow fever	Neut.	2	3	8	7	2					22	17.0
	HI				1	4	3	5	9			218.2

smallpox, and 22 who had received measles-smallpox-yellow fever. Appraisal of the GMT values and the distribution of titers obtained with individual specimens reveals that the HI antibody values for the three groups were similar. The neutralizing antibody values for the first two groups—measles vaccine alone and measles-smallpox vaccine—were also similar. However, the measles-neutralizing antibody response of children receiving the trivalent vaccine was lower. These differences are reflected in the GMT of neutralizing antibodies of 43.7, 54.4, and 17.0, respectively.

Neutralizing and HI antibody tests for vaccinia virus antibodies were performed on a selected number of paired sera from children receiving vaccine alone or in combination (Table 3). The highest vaccinia-neutralizing and HI antibody titers were obtained in the group injected with the monovalent vaccine. Combining either measles or yellow fever viruses in the vaccine seemed to have a suppressive effect on the

TABLE 3. VACCINIA NEUTRALIZING AND HEMAGGLUTINATION-INHIBITING ANTIBODY RESPONSE OF CHILDREN INOCULATED WITH SMALLPOX VACCINE ALONE OR IN COMBINATION

Type vaccine	Antibody test	Vaccinia antibody titer										No. vaccinated	Geometric mean titer
		<8	8	16	32	64	128	256	512	1024	2048 or>		
Smallpox	Neut.					4	4	9	3			20	187.4
	HI						1	4	5	4	6		724.
Measles-smallpox	Neut.	4		2	1	1	1					9	7.41
	HI				1	4	1	2	1				109.1
Measles-smallpox-yellow fever	Neut.	4	4	5	3	2	2	1				21	13.9
	HI					1	4	10	3	3			282.

vaccinia-neutralizing antibody response. The GMT of vaccinia-neutralizing antibodies in the three groups were 187.4, 7.41, and 13.9, respectively.

DISCUSSION

In our studies with combined measles-smallpox and measles-smallpox-yellow fever vaccines (14, 15), no accentuated clinical reactions were noted. Moreover, in the broad experience of others using vaccines such as smallpox and yellow fever (3, 6, 11, 12, 19), bivalent and trivalent poliovirus (7, 23), measles and smallpox (25), measles and mumps (29), smallpox and BCG (10), and monovalent poliovirus and measles (5), there was no evidence that the inoculation of these agents either simultaneously or in combination resulted in any untoward clinical responses. Considering the span of years and multiplicity of agents involved, this is a remarkable negative experience.

There have been two separately reported instances in England of encephalitis in association with the inoculation of smallpox vaccine followed several days later by the administration of yellow fever vaccine. While these cases have been referred to in the literature (3) as possible vaccine reactions and have, indeed, influenced smallpox and yellow fever vaccine policy (16), there is no real evidence that the dual inoculation of these agents was the actual cause of the ensuing encephalitis.

In view of the obvious need in many areas of the world for mass vaccination against a number of preventable diseases, the possibilities for increased use of combined vaccines must continue to be explored. For example, for more than 25 years the use of combined smallpox and yellow fever vaccines in West Africa has provided a practical solution to the ever-present threat posed by these two major diseases.

Of course, an important question raised by the use of combined vaccines concerns the possibility that the efficacy of one or more of the vaccine components will be reduced as a result of viral interference. There is some apparent conflict in the literature on this point. Mixtures of smallpox vaccine and French neurotropic yellow fever vaccine (19) or 17-D yellow fever vaccine (6) were found by some to be fully effective when administered by scarification. However, others have reported that vaccinia virus interferes with yellow fever seroconversion when they are inoculated *in duo*, but no reduction in antibody response was observed when the viruses were inoculated at separate sites (3, 11, 12). In studies with attenuated polioviruses, Sabin (23) and Horstmann (7) have indicated that these viruses may interfere with one another or may be rendered less effective if the intestinal tracts of recipients are colonized by other enteroviruses.

In our own study there was some indication of interference by measles and yellow fever vaccines, both with each other and with the vaccinia moiety. One possible explanation of this, and of certain other recent examples of interference between attenuated viruses, relates to interferon production (8). Interferon induction in cell cultures by measles and vaccinia viruses is well known (2), and recently in our laboratory it was found that interferon is produced in tissue cultures infected with yellow fever virus (Hopps *et al.*, unpublished data).

Circulating serum interferon can be detected during the course of attenuated measles (20) and yellow fever (27) infections in man. However, smallpox vaccination of susceptible persons results in interferon production that can be detected locally but not systemically (26). These characteristics are summarized in Table 4. It may be noted that in our study the two viruses shown to produce circulating interferon caused interference with the other vaccine components, whereas smallpox vaccine, which produces only locally detectable interferon, did not cause demonstrable interference with the other viruses in the mixtures.

TABLE 4. INTERFERON INDUCTION BY VIRUSES USED FOR HUMAN VACCINATION

Infecting virus	Interferon detected		
	In vitro	In vivo	
		Circulating	Local
Attenuated measles...	+*	+*	N.T.
17-D Yellow fever....	+	+*	N.T.
Vaccinia.....	+*	0	+

*See text for references.
N.T.—Not tested.

Several practical conclusions can be derived from these observations. If circulating interferon occurring in response to measles or yellow fever vaccination is of cardinal importance in interference, then simultaneous inoculation of vaccines at different sites may offer no advantage over the use of a combined product. Moreover, the practice advocated by some groups in the past (16) of delaying smallpox vaccination until four or more days after yellow fever inoculation seems ill-advised, since maximum interference might be expected. By delaying smallpox vaccination until several days after inoculation with attenuated measles virus, it was possible to block completely the cutaneous response to vaccinia virus (21). The period of effective suppression of smallpox vaccination corresponded to the period of circulating measles-induced interferon.

Recent work has indicated that the degree of attenuation of a virus may be correlated with its interferon-inducing capacity (17, 18). If so, more attenuated strains of the same virus might be expected to be less satisfactory for use in combined vaccines than other strains. For example, the clinical experience obtained by concurrent administration of Enders B-level measles vaccines and vaccinia virus should not be assumed to apply to simultaneously inoculated Schwarz-type measles vaccine and smallpox vaccine.

Since some degree of interference may be common in dual infections, it becomes important to test each new combined preparation carefully, bearing in mind that small differences in vaccine dose, strain, and route of inoculation may have a pronounced effect on efficacy.

In the present discussion, reduction in vaccine efficacy as a result of combining virus vaccines has been evaluated solely on the basis of reduced antibody levels. Actually, the significance of this observation is not entirely clear, since few data are available on the correlation between antibody level and protective immunity against many agents. Certainly in the case of measles virus it would appear that any demonstrable level of antibody is an indication of solid immunity; this same thing may also hold true for rubella virus. Moreover, in evaluating antibody response data with combined vaccines, the time at which the sera are tested may be extremely important. It is possible that antibody production may be delayed during the period when interferon is present but occur at a later time. In our own studies it was necessary to obtain serum specimens between 18 and 20 days after vaccination; one wonders whether increased levels of smallpox antibody would have been observed in specimens taken several weeks later. Other studies in the literature shed little light on this question, since in many of them antibody assays were not done and in others antibody determinations were made at only one fixed postinoculation period.

In making a general assessment of the feasibility of combined vaccine usage, the factor of practical field need must be carefully weighed against that of possible interference between the vaccine components. It would seem that a judicious selection and balancing of vaccines intended for mixed or simultaneous use, coupled with attention to inoculation routes and techniques, should make possible the realization of maximum benefits from future preventive medicine programs. Today there are many prospects for useful combined vaccines; tomorrow will bring still more.

Summary

The over-all experience in studies of the use of combined vaccines throughout the world has been reviewed. Detailed information has been given on the administration of live attenuated measles, smallpox, and yellow fever vaccines, alone and in combinations, to 545 children in West Africa. No alteration of the characteristic clinical reactions was noted in children receiving the bivalent or trivalent vaccines. Some suppression of neutralizing and, to a lesser extent, of HI antibodies occurred. Factors that might influence antibody response to combined vaccines have been discussed.

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SECTION A. RUBEOLA

DISCUSSION

CHAIRMAN BAROYAN: So now we come to a very important section of our day's meeting, and that is the discussion. Our first speaker is Dr. W. Chas. Cockburn, Virus Diseases, Division of Communicable Diseases, World Health Organization, Geneva, Switzerland.

DR. COCKBURN: Dr. Enders and his colleagues must be gratified and impressed by the reports made today on the extensive and successful use of live measles vaccines in North America, the USSR, Europe, Africa, Latin America, Japan, and elsewhere.

There is good evidence that the Enders Edmonston B strains give solid long-lasting immunity, and it seems reasonable to believe that the other strains in current vaccines will also do so. If used in well-planned, long-term vaccination programs, the available vaccines will rapidly bring the disease under control. However, as Dr. Labusquière has pointed out, such programs must ensure the regular vaccination of a high proportion of children as they approach one year of age, or the results will be disappointing.

We should not let the successes achieved so far blind us to the problems that are still unsolved. Vaccines are acceptable at the present time, while parents and physicians still have before them evidence of the severity of the disease in many developing countries and an evidence of the risk of complications in developed countries. Compared with the clinical manifestations of the natural disease and its sequelae (rare though these may be), the reactions currently associated with vaccination—pyrexia of 103°F for 24 to 48 hours in 10 to 20 per cent of the vaccinated children and convulsions in less than 1 per cent—seem relatively unimportant. But when the disease has become rare, thanks to vaccination, the reactions to the vaccine will loom much larger in the mind and acceptance rates will certainly fall. Experience with other vaccines has shown that adequate evaluation of new or improved products is difficult when the incidence of the disease has reached low levels, and the acceptance of these new vaccines by national control authorities may consequently be long delayed. Thus the search for more acceptable measles vaccines should be continued and intensified.

Concern should also be given to reports of unexpected reactions when inactivated vaccines are followed by the injection of live vaccines or by the exposure of vaccinated persons to the natural virus. I shall leave this question for Dr. Katz to deal with and shall end by drawing attention to the paper by T. A. McNeil,* in which he records the occurrence of severe local reactions in rabbits vaccinated first with inactivated and later with live vaccinia virus vaccines. His observation that there may be an association between these reactions and certain inactivating agents and adjuvants may be important to further study of the subject.

CHAIRMAN BAROYAN: Thank you very much, Dr. Cockburn. The next speaker is Dr. Samuel L. Katz,† Research Division of Infectious Diseases, Children's Hospital Medical Center and the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts.

DR. KATZ: I should like to report on "Unusual Manifestations of the Interaction of Inactivated Measles Antigens with Live Measles Viruses."

* "The Development of Skin Resistance and Hypersensitivity Following Inactivated Vaccinia Virus Vaccines in Rabbits." *J Hyg (Camb)* **64**:23–31, 1966.

† Recipient of a Research Career Development Award of the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service, Bethesda, Maryland.

Early investigations of the immunologic responses of simian and human hosts to inactivated and live measles viruses disclosed consistent quantitative differences in the peak antibody titers and the persistence of detectable antibody following stimulation by the respective antigens. The rapid decline of antibody observed in the six-to-eighteen-month period after completion of three monthly injections of inactivated vaccine was in marked contrast to the indefinite persistence of measles antibodies noted after a single dose of live attenuated virus (1). Subsequent studies in monkeys revealed qualitative differences as well. Monkeys with high levels of circulating antibody resulting from immunization with inactivated measles antigens were quite surprisingly able to support a prolonged viremia when challenged with live virus (2), whereas those immunized with attenuated rubeola virus would not support a successful reinfection on challenge with virulent virus (1). Whether these contrasting results reflected a difference in type, configuration or avidity of antibody was never explained.

With the clinical use of inactivated antigens, either alone or in combined schedules with live attenuated virus, a distressing spectrum of unusual manifestations has been recorded in some children who have been infected with naturally-occurring or attenuated rubeola strains six or more months after the "killed" vaccine.

Inactivated Antigen and Natural Measles Virus

Rauh and Schmidt (3) first called attention to eight children in Cincinnati who developed severe atypical measles on contact with wild rubeola virus two to two and one half years after completing a course of three monthly injections of inactivated virus. Five patients were sufficiently ill, with fever and signs of pneumonia, to warrant hospitalization. The exanthemata were most unusual, characterized by urticarial lesions, purpura, and petechiae and a predilection to begin and concentrate on the extremities. Edema of the hands and feet was also recorded. More recently, a cluster of very similar cases occurred when six children in Riverton, Wyoming, developed measles four years after immunization with three injections of killed rubeola vaccine (4). As in the Ohio cases, respiratory-tract signs and symptoms were exaggerated; there was cough and striking tachypnea disproportionate to the height of fever. Several children had severe myalgia and marked hyperesthesia of muscle and skin. Generalized, unexplained edema was apparent for four days in one youngster. Rashes again commenced and were more profuse on the feet and legs. These children had a definite vesicular component superimposed on the initially macular or maculopapular exanthem. Norrby has described two similar cases observed among 38 vaccinees (9).

Inactivated Antigen and Attenuated Measles Virus

When live attenuated virus was administered to children who had previously received two, three, or four doses of inactivated antigen, bizarre local reactions occurred at the site of live virus injection during the next two to seven days. These have been described by Buser in Bern, Switzerland (5), Scott in Philadelphia (6), Fulginiti in Denver (7), and Krugman in New York (8). Similar examples have been brought to our attention by physicians in other areas. In general, the local manifestations included marked swelling, erythema, tenderness, warmth and, in some cases, hemorrhage and/or vesiculation. Fevers as high as 105°F were recorded and enlargement of regional lymph nodes was striking. In all children, those unusual findings subsided spontaneously in approximately one week.

The incidence of these aberrant responses after either sequence, i.e., killed followed by attenuated or killed followed by natural measles infection, ranged from 4 to 30 per cent. Since antigens prepared in three different substrates—chick embryo cells, simian renal cells, or canine kidney cells—were involved in varying combinations, the possibility of sensitization to the host cell of the vaccine is not likely. More probable is a hypersensitivity to some component of the measles virion which is concentrated in the inactivated vaccines. It is equally interesting and reassuring that no such reactions were observed after the administration of live attenuated virus to naturally immune individuals, and we may conjecture that previous natural measles infection does not result in any similar sensitization

which might induce the capacity to interact with attenuated virus in the same manner.

Two other variables in this problem are not completely elucidated at present. The quantity and frequency of the primary sensitizing dosage is uncertain, but a minimum of two injections of inactivated measles vaccine was administered previously to each child who underwent aberrant local or systemic response on subsequent infection with replicating measles virus. The requisite interval between initial sensitization and exposure to live virus has not been defined. However, in all cases to date, in which the appropriate information has been obtainable, the minimum time that elapsed was six months, while periods as long as four years separated the pertinent events for some children.

The mechanism of these unusual reactions is at present obscure, but similarities to Arthus's phenomenon are suggestive. Aside from their role as another known liability to the use of inactivated antigens, the events provide a unique opportunity to study the immunochemical and cellular mechanisms involved in man's response to foreign nucleoproteins or other definable antigenic materials.

Summary

Attention has been directed to a series of reports, from widely separated areas, of unusual reactions that have followed the exposure to live attenuated or natural measles virus of children previously immunized with inactivated measles antigens. These aberrant responses have been of sufficient concern and frequency to warrant careful reconsideration of any further use of schedules for measles immunization that include more than a single dose of inactivated vaccine. The decision to use available, safe, effective, attenuated, live measles virus as the sole immunizing experience to prevent the natural disease, seems further justified by these adverse reports.

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CHAIRMAN BAROYAN: Our next speaker is Dr. Saul Krugman, Department of Pediatrics, New York University School of Medicine, New York, N. Y.

Dr. Krugman presented a summary of a paper entitled "Studies on Immunity to Measles," which appears in full as Annex 1 to this section, pages 353–360.

CHAIRMAN BAROYAN: The next speaker is Dr. M. V. Milovanović, who is with the Pan American Health Organization in Rio de Janeiro, Brazil.

DR. MILOVANOVIĆ: After listening to the reports presented, all of us must agree that tremendous progress has been achieved in the specific control of measles infections. The credit undoubtedly goes to Enders, in whose laboratory most of the basic pioneer work has been done.

Many people have the impression that the problem of live measles vaccine is almost completely solved. Indeed, there are some who believe that if sufficient funds were available and public health services were prepared for action measles could even be eradicated.

In my opinion, one of the main reasons—and in some countries the only reason—why live measles vaccine has not yet been introduced as

a routine procedure on a nationwide scale is that the vaccine may induce reactions, which are usually mild but nonetheless quite frequent.

Of the live measles vaccines that are being used so far, a number have emerged with specific names—live Edmonston B, further attenuated, Leningrad, Beckenham, Belgrade, and so on. It is true that a number of these vaccines have been shown to be more or less attenuated. I would think that the common denominator for all of them is the adaptation of the virus to grow in the developing chick embryo and in the chick cells. Thus attenuation, as a new quality, is the result of adaptation in chick cells. The difference, if any, in the level of the attenuation of the various vaccines is due to what one might call the additional refinement of the cultivation methods.

Now the question may arise whether further attenuation may be achieved by adapting the virus to grow in the tissue of a species phylogenetically lower than the chicken—for instance, in amphibian tissue. One cannot predict whether any further attenuation would occur or, if so, whether the immunogenic capacity would be preserved, but it seemed to me that it would be worth trying. The first step in this project was to adapt the virus to grow in amphibian tissue. This is the subject of my discussion—the work I have done this year in the Oswaldo Cruz Institute in Rio de Janeiro, where I am temporarily assigned as a WHO virologist.

Using the plasma clot technique, primary cultures were made from the tails and legs of tadpoles and from the kidney of adult frogs. Enders or Melnick medium supplemented with 25 to 30 per cent bovine serum was used. The cultures were kept at 28°C. When the growth of the cells was established, the Belgrade line of the Enders Edmonston B strain was introduced as the inoculum. A definite growth was established in this system during three consecutive passages. So far, no CPE was observed, and evidence of viral growth was obtained by testing the tissue fluid in cultures of chick cells and human amnion.

The second frog tissue passage showed the virus in the fluid for a period of six weeks (the length of time the tissue was kept), the fluid being tested once a week. This work is still in progress and I hope the virus will continue to grow. Of course, frog tissue is not the tissue for making the vaccine, but only for effecting the changes in the virus; the vaccine could be made in chick cells.

Also worth noting is the work being carried out by one of my associates, Dr. A. Klasnja, in my old laboratory in Belgrade. He grows the virus in chick cells derived from embryos that have been given measles globulin in the yolk sac four to five days before trypsinization. When the vaccinal virus grown in this way is given as a vaccine it induces less severe reactions than the usual vaccinal strain. He tested this in a rather small number of children, and on the basis of these preliminary results he is continuing his work.

I would like to stress that all these efforts are being made with the idea in mind that something already proved to be very good can be made even better.

CHAIRMAN BAROYAN: The next speaker is Dr. Minoru Matumoto, Institute for Infectious Diseases, University of Tokyo, Tokyo, Japan.

DR. MATUMOTO: I would like to comment on vaccination against measles as practiced in Japan. Both live and killed vaccines were licensed in 1965 and now are commercially manufactured. The killed vaccine is a formalin-inactivated, alum-adsorbed preparation made from virus grown in primary cultures of monkey kidney cells. Two strains are used for the preparation of live vaccine: the Toyoshima strain of Okuno and his associates,* and our own Sugiyama strain.† Both are derived from local strains of measles virus. The Toyoshima strain was adapted to chick embryos and tested in field trials. It was subsequently transferred to primary monkey kidney cell cultures and is now used for the preparation of vaccine in this host

* Okuno, Y., Sugai, T., Toyoshima, K., Takahashi, M., Yamamura, T., Hata, S., Miki, T., Nakamura, K., Ueda, S., and Kunita, N. "Studies on the Prophylaxis of Measles with Attenuated Living Virus. IV. Inoculation Tests in Children with Chick Embryo Passage Measles Virus in 1960." *Biken J* **3**:293–300, 1960.

† Matumoto, M., Mutai, M., Saburi, Y., Fujii, R., Minamitani, M., and Nakamura, K. "Live Measles-Virus Vaccine: Clinical Trial of Vaccine Prepared from a Variant of the Sugiyama Strain Adapted to Bovine Kidney Cells." *Jap J Exp Med* **32**:433–448, 1962.

system. The Sugiyama live vaccine is prepared in primary cultures of calf kidney cells with the strain adapted to these cells.

In a comparative study conducted in 1962 by the Committee on Measles Vaccines, which was supported by WHO and Japanese Government, the Toyoshima and Sugiyama live vaccines proved very effective and quite safe. They were found to induce clinical reactions similar to Edmonston B vaccine.* Mainly for administrative and economic reasons, killed vaccine rather than immune gamma globulin is recommended in Japan for reducing clinical reactions. Killed vaccine significantly reduces the clinical reactions to live vaccine given one or two months later. The immunogenic effect of live vaccine is little impaired by one dose of killed vaccine, but significantly by two doses.† Hence, the former regime is recommended in Japan.

Further attenuation of the Sugiyama strain has been achieved by continuation of serial passage in calf kidney cells. The evaluation of the further attenuated vaccine in field trials is still in progress. It should be noted here that the Sugiyama live vaccine is unique in that it is prepared in calf kidney cell culture. This host system is not only suitable for the propagation of this particular strain, it is also practically free of latent virus, as contrasted with chick embryo or monkey kidney tissue cultures. The Sugiyama strain grown in calf kidney cell culture is also a suitable viral material for the preparation of inactivated vaccine (Sasai, *op. cit.*).

CHAIRMAN BAROYAN: The next speaker is Dr. F. Kalabus, of the Ministry of Health of the Province of Quebec, Rouyn, P. Q., Canada.

DR. KALABUS: Following the report of Dr. Meyer, I am glad to present a brief summary of our recent work entitled "Mass Application by Jet Gun of Combined Measles-Smallpox Vaccine in Upper Volta," co-authored with Drs. H. Sansarricq, P. Lambin, J. Proulx, and M. R. Hilleman.‡

While increasing numbers of vaccines are becoming available for human use, there is at the same time a shortage of health personnel in many areas of the world. To reduce this gap, vaccine administration must be made more efficient—costs lowered, the number of injections reduced, speed improved, and methods developed whereby routine application can be carried out by medical aides after a minimal training period.

Following initial studies conducted elsewhere,§ a mass campaign was carried out in the Republic of Upper Volta in 1965–1966,¶ in the course of which 18,464 children were administered combined live Enders Edmonston B measles vaccine with ordinary calf lymph smallpox vaccine via jet gun. The gun was adjusted to deliver 0.1 ml intradermally and 0.4 ml into the deeper tissues. Some of the vaccine was premixed before drying and the remainder was mixed in the field immediately prior to use.

Clinical follow-up and surveillance showed conclusively that the measles-smallpox vaccine given in combined form was safe and did not cause serious clinical reactions or increase in febrile reactions above that expected for either vaccine given alone.

Immunologic responses in the children were in the range of 98 per cent for both vaccine components. Table 1 shows that 13,053 of 13,254 children with negative smallpox vaccination history and 5,095 of 5,210 children with positive smallpox vaccination history showed a primary vaccinoid or immune response, giving an over-all

* Committee on Measles Vaccines (Japan). *Reports on Studies of Measles Live Vaccine. I, 1963.* In Japanese.

† Committee on Measles Vaccines (Japan). *Reports on Studies of Measles Live Vaccine. III. 1965.* In Japanese.

Sasai, S., Kawana, R., Wako, H., Iwai, T., Kaneko, M., and Matumoto, M. "Response of Children to Inactivated Measles Vaccine Prepared in Bovine Renal Cell Culture." *Jap J Microbiol.* In press.

‡ Ministry of Health of the Province of Quebec, Rouyn, P.Q., Canada; the Service des Grandes Endemies, Ministère de la Santé, Ouagadougou, Upper Volta; and the Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania.

§ Meyer, H. M., Jr., Hostetler, D. D., Jr., Bernheim, B. C., Rogers, N. G., Lambin, P., Chassary, A., Labusquière, R., and Smadel, J. E. "Response of Volta Children to Jet Inoculation of Combined Live Measles, Smallpox and Yellow Fever Vaccines." *Bull WHO* **30**:783–794, 1964.

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¶ Kalabus, F., Sansarricq, H., Lambin, P., Proulx, J., and Hilleman, M. R. "Standardization and Mass Application of Combined Live Measles-Smallpox Vaccine in Upper Volta." *Amer J Epid*, in press.

TABLE 1. SUMMARY APPRAISAL OF SMALLPOX VACCINE EFFICACY IN COMBINED FORMULATION*

Previous smallpox vaccination history	No. of children vaccinated	Positive reactions (Primary, vaccinoid, immune)	
		No.	Percentage of positive reactions
Negative.....	13,254	13,053	98.4
Positive	5,210	5,095	97.8
Total.....	18,464	18,148	98.3

*Calculations based on observations of approximately half the children.

positive dermal reaction rate of 98.3 per cent. Children who received the combined vaccine were solidly immune to vaccinia challenge. A group of 102 previously unvaccinated children were given combined vaccine and were challenged with smallpox vaccine one month later by the usual multiple-puncture procedure. One hundred of the 102 children gave a vaccinoid or immune reaction, showing that the initial vaccination was effective in 98 per cent of the cases. The remaining two children showed primary takes on challenge.

Antibody responses in children who received the combined vaccine were roughly the same as when either vaccine was given alone. As is shown in Table 2, the mean neutralizing and hemagglutination-inhibiting vaccinia antibody titers in the sera taken after vaccination were roughly the same whether the combined vaccine or only smallpox vaccine was given. The hemagglutination-inhibiting antibody responses to the measles virus component of the combined vaccine was 98 per cent or greater in initially seronegative children and the titers were of the same order of magnitude as found when measles vaccine is given alone.

TABLE 2. VACCINIA ANTIBODY RESPONSES IN A SAMPLE OF INITIALLY SERONEGATIVE CHILDREN WHO RECEIVED COMBINED MEASLES-SMALLPOX VACCINE COMPARED WITH SMALLPOX VACCINE GIVEN ALONE

Vaccine	No. of children	Geometric mean titer	
		Neutralizing antibody	HI antibody
Measles-smallpox (jet)			
Premix.....	22	1:99	1:24
Field mix.....	22	1:106	1:30
Smallpox (mult. punct.).	25	1:132	1:35

The data obtained in the mass trial established the safety, efficacy, and efficiency of administering a combined live measles-smallpox vaccine under natural field conditions. Such a combined vaccine may be of great usefulness in the emerging nations of the world and may also offer advantages for mass vaccination of young children in the well developed countries.

CHAIRMAN BAROYAN: The next speaker is Dr. A. J. Schwarz, Human Health Research and Development Laboratories, the Dow Chemical Company, Indianapolis, Indiana.

Dr. Schwarz presented a summary of a paper entitled "Studies on the Persistence of Antibodies after Further Attenuated Live Measles Vaccine," which appears in full as Annex 2 to this section, pages 361–365.

CHAIRMAN BAROYAN: The statements concerning measles are now terminated, but there are several persons who wish to make comments.

DR. STRAUSS: I should like to give you a brief report on a WHO collaborative study on the reactivity and antigenicity of four different live measles vaccines conducted on about 500 children in Czechoslovakia in 1964.

Table 1 shows the geometric mean of HI antibodies two years after vaccination and two years after natural measles in a group of 122 children. The 92 vaccinated children received four different live viruses: Enders Edmonston B (with and without gamma globulin), Schwarz, Beckenham 20, and Milovanović. The remaining 30 children, from the same institutions, had normal measles infection and disease at the time of the vaccination program and were observed simultaneously.

The results of tests conducted two years later, similar to the findings of Dr. Enders, showed no substantial difference between the antibody

TABLE 1. GEOMETRIC MEAN TITERS OF MEASLES ANTIBODIES TWO YEARS AFTER VACCINATION AND TWO YEARS AFTER NATURAL MEASLES

Group	No. of children		Geom. mean titers (HI test)
Enders Edmonston B+GG.	21		75
Schwarz	22		81
Beckenham 20	27	92	87
Milovanović	11		192
Enders Edmonston B−GG.	11		121
Measles patients	30		122

levels of the vaccinated children and those of the children infected by wild measles strains.

DR. FOX: I should like to bring up a subject that I have not heard discussed yet—namely, the use of measles vaccine in adults. Since the natural disease is often more severe in adults, we cannot accept measures of vaccine reactivity in children as an adequate basis for judging the safety of vaccine use in adults. I hope that someone can speak on the current state of knowledge with regard to adult response. I also hope that plans exist for the further accumulation of knowledge in this area. The subject may be of minor importance at the moment—although there are parts of the world in which it would be of current importance—but it is certainly a matter that will take on greater significance in the future as opportunities for childhood exposure decrease by the use of vaccine and, perhaps, as vaccine-induced immunity disappears in some cases.

I should also like to direct a remark to Dr. Cockburn. Although I certainly would agree that better vaccines—that is, vaccines that produce less reaction—would be desirable, I am less concerned than he is about the problem of continuing acceptance of vaccines with the current level of reactivity. Indeed, in the United States we have continued to accept vaccinia as a routine immunizing agent, even though when it is successful it induces 100 per cent significant clinical response.

DR. CHUMAKOV: It is well known that measles is characterized by almost 100 per cent susceptibility in children and by the development of a stable, lifelong immunity in those who recover from the infection. This leads us to believe that we should be able to successfully vaccinate all of the child population with a suitable vaccine and thus to control measles to the same extent that it has been possible to prevent poliomyelitis. Since in a number of countries leukosis-free chickens are difficult to obtain and other primary tissue cultures have had to be used (such as guinea pig embryo kidney tissue and tissues from young dogs), the production of a measles virus vaccine that could serve for mass vaccination is still very difficult.

Studies have been carried out during the past two years at the Institute of Poliomyelitis and Viral Encephalitides of the USSR Academy of Medical Sciences in Moscow with a view to adapting the well-known Enders strain, Schwarz variant, to multiplication in serial passages in primary green monkey kidney tissue cultures.

The cultures were tested in advance for contamination by extraneous viruses. We found that in the course of 7 to 17 passages in monkey kidney tissue cultures there occurred not only an adaptation but a further attenuation of the Schwarz variant of the Edmonston strain (isolated by Dr. Enders and his assistants in 1954). All lots of our variant of live measles vaccine of the Edmonston strain (the so-called Edmonston-Schwarz-green monkey strain) have passed all tests and have been found to be in line with national and international requirements for live measles vaccines.

Our variant of the Edmonston strain adapted to monkey tissue multiplied satisfactorily after seven passages in primary green monkey kidney tissue cultures and retained a high titer in freeze-dried preparations. Thus, for instance, in the first 20 lots of the freeze-dried vaccine, only 4 had rather low titers—from 4.5 to 4.7 ($\log_{10}$); 13 had titers of from 5.0 to 5.6 ($\log_{10}$); and 3 had titers of from 5.7 to 6.1 ($\log_{10}$). Such high titers in the finished measles vaccine enabled us to use large and varying concentrations of the vaccine virus for vaccination against measles and thus to evaluate the action of different doses of the antigenic substance for the creation of immunity against measles. The doses varied between 1,000 and 5,000, and between 50,000

and 100,000 virus culture units per vaccination.

The study of the clinical reactogenicity of the first seven lots of our live measles virus vaccine, when introduced subcutaneously (without gamma globulin) to 700 children between one and two years of age who had not previously had measles, showed that our vaccine prepared in monkey kidney tissue cultures apparently produced reactions more readily than the Schwarz variant of the Edmonston strain grown in chick embryos.

The number of children whose temperature rose to 39.0°C—that is, who showed a severe febrile reaction—varied in the different series, depending on the virus dosage given, between 0 and 2.4 to 5.6 per cent. The total number of children whose temperature remained for one to two days at more than 37.0°C varied in the different lots, depending on the vaccine virus dosage used, between 16.6 and 57 per cent. The number of cases of pronounced rash in vaccinated children ranged from 3.7 to 5.9 per cent. Although in individual studies some clinical workers have reported an atypical ephemeral rash in as many as 50 per cent of the cases, not all of these were considered to be associated with live virus vaccinations.

Among the children vaccinated with our vaccine there was not a single case of neurologic disturbance—such as convulsions—or of any other complication or severe reaction. Medical workers who carried out antimeasles vaccinations were extremely satisfied with the very mild reactions and the absence of disturbances in the children's state of health.

As for seroconversion, the data obtained from studies of 256 vaccinated children who had no detectable antibodies before vaccination showed antibody in 250 of them (97.6 per cent), with a geometric mean antibody titer for the total group of 1:157. Another study of children vaccinated with our vaccine showed that antibody titers to the measles virus still persisted in most of the children three months after vaccination.

We consider that the use of primary green monkey kidney tissue cultures may permit the rapid development and production of a safe, effective live measles vaccine that many countries can afford and that can be used for the immunization of all susceptible children.

DR. ANDZAPARIDZE: Dr. Bolotovsky has told you about the interesting results of the comparative study of different vaccines. I should like to

TABLE 1. PATHOGENIC AND IMMUNOGENIC PROPERTIES OF LIVE MEASLES VACCINES, LENINGRAD 16 STRAIN, PRODUCED BY THE MOSCOW RESEARCH INSTITUTE OF VIRUS PREPARATIONS

Series No.	No. of doses used for vaccination per series	Febrile reaction (%)		Immunogenicity	
		Total	38.5° C. and above	Seroconversion (%)	Mean antibody titers
1/28	52,000	42	6	98	166
2/29	81,000	60	8	100	234
3/30	176,000	36	12	100	237
4/31	104,000	40	8	95	131
5/32	158,000	12	0	100	142
6/34	175,000	44	8	100	119
7/35	324,000	28	8	100	90
8/37	120,000	40	8	95	87
9/41	193,000	20	8	85	89
10/43	320,000	48	12	100	156
11/44	133,000	36	4	100	120
12/45	112,000	20	4	85	249
13/46	369,000	32	8	95	192
14/49	13,000	44	8	89	126
15/56	41,000	48	4	95	241
16/59	118,000	32	12	94	247
17/60	138,000	24	4	91	125

report on an experiment in which we prepared and tested vaccines from Smorodintsev's Leningrad-16 strain at the Moscow Research Institute of Viral Preparations.

We at our Institute, together with our Leningrad colleagues, have been carrying out tests with a view to improving this vaccine. We have studied its properties in small-scale experiments and also under mass vaccination conditions. Each of the vaccine series prepared at our Institute contains between 100,000 and 350,000 doses for vaccination purposes.

In each series in the small-scale experiments we have vaccinated 20 to 25 seronegative children and compared our results with those of mass immunization studies carried out in various areas and regions of our country.

Up to 60 per cent of the children vaccinated have had a slight febrile reaction (Table 1, p. 350). A temperature of 38.5°C has been recorded in several children (4 to 12 per cent). Many of the series showed seroconversion of 95 to 100 per cent. HI antibody titers have ranged between 1:86 and 1:250.

Information received from various republics and provinces of the Soviet Union where mass immunization experiments have been conducted has confirmed the rather limited reactogenicity and the high immunogenicity of the Leningrad-16 strain.

I think that as a result of our data we shall be able to adopt a national program of measles vaccination in our country in the very near future.

DR. SOLOVIEV: I should like to address a comment to Dr. Meyer and perhaps to Dr. Kalabus regarding the combined vaccination against smallpox and measles.

We know very well from the results of experiments and research on smallpox that antibodies do not always determine immunity against this disease. I therefore feel that before we can recommend a combined vaccination against smallpox and measles—that is to say, simultaneous vaccination—we should first of all, at the very least, study the reactions of vaccinated children after they have had a second potent smallpox vaccination at least one year after the first vaccination. We should also carry out epidemiological surveys and research on immunity against natural smallpox.

There is a second point. We know that sometimes vaccination against smallpox produces undesirable side effects, and in particular we are very much afraid of any disturbances of the central nervous system. I wonder whether such a combined vaccine would not be more dangerous from this point of view.

DR. CORIELL: I should like to comment briefly on the use of the tuberculin test prior to vaccination with a live measles preparation. This has been recommended as a routine practice in the United States.

By way of background, I should point out that we recommend yearly use of the tuberculin test in this country on normal, healthy preschool infants in order to detect tuberculosis soon after infection so that treatment with isoniazid can be initiated.

Obviously the test should not be performed immediately after vaccination with a live measles preparation, since the latter will create tuberculin anergy in a certain number of patients and may persist for a month or even longer in a few of them.

Another reason for scheduling the tuberculin test before the measles vaccination is that three cases of tuberculous meningitis have occurred among those who had received live measles vaccine three to four months previously. We know from experience that an attack of attenuated measles can cause the spread of tuberculosis in some individuals. This phenomenon has been frequently observed in institutionalized tuberculosis patients. I agree with Dr. Enders that the observation of three cases of tuberculous meningitis after measles vaccination does not really establish an association between the two events. Such a small number could be entirely the result of chance.

Nevertheless, if a tuberculin test is going to be performed at approximately one year of age, it should be scheduled before the measles vaccination. Positive reactors would then, of course, be put on antituberculosis therapy. This has been

the recommendation of the American Academy of Pediatrics for the routine immunization of healthy infants.

Does this recommendation apply to mass community-wide use of measles vaccine to combat a measles epidemic? I think a distinction should be made here, since the risk of extending a tuberculous infection with the measles vaccine is very, very much less than the risk involved in infection by wild measles virus. The control of measles comes first in planning the massive community-wide use of measles vaccine. The control of tuberculosis will entail much more time and a much greater effort.

ANNEX 1 TO SECTION A. RUBEOLA

STUDIES ON IMMUNITY TO MEASLES*

SAUL KRUGMAN, JOAN P. GILES, AND HARRIET FRIEDMAN

Department of Pediatrics, New York University School of Medicine, New York, and the Willowbrook State School, Staten Island, New York, U.S.A.

DR. KRUGMAN (*presenting the paper*): The studies on immunity to measles have been in progress since 1960. The present account is a supplement to a previous publication that reported on our experience during the four-year period 1960–1964 (1).

All measles antibody studies were performed by the highly sensitive hemagglutination-inhibition (HI) test described by Norrby (2). As he has demonstrated, this test is more sensitive than the neutralizing antibody test.

MEASLES IMMUNITY DURING THE FIRST YEAR OF LIFE

The persistence of passively acquired measles HI antibody was studied in a group of 107 infants. In this longitudinal study four to five serial samples of blood were obtained from each infant during the first year of life.

The results of serial determinations of measles HI antibody are shown in Figure 1. Only one infant had no detectable antibody at one month of age. Passively acquired antibody persisted as late as 9, 10, and 11 months in a small number of infants. There was a high correlation between the level of the antibody titer and its persistence.

The persistence of passively acquired antibody after six months of age was effective in modifying the disease in six of eight infants who were exposed to measles. Classical measles with rash was observed in only two of the eight infants who had a fourfold or greater antibody level. On the other hand, the presence of antibody between 9 and 11 months of age may have a suppressive effect on the immunogenic response to live measles virus vaccine.

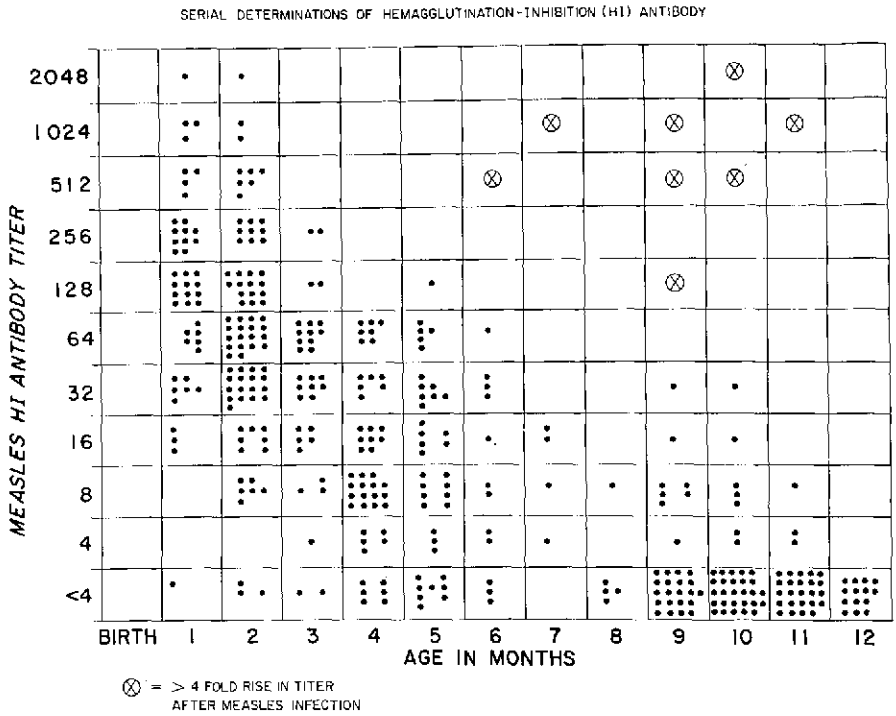
A LONGITUDINAL STUDY ON THE PERSISTENCE OF MEASLES ANTIBODY AFTER NATURAL INFECTION AND VACCINATION

Studies have been in progress in two areas: in an institution where measles has not occurred during the past four years (Willowbrook State School), and in a community where vaccinees have been repeatedly exposed to measles over the past four and one half years.

Institutional Studies. The results of a six-year follow-up of 46 children who had natural measles infection are shown in Figure 2. A similar six-year follow-up of 43 children who received live attenuated measles virus vaccine, Edmonston B type (LAV-ED), is shown in Figure 3. A five-year follow-up of 41 children who received LAV-ED plus gamma globulin is shown in Figure 4. A four-year follow-up of 75 children who received the live further attenuated Schwarz strain measles virus vaccine (FAV) is shown in Figure 5. A comparison of the geometric mean antibody titers observed after natural infection and after the three measles vaccine regimens is shown in Figure 6.

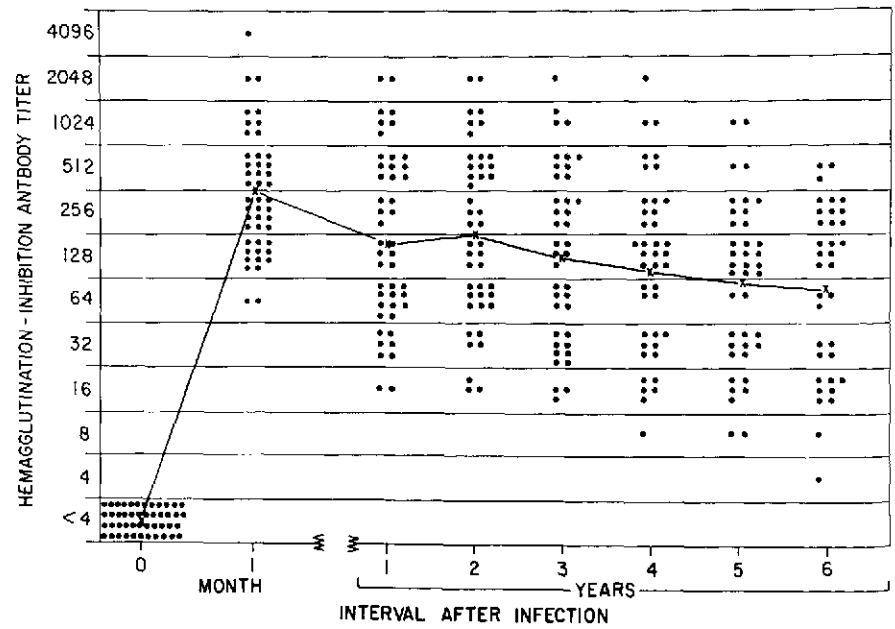
The HI antibody titer six years after natural infection ranged from a low of 1:4 to a high of 1:512 (Fig. 2). Individuals with low an-

* The studies reported in this discussion were supported by the Health Research Council of the City of New York under Contract No. V-1056.



Note: Each black dot represents one of the serial HI antibody determinations performed by the Norrby method (2). Note the persistence of detectable HI antibody as late as 11 months of age. Of 8 infants who had a greater than fourfold rise in antibody titer after natural measles infection, only 2 had the typical disease with rash, whereas the remaining 6 had a modified illness or a subclinical infection.

Fig. 1. Longitudinal study of measles immunity during the first year of life.



Note: Each black dot represents one of the serial HI antibody determinations performed by the Norrby method (2). Solid line indicates the geometric mean antibody titer.

Fig. 2. Pattern and persistence of HI antibody response after natural measles infection—Six-year longitudinal study in 46 institutionalized children who were not subsequently exposed to measles.

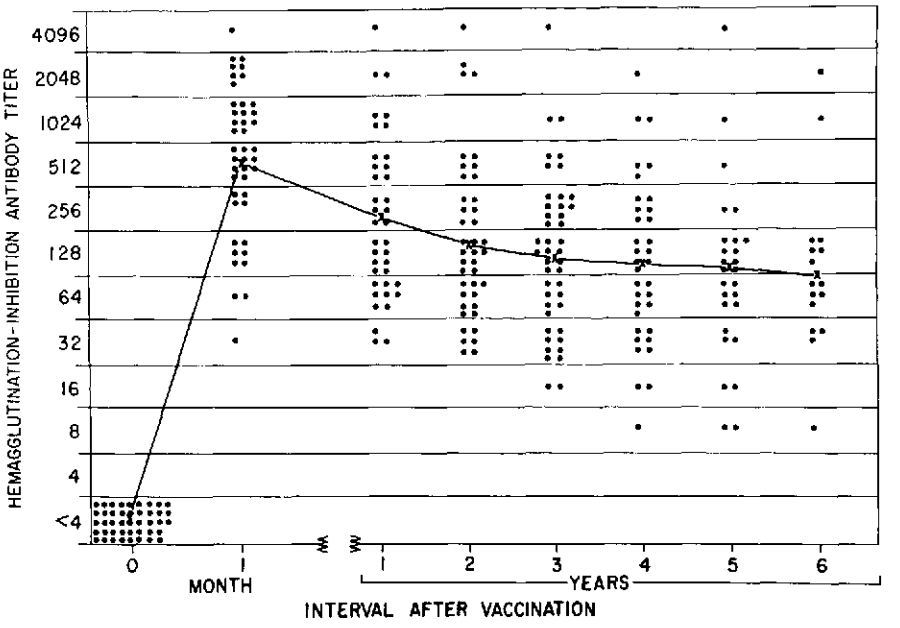


Fig. 3. Pattern and persistence of HI antibody response in 43 institutionalized children who received attenuated measles vaccine, Edmonston B type—Six-year longitudinal study.

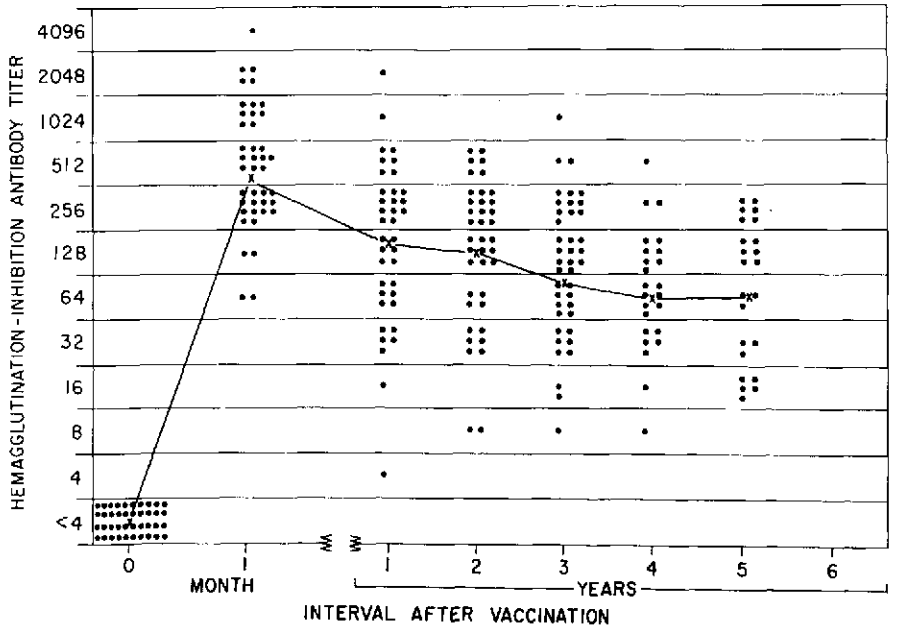


Fig. 4. Pattern and persistence of HI antibody response in 41 children who received live attenuated measles virus vaccine, Edmonston B type, plus gamma globulin—Five-year longitudinal study.

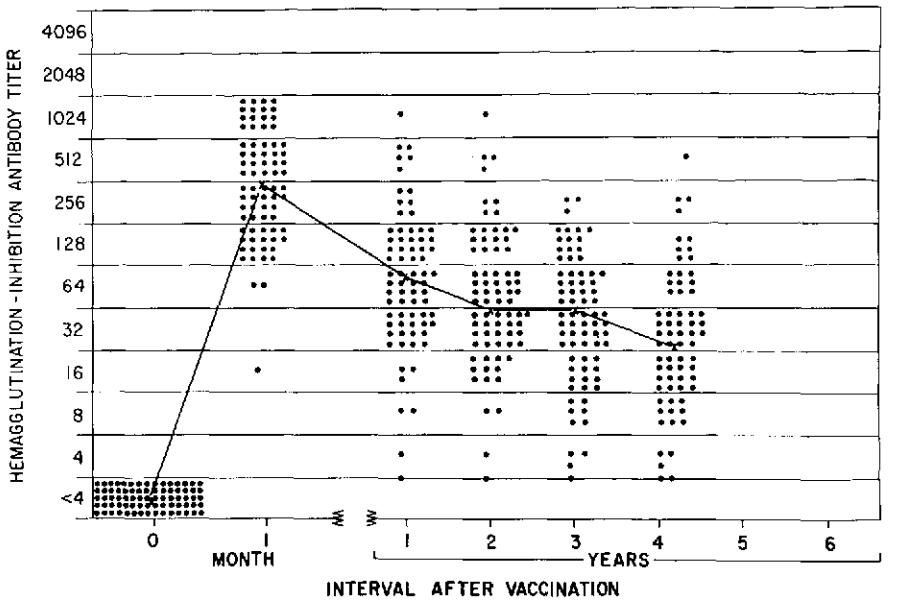


Fig. 5. Pattern and persistence of HI antibody response in 75 children who received live further attenuated vaccine, Schwarz strain—Four-year longitudinal study.

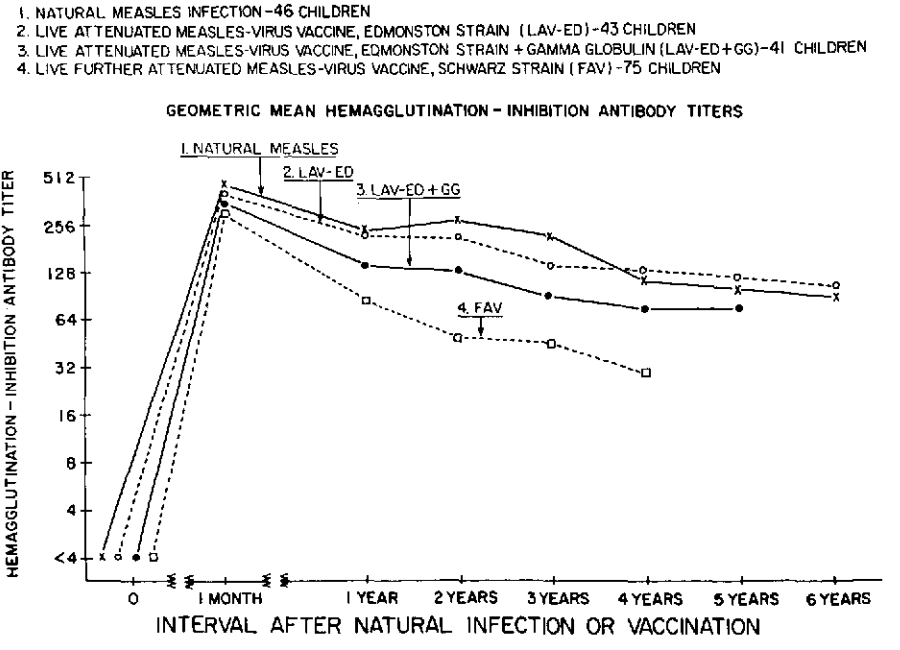


Fig. 6. Comparison of the pattern and persistence of measles HI antibody response after natural infection and after vaccination in institutionalized children who were not subsequently exposed to measles.

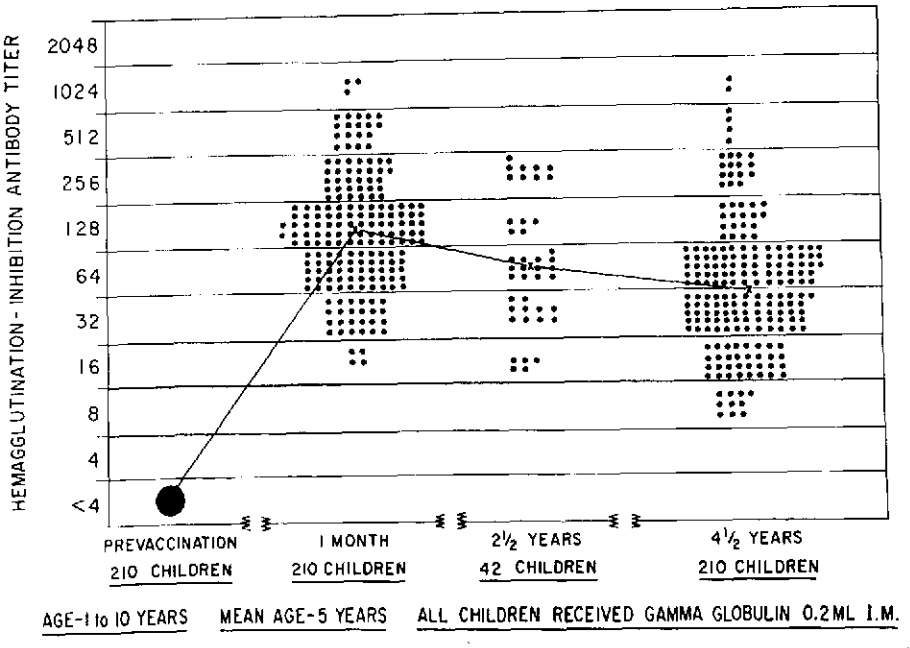
tibody titers after natural infection are undoubtedly just as solidly protected against the disease as those with high antibody titers.

In spite of lack of exposure to measles, all vaccinees had detectable HI antibody four to six years after vaccination (Figs. 3, 4, and 5). As Figure 6 shows, the pattern and persistence of the antibody response was essentially the same after natural infection and after vaccination. The geometric mean antibody titer after vaccination with further attenuated measles vaccine was consistently lower than after vaccination with Edmonston B vaccine or after natural infection.

Community Studies. The results of a four and one-half year follow-up of 210 children who received the live further attenuated Schwarz strain are shown in Figure 7. All the children received a minimal dose of gamma globulin (0.2 ml, total dose). During the four and one-half years 37 per cent of these children were exposed to measles, but no cases occurred in this vaccinated group. All 210 children had detectable HI antibody, ranging in titer from 1:8 to 1:1024.

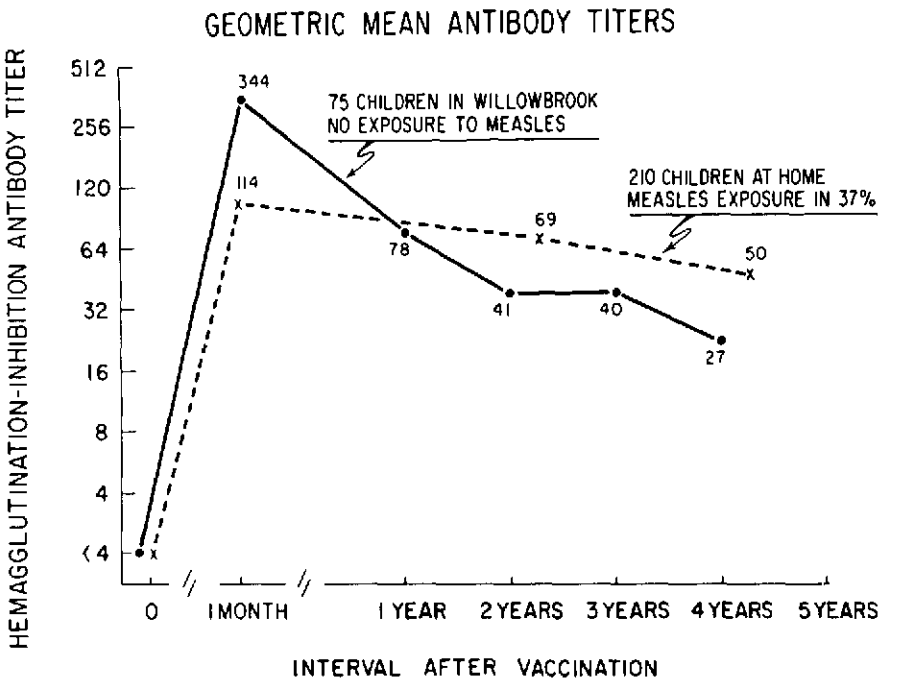
A comparison of the geometric mean antibody titers (GMAT) of institutionalized children who received FAV and home-dwelling children who received FAV and a small dose of gamma globulin is shown in Figure 8. The home-dwelling children had a lower GMAT at one month and a higher GMAT four and one-half years later. The lower titers at one month are probably due to a suppressive effect of gamma globulin. The higher GMAT titers at four and one-half years are probably due to a booster response after exposure to measles.

Comment. The data from these studies support the hypothesis that a single inoculation of live attenuated measles vaccine, either Edmonston B or the Schwarz further attenuated type, will probably induce the same type of lasting immunity that follows natural measles infection. Both vaccines are highly immunogenic. The lower antibody titers observed after vaccination with the Schwarz strain vaccine have had no practical significance. As is indicated in a previous publication (1), vaccinees who had low antibody titers were solidly protected against measles. The classical booster type of response



Note: A history of exposure to measles was obtained in 37 per cent of this group of home-dwelling children. All children were solidly protected against the disease. Note the persistence of HI antibody four and one half years after vaccination.

Fig. 7. Pattern and persistence of antibody response in 210 children who received live further attenuated vaccine and gamma globulin (0.2 ml, total dose).



Note: The institutionalized children (solid line) were observed for four years; the home-dwelling children (interrupted line), for four and one half years. Note the higher antibody levels after exposure to measles in the community.

Fig. 8. Comparison of geometric mean HI antibody titers in 75 institutionalized children and 210 home-dwelling children who received live further attenuated vaccine, Schwarz strain.

after re-exposure to live measles virus is shown in Figure 9. This response has been observed in children with low antibody titers but not in those with high levels of antibody.

It is important to emphasize that the standard HI test described by Rosen (6) is not sensitive enough to detect low levels of measles antibody. Dr. Victor J. Cabasso provided us with coded samples of the following selected serum specimens:

1. Sera from 20 patients who received the Edmonston B vaccine: all specimens had HI antibody titers ranging between 1:32 and 1:64 by the Rosen HI test (6).

2. Sera from 20 patients who had received Schwarz strain vaccine one year previously: all 20 sera had a titer of less than 1:8 by the Rosen HI test.

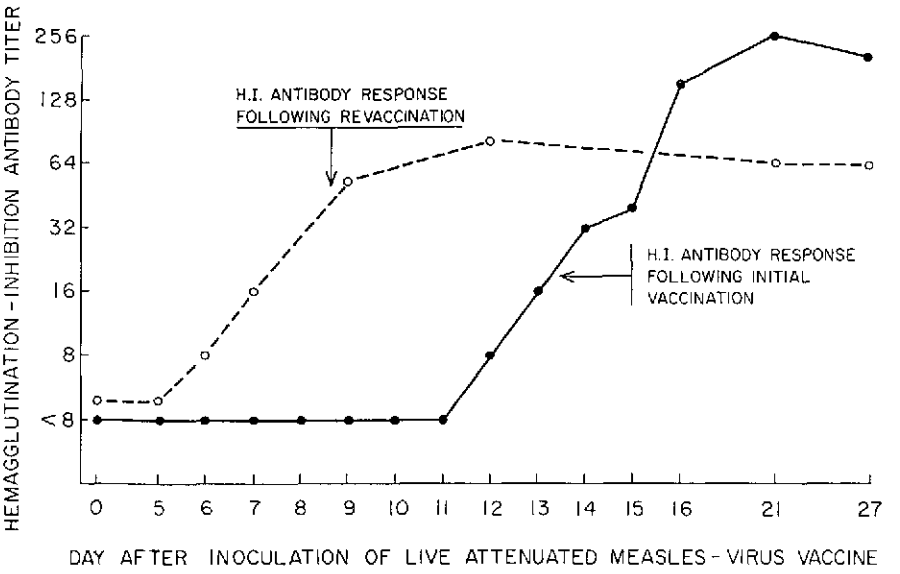
Our laboratory tested the 40 coded serum specimens using the Norrby HI antibody test (2). Dr. Cabasso's laboratory had performed measles neutralizing antibody tests. When the code was broken, it was apparent that all 40 specimens had detectable neutralizing and HI (Norrby) antibody. The range in HI antibody titer was from 1:8 to 1:128 for the Schwarz strain vaccine and from 1:128 to 1:1028 or more for the Edmonston B vaccine.

LOCAL REACTIONS IN CHILDREN IMMUNIZED WITH COMBINED KILLED-LIVE MEASLES VACCINE REGIMENS

Reports by Buser (3), Scott (4), and Fulginiti (5) have indicated that local reactions of varying severity may occur at the site of live measles virus inoculation in children previously vaccinated with inactivated measles virus vaccine. This phenomenon was recently studied by us in the following four groups.

Group I. Live further attenuated vaccine was administered to 14 children who had received two inoculations of inactivated measles vaccine* at monthly intervals approximately one year

* Inactivated measles vaccine prepared in monkey kidney tissue culture, Chas. Pfizer and Co.



Note: After initial vaccination (solid line), antibody was first detected on day 12 and peak levels were observed on day 21. After revaccination with live measles vaccine (interrupted line), antibody appeared on day 6 and peak levels were reached by day 12. Assay for HI antibody was performed by the Rosen method (6). *Source:* Krugman, S., *et al., J. Pediat* **66**:471, 1965.

Fig. 9. Comparison of the pattern of HI antibody response after initial vaccination with live attenuated measles virus vaccine and after subsequent vaccination of children whose antibody titers had declined to nondetectable levels.

earlier. A severe (4+) or moderately severe (3+) reaction was observed in 4 of 14 vaccinees seven to ten days after they received the live vaccine.

Group II. Live further attenuated vaccine was administered to 17 children who received three inoculations of killed vaccine at monthly intervals approximately one year earlier. A moderate (2+) or mild (1+) local reaction was observed in 5 of 17 children five to seven days after they received the live vaccine.

Group III. Live further attenuated vaccine was given to 17 susceptible children who had never received any measles vaccine. Only one child in this group had a minimal (<1+) local reaction nine days after receiving live measles vaccine.

Group IV. Live further attenuated measles vaccine was given to 16 children who had received the same live vaccine two to four years previously. There were no local reactions in this group.

The local reactions were characterized by erythema, induration, marked swelling, and vesiculation.

Summary

Studies on immunity to measles after natural infection and after vaccination with live attenuated measles virus vaccines have revealed a similar pattern and persistence of HI antibody response. Both the Edmonston B and the Schwarz strain vaccines are highly immunogenic. Although the antibody titers observed after vaccination with the Schwarz strain vaccine are significantly lower, this phenomenon has not altered the protective effect. The evidence available indicates that a single inoculation with one of the currently licensed live attenuated measles virus vaccines will probably confer lifelong immunity.

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(2) Norrby, E. "Hemagglutination by Measles Virus: 4. A simple Procedure for Production of High Potency Antigen for Hemagglutination-Inhibition (HI) Tests." *Proc Soc Exp Biol Med* **11**:814, 1962.

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(5) Fulginiti, V. A., Arthur, J., Pearlman, D. S., and Kempe, C. H. "Serious Local Reactions Following Live Measles Virus Immunization in Previous Killed-Vaccine Recipients." *J Pediat* **69**:891–892, 1966.

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ANNEX 2 TO SECTION A. RUBEOLA

STUDIES ON THE PERSISTENCE OF ANTIBODIES AFTER FURTHER ATTENUATED LIVE MEASLES VACCINE

A. J. SCHWARZ, J. L. BITTLE, A. E. CASTRO, AND A. Y. ELLIOTT

Human Health Research and Development Laboratories
Dow Chemical Company, Indianapolis, Indiana, U.S.A.

DR. SCHWARZ (*presenting the paper*): The safety of the live virus measles vaccines has been proved quite conclusively in many different field trials and has again been confirmed by the prior speakers. Controlled comparative tests (1, 2, 4, 5) have shown that the further attenuated vaccine (Schwarz strain) causes less clinical reaction than the Edmonston B vaccine when used without gamma globulin. Although the excellent effectiveness of both vaccines, as determined by serum conversion and protection, has also been demonstrated in many trials, certain differences have been described by some investigators studying the levels and persistence of antibodies after vaccination. Since different investigators have been using varied procedures for demonstrating antibodies, we have studied the influence of the variables involved on determining persistence of antibodies after vaccination with further attenuated live virus measles vaccine.

MATERIALS AND METHODS

Vaccine

The live measles virus vaccine, Schwarz strain (LIRUGEN ®),* used in these tests was derived from the Edmonston strain by 85 additional special passages in chick embryo tissue culture (8). Individual doses of this lyophilized vaccine were reconstituted to 0.5 cc with sterile distilled water just prior to vaccination. Each dose contained no less than 1,000 $TCID_{50}$ of measles virus. It was administered in the upper deltoid region.

Neutralization test

The measles virus used was low-passage Edmonston strain virus grown in canine kidney tissue culture. All sera were inactivated at 56°C for 30 minutes. Twofold dilutions of serum were mixed in equal amounts with virus calculated to contain 100 $TCID_{50}$. Virus control titrations were diluted in half-log steps. The virus control and serum-virus mixtures were incubated at room temperature for one hour, and 0.2 ml of each dilution was inoculated into each of four FL amnion (FLA) tissue culture tubes maintained in Eagle's medium with 2 per cent agamma calf serum. Tissue cultures were incubated in roller drums at 35°C. At the end of 14 days, the test was read and serum neutralization endpoints were determined by the Reed-Muench method.

Hemagglutination inhibition test (HI)

Preparation of hemagglutination antigen. The Norrby-type (6) measles hemagglutination antigen was prepared by inoculating FLA cultures maintained in Eagle's medium, 2 per cent agamma calf serum, with low-passage Edmonston strain measles virus. Ten days after inoculation all fluids from the culture bottles were transferred to new cultures and absorbed for one

* Registered trademark, Pitman-Moore Division of the Dow Chemical Company.

hour. Half of the medium was then replaced in the new cultures with fresh maintenance medium. The bottles were harvested four to eight days later. The antigen was further concentrated by Tween-80 treatment and ether extraction as described by Norrby (6). The hemagglutination titer of this antigen was 1:128.

Commercial antigen. A commercial antigen, lot number 30–811, was purchased from Microbiological Associates. The titer of this antigen was found to be 1:64.

Procedure. All sera were inactivated at 56°C for 30 minutes, extracted with an equal volume of 25 per cent kaolin, and adsorbed with a 50 per cent suspension of red blood cells (RBC) from green African monkeys.

A preliminary hemagglutination (HA) titration was performed prior to each HI test. Twofold serial dilutions were prepared in phosphate buffer, pH 7.3–7.4. A dilution of antigen was prepared to give four HA units for each tube in the test. Each serum was diluted serially using twofold dilutions in phosphate buffer, pH 7.3–7.4. An equal volume of antigen was then added to each tube and incubated for one hour at room temperature (25°C). At the end of the incubation period, 0.2 ml of 5 per cent RBC from green African monkeys was added to each tube and incubated in a 37°C water bath for two hours. Serum, RBC, and antigen controls were included in each test. Titers were expressed as the negative reciprocal of the dilution.

Sera

Sera were obtained from children who were involved in a large-scale clinical evaluation of the further attenuated measles vaccine in 1964 (9). For this particular study only children who had no measles antibodies prior to vaccination were included. Serum samples were obtained at four weeks, and approximately one year and two years after vaccination. The sera were kept frozen at −70°C until tested. Because of the mobility of the population used in the clinical trial, serum samples could be obtained from only a relatively small number of children after one and two years.

RESULTS

Table 1 shows the neutralizing antibody titers and the geometric means from 50 of these children four weeks, one year, and two years after vaccination. These data show that all the children had significant serum neutralizing antibodies even two years after vaccination and that generally little change could be detected during this period. All the serum specimens from each person were tested simultaneously, except those for which the titers appear in parentheses. Only the results of simultaneous tests were considered in calculating the geometric mean titer.

Serum samples from 12 vaccinated children were selected at random and tested for HI antibodies with two different HA antigens. The results of these tests demonstrate very clearly the significant differences between antibody titers obtained using Norrby antigen and those obtained with the commercial antigen (Table 2). In all instances where the Norrby antigen was used the children had significant antibody levels even after two years, whereas with the commercial antigen antibodies of 1:4 or greater were detected in only 9 of the 12 sera. The geometric mean was four- to tenfold higher when tested with the Norrby HI antigen than with the commercial HI antigen.

These differences are graphically illustrated in Figure 1, which shows a comparison of the geometric mean titers obtained by the serum-neutralization test and by the HI test using Norrby and commercial antigens. The HI test using the Norrby antigen gave the highest antibody level—higher than the HI test with the commercial antigen or the neutralization test. However, the drop of antibody titer after one year was very distinct in this test, but leveled off after one year and then compared quite well to the neutralizing antibody level. In the commercial antigen tests the antibody decline after the first year was not as marked, but since the titers were generally lower the result may reflect a leveling off of some titers below detectable levels, which creates the impression that antibodies are no longer present. The neutralizing antibody level demonstrated the least change. A slight increase after one year was noted, which was probably due to the fact that the first post-vaccination serum sample was taken at three to

TABLE 1. SERUM NEUTRALIZING ANTIBODY TITERS FOUR WEEKS, ONE YEAR, AND TWO YEARS AFTER VACCINATION WITH FURTHER ATTENUATED MEASLES VACCINE

Serum No.	Postvaccination serum neutralizing antibody titers		
	4 weeks	1 year	2 years
1	(1:100)	1:69	1:40
2	1:25	1:45	1:22
3	1:10	1:6	1:6
4	1:45	1:355	1:80
5	>1:64	1:11	1:11
6	(1:25)	(1:25)	1:25
7	—	—	1:89
8	≥1:64	1:64	1:178
9	—	—	1:50
10	—	—	1:256
11	1:100	—	1:89
12	—	—	1:80
13	—	—	1:178
14	(≥1:6)	1:40	1:64
15	1:25	(1:40)	1:22
16	1:50	(1:100)	1:45
17	1:22	(1:100)	1:20
18	(1:32)	1:32	1:22
19	—	1:22	1:20
20	—	1:25	1:8
21	—	1:80	1:80
22	(1:40)	1:128	1:178
23	(1:100)	1:50	1:45
24	—	(1:25)	1:22
25	—	1:50	1:64
26	1:45	1:45	1:45
27	1:100	1:40	1:45
28	1:20	—	1:40
29	1:16	1:32	1:20
30	1:8	—	1:20
31	1:11	1:40	1:64
32	—	1:89	1:80
33	1:22	1:64	1:16
34	1:20	1:13	1:20
35	1:11	1:13	1:6
36	1:16	1:16	1:13
37	—	—	1:20
38	—	1:50	1:32
39	1:200	1:32	1:159
40	1:80	≥1:100	1:128
41	1:45	1:64	1:64
42	1:69	1:32	1:32
43	1:16	1:16	1:16
44	1:16	1:32	1:20
45	1:6	1:16	1:256
46	1:40	1:32	1:45
47	1:178	1:40	1:50
48	1:64	1:40	1:50
49	1:159	≥1:256	1:355
50	1:32	1:80	1:45
Log_{10} geometric mean titer	1.493	1.594	1.619
Geometric mean titer	1:32	1:40	1:40

Figures in parentheses were not in calculation of geometric mean titers, since the tests were not done simultaneously.

four weeks before the antibody response had reached its maximum level.

DISCUSSION

The data presented in this report indicate that the neutralization test is the most reliable measure for determining the persistence of measles antibodies. The HI test using the Norrby-type antigen is also suitable for this purpose, but the HI test with nonextracted antigen has to be considered of questionable value. This confirms the report by Fulginiti that caution should be exercised in interpreting HI data obtained with a nonextracted antigen in studying the persistence of antibodies (3). The reason for the significant difference in detecting HI antibodies using the Norrby antigen or the commercial antigen is not completely clear, but it is probably related to the two different hemagglutinins described by Norrby (7). Apparently a preparation consisting of smaller hemagglutinins is more reliable and more sensitive for the detection of antibodies than one containing the large hemagglutinins. Such a superior antigen can be produced by the Norrby procedure of extracting hemagglutination antigens, since it results in a relatively pure preparation of small hemagglutinins.

To realistically determine the persistence of antibody levels after vaccination, it is of the utmost importance to:

1. Utilize a sensitive test, such as the serum-

TABLE 2. COMPARATIVE STUDY OF TWO HA ANTIGENS IN DETECTING MEASLES ANTIBODY

Code No.	HI antibody titers					
	Norrby antigen			Commercial antigen		
	4 weeks	1 year	2 years	4 weeks	1 year	2 years
1		1:32	1:64		1:8	1:8
5		1:16	1:16		<1:4	<1:4
6		1:8	1:32		1:4	1:8
14		1:128	1:64		1:32	1:16
18		1:128	1:256		1:16	1:16
20		1:32	1:16		1:8	<1:4
22		1:512	1:256		1:32	1:32
29	1:128	1:128	1:64	1:16	1:16	1:16
32		1:64	1:128		1:16	1:8
34	1:256	1:8	1:128	1:16	<1:4	1:16
35	1:128	1:32	1:32	1:16	ND	1:4
36	1:256	1:64	1:64	1:8	1:8	1:8
Log_{10} geometric mean titer	2.2577	1.6807	1.8062	1.1289	1.0126	0.9784
Geometric mean titer	1:181	1:48	1:64	1:13	1:10	1:10

neutralization test or the hemagglutination-inhibition test using a Norrby-type antigen.

2. Test all the serum specimens from one person simultaneously in order to avoid variation from one test to another.

3. Consider the time when the first postvaccination serum sample is taken in relation to the time when the highest antibody level is reached after vaccination. This is important since the results obtained from this sample provide the base line for further investigation and since it has been reported that HI antibodies reach their highest point four to five weeks after vaccination, whereas the maximum neutralizing antibody response may not occur until six weeks after vaccination (10).

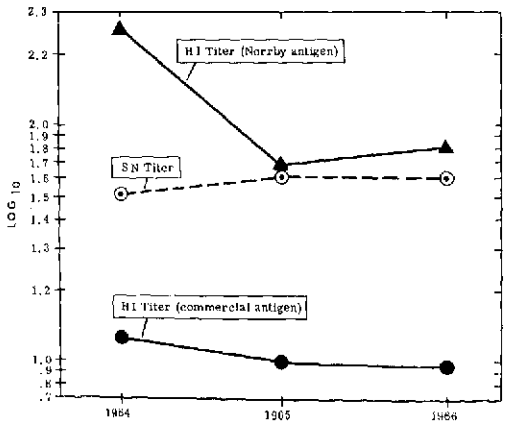

Fig. 1. Comparison of HI and SN measles antibody titers, one and two years after vaccination with further attenuated vaccine.

Dr. Krugman has very eloquently pointed out that the quantitative difference in antibody titer has no practical significance and that all the data indicate that vaccination with further attenuated live virus vaccine will give long-lasting protection.

This study and others previously reported clearly demonstrate that the further attenuated vaccine results in long-lasting immunity with a single inoculation. Moreover, the reaction rate of the vaccine is extremely low. For these reasons it appears to be very well suited to large-scale immunization programs.

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SECTION B. RUBELLA

EPIDEMIOLOGY OF RUBELLA

JOHN L. SEVER

Section on Infectious Diseases, Perinatal Research Branch, National Institute of Neurological Diseases and Blindness, and Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Disease, Bethesda, Maryland, U.S.A.

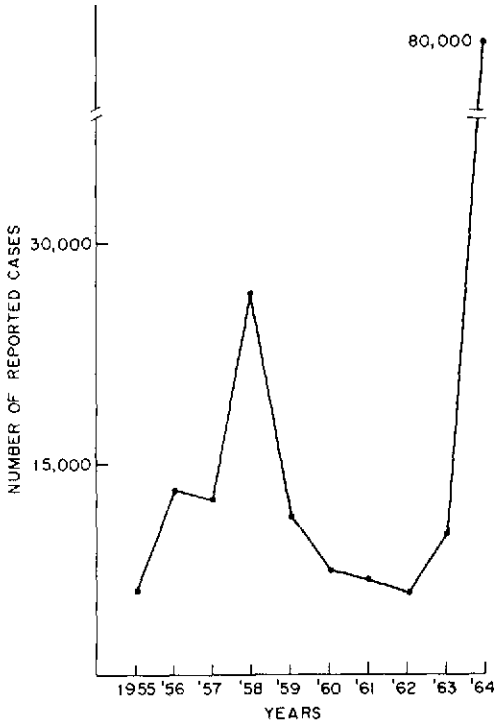
DR. SEVER (*presenting the paper*): Rubella was first recognized as a cause of defective children 25 years ago (1). It was after an epidemic in Australia that the ophthalmologist Sir Norman Gregg noted a high frequency of cataracts in children and recognized the association between rubella in the first trimester of pregnancy and damage to the eye (1). Since that time a number of studies have confirmed and extended these observations. The frequency of abnormal pregnancy outcomes is approximately 50 per cent if rubella occurs in the first month of pregnancy, 22 per cent if it occurs in the second month, and 6 per cent if it occurs in the third (2, 3). In the past few years we have become aware that, in addition to the malformations of the eyes, heart, deafness, microcephaly, and mental retardation, congenital rubella may also result in hepatosplenomegaly, thrombocytopenia, jaundice, pneumonitis, radiolucency of the long bones, and chronic infection during the newborn period (4, 5, 6).

EPIDEMIC CYCLES

In most countries rubella occurs primarily in the spring of each year. Epidemics generally occur at intervals of about seven years (7). Some of the largest epidemics have coincided with periods of military mobilization. The majority of infections occur in children under 15 years of age, and most of these are among school-age children (7). Rubella is less communicable than rubeola or varicella; populations in semi-isolated areas frequently contain a high proportion of susceptibles and may experience extensive epidemics at infrequent intervals. There is considerable variation among different countries and cities in the reporting of rubella. This is due mainly to the mildness of the disease and to the fact that in most areas it is not a reportable infection.

In the United States data from the Communicable Disease Center since 1955 have been analyzed (8). Significant epidemics were noted in 1958 and in 1964–1965 (Fig. 1). In the 1964–1965 epidemic there was a clear movement from the Northeast to the West and South. For the most part the West Coast, Hawaii, and Puerto Rico were not involved until 1965, and the epidemic was more intense in 1964 than in 1965.

The intensity of the 1964 epidemic and its effect on pregnant women in the United States were studied by 11 institutions participating in the prospective Collaborative Study of Cerebral Palsy. In this study detailed clinical data were obtained for 6,161 pregnant patients and their children, and serial serum specimens were taken throughout pregnancy and subsequently tested for antibody to rubella. Data now available from this study indicate that 10 per cent of the women were exposed during the first trimester and 26 per cent during the second and third trimester (Fig. 2) (9). Two per cent developed clinical rubella, 40 per cent of them in the first trimester. Ten per cent of the women with clinical rubella in the first trimester had a child with congenital rubella syndrome

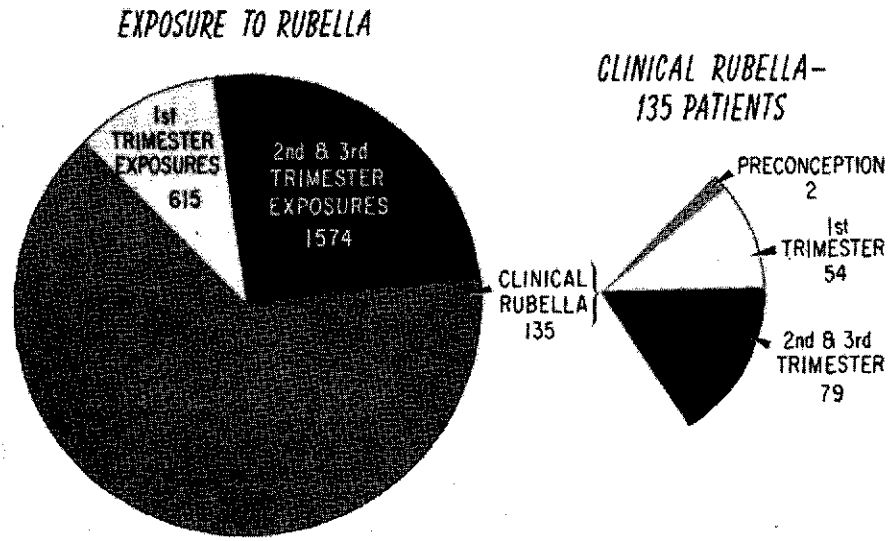


Source: Communicable Disease Center. *Morbidity and Mortality Weekly Report* (Vol. 14, No. 16), Week ending 24 April 1965.

Fig. 1. Reported rubella for the United States, by year.

recognized within the first month after birth. Clinical rubella was more frequent among white patients. Nonhousehold exposures were as likely as household exposures to lead to clinical rubella. The serological data corroborated the diagnosis of clinical rubella in 74 per cent of the cases tested. Inapparent infection occurred in 6 per cent of the women studied who were exposed to rubella in the first trimester of pregnancy but did not develop clinical disease.

Studies in military populations in the United States have further substantiated the seasonal occurrence of rubella (10). In general, it occurs among recruits primarily in the spring. Of the recruit training centers in the United States, the one at Fort Ord, California, consistently reports more rubella than any other training station. Peak years at Ford Ord were 1955, 1958–1959, and 1964–1965. The over-all rubella rate reported in U.S. military recruits is 18 per thousand per year (10). One of the factors unique to Fort Ord is the high frequency of rubella among individuals from Hawaii. In 1960, for example, 77 per cent of the recruits from Hawaii had rubella while at Fort Ord. The high frequency among recruits from other western states varied between 5 and 20 per cent. In short, rubella occurs rather frequently among military recruits, particularly at Fort Ord, and



Source: Sever, J. L., Nelson, K. B., and Gilkeson, M. R.: "Rubella Epidemic, 1964: Effect on 6,000 Pregnancies." *Amer J Dis Child* 110:395, 1965.

Fig. 2. Study of 6,161 pregnant women, January June 1964.

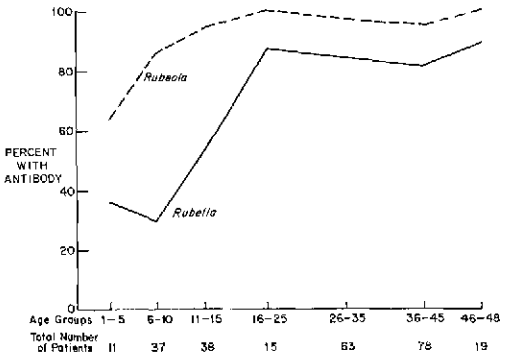
is of concern in military recruit training programs (10).

SUSCEPTIBILITY—SPREAD OF INFECTIONS

The epidemiology of rubella is undoubtedly influenced by the susceptibility of the population. This susceptibility can be determined by serological studies of the frequency of antibody among individuals.

In a serological study of 268 members of the general population of Montgomery County, Maryland, in 1957 (11), the frequency of rubella antibody was found to increase from approximately 35 per cent among children 1 to 10 years of age to 85 per cent in the 16-to-25 and older groups (Fig. 3). This indicates that approximately 15 per cent of the adults sampled were susceptible to infection with rubella. Rubella antibody was acquired most notably by school-age children; this occurred several years later than with rubeola antibody. At all ages, there were fewer people with antibody to rubella than to rubeola.

In studies of pregnant women we have found that the frequency of women without antibody to rubella in the continental United States has varied between 19 to 32 per cent in 11 collaborating study hospitals (12). There was a significantly greater frequency of individuals without neutralizing antibody among pregnant Negroes than among white patients and the number of women without antibody



Source: Sever, J. L., Schiff, G. M., Bell, J. A., *et al.* "Rubella: Frequency of Antibody among Children and Adults." *Pediatrics* 35:996, 1965.

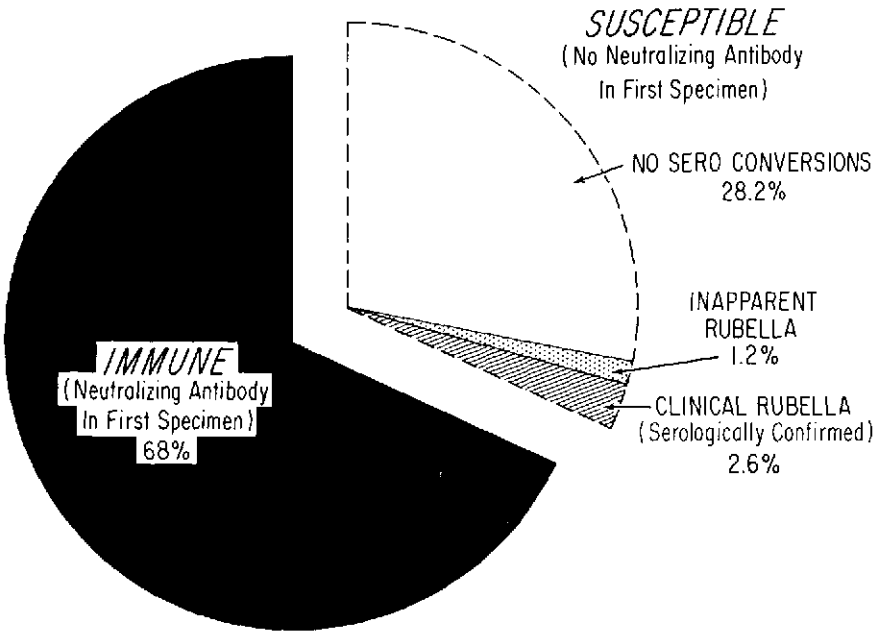
Fig. 3. Frequency of patients with antibody to rubella and rubeola, Montgomery County, Maryland, 1957.

decreased significantly with increasing age. There was no correlation between a reported history of rubella and the presence or absence of antibody (12).

In Hawaii the frequency of pregnant women without antibody was considerably higher than among groups studied in the continental United States. At the Kaiser Hospital in Honolulu, 58 per cent of the women tested did not have neutralizing antibody; in a similar group studied in Hilo, Hawaii, 71 per cent did not have antibody (13). Our recent studies in southern Japan indicate that 34 per cent of the pregnant women in the population studied did not have neutralizing antibody (14).

Studies of isolated populations have indicated that when rubella occurs in these groups almost all susceptible individuals become infected. In an epidemic we studied on the Pribilof Islands, off the coast of Alaska, for example, almost everybody over 19 years of age had neutralizing antibody, whereas none of those under that age had any (15). When rubella was brought to the island by several girls returning from the mainland, the epidemic involved almost every person under 19.

We have recently completed a study of the epidemiology of rubella in a general population of pregnant women. In this investigation, tests were made of paired serum samples from 500 pregnant women selected from the more than 6,000 who were under study in the Collaborative Perinatal Research Investigation during the 1964 rubella epidemic (Fig. 4) (16). In each case, the first sera was taken before the epidemic and the second, approximately six months later, after the epidemic. The frequency of neutralizing antibody was determined with both sera. According to these data, 68 per cent (340) of the women had neutralizing antibody in the first specimen. There was no clinical or serological evidence of rubella in those with neutralizing antibody. Clinical rubella, serologically confirmed, was found in 2.6 per cent of the population; 1.2 per cent had inapparent rubella, serologically detected. The data thus indicate that in this population approximately 4 per cent of the pregnant women experienced rubella and that the over-all ratio of clinical rubella to inapparent infection was 2:1. It also demonstrates that a considerable reservoir



Source: Sever, J. L., Fuccillo, D. A., Huebner, R. H., Traub, R., Ley, A., and Gilkeson, M. R. "Clinical and Subclinical Rubella in Pregnant Women" (in press).

Fig. 4. Frequency of rubella in 500 pregnant women during 1964 epidemic.

of susceptible women of childbearing age remained after the 1964 epidemic.

A survey of small groups of young adult women in the Philippines, Eastern Nigeria, Togo, Thailand, and Czechoslovakia was initiated in conjunction with the World Health Organization and the Prague Serum Reference Bank. The collaborators included Drs. K. Raska and P. Yekutiel of the WHO in Geneva and Drs. L. Syrůček and Záček of the WHO in Prague. The initial data are as follows:

Country	*Number with antibody*	*Per cent*
Philippines	27/29	93
Eastern Nigeria	15/19	79
Togo	24/27	89
Thailand	21/24	87
Czechoslovakia	29/30	97

The level of immunity in these particular groups thus seems high, but this must be confirmed by additional testing. Naturally, these are very preliminary studies, and they are being extended to include significantly larger numbers of patients. The investigations point up the need for seroepidemiological studies to determine the susceptibility of various populations, particularly in connection with the study of vaccine preparations.

Summary

Serious fetal damage is caused by rubella and particularly by epidemics of it. Rubella occurs primarily in the spring of the year and epidemics occur approximately every seven years. Seroepidemiological data are useful for determining the effects of epidemics, the frequency of infection, and the susceptibility of the population and are needed for vaccine studies. It is obvious that intensive programs are necessary to develop and test successful vaccines so that rubella may be prevented.

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SECTION B. RUBELLA

GAMMA GLOBULIN PROPHYLAXIS OF RUBELLA

J. C. McDONALD

Department of Epidemiology and Health
McGill University, Montreal, Canada

DR. McDONALD *(presenting the paper)*: Since rubella is a widespread human infection against which most adults are immune (6, 10, 20, 22), it is not unreasonable to hope that gamma globulin from large donor pools may contain antibody in sufficient concentration to have a prophylactic effect. There is some evidence, moreover, that infants in the first six months of life are resistant to infection (22), presumably as a result of maternal antibody passively acquired (4). The presence of neutralizing antibody in gamma globulin was first demonstrated by Krugman and Ward in 1958 (12), and since the isolation of rubella virus in tissue culture it has been confirmed more directly in further studies by Schiff, Sever, and Huebner (19). Schiff and his colleagues examined 19 lots prepared commercially by seven manufacturers and found titers of neutralizing antibody ranging from 256 to 2048 for pooled gamma globulin and from 512 to 4096 for convalescent gamma globulin. These titers were about 20 times higher than those of serum specimens from patients convalescing from natural or experimental infections. A small group of men who had had rubella 10 to 15 years earlier had titers in the same range. The question is not, however, whether gamma globulin ought to prevent rubella but whether it does and, more particularly, whether any such effect in pregnant women lowers the risk of fetal defect.

CONTROLLED TRIALS

There have been few adequately controlled trials of gamma globulin in rubella and none in pregnant women. The salient features of those I am aware of (1, 3, 8, 9, 11, 13, 17) are set out chronologically in Table 1. In nine trials pooled gamma globulin was used in doses ranging from about 0.03 to 0.25 ml per pound of body weight, and in all but one the attack rate was reduced. Most of the trials were small and the results varied in statistical significance, but over-all it is hard to avoid the conclusion that gamma globulin appeared to have a substantial effect. Convalescent gamma globulin was tested in three trials: one in Australian adolescents showed protection (1), whereas two in Taiwan school children showed none (8). It may be relevant that the gamma globulin that failed to protect in the trial reported by Korns (11) was four years old and had been considered outdated for three years; the convalescent material of Grayston and Watten (8) was also old and had been stored six years before use.

In the trial of Houser and Schalet (9) the injections were given to military recruits before exposure, but the remaining studies were made during epidemics and the gamma globulin would therefore have been received by some subjects before and in others after contact. It was suggested by Brody *et al.* (3) that their high rates of protection might have been due to the fact that the gamma globulin was given before exposure, but neither in their trial nor in any of the others was protection significantly less when based on cases occurring within 16 days of injection. On the other hand, the exclusion of cases occurring within two or three days of injection tended to increase the difference between inoculated and control groups.

TABLE 1. CONTROLLED TRIALS

Reference	Place	Age group	Dose (ml/lb)		Control group No.	Attacked	(%)	Treated group No.	Attacked	(%)	Reduction in attack rate (%)
Pooled gamma globulin											
Landon *et al.* 1949 (13)	Institution, USA	Children	0.1*		129	24	(18.6)	133	8	(6.0)	68
Korns 1952 (11)	Institution, USA	Children	0.1	(a)	199	26	(13.1)	201	26	(13.0)	0
				(b)	60	35	(58.3)	45	9	(20.0)	66
				(c)	46	18	(39.1)	38	11	(28.9)	24
Houser and Schalet 1958 (9)	Military base, USA	Adults	0.03*		300	7	(2.3)	300	2	(0.7)	70
			0.1*					300	0	(0)	100
Grayston and Watten 1959 (8)	Primary schools, Taiwan	Boys, 7–10	0.1*	(a)	96	12	(12.5)	92	6	(6.5)	48
				(b)	107	21	(19.6)	105	11	(10.5)	46
McDonald 1963 (17)	Institution, England	Children	0.03*		89	11	(12.4)	94	5	(5.3)	57
Brody *et al.* 1965 (3)	Primary school, Alaska	Children, 7–10	0.25		69	60	(86.9)	49	9	(18.4)	79
Convalescent gamma globulin											
Anderson and McLorinan 1953 (1)	Suburban community, Australia	Boys, 15–18	0.03*		46	8	(17.4)	45	3	(6.7)	61
Grayston and Watten 1959 (8)	Primary schools, Taiwan	Boys, 7–10	0.04*	(a)	96	12	(12.5)	93	15	(16.1)	0
				(b)	107	21	(19.6)	103	23	(22.3)	0

*A flat rate of dosage unrelated to body weight was used and the figure shown is an approximation only.

The Brody *et al.* study was of particular value because it was accompanied by serological tests on the children immediately before the administration of gamma globulin and one month later. These tests showed that illness was prevented much more effectively than infection. Whereas the clinical attack rate was reduced by about 80 per cent, the infection rate, judged serologically, was only halved.

Though the dosage range used in these trials was wide (0.03 to 0.25 ml per pound), there was little correlation with the percentage reduction in attack rate. This may have been due to chance or to the small size of the trials, but variation in antibody titer from lot to lot may also have been a contributory factor. Further trials using lots of known potency are needed to clarify this question; in the meantime it would seem reasonable and economical to employ modest dosage but to demand that gamma globulin for rubella prophylaxis meet a high standard for antibody content.

OPERATIONAL SURVEYS

Despite the uncertain value of gamma globulin, women exposed to infection in early pregnancy have been given the benefit of the doubt in many countries. Several analyses of the results have been published (1, 14, 17, 18, 21), but some of the figures are difficult to interpret because there was no estimate of what would have happened if gamma globulin had not been given. Two large surveys less subject to the criticism, based on experience in England and Wales in the period 1954–1962 (17, 18) and in the United States in 1964 (21), are presented in Table 2.

TABLE 2. POOLED GAMMA GLOBULIN IN THE PROTECTION OF PREGNANT WOMEN

Reference	Place	Dose	No. treated	Attack rate within 28 days (%)	Attack rates in untreated women exposed to rubella during same period of time
McDonald 1963 (17)	Britain, 1956–1961 (family contacts)	5 ml	2,700	1.5	3.7% of 461 adult female family contacts
		10 ml	3,278	1.1	
McDonald and Peckham 1966 (18)	Britain, 1962 (family contacts)	5 ml	7,730	2.5	15.6% of 250 adult female family contacts*
		10 ml	999	2.4	
Sever *et al.* 1965 (21)	USA 1964 (all types of contact)	15–20 ml	145	1.4	11.1% of 524 pregnant women in same epidemic not given gamma globulin

*Dr. John Fry, personal communication.

In the British survey, records in relation to dose, type of contact, and other details were collected systematically on all women given gamma globulin. During the nonepidemic years 1954–1961, the attack rate in some 6,000 women exposed within the family was 1.3 per cent—appreciably lower than the 3.7 per cent that was the lowest figure for untreated women of similar age from a survey conducted during the same period. In the severe rubella epidemic of 1962 (5) the attack rate in nearly 9,000 family contacts rose to 2.5 per cent, but the comparable figure for untreated women was apparently much higher (J. Fry, personal communication).

The American survey reported by Sever *et al.* (21) was based on observations made during 1964 on some 6,000 pregnant women included in the Collaborative Study of Cerebral Palsy. A tenth of the women (669) were exposed to infection during the first trimester; 145 were given gamma globulin and 524 were not. The rubella attack rates within one month in two groups—1.4 and 11.1 per cent, respectively—were significantly different ($p < .01$). Surveys of this kind cannot be conclusive, but the results suggest that without gamma globulin the attack rates would have been higher.

EXPERIMENTAL STUDIES

The few studies in which subjects have been deliberately exposed to infection in various ways are also difficult to interpret (Table 3). Certainly they have failed to provide evidence that under these conditions gamma globulin was protective. Anderson and McLorinan (1) in Australia came close to simulating the clinical situation: they took women of childbearing age who had a past history of rubella, infected them by nasopharyngeal instillation, and gave 4 ml of convalescent gamma globulin 72 hours later. A higher proportion of the treated women than of the controls developed rubella, so perhaps the groups were not comparable, but the experiment was too small to have much statistical significance.

The more recent experiments of Green and associates (7) in an institution for mentally retarded children in New York State were extensive and included detailed virological tests. These inquiries illustrated the difficulty of obtaining statistically meaningful results with a relatively small total number of subjects and a large number of relevant variables—susceptibility, gamma globulin dose and potency, type of exposure, timing of signs of infection, and so on. The few figures selected for presentation in Table 3 do not fully represent the findings from these inquiries, which must be examined in detail. It can be seen, however, that gamma globulin appeared to have little effect on the rate of clinical or subclinical infection when virus was given parenterally or by spray, though it may possibly have afforded some protection against infection by contact.

An interesting finding was that viremia in patients with rubella was as frequent in those who had received gamma globulin as in those who had not, though of shorter duration. It remains difficult to assess the contribution made

TABLE 3. EXPERIMENTAL STUDIES

Reference	Subjects	Type of exposure	Dose (ml/lb)	Control group			Treated group		
				No.	No. attacked*		No.	No. attacked*	
					Clin.	Subclin.		Clin.	Subclin.
Convalescent gamma globulin									
Anderson and McLorinan 1953 (1)	Female students (18–22 years), Australia, 1951	Nasopharyngeal instillation	About 0.03	9	3	Not known	15	8	Not known
Pooled gamma globulin									
Green *et al.* 1965 (7)	Retarded children (1–10 yrs), New York, 1961–1964	(a) Intramuscular injection	0.12	5	0	2	5	2	0
		(b) Pharyngeal spray	0.12	10	4	2	6	2	2
		Injection or spray		15	4	4	11	4	2
		(c) Prolonged contact	0.15 –0.2	12	6	0	11	4	1
		(d) Brief contact	0.2	5	1	0	6	0	0
		Contact		17	7	0	17	4	1

*Within 12–24 days of first exposure.

by chance and by known and unknown selective factors to the results of these experiments. There is also some question whether the gamma globulin used, much of which was well beyond expiry date and of low titer, was up to optimum potency.

EFFECT ON THE FETUS

Even if gamma globulin reduces the attack rate of clinical rubella in women exposed to infection in early pregnancy, it cannot be assumed that the risk of fetal defect is also less. Passive immunization may simply change clinically apparent illness into subclinical infection that may be no less dangerous to the child. The work of Brody *et al.* (3) and Green *et al.* (7) suggests that subclinical infection is common after gamma globulin; but whether it is more than usually so is uncertain, since perhaps half the rubella infections ordinarily pass unnoticed. The risk of fetal damage from inapparent infection has yet to be fully assessed but it is already clear that many rubella-type defects have been caused in this way (2, 16). Unrecognized rubella is in some ways more serious than the disease itself because it provides no opportunity for termination of pregnancy.

Without a well-controlled trial of gamma globulin in pregnant women exposed to rubella, which is virtually impossible, no certain answer can be obtained. For this reason, the results of a large survey recently completed in Britain (18) have some value. This investigation involved following up some 30,000 pregnancies in which gamma globulin prophylaxis was attempted, to ascertain the outcome and congenital defects in the children. The main findings are set out in Tables 4, 5, and 6.

It may be seen in Table 4 that infants exposed to maternal rubella suffered to much the same extent as in other published series (15). The absence of cataract was probably due to the fact that in only 13 per cent of the women did

TABLE 4. FETAL SURVIVAL AND DEFECTS AFTER RUBELLA IN PREGNANCY IN WOMEN GIVEN GAMMA GLOBULIN*

	No. of cases of rubella	Total births (live and still)	Stillbirth rate (%)	Children with rubella-type defects				Other children with defects (%)	Total rate of children with defect (%)
				Deafness (%)	Heart (%)	Cataract (%)	Other (%)		
Failures of prophylaxis	467	319	2.2	6.0	2.8	0	8.2	4.1	12.2
Mother given gamma globulin after onset of rubella	143	105	4.8	9.5	3.8	0	12.4	3.8	16.2
Total	610	424	2.8	6.8	3.1	0	9.2	4.0	13.2

**Source:* McDonald and Peckham 1966(18).
Note: All rates are expressed as a proportion of total births—live and still—and are based on information from two postal inquiries, one made shortly after the birth and the other when the child was at least two years old. A large sample of children was later examined in greater detail and it was estimated from this that 12.5 per cent probably had hearing defects.

the onset of rubella occur during the first eight weeks of gestation. It is noteworthy that stillbirth and defect rates were lower for women who developed rubella despite prophylaxis than for those given gamma globulin after the onset of the disease. This suggests that rubella in women recently injected with gamma globulin may be slightly less dangerous than the normal disease. In contrast, with women who escaped rubella the outcome of pregnancies observed at or soon after birth was very much more favorable (Table 5). The absence of deafness has no significance at this early stage, but the rate for congenital heart disease (0.37 per cent) was well within normal limits and there was no excess of patent ductus arteriosus. There was only one case of cataract reported in nearly 24,000 births. Although the rubella attack rate for family contacts was four times higher than in those exposed outside the family, there was no difference in outcome between these two groups.

Children whose mothers had been in contact with rubella in the family during the first twelve weeks of pregnancy, and who were therefore at maximum risk, were followed until they were at least two years of age and then subjected to an inquiry aimed specially at the detection of deafness, cataract, and heart defects. The findings for this group are compared in Table 6 with those from another large prospective survey of normal pregnancies conducted during the same period by fairly similar methods (J. C. McDonald, unpublished data). Deafness was slightly more common in the rubella contacts—3 as against 1.2 per thousand—but

TABLE 5. FETAL SURVIVAL AND DEFECTS REPORTED SOON AFTER BIRTH IN CHILDREN OF WOMEN EXPOSED TO RUBELLA IN PREGNANCY WHO WERE GIVEN GAMMA GLOBULIN AND ESCAPED THE DISEASE*

	No. of women inoculated	Total births (live and still)	Stillbirth rate (%)	Children with rubella-type defects				Other children with defects (%)	Total rate of children with defect (%)
				Deafness (%)	Heart (%)	Cataract (%)	Other (%)		
Family contacts	14,840	11,678	1.7	0.03	0.33	0	0.36	2.1	2.4
Other contacts	15,457	12,253	1.5	0.04	0.41	0.01	0.46	2.3	2.7
All contacts	30,297	23,931	1.6	0.04	0.37	0.004	0.41	2.2	2.6

**Source:* McDonald and Peckham, 1966 (18).

TABLE 6. DEFECT RATES IN STILLBORN AND LIVE-BORN CHILDREN OBSERVED UNTIL AT LEAST TWO YEARS OF AGE—COMPARISON OF TWO SERIES*

Mothers	Total births (live and still)	Stillbirth rate (%)	Children with rubella-type defects				Other children with defects (%)	Total rate of children with defect (%)
			Deafness (%)	Heart (%)	Cataract (%)	Other (%)		
Exposed to rubella in family in first 12 weeks of pregnancy, given gamma globulin and escaped disease (1956–1962)....	7,505	1.7	0.30	0.38	0	0.67	2.9	3.6
Normal women in a prospective pregnancy survey (1959–1960).	11,192	2.0	0.12	0.45	0.01	0.58	4.5	5.1

**Source:* McDonald and Peckham 1966 (18).

other types of defect were less common, mainly because of fewer minor defects, which were probably underreported in the rubella series. The findings from this survey suggest but do not prove that gamma globulin prevented congenital defects due to rubella; they do make clear, at least, that it cannot be accused of giving a false sense of security.

CONCLUSION

Controlled trials of gamma globulin prepared from pooled plasma, though small and mainly in children, have almost all shown a substantial reduction in the attack rate of clinical illness under natural conditions of exposure to rubella. The lot responsible for the only notable failure may have been below normal quality. Large surveys in Britain and the United States into the results obtained from the use of gamma globulin in pregnant women also suggest that protection has been afforded. In studies using experimental methods of infection, gamma globulin had little or no effect. Even after natural exposure, serological tests have shown that some of those who apparently were protected from clinical illness had symptomless infection.

It therefore cannot be assumed that the prevention of rubella in pregnant women necessarily implies protection of the fetus, but the findings from a follow-up recently completed in Britain of 30,000 women given gamma globulin early in pregnancy are reassuring. A quarter of the children whose mothers had rubella in the first trimester despite gamma globulin had rubella-type defects, but there was no excess of cataract or congenital heart lesions, and probably no excess of deafness, in the children of women who did not develop the disease. Subclinical infection either carried little risk to the fetus or did not occur often enough for any adverse effect to be detected. Subject to the usual limitations of passive immunization, gamma globulin thus appears to offer useful protection to mother and child. More information is needed on dosage, and in the meantime preparations intended for use in rubella should be required to meet a high standard of antibody content.

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SESSION VI

EXANTHEMS AND MUMPS (*continued*)

Wednesday, 9 November 1966, at 2:00 p.m.

CHAIRMAN
DR. D. J. DAVIS

RAPPORTEUR
DR. M. R. HILLEMAN

Section B.

Rubella (*continued*)

Presentation of Papers by:

Dr. Paul D. Parkman
Dr. Harry M. Meyer, Jr.
Dr. Saul Krugman

Discussants:

Dr. Joseph Stokes, Jr.
Dr. Stanley A. Plotkin
Col. Edward L. Buescher
Dr. Thomas H. Weller
Dr. Frederick C. Robbins

Section C.

Mumps

Presentation of Papers by:

Dr. Frederick C. Robbins
Dr. Victor J. Cabasso
Dr. A. A. Smorodintsev
Dr. Robert E. Weibel

Discussants:

Dr. Friedrich W. Deinhardt
Dr. Eugene B. Buynak
Dr. Werner Henle

SECTION B. RUBELLA (*continued*)

LABORATORY STUDIES WITH AN ATTENUATED RUBELLA VIRUS

P. D. PARKMAN, H. M. MEYER, JR., H. E. HOPPS, AND R. L. KIRSCHSTEIN

Division of Biologics Standards, National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland, U.S.A.

DR. PARKMAN (*presenting the paper*): The need for an effective rubella virus vaccine has been recognized in the 25 years that have elapsed since the original epidemiologic observations of Gregg (3) concerning the teratogenic effect of rubella infection. It was not until 1961, however, that laboratory methods for studying rubella virus became available (10, 24) and made the development of potential immunizing agents feasible.

Early attempts in our laboratory to produce a killed virus vaccine were discouraging. The development of a live virus vaccine seemed possible, however, and approaches that have been used to attenuate other viruses were explored. Simultaneously, attempts were made to identify changes in the biological characteristics of the virus strains that would serve as markers of attenuation in advance of their use in clinical trials in man.

This report describes the production of an attenuated strain of rubella virus (HPV-77), the laboratory marker tests that assisted in its development, and studies of the effect of attenuation on the pathogenesis of experimental infections in monkeys.

MATERIALS AND METHODS

Viruses

The M33 (10, 11, 12A, B) and ML (12A, B) strains of rubella virus were used at several levels of tissue culture passage. The second to fifth passages were made in primary African green monkey kidney cells (GMK) or, for certain experiments, in BS-C-1 (4) continuous African green monkey kidney cells. Higher passage levels of virus were made only in GMK.

Tissue Cultures

Cell cultures of GMK, BS-C-1, the RK_{13} line of continuous rabbit kidney cells (8), and BHK21 baby hamster kidney cells* (21) were obtained from the Tissue Culture Section, Division of Biologics Standards. Methods for the maintenance of these cultures have been described previously (14). Briefly, the GMK and BS-C-1 cell cultures were maintained at 35°C in Eagle's basal medium (BME) containing 2 per cent heat-inactivated chicken serum. The cultures of BHK21 cells, which were used for the production of hemagglutinating antigen (HA), were maintained at 35°C in BME containing 2 per cent kaolin-treated fetal bovine serum. The RK_{13} cultures were incubated at 33°C for use in neutralization tests, or at 35°C for use in cytopathic (CPE) marker tests; in either case the cultures were maintained in Medium 199 containing 0.4 per cent bovine plasma albumin (BPA). For plaque marker experiments, RK_{13} cells were grown in two-ounce glass prescription bottles and overlaid with M199 containing Nobel's agar.

* Two lines of BHK21 cells were employed, one obtained from Dr. Stanley Plotkin of the Wistar Institute, the other from Mr. Monroe Vincent, of Microbiological Associates, Inc.

Laboratory Animals

Methods for the care and inoculation of monkeys for experimental use have been described (14, 15). Commercially procured Indian rhesus monkeys were caged singly or in pairs, depending on size, and fed a standard primate diet. For surgical procedures or sacrifice, animals were anesthetized intravenously or intraperitoneally with sodium pentobarbital (15–50 mg/kg of body weight).

Specimens for attempted virus recovery were inoculated into GMK cultures. Procedures for the collection, processing, and inoculation of pharyngeal, nasopharyngeal, and rectal swab specimens and of blood and tissue specimens are described in detail elsewhere (14). Swab specimens were collected with dry, sterile cotton swabs, which were then immersed in 6 ml of BME containing 1 per cent BPA, penicillin, streptomycin, and amphotericin B. Blood was obtained by femoral venipuncture and was either heparinized or allowed to clot and the serum then removed. Tissue specimens were weighed, ground in Ten Broeck grinders, and prepared as 10 per cent suspensions in Hanks's balanced salt solution (HBSS) containing 1 per cent BPA and antibiotics.

The swab specimens, tissue suspensions, and heparinized bloods were inoculated in 0.5 ml volumes into each of three to five GMK tube cultures from which the medium had been removed. After an adsorption period of one hour at room temperature, the inocula were removed, 1 ml of fresh maintenance medium was added, and the cultures were incubated at 35°C. Both the original inoculated culture and a subpassage were tested for the presence of interfering agents. Representative isolates were identified as rubella virus by means of a specific immune serum.

Virus Titrations

Infectivity titrations were performed by the usual methods (5). The specimens were clarified by centrifugation at 1500 rpm for 10 minutes. Each of five to ten tube cultures of GMK or RK_{13} was inoculated with 0.1 ml of serial tenfold dilutions of the specimen made in HBSS containing 1 per cent BPA. After 10 days' incubation, the inoculated GMK cultures were tested with echovirus Type 11 (E-11) for evidence of interference. The RK_{13} cell cultures were observed for CPE for 20 or 21 days. Fifty per cent endpoints were calculated by the Karber method (6) and expressed as the interfering $dose_{50}$ (InD_{50}) or the tissue culture cytopathic $dose_{50}$ (TCD_{50}). For plaque studies, serial 0.5 log dilutions were inoculated in 0.2 ml volumes into two-ounce bottle cultures of RK_{13} cells. After one hour, the inocula were removed and the cultures overlaid with agar medium.

Interferon Assay

Interferon assays were performed in homologous cell cultures. After 24 hours' exposure to serial twofold dilutions of the interferon preparation, the cultures were challenged with 50 plaque-forming units of vesicular stomatitis virus (VSV). Plaques were counted after 48 hours, and the interferon titer was expressed as that dilution resulting in a 50 per cent reduction in the number of VSV plaques formed.

Serologic Tests

The procedures used for measuring rubella virus neutralizing and complement-fixing antibody have been described previously (13, 14, 19). Hemagglutination-inhibition (HI) tests were performed using a slight modification of the procedure previously reported (20). Here, heat-inactivated serum samples were first adsorbed with erythrocytes from newly hatched chicks and then treated with 25 per cent acid-washed kaolin in order to remove nonspecific agglutinins and inhibitors of hemagglutination (1).

Rubella virus hemagglutinating antigens (HA) were prepared in monolayer cultures of BHK21 cells. Equal volumes of serial twofold dilutions of sera were mixed with antigen containing four HA units. After incubation for one hour at room temperature, a volume of 0.16 per cent chick red blood cells equivalent to the volume of antigen employed was added; the test was then refrigerated for one hour. Antibody endpoints were read as the highest dilution of serum producing complete inhibition of hemagglutination. Each test included an antigen titration, positive and negative control sera, and serum-RBC and diluent-RBC controls.

RESULTS

In an effort to produce attenuation, rubella virus strains were serially subcultured in GMK

cells. Supernatant fluids and cells from rubella-infected cultures were passed at intervals of 7 to 19 days. Because rubella virus does not produce cytopathic changes in GMK cultures, each passage was tested for interference with E-11 to confirm the presence of virus. At intervals of 10 to 20 passages these viruses were tested for evidence of modification of their biologic characteristics. A summary of these marker tests is presented in Table 1.

Tissue Culture Markers of Attenuation: Cytopathic Effect and Plaque Markers in RK_{13} Cells

In most tissue culture systems the presence of rubella virus is detected by its ability to produce interference with superinfection by a second virus. This property has been used as a standard technique for the titration of rubella virus. However, the virus can be adapted to produce complete cytopathic changes in RK_{13} cell cultures but only after 3 to 10 passages in homologous cells. In this system infectivity can be expressed in terms of tissue culture cytopathic changes (TCD_{50}). It was surprising to find that instead of following this established pattern, high-GMK-passage viruses produced marked cytopathic changes in the heterologous RK_{13} system upon first passage. This unique characteristic of the high-passage virus provided one of the laboratory markers. Comparative titrations of several passage levels of the M33 and ML strains of virus were performed in RK_{13} and GMK cell cultures. Striking differences were noted between low-passage and high-passage viruses. With viruses passaged less than 10 times in GMK the onset and progression of cytopathic effect in RK_{13} cells was delayed and the cytopathic endpoint titers (TCD_{50}) were consistently 1.3 to 4.0 $\log_{10}$ units lower than the corresponding interference titration value (InD_{50}). With higher passage levels this relationship changed; here, the titers indicated by the cytopathic system were always equal to or higher than those shown by the interference method.

Similar changes were noted in the ability of various passage levels of virus strains to produce plaques in RK_{13} cells. Comparative titrations of several GMK passage levels of the M33 and ML strains were performed by the interference technique and by plaque formation in RK_{13} cells. The M33 strain in the 5th to the 23rd passage and the ML strain in either throat washing or 5th passage failed to produce plaques. Higher passage levels of both virus strains produced discrete round plaques approximately 2 mm in diameter, after 10 to 14 days.

TABLE 1. CHARACTERISTICS OF RUBELLA VIRUS: IN VITRO AND IN VIVO "MARKERS"

Type of marker	Rubella virus	
	Virulent	Attenuated HPV-77
Tissue culture		
Cytopathic effect on RK_{13} cells	0	+
Interferon production	0 to ±	+
Rhesus monkey		
Viremia	+	0
Virus shedding	+	0 to ±
Communicability	+	0

Interferon Marker

Infection of GMK cell cultures with a variety of rubella virus passage levels induced the production of interferon. The interferon titer increased with the passage level; thus, the titers induced by 74th- to 77th-passage virus were consistently two- to fourfold higher than those observed with 2nd- to 4th-passage virus preparations.

Marker Tests Performed in Rhesus Monkeys

Earlier work showed that the rhesus monkey was a useful laboratory host for the study of experimental rubella infections (14, 15). Studies with several passage levels of virus indicated that repeated serial GMK passage significantly altered the pattern of these simian infections. Animals were inoculated intravenously or intramuscularly and specimens of blood and nasopharyngeal, pharyngeal, and rectal swabs were collected at two- to three-day intervals for 21 days. The monkeys were examined for signs of illness on the days when specimens were collected. None of the animals developed clinical illness.

While antibody developed in 100 per cent of

the inoculated monkeys given low passage virus and in 93 per cent of those given high-passage, modified virus, the pattern of virus recovery in the two groups was strikingly different. Of 27 animals inoculated with 3rd to 5th passage, 13 (48 per cent) showed viremia and 25 (93 per cent) showed virus excretion from the respiratory tract. Rectal excretion of virus occurred in 41 per cent.

In contrast, monkeys receiving comparable doses of high-passage-level virus did not show viremia or virus excretion from the respiratory or intestinal tract. No virus recoveries were made from any of the specimens collected from 14 animals parenterally inoculated with the attenuated virus. Uninoculated susceptible control animals were caged with infected animals in each experiment. Serologic evidence of infection was commonly observed in the cage contacts of monkeys receiving low passage virus. With the high-passage attenuated virus, experimental infections were not transmissible.

Antibody Response and Immunity

Until a short time ago, only neutralization, complement-fixation, and fluorescent-antibody tests were available for detecting rubella antibodies. However, recent studies in our laboratory have provided methods of demonstrating rubella HA antigen production and of performing a highly specific HI antibody assay (20). The new test procedures are summarized in Table 2.

Hemagglutinins for erythrocytes of several avian and mammalian species were produced in the supernatant fluids of rubella-infected BHK21 cells. Consistent yields of HA antigen with a titer of 1:16 to 1:64 required the use of the BHK21 cell line described by Stoker and Macpherson (21) and a high-multiplicity virus inoculum. When BME medium containing fetal bovine serum was employed, the removal of nonspecific inhibitors of HA was accomplished by kaolin treatment of the fetal bovine serum before it was incorporated into the medium. The HA antigen was also produced in cells maintained in serum-free BME.

Erythrocyte suspensions from newly hatched baby chicks provided the most satisfactory agglutination patterns and the highest HA titers. While HA was demonstrable in a variety of

TABLE 2. HEMAGGLUTINATION BY RUBELLA VIRUS

HEMAGGLUTINATION (HA)	
HA production	BHK 21 cell cultures
Red blood cells	Newly hatched chick Adult chicken Goose Sheep
Incubation temperature	4°C, 22°C, 37° C
Test diluent	Dextrose-gelatin-veronal with 0.2% bovine serum albumin
HA titer	1:1 to 1:64
HEMAGGLUTINATION-INHIBITION (HI)	
Treatment of sera	Red blood cell and kaolin adsorption
Specificity	Inhibition by sera containing rubella neutralizing antibody
HI antibody response	Parallels neutralizing antibody; ≥8-fold higher

commonly used diluents, those containing organic buffer systems (veronal, tris) resulted in titers four- to eightfold higher. The rubella HI was similar to that of rubeola. Kaolin adsorption of test sera was particularly important in order to remove nonspecific inhibitors of HA. The HI antibody was specific; antibody responses occurred in rubella-infected animals and humans but were not observed in sera from hosts infected with other viruses. In general, the HI antibody levels were eightfold or more higher than the corresponding neutralizing-antibody levels.

Data comparing the neutralizing, CF, and HI antibody responses observed in sera obtained from 18 rhesus monkeys five to six weeks after inoculation with virulent or attenuated rubella virus are shown in Table 3. All the animals developed both neutralizing and HI antibody. Tests on serial serum samples from each group indicated that the neutralizing antibody was first detectable between the tenth and twentieth day after inoculation and increased to stable maxi-

TABLE 3. ANTIBODY RESPONSES IN RHESUS MONKEYS FOLLOWING INOCULATION WITH VIRULENT OR ATTENUATED RUBELLA VIRUS

Rubella virus	Antibody response					
	Neutralizing		CF		HI	
	Positive/total	GMT*	Positive/total	GMT*	Positive/total	GMT*
Virulent	9/9	17.0	6/9	5.4	9/9	298.0
Attenuated HPV-77	9/9	7.5†	0/9	—	9/9	32.0

*Geometric mean antibody titer.
†Based on antibody assay of sera from eight monkeys.

mum titers by one to two months. Both neutralizing and HI antibodies persisted for at least four months after inoculation. The production of CF antibody in these animals was less consistent; only six of the nine monkeys receiving low-passage virulent virus showed antibody in sera collected five to six weeks after inoculation, and none was demonstrable in the nine animals given attenuated virus. In infections with low-passage virulent virus, the neutralizing antibody titers ranged from 1:4 to 1:32, with a geometric mean titer of 1:17. Although there was considerable overlapping of values, the neutralizing-antibody titer in the animals given attenuated virus tended to be lower, ranging from 1:2 to 1:16. With sera from eight monkeys in which technically satisfactory tests were done, the geometric mean titer was 1:7.5. The results of HI tests were comparable to the neutralization test results. However, the HI antibody titers were significantly higher, reflecting the greater sensitivity of the test. The differences in geometric mean HI titer between the two groups were magnified; the monkeys receiving high-passage virus developed antibody levels about tenfold higher.

Animals that have antibody as a result of previous infection with either virulent or attenuated rubella virus are protected against reinfection. Groups of monkeys originally infected with high- and low-passage levels of virus were challenged intravenously or intramuscularly several months later with 4th-passage-level virus of the M33 strain. The challenge inoculation produced neutralizing-antibody increases in animals with low levels of pre-existing antibody. These "booster" responses in neutralizing antibody were observed in one out of eight animals previously infected with virulent virus, and in two out of four with antibody as a result of infection with attenuated virus. Sera from all four animals in this latter group showed increases in HI titer ranging from 4- to 64-fold. Despite parenteral challenge of these animals with $10^{2.9}$ to $10^{6.6}$ InD_{50}, rubella virus could not be recovered from isolation specimens. A total of 68 pharyngeal, nasopharyngeal, rectal, and blood specimens collected from animals originally infected with low-passage virus were negative. Virus isolation specimens obtained from the four monkeys previously infected with high-passage virus (and showing HI antibody increases described above) did not yield virus after challenge inoculation. None of the respiratory or intestinal-tract swabs or heparinized blood specimens collected at intervals of two to three days during the 21 days after challenge were positive for rubella virus. None of the four cage contact control monkeys showed serologic evidence of transmitted infection. Thus, the presence of detectable rubella-neutralizing antibody appeared to protect these animals against viremia or virus shedding irrespective of the passage level of the original infecting virus.

Viral Pathogenesis of Experimental Rubella Virus Infections

Recently, other *in vivo* experiments in rhesus monkeys were performed to determine whether the marked differences observed in the pattern of infection with high- and low-passage rubella virus were accompanied by alteration in the pathogenesis of infections in monkeys. Groups of animals were inoculated intravenously with

either virulent 4th-passage-level or attenuated 77th-passage-level virus. Respiratory- and intestinal-tract specimens and bloods were obtained at two- to seven-day intervals during the month following inoculation. The animals were then anesthetized, sacrificed by exsanguination, and autopsied. Specimens of organs and tissues were removed and processed for attempted virus recovery.

There was no clinical or gross pathological evidence of disease in any of these animals. Data concerning the virologic events occurring in these experimental infections are summarized in Figure 1. In the group of 14 monkeys inoculated with low-passage virus, two animals were sacrificed at each time interval. Viremia was detected in eight of these, and the typical pattern of virus shedding was observed. All the surviving animals had developed antibody by the seventeenth day after infection. The presence of virus was detected in the spleen and the inguinal lymph nodes of animals sacrificed on days 2, 7, 11, and 17. The mesenteric lymph nodes yielded virus in one of the pair of animals studied on days 2 and 7 and in both animals sacrificed on days 11 and 17. The last specimen positive for virus was a mesenteric node obtained from an animal 24 days after inoculation. The respiratory-tract tissues first yielded detectable amounts of virus in animals studied on the seventh day after inoculation; the nasal turbinates, pharyngeal and nasopharyngeal mucosa, and lung continued to contain virus through the seventeenth day. Rubella virus was also recovered consistently from specimens of colon during the same period. Widespread infection of other tissues, including pectoralis major muscle, liver, kidney, and adrenal was detected on the seventh, eleventh, and seventeenth days. No rubella virus recoveries were made from

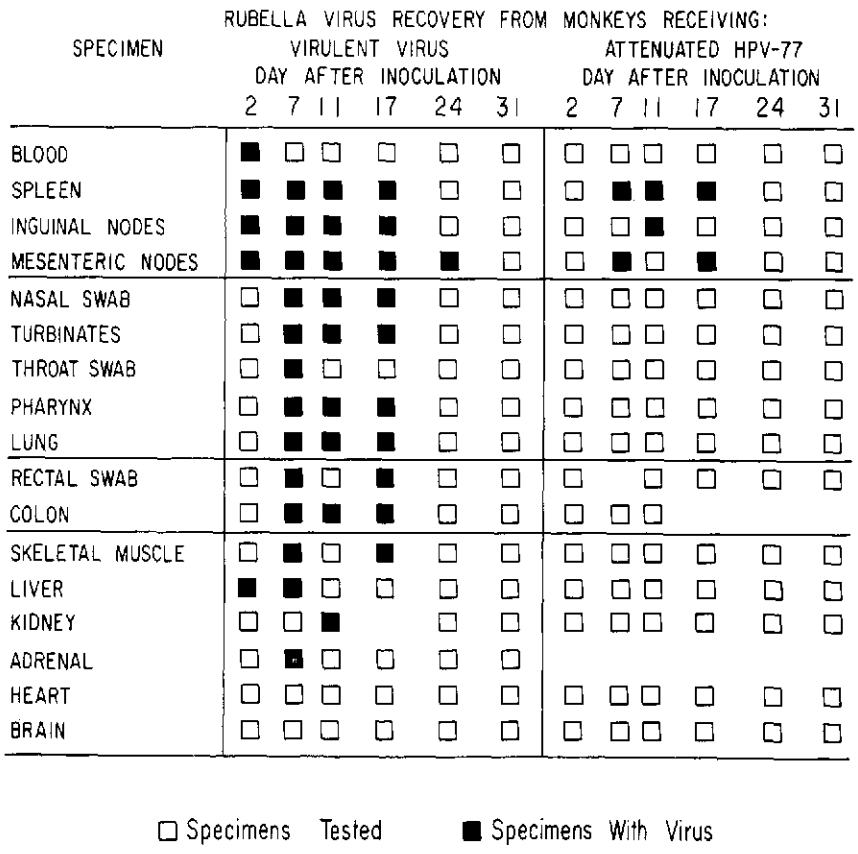

Fig. 1. The effect of rubella virus passage level on virologic events occurring in experimentally-infected rhesus monkeys.

tissue obtained 31 and 72 days after inoculation.

The results of a similar study of seven rhesus monkeys inoculated with the HPV-77 attenuated strain are also summarized in Figure 1. In this group, one animal was sacrificed on each of the days indicated. As had been noted previously, in experimental infections with the attenuated strain, blood and respiratory- and intestinal-tract swab specimens were uniformly negative for virus. The paucity of virus isolates in the tissues of these monkeys provided a striking contrast with the findings in infection with the virulent low-passage strain. While spleen and, to a lesser extent, lymph nodes were sometimes positive, virus was never detected in respiratory or other tissues. Marked quantitative differences in high- and low-passage rubella infections were also shown. Lymph node and splenic tissues of animals infected with the 4th passage level strain contained geometric mean virus titers of 4.2 $\log_{10}InD_{50}$ per gram of tissue. In contrast, similar specimens from animals infected with HPV-77 yielded only 2.0 $\log_{10}InD_{50}$.

A similar comparison of experimental infections in pregnant rhesus monkeys is illustrated in Figure 2. Nine animals were inoculated parenterally with rubella virus during the fourth week of gestation; six of these were given low-passage virulent virus, and three the attenuated HPV-77 strain. Ten to 33 days after inoculation, the pregnancies of these monkeys were interrupted surgically. The procedures for the collection and processing of maternal and fetal specimens have been described in detail previously (15). All the animals developed rubella virus antibodies.

In monkeys receiving the low-passage strain, virus was frequently detected in maternal blood, nasal, pharyngeal, and rectal swabs; and in mesenteric node, lung, and, in one instance, uterine decidua. The transmission of rubella-virus infection to the products of conception was demonstrated in five of these six animals. In the three animals given the attenuated strain all attempts at virus isolation from specimens collected were uniformly negative. Maternal operative specimens (mesenteric node, peritoneal

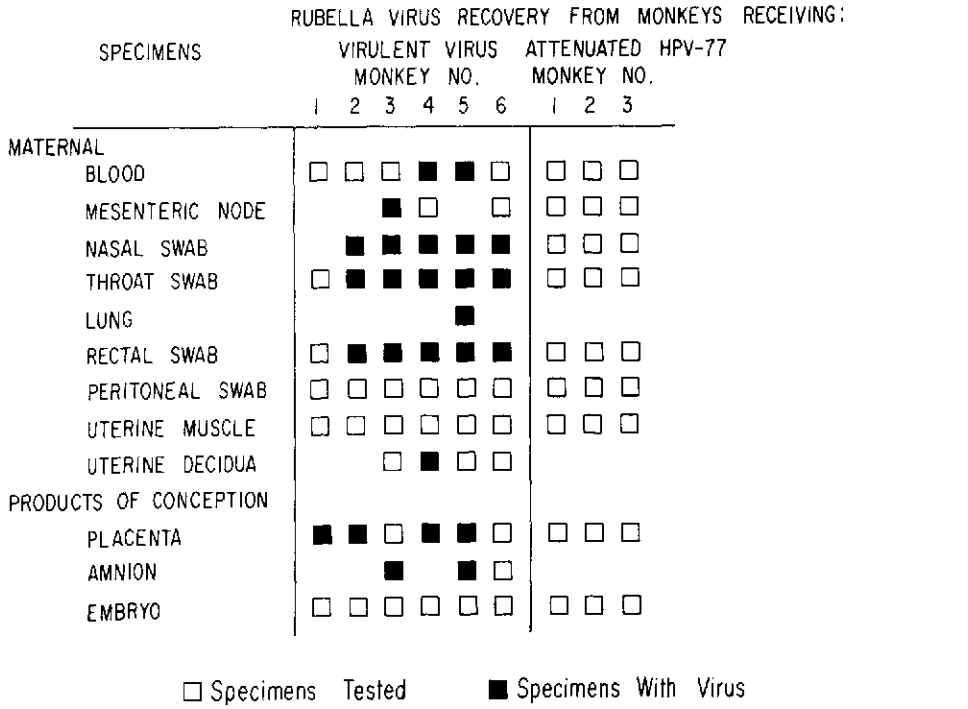

Fig. 2. The effect of rubella virus passage level on virus recovery from pregnant rhesus monkeys inoculated during the fourth week of gestation.

swabs, uterine muscle, and fetal and placental specimens) obtained 17, 21, and 28 days after inoculation failed to yield detectable virus.

DISCUSSION

In the past, the attenuation of several viruses, including rubeola and polioviruses, has been attended by significant changes in their biological properties. These alterations in virus characteristics have served as markers to differentiate the attenuated from the virulent virus strains (9, 22, 23). In our program strong emphasis was placed on the development of *in vitro* and *in vivo* laboratory methods that might provide markers of rubella virus modification prior to clinical trial in man. This approach was productive. *In vitro* tests indicated that definite modification had occurred in the high-passage virus; these viruses produced CPE and plaques in cultures of RK_{13} cells. The two- to fourfold increases in interferon titer induced by the high-passage virus was particularly encouraging, since increased interferon production has been described as characteristic of attenuated measles and polioviruses (2) and has also been shown for several other mammalian viruses (17, 18). To our knowledge, this is the first time that the interferon-inducing capacity of a virus has been used in advance of clinical trial to predict attenuation for man.

In vivo experiments in rhesus monkeys showed differences in antibody production and virus shedding in infections produced by low- and high-passage virus. In general, less antibody was produced in infection with the attenuated rubella virus. Neutralizing-antibody responses following infection with the attenuated strain were qualitatively similar to those observed with virulent virus, but the titers were twofold lower on the average. Complement-fixing antibody was not produced in animals inoculated with the high-passage virus strain. This finding is not surprising; failure to induce CF antibody is a characteristic of certain other live viruses used for immunization (7, 16). Hemagglutination-inhibiting antibody developed after infection with either virus passage level, and titers appeared to persist unchanged for several months after infection. Indeed, the HI test promises to be the most useful of the serologic methods for detecting rubella virus antibody. HA antigen is easily and inexpensively produced and the HI test itself is specific, sensitive, and simple to perform.

The most dramatic difference between infections with low- and high-passage viruses concerned the patterns of virus recovery; the high-passage rubella viruses failed to produce the typical pattern of viremia, virus shedding, and communicability characteristic of virulent low-passage strains. These changes provided the most suggestive evidence that the high-passage virus might, in fact, be attenuated and noncommunicable in man.

Recent studies of the pathogenesis of experimental infections indicated that infection with attenuated virus exhibited a reduced level of dissemination and tissue multiplication. This decreased invasiveness of the attenuated virus may be of importance in estimating the degree of risk associated with vaccine inoculation during pregnancy. Although the numbers of pregnant animals studied are small, the results suggest that the attenuated virus infections are less likely to involve the products of conception.

The observation that animals immunized with high-passage rubella virus were protected against viremia and virus shedding after challenge with low-passage virulent virus was of particular interest. Monkeys with low titers of antibody had serologic responses but, like the animals with higher titers, did not shed virus and were not viremic. It seems probable that since rubella virus infections appear to be transmitted to the conceptus by way of the maternal blood, an absence of viremia would be expected to preclude fetal infection and the resulting congenital defects.

Summary

In vitro and *in vivo* changes in the biological characteristics of a high-passage-level rubella virus (HPV-77) provided a firm experimental basis for the use of this strain as a live rubella virus vaccine in man. These changes included increased cytopathic effect and induction of interferon in cell cultures and the production of modified infection in the simian host. The HPV-77 strain was immunogenic and conferred pro-

tection against reinfection in immunized monkeys.

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SECTION B. RUBELLA

CLINICAL STUDIES WITH ATTENUATED RUBELLA VIRUS

H. M. MEYER, JR., P. D. PARKMAN, T. C. PANOS, G. L. STEWART, T. E. HOBBINS, AND F. A. ENNIS*

DR. MEYER (*presenting the paper*): There has been a considerable research interest in developing a vaccine that could be used to prevent rubella and rubella-associated fetal defects.

Our laboratory found that protracted tissue culture passage of rubella viruses resulted in modification of their biological characteristics (14, 15). One of the high passage viruses serially propagated 77 times in primary green monkey kidney cultures (GMK), referred to as the HPV-77 strain, appeared particularly promising. This virus material was prepared as an experimental live vaccine and subjected to clinical testing (11, 12). The results of the initial and several subsequent clinical studies were favorable and will be summarized here.

MATERIALS AND METHODS

Preparation of Vaccine

The vaccine was produced in 32-ounce flasks of GMK cells and represented the 77th GMK passage of the M-33 strain of rubella virus. The production and testing methods employed conformed in general to the rigorous requirements applying to live measles and poliovirus vaccines commercially produced in the United States (20).

Cultures of GMK maintained in Eagle's minimum essential medium containing 1 per cent fetal bovine serum and antibiotics were inoculated with the high-passage virus. Before the period of virus harvest, the monolayers were washed three times with 50 ml of Hanks's balanced salt solution (HBSS), refed, and maintained on serum-free Medium 199 containing 25 μg/ml of neomycin as the only antibiotic. Daily harvests of the infected fluids were made from the eighth to the fifteenth days after inoculation. Pooled fluids were centrifuged to remove intact cells, and commercially purchased human serum albumin was added to the fluids (final concentration 2 per cent) as a virus stabilizer. This final bulk suspension after distribution in single-dose containers was frozen at $-60°$C and represented the final vaccine. When thawed and assayed in cell cultures the vaccine contained $10^{3.9}$ tissue culture cytopathic $dose_{50}$ (TCD_{50})/0.5 ml. Under these conditions of storage the experimental vaccine has been stable for one year.

Selection of Study Area

One of the primary concerns in the initial clinical study with the virus was that susceptible persons not participating in the work be shielded from possible exposure. This theoretical risk can be reduced in institutions. Here the population is relatively stable and the entry of personnel into a study area can be rigorously controlled.

After careful evaluation of all pertinent factors, the Arkansas Children's Colony, a school for the mentally retarded, was selected. This institution has been used in each of our rubella clinical studies. The Colony is in a rural setting near Conway, Arkansas. Approximately 700 students reside in widely scattered cottages that are functionally independent. Since 32

* Drs. Meyer, Parkman, Stewart, Hobbins, and Ennis (Division of Biologics Standards, National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland); Dr. Panos (Department of Pediatrics, University of Arkansas, Little Rock, Arkansas).

children are assigned to each cottage, it is possible to isolate small groups from other rubella-susceptible persons with very little interruption in the normal program. The immunity status of the 700 children and 300 employees to rubella was ascertained by virus-neutralization (13) and hemagglutination-inhibition tests (19).

Study Groups

The parents or legal guardians of the study participants were fully acquainted with all details of the project and gave their written permission.

Sixteen rubella-susceptible girls were involved in the initial study begun in October 1965. Eight of these were inoculated subcutaneously with 0.5 ml of experimental vaccine containing approximately 10,000 TCD_{50} of the live, modified virus; the remainder of the group served as uninoculated rubella-susceptible contact controls. All 16 girls were isolated in their cottage for the eight-week period of clinical observation.

Three subsequent studies, initiated in February, June, and September 1966 at the Children's Colony, have been designed to supplement the information obtained in the initial trial.

Clinical and Virologic Surveillance

In the first two studies, the children were examined daily and their temperatures recorded twice daily for approximately eight weeks. Throat swabs were collected daily from both the inoculated and the control groups and heparinized whole blood samples were obtained frequently from the vaccinated children between the sixteenth and twenty-first days. Clotted blood samples for serologic examination were collected weekly from all the participants. Throat swabs and heparinized blood samples for an attempt at virus isolation were frozen immediately in dry ice. The specimens for virologic testing were shipped by air to our laboratory at the National Institutes of Health.

Virologic Tests

The throat swabs, originally collected and frozen in 6.0 ml of HBSS containing 1 per cent bovine plasma albumin and antibiotics, were thawed and individually inoculated in 0.5 ml volumes into each of 3 GMK culture tubes from which the medium had been removed. After a one-hour adsorption period, the inoculum was replaced with 1 ml of maintenance medium and the tubes were incubated at 35°C. Tests for interfering viruses were made after 10 days, both in the original inoculated cultures and in a subpassage. Interfering agents were identified as rubella virus by neutralization tests with specific serum. Heparinized blood samples were inoculated similarly. After one hour the blood was removed and the cultures were washed twice before refeeding. Again, tests for the presence of virus were made by the interference technique.

To quantitate the amount of virus present, positive throat swab or blood specimens were titrated in tenfold dilutions in GMK and continuous rabbit kidney (RK_{13}) (9) tube cultures. Virus titers in GMK were expressed as the interfering $dose_{50}$ (InD_{50}) and titers in RK_{13} as the cytopathic $dose_{50}$ (TCD_{50}).

Neutralization tests were performed in RK_{13} tube cultures by methods previously described (13). Neutralizing-antibody titers were expressed as the twofold dilution of serum that protected 50 per cent or more of cultures from rubella virus cytopathic effect (CPE). Specimens that in the initial 1:2 dilution failed to neutralize virus were considered devoid of antibody.

Hemagglutination-inhibition (HI) tests for rubella virus antibodies were performed by methods recently described (19). The HI antibody endpoints were expressed as that twofold dilution of serum that completely inhibited rubella virus hemagglutination. Specimens that in an initial 1:8 serum dilution failed to inhibit hemagglutination were considered negative. Persons without rubella virus neutralizing antibody at a 1:2 serum dilution and without HI antibody at a 1:8 dilution were classified as susceptible to rubella.

RESULTS

Clinical and Epidemiological Characteristics of Attenuated Rubella Virus Infections

The clinical and epidemiological characteristics of attenuated rubella virus infection in the participants in each of our studies have been similar. A summary of the findings as com-

pared to those in natural rubella occurring under the same environmental circumstances appears in Table 1. None of the 8 girls vaccinated in the first trial nor any of the 43 children immunized in subsequent studies have manifested any evidence of vaccine reaction. Thus, we have infected a total of 51 children with HPV-77 attenuated rubella virus without observing vaccine-related fever, rash, or lymphadenopathy. None of the 49 rubella-susceptible cottage contacts of the vaccinees developed rubella. Detailed virologic surveillance for seven to eight weeks confirmed that the cottage contacts remained uninfected. Their serum samples remained negative for antibody and their throat-swab specimens contained no rubella virus. This experience is in contrast to that reported by others who have studied natural or experimentally induced rubella (1, 4, 5, 7, 8, 17, 18). Unmodified preparations of rubella virus commonly produce rash and lymphadenopathy in infected recipients.

We were able to study an outbreak of natural rubella at the Children's Colony. The outbreak was confined to a single cottage not involved in the vaccine studies. The virus was introduced by a girl exposed to rubella during Christmas vacation. Ten other rubella-susceptible and 22 rubella-immune persons lived in the same cottage. Certain features of the ensuing localized rubella outbreak are summarized in Table 1. All 10 susceptible contacts were infected during the following weeks; of the 11 persons with rubella, 9 manifested a rash disease and the other cases were clinically inapparent.

TABLE 1. CLINICAL AND EPIDEMIOLOGICAL FEATURES OF NATURAL AND ATTENUATED (HPV-77) RUBELLA VIRUS INFECTIONS

Infecting virus	Clinical illness with rash	Inapparent infection	Spread of infection to cottage contacts
Natural virus.....	9/11*	2/11	10/10†
HPV–77 strain.....	0/51	51/51	0/49

*Numerator denotes numbers of persons experiencing designated type of infection; denominator denotes number of persons in study group.
†Numerator denotes number of susceptible contacts infected; denominator denotes number of susceptible cottage contacts exposed.

Virologic Characteristics of Attenuated Rubella Virus Infection

The virologic events associated with HPV-77 attenuated rubella virus infection are summarized in Figure 1. The 51 children infected developed neutralizing and HI antibodies by the fourth week after inoculation. None of the sera collected from the vaccinees have contained detectable levels of complement-fixing antibody. The appearance of HI antibodies correlates well with the appearance of neutralizing antibodies. Both types of antibody can be detected in some persons by the end of the second week and are present in most instances by the third week after inoculation.

Numerous attempts were made to detect viremia. Although 7 to 18 specimens of heparinized blood were collected daily from the sixth through the twenty-first days and tested, none contained detectable virus. This represents a total of 177 blood samples negative for virus.

Pharyngeal excretion of virus occurred in about 75 per cent of the vaccinees. Virus was recovered as early as the seventh day and as late as the twenty-first. The period of maximum virus shedding was between 10 and 20 days after inoculation. On any given day, even during this period, only about one third of the throat swabs were positive for virus.

The level and duration of neutralizing and HI antibody are depicted in Figure 2. The geometric mean titers of neutralizing and HI antibodies one to two months after vaccination were 1:8 and 1:64, respectively. These antibody levels have remained stable, as is shown by the results of tests on serum samples collected four, eight, and twelve months after vaccination.

Although not shown in the figure, the levels of antibody in the girls involved in the natural rubella outbreak at the Children's Colony are also being monitored. The titer of neutralizing antibody in sera from the cases of natural rubella is about four times higher than that obtained in tests on samples from the vaccinated group. Similarly, there is an eight- to sixteenfold difference in HI antibody level between the groups infected with natural and attenuated rubella viruses. The greater difference is a reflection of the considerably greater sensitivity of the rubella HI test than of the neutralization method.

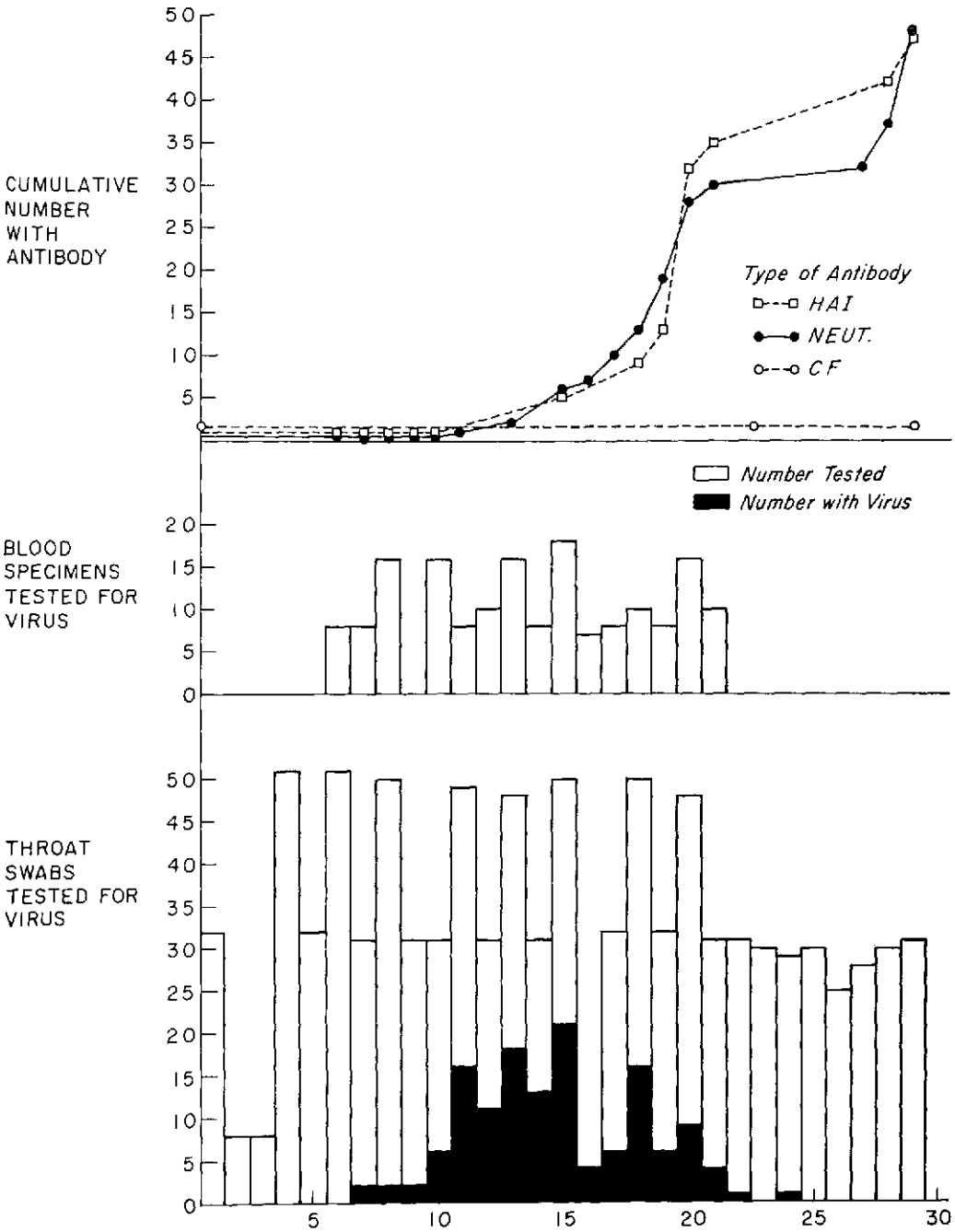

Fig. 1. Virologic events after HPV-77 rubella virus inoculation of 51 susceptible children.

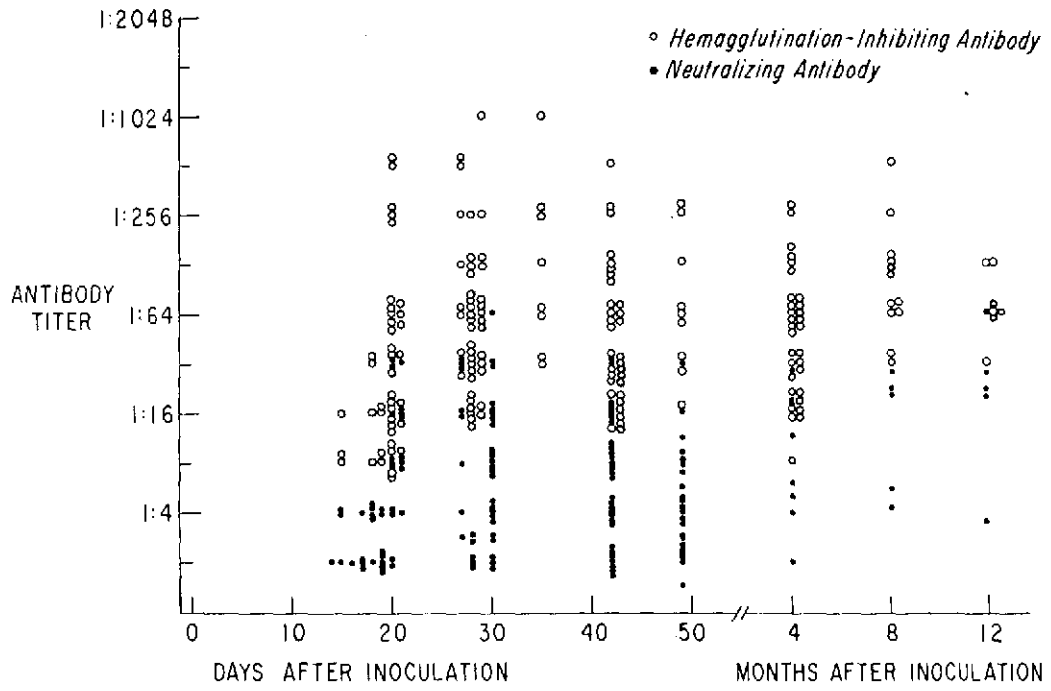

Fig. 2. Neutralizing and hemagglutination-inhibiting antibody response in 51 persons inoculated with attenuated (HPV-77) rubella virus.

Study of Natural Rubella at the Children's Colony

The results of virus studies on the 11 cases of natural rubella occurring in the isolated cottage outbreak mentioned earlier are presented in Figure 3. Neutralizing and HI antibodies were first detected at the time of the onset of rash, and by early convalescence all sera tested were positive. Complement-fixing antibodies rarely appeared before the second week of convalescence. Three of the eight cases of rubella with rash failed to develop CF antibodies.

To demonstrate rubella viremia it is desirable to obtain blood samples before the onset of rash. Of four heparinized blood samples collected one to four days before the onset of rash, three were positive for virus. Two out of seven specimens obtained after rash had appeared also yielded virus. There was a high efficiency of virus recovery from throat-swab specimens. During the period from seven days before the onset of rash to four days afterward, 26 of 28 throat swabs contained virus. For several consecutive days, 100 per cent of the swabs were positive.

This is the typical virologic picture of natural rubella and stands in contrast to our experience with vaccinated children studied under the same conditions. In the attenuated virus infections, viremia was never shown and throat swabs were positive far less frequently.

Quantitation of Virus Excretion in Natural and Attenuated Rubella Virus Infections

Because of the obvious differences in the communicability of the virulent and attenuated virus infections, attempts were made to quantitate the amount of virus excreted. Thirteen virus-positive throat-swab specimens from the virulent rubella outbreak and 22 positive throat swabs from vaccinated children infected with the attenuated virus were titrated in tissue cultures. The geometric mean titer of virus in swabs from the virulent cases was $10^{-2.5}$ per 0.1 ml and that from the vaccinated children was only $10^{-0.4}$ per 0.1 ml, representing a hundredfold difference—that is, 320 as compared to 2.5 infectious doses.

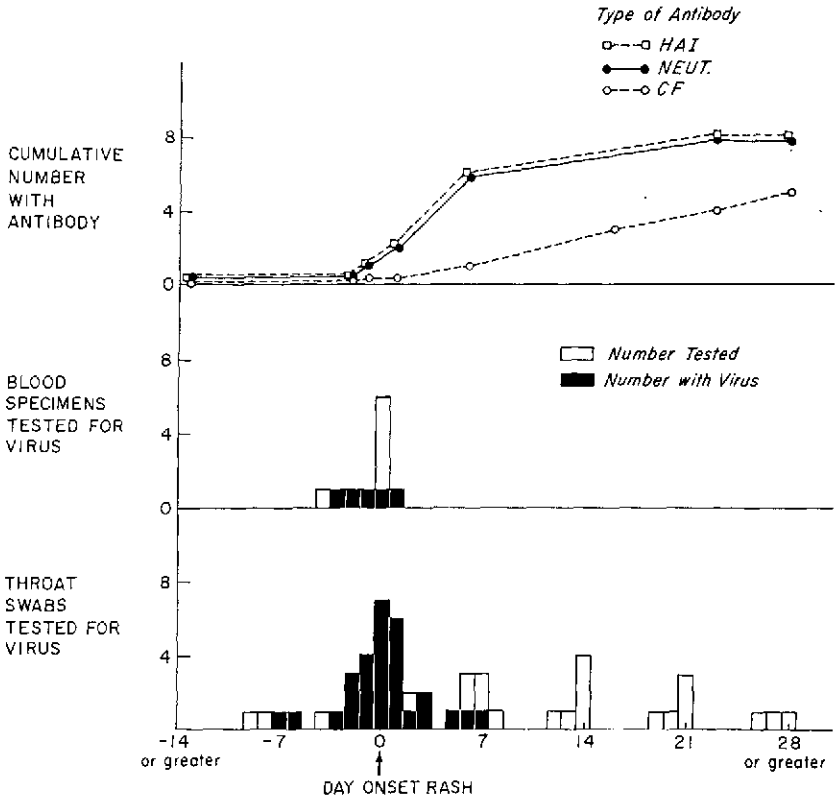

Fig. 3. Virologic events in natural rubella showing the results of virus studies on specimens collected from 8 of the 11 girls involved in the cottage outbreak.

Laboratory Characterization of Natural Rubella Virus and Virus Excreted by Vaccinees

The laboratory marker tests that had indicated modification of the high-passage virus (14, 15) were used to characterize the viruses excreted by the vaccinated children (Table 2). A representative isolate from a vaccinated girl (J. L.) was subjected to the entire gamut of tests. Each of the marker tests indicated that the isolate possessed the properties of the vaccine strain and not of virulent rubella virus. The J.L. isolate produced cytopathic changes and plaques in RK_{13} cultures and induced the production of interferon *in vitro*. Interferon titers in GMK cultures infected with the J.L. isolate were 1:13, as compared to less than 1:4 with low-passage virus. Five rhesus monkeys inoculated intravenously with the virus from the vaccinated girl were not viremic, did not shed virus, and did not transmit their infections to uninoculated cagemates.

TABLE 2. BIOLOGICAL CHARACTERISTICS OF RUBELLA VIRUSES: VIRULENT, ATTENUATED, AND STRAIN ISOLATE FROM VACCINATED CHILD

Virus characteristics	Rubella virus type		
	Virulent	HPV–77	JL isolate
In vitro			
Cytopathic effect in RK_{13} cells	0	+	+
Interferon production	0 to ±	+	+
In vivo			
Monkey			
Viremia	+	0	0
Virus shedding	+	0 to ±	0
Communicability	+	0	0

With the use of the RK_{13} cytopathology marker, 22 other representative pharyngeal isolates from eight vaccinated children were compared with 16 isolates from the pharynx and blood of four children involved in the cottage outbreak of virulent rubella. None of the viruses from the cases of natural rubella produced CPE in RK_{13} cultures, whereas all the strains from the vaccinated children did. Thus, the viruses recovered from vaccinated children uniformly exhibited the properties characteristic of the high-passage attenuated strain.

Titration of Attenuated Rubella Virus by Subcutaneous and Intranasal Inoculation of Susceptible Children

Earlier we noted that the attenuated virus was less likely to induce infection in rhesus monkeys after intranasal inoculation (11, 12). Following this lead, a clinical study was performed in which dilutions of the attenuated virus vaccine were inoculated either subcutaneously or intranasally into groups of children (Table 3). Blood specimens for serologic testing were collected at weekly intervals from each of the 48 participants to determine whether infection had occurred. The results indicated that one tissue culture infectious dose of the attenuated virus represents approximately one human infectious dose when the vaccine is given subcutaneously. However, the high-passage strain is inefficient in producing infection by the natural route of rubella infection. Only 2 of the 26 children inoculated intranasally developed antibodies. These same characteristics, a high efficiency of infection by the subcutaneous route of inoculation and considerably reduced efficiency by respiratory routes, have been observed with Enders B-level live attenuated measles vaccine (2, 10).

TABLE 3. SENSITIVITY OF SUSCEPTIBLE PERSONS TO INFECTION WITH ATTENUATED (HPV-77) RUBELLA VIRUS

TCD_{50} virus inoculated	Route of inoculation	
	Subcutaneous	Intranasal
10^4	N.T.*	1/3†
10^3	5/5	0/5
10^2	4/4	1/5
10^1	5/5	0/5
10^0	1/5	0/5
10^{-1}	0/3	0/3

*Not tested.
†Numerator denotes number persons developing antibody; denominator denotes number of susceptible persons inoculated.

DISCUSSION

A rubella virus strain used in the preparation of a live virus vaccine should ideally be attenuated, immunogenic and noncommunicable. The results of our studies, which involved 51 vaccinees and 49 susceptible controls, suggest that the HPV-77 strain may satisfy these criteria.

The absence of discernible clinical reaction in the vaccinated persons establishes that the high-passage virus is highly attenuated for man. An unmodified virus would be expected to produce rubella with rash in the majority of recipients inoculated subcutaneously.

The absence of communicability in these studies indicates that if communicability does exist it must be exceedingly rare. The attack rates of communicable diseases in institutionalized children are generally high, because of the conditions of intimate contact. The cottage outbreak of natural rubella in which all ten susceptible contacts became infected demonstrates the high secondary attack rate of virulent rubella virus infections under environmental circumstances similar to those present in the vaccine studies.

The quantitative differences in virus-excretion patterns between virulent and attenuated rubella infections may well be important in explaining the lack of communicability observed in the high-passage virus infections. On the basis of tissue culture assay data it appears that about 100 times more virus is shed in natural rubella.

During the cell culture passages that resulted in attenuation, the high-passage virus has lost much of its normal infectivity for the human respiratory mucosa. This feature, coupled with the quantitative reduction in the excretion of attenuated virus, suggests that the theoretical risk that infection will spread to uninoculated contacts should be minimal.

The laboratory marker tests indicated that the properties of the viruses excreted by vaccinees were the same as those of the attenuated strain. Thus, it seems likely that if a secondary case did

occur it would also be attenuated and offer little risk of further spread.

This matter of communicability concerns us primarily because of the possible hazard accompanying inadvertent infection of pregnant women. Our recent work on the pathogenesis of attenuated and natural rubella virus infections in pregnant and nonpregnant rhesus monkeys (16) suggests that while attenuated rubella virus may be capable of inducing fetal infection, it would be surprising if it did so with the efficiency of the virulent virus.

The HPV-77 strain infections do not evoke antibody responses of the same magnitude as are observed in natural rubella. However, in both attenuated and natural infections neutralizing and HI antibodies appear early and persist with little change for relatively long periods. Complement-fixing antibodies are apparently not produced in the attenuated infections.

Many studies have established that the presence of rubella-neutralizing antibodies prevents clinical and subclinical infections in man (3, 4, 6, 17, 18) and experimentally infected monkeys (13, 14, 15). Will the lower levels of antibody induced by vaccination be equally protective? Preliminary observations from a challenge study currently in progress indicate that the HPV-77 strain does confer a solid immunity.

Summary

Controlled trials of experimental live rubella virus vaccine were performed in groups of institutionalized children. The results indicated that the high passage virus strain was attenuated and immunogenic. There was no evidence of communicability; 49 susceptible intimate contacts of 51 successfully vaccinated children remained uninfected. Virologic data comparing the attenuated and natural rubella virus infections are presented and discussed.

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SECTION B. RUBELLA

PROSPECTS FOR VACCINATION AGAINST RUBELLA

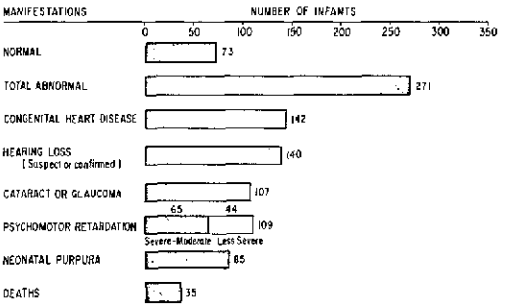
SAUL KRUGMAN

Department of Pediatrics, New York University School of Medicine, New York, N. Y., U.S.A.

DR. KRUGMAN (*presenting the paper*): On 6 May 1965, I had the privilege of being the moderator of a rubella symposium at a joint session of the American Pediatric Society and the Society for Pediatric Research. At this symposium, 18 speakers representing rubella research groups from various parts of the United States summarized their observations following the extensive epidemic of rubella that swept the country in 1964. In his introduction to the published version of the symposium, which appeared in the October 1965 issue of the *American Journal of Diseases of Children*, Dr. Thomas H. Weller wrote:

> The overwhelming personal tragedy, the extent of fetal wastage, and the expense accruing as a consequence of the recent rubella epidemic will probably not be subject to accurate assessment, for available statistics are deplorably inadequate. Yet the documentation is sufficient to indict rubella as a major medical and social problem. The evidence should stimulate an increasing effort toward the development of effective prophylactic procedures. Although some of the problems to be faced in the development of a rubella vaccine are unique and difficult, the need is all too apparent, and it is hoped that the next chapter in the rubella story will not be long in coming forth.

In the wake of the 1964 epidemic our group has observed more than 400 infants with congenital rubella infection (2). The data derived from a follow-up of 344 infants for 8 to 18 months after birth are shown in Figure 1. It is important to note that this population was selected and heavily weighted toward infants with congenital disease because the history of maternal rubella was obtained retrospectively. As Figure 1 indicates, the major manifestations of congenital rubella among 271 abnormal infants



Source: Cooper, L., and Krugman, S. *Arch Ophthal* (in press).

Fig. 1. Congenital rubella in infants—Clinical status of 344 infants grouped according to the major manifestations of congenital disease observed after a follow-up period 8 to 18 months after birth.

included congenital heart disease in 142 (52 per cent); hearing loss in 140 (52 per cent); cataract or glaucoma in 107 (40 per cent); psychomotor retardation, severe or moderately severe in 65 (24 per cent) and less severe in 44 (16 per cent); and neonatal purpura in 85 (31 per cent). The mortality rate in this group of 271 infants was 13 per cent; in infants with thrombocytopenic purpura it was 40 per cent. The mothers of 40 abnormal infants had a subclinical infection as indicated by no history of rubella with rash during pregnancy. In addition, 31 infants contracted rubella in spite of administration of gamma globulin after exposure. These observations confirm Dr. Weller's indictment of rubella as a major medical and social problem.

Today we have heard Drs. Parkman and Meyer add a new chapter to the rubella story. They have demonstrated that their live rubella vaccine (HPV-77) is indeed attenuated. Their studies have revealed that the vaccine virus has

not provoked clinical reactions such as fever, rash, or lymphadenopathy and has not spread from vaccinated persons to susceptible contacts after intimate, prolonged exposure. The absence of contact infection has been observed in spite of the presence of rubella virus in the pharynx of vaccinated subjects. Preliminary observations have indicated that the vaccine is immunogenic.

In spite of the absence of contact infection, it is important that the rubella virus be further attenuated to eliminate pharyngeal shedding, if possible. However, further attenuation may conceivably result in a decrease in antigenicity. Under these circumstances, it may be more feasible to develop a nonspreading, pharyngeal-shedding, immunogenic rubella virus than a nonpharyngeal-shedding vaccine virus that is ineffective.

The prospects for the development of a safe and effective live rubella vaccine are excellent. Rubella, like measles, is a monotypic virus; one attack of the disease is followed by permanent immunity. The presence of serum rubella-neutralizing antibody following naturally acquired infection is indicative of immunity to the disease. The data presented in Table 1 support this observation. When the serum neutralizing antibody was less than 1:4 before exposure, the attack rate of rubella infection in 49 subjects was 94 per cent. In contrast, 35 subjects with an antibody titer of 1:4 or greater were solidly protected against a comparable exposure (3). The availability of a sensitive, specific rubella hemagglutination-inhibition antibody test will be a convenient tool for following the pattern and persistence of antibody after vaccination with live attenuated rubella virus vaccine.

Additional, extensive controlled trials are needed to confirm the lack of communicability and the protective capacity of live attenuated rubella vaccine. If the encouraging preliminary observations are confirmed, a safe, effective vaccine will be available for the solution of the rubella problem.

The timetable for the development of a live attenuated rubella vaccine has been remarkably similar to past experience with measles vaccine. Measles virus was cultivated in tissue culture in 1954 (4) and was successfully attenuated four years later in 1958 (5). Rubella virus, which was cultivated in 1962 (6, 7), was successfully attenuated in 1966 (8, 9)—also four years later. The interval between the attenuation and the licensure of measles vaccine was five years, from 1958 to 1963. If this schedule is a reliable forecast, a licensed product for vaccination against rubella should be available by 1971.

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TABLE 1. EXPERIMENTAL TRANSMISSION OF RUBELLA: RELATIONSHIP OF SERUM ANTIBODY TO INFECTION*

Serum antibody before exposure	Type of exposure		No. of subjects	No. with rubella		Attack rate (%)
				Clinical†	Subclinical‡	
<1:4 (49 Subjects)	Inoculation of virus (serum)	IM	22	22	0	94
		Pharynx sprayed	10	4	3	
	Contact with rubella	Prolonged	17	11	5	
≥1:4 (35 Subjects)	Inoculation of virus (serum)	IM	26	0	0	0
	Contact with rubella	Prolonged	9	0	0	

*Modified from Green, *et al.*, *Amer J Dis Child* 110:348, 1965.
†Rash and antibody rise with or without virus isolation.
‡No rash but antibody rise with or without virus isolation.

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SECTION B. RUBELLA

DISCUSSION

CHAIRMAN DAVIS: We shall now have the discussion of these papers. I call upon Dr. Joseph Stokes, Jr., the Henry Phipps Institute, University of Pennsylvania, Philadelphia, Pennsylvania.

DR. STOKES: My report is entitled "Clinical and Laboratory Tests of Merck Strain Live Attenuated Rubella Virus Vaccine," co-authored with Drs. R. E. Weibel, E. B. Buynak, and M. R. Hilleman.* Rubella stands high on the priority list of infections for which an effective vaccine is needed, because of its marked teratogenic potential in pregnancy, especially during the first trimester. For children also such vaccine might be of considerable use, reducing morbidity and such complications as neuritis, rubeola-like encephalitis, arthritis, and thrombocytopenia. To be acceptable for routine use, the vaccine should induce lasting immunity. The greatest chance for success, therefore, resides with a live virus preparation—but it must be one that produces a noncontagious infection, to prevent chance spread to pregnant women.

During the past three years, Dr. Robert Weibel and I have been collaborating with Drs. Maurice Hilleman and Eugene Buynak in tests of a live rubella virus vaccine prepared using virus attenuated and propagated in cell cultures of duck embryo.† The vaccine was developed by the latter workers at the Merck Institute for Therapeutic Research. The studies by our group have been aimed at developing a vaccine that would cause little or no clinical illness, would induce a substantial amount of rubella antibody and durable protection, and would cause a noncontagious infection in vaccinated persons. To provide an extra safeguard, we deemed it advisable to develop a virus for vaccine that would not be excreted from vaccinated persons.

With appropriate consent, we have carried out tests in children using the Merck strain of rubella virus at progressive levels of attenuation in duck embryo cell culture, which we have called levels A, B, and C. The vaccines were given subcutaneously in 0.5 or 1.0 ml amounts, and blood and throat specimens were taken at appropriate intervals for laboratory tests.

The studies were conducted in four institutions for retarded children under conditions of contact similar to those of a crowded family.

Table 1 shows the findings in children given the Merck strain rubella vaccine at level A. The virus was only partially attenuated, as was evidenced by the appearance of mild clinical rubella in the vaccinated children. All the children developed rubella antibody and all excreted the virus, which was contagious to essentially all susceptible contacts.

Vaccine at level B was far more attenuated than level A vaccine. There was no detectable illness whatever, and only five out of seven children excreted virus (see Table 2). All the children developed rubella antibody at a lower titer than the level A vaccinees. The infection was not contagious to exposed susceptibles, who failed to show serologic responses. This level B vaccine appeared to be similar to that of Meyer and Parkman.‡

* From the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, and Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania.

† Hilleman, M. R. "Advances in Control of Viral Infections by Nonspecific Measures and by Vaccines with Special Reference to Live Mumps and Rubella Virus Vaccines." Presented at the 67th Annual Meeting of the American Therapeutic Society, Chicago, Illinois, 25 June 1966. *Clin Pharmacol Ther* **7**:752–762, 1966.

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TABLE 1. VIRUS SECRETION AND SEROLOGY IN SUSCEPTIBLE CHILDREN GIVEN MERCK STRAIN LIVE ATTENUATED RUBELLA VIRUS VACCINE AT ATTENUATION LEVEL A

Patient	Age (years)	Virus in throat secretions on day											Rubella serum neut. titer		
													Day		
		0	6	8	10	12	14	16	18	20	22	24	0	30	60
Vaccinated															
61–1	3	0	0	0	+	+	+	+	+	+	0	0	0*	32	64
61–2	4	0	0	+	+	+	+	+	+	+	+	0	0	32	32
61–3	3	0	0	+	0	+	0	0	0	0	0	0	0	32	64
61–4	9	0	0	0	+	+	+	+	0	0	0	0	0	16	32
61–5	8	0	0	0	0	0	+	+	+	+	+	0	0	64 or >	64 or >
61–6	6	0	0	+	+	+	+	+	+	+	0	0	0	16	32
61–7	1	0	0	0	0	0	+	+	+	+	0	0	0	16	32
Contact controls 61–8 through 14 (7 children)	2 to 8	6/7 positive, days 27–36											0	0	16–32

Note: Test started 25 January 1965, Lot 150.
*0 = <1:1

TABLE 2. VIRUS SECRETION AND SEROLOGY IN SUSCEPTIBLE CHILDREN GIVEN MERCK STRAIN LIVE ATTENUATED RUBELLA VIRUS VACCINE AT ATTENUATION LEVEL B

Patient	Age (years)	Virus in throat secretions on day											Rubella serum neut. titer		
													Day		
		0	6	8	10	12	14	16	19	21	23	25	0	28	59
Vaccinated															
68A–18	2	0	0	+	+	+	+	0	0	0	0	0	0*	2	16
68A–17	1	0	0	0	0	+	+	+	0	0	0	0	0	8	4
68A–21	3	0	0	0	0	0	+	0	0	0	0	0	0	8	4
68A–20	3	0	0	0	0	0	0	0	+	0	0	0	0	4	16
68A–19	2	0	0	0	0	+	+	0	0	0	0	0	N.D.	N.D.	N.D.
68A–22	6	0	0	0	0	0	0	0	0	0	0	0	0	2	8
68A–15	9 mos.	0	0	0	0	0	0	0	0	0	0	0	0	8	8
Contact controls 68A–1, 2, 4–7, 16, 24 (8 children)	1 to 3	Not tested											0	N.D.	0

Note: Test started on 14 January 1966, Lot 214.
*0 = <1:1.

TABLE 3. VIRUS SECRETION AND SEROLOGY IN SUSCEPTIBLE CHILDREN GIVEN MERCK STRAIN LIVE ATTENUATED RUBELLA VIRUS VACCINE AT ATTENUATION LEVEL C, TEST 1

Patient	Age (years)	Virus in throat secretions on day											Rubella serum neut. titer		
													Day		
		0	6	8	10	12	14	16	18	20	22	24	0	28	63
Vaccinated															
78A–1	5	0	0	0	0	0	0	0	0	0	0	0	0*	8	4
78A–3	8	0	0	0	0	0	+	0	0	0	0	0	0	32	8
78A–5	6	0	0	0	0	0	0	0	0	0	0	0	0	4	2
78A–7	9	0	0	0	0	0	0	0	0	0	0	0	0	4	4
78A–9	3	0	0	0	0	0	0	0	0	0	0	0	0	2	2
78A–11	3	0	0	0	0	0	0	0	0	0	0	0	0	2	8
78A–13	3	0	0	0	0	0	0	0	0	0	0	0	0	8	4
Contact controls 78A–2, 4, 6, 8, 10, 12, 14 (7 children)	1 to 9	Tested day 20 only; all negative											0	0	0

Note: Test started on 30 June 1966, Lot 215.
*0 = <1:1.

TABLE 4. VIRUS SECRETION AND SEROLOGY IN SUSCEPTIBLE CHILDREN GIVEN MERCK STRAIN LIVE ATTENUATED RUBELLA VIRUS VACCINE AT ATTENUATION LEVEL C, TEST 2

Patient	Age (years)	Virus in throat secretions on day											Rubella serum neut. titer		
													Day		
		0	6	8	10	12	14	16	18	20	22	24	0	28	60
Vaccinated															
78B–15	2	0	0	0	0	0	0	0	0	0	0	0	0*	16	8
78B–19	2	0	0	0	0	0	0	0	0	0	0	0	0	8	4
78B–20	1	0	0	0	0	0	0	0	0	0	0	0	0	8	16
78B–22	1	0	0	0	0	0	0	0	0	0	0	0	0	16	8
78B–24	2	0	0	0	0	0	0	0	0	0	0	0	0	16	8
78B–25	4	0	0	0	0	0	0	0	0	0	0	0	0	16	8
78B–26	5	0	0	0	0	0	0	0	0	0	0	0	0	8	16
Contact controls 78B–16–18, 21, 23, 27 (6 children)	1 to 5	Tested day 20 only; all negative											0	0	0

Note: Test started on 19 August 1966, Lot 215.
*0 = <1:1.

Vaccine at attenuation level C was assayed in two tests. In the first, the results of which are shown in Table 3, only one child out of seven excreted virus and on only one day. There was no virus spread to susceptible contacts and all the vaccine recipients developed rubella antibody. In a repeat test, the findings were similar except that none of the children excreted virus at any time (see Table 4). These findings showed that rubella virus may be progressively attenuated to the point of causing no clinical effect and of being noncontagious even though it regularly induces rubella neutralizing antibody. Vaccine at attenuation level C caused only minimal excretion on one person on one day in one of two clinical trials. Tests are in progress of a further-attenuated vaccine—at level D, which we hope will cause no virus excretion whatever. Vaccine of such nonexcreting quality seems desirable in providing an extra margin of safety in contacts between the vaccinated person and pregnant women.

CHAIRMAN DAVIS: The next discussant is Dr. Stanley A. Plotkin,* Wistar Institute, Philadelphia, Pennsylvania.

DR. PLOTKIN: The paper that serves as the basis for this discussion † has been co-authored with Drs. John D. Farquhar, Michael Katz, and Theodore H. Ingalls.‡

Because rubella virus grows in many cell systems, choices other than monkey kidney tissue culture may be considered as substrates for a live-virus vaccine. We have recently performed controlled clinical trials of two rubella virus variants, one grown in primary rabbit kidney cells and the second grown in WI-38 human diploid lung cells.

The first strain is the Cendehill strain isolated by Dr. Abel Prinzie of the Rega Institute in Louvain from the urine of a 10-year-old child with typical rubella. The virus was isolated in African green monkey kidney tissue culture and passaged four times in that cell type before a passage series was begun in primary rabbit kidney cells. Dr. Prinzie made the strain available to us in its twenty-first rabbit kidney passage. Its properties may be summarized as follows: it grows to a similar titer in African green monkey kidney and primary rabbit kidney; plaques are produced in RK_{13}§ and in BHK_{21}‖ cells; it produces a tenfold greater amount of interferon than low-passage virus; and a cytopathic effect is produced in primary rabbit kidney tissue culture.

This twenty-first passage was tested in normal children aged one to five years in two separate institutions for child care in Europe. The results, as reported to us by Dr. Prinzie, are shown in Table 1. All 18 seronegative children inoculated with the virus responded with antibodies, whereas 17 seronegative children serving as contacts failed to develop antibodies when tested eight weeks later. These studies were encouraging, but gave little information on virus excretion.

Our own trial was performed in a ward of a home for retarded children. Seronegative children, about 75 per cent of whom were ambulatory, were selected. Seven children received subcutaneously 80 TCD_{50} of the twenty-first passage of Cendehill strain and were kept together with seven contact children for six weeks. A program ensuring intimate daily physical contact among the children was carried out during this period.

Nasal and pharyngeal swabs were obtained; the results of attempts to isolate virus from them in African green monkey kidney are shown in

* Joseph P. Kennedy, Jr. Foundation scholar.

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‡ From the Wistar Institute, the Children's Hospital of Philadelphia, and the Departments of Pediatrics and Public Health and Preventive Medicine of the University of Pennsylvania.

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TABLE 1. CENDEHILL STRAIN—21ST RABBIT KIDNEY PASSAGE: EUROPEAN TRIALS

Institution	Antibody responses			
	Vaccinees		Contacts	
	Ratio pos.	Median titer	Ratio pos.	Median titer
A	13/13	32	0/10	<4
B	5/5	32	0/7	<4

TABLE 2. CENDEHILL RABBIT KIDNEY VIRUS—21ST PASSAGE: SEROLOGIC RESPONSE

Vaccinees	Pre	Post	Contacts	Pre	Post
2	<4	16	4	<4	<4
24	<4	8	22	<4	<4
26	<4	16	23	<4	<4
27	<4	16	29	<4	<4
31	<4	4	30	<4	<4
32	<4	16	33	<4	<4
34	<4	8	35	<4	<4

Table 2. Rubella virus was isolated from the pharynx of five of the seven vaccinees between the eleventh and the sixteenth days after inoculation. The duration of virus excretion by the positive children varied from one to four days. Titration of some of the positive swabs revealed virus in quantities ranging from 2.5 to 15 TCD_{50}. To exclude the possibility that de-adaptation to monkey kidney was the reason for the failure to isolate virus from two children, all swabs obtained 13 days after inoculation were tested in rabbit kidney. No additional isolations were made through the use of rabbit kidney.

Heparinized blood was obtained on the eleventh day after inoculation, in a search for viremia; specimens from six vaccinees were negative. Interference was shown on first passage of blood from one of the seven vaccinees, but was not demonstrated on a second passage. We are now attempting virus isolation from the original specimen again.

None of the seven contacts showed any virus in the swabs.

Close and repeated clinical examination of the children disclosed a definite rubelliform rash in one vaccinee and equivocal rashes in two others. Postauricular or suboccipital lymphadenopathy was seen in five vaccinees but not in the control subjects. A slight fever was recorded in two vaccinees.

The serologic responses of all 14 children are presented in Table 3. The seven vaccinees developed neutralizing titers ranging from 4 to 16, with a median of 16, while none of the contacts showed any antibody response. Thus no spread had occurred despite the intimate exposure of the contact children.

The second rubella variant strain tested was

TABLE 3. CLINICAL TRIALS OF STRAIN RA 27-3

WI-38 passage	Pharyngeal virus median duration (days)	Rash	Antibody response	Spread to contacts
4	12	10/11	11/11	4/12
8	7	5/13	12/13	1/9
17	0	0/2	2/2	N.D.

N.D. = Not done.

the RA 27-3 strain, isolated by us from a fetus surgically aborted because of maternal rubella. Rubella virus had been identified in the mother's throat before abortion. The strain was isolated from an explant of fetal tissue* and passaged exclusively in WI-38 human diploid lung cells, first at 35°C, then at 33°C, and finally at 30°C. Two terminal dilutions were included in the passage series. Clinical trials were performed at different passage levels, the results are indicated in Table 4. Progressively less rash, less pharyngeal excretion, and less spread occurred as the passage level increased. At the seventeenth passage no virus could be demonstrated in the throats of two vaccinees, but contagiousness was not studied in this experiment.

With this evidence for progressive attenuation, an experiment was performed at the same institution and under exactly the same conditions as those used for the rabbit kidney virus. Again 14 seronegative children were included, seven of whom received the twenty-first WI-38 passage of the RA 27-3 strain. One thousand TCD_{50} were administered subcutaneously. Though our labo-

* Plotkin, S. A., Cornfeld, D., and Ingalls, T. "Immunization against Rubella with Living Virus: Studies in Children of a Strain Obtained from an Aborted Fetus." *Amer J Dis Child* **110**:381–389, 1965.

TABLE 4. RA 27-3 IN WI-38, PASSAGE 21

Vaccinees	Pre	Post	Contacts	Pre	Post
30	<4	64	4	<4	<4
33	<4	8	22	<4	<4
35	<4	16	23	<4	<4
43	<4	32	29	<4	<4
45	<4	32	49	<4	<4
48	<4	64	52	<4	<4
51	<4	8	53	<4	<4

TABLE 5. TRIAL 5—CENDEHILL RK P 21

		Days post inoculation													
		0	7	9	10	11	13	14	16	18	21	24	28	31	34	42
Vaccinees	2V	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0
	24V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	26V	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0
	27V	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0
	31V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	32V	0	0	0	0	0	+	+	+	0	0	0	0	0	0	0
	34V	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0
Contacts	4C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	22C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	23C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	29C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	30C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	33C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	35C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

ratory studies are as yet incomplete, they seem sufficiently interesting to report here today.

Table 5 shows the information on virus excretion now available on the basis of one blind passage. During the first three weeks after inoculation, no virus has been isolated from the nose or throat of any child. Heparinized blood collected at 11 days also failed to show virus. Nevertheless, one vaccinee developed a typical rubella rash with fever and three had mild lymphadenopathy. The contacts were unaffected.

The antibody data are shown in Table 6. All the vaccinees developed antibodies ranging from 1:8 to 1:64, while in contrast none of the controls became seropositive.

These studies are still rudimentary with respect to numbers of subjects. Future work may modify our conclusions. Nevertheless, it appears

TABLE 6. TRIAL 6—RA 27–3 IN WI–38, PASSAGE 21

		Days post inoculation							
		0	7	9	11	13	15	17	21
Vaccinees	30V	0	0	0	0	0	0	0	0
	33V	0	0	0	0	0	0	0	0
	35V	0	0	0	0	0	0	0	0
	43V	0	0	0	0	0	0	0	0
	45V	0	0	0	0	0	0	0	0
	48V	0	0	0	0	0	0	0	0
	51V	0	0	0	0	0	0	0	0
Contacts	4C	0	0	0	0	0	0	0	0
	22C	0	0	0	0	0	0	0	0
	23C	0	0	0	0	0	0	0	0
	29C	0	0	0	0	0	0	0	0
	49C	0	0	0	0	0	0	0	0
	52C	0	0	0	0	0	0	0	0
	53C	0	0	0	0	0	0	0	0

that there are at least two strains not grown in monkey kidney cells that could qualify as vaccine strains. The rabbit kidney strain has been tested successfully in 25 vaccinees and 24 contacts. In the case of the strain grown in WI-38, preliminary evidence has been presented for low or absent nasopharyngeal virus excretion in 7 vaccinees and 7 contacts.

ACKNOWLEDGMENT

The authors are pleased to thank Dr. Benjamin P. Clark, Superintendent of the Hamburg State School, for his excellent cooperation.

CHAIRMAN DAVIS: Thank you, Dr. Plotkin. The next discussant will be Col. Edward L. Buescher, Walter Reed Army Institute of Research, Washington, D. C.

COL. BUESCHER: Once again I should like to take this opportunity to generalize, rather than attempt to review in detail the observations on rubella presented until now. It is rather clear, I believe, that efforts to control rubella stem entirely from the need to minimize the effects of infection on the developing human fetus. Classical rubella is a mild febrile exanthematous disease of short duration, with practically no sequelae. This makes it, as a disease, hardly worth the effort involved in developing attenuated or nonreplicating immunizing preparations. On the other hand, the impact of wild virus on the developing fetus more than justifies investigations such as have been described today.

This effect on the fetus, however, must be borne in mind by all of us who would encourage the development and evaluation of replicating vaccines, for in the last analysis the ultimate test of safety for any preparation lies in its inability to injure embryonic tissue. Thus, control of the age and circumstances under which susceptibles are infected could conceivably minimize the potential teratogenic effects. Yet such administrative restriction of vaccines is hardly feasible as a continuing requirement for immunization. It therefore goes without saying that considerable thought and attention should be given to a plan whereby the potential teratogenic effects of candidate rubella vaccine viruses can be evaluated. Though this will not be easy to do, the observations of Dr. Parkman on the distribution of HPV-77 in experimentally infected monkeys is a step in the right direction. Perhaps the approach would become clearer if we knew more about the natural process of human fetal infection or of the nature of *in vitro* infection in human embryonic cells.

This line of investigation now assumes increasing significance in the light of the encouraging results at attenuation described here today. Again let me say that unless there is complete assurance of the safety of vaccine viruses for the fetus, the introduction into general practice of vaccines against rubella is not ethically justified. Before we can have this assurance we must have more information on the natural history and pathogenesis of rubella. I have no doubt that eventually, though perhaps not in accordance with Dr. Krugman's schedule, success will crown these endeavors.

CHAIRMAN DAVIS: Thank you, Col. Buescher, for bringing these thoughts to our attention. The next discussant will be Dr. Thomas H. Weller of the Department of Tropical Public Health, Harvard University, Boston, Massachusetts.

DR. WELLER: Information on rubella has accumulated rapidly over the past four years. At least four attenuated rubella virus vaccines have now been mentioned publicly as undergoing preliminary testing; undoubtedly other products are at the same stage of development.

It is gratifying that Dr. Parkman, a co-discoverer of rubella virus, has continued to pioneer and is responsible for one promising product. Successes to date have led Dr. Krugman—whose intimate knowledge of rubella stems from years of work—to outline a rubella-vaccine timetable analogous to that for measles vaccine.

In this atmosphere of progress and of optimism it seems not particularly appropriate to note in a low key that our ignorance regarding rubella virus is considerable. Some might question whether or not rubella virus has been properly characterized and classified. We assume that it is monotypic and antigenically stable, and indeed there is evidence to support this view, but definitive conclusions must await the study of isolates that are widely separated in time.

Protocols for the safety-testing of vaccines are based on what is known, and provide no refuge from the unknown. In all probability the parenteral administration of a new product designed

to vaccinate against teratogenesis will unmask now-unrecognized hazards that for solution will require a revision of existing safety standards.

On the first day of this Conference Dr. Zhdanov aptly outlined three evolutionary stages of assurance with respect to the development of a viral vaccine. He described an initial stage of enthusiastic assurance based on ignorance, a subsequent stage of disillusionment, and finally the growing assurance that comes as knowledge replaces assumption. The passage of time will define the characteristics of our present stage of assurance with respect to rubella vaccines.

CHAIRMAN DAVIS: Thank you, Dr. Weller. Dr. Frederick C. Robbins, Department of Pediatrics, Western Reserve University, Cleveland, Ohio, will continue the discussion.

DR. ROBBINS: Our group in Cleveland, Ohio, headed by Dr. Martha L. Lepow and including Drs. Donald D. Hostelter and Jorge Veronelli, has conducted a small trial with the HPV-77 attenuated rubella virus vaccine of Parkman and Meyer. Fourteen susceptible young children in an institution were vaccinated, and 11 received a placebo. The vaccinees and controls were allowed to associate freely. The data obtained are superimposable upon those of Parkman and Meyer in regard to development of antibody, presence of virus in the nasopharynx, and failure of the virus to be transmitted to the control contacts. In addition, the vaccinees were tested for the presence of virus in feces and urine; out of 73 fecal specimens collected between the tenth and the nineteenth day after vaccination, rubella virus was isolated from only one. This specimen was obtained on the twelfth day from a two-year old female who had positive nasopharyngeal cultures for a period including the twelfth day. No virus was isolated from any of 27 urine specimens collected from the same children during the same period of time. Thus it would appear that the feces and urine of children who have received the HPV-77 strains are rarely if ever infectious.

As for the possibility of using it on a large scale, the present HPV-77 vaccine seems to fall somewhat short of what might be considered ideal.

First, even though we have good evidence that the virus in the nasopharynx of vaccinated children is not disseminated to other children, one hesitates to assume, without more evidence, that a pregnant woman would be safe if exposed to a vaccinee. A vaccine that was not excreted at all would obviously be much more desirable. Proof that the vaccine virus is not teratogenic for man would of course be reassuring. This is clearly a very difficult question, but is one that we should probably try to answer if the opportunity to do so can be found.

Secondly, the antibody titers resulting from the HPV-77 infection are somewhat lower than those that follow natural infection. Whether a shorter period of immunity after vaccination is therefore to be expected cannot necessarily be inferred, but it is a matter of some concern.

Finally, the cell in which the vaccine is produced presents a problem when it is considered for use on a large scale by the parenteral route, particularly in children. The adaptation of the rubella virus to avian cells is an important advance. One would feel much more secure with material produced in chick cells, a system with which there is now much accumulated experience.

Although the state of the art is not such that we can deliberately produce a viral strain to meet particular specifications, it has been shown that rubella virus can be propagated in a variety of cell types, does become less virulent upon passage in tissue cultures, and loses much or all of its capacity to be transmitted from one person to another. These are great advances and lead to the hope that, although many difficult problems remain, the ideal or a more nearly ideal vaccine strain will be found that is safe, avirulent, nontransmissible, nonteratogenic, and protective at least long enough to span the usual childbearing period.

CHAIRMAN DAVIS: Thank you very much, Dr. Robbins. We have some time left for free discussion, and I should like to call on Dr. Cockburn from the World Health Organization.

DR. COCKBURN: I simply wanted to report that a collaborative study under the coordination of Dr. Melnick, who is Director of the WHO International Reference Center for Enteroviruses, Houston, Texas, is at present being made by WHO. The aim was first of all to standardize methods of examination in different laboratories. The tissue cultures being used are green monkey kidney for the indirect neutralization test and

SARC cells for the direct test. The WHO provisional standard gamma globulin has been included in these studies and also a whole serum that will be designated as a WHO reference serum if found satisfactory. This has now been done, and the next step is to test from each of the test areas 250 sera from women five to thirty years of age living in urban settings. The studies are being made in laboratories in London (Colindale), Copenhagen, Tokyo, Lyons, Prague, Melbourne, Ottawa, and Houston. Additional sera are being obtained from Trinidad, Jamaica, Argentina, and Chile.

CHAIRMAN DAVIS: Are there questions or remarks by the participants?

DR. CABASSO: I should like to add our experience to that presented by others on attenuated rubella virus. We used the virus isolated by Dr. Parkman, the M-33 strain, which we received as the 19th passage in green monkey kidney. We continued passage of this virus in monkey kidney first at 35° and then at 32°C. Small trials in children were carried out, with all appropriate precautions and controls, with the 25th- and 50th-passage levels. The 25th level resulted in mild clinical rubella with abundant transfer of the virus to contact children. In contrast, the 50th-passage level gave results superimposable on those obtained by Drs. Parkman and Meyer.

I should also like to add a word of caution about the markers suggested by Dr. Parkman as a possible indication of attenuation in terms of nontransmissibility. We have preliminary information suggesting that our 25th-passage virus, which was abundantly transmitted among the control children, possesses more nearly the markers of the attenuated or 77th-passage than of the 3rd- or 4th-passage virus.

DR. KATZ: Although I share the optimism of all the gentlemen who have discussed rubella vaccines, I have the fear that—as happens with so many other programs of this sort when an exciting possibility like a vaccine comes along—many questions lurking in the background are never resolved. Despite the data presented by Dr. McDonald this morning, we are still lacking the answer to a question that will arise repeatedly until the vaccine situation is further developed. That is, Has gamma globulin a role in protecting the pregnant woman exposed to rubella? I hope that those familiar with the techniques that have been described—the most recent one being a hemagglutination-inhibition test easily adaptable to many laboratories—will now provide definitive answers by making possible studies in areas where there are women susceptible to rubella. I should like to ask Dr. Sever whether he can answer this from his prospective study, which does include data to document rubella infection not just as a rash disease but as serologically proven.

DR. SEVER: The data on the use of gamma globulin in the Collaborative Perinatal Study is summarized in Dr. McDonald's Table 2 (see p. 373). This prospective study, involving over 650 women who were exposed during the first trimester of pregnancy, included 145 who received gamma globulin. A decreased rate of clinical and inapparent infections and a decreased rate of abnormal pregnancy outcomes was found for this group. The information from the one- and two-year follow-up is being analyzed, and the trend of a decrease in the frequency of abnormalities persists.

I now have a question for Dr. Cabasso. The markers of your 25th-passage virus, which was transmissible, were more like HPV-77 than very low-passage virus. It would be helpful to know what markers were found with the 50th-passage, attenuated virus.

DR. CABASSO: As I have indicated, these are preliminary observations, which I want to confirm. However, the markers of the 50th, more attenuated passage were quite similar to those of the 25th.

DR. EDSALL: I think that a point mentioned very briefly by Dr. McDonald deserves to be reemphasized: the possibility that there may be greater differences in gamma globulins than we are at present accustomed to testing for. The two that he spoke of that may have been over-age when used remind us that recently it has been shown that some gamma globulin preparations deteriorate with time; that although their antibody titers may appear to be normal, the antibody molecules are degraded and may be ex-

creted more rapidly than others. It is important, in view of the comments made by Drs. Katz and McDonald, to bear in mind the critical necessity of finding ways to insure that the gamma globulins used in rubella studies are comparable in their characteristics.

CHAIRMAN DAVIS: Let us turn now to mumps. Mumps is one of the earliest-recognized infectious diseases. Indeed, it is referred to in the Hippocratic writings, and is unlike most of the infections we have been discussing here in this Conference.

SECTION C. MUMPS

EPIDEMIOLOGY AND CLINICAL IMPORTANCE OF MUMPS INFECTION IN MAN

FREDERICK C. ROBBINS

School of Medicine, Western Reserve University, Cleveland, Ohio, U.S.A.

DR. ROBBINS (*presenting the paper*): My purpose is to review briefly certain aspects of the epidemiology and the clinical features of mumps as a background for consideration of the need for immunization, what would be required of an immunizing agent, and how it might be employed if available.

Epidemiology (8–10, 20)

Mumps occurs throughout the world except in occasional isolated communities. Although cases occur at any time during the year, they are more frequent during the late winter and early spring and the incidence reaches epidemic proportions in cycles of seven to eight years. Localized epidemics occur among groups of people living closely together in places such as schools, institutions, and military establishments. During World War I mumps was an important military problem, particularly among recruits. Men from rural areas were more susceptible than those from the cities; under the conditions of the military camps they often acquired mumps and other common communicable diseases. In World War II the problem was much less serious, presumably because of the greater urbanization of the country that had occurred between the two conflicts.

Mumps is generally considered to be spread by the respiratory route. Close contact is probably important; large droplets and occasionally fomites may play a role. The virus can be recovered from the saliva of an infected person for about six days before the onset of the disease (11) and up to nine days afterward (11, 20). It has also been recovered with some regularity from the urine, where it may be present for as long as 14 days (31). The epidemiologic significance of viruria is not known. The attack rates in epidemics vary greatly, but in a virgin population it can be as high as 88 per cent. Among susceptibles exposed in the family, the rates depend to a significant degree on age. In a recent study by Meyer, Stifler, and Joseph (21), the rate among family contacts with a negative history was 17 per cent for children under one, but 64 per cent for two-to-four-year-olds. The rate for parents without neutralizing antibody was approximately 16 per cent and mothers were affected twice as often as fathers. The young infant is probably less susceptible because of antibody acquired from the mother, which, like other such passively acquired antibodies, persists for the first few months of life.

Mumps is most common in the young child. In urban societies most children acquire infection during the school years and the maximum level of immunity is achieved by the age of about 15. A survey of the frequency of mumps complement-fixing antibody in the sera of children in Stockholm, Sweden (6), revealed that 15 per cent of those between one and three and 70 per cent of those from nine to twelve possessed antibodies. Approximately 60 per cent of adults will give a history of having had mumps. However, when specific serologic or skin tests are applied, about 50 per cent of those with negative histories are found to be immune. Thus, in urban societies approximately 80 per cent of adults are immune to mumps even though in a significant number of them the infection was inapparent or

unrecognized. This figure can be much lower in rural communities or in situations where contacts are limited.

The immunity following mumps infection is long-lasting, but second attacks apparently do occur occasionally. Parenthetically, it might be mentioned that the syndrome of recurrent parotitis is rarely, if ever, due to the mumps virus (27). Man is the only natural host for the virus, and thus, as with measles or rubella, it will disappear from an isolated population and the disease will not reappear until it is introduced from the outside. So far as is known, recurrences do not occur, nor are there chronic virus carriers. The subclinical case is probably an important mechanism for maintaining the virus in the community (20).

An important consideration related to the possibility of vaccination is that neither epidemiologic nor direct laboratory observations have indicated significant antigenic differences among mumps-virus strains.

The Clinical Disease

It seems hardly necessary to describe in any detail the characteristic clinical features of mumps. However, certain points may be made.

Mumps is a disease with a relatively long incubation period—18 to 21 days. Inapparent infections are common and may account for as many as 25 to 40 per cent of the total. Although the pathogenesis is not well understoood, it has been shown that viremia occurs (15, 26); thus mumps infection is systemic, not limited to the salivary glands alone. Indeed, symptomatic infection not infrequently occurs without evident parotitis. A variety of other organs, such as the central nervous system, testis, pancreas, and others may be affected.

The disease in children is less severe than in adults, and children experience fewer complications. Nonetheless, the average patient with mumps is disabled for a week or more. If he has salivary-gland enlargement, he should be isolated as long as this is evident, seldom less than one week and often longer. I could find no evidence indicating that malnutrition might affect the outcome in mumps as it does in measles.

Death rarely results directly from mumps-virus infection. Between 40 and 50 deaths are ascribed to mumps each year in the United States (23). However, no specific information on these cases is available.

Neurological Complications

Neurological manifestations are the most common complications of mumps (1, 10, 22, 34). Estimates of their frequency vary widely from less than 1 per cent of cases of mumps-virus infection to over 50 per cent. There is some suggestion that adults are more susceptible to these complications than children and that males are affected approximately five times more often than females. The commonest form of central nervous system involvement is simple aseptic meningitis. However, there may be evidence of encephalitis with or without convulsions. A spinal-fluid pleocytosis may be found in the absence of any clinical evidence of central nervous system disease. Neurological symptoms may precede, coincide with, or follow other symptoms of mumps-virus infection or may be the only manifestations of it. Almost without exception, the cases that present themselves as aseptic meningitis or mild to moderate meningo-encephalitis have a benign course and recovery takes place with no evident residual manifestations. However, infrequently muscle weakness or paralysis, indistinguishable from that of poliomyelitis, occurs (16). Behavior disturbances and other less-well-defined sequelae have also been described (25).

In contrast to the rather benign disease just described is an encephalomyelitis that clinically and pathologically resembles that seen in association with rubeola. It is estimated to occur in about 1 in every 6,000 cases of mumps and has accounted for a small number of deaths (2, 34).

Other neurological complications associated with mumps include occasional cases of transverse myelitis and peripheral neuritides usually involving the cranial nerves. These conditions are transient, with full recovery the rule.

Deafness. The deafness that follows mumps is not strictly a neurological complication, since the pathology appears to be that of an endolymphatic labyrinthitis with destruction of the organ of Corti and supporting structures (17). Deafness is not common; it probably occurs no oftener than once in every 300 to 400 cases of mumps and is seldom bilateral when it does

occur. However, the loss of hearing due to mumps is almost always severe and permanent.

Orchitis. Certainly one of the most unpleasant and dreaded manifestations of mumps is orchitis. This occurs almost exclusively in postpuberal males, although occasional cases have been reported in prepubescent children. Great differences in the incidence of orchitis in various epidemics have been reported—from as low as 12 per cent to as high as 66 per cent. However, on the average approximately one in five mature males who become infected with the mumps virus will develop orchitis. About a third of these cases will be bilateral. The patient is often severely ill and acutely uncomfortable, and may require hospitalization for a week or more. It has been popularly assumed that mumps orchitis is often the cause of sterility. However, this has proved to be a needless fear; although testicular atrophy is recorded following mumps orchitis in about 30 per cent of the cases, it is rarely bilateral and would appear to be an insignificant cause of infertility (33). Oophoritis does occur, but less often than orchitis, and does not appear to have any serious consequences.

Miscellaneous Complications

Reports can be found in the literature describing the involvement of almost every organ in the body during the course of mumps.

Pancreatitis is one of the more frequently mentioned complications. Overt disease is not particularly common, but it probably complicates about 5 per cent of infections with mumps virus. It has been proposed by many authors that mumps may be the cause of some cases of diabetes mellitus, but the evidence is no more than anecdotal.

Thyroiditis can also be caused by mumps virus, and epidemiologic evidence in Israel has suggested that subacute thyroiditis may follow the acute viral inflammation of the gland (3). A few additional cases of subacute thyroiditis following mumps have been recorded (4, 19), but the role of the virus in the more chronic process is still to be substantiated.

Nephritis, prostatitis, epididymitis, uveitis, hepatitis, arthritis, myocarditis, and thrombocytopenia have all been attributed to the mumps virus, but none are sufficiently common to constitute major problems. The nephritis can apparently be severe, and indeed a few fatalities have been described (13). However, no evidence for chronic renal involvement has been obtained in spite of the frequent viruria that has been demonstrated.

Mumps in Pregnancy

The effect on the fetus of mumps occurring during pregnancy is a matter about which the data are not entirely conclusive (12, 14, 18). When mumps occurs early in gestation it may increase the risk of abortion or miscarriage, but the effect does not appear to be striking and may well not be specific. In spite of many individual reports in the literature, there is no convincing evidence that the mumps virus causes congenital anomalies in man. Holowach and associates (12) present circumstantial evidence of a single instance of fetal infection with mumps virus resulting in chorioretinitis. It is interesting that at 18 months of age this patient was found positive in a mumps skin test but lacked serum complement-fixing antibodies. However, there is no direct proof that fetal infection did occur or that the chorioretinitis was caused by mumps-virus infection.

A recent observation that has aroused much interest is an apparent correlation in infants between reactivity to mumps skin test antigen and subendocardial fibroelastosis—a rare but almost universally fatal congenital cardiac abnormality (24, 28–30, 32). Controls have shown a much lower frequency of positive reaction. A history of maternal mumps or exposure to mumps has been elicited in some—but a minority—of the cases. A puzzling feature has been that the infants with positive skin tests have not had antibodies to mumps virus in their sera. It has been hypothesized that subendocardial fibroelastosis is caused by intrauterine infection of the fetus with mumps virus and that the dissociation between skin reactivity and development of serum antibodies is to be explained by a state of partial immunologic tolerance. The hypothesis seems reasonable, but unfortunately one of the most recent studies on this subject has failed to confirm the basic observation (7). It would seem necessary to reserve judgment on the causative role of intrauterine mumps-virus infection in subendocardial fibroelastosis.

Summary

Mumps is one of the common acute communicable diseases of man. In its epidemiology it resembles measles, differing in that inapparent infection is more frequent, particularly in children under three years of age, and the incubation period is longer by about one week. Since man is the only natural host, immunity is solid and long lasting and there are no true carriers. The persistence of the virus in the community depends on a sufficient density of population for dissemination to occur and on the continual introduction of new susceptibles. In urbanized societies the infection occurs chiefly in young schoolchildren and is disseminated by them. By adulthood 80 per cent or more are immune. In rural societies immunity in adults is lower and a relatively high rate of mumps can be expected when, for whatever reason, they are brought into close contact with city dwellers.

The clinical disease in children is relatively benign, although it does account for considerable loss of time from school. Invasion of the central nervous system occurs with considerable frequency. Recognizable sequelae are rare, although no satisfactory studies have been conducted to determine whether or not less obvious effects on cerebral function occur. One could reasonably adopt the position that anything that causes inflammation of the central nervous system is undesirable. Hearing loss is not common; when it does occur it is usually severe and permanent.

Thus it would seem that, although the point might be disputed, a substantial case can be made for preventing mumps in childhood. If this were to be done, the immunizing agent probably should be administered between two and three years of age. It should have no significant reaction and ideally it should provide immunity comparable to that conferred by natural infection, without requiring repeated boosters. This would seem to limit the choice to a live attenuated vaccine.

The disease is more severe in adults and the complications—particularly orchitis—are more frequent and serious. Therefore, immunization before puberty, especially of males, would seem particularly desirable. If immunization was delayed until this age, a large proportion of the population would already be immune, but unfortunately our present methods of identifying the susceptibles are not accurate enough or sufficiently easy to perform to be practical on a large scale. Likewise, reliance on immunization after recognition of exposure does not seem to be a desirable solution, even if an agent effective at this time were available, since inapparent infections are so frequent that many exposures would not be recognized. It would seem, therefore, that protection of the adult would be most effectively achieved by immunization in childhood. A special circumstance is groups of young adults, such as military recruits, particularly when persons from urban areas mingle with those from rural settings. The process of urbanization in the developing countries will present similar problems. Under these circumstances, immunization would be most desirable.

It is theoretically possible to eradicate the mumps virus from the population by widespread immunization of children, which would reduce the number of susceptibles to a level below that required for circulation of the virus. In this regard mumps resembles measles, poliomyelitis, and smallpox.

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SECTION C. MUMPS

PRESENT KNOWLEDGE OF KILLED MUMPS VACCINE

VICTOR J. CABASSO

Viral and Rickettsial Research, Lederle Laboratories
American Cyanamid Company, Pearl River, New York, U.S.A.

DR. CABASSO (*presenting the paper*): Although the incidence of mumps is very high in children and young adolescents (26), the disease is usually benign in these age groups. Because of its mild nature in the young, there is general agreement that immunologic control should not be practiced routinely in early life unless and until a method of vaccination providing durable immunity becomes available. The reports to follow will afford an opportunity to assess the value of live mumps vaccines in this regard.

Mumps, however, often takes a more severe form in adults, with involvement of the testes and of the central nervous system the most dreaded complications. Thus for susceptible older persons who have been or who are likely to be exposed, immunization, even though transient, is justified if demonstrated to be effective when applied before, at the time of, or shortly after exposure. Preformed mumps antibody preparations derived from gamma globulin of hyperimmunized humans have been reported to be useful in adult males when given before the onset of orchitis, but available information is too limited to allow definite recommendations (17). The present report will be limited to an assessment of the value of immunization with inactivated mumps vaccine.

DEVELOPMENT OF INACTIVATED MUMPS VACCINES

An early attempt to develop an inactivated mumps vaccine was made in 1945 by Enders and co-workers (8). They prepared formalin-inactivated suspensions of infected monkey parotid glands, some of which were precipitated with alum. Vaccine of either type elicited complement-fixing antibody in rhesus monkeys, particularly when injected in two doses at an interval of five days. Sixty per cent of the vaccinated animals gave evidence of increased resistance on challenge in the parotid gland with active mumps virus. As a result of these findings, Stokes and co-workers (25) used a formalin-inactivated vaccine made from monkey parotid glands in humans.

In children given two or three doses five days apart, a 50 per cent protection was recorded after challenge with active virus through Stensen's duct five to ten days after the last vaccine injection. In children or adults given two vaccine injections five days apart after exposure the incidence of disease was the same as in unvaccinated controls. However, vaccination after exposure seemed to reduce the severity of the simple disease and may have prevented or diminished complications. Although the results demonstrated the possibility of obtaining antigenic preparations of inactivated mumps virus, further progress in developing a vaccine and applying it clinically would probably have been impeded by the necessity of using the rhesus monkey. However, the cultivation of mumps virus in chick embryos was achieved, and thus the pace of vaccine development was accelerated.

Adaptation of mumps virus to the chick embryo was first reported by Habel (11) who grew the virus in the yolk sac, the amniotic sac, and the allantoic cavity of embryonated eggs. His work was promptly confirmed by Levens and Enders (20), who found the amniotic sac to be

the best source of virus. Habel showed the chick embryo to be a suitable source of complement-fixing and skin antigens and demonstrated its usefulness in the performance of a serum-neutralization test with mumps virus. Levens and Enders discovered the hemagglutinating ability of the virus and carried out hemagglutination-inhibition (HI) tests with monkey and human serums. Thus, not only was the viral raw material made abundantly available, but convenient tools for its investigation in the laboratory were at hand as well. In a short time Beveridge and his co-workers (3) in Australia made three isolations of mumps virus from human saliva in chick embryos. They found the virus to multiply readily after yolk-sac or amniotic-sac inoculation and somewhat less readily after allantoic inoculation.

Inactivated mumps vaccines were prepared subsequently by a number of workers. Habel (12) made his from infected yolk sacs and allantoic fluid, carrying out concentration and clarification by ether extraction, precipitation on urates, or by pH change; he effected inactivation using ether or ultraviolet light. These vaccines were immunogenic in monkeys when given three times at weekly intervals, as demonstrated by the serum-neutralization test or by challenge with virulent virus. Habel felt that serum-neutralizing antibody was of greater significance than complement-fixing antibody in denoting immunity, a belief later confirmed by Bashe and co-workers (2). Habel also found that vaccines with beeswax in oil as adjuvant were definitely more immunogenic than the same antigens with saline. Beveridge and Lind (4, 5) inactivated infected amniotic or allantoic fluids with 0.1 per cent formalin and noted a good HI response in rabbits after single 2 ml intravenous injections. The antigenicity of their inactivated preparations remained unimpaired after five months at 4°C. Given to children or young adults, the vaccines elicited increasingly higher antibody levels after each of two 2 ml subcutaneous doses in only 4 of 40 subjects, all the others responding with higher HI titers after the first dose but no further elevation after the second. In all probability, the four children were the only ones that lacked basic immunity to mumps at the initiation of the studies. Later reports included those in which heat-, formalin-, or ultraviolet-inactivated vaccines induced CF or HI antibody formation in mice (23), in guinea pigs (7), or in humans (7, 22, 18, 19, 9).

The investigations cited showed that inactivated mumps vaccines of chick embryo origin could induce the formation of antibodies in previously antibody-free animals or humans, particularly when given in multiple doses. However, the ability of the vaccines to prevent disease was not demonstrated. The remainder of this presentation will summarize the results of administering vaccine before exposure to mumps or after exposure in epidemic or individual situations.

EFFICACY OF INACTIVATED MUMPS VACCINE ADMINISTERED BEFORE EXPOSURE

Using concentrated vaccines inactivated by formalin or ultraviolet light in a population with a high percentage of persons with no record of past mumps, Henle and co-workers (15) noted only insignificant reactions in human subjects given as much as 4 ml of vaccine at one time. After subcutaneous doses of 1, 2, or 4 ml, maximal antibody levels were reached two to three weeks after vaccination. A second injection did not increase the CF antibody level reached after the first dose. However, the frequency of response and the titer levels depended on the amount of vaccine given in the first dose. Only with 4 ml of vaccine did the antibody response occur in 100 per cent of the recipients. When a mumps epidemic broke out in the study population nine months to a year after vaccination, the incidence of mumps among unvaccinated controls was twice that in the vaccinated groups. The incidence was correlated to the level of antibody attained four to five weeks after vaccination: it was highest in those who had failed to respond with measurable antibody and it decreased markedly with increase in antibody levels. No cases of mumps were confirmed in individuals who had developed CF titers of 1:16 or higher after vaccination. Thus the duration of immunity induced by subcutaneous vaccination with inactivated mumps vaccine appeared to be related to the antibody levels reached.

Continuing their evaluation, Henle and co-workers (16) later found that two or three doses of vaccine given one to two weeks apart afforded significant protection of susceptible children dur-

ing an outbreak of mumps that occurred three to eight months after vaccination. However, the incidence of inapparent infections in the vaccinated children was high, so that the total rate of infection (inapparent and overt) was of the same order as in the unvaccinated group. As for dosage schedules, many children formed some antibody within two weeks after a 1 ml dose of vaccine, and nearly all did so after a second dose. Most of them had lost detectable antibody three to six months after the initial dose, but a booster of 1 ml six to twelve months after the primary course rapidly induced antibody levels higher than those recorded after the first immunization. Following administration of the booster, neutralizing antibody was found in the majority of children for six to twelve months.

Other studies undertaken to assess the value of inactivated mumps vaccine before exposure to infection were those of Blitz and Eisenoff (6), Penttinen and co-workers (24), and Anderson and Lind (1).

Blitz and Eisenoff reported no significant protection of susceptible children given formalin-inactivated mumps vaccine in two doses of either 0.1 ml intracutaneously or 0.2 to 0.5 ml subcutaneously at a three-month interval. The children were exposed to mumps seven to nine months after vaccination.

Penttinen and his co-workers vaccinated troops with two subcutaneous doses of inactivated vaccine one week apart, the first dose consisting of 0.1 ml and the second of 0.5 ml. Over an eight-month period after vaccination the incidence of parotitis in the vaccinated men was only less than half that in the unvaccinated controls, but that of orchitis was significantly less—2 per cent in the vaccinated recruits as against 21 per cent in the controls. Vaccination also appeared to reduce the height and duration of fever.

Over a period of five to six years, Anderson and Lind found no significant difference in the total or time incidence of cases of parotitis between a control group and boys given two 2 ml subcutaneous injections of formalin-inactivated vaccine at intervals of three to ten weeks.

The investigations summarized above varied too greatly in design and in dosage schedules to afford a definitive conclusion regarding the efficacy of inactivated mumps vaccine before exposure or concerning the recommended immunization regimen. As a result of more comprehensive studies, however, Henle and co-workers concluded that formalin-inactivated mumps vaccine was effective when administered before exposure, provided that it was given in two initial subcutaneous doses of 1 ml each, one to four weeks apart, with a booster dose of 1 ml six to twelve months later. They also believed that further doses would most likely be needed at one- or two-year intervals to maintain a resistant status.

EFFICACY OF INACTIVATED MUMPS VACCINE IN EPIDEMIC SITUATIONS OR AFTER EXPOSURE

Only a few studies have been reported in which inactivated mumps vaccine was administered to large groups of persons after epidemic outbreaks or after individual exposures to mumps.

The classic investigation of Habel (13, 14) was carried out in Florida in a large group of highly susceptible West Indians imported to work on sugar plantations. The vaccine consisted of a concentrated, formalin-inactivated preparation incorporated in mixtures of beeswax and peanut oil. In a population of 2,500 men, 10 primary and 32 secondary cases of mumps had occurred before vaccination began. After a single 1 ml subcutaneous injection of vaccine, a perceptible reduction in the incidence of mumps was observed in the vaccinated group—a reduction that became most evident by the sixth week after vaccination. At that time, the ratio of attack between control and vaccinated individuals was three to one. There was also evidence that when mumps occurred in vaccinated subjects the clinical manifestations were milder and complications were fewer than in control cases. The net effect of vaccination on the epidemic was most apparent in a comparison of camps where no vaccine was used with those in which 90 per cent of the population had been vaccinated. In the former the epidemic continued for 14 weeks, whereas in the latter it lasted for only one week after the initiation of vaccination. Habel felt that the beeswax-in-peanut-oil preparations delayed somewhat the onset of an immune response and suggested that two injections of an aqueous vaccine given two weeks apart might have yielded more favorable results.

In this connection it may be appropriate to mention the attempt of Friedman and co-workers

(10) to induce as rapid an immune response as possible to cope with incipient infection in persons already exposed. These workers were administered single 1, 3, or 10 ml doses of a formalin-inactivated commercial vaccine. Antibody was measured one week later. Over-all conversion rates of 5 per cent after 1 ml, 6 per cent after 3 ml, and 17 per cent after 10 ml led the authors to conclude that the use of single large doses is not recommended with the currently available vaccines. They suggest that future vaccines, more highly purified and concentrated, may induce more rapid immunity.

The favorable effect of inactivated mumps vaccine on the course of a developing epidemic contrasts disappointingly with its effect in individual adults vaccinated after exposure to their sick children. Meyer and co-workers (21) gave formalin-inactivated commercial vaccines subcutaneously in two 1 ml injections one week apart to 173 parents. These adults had negative mumps histories, and 76 per cent of them received the first vaccine injection on the day of, or on the day after, the onset of mumps in a child. The rationale on which these studies were based was that, as in the case of rabies, the long incubation period of the virus and the observed rapid appearance of antibody after vaccination might possibly prevent clinical illness or at least lessen the severity of symptoms. Mumps attack rates were low among exposed parents, whether vaccinated or not, amounting to 8 per cent in the former and 3.9 per cent in the latter. The authors conclude that the use of inactivated mumps vaccine in exposed adults is not recommended, as it may even cause a transient reduction in resistance, a so-called "negative phase," at a time when the body must call on its maximum defense mechanism. An alternative is that the question of efficacy of inactivated vaccine after exposure to mumps is still open and that further information is needed. Be that as it may, Meyer and her co-workers offered a solution to the problem of parental exposure that may have merit. She points out that, since parents whose children are entering school may be at highest risk of exposure, the true susceptibles among them can be detected with considerable reliability by available serological method. They can then be vaccinated before exposure and have their immunity reinforced by periodic booster doses of vaccine.

Summary and Conclusions

Cultivation of mumps virus in the chick embryo made practical the development of an inactivated mumps vaccine. Vaccines inactivated with heat, formalin, or ultraviolet light have been prepared and have been shown to elicit antibodies when given in at least two properly spaced injections.

Formalin-inactivated vaccines have been commercially available since the early 1950's. They were demonstrated to induce significant protection against the disease in susceptible persons given two 1 ml subcutaneous doses one to four weeks apart, but resistance appeared to be short-lived and had to be reinforced six to twelve months after the initial course. Inactivated mumps vaccine was also shown to be of value in shortening an epidemic and modifying the severity of illness in a highly susceptible group of young adults, even when given after several secondary cases of mumps had appeared. The question of efficacy of inactivated mumps vaccine in individual parents after exposure is still in doubt and remains to be elucidated by further studies.

The concensus of workers in the field is that inactivated mumps vaccine should not be used in routine pediatric practice. It is conceded, however, that pre-exposure vaccination would be beneficial in children under particular circumstances and in special groups of adults such as military and labor recruits, students, medical personnel, and patients confined to hospitals for long periods of time.

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SECTION C. MUMPS

EXPERIENCE WITH LIVE MUMPS VACCINE IN THE USSR

A. A. SMORODINTSEV, N. S. KLYACHKO,
M. N. NASIBOV, AND E. S. SHICKINA

Measles Laboratory, Pasteur Institute of Epidemiology
and Microbiology, Leningrad, USSR

DR. SMORODINTSEV (*presenting the paper*): Success in the prevention and treatment of the most widespread droplet infections of childhood (diphtheria, pertussis, measles, and scarlet fever) in recent years has substantially increased the relative importance of the so-called "minor" infections of infants (mumps, varicella, rubella). This is especially true of mumps, which causes continuous mass infections among children and often attacks adults, producing complications such as meningitis, pancreatitis, and orchitis.

Successful control of mumps is possible only through active immunization, and this has now become more of a reality through our development since 1954 of an attenuated live vaccine (1–3). This live vaccine assures on the average an eight to tenfold reduction of the mean incidence of mumps in vaccinated children within at least the first five years after vaccination. In 1952 we started our work with five laboratory strains attenuated by 17 to 40 passages through developing eggs and combined into a single vaccine strain, which was then preserved in a freeze-dried state from additional passages. This vaccine strain proved to be safe and highly immunogenic for children between one and twelve years of age after a single intradermal or subcutaneous injection of nearly 100,000 infectious doses for the developing egg.

During the first period of our work (1954–1961) freeze-dried egg vaccine from pooled amniotic and allantoic fluid of infected developing chick embryos seven to eight days old was used. In the period 1961–1965 the same vaccine strain was also used for the production of a cheaper tissue culture vaccine in primary monolayer of chicken fibroblasts (6).

The vaccine strain of mumps virus multiplies well in many primary and continuous tissue cultures and provokes typical morphological changes in the susceptible cells, such as formation of a syncytium and cytoplasmic oxyphilic inclusions.

The highest yields of mumps virus in primary tissue cultures were obtained when the cell suspension was infected immediately after trypsinization of the tissue and the infected cultures were incubated at 34°C for five days. The infected fluids can be preserved in 1 per cent gelatin at 4°C for two to three months, and in the lyophilized state for a year, without substantial decrease in the virus titer (5.5 to 6.0 lg/ml).

Live mumps vaccine induces no clinical symptoms or rise of specific antibodies after pulverization in the upper respiratory tract or rubbing into the mucous membranes of the oral cavity around the Stensen's duct of susceptible children.

On the basis of clinical observations, it appears that live mumps vaccine is safe and nonreactogenic even for small children one to three years of age. No signs of parotitis, meningitis, orchitis, or serious toxic reactions were seen in more than 100,000 children observed in Leningrad between 1955 and 1959. No evidence has been found that the attenuated virus can multiply in the parotid gland after intradermal or subcutaneous immunization or that it is excreted in the saliva of vaccinated children. The development of mild general toxic reactions, such as

short-lasting subfebrile temperature, headache, or vomiting, is observed in less than 0.5 per cent of the vaccinated children.

Serological and allergic evidence of immunity has been observed 14 to 21 days after the administration of single subcutaneous injections of live mumps vaccine. For testing the immunogenic efficiency of live mumps vaccine a simple and sensitive neutralization test in monolayer cultures of chick embryo cells can be recommended. The endpoint of the reaction has been detected more precisely by the hemadsorption test, using chicken erythrocytes (5, 7).

In evaluating immunological changes in paired sera of vaccinated children the serum-neutralization test was shown to be superior to the hemagglutination-inhibition test: twice as many paired sera showed a fourfold or greater rise of virus neutralizing antibody, with much higher quantitative titers than those of antihemagglutinins (Fig. 1).

According to the results of the neutralization reaction, a fourfold or greater increase in specific antibodies occurred in the majority (80 to 90 per cent) of vaccinated children who had not had antibodies in the serum obtained before immunization. This test was found to be even more sensitive and stable than the skin allergic reaction test, which remained positive in 60 per cent of the subjects for as long as 18 to 30 months after vaccination. The neutralization test, however, clearly differentiates vaccinated from unvaccinated children in the majority of cases for at least five years.

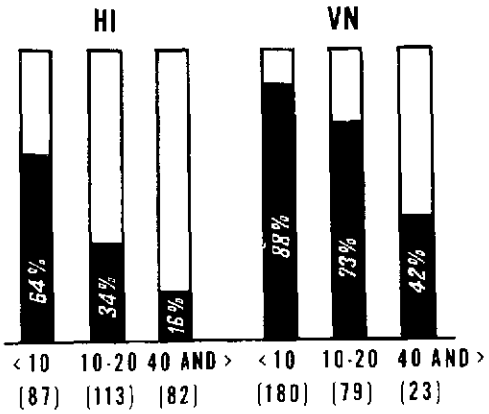


Fig. 1. Increase of antibody levels fourfold or higher (dark area) in sera of 282 children vaccinated against mumps as determined by hemagglutination inhibition (HI) and virus neutralization (VN) tests.

The long duration of seroimmunity after the use of live mumps vaccine is quite different from the situation with the killed vaccine, where both the serological changes and the very limited epidemiological effectiveness have disappeared one to two years after repeated immunization (11).

We have not yet succeeded in isolating mumps virus from the blood or urine of vaccinees. Similarly, negative results have been obtained in many laboratories with much more potent modern live vaccines against measles, especially among children, who had no febrile reaction to vaccination, in spite of all evidence for virus multiplication.

The valuable immunogenic activity of the live mumps vaccine is shown in a most convincing manner by epidemiological observations, which have thus established the high effectiveness of this method.

Figure 2 represents our data on 157 nursery schools and kindergartens in Leningrad in which there were 4,116 vaccinated and 5,567 unvaccinated children from three to six years of age with no past history of mumps. During the 30-month observation period single or repeated outbreaks of mumps were recorded in 103 of 157 groups, in which nearly 42 per cent of children had been vaccinated and 58 per cent were being observed as controls. On the basis of total morbidity in the vaccinated and control groups, there was a consistent tenfold reduction of the morbidity rate among the vaccinated children during the first 18 months and a sixfold reduction during the next two six-month periods studied.

The effectiveness of the vaccine in this field trial is also confirmed by an analysis of age-linked morbidity among the vaccinated and control groups. The effectiveness was regularly confirmed in all age groups and resulted in a 7.0- to 19.6-fold reduction in mumps morbidity (2, 3).

Figure 3 shows morbidity data for 30 of the most severely affected school groups (those having 5 or more cases of mumps), as reported during the 12 months after vaccination. In 20 of

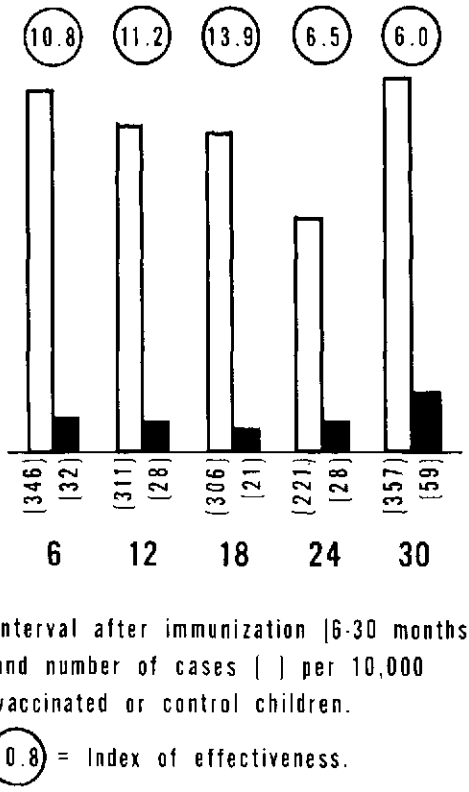


Fig. 2. Incidence of mumps in kindergartens among vaccinated (3,449) and control (4,452) children at different intervals of a 30-month period after immunization.

these school groups 5 to 13 cases were reported among the control children and none in the vaccinated. This means that in these groups vaccination had been 100 per cent effective. In 10 school groups single cases occurred among the vaccinated children, but the incidence of mumps among the unvaccinated control children was definitely much higher. The sporadic infections in the vaccinated children gave the picture of mild, abortive mumps.

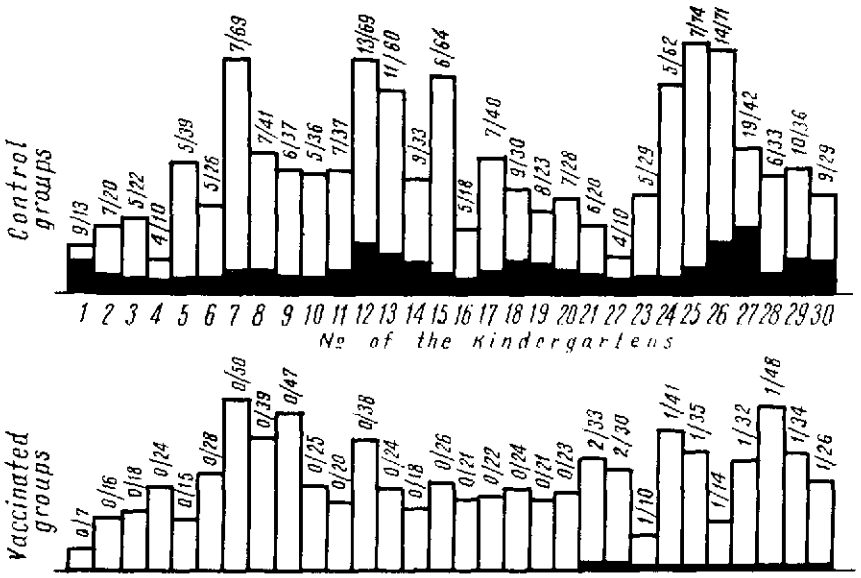
When the first infection occurred in a vaccinated infant, further spread of mumps either was not reported at all or was limited to only one additional case in the unvaccinated group (Table 1). Thus it is evident that the vaccinated children if they became infected were of only low contagiousness or none at all.

Table 2 summarizes the results of six field trials organized in Leningrad, Kiev, and Pskov over the period 1955–1962 by N. S. Klyachko and co-workers to study the epidemiological effectiveness of live mumps vaccine. In half of these trials the number of vaccinated children and internal contacts (in the same nursery schools and kindergartens) was equal to or even greater than the number of children in the corresponding control group. The morbidity rates among the vaccinated children varied between 0.9 to 9.8 per 1,000, whereas in the control group the range was between 11.4 and 92.1 per 1,000. The index of effectiveness was quite similar for the various field trials and showed an 8.1- to 12.6-fold reduction in morbidity.

In all, 127 mumps cases were recorded among the 38,542 vaccinated contact children (3.3 per 1,000), in contrast to 922 cases among the 20,972 children in the unvaccinated control group (43.9 per 1,000). This represents more than a tenfold average decline in the incidence of mumps after vaccination. During the last 10 years more than 1 million children in the USSR have been vaccinated against mumps with the most favorable results. The persistence of antimumps immunity in vaccinated children is distinctly evident five years later, and it gradually diminishes thereafter.

According to our experience, revaccination by repeated injection of the same vaccine five years or more after the first immunization is desirable.

The live mumps vaccine offers the most favorable prospects for the elimination of epidemic parotitis as a mass infection by the intensive immunization of susceptible children. This has recently been shown by N. S. Klyachko and co-workers in field trials in Pskov, where 12,477 of a total group of 15,642 susceptible children, that is more than 80 per cent of all susceptible children between the ages of two and twelve, were vaccinated against mumps. During the 12 months after immunization not only did the morbidity in vaccinated children drop 12 times in comparison to that in a small group of unvaccinated children, but all fundamental epidemiological features of mumps, such as seasonal and age prevalence and intensive contact spread, were markedly changed as well. Throughout the 12 months after the mass immunization program only sporadic cases of mumps were recorded, and the typical winter-spring seasonal peak was not nearly as pronounced (Fig. 4). The characteristic prevalence of mumps in the two-to-nine age group was also entirely lost,



White columns = Total no. of children in group; black columns = No. of diseased.

Fig. 3. Incidence of mumps among vaccinated and control children in 30 kindergartens during outbreak of the disease (four or more cases of mumps in one kindergarten).

and the sporadic cases were equally distributed among all the age groups in question. (Fig. 5).

The vaccination of children and adults already exposed to mumps has also been shown to be effective. Even under such difficult conditions as when the first cases have already been reported, the rapid complete vaccination of all healthy contacts regularly eradicates the outbreak of mumps during the next three to four weeks (Klyachko, 1959; Shaposhnikova *et al.*, 1961).

Table 3 gives an example of the effectiveness of the live mumps vaccine when extra immunization of contact children in Leningrad nursery schools and kindergartens was performed. During the first three weeks after extra immunization of 65 per cent of the susceptible preschool children, the morbidity rate was quite similar in both the vaccinated and control groups. Thereafter, in those institutions in which epidemic parotitis was still present the morbidity rates differed widely between the vaccinated groups (2.1 cases per 1,000) and the control groups (20.8 per 1,000). Thus our results demonstrate not only intensive reduction of mumps among the contact children but also regular climination of the epidemic three to four weeks after the complete immunization of all contact children.

To increase the program of immunization against mumps it is very useful to combine this preparation with a live measles vaccine—for ex-

TABLE 1. DEVELOPMENT OF MUMPS OUTBREAKS IN KINDERGARTENS IN WHICH INFECTION WAS INTRODUCED BY VACCINATED AND CONTROL CHILDREN

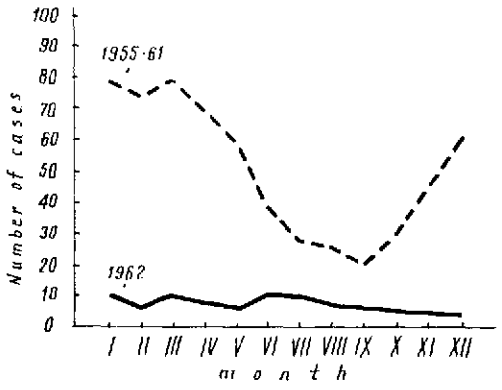
Source of infection	Total No. of kindergartens	Kindergartens in which additional infection occurred	Kindergartens with additional cases			Percentage of kindergartens with 5–36 cases of infection
			1	2–4	5–36	
Vaccinated	10	1	1	—	—	0.0
Control	43	26	5	6	15	34.9
Newly arrived	28	14	5	2	7	25.0

TABLE 2. EPIDEMIOLOGICAL EFFECTIVENESS OF THE LIVE MUMPS VACCINE IN SIX FIELD TRIALS IN LENINGRAD, KIEV, AND PSKOV, 1955–1962

No. of field trial	Period of vaccination	Place of vaccination	Period of subsequent observation (months)	Vaccinated children			Nonvaccinated children (internal controls)			Index of effectiveness
				Total no. in contact	Cases of mumps	Morbidity rate per 1,000	Total no. in contact	Cases of mumps	Morbidity rate per 1,000	
1	XI. 1955–III. 1956	Leningrad	18	4,116	24	5.8	5,567	333	59.8	10.3
2	XI. 1956–III. 1957	Leningrad	15	6,773	35	5.2	6,127	260	42.4	8.1
3	1959	Kiev	12	1,415	14	9.8	1,390	128	92.1	9.4
4	I. 1960–VI. 1961 (extra vaccination)	Leningrad	3	6,412	18	2.8	3,430	113	32.9	11.7
5	I. 1961–VI. 1961	Leningrad	18	7,349	24	3.4	1,293	52	40.2	11.8
6	X. 1961–XI. 1961	Pskov	12	12,477	12	0.9	3,165	36	11.4	12.6
	Total			38,542	127	3.3	20,972	922	43.9	

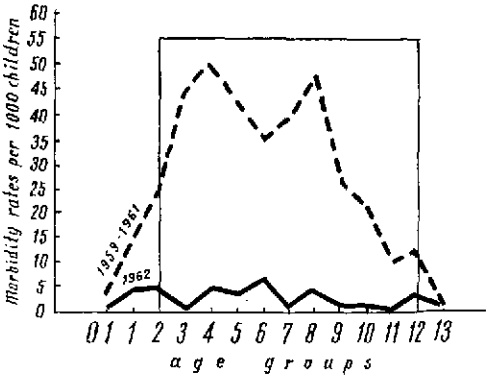
ample, the Leningrad-16 strain, which is obtained by the direct adaptation of measles virus from a patient to monolayer primary guinea pig kidney tissue culture. This live vaccine provokes very mild febrile reactions in not more than 50 per cent of the susceptible children and it does not require the use of gamma globulin, which suppresses the immunogenic activity of our mumps vaccine (7).

The Leningrad-16 live measles vaccine is very effective, according to the results of mass field trials organized in various parts of the USSR. In many hundreds of outbreaks in which vaccinated children were exposed to measles a regular ten- to twentyfold decrease in morbidity indices was observed among vaccinated children as compared to equivalent unvaccinated children in the same institutions or family groups. The epidemiological effectiveness of the live measles vaccine from the Leningrad-16 strain was especially pronounced when 90 per cent or more of the susceptible children between the ages of one and eight were immunized. A similarly high level of immunization is being reached now in the Moldavian and Kirgiz Republics and also in some of the large cities such as Leningrad.



---- = Average rates for seven years preceding period (1955-1961).
——— = Same for 1962, after vaccination.

Fig. 4. Monthly morbidity rates of mumps in the city of Pskov before and after vaccination of 80 per cent of the susceptible children between one and twelve years of age.



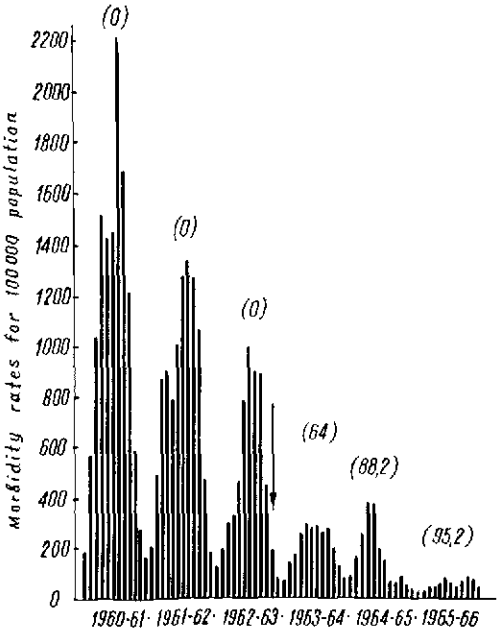
---- = Average morbidity rates during the three years before immunization.
——— = Same in 1962, after immunization.

Fig. 5. Effect of intensive (80 per cent) immunization against mumps in Pskov in 1962 on the age distribution.

TABLE 3. EFFECTIVENESS OF LIVE MUMPS VACCINE UNDER CONDITIONS OF EXTRA IMMUNIZATION OF CONTACT GROUPS IN KINDERGARTENS AND NURSERY SCHOOLS

Groups	Mumps morbidity during the indicated period of outbreak							
	The first three weeks				After three weeks			
	Total no. of children observed	No. of mumps cases	Rate per 1,000	Index of effectiveness	Total remaining no. of children observed	No. of mumps cases	Rate per 1,000	Index of effectiveness
Vaccinated.....	2,002	185	9.2	1.2	854	18	2.1	9.8
Control.....	1,066	123	11.5		598	113	20.8	

Figure 6 shows an example of a highly effective measles suppression in a large city (Alma-Ata, the capital of Kazakh Republic, USSR), where since 1963 the majority of susceptible children have been vaccinated with Leningrad-16 live measles vaccine. As a result of regular and intensive immunization of 90 per cent and more of the susceptible children, including the new generations of infants, measles has been shown to be almost fully suppressed in this large town of more than half a million people. The live vaccines against mumps and measles were combined just before immunization in doses of 300 to 1,000 infectious tissue units for measles and 100,000 infectious units for mumps. This less virulent Leningrad-16 measles vaccine is very suitable for combination with live mumps vaccine and provides the most convenient method for large-scale use of mumps vaccine. No changes in clinical reaction rates were noted when this combined vaccine was used.



( ) = Vaccinees in the years indicated.
→ = Period of first vaccination.

Fig. 6. Monthly morbidity rates of measles in the city of Alma-Ata before vaccination (1960–1962) and after vaccination (1963–1966).

Our laboratory studies showed complete maintenance of the immunogenic activity of the measles and mumps components in the combined preparation on the basis of antibody rise in tests of paired sera (Table 4).

Similar favorable results were obtained in field trials with combined measles and mumps live vaccines, which achieved the same rate of protection against both diseases as did the corresponding monovalent preparations (Table 5).

One of the matters for further study with regard to live mumps vaccine is the improvement of its immunogenic activity. At the present time the human virulence of the vaccine strain appears to be comparatively low and the preparation seems to be hyperattenuated. We plan to increase the existing level of virulence to stimulate the duration of postvaccinal immunity and to decrease the minimum inoculation dose, which is now too large considering the limited reproduction capacity of mumps virus in chicken fibroblasts monolayer.

TABLE 4. INCREASE OF ANTIBODY LEVEL IN SERA FROM CHILDREN AFTER IMMUNIZATION AGAINST MUMPS AND MEASLES BY MONOVALENT OR COMBINED LIVE VACCINES

Type of antibodies	Live vaccines	No. of children in group	Percentage of mild febrile reactions	Fourfold or greater antibody rise		Mean titer
				Total	Percentage	
Against mumps	Mumps (monovalent)	102	0	81	79.4	80
	Mumps and measles	128	69	107	83.5	76
Against measles	Measles (monovalent)	124	75	116	93.5	116
	Measles and mumps	136	73	121	88.9	106

TABLE 5. INCIDENCE OF MUMPS AND MEASLES AMONG CHILDREN VACCINATED BY MONOVALENT OR COMBINED LIVE VACCINES IN COMPARISON TO NONVACCINATED GROUPS

Nature of infection	Groups	Vaccination by monovalent vaccines					Vaccination by combined preparation (live vaccines against mumps and measles)				
		Morbidity			Effectiveness		Morbidity			Effectiveness	
		No. in contant	No. of cases	Percentage	Index	Coefficient	No. in contact	No. of cases	Percentage	Index	Coefficient
Mumps	Vaccinated	201	6	3.0	10.1	90.1	251	6	2.4	9.7	89.7
	Nonvaccinated	243	81	33.3			279	64	23.3		
Measles	Vaccinated	167	3	1.8	9.5	88.4	184	0	0	10.8	100
	Nonvaccinated	151	26	17.2			231	25	10.8		

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SECTION C. MUMPS

EVALUATION OF LIVE ATTENUATED MUMPS VIRUS VACCINE, STRAIN JERYL LYNN

R. E. WEIBEL, E. B. BUYNAK, J. STOKES, JR., J. E. WHITMAN, JR., AND M. R. HILLEMAN *

DR. WEIBEL (*presenting the paper*): Mumps is a common childhood disease that may be severely and even permanently crippling when it involves the brain, testes, ovaries, auditory nerves, or pancreas. Adult males may be permanently sterilized by mumps. The personal discomfort and the time loss from productive effort resulting from ordinary mumps and the serious or even fatal aspect of the extraordinary case appear to justify the development and application of a safe and effective live virus vaccine.

Early clinical studies (4, 5) with live mumps viruses in the United States showed that the agent was rapidly attenuated for man by passage in embryonated hens' eggs and that administration of the virus by oral spray could elicit antibody and a degree of protection without causing typical mumps. It was apparent, however, that the virus became rapidly overattenuated for man (6) and that a narrow range of passage level might be required for a safe and effective vaccine. Smorodintsev and co-workers (11, 12) conducted investigations of live mumps virus vaccines for several years in the USSR. At first they employed a virus prepared in embryonated hens' eggs but more recently they have used a preparation grown in chick-embryo cell culture. The virus was given by the intradermal or the subcutaneous route and afforded an apparent protective efficacy of 90 per cent or greater. The immunity was reported to last for at least five years.

* From the Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, and the Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania.

Buynak and Hilleman (1, 7) have recently recorded the development of a live attenuated mumps virus vaccine referred to as the Jeryl Lynn strain, named after the child from whom the virus was derived. The virus was attenuated by passage in embryonated hens' eggs and chick-embryo cell culture, and the vaccine was prepared from virus grown in the latter medium. The vaccine was prepared as a stable dried product and was tested for safety and potency by procedures consistent with the standards for live attenuated measles virus vaccine. During the past two years, our clinical laboratory team —Drs. Stokes, Buynak, Whitman, Hilleman, and I—has conducted extensive investigations of the safety, efficacy, and utility of the vaccine. I should like to summarize the pertinent highlights.

EARLY CLINICAL STUDIES

First tests (14) of the vaccine were carried out in Pennsylvania institutions for the mentally retarded. The investigations were performed with the concurrence of the medical and supervisory staffs of the institutions and with the approval of the Pennsylvania Association for Retarded Children.

The findings in a representative trial started in June 1965 are shown in Table 1. All of 14 initially seronegative children one to ten years of age (mean five years) who were given 1.0 ml of vaccine subcutaneously developed neutralizing and hemagglutination-inhibiting antibody as measured in serum samples taken 28 days later. The mean neutralizing and hemagglutination-inhibiting antibody titers were 1:11 and 1.12, re-

TABLE 1. CLINICAL AND LABORATORY FINDINGS IN FIRST TESTS OF JERYL LYNN STRAIN LIVE ATTENUATED MUMPS VIRUS VACCINE IN INSTITUTIONALIZED CHILDREN

Child No.	Mumps excretion (days 0, 8, 10, 14, 17, 21)	Parotid swelling	Antibody responses				Serum amylase on day		
			Neutralizing titer		HI titer				
			Before vaccination	28 days after	Before vaccination	28 days after	0	14	28
Vaccinated									
1	None	None	0*	32	0*	40	12	12	14
2	None	None	0	16	0	40	–	24	12
3	None	None	0	16	0	20	14	12	14
4	None	None	0	16	0	10	10	18	14
5	None	None	0	16	0	10	8	24	18
6	None	None	0	16	0	10	8	24	8
7	None	None	0	16	0	20	22	20	12
8	None	None	0	16	0	10	2	10	2
9	None	None	0	8	0	10	2	8	12
10	None	None	0	8	0	10	10	2	14
11	None	None	0	8	0	10	14	40	16
12	None	None	0	8	0	5	4	6	14
13	None	None	0	4	0	10	2	14	8
14	None	None	0	4	0	5	16	24	16
		Mean titer:	0	11	0	12	10	17	12
Unvaccinated controls				28 and 84 days after					
1–13	None	None	0	0	0	0	–	–	–
		Mean titer:	0	0	0	0	12	13	14

*0 = <1:1 in the neutralization test and <1:5 in the hemagglutination-inhibition test.

spectively. None of the children developed fever or any other clinical sign or symptom referable to the vaccine and none excreted the virus. The serum amylase values remained normal and there was no evidence of contagiousness of the mumps virus infection—that is to say susceptible children who were held as unvaccinated controls did not display clinical illness and did not develop antibody against mumps. These results were in contrast to previous investigations by our group with less attenuated mumps virus strains in which mild clinical mumps was produced and the virus was excreted.

The findings in the early studies with the Jeryl Lynn strain were sufficiently promising to justify extensive trials to evaluate the safety and protective efficacy of the vaccine under natural conditions of exposure to mumps in the community. The results of the trials have been recorded in detail elsewhere (7, 9, 16), and only the important highlights are given here.

LARGE-SCALE FIELD TRIAL

Conduct of the trial. The studies were carried out during the fall, winter, and spring of 1965–1966 among 1,337 children in families or in nursery and kindergarten classrooms in the Havertown-Springfield suburb of Philadelphia (Table 2). Those families in which one or more siblings attended a study classroom were designated as a classroom-family group. Classroom children with a negative history of clinical mumps were alternately selected to receive vac-

TABLE 2. COMPOSITION OF THE STUDY GROUPS IN WHOM THE MUMPS VACCINE WAS EVALUATED

Group of children	Number vaccinated	No. of controls	Total number
Classroom	253	366	619
Family	113	225	338
Classroom-Family	116	264	380
Total	482	855	1,337
No. initially seronegative to mumps	362	505	867

cine or to serve as controls. In addition, a number of children with a positive or uncertain clinical history of mumps were also included among the unvaccinated controls. Among the children in the families, an effort was made, whenever possible, to vaccinate one susceptible in a given family and to retain two susceptibles as controls. Altogether, 482 children received vaccine and 855 served as unvaccinated controls. In the total group, 867 children were without mumps antibody initially; of these, 362 were given vaccine and 505 were held as controls. In the analyses for clinical reaction and efficacy, those particular children in the families who also attended classrooms were counted twice, once in the classroom group and once in the family group. This was done in order to achieve the fullest possible use of the data in ascertaining whether contact transmission of the mumps vaccine infection had occurred in either habitat and in assessing the rates of clinical reactions, if any, in the largest size vaccinated and control populations.

The children in the schools ranged from three to five years of age and those in the families ranged from 11 months to 11 years of age. The vaccine dose was 1.0 ml, given subcutaneously. Blood samples were taken just prior to vaccination and again six to eight weeks later from all vaccinated children and from all but 191 controls (from whom only a single sample was drawn). The serum samples were properly spaced to demonstrate antibody response in the vaccinated persons and to permit detection of antibody rise in the controls in the event the vaccine infection had been contagious.

The mothers were given cards on which to record fever and other clinical reaction in each child during the first 28 days after vaccination or establishment as a control, and all patients with significant reported illness were visited by a physician. Reports of cases of natural mumps occurring in the study children were made by mothers, family physicians, and visiting nurses. All children in classrooms were visited twice weekly by nurses. If they were ill they were visited at home. All children with suspected mumps were seen by a qualified physician, who made clinical observations and took throat and blood samples for laboratory diagnostic purposes.

The incidence of natural mumps was low in the study populations at the time the investigation was initiated. This caused no difficulty except in a few instances in which a child had been exposed to mumps prior to vaccination but not long enough to have developed antibody and to be considered immune. Such children usually developed mumps as a result of natural exposure and for this reason the few cases of mumps that occurred in vaccinees within 16 days after vaccination were disregarded in the study. In each instance the source of the exposure to natural mumps in a sibling or other close contact was established and the occurrence of mumps as a result of vaccination was ruled out.

SEROLOGIC RESPONSE TO VACCINATION

The serologic responses among the seronegative children who received the mumps vaccine are reported in Table 3. The findings are based on the results of both hemagglutination-inhibition and serum-neutralization tests. The serum-neutralization test was far more sensitive for detecting antibody than was the hemagglutination-inhibition test, and it was used as the final assay method for all sera in which the hemagglutination-inhibition titers were low or equivocal.

Of the 402 children who were initially seronegative for mumps, 395 developed antibody after vaccination. The seroconversion rate was 98 per cent. By contrast, none of 407 contact controls developed mumps antibody, which indicated that the vaccine infection was not contagious.

Children who had antibody prior to vaccination sometimes showed an increase in the level of mumps antibody. Data on five instances of increased titers among initial seropositives are

TABLE 3. SEROLOGIC RESPONSE RATES IN CHILDREN WHO RECEIVED THE JERYL LYNN STRAIN LIVE ATTENUATED MUMPS VACCINE AND LACK OF RESPONSE IN UNVACCINATED CONTACT CONTROLS

Group of children	Vaccinated		Unvaccinated	
	No. developing antibody / Total	Percentage developing antibody	No. developing antibody / Total	Percentage developing antibody
Classroom	224/225	99.6	0/189	0
Family	171/177	97.0	0/218	0
Total group	395/402*	98.0	0/407	0

Note: All children were initially seronegative.
*Forty of the vaccinated and 42 of the unvaccinated children were members of both the classroom and family groups and were counted in both categories.

shown in Table 4. These titer increases ranged from four- to sixteenfold and indicated stimulation of mumps antibody production, presumably as a result of mumps vaccine infection, in spite of pre-existing antibody.

Figure 1 compares the height of the neutralizing and hemagglutination-inhibiting antibody titers attained among a group of randomly selected persons who received the mumps vaccine with those of persons convalescent from natural mumps. The neutralizing antibody titers observed after natural mumps were usually higher than those observed after vaccination, with a ratio of mean titer of 6.7:1. The hemagglutination-inhibiting antibody titers were more nearly alike—1:9 for the persons who had had natural mumps and 1:5 for the vaccinees.

TABLE 4. EXAMPLES OF SEROLOGIC RESPONSES TO THE JERYL LYNN STRAIN LIVE MUMPS VIRUS VACCINE AMONG CHILDREN WHO WERE INITIALLY SEROPOSITIVE

Case No.	Neutralization titer		HI titer	
	Before vacc.	After vacc.	Before vacc.	After vacc.
2070	64	512	10	40
1111	16	128	10	40
2233	8	128	5	40
3301	16	64	10	40
365	...	...	5	20

CLINICAL REACTIONS

None of the children who were vaccinated developed clinical symptoms or signs that were significantly different in number or kind from those observed in the unvaccinated controls. The occurrence of fever among the vaccinated and control children in the classroom and family groups is summarized in Figure 2. It is seen that the entire group of children was relatively free of fever during the period of observation and that the distribution of fever in the vaccinated and control groups was essentially the same. Moreover, there was no appreciable difference in the height of fever during any particular period after vaccination, as would have been expected for vaccine-caused fever. The findings indicated failure of the vaccine to cause any detectable illness.

PROTECTIVE EFFICACY AGAINST NATURAL MUMPS CHALLENGE

Natural mumps occurred in endemic form in the study population during the fall and early winter of 1965–1966. It reached a peak during the week of 14 March and then fell off to a level of low endemic prevalence. The occurrence of natural mumps in the study population permitted evaluation of the vaccine for preventing mumps acquired in nature. Evaluation of protective efficacy of the vaccine was based on attack rates of laboratory-proved cases of mumps in the vaccinated and control groups of children. Outbreaks of natural mumps occurred in 14 of the classrooms under study; 86 vaccinated and 76 control children were placed at risk. In all, 39 proved cases of mumps occurred in the controls, whereas only 2 cases occurred in the vaccinees (Table 5). The attack rates were 51 and 2 per cent, respectively, and the vaccine afforded a protective efficacy of 96 per cent. Natural mumps occurred in 13 of the families studied. There were 22 proved cases among 24 controls at risk in the families, whereas there were none among the 14 vaccinated children at risk. Thus the protective efficacy was 100 per cent. By the end of September 1966 the incidence of natural

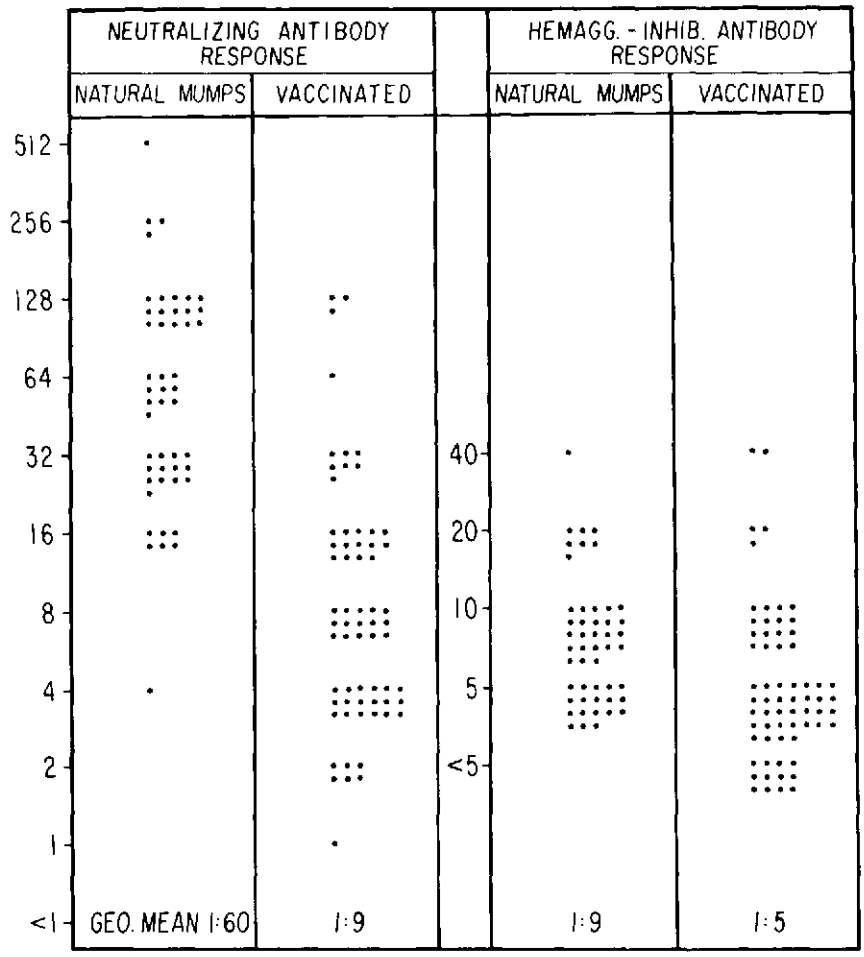

Fig. 1. Comparison of height of neutralizing and hemagglutination-inhibiting antibody titers following vaccination with those following convalescence from natural mumps.

mumps was approximately double what it had been at the end of April. Laboratory diagnostic tests are now nearly completed on the additional cases, and the same high level of protective efficacy is being shown.

In additional studies it was found that the titer of mumps neutralizing antibody in lots of human and immune globulin of diverse origin ranged from 1:64 to 1:2048, with a median of 1:256. Human immune globulin, which titered 1:512 for mumps-neutralizing antibody, was given to children simultaneously with mumps vaccine in opposite arms in a dose of 0.01 or 0.02 ml per pound of body weight. There was no suppression of antibody response to mumps.

The minimum age for mumps vaccine administration is being studied, but it can be said now that one year of age or more is acceptable. The vaccine can be given to adults without untoward effect. The minimal dose of mumps virus required for immunization is no more than 317 tissue culture infectious doses.

COMMENT

Mumps virus infection is analogous to measles (rubeola) in many respects. Both illnesses are caused by viruses of the myxovirus group, and both have systemic involvement. In both cases the virus has only a single serotype and natural infection provides lifelong immunity. Both viruses are neurotropic, although clinically

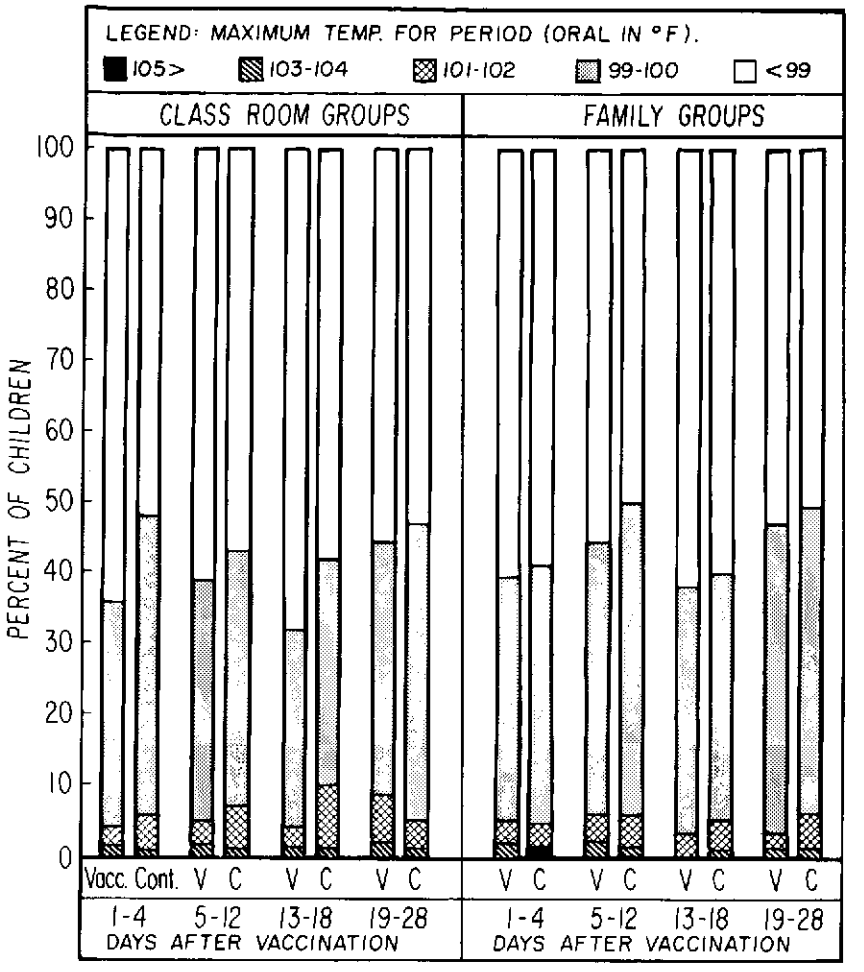

Fig. 2. Maximum temperature in vaccinated (V) and control (C) children according to time after vaccination.

TABLE 5. PROTECTIVE EFFICACY OF THE JERYL LYNN STRAIN LIVE ATTENUATED MUMPS VIRUS VACCINE BASED ON FINDINGS IN LABORATORY PROVED CASES

Group of children	Vaccinees at risk		Controls at risk		Protective efficacy (%)
	No. of cases / Total group	Attack rate %	No. of cases / Total group	Attack rate (%)	
Classroom	2/86	2	39/76	51	96
Family	0/14	0	22/24	92	100

apparent involvement of the central nervous system appears to be more frequent but less devastating, on the average, in mumps than in measles. Both viruses are attenuated in virulence by passage in embryonated hens' eggs and both propagate well in chick-embryo cell cultures. Both are stable on drying. Neither of the infections established by Enders live attenuated measles virus vaccine (3) or by the Jeryl Lynn strain mumps virus vaccine is contagious to susceptible human contacts. The infection induced by the mumps vaccine is clinically inapparent, whereas that associated with the measles vaccine is apparent in a portion of the recipients and may be accompanied by fever and rash (3,

15). The height of the neutralizing antibody responses to mumps and measles virus vaccines are considerably lower than those that follow the respective natural infections (8). Both Enders live attenuated measles virus vaccine and the Jeryl Lynn strain live mumps virus vaccine induce antibody in approximately 98 per cent of susceptible contacts, and the protective efficacy of the two vaccines after natural challenge is about 97 per cent. Circulating antibodies resulting from prior natural infection often preclude antibody responses to both vaccines, though antibody increases do occur (10, 13). Both vaccines appear to induce neutralizing antibody that either prevents natural virus infection entirely or which prevents viremic spread in the body. The pattern of antibody retention after administration of Enders live attenuated measles virus vaccine is similar to that occurring after the natural disease, and the immunity developed after use of the unmodified Enders-Edmonston vaccine appears to be lifelong. The pattern for the antibody retention following mumps vaccination, which will be reported on by Buynak and co-workers at this Conference, provides a basis for anticipating that this immunity following Jeryl Lynn strain live attenuated mumps virus vaccine will also be lifelong. A neutralizing antibody titer of 1:1 or less may not be protective against natural challenge with either measles or mumps virus, but a titer of 1:2 or greater affords solid immunity. However, protection against natural measles (8) or mumps challenge has been observed in individuals with homologous neutralizing antibody titers of less than 1:1—that is, titers too low to be detected by ordinary procedures. These close resemblances between the live measles and mumps vaccines strongly suggest that their characteristics of performance should be much the same.

Conclusions

The data presented here, which are reported in greater detail elsewhere (1, 7, 9, 14, 16), have conclusively established the safety, the high level of antibody response, and the excellent protective efficacy of the Jeryl Lynn strain live attenuated mumps virus vaccine. The vaccine was used as a dried product and was sufficiently stable both in dried form and after rehydration to permit convenient use in routine practice. The vaccine caused no detectable clinical illness and the serologic and protective efficacy was of the order of 97 to 98 per cent. The Jeryl Lynn strain live attenuated mumps virus vaccine should find considerable application for eliminating morbidity, patient discomfort, and time loss from productive activity resulting from mumps; in preventing the serious complications that sometimes occur in natural mumps infections; and in relieving personal or parental distress. The patterns of antibody retention observed after vaccination (2) provide a basis for anticipating that immunity will be lifelong.

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SECTION C. MUMPS

DISCUSSION

CHAIRMAN DAVIS: We shall now proceed with a discussion of the preceding papers. I shall call first on Dr. Friedrich W. Deinhardt, Department of Microbiology, Presbyterian-St. Luke's Hospital and Medical and Graduate Schools, University of Illinois, Chicago, Illinois.

DR. DEINHARDT: It is clear from the data presented here today that mumps viruses attenuated by serial passages in chick-embryo tissues are both safe and immunogenic. However, three main questions remain unanswered.

The first question is practical. How long does immunity last?

Smorodintsev recommends revaccination after four or five years, and Raikhshtat and co-workers* recently suggested that loss or fading of immunity may occur one and a half to two years after vaccination with live attenuated mumps virus vaccine. I should like to ask Dr. Smorodintsev to comment on this. Will it be necessary to vaccinate every five years? If so, for how long? Will it be necessary to continue vaccination throughout the life of an individual?

The second question is also practical. What is the optimal route of inoculation?

Henle and Stokes and co-workers† and also Enders‡ showed more than 15 years ago that egg-adapted viruses administered by oral spray induced quite good immune responses in man. In confirmation of this, I wish to point to the results of studies on animals conducted by Dr. Grace Shramek in our laboratory, in which the parenteral route is compared with the intranasal route of inoculation.

Table 1 shows a typical example of the responses of guinea pigs to parenteral inoculation of four different mumps virus preparations. The first had undergone 9 passages in the amniotic cavity; the second, 30 passages in the amniotic cavity; the third, 15 passages in the amniotic cavity and 20 additional passages in the allantoic cavity. The fourth was a "killed" virus preparation. All four were adjusted to contain the same antigenic mass as measured by complement-fixation or hemagglutination tests. A 0.2 ml dose was injected subcutaneously into the guinea pigs on day 0 and again on day 35. The antibody responses were then measured by standard techniques. Low antibody titers had developed after the first injection with the virus least adapted to embryonating hens' eggs (amniotic-9), and even lower titers were stimulated by the preparation adapted to the allantoic cavity. No antibodies were produced after inoculation of the vaccine containing only inactivated virus. After the second inoculation with the same respective virus preparations as used for the same inoculum, high levels of antibodies were produced by all animals that had received the two types of live virus preparations, regardless of the passage histories of these viruses. On the other hand, only low and short-lived titers were observed in the group receiving "killed" virus mumps vaccine.

The results obtained when the same types of viruses were used for intranasal inoculation of guinea pigs is seen in Table 2. By this route of inoculation even the virus preparations with higher numbers of egg passages induced antibody responses after the first inoculation, and the titers with all preparations were significantly

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TABLE 1. SERUM ANTIBODY TITERS OF GUINEA PIGS AFTER TWO SUBCUTANEOUS INOCULATIONS OF VARIOUS MUMPS VIRUS PREPARATIONS

Virus used for initial inoculation	Type of antibody	Days after initial inoculation 0	14	21	28	35	Virus used for second inoculation on day 35	Type of antibody	Days after second inoculation 14	21	35
Amniotic 9	V	<8*	29	64	27	44	Amniotic 9	V	443	160	200
	S	<8	19	36	19	24		S	347	320	172
	N	<8	9	10	8	24		N	725	896	384
Allantoic	V	<8	<8	<8	<8	<8	Allantoic	V	93	174	269
	S	<8	<8	11	<8	<8		S	198	292	269
	N	<8	<8	<8	14	23		N	1,078	249	203
Killed virus vaccine	V	<8	<8	<8	<8	<8	Killed virus vaccine	V	<8	<8	<8
	S	<8	<8	<8	<8	<8		S	<8	<8	<8
	N	<8	<8	<8	<8	9		N	34	18	8

V = Complement-fixing antibody against mumps-V antigen.
S = Complement-fixing antibody against mumps-S antigen.
N = Neutralizing antibodies.
*Reciprocal of serum dilution giving a 50 per cent endpoint. Arithmetic mean titer of groups of 10 animals.

TABLE 2. SERUM ANTIBODY TITERS OF GUINEA PIGS AFTER TWO INTRANASAL INOCULATIONS OF VARIOUS MUMPS VIRUS PREPARATIONS

Virus used for initial inoculation	Type of antibody	Days after initial inoculation 0	14	21	28	35	Virus used for second inoculation on day 35	Type of antibody	Days after second inoculation 14	21	28	35
Amniotic 9	V	<8*	58	215	88	116	Amniotic 9	V	113	68	110	43
	S	<8	67	302	110	61		S	337	314	244	42
	N	<8	21	77	64	120		N	2,513	1,815	873	587
Amniotic 30	V	<8	56	122	73	80	Amniotic 30	V	108	53	53	16
	S	<8	45	170	96	40		S	691	469	298	84
	N	<8	<8	<8	10	9		N	924	554	156	177
Allantoic	V	<8	37	80	72	45	Allantoic	V	52	58	145	32
	S	<8	97	197	139	61		S	244	267	244	40
	N	<8	11	<8	<8	9		N	1,147	656	188	126
Killed virus vaccine	V	<8	<8	<8	<8	<8	Killed virus vaccine	V	<8	<8	<8	<8
	S	<8	9	<8	<8	<8		S	<8	<8	<8	<8
	N	<8	10	13	<8	<8		N	12	11	<8	<8

V = Complement-fixing antibody against mumps-V antigen.
S = Complement-fixing antibody against mumps-S antigen.
N = Neutralizing antibodies.
*Reciprocal of serum dilution giving a 50 per cent endpoint. Arithmetic mean titer of groups of 10 animals.

higher than after subcutaneous inoculation. Again, only the "killed" virus preparations gave very short-lived antibody responses or none at all. This is understandable if the small amount of antigenic material used for inoculation is considered.

In view of these results, I feel that the intranasal or oral route of administration, as well as the parenteral route, should be considered. We will soon begin to evaluate both types of vaccination in children. We plan to use attenuated live virus vaccine that has been prepared in collaboration with Drs. A. Schwarz and J. Biddle of the Pittman-Moore Research Center from a mumps virus strain isolated in our own laboratories.

My third question is academic. What happens to viruses during adaptation to an heterologous host?

Loss of virulence for the cells of a particular species can in some instances be explained by an increased stimulation of interferon production. This seems to be true in the case of mumps viruses. Preliminary data obtained in our laboratory indicate that the ability of mumps viruses to induce interferon production in mammalian cells might give us an accurate marker for determining the virulence of mumps viruses of various degrees of attenuation for man. However, this still does not explain why viruses after serial passages in avian tissues behave differently in mammalian cells than the original virus does—that is, why they stimulate the production of larger amounts of interferon. If we can learn more about the basic mechanisms involved in these changes, then we might be able to approach the question of the ideal degree of attenuation rationally rather than by trial and error.

CHAIRMAN DAVIS: Dr. E. B. Buynak of the Merck Institute for Therapeutic Research, West Point, Pennsylvania, will continue.

DR. BUYNAK: I should like to report on "Measurement and Durability of Antibody Following Jeryl Lynn Strain Mumps Virus Vaccine," co-authored with Drs. M. R. Hilleman, J. E. Whitman, R. E. Weibel, and J. Stokes, Jr.*

* From the Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania, and Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

The B-level Jeryl Lynn strain live attenuated mumps virus vaccine has proved highly effective both in stimulating antibody against mumps in susceptible children and in affording protection against the disease after exposure to natural infection (1–3, 6–8). A prime factor in judging the value of any vaccine is the durability of the immunity it induces.

Our clinical-laboratory team has carried out two studies (6) in which it has been possible to measure mumps antibody levels one year after vaccination. All children studied were initially seronegative to mumps and all responded serologically to the vaccine.

Figure 1 shows a comparison of the serum-neutralizing antibody titers in samples of blood taken at one month and at one year after vaccination from the children in the first study. Titer values that fall on the line are unchanged and those that lie at one twofold dilution step above or below the line are within the range of variation in the test procedure. It is seen that the neutralizing antibody titers in all but 4 of the 23 sera (17 per cent) were unchanged at one year compared with one month after vaccination.

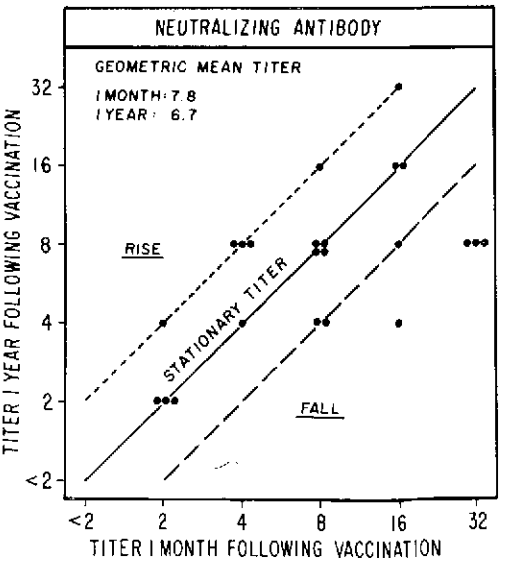

Fig. 1. Neutralizing antibody titers in individual children at one year compared with one month after vaccination with B-level Jeryl Lynn strain mumps virus vaccine (Study 62D).

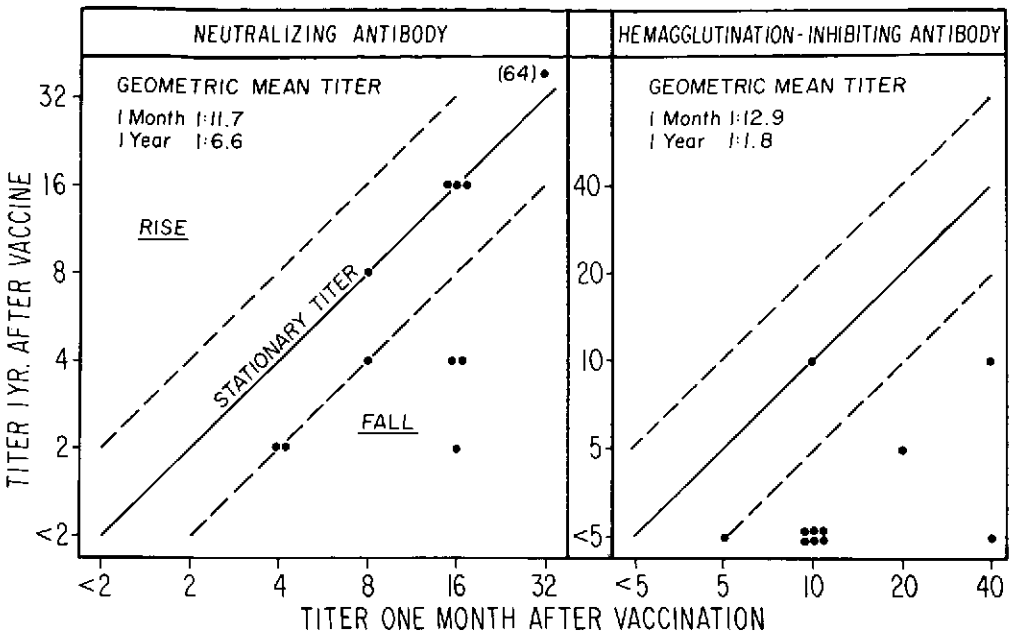

Fig. 2. Comparison of neutralizing and hemagglutination-inhibiting antibody titers in individual children at one year compared with one month after vaccination with B-level Jeryl Lynn strain mumps virus vaccine (Study 62C).

Similar findings are shown in Figure 2, which summarizes the results of the second study. Within the range of variation in the test, the neutralizing antibody titers in the sera of all but 3 of the 11 children were the same as noted 11 months earlier. The ordinary hemagglutination-inhibition test was far less sensitive than the serum-neutralization test for measuring mumps antibody. In addition, the hemagglutination-inhibiting antibody titer tended to decline during the year following vaccination.

The most sensitive and most reliable test for mumps antibody is by neutralization employing chick embryo cell cultures as indicators. Recently Stewart and co-workers (5) applied Norrby's ether-tween procedure (4) for releasing individual hemagglutinating antigens from intact mumps virus. Employment of such antigen greatly increases the sensitivity of the hemagglutination-inhibition test for detecting mumps antibody.

Table 1 shows the mumps antibody titers in 14 representative postvaccination sera as measured by the conventional hemagglutination-inhibition test, by the same test using soluble mumps hemagglutinin, and by the serum-neutralization method. The titers obtained in the conventional hemagglutination-inhibition test were typically far lower than those obtained by the serum-neutralization procedure; moreover, sera that were seronegative by the hemagglutination procedure were indeed positive when assayed in the neutralization test. Employment of the ether-tween split soluble hemagglutinating antigen greatly improved the sensitivity of the hemagglutination-inhibition method and permitted detection of nearly all seropositive sera. The mean titers obtained in tests of a group of 73 sera by the three procedures were 1:3.53 by the ordinary hemagglutination test, 1:9.05 by tests employing soluble hemagglutinin, and 1:13.74 by the neutralization procedure.

In summary, the retention of mumps antibody without appreciable loss for at least one year closely resembles the findings in natural infection and indicates that immunity will be lasting. The simple hemagglutination-inhibition test employing soluble hemagglutinin is far more reliable for detecting mumps antibody than the ordinary procedure and approaches the sensitivity of the serum-neutralization test method.

TABLE 1. ANTIBODY TITERS IN CHILDREN ONE MONTH FOLLOWING JERYL LYNN B MUMPS VIRUS VACCINE AS MEASURED BY HEMAGGLUTINATION TESTS WITH WHOLE VIRUS OR ETHER-TWEEN SPLIT VIRUS OR BY SERUM NEUTRALIZATION TEST

Patient	Reciprocals of serum antibody titers in tests		
	Hemagglutination-inhibition		Serum neutralization
	Whole virus	Ether-tween split virus	
4279	40	80	128
1002	10	40	32
4109	10	20	64
74	5	10	32
386	5	40	32
3250	5	20	16
4043	5	10	8
315	0*	5	16
448	0	10	8
4029	0	5	4
373	0	5	2
236	0	0	8
347	0	0	4
382	0	0	2
Geometric mean: 73 Sera	3.53	9.05	13.74

*0 = <1:5.

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CHAIRMAN DAVIS: Dr. Werner Henle, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, will continue the discussion.

DR. HENLE: There is no need to re-emphasize the advantages of live mumps vaccines as compared to inactivated ones. The data presented this afternoon speak for themselves. It is clear that the live vaccines afford a considerable degree of protection for at least three to five years, whereas according to all available information the resistance induced by inactivated vaccines is of shorter duration unless reinforced by booster injections. It has become apparent, however, that also with live vaccines protection may be transitory in at least a few individuals. The suggestion has been made today that reimmunization may be desirable, or perhaps even necessary, after an interval of several years.

It is conceivable that individual responses to a live vaccine may differ to some extent. In most cases the virus presumably infects and replicates in the vaccinees, thus providing a lasting immunity similar to that occurring after natural infection. In a few cases antibodies may be elicited, however, merely in response to the antigenic mass injected, and in that event resistance might be as transitory as it appears to be after the injection of an inactivated vaccine. There might be a way to differentiate between these two types of possible responses.

A natural infection by mumps virus generally calls forth appreciable titers of complement-fixing antibodies to the soluble or S antigen, whereas anti-S responses of initially seronegative individuals to the administration of inactivated virus vaccines are often absent and at most of a low order. Determination of anti-S levels after

the injection of live virus vaccines and correlation of these levels with respect to the duration of immunity might well clarify this point and possibly indicate further steps for the protection of persons who now fail to develop a long-lasting immunity.

CHAIRMAN DAVIS: Thank you. We will now throw the subject open for discussion from the floor.

DR. SMORODINTSEV: Dr. Deinhardt asked me a question concerning the period of immunity produced by live vaccine. I do not think that anyone knows exactly what this period is, even in regard to live measles vaccine.

We have observed vaccination immunity for six to seven years, and even though the results have been quite good, we cannot say the immunity will continue throughout the person's lifetime. I can only say that on the basis of serological data we can observe immunity five years later in children vaccinated with live vaccine and we can distinguish them from the children who have not received live vaccine.

But the indices are rather weak. I think that only epidemiological surveys will be able to show us the results. However, epidemiological data are difficult to obtain because the children we vaccinated when they were still very small go to different schools now. Thus I feel that if we had a possibility of revaccinating against measles and against mumps ten years, let us say, after the first vaccination this would be an extremely useful measure, and a most suitable one.

As for the combination of vaccinations and the production of more active interferons, such a direct connection cannot exist, because this is a vaccine strain that has changed its biological character. I mentioned before that we tried to rub this material into the oral cavity and we saw that the virus did not develop in the gland, so that we were unable to isolate the active virus from the sputum of the child. The only explanation we can offer for the biological changes in the virus is that it produces more interferons than wild virus and then becomes a sort of avirulent virus.

DR. HILLEMAN: The question of lifelong immunity attending the use of any vaccine is, of course, never answerable in a definitive and proved sense until a lifetime has passed. From the practical point of view, however, one has to look at the data available for a limited time after vaccination and compare the findings with those observed after the natural disease. By analogy one should be able to make reasonably reliable appraisals and predictions and at least arrive at a decision. The pattern of antibody level following Jeryl Lynn strain mumps virus vaccine resembles that seen after the natural infection. I think we have reason to expect lasting immunity after use of this strain. The relationships observed for mumps and mumps virus vaccine are not markedly different from those seen for measles and measles virus vaccine.

I think it is important to note that there are marked differences between the various mumps vaccine strains and that one must not fall into the trap of judging all vaccines on the basis of findings for one particular strain. It is well known that mumps virus attenuates extremely rapidly on passage in embryonated hens' eggs and that overattenuation can be reached quite quickly. In the process of developing the Jeryl Lynn strain we were very careful to take the vaccine only a few passages beyond the point at which the virus no longer caused illness and was no longer excreted after vaccination. In doing this, we minimized the possibility of overattenuation. Dr. Smorodintsev has indicated that his vaccine is at high passage level and has stated his belief that the strain is so overattenuated as to require revaccination after five years.

There is another point to note with regard to natural mumps, and that is that by adulthood many or perhaps most persons have low and barely detectable antibody. Yet these persons are still solidly immune.

The matter of optimal route for vaccination is a question that invariably arises in mumps and measles vaccine discussions, and one that seems to be answered so well in terms of parenteral injections. There are too many vagaries and variabilities in trying to get these viruses to take uniformly in the respiratory tract at this time and there is really no need for the nasal route when the parenteral route works so well.

Finally, I might close by saying that one might hope that diseases such as mumps and measles can be eliminated before the many problems mentioned today have been solved.

DR. FOX: I raised a question this morning with respect to use of measles vaccine in adults and got a private answer later on. I am not exactly hipped on this subject of adults, but it is true that in the immediate future one of the greatest demands for mumps vaccine is likely to be in adults.

Dr. Weibel commented that the vaccine could be used safely in adults but he did not elaborate on this point. I would be interested in knowing a little bit more about the extent to which the mumps vaccine has been used in adults. Also, there is the question of the possible effectiveness of vaccine after exposure, which is the usual stage at which the immunity of a father is brought into question.

Is there information on the rapidity with which antibody response is developed to mumps vaccine of the Jeryl Lynn strain or to strains employed by Dr. Smorodintsev? Does it develop fast enough to possibly modify an infection that actually has been acquired by a father exposed to his infected child?

DR. HILLEMAN: As regards the first question, the vaccine can be safely administered to adults with full serologic response. Only about 30 susceptibles have been vaccinated to date, but the number is being increased.

In answer to the second question, antibody appears about 14 to 17 days after the vaccine is given. The limited experience in field trials to date shows that the vaccine will not prevent mumps once the susceptible person has been exposed.

DR. STOKES: I think it should be recalled that during World War II Dr. Gellis and his group utilized mumps convalescent gamma globulin for prevention of orchitis, and quite successfully so. Thus we should remember that it can be used with a father or other susceptible adult who may be exposed until immunization can be carried out. Such gamma globulin can be obtained in quantity by plasmapheresis of convalescent donors.

DR. FABIYI: I do not seem to share the view expressed by Dr. Hilleman and also by Dr. Smorodintsev regarding the duration of immunity from these vaccines.

By looking at the data presented by Dr. Buynak one can probably estimate how long the immunity will last. In one of the figures shown there was roughly a 20 per cent drop-off one year after immunization. If one were to project this rate over five years, depending on the original level of the antibody titer, most of the measurable antibodies would probably be found to have disappeared. I think from the figures in Dr. Buynak's paper one can roughly estimate how long the immunity will last. This, of course, would depend on whether or not when one fails to measure antibody the level as such is still enough to give protection.

DR. HILLEMAN: One cannot draw curves of rates of decline in the absence of the points needed to create the curve. All one can say at this time is that the neutralizing antibody levels are remarkably well preserved and that they resemble the patterns seen after natural mumps. So far as we can predict, immunity will be lasting.

DR. SMORODINTSEV: It is my understanding that in determining measles and mumps immunity it is not so important to know the quantitative indices as it is to know the percentage of children having antibodies and the percentage of children not having antibodies, since in epidemic parotitis, as in measles, there is great immunity as long as there are virus antibodies.

We have observed children for five years after vaccination and in many of them we have seen a considerable difference in antibody levels as compared with those of unvaccinated children, but the quantitative indices were low.

We can have excellent results for epidemiologists after these children have been exposed to a contact with mumps. Thus we must continue further studies on the duration of immunity not only on the basis of serologic data but also on the basis of epidemiological data, since the latter determine more conclusively the difference in morbidity between the vaccinated and the unvaccinated groups.

The purpose in evaluating the different vaccine strains is not only to determine their immunogenicity but also to establish their safety. We must vaccinate many thousands of children and we must be able to say whether this vaccine is worthwhile from the point of view of the fate of the children vaccinated. Since we have vac-

cinated more than 1 million children, I think we are in a good position to say this vaccine is indeed safe. We have been surveying these groups of children for ten years and we have seen no ill effects.

DR. DEINHARDT: I would first like to say that I agree with Dr. Hilleman and Dr. Smorodintsev. As the latter said, circulating antibodies are not absolutely necessary as an indication of immunity to mumps—even very low neutralizing antibody titers still can mean immunity. In this respect the mumps skin test certainly seems to be very much more sensitive.

In regard to Dr. Hilleman's remark about how careful one must be not to overattenuate mumps viruses, I would like to go back to the animal data that I discussed earlier. After one inoculation of 1,000 to 5,000 tissue culture infectious units of a completely overattenuated mumps virus strain that has undergone more than 50 or 60 passages in chick embryo, guinea pigs, and nonhuman primates develop barely detectable antibody levels. However, if these subjects are challenged a month or two later with exactly the same overattenuated strain they react as if they had been inoculated with fully virulent virus. In other words, the second inoculation really reveals that the first one induced immunity despite the fact that only barely detectable circulating antibodies, or none at all, developed.

I also would like to underscore Dr. Henle's comment—admitting that I come from his school—that complement-fixation with soluble antigen certainly can help in distinguishing between stimulation by killed antigen and by live infection.

SESSION VII

SMALLPOX, RABIES, AND HEPATITIS

Thursday, 10 November 1966, at 8:30 a.m.

CHAIRMAN
DR. J. STOKES, JR.

RAPPORTEUR
DR. F. O. MACCALLUM

Section A.
Smallpox

Presentation of Papers by:
Dr. M. F. Polak
Dr. J. D. Millar
Dr. J. Å. Espmark

Discussants:
Dr. A. W. Downie
Dr. C. Henry Kempe
Dr. O. G. Andzaparidze
Dr. K. Raska

Section B.
Rabies

Presentation of Papers by:
Dr. J. F. Bell
Dr. H. Koprowski

Discussants:
Dr. Paul Fenje
Dr. Martin M. Kaplan
Dr. Harald N. Johnson

Section C.
Hepatitis

Presentation of Paper by:
Dr. Robert W. McCollum

Discussants:
Dr. J. Wm. McLean, Jr.
Dr. Saul Krugman
Dr. Thomas G. Ward
Dr. Friedrich W. Deinhardt
Dr. Werner Henle
Dr. Frederick O. MacCallum

SECTION A. SMALLPOX

PROBLEMS WITH SMALLPOX VACCINES AND POSSIBLE SOLUTIONS

M. F. POLAK

Rijks Instituut voor de Volksgezondheid
Utrecht, Netherlands

DR. POLAK (*presenting the paper*): The present WHO requirements (55) for smallpox vaccine stipulate a log pock count (pock-forming units on chick chorio-allantoic membrane [CAM]) of more than 8 for 1 ml of undiluted vaccine. An upper limit has not been fixed. The question arises whether a considerable excess of infectious units means merely a waste or a more effective preparation. Furthermore, it might be suggested that the discomforts, even risks, connected with smallpox vaccination are correlated with the concentration of viable particles in the vaccine.

Considering the usual method of smallpox vaccination—that is, the application of some fluid or resuspended lyophilized vaccine to a small slightly injured spot of the skin—the concentration of infectious units in the vaccine is clearly just one of the factors that determine the success or failure of infection. The local dermal reaction indicating success has been described as follows:

> A successful primary vaccination is one which, on examination after one week, shows a typical Jennerian vesicle.
>
> A successful revaccination is one which, on examination one week (six to eight days) later, shows a vesicular or pustular lesion *or* an area of definite palpable induration or congestion surrounding a central lesion, which may be a scab or ulcer. These reactions should be termed "major reactions"; all others should be termed "equivocal reactions" (53).

The appearance of such a primary take or such a major reaction at revaccination is accepted as evidence of a good, but slowly subsiding, protection against smallpox conferred by inoculation.

Next to the virus concentration, it is the technique of the vaccinator that most influences the dose available for infection of susceptible cells. The vaccinator decides on the size of the skin area to be injured: whether to inoculate at one or more sites, how long or deep (10) the scratch. A proper vaccination technique is, however, in large measure a matter of skill (4, 5, 10). A discussion of virus dose must therefore include some consideration of the dermal lesions to be made. I do not intend to argue the matter of scratch versus multiple pressure, because I feel that a vaccinator must find out for himself which method gives him the best results.

In the course of many decades of smallpox vaccination, the number of inoculation sites per individual has gradually been reduced. This trend seems to be partly a concession to public feeling. Several studies have shown a correlation between protective effect and number of scars (23, 27, 35, 48). The vaccination illness, however, would seem to be milder in the case of one take (23, 35).

At present a single inoculation (6 mm linear scratch or multiple pressures on an area 3 mm in diameter) is recommended for routine primary vaccination (53). Such a policy will probably meet with few objections from the population and may therefore tend to promote smallpox control. It is a stringent necessity that, presupposing a proper vaccination technique, the virus content of the vaccine still guarantees a primary take rate of virtually 100 per cent. We found

that a log pock count (CAM) of 8 will do for this purpose, at least for some strains of vaccinia virus (43).* It is wishful thinking to assume without further study that all vaccine strains in use will behave similarly. A log titer of 8 at delivery from the manufacturer still leaves a margin for a subsequent slight loss of infectious units, but failures will certainly result from substantially lower values.

What effects are to be expected from variation in virus titer? In discussing this question I deliberately leave out of consideration the consequences of discrete quantitative response. This means the site take rate per person when inoculation is performed in man at multiple sites, or the number of lesions in rabbits when vaccination is performed for the purpose of titration, for instance, on a large scarified area or by multiple intracutaneous injections of serial dilutions (22, 49). The discussion is thus restricted to the effect of virus titer in the case of uniform response—one take when inoculation is done at one site or two takes if two sites are chosen.

A take from a vaccine with a relatively low titer appears to be of full value with regard to antibody formation (9, 17) and skin resistance (9). There is, however, some delay in the start of pustule formation (9, 16, 41), skin resistance (9), and vaccination illness (16, 42). This may be of importance if vaccination is performed under an actual threat of smallpox. Otherwise there is no reason to require vaccines of extra strength for the sake of protection.

The data on correlation between virus titer and ensuing vaccination illness are somewhat conflicting. From our observations in primary vaccination of young adults (42) such a correlation could not be inferred. Espmark's study (16) of primary vaccination of infants and children four to ten years old, suggests, however, that the titer has some significance. It is not clear how the number of infectious units applied on and in the skin could unfavorably influence the course of subsequent fever provoked by an autonomous focus of infection, unless through the size of the focus and the timetable of its development. It would seem that there must be an upper limit to such an effect, and from a practical point of view our main interest should be focused on a possible pathogenic gradient within the range of full-strength titers that ensure a take rate of virtually 100 per cent. Such a gradient, if demonstrated by the use of weaker vaccines, is of biological interest but has no impact on the use of vaccine of the required virus titer. Our observations in two groups of 66 recruits after primary vaccination with calf lymph (Copenhagen strain) are summarized in Figure 1. Undiluted vaccine was applied in one group (log pock counts 9.0 and 8.5 for 56 and 10 vaccinees, respectively) and 1:10 diluted vaccine in the other (log pock counts 8.0 and 7.5 for 56 and 10 vaccinees, respectively). Only minimal differences between the undiluted and the diluted vaccines are discernible. The frequency of illness is slightly lower for diluted lymph, but the length of fever and of medical care is a little more favorable for undiluted vaccine.

In conclusion, there is as yet little or no reason to set an upper limit to the virus concentration of smallpox vaccine to ensure a more benign course of the vaccination illness. There is no evidence that the nature and frequency of complications can be limited simply by setting a maximum level for virus concentration. Inoculation at more than one site is indicated, as a matter of safety, only for smallpox contacts (53).

THE DOSE FOR REVACCINATION

It might seem from the foregoing that pock counts substantially above 10^8 would mean waste of a vaccine that is so much needed in many parts of the world. However, successful results are more difficult to obtain in revaccination and in primary vaccination of infants than in primary vaccination of adults.

Log concentration values of infectious virus units for several groups of vaccinees for 80 per cent response—the highest level common to all groups—are shown in Table 1. These data were derived from dose-response lines of studies in Sweden (14, 15, 18) and in the Netherlands (43, to be published). In view of the differences between the two countries in units of virus concentration and in modes of vaccination, it seems better to compare the log values only within

* In this trial vaccination has been performed by two 3 mm linear insertions.

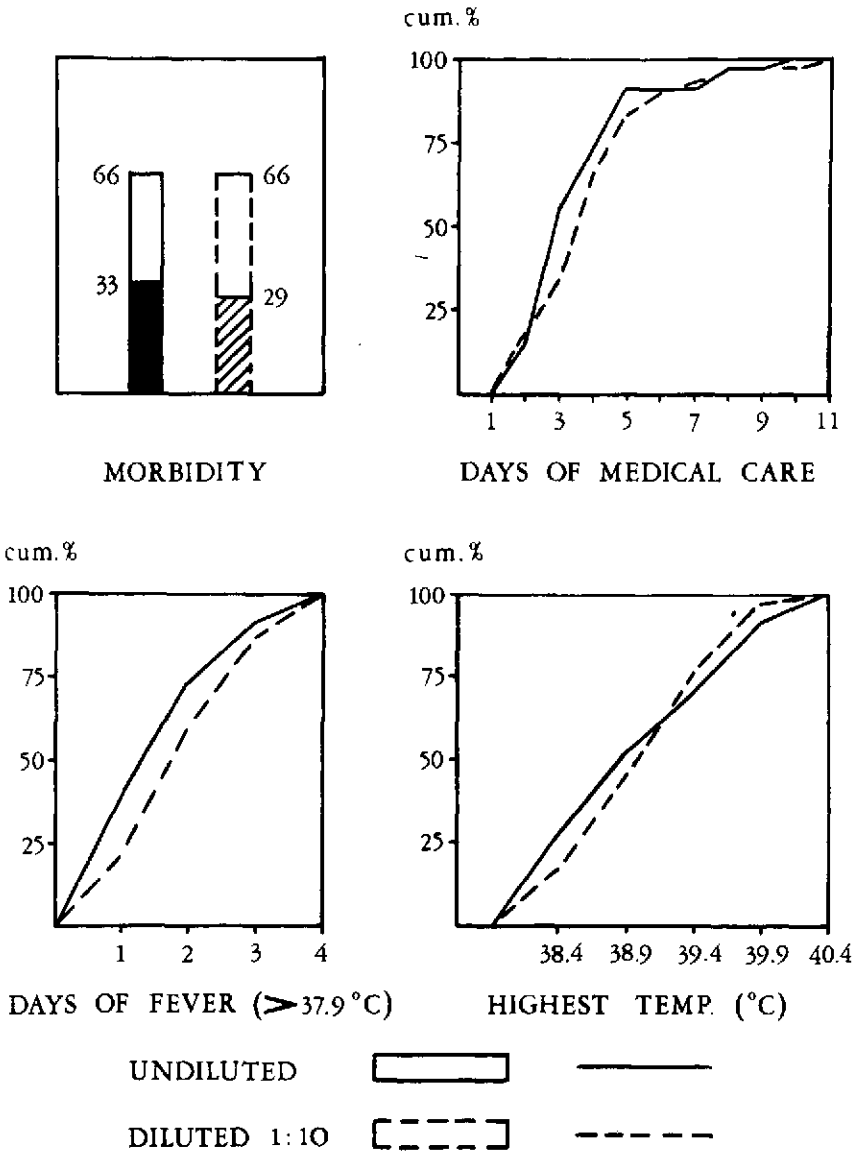


Fig. 1. Clinical observations after primary vaccination (two takes) of young adults inoculated with undiluted or 1:10 diluted calf lymph, Copenhagen strain.

TABLE 1. APPROXIMATE LOG DOSE FOR 80 PER CENT RESPONSE*

Country of study and dose unit	Primary vaccination			Revaccination (years since last vaccination)			
	Young adults	Infants 5–12 months	Infants <10 weeks	10–12	>30	13–29	6–12
Sweden† monkey kidney cells ID_{50}/ml	5.6	6.2	7.1	6.6	—	—	—
Netherlands‡ CAM PFU/ml	6.7	—	—	—	7.4	7.7	8.2

*Unit of quantal response for Sweden: Site of multiple pressure; for the Netherlands: vaccinee with two linear insertions 3 mm each in primary vaccination, 7–10 mm each in revaccination.
†References 14, 15, 18.
‡Reference 42 and to be published.

countries.* From this comparison the conclusion emerges that at least a tenfold increase in virus titer would be required to obtain results in revaccination that are as satisfactory as those in primary vaccination. This is quite apart from the relative flatness of the dose-response line that may be expected and that indeed is observed in revaccination because of the large variation in the immune status of people being revaccinated.

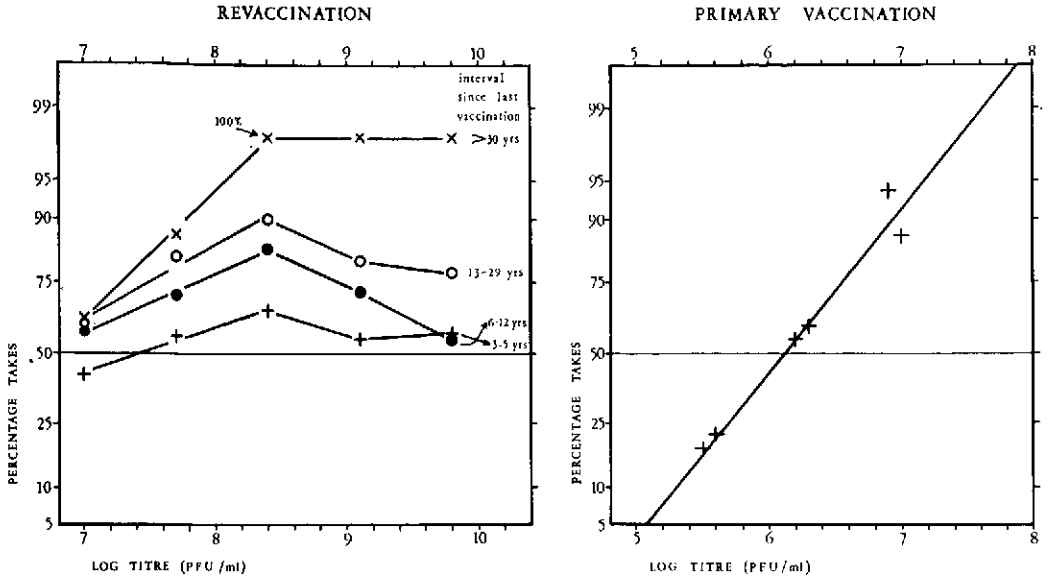
The figures in the right hand column of Table 1 were derived from an experiment on primary vaccination (43) and from a recent study on revaccination that was done in collaboration with Dr. J. Huisman of the Municipal Health Service in Rotterdam. As might be expected in a large seaport town, the population to be revaccinated was heterogeneous—from ship crews to couples going to visit children settled abroad. Undiluted calf lymph, Elstree strain of an exceedingly high log pock count of 9.8 was used, together with four fivefold serial dilutions, all on a random basis. Since two of the dilutions had a log pock count below the required limit of 8.0 (7.7 and 7.0), all negative readings were followed by a repeat revaccination, so that an international certificate could be issued.

Figure 2 shows, on a log-probit scale, the take percentages (major reactions) obtained with two 7 to 10 mm linear scratches in 890 revaccinations in four groups distinguished by length of interval since the preceding vaccination. For the sake of comparison, data from our earlier study on primary vaccination are also given, with results for the Copenhagen and Elstree strains combined.

In the dosage range from 7.0 to 8.4 a nearly linear dose-response relation can be observed. The steepness of the slope increases with the interval since the preceding inoculation. Distinct response differences can be observed between the various interval groups when a vaccine of log titer 8.4 is used. Vaccine titers of 9.1 and 9.8 seem to do no better than 8.4. The response even decreases somewhat for all intervals below 30 years. The reason for this reduction is not clear. It may be that there is a mutually inhibiting effect between virus units in high concentration, or that an inhibiting factor (interferon induction?) is present, the effect of which disappears with dilution. This subject needs further study. However this may be, there appears to be a limit to the response percentage. This conclusion had been reached earlier in a study with Dr. Huisman (24). In 675 revaccinations virtually no differences were found between vaccines with log pock counts of 9.2 (undiluted), 8.7, and 8.2.

In these studies, response was judged positive

* Since many different titration techniques are used in virus laboratories it is highly desirable that in all publications the strength of the International Reference Preparation of Smallpox Vaccine (30), as indicated by the local method of titration, be given. The titrations of all vaccines used in the Netherlands studies mentioned in this report were performed by Miss J. M. Bos in the Smallpox Vaccine Laboratory of the Rijks Instituut voor de Volksgezondheid, Utrecht. The strength of the International Reference Preparation of Smallpox Vaccine has been assayed by the pock count method on CAM; a log pock count of 8.4-8.5 per ml was found.



Note: The primary vaccination data summarize observations obtained with two lymphs (Copenhagen and Elstree strains); two observations at zero response level not entered.

Fig. 2. Relationship between pock count and response in primary vaccination (43) and in revaccination (to be published) on log-probit scale.

or negative on the basis of the local skin reaction after one week. We know, however, that the absence of a major reaction in revaccination does not mean that serum antibody is not being produced. Some of the so-called immediate—second- or third-day—reactions are also coupled with an increase in antibody (5, 10, 31, 32, 45). A reading of major reactions therefore underestimates the immune response. Whatever this may contribute to the explanation of the observed limit in response, on the basis of our present data no useful purpose would seem to be served by applying vaccines of a log count above 8.4. Some improvement in the group take rate may be expected from inoculation at multiple sites.

In conclusion, except perhaps for very young infants of vaccinated mothers, log pock counts of some tenths above 8.0 would seem a good choice both for primary vaccination and for revaccination. As a rule the use of such a vaccine is guaranteed if the WHO requirements are fulfilled and the vaccine is handled with care. Under adverse conditions lyophilized vaccine is preferable.

VIRUS STRAIN

Local cutaneous vaccinia confers protection against smallpox. The protection is not durable, but in a well-organized and well-conducted campaign general vaccination will lead to the elimination of endemic smallpox. Thus was the situation 30 years ago in Indonesia, where dried vaccine was in use (21). The inconveniences, even the small risk of serious complications, associated with vaccination will not weigh heavily in a country on the road to effective smallpox control.

In many parts of the world, however, smallpox is merely a menace from outside that is met by border controls as a first line of defense and vaccination of specially exposed groups as second line; routine vaccination of the general population has become in these areas a matter for argument (11, 29). The main objections raised have been to the direct and indirect pathogenic properties of vaccinia virus. The somewhat cumbersome cutaneous mode of administration is another weakness in the present procedure, since it leaves ample room for technical failures (4, 10). The discussion centers on the question of balance—or imbalance—between the actual and continual burden imposed on a population by routine vaccination and the benefit from strengthened protection against the ever-present threat and potential spread of a terrible disease. The relative significance of these two aspects of the

problem varies from one country to the other and cannot be easily quantified.

Certainly, the present situation is unsatisfactory from two points of view. First, among the present-day assortment of generally accepted immunizing agents, smallpox vaccine stands out as a cause of frequent illness and of less frequent but serious complications. Second, whereas in the development of a new vaccine it is customary to choose the antigen most appropriate from the standpoint of innocuity and effectiveness, the vaccinia strains cultivated in vaccine institutes are in a sense legacies of the nineteenth or even eighteenth century, of ill-defined origin.

It would thus seem useful, as a first step toward improvement, to determine the innocuity and effectiveness of the various vaccine strains. Subsequent screening procedures might then lead to the selection of a limited number of strains, if not to a single strain, for vaccine production. Soundly based requirements with regard to protective qualities could then open the way for attenuated and killed vaccines of equal value. We are as yet far from this situation.

Pathogenicity

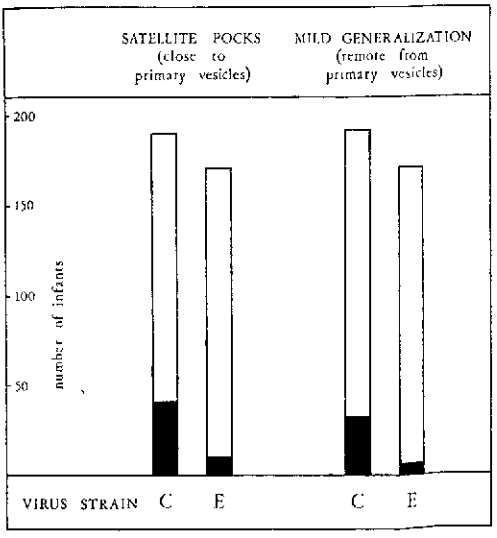
As a first approach to the selection of a strain of low pathogenicity, four vaccinia strains were investigated in a comparative trial of primary vaccination of young adults in the Netherlands (42). Distinct differences between strains were observed in respect to morbidity and the course of fever. These may be summarized as index numbers indicating the morbidity and the number of days of high fever per vaccinee (Table 2). A standard value of 100 was given to the results with the Copenhagen strain, which at the time of the trial was in use in the Netherlands. The findings brought about a change in the vaccine strain, particularly since a comparative study in infants also showed a more favorable course of fever for the Elstree strain than for the Copenhagen strain (44). Furthermore, 134 mothers in one study group were questioned about the severity of their children's infection and the Elstree strain clearly emerged as the most benign. Finally, these strains showed a marked difference with regard to the occurrence of secondary pocks (Fig. 3). If these secondary pocks appear close to the primary lesion they are

TABLE 2. COMPARATIVE PATHOGENICITY OF VACCINIA STRAINS IN PRIMARY VACCINATION OF YOUNG ADULTS*

Vaccinia strain	Morbidity†	Days with fever >38.7° C †
Copenhagen‡	100	100
Bern	96	94
Ecuador	63	56
Elstree	41	27

*From data observed in comparative study groups.
†Calculated on vaccinees with two pocks.
‡Given an index value of 100 as standard.

called satellite pocks; if elsewhere on the skin they may be considered as a symptom of predominantly hematogeneous, mild generalization. This mild generalization is certainly not a major complication (the efflorescences appear about the tenth day, remain small, and do not develop to real pustules), but it is a source of anxiety to parents and perhaps of infection to susceptible contacts. If the frequency of mild generalization seems rather high in this series of observations, it must be taken into account, first, that vaccinia virus need not be the true cause of *all* reported cases and, second, that this symptom is most frequently seen in two-to-three-month-old infants of unvaccinated mothers (51). In the Netherlands infant vaccination is performed at a rather early age, and many women of childbearing age



C-Copenhagen strain; E-Elstree strain.

Fig. 3. Frequency of secondary pock counts in young infants.

were born in a period of low infant vaccination rates.

The distribution and simultaneous appearance of the crop in mild generalization strongly suggests virus dissemination through the bloodstream. The Copenhagen and Elstree strains thus seem to differ in their viremic tendency. A similar difference recently claimed by Brandis *et al.* (6) for the Bern and Elstree strains on the strength of laboratory methods is not convincing, since the virus isolates were not sufficiently identified. McCarthy *et al.* (32) found rather high neutralizing-antibody levels in sera from three cases of generalized vaccinia after primary vaccination. It may be, therefore, that a viremic phase contributes to the immune response. In view of the concomitant clinical symptoms, this is hardly an advantage.

Since 1963, when the Elstree strain replaced the Copenhagen strain in the manufacturing of smallpox vaccine in the National Institute of Public Health in the Netherlands, there have been few complaints from the medical profession about mild generalization after primary vaccination of infants. The situation with regard to the occurrence of more serious complications since the introduction of the Elstree strain is not yet clear. The data currently available are not sufficient for a considered judgment.

A useful lead to strains of low pathogenicity for humans would be given if the different index values in Table 2 had a counterpart in laboratory experiments. From the results obtained in the international assay on behalf of the International Reference Smallpox Vaccine (30) it would seem that no accurate indication can be expected either from the LD_{50} in chick embryos or from that in mice. In comparison with the Elstree strain, the Ecuador strain is of very low pathogenicity for infant mice (1). This finding does not correspond with the figures in Table 2.

Protective Quality

What really matters in considering the protective quality of any strain of vaccinia virus is its effect under conditions of natural smallpox. A smallpox vaccine is expected to give a substantial decrease in morbidity and in case fatality and to create a herd immunity that causes variola virus to lose its foothold in the various regions where it is endemic so that in the end global eradication is attained. Numerous strains of vaccinia virus are used to realize these expectations.

It has been suggested that, over the long history of smallpox vaccine, strains clearly deficient in affording protection have been eliminated from vaccine production. This may be true, but it is not certain that equally and optimally effective strains were thereby selected (29).

So far as analyzing the mechanism of immunity conferred by vaccination is concerned, the following elements of the pathogenesis of smallpox seem to be pertinent. From an initial focus of infection in the respiratory tract, presumably followed by virus multiplication at various internal sites, variola virus is disseminated throughout the body by the bloodstream. This marks the onset of disease, after an incubation period of about 12 days (13). Acquired immunity seems, therefore, to have several potential points of operation, and incubation takes long enough to elicit an effective secondary immune response by the invading virus. The protective effect of a previous vaccination could be brought on by interference at the stages of preliminary virus multiplication and viremia.

Challenge of skin resistance after vaccinia infection is a time-honored procedure for testing acquired immunity. In Jenner's day, human beings could be chosen as test subjects and variola virus as the challenge. Although similar studies have been done in monkeys (47), for a long time now the procedure of skin revaccination of humans with a potent vaccinia preparation has been applied, not just to boost immunity but also to test the immunity remaining from the preceding primary vaccination.

In this way a comparison can be made between two or more vaccines used in primary vaccination. The groups of vaccinees should not, of course, differ in any other respect that might influence the remaining immunity, such as the number of takes in primary vaccination, the interval since primary vaccination, and the challenge procedure. Not all these conditions were fulfilled in a study by Pandit and Venkatraman (37), who challenged four years after primary vaccination performed with calf lymph and CAM lymph respectively. According to the data given, it is highly probable that the number of primary takes per individual differed for the two groups; furthermore, challenge with heterologous lymph

(calf vaccine after primary vaccination with membrane vaccine) was done at a later date than with homologous lymph (calf vaccine after primary vaccination with calf vaccine), so that the challenge doses may have varied.

In another trial (7) two vaccines of calf and chick embryo origin were tested in a large population of children with a past history of vaccination that was not reliably known; many of the children had scars of older vaccinations. Challenge with calf lymph was done after one year, and no differences between the two groups emerged if the first vaccination in the trial had resulted in a take. This does not necessarily mean that the two vaccines tested would have been equivalent with respect to skin resistance after primary vaccination.

These examples are given to demonstrate the difficulty of obtaining in actual practice, truly comparative results for large groups. This holds even truer if we wish to test the long-term effects of a previous inoculation. With present-day vaccination procedures the smallpox attack rate is still markedly reduced after ten years (12).

A further point about the interpretation of revaccination results must be made. Primary, accelerated, and immediate types of response are generally considered to serve—in that order—as indicators of low, medium, and high degrees of immunity. This may be true insofar as this classification reflects the absence or presence of a focus of infection and its size. Mention has been made earlier, however, of the equivocal immunological significance of a response that is not a "major reaction." Pirquet's classic observations (41) demonstrated long ago the allergic component that contributes to the cutaneous lesion, which nowadays is understood as delayed hypersensitivity. There is further recent experimental evidence that delayed hypersensitivity is of paramount importance in the formation of the primary lesion (19, 38, 39, 40). The picture of the challenge lesion thus seems to be a complex of virus multiplication and allergic reaction due to virus constituents.

The significance of hypersensitivity in acquired immunity to smallpox, as well as other virus diseases, is a matter for argument (46, 54). The waning of clinical immunity after smallpox vaccination somehow runs parallel with alterations in local skin lesions by vaccinia virus that are interpretable as degrees of hypersensitivity. This suggests, but does not prove, a causal relationship. However, from experiments of Turk *et al.* (52) a slight depressive effect on vaccinia virus multiplication at the site of inoculation can be inferred. Such an effect was also found if delayed reaction was provoked by an unrelated antigen such as tuberculin. I hesitate to endorse the conclusion that "it is unlikely that this slight effect on virus multiplication plays a significant part in spontaneous remission of vaccinia virus infection." On the other hand, Baldridge and Kligman found that desensitization of previously infected guinea pigs by daily injections of killed vaccinia virus did not result in a loss of immunity (3).

Clinical cases of malignant progressive vaccinia coupled with absence of delayed hypersensitivity (20, 27) suggest that this characteristic has some significance for recovery from disease and also for the inhibition of the preclinical development of infection in a sensitized person.

Postvaccinal hypersensitivity in humans and rabbits has been studied by Stickl and Engelhardt (50) by means of a leukocytolysis test. The white blood cells of vaccinated individuals are impaired by vaccinia virus, and semiquantitative estimates show a correlation with the interval since the preceding inoculation. If the results of these tests are reproducible they may, as the authors suggest, reveal differences between various smallpox vaccines and between variola and vaccinia viruses.

Also on the subject of hypersensitivity, the study of McNeill (34) must be mentioned. Semiquantitative assessment of skin resistance in rabbits following immunization with inactivated vaccinia virus vaccines demonstrated no correlation with semiquantitatively estimated hypersensitivity.

It must be said, as a general remark, that the antigens used in testing for hypersensitivity are far from uniform and may not be equivalent when it comes to evaluating a possible relationship between acquired resistance to infection and delayed hypersensitivity.

The role of virus-neutralizing serum antibodies in acquired immunity to smallpox and vaccinia is somewhat less controversial than the subject of hypersensitivity. The stage of viremia and other phases of extracellular virus dissemination would give ample opportunity for antibody to

exert a protective effect. Even more convincing is the demonstrated preventive effect of vaccinia-hyperimmune gamma globulin in contacts of smallpox cases (28).

The extent of correlation between virus-neutralizing antibody and skin resistance as indicated by the minimum infecting virus dose is not clear. McNeill (33, 34), using inactivated virus vaccines in rabbits, found both factors to be dependent on the dose of antigen. Though there was some relationship between antibody titer and skin resistance, it was not very precise. A similar conclusion may be drawn from several other studies (2, 25, 31, 45). This may perhaps be due in part to a rather crude method of testing for skin resistance. Kaplan *et al.* (26) found a take in 18 out of 20 infants and young children after two doses of ultraviolet-irradiated noninfectious vaccinia virus when challenged with undiluted live smallpox vaccine. Prechallenge sera from these children contained neutralizing antibody on the same level as in a control group after inoculation with live vaccinia virus. It is remarkable that all takes after challenge are described as typical primary reaction, so that seemingly this noninfectious vaccine lacked sensitizing capacity. Nevertheless, a take rate of 90 per cent might still be the expression of a substantial skin resistance, depending on the technique of inoculation and the titer of the challenge virus.

The proper role of neutralizing antibody in acquired immunity cannot yet be defined. The evidence available indicates that in some way these antibodies are connected with the state of passive and active acquired immunity. On the basis of our present knowledge, a virus strain evoking comparatively low levels of neutralizing antibody one year after primary vaccination, as was found for the Rivers vaccinia strain propagated on CAM (36), cannot be recommended as a substitute for a "classic" vaccinia strain in the manufacturing of smallpox vaccine.

This summary of the present knowledge of the factors responsible for acquired immunity against smallpox is rather sketchy. Certainly our knowledge is unsatisfactory. It is not yet possible to indicate how the protective qualities of various vaccinia strains can be validly compared. Tests of skin immunity in humans and neutralizing-antibody response are used more or less tentatively. Until more is known about interpreting the results obtained with these methods, the use of strains that have been shown to give a solid immunity under conditions of exposure to natural smallpox is indicated.

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SECTION A. SMALLPOX

PROBLEMS OF MASS VACCINATION PROGRAMS

J. D. MILLAR

Smallpox Eradication Program, Communicable Disease Center, Atlanta, Georgia, U.S.A.

DR. MILLAR (*presenting the paper*): Many countries have conducted mass smallpox vaccination programs since World War II. These have embraced a wide range of technical and administrative aspects. Under the title of this paper one might properly discuss anything from epidemiologic problems to the timely arrival of personnel pay checks. Instead I shall discuss briefly four major difficulties that have troubled many mass programs: (1) maintenance of vaccine potency under field conditions, (2) individual vaccinator variability, (3) insufficient population coverage, and (4) gradual attrition of interest and support. The first three might be considered fundamental problems; the fourth is generally a product of the other three.

VACCINE POTENCY IN THE FIELD

Cockburn and others (1) and Espmark (2) showed that essentially universal success in primary vaccination and in revaccination may be expected with vaccines of titer greater than 10^8 PFU/ml. Since both are involved in mass campaigns, this level of potency must characterize the vaccine that actually reaches the skin of the persons inoculated.

This is difficult where heat-labile lymph vaccine has been used. As one example, Indian calf lymph vaccine, adequate for primary vaccination and well refrigerated until use, was compared with English freeze-dried vaccine in revaccination (3). The results showed a take rate of 67 per cent for the English vaccine and 27 per cent for the Indian lymph. The normal revaccination take rate for Indian lymph was 7.5 per cent in field use. Differences in initial titers probably accounted for the 67/27 per cent difference observed between Indian and English vaccines, but the difference between the usual 7.5 per cent rate for Indian lymph and the 27 per cent rate observed in the study illustrates the capriciousness of liquid lymph potency under field conditions. This problem has been satisfactorily resolved only by avoiding the use of lymph vaccine in the field and employing the more stable freeze-dried product.

VACCINATOR VARIABILITY

A recurrent problem of mass programs has been variation in the techniques of individual vaccinators. Multiple pressure, scratch, and rotary lancet methods are satisfactory in the hands of good operators. All are slow, however, and a vaccinator performs only 75 (4) to 200 (5) such vaccinations in a day. Therefore, large numbers of vaccinators are required if a reasonable rate is to be maintained; generally a low-level public health employee is put to this task, and wide variation in techniques is the common result.

This is illustrated in Table 1. In five Indian districts primary take rates varied over a range of more than 25 per cent (4). The same vaccine was used in all areas: freeze-dried vaccine provided by the USSR. The differences may be attributed to differences in vaccinator technique. The impact of these differences on the proportion of persons actually protected is shown dramatically in the final column of the table. Even though Ganganagar led the group in the pro-

TABLE 1. SMALLPOX VACCINATION STATUS FOLLOWING CAMPAIGNS IN FIVE INDIAN DISTRICTS, 1963–1965

District	Vaccinated (%)	Primary vaccination take (%)	Estimated max. immunized (%)
Mysore	79.5	92.4	73.4
Palghat	79.2	86.2	68.3
Chingleput	65.6	94.2	61.8
Varanasi	66.4	80.8	53.7
Ganganagar	81.9	68.9	56.4

Source: Gelfand, H. M. "A Critical Examination of the Indian Smallpox Eradication Program," *Amer J Public Health* 56:1634–1651, 1966.

portion vaccinated, the low take rate there resulted in a next-to-lowest level of protection.

This situation is particularly unfortunate; the considerable labor involved in reaching the people is often wasted by unnecessary inadequacies in vaccinator technique. A hopeful solution to this problem lies in the newer jet-injection techniques that will be discussed.

POPULATION COVERAGE

It appears impossible to define a minimum proportion of population coverage that is broadly applicable. The necessary level depends on many local factors—population density, disease patterns, migration, and so on.

A frequently mentioned goal is 80 per cent coverage. It is true that some countries, such as Iraq (6) and Bolivia (7), have eradicated smallpox after vaccinating approximately 80 per cent of their populations. On the other hand, although India reached the 80 per cent level in October 1965 (8) more than 3,000 cases of smallpox and 800 deaths occurred during the remainder of the year (9). In parts of Brazil such as Rio de Janeiro and Maceió, dramatic reductions of disease followed campaigns that resulted in only 50 per cent population coverage (10). There seems to be little magic in the 80 per cent figure; it probably reflects only the fact that it is always difficult, as Hinman puts it, "to reach more than 8 out of 10 people" (11). The only reasonable criterion is the interruption of transmission. Until this is achieved, coverage is inadequate.

Table 2 illustrates the wide variation in coverage rates that occurred in several Brazilian campaigns. With older techniques it would appear that, in general, vaccination-post-oriented campaigns are less satisfactory than house-to-house methods. The one post-oriented jet-injection campaign resulted in a high coverage level.

ATTRITION OF INTEREST AND SUPPORT

Reduced vaccine potency, vaccinator error, and inadequate coverage frequently combine to preclude interruption of transmission despite vigorous and costly activities. The result has frequently been delay in achieving objectives (if not failure to do so), repeated postponements of completion deadlines, rising expenditures, and occasionally the need to repeat entire programs.

PROSPECTS FOR RESOLUTION OF PROBLEMS

Freeze-Dried Vaccines

The most important advance in smallpox control since Jenner's introduction of vaccination,

TABLE 2. COVERAGE OF COMPLETED CAMPAIGNS IN SELECTED AREAS (BRAZIL, 1963–1965)

Area	Population	Campaign methodology	Coverage (%)
Belém (Pará)	475,000	MP	
		House-house	86.3
Rio de Janeiro (G.B.)	3,800,000	MP-Post	50.3
Rio Grande do Norte State	1,200,000	MP-Post and	
		House-house	72.4
Macapá (Amapá)	37,000	Jet-Post	92.3

Source: Campanha Nacional contra a Varíola, Ministerio da Saúde, Rio de Janeiro, Brazil.

has been the development and widespread administration of highly stable freeze-dried smallpox vaccines. The proper use of these products virtually eliminates problems of maintaining vaccine potency under field conditions. As the WHO Expert Committee on Smallpox advised, "In all programs . . . very high priority must be given to providing adequate quantities of potent freeze-dried vaccine" (12).

Jet-Injection Vaccination Techniques

The introduction of intradermal jet injection offers hope for resolving other major problems. This technique, now extensively tested by the Communicable Disease Center in the United States, Jamaica, Tonga, Brazil, and West Africa, promises a near-revolutionary change in concepts of mass vaccination.

Among the fundamental advantages of jet injection are a consistency of technique virtually impossible by other methods and a dramatic increase in speed of operation. Further, since 0.1 cc of vaccine is injected intradermally, it is possible to dilute the vaccine as much as 10 times without jeopardizing high take levels. Table 3 presents data on vaccination effectiveness with jet injection. Vaccines diluted 10 times and administered with jet injector produced highly consistent primary and revaccination take rates irrespective of the local area or the personnel involved. This is because vaccine delivery is consistent and vaccinator variability is virtually eliminated. The operator has only to place the apparatus next to the subject's skin and pull the trigger. Opportunities for error are quite limited.

That this consistency makes possible a high effective vaccination rate is illustrated in Table 4. Campaigns were conducted in two small Brazilian towns—one by house-to-house multiple-pressure vaccination, the other by jet injection. The house-to-house technique reached a greater proportion of the people; the jet-injector technique showed a consistently higher take rate. Thus, in both towns the proportion of persons effectively vaccinated was about equal. Eight times as many people were required to do the job by house-to-house multiple-pressure vaccination as were required for the larger campaign done by jet injector.

With respect to population coverage the jet injector offers striking effectiveness in urban areas. In a campaign in Macapá, Amapá Territory, Brazil, 92 per cent of the 36,000 inhabitants were vaccinated in three days with seven jet injectors (13).

Table 5 contrasts logistical and cost data for two urban campaigns; the Macapá campaign was conducted by jet injection and the Belém effort by house-to-house multiple-pressure methods (14). These data suggest that conducting urban campaigns by jet injection would result in substantially reducing present costs.

The estimated cost of seven jet injectors used in the Macapá campaign cited in Table 5 is a prorated cost of the injectors over the course

TABLE 3. TAKE RATES FOLLOWING JET INJECTION OF 0.1 CC OF DILUTED SMALLPOX VACCINES (CDC STUDIES, 1963–1965)

Category	Place	Vaccine	Dilution*	No. vaccinated	Take rate
Primary	Jamaica	U.S. dried	1–10	165	97.6
	Jamaica	U.S. dried	1–50	112	97.3
	Tonga	U.S. dried	1–10	6,358	99.6
	Tonga	U.S. dried	1–50	29,414	98.8
Revaccination					
Late	Georgia	U.S. dried	1–10	23	100.0
	Brazil	Brazil-calf	1–50	634	86.7
Early	Georgia	U.S. dried	1–10	28	96.4
	Jamaica	U.S. dried	1–10	161	97.5

*1–10 has titer of about 1×10^7 $TCID_{50}$/ml.
1–50 has titer of about 2×10^6 $TCID_{50}$/ml.

TABLE 4. COMPARISON OF HOUSE-TO-HOUSE CAMPAIGN USING MULTIPLE-PUNCTURE TECHNIQUE WITH JET-INJECTOR CAMPAIGN IN TWO SMALL BRAZILIAN CITIES

	Mazagão	Amapá
Urban population	974	1638
Campaign method	House-to-house	Central medical post street-by-street mop-up
Method of inoculation	Multiple puncture	Jet injector
No. of personnel utilized	38	5
Vaccination per man/hour	8.0	40.5
Per cent of total population vaccinated*	89.6	78.6
Over-all take rate (%)*	80.8	90.1
Primaries (%)*	84.6	95.3
Revaccinees (%)*	76.1	86.7
Per cent effective vaccination of available population	72.4	70.8

*Based on immunization survey conducted seven days following day of vaccination.

of this particular campaign based on a conservative estimate of the life expectancy of the instrument. This figure was derived as follows:

1. For purposes of calculation, the jet injector is considered to have a life expectancy of one year. (With proper use and maintenance, it should last far longer, and subsequent costs would be only for necessary spare parts.)

2. During a year of reasonably continuous use, the average number of vaccinations expected daily from a single gun is 2,500. A year is assumed to consist of 200 working days; thus, 500,000 vaccinations would be performed in a year with each gun.

3. The cost for a single jet injector, including a liberal supply of spare parts, would be as follows:

$600	jet injector, spare-parts kit, and two subcutaneous heads
170	two intradermal heads
230	additional spare parts
$1,000	

4. Charging the entire cost of the jet injector against a single year's operation indicates a cost of $1.00 for every 500 vaccinations. For the more than 35,000 vaccinations done in Amapá,

TABLE 5. COST ANALYSIS OF TWO URBAN SMALLPOX VACCINATION PROGRAMS IN NORTHERN BRAZIL

Campaign data	Macapá*		Belém†	
	No.	Cost ($)	No.	Cost ($)
Population	35,700		450,000	
No. of vaccinations	32,700		411,000	
Professional man/days	6	72.00	10	120.00
Local man/days	120	480.00	6,000	24,000.00
Vehicle days	6	60.00	250	2,500.00
No. of tubes of vaccine	30	9.00	3,000	900.00
Jet injectors	7	70.00	0	
TOTAL COST		691.00		27,520.00
Cost per vaccination		0.021		0.067

*Mass immunization program utilizing jet injectors.
†House-to-house multiple-pressure program.

the prorated jet-injector cost thus approximates $70.

If anything, the cost figure used is higher than might be expected under continued field use.

Implications of Jet Injection

As do all innovations, jet injection brings in its train new problems, but of a different nature from the old. To realize the advantages of the technique, the accepted principles of campaign organization require some change. Since people must come to "collecting points" for jet-injection vaccination, campaign publicity should capture the public imagination at every level. A reappraisal of priorities and timing is also necessary. Jet injection is best used in urban areas where people readily congregate (and where, in most instances, the mainstreams of disease are). Cities deserve the highest priority in national jet-injector programs so that a large number of immunes can be rapidly accumulated and public and official enthusiasm aroused. Of course, there is a significant role for multiple-pressure and other techniques in "mop-up" activities and in areas that are sparsely populated.

These and other new issues often have to do with what the business world calls "advertising and marketing." While such concepts have imaginatively developed in other fields, the traditional conservative attitudes in public health will require reorientation to accommodate this new approach.

OBJECTIVES OF MASS VACCINATION PROGRAMS

Vaccination programs are not an end in themselves. Even when conducted by jet injection, they are expensive, time consuming, and demanding of a country's health resources. Except for epidemic control activities, major programs should be undertaken only with the goal of smallpox eradication in mind. In this context, mass vaccination programs may be viewed as only one part of a plan that must include other usually important components.

In 1954 Peru completed a highly successful national vaccination campaign and completely stopped the transmission of smallpox (15). The country was reinfected in 1963. A major epidemic resulted in the interior that necessitated an extensive vaccination program. The misfortune of Peru emphasizes two points: mass campaigns must be accompanied by adequate disease surveillance to prevent unrecognized introductions and spread from small foci; similarly, mass campaigns must be coordinated on a regional basis to reduce risks of spread across international boundaries. Without surveillance and regional coordination, the prospect is gloomy —endless repetition of mass vaccination programs.

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SECTION A. SMALLPOX

SMALLPOX VACCINES: ATTRIBUTES OF EGG AND CALF LYMPH VACCINES

J. Å. ESPMARK

National Bacteriological Laboratory
Stockholm, Sweden

DR. ESPMARK (*presenting the paper*): In most countries special institutes were established between 1860 and 1885 for the large-scale production of smallpox vaccine in the scarified skin of calves (36). During the next 50 years several improvements in vaccine control and preservation were introduced, but very few major changes in the production procedure were considered.

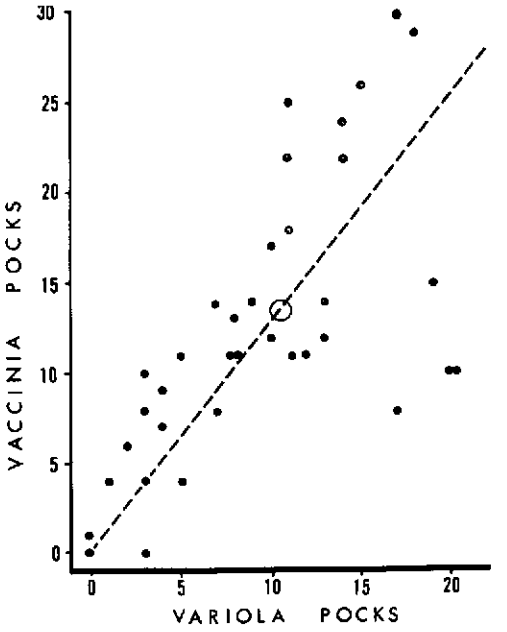
In the early 1930's some of the rapidly developing virological techniques were also tried for smallpox vaccine production on an experimental scale. Rivers and his co-workers (38, 44) used suspended-fragment cultures of chick embryo tissue, and Goodpasture and Buddingh and their co-workers (29, 31) used embryonated eggs for cultivation of vaccinia virus. Their reports also included the results of successful vaccination trials. The results of later work by the same groups of authors suggested that long-term passage in chick embryo tissue made vaccinia virus less pathogenic in rabbit and human skin than low-passage material (5, 45). On the assumption that this suggested attenuation might also imply reduced immunogenicity, most of the egg vaccine trials subsequently reported upon have been carried out with low-passage vaccinia virus (6, 7, 8, 12, 16, 18, 32, 50).

Egg vaccine has been officially approved and used in Texas since 1939 and by now almost 6 million doses have been issued for public vaccinations in that state (Dr. J. V. Irons, personal communication). The Lederle Laboratories have distributed chick embryo vaccine for about 10 years (Dr. V. J. Cabasso, personal communication). In Sweden about 4 million doses of egg vaccine have been issued since 1961 (18). Egg vaccine has also been in general use in New Zealand since 1959 (Dr. J. D. Manning, personal communication).

The production of smallpox vaccine in chick embryo offers certain definite advantages: low cost, sterility, the possibility of producing vaccine on short notice in emergency situations. On the other hand, certain alleged disadvantages have often been mentioned and have created some reluctance to use it. Some of these objections will be discussed later in this paper—questions of heat stability, infectious and immunological responses, and the innocuity of the egg vaccine as compared to calf lymph vaccine.

METHODOLOGICAL CONSIDERATIONS

The infectious response of chorio-allantoic membrane (CAM) as estimated by pock counts on eggs infected with vaccinia virus is subject to excessive variation even under good experimental conditions (2). That this variation is due not only to titration error *per se*, but also to variation in the susceptibility of eggs is demonstrated in Figure 1. Eggs were prepared for CAM inoculation by the same method as is used in full-scale vaccine production (7, 11, 18), except that two separate holes were made in the artificial air sac for separate inoculations of two distinguishable pox viruses, vaccinia and variola. Thirty-six eggs were double-infected and pock counts were taken after two days' incubation at 37°C. The counts are plotted in a



Note: Degree of correlation between the number of vaccinia and variola pocks in individual eggs reflects importance of variation in host source.

Fig. 1. Double infection experiment in chorioallantoic membrane (CAM) of embryonated eggs with vaccinia and variola viruses.

correlation diagram in Figure 1. Evidently there is a covariation of the vaccinia and variola pocks. Assuming that the two viruses have similar growth requirements, it can be estimated from the degree of correlation how much of the count variation is due to variation in eggs. Under the conditions of this experiment, the host variation is apparently important; it can probably be separated into one source that is inherent and one that is connected to egg damage during preparation. Since the procedure used in large-scale vaccine production cannot easily be much more refined than in this example, and since 200 to 400 pocks are required to make a confluent membrane, an inoculum of about 1000 pocks per CAM should be needed to ensure a good virus yield in most eggs. However, if the allantoic sac route is used, an inoculum a thousandfold larger is needed to achieve the same vaccine titers as by the CAM route (18, 33). The allantoic sac inoculation route is the most time-saving in routine vaccine production. If the seed virus contains traces of a contaminant, however, this procedure will increase the chance of carrying the contaminant over into the vaccine batch.

The egg passage level of the vaccine should be kept as low as possible because repeated passages will increase the probability that hidden agents accumulate in the material. In our hands satisfactory vaccinia titers have been obtained using seed virus of the first or second egg passage.

Since several comprehensive technical descriptions of the currently used egg vaccine production methods have been published in the past (6, 7, 11, 12, 18, 34), they will not be further treated in detail here. An especially valuable comparison between egg and calf lymph vaccines from the production standpoint has been presented by Cabasso and his co-workers (7).

STABILITY

The need for a stable smallpox vaccine, especially in tropical areas, has evoked numerous attempts in the past to find stabilizing agents or procedures. An extensive review of these efforts has been made by L. H. Collier (9). Egg vaccines have been reported by some authors to possess poor stability in comparison to calf lymph, whether stored in the liquid state or subjected to lyophilization (26, 28, 34). Several methods have been tried to improve their stability. Among the more successful have been the addition of heated serum (4, 11, 18, 30), sorbitol (8, 18), or peptone (34). Glycerol, on the other hand, does not seem to offer any significant protection (9; Espmark, unpublished data).

The sorbitol-preserved egg vaccine has been used for several years in Sweden with favorable results. A series of vaccines stored at −25°C for periods of up to four years were titrated in monkey kidney cultures at intervals (Table 1). Except possibly in one vaccine (No. E-21), no appreciable drop in titer was observed during this storage.

Of further practical interest are the following data reflecting the stability of vaccines after being issued for public vaccination (19). Vaccines are sent to vaccinators by regular mail without refrigeration and with an expiration time

TABLE 1. INFLUENCE OF STORAGE AT −25°C UPON POTENCY OF SORBITOL-TREATED EGG VACCINES (LOG $TCID_{50}$ PER ML IN MONKEY KIDNEY TISSUE)

Vaccine batch No.	Initial titers after harvest	Titers after freeze-storage for			
		1 year	2 years	3 years	4 years*
E–20	7.6	—	8.1	7.7	7.4
E–21	7.8	—	7.6	7.4	7.4
E–26	8.3	—	8.3	—	8.2
E–27	8.8	—	8.0	—	8.5
E–28	8.4	—	8.2	—	8.3
E–35	8.2	8.3	—	7.8*	
E–36	8.2	8.1	—	8.0*	
E–42	8.7	8.5	—	8.5*	
E–44	Stored as membranes		8.5		
E–45	for 2 yrs		8.6		

*Vaccine from freeze-stored final containers—plastic ampules (23)—filled soon after the previous titration.

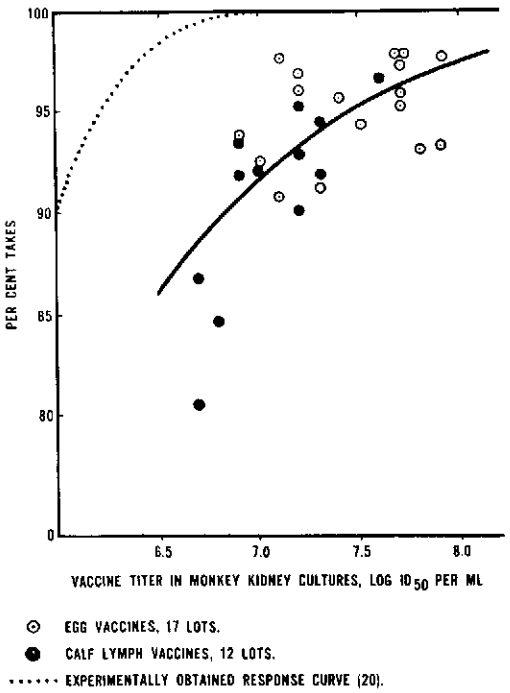


Fig. 2. Reported public vaccination take rates in about 20,000 primary vaccinations in relation to laboratory titers of egg vaccines and calf lymph vaccines issued in Sweden, 1958–1962.

of one month. In Figure 2 the reported take rates with 17 egg vaccines (circles) and 12 calf lymph vaccines (large dots) are plotted against their final laboratory titers. The solid line is drawn as the best-fitting response curve. The points representing take rates with the egg vaccines are distributed at approximately the same level in relation to the dose-response curve as those of the calf vaccines. This means that if the vaccines underwent any deterioration after issuance the egg vaccines would seem to have suffered no more than the calf lymph vaccines.

The lyophilization of egg vaccine has not yet been extensively applied in practice. Jackson and co-workers (34) reported an average initial potency loss of more than 90 per cent using egg vaccine in 5 per cent peptone. This is higher than the loss reported by L. H. Collier with sheep lymph. Better results seem to have been obtained by Cabasso and co-workers (7) with egg vaccine in 5 to 7 per cent sorbitol. It is probable that at present, with some difficulty, a lyophilized egg vaccine could be produced that after rehydration would meet the international potency recommendation (52) of at least 10^8 PFU per ml. Further improvements of the preservation techniques seem desirable, however, to make the egg vaccine economical in hot-climate areas.

RESPONSES TO EGG AND CALF LYMPH VACCINES

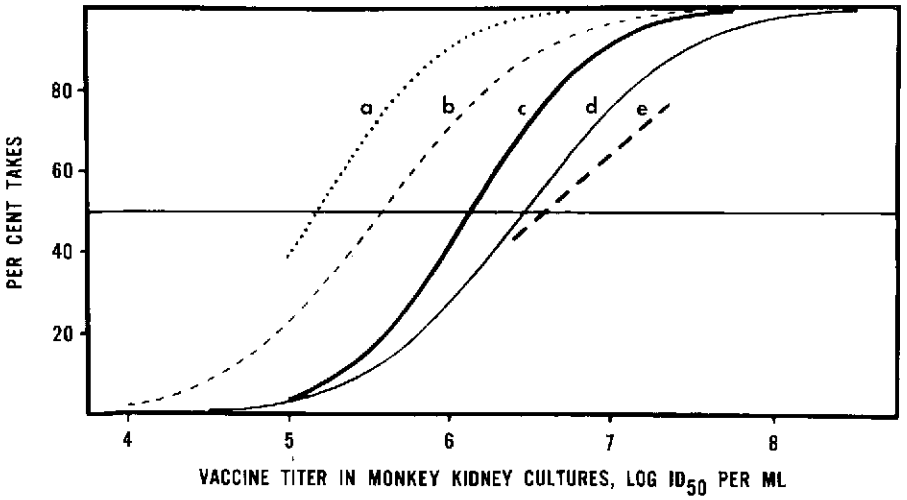
The question whether infectious and immunological responses with chick embryo vaccine can be predicted from laboratory potency data to the same extent as responses with calf lymph was brought up by the findings of Rivers *et al.* (45) and Buddingh (5). Long-term passage in chick embryo tissue seemed to attenuate the vaccinia virus so as to give relatively weak reactions in man and possibly a weaker antigenic stimulation than is found with calf lymph.

Although no such deficiencies have been noted in practice with low-passage egg vaccine (see below), a series of experiments was carried out to further clarify this point and related problems (17, 20–22, 24, 25).

When serial dilutions of vaccine were inoculated into people of several different categories, the sigmoid quantal dose-response curves obtained were essentially parallel but had different positions on the dosage scale, reflecting the varying levels of susceptibility of the groups (Fig. 3). The middle curve, representing 2,489 inoculations into 20-year-old males previously vaccinated as children (more than 10 years earlier), will be further analyzed with respect to responses obtained separately with four different vaccines.

Figure 4 shows the probit-transformed (27) percentages of takes in late revaccinations with serial dilutions of one calf lymph vaccine (CL 8/51), one tissue culture vaccine (TC-341) derived from bovine embryonic skin by the method of Wesslén (51), and two egg vaccines (E-13 and E-29). The virus strain was the same for all vaccine batches (Beaugency), and the tissue culture and egg vaccines were only two or three passages away from a calf-skin passage.

Each separate point in Figure 4 represents the results of 92 to 287 inoculations (usually about 100). All the vaccines gave very similar quantal response patterns. Statistical analysis (21) showed no significant deviation from normality or parallelism. The ID_{50} for each vaccine can be determined by interpolation in the diagram. These "human titers" have been entered in Table 2 for comparison with laboratory titers of the vaccines. From the last two columns it appears that the relationship between the qualitative vaccination response and the labora-



a. Primary vaccinations in adults.
b. Primary vaccinations in children aged 5–12 months.
c. Late revaccination in adults, more than 10 years after previous vaccination.
d. Primary vaccinations in infants less than 10 weeks old.
e. Early revaccinations in adults 1–3 years after previous vaccination.
Note: Data from two separate trials (20, 25).

Fig. 3. Types of quantal dose-response curves obtained with serial dilutions of smallpox vaccine in five populations differing with respect to vaccination history or age.

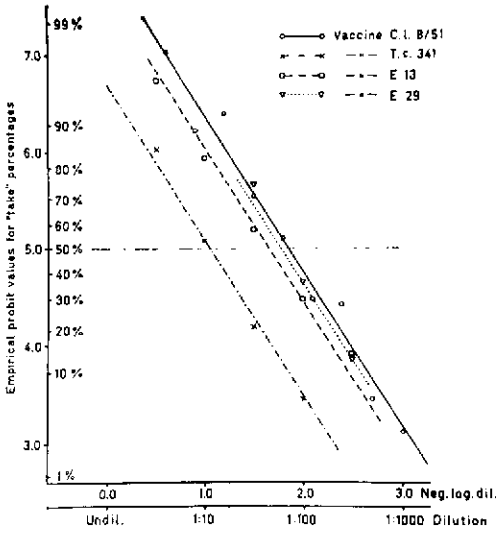


Fig. 4. Dose response curves obtained in late revaccinations (more than 10 years after previous vaccination) in young adults using four different vaccines: one calf lymph vaccine (CL 8/51), one vaccine from bovine embryonic skin cultures (TC 341), and two egg vaccines (E-13 and E-29). Success rates expressed in probits are plotted against vaccine dilutions.

tory titers is essentially the same for the calf lymph and egg vaccines.

A small trial was performed to estimate possible differences in quantitative responses to the different vaccines. Twenty-year-old males, previously vaccinated more than 10 years earlier, were each inoculated on three sites, and the local reactions, read after seven days, were scored according to an arbitrary scale (20) as large, medium, or small positive reactions or as negative reactions. It had been shown previously that sites in the same individual were biologically and statistically independent (20, 21). The first group of 85 individuals (Table 3, group A) were inoculated with three eightfold dilutions of the same vaccine. The dose dependence of the graded reactions was found to be rather pronounced, as was further verified in a later trial (22). The lower part of the table (group B) gives the results in a similar group of 52 individuals each inoculated with three undiluted vaccines—one calf lymph and one egg vaccine of equal potency and a tissue culture vaccine with a titer 10 times lower. It appears that the graded responses for the calf lymph and egg vaccines are very similarly distributed, whereas the weaker culture vaccine gave correspondingly smaller reactions.

In summary, these response studies supported the assumption that quantal and quantitative responses obtained with egg vaccine and calf lymph vaccines derived from the same virus strain are similar and are determined only by the titer of the infectious virus, not by the culture medium used for production.

The immunogenic capacity of low-passage egg vaccine has been found indistinguishable from that of calf lymph in the few cases where this has been studied. Elisberg and co-workers (16) found no significant defect in antibody production, and Cabasso and co-workers (8) obtained similar revaccination responses in two

TABLE 2. COMPARISON BETWEEN LABORATORY TITERS AND RESPONSES TO VACCINATION OBTAINED WITH CALF LYMPH, TISSUE CULTURE, AND CHICK EMBRYO VACCINES

Vaccine lot and origin	Laboratory titers		Human titers	Laboratory titers corresponding to 1 ID_{50} in late revaccinations	
	Chick embryos (CAM) log PFU/ml	Monkey tissue cultures log ID_{50}/ml	Log ID_{50} in late revaccination	Log PFU	Log ID_{50}
CL 8/51 (calf lymph)	7.78	8.00	1.86	5.92	6.14
TC 341 (embr. calf skin)	6.52	6.95	1.05	5.47	5.90
E–13 (chick embryo)	7.56	8.05	1.66	5.90	6.39
E–29 (chick embryo)	7.88	8.15	1.78	6.10	6.37

TABLE 3. SEMIQUANTITATIVE ESTIMATE OF INFLUENCE OF VACCINE DOSE ON SIZE OF LOCAL REACTION AND COMPARISON BETWEEN REACTIONS OBTAINED WITH VACCINES FROM DIFFERENT SOURCES

Group and No. of vaccinees	Inoculation site No.	Vaccine dilution	Titer $TCID_{50}$ per ml	Percentage of positive reactions			Negative reactions (%)
				Large	Medium	Small	
	1	CL 8/51 1:4	$10^{7.4}$	18	56	25	1
A (85)	2	CL 8/51 1:32	$10^{6.5}$	3	35	29	33
	3	CL 8/51 1:256	$10^{5.6}$	0	8	20	72
	1	CL 8/51 1:1	$10^{8.0}$	44	44	10	2
B (52)	2	E–13 1:1	$10^{8.0}$	46	52	2	0
	3	TC 341 1:1	$10^{7.0}$	8	58	19	15

CL 8/51: Calf lymph vaccine, titer about $10^{8.0}$ $TCID_{50}$/ml.
E–13: Egg vaccine, titer about $10^{8.0}$ $TCID_{50}$/ml.
TC 341: Calf tissue culture vaccine, titer about $10^{7.0}$ $TCID_{50}$/ml.
Readings taken after seven days.

groups of vaccinees that had been inoculated one year previously with egg vaccine and calf lymph, respectively. From an epidemiological study in India, Pandit (41) reported the same degree of protection against smallpox in persons vaccinated with egg vaccine and with lymph vaccine. During the 1963 smallpox outbreak in Stockholm (27 cases) approximately 600,000 vaccinations were performed in the Stockholm area, practically all with egg vaccine. Although no comparisons with other vaccines could be made at that time, not a single contact case was observed in which an adequately performed vaccination with egg vaccine failed to produce the expected protection (Zetterberg *et al.*, to be published). Similar experiences were obtained during the smallpox outbreak in Texas in 1949 (32).

COMPLICATIONS

The frequency of encephalitis and other serious complications to smallpox vaccination does not seem to have changed in either direction in areas where egg vaccine has been introduced into common use. Comparative data from Sweden are shown in Table 4. Complications due to egg allergy have not been reported so far in either Sweden or the United States (E. B. M. Cook, V. J. Cabasso, personal communications). It is now commonly felt that the egg material in itself does not require special restrictions for percutaneous use in cases of suspected egg allergy.

The study of vital-statistics data for an area (Texas) where egg vaccine has been used extensively for more than 25 years (48) does not indicate overrepresentation of any cause of death, including the leukemia-lymphoma group.

TABLE 4. INCIDENCE OF POSTVACCINAL ENCEPHALITIS IN SWEDEN IN RELATION TO TYPE OF SMALLPOX VACCINE PREDOMINANTLY USED

Period	Vaccine issued	No. of postvaccinal encephalitis cases
January 1958–February 1961	Calf lymph vaccine, 1.43 million doses	12
March 1961–February 1963	Egg vaccine, 1.2 million doses	16*

*Eight of these cases occurred during January–March 1962, when European outbreaks of smallpox led to more vaccinations than usual in Sweden.

INNOCUITY PROBLEMS

A special aspect of the innocuity problem that is steadily attracting increased attention is connected with the accumulating findings within the field of viral oncology. The viral nature of the fowl leukosis agents has been recognized for a long time (49) and some evidence is being accumulated that bovine leukosis is also induced by viruses (14, 15).

It has recently been demonstrated that certain virulent strains of Rous chicken sarcoma virus can induce malignant transformation of cultured human cells *in vitro* (35, 53) and induce progressive tumor growth when injected into newborn, but not into adult, monkeys (13, 40). On the other hand, it has been suggested by some authors (1, 37, 43) that a connection exists between the incidence of leukemia in cattle and in man. Probably there is still a need for more evidence to support such conclusions. The possible means by which hypothetically oncogenic agents from domestic animals can be transmitted to man seem numerous in every-day life—professional care of animals; the processing of meat, dairy products, eggs, and so on; and the consumption of such materials. By comparison, smallpox vaccination seems to represent a small and very infrequent contact with bovine or avian material.

The percutaneous route of inoculation is relatively very inefficient—in the case of vaccinia, a virus concentration at least 100,000 times higher is needed for a cutaneous take than for a response in tissue culture. Some recent findings suggest that the fowl leukosis agents lack the ability to infect man and multiply enough to elicit a serological response (39, 46). Transformation of cells *in vitro* with nonmultiplying viruses as a rule requires large virus doses. It thus seems unlikely that a transforming dose of a potentially oncogenic virus would be introduced by the ordinary smallpox vaccination procedure.

In spite of this reasoning, all adequate precautions should be taken to control the leukosis agents in smallpox vaccines. No useful methods other than the clinical are as yet available for the bovine leukosis, but for the fowl leukosis several practicable techniques have been described in recent years (3, 42, 47, 49).

The source of eggs for vaccine production should be selected after screening several isolated flocks for the presence of immune birds and virus-shedders. All virus-shedders should be removed and, if possible, only immune birds used for egg production.

The testing of vaccine batches, and especially seed viruses, may be performed along the following lines. The material is first assayed for gross contamination by the complement-fixation (CF) test, using serum from hamsters bearing tumors induced by the Schmidt-Ruppin or equivalent strains of Rous sarcoma virus (47). If the outcome of this test is negative, samples of vaccine are mixed with a potent antivaccinia serum and subcultured in chick embryo cultures which are then assayed by immunofluorescence (42) or the CF test using hamster sera. These tests, and the less reliable immunodiffusion test (3) as well, possess the same fowl leukosis-group specificity. However, the achievement of a satisfactory CF antigen titer will often be hampered by the incomplete neutralization of the vaccinia virus with subsequent early destruction of the culture. Since most of the larger vaccine institutes are by now experienced in the use of the immunofluorescence method, this may be the technique of choice for the leukosis test.

Summary

It has been known for more than 30 years that the yield of vaccinia virus in embryonated eggs may be quite adequate so far as *in vitro* potency is concerned, but only in a few areas of the world (Texas and Sweden, for example) has this vaccine source been utilized for human vaccinations on a large scale.

Only a few of the more important details in the egg vaccine production, such as passage level and concentration of seed virus and route of inoculation, are mentioned. The effect of variation in the eggs and laboratory techniques is exemplified by the results of tests involving double infection of eggs with two distinguishable pox viruses. Egg vaccines, especially after lyophilization, appear less heat-stable than calf lymph. Various attempts to overcome this problem are reviewed.

Comparative titrations of egg and calf vaccine in various host systems failed to reveal differences with respect to response or degree of

adaptation. Dose-response studies in humans suggest that, for a given virus strain, the virus titer is the only factor that influences the quantitative and qualitative response in immunologically homogenous populations. The immunogenic capacity of egg and calf lymph vaccine appears to be similar, as estimated by vaccine challenge experiments and epidemiological observations. Postvaccinal complications are not significantly different in character or frequency for the two vaccines. Serious complications due to egg allergy have not been reported.

The innocuity problem is discussed with special references to extraneous agents hypothetically oncogenic for man.

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SECTION A. SMALLPOX

DISCUSSION

CHAIRMAN STOKES: The first discussant of this section on smallpox will be Dr. A. W. Downie, who recently retired as Professor of Bacteriology at the University of Liverpool and is now at the University of Colorado Medical Center in Denver, Colorado.

DR. DOWNIE: I wish to consider very briefly the question of routine infant vaccination in non-endemic countries to protect the community against smallpox. The two figures illustrate one point concerning the immunity resulting from smallpox that is fairly generally accepted. Figure 1 shows the incidence and mortality, by age, of unvaccinated smallpox patients admitted to the Madras infectious diseases hospital in 1962–1963. The incidence occurred mostly in children. Mortality was approximately 50 per cent at the extremes of life. Figure 2 shows the same data for smallpox patients who bore scars from infant vaccination. The resistance to infection had apparently largely disappeared after 15 or 20 years, although—as is indicated by the low mortality—some immunity remained.

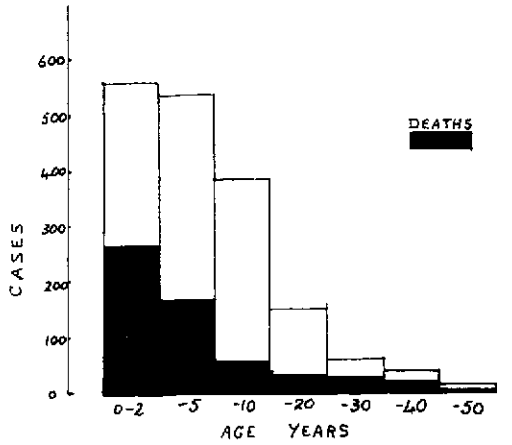

Fig. 1. Incidence and mortality, by age, of unvaccinated smallpox patients, Madras, 1962–1963.

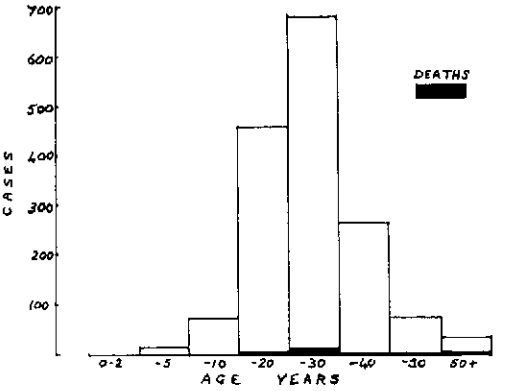

Fig. 2. Incidence and mortality, by age, of vaccinated smallpox patients, Madras, 1962–1963.

These observations are not new, of course. The data bear a striking resemblance to those available for Britain and elsewhere early in this century, when epidemics of variola major were still occurring. On such data is based the belief that to maintain an immune population vaccination must be regularly repeated every five to ten years. In some countries of the West, at any rate, this is very difficult to achieve. If such a situation is impracticable the question arises, Is it worthwhile to persist with infant vaccination by present methods? The relatively poor community protection that infant vaccination alone would confer has to be set against the risk associated with the immunizing procedure. Exact information on vaccination complications is difficult to obtain. Perhaps that available for England, although admittedly incomplete, is as good as any (Table 1).

In this ten-year period there were 33 deaths from complications in over 5 million vaccinations, and 22 deaths from smallpox in five out-

TABLE 1. COMPLICATIONS OF VACCINATION IN ENGLAND AND WALES, 1951–1960 (5,061,013 VACCINATIONS)

	Generalized vaccinia	Eczema vaccinatum	Progressive vaccinia	CNS illness
Cases	162	16	8	64
Deaths	0	4	7	22

Smallpox deaths: 22.

Source: Modified from E. T. Conybeare in *Monthly Bulletin*, Ministry of Health and Public Health Laboratory Service 23:126, 150, 182, 1964.

breaks resulting from importations. The matter is, of course, not quite as simple as this comparison suggests. If routine infant vaccination is omitted, regular vaccination will still be required for those at special risk—doctors, hospital staff, public health employees, members of the armed forces, and of course contacts of cases when the disease is introduced from elsewhere. Primary vaccination at an older age may still be required for purposes of travel abroad.

The question of rapid spread in a totally unprotected community is often raised in favor of retaining vaccination in childhood (the present acceptance rate for vaccination of children in Britain is about 30 per cent). From our experience in Britain since the war, however, I believe with others that spread in a nonendemic country with an efficient public health service can be readily controlled by the energetic application of epidemic-control measures involving, among others, early detection and isolation of cases; vaccination and surveillance of contacts; and disinfection of rooms, clothing, bedclothes, and articles used by smallpox patients.

In a Conference of this kind, I am perhaps a little out of step in suggesting partial withdrawal, in its country of origin, of the first and one of the most effective virus vaccines—a vaccine, however, that if it were being introduced today, might not easily be accepted by the licensing authorities.

CHAIRMAN STOKES: Thank you, Dr. Downie. The next discussant is Dr. C. Henry Kempe, Chairman, Department of Pediatrics, University of Colorado Medical Center, Denver, Colorado.

DR. KEMPE: While a major effort of the World Health Organization is now being directed toward smallpox eradication in endemic regions, countries that have already eradicated the disease must increasingly question the use of traditional and needlessly virulent strains for routine primary vaccination of all infants. Prevaccination with inactivated vaccinia is under study by Kaplan in Great Britain and by Herrlich in West Germany. An attenuated strain of vaccinia (Rivers CV-2) has been used by van der Noorda in Dutch recruits, and we have used a related strain (Rivers CV-1) that has undergone 72 additional chorio-allantoic passages to vaccinate eczematous children who are generally not vaccinated and are at risk from accidental vaccinia infection. Ninety thousand such children each year are currently exempted from vaccination, and there is a specific need on their part for protection from a public health measure used for the rest of the child population.

Of the first 519 primary vaccinations of eczematous children with CV-1-72, 366 were carried out with the standard multiple pressure technique; the rest received subcutaneous inoculations (Table 1). The local reactions, systemic reactions, and febrile responses were milder than those encountered with standard vaccine in normal children, and none of the eczematous children suffered general spread of virus (Fig. 1, Tables 2 and 3). A single case of mild erythema multiforme occurred in this highly allergic group.

The neutralizing antibodies obtained were similar to those resulting in normal children from standard vaccine and were not related in level to febrile response (Tables 4 and 5).

Revaccination with standard strain three to six months after the CV-1 vaccination showed

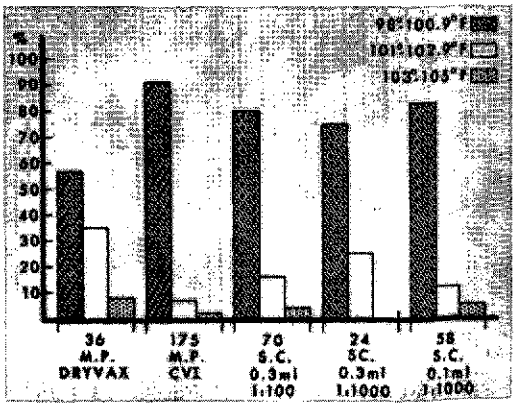

Fig. 1. Primary vaccinations fever chart.

TABLE 1. PRIMARY VACCINATIONS OF ECZEMATOUS CHILDREN WITH CV-72

Method	Primary	Revaccination	Total
M.P.	366	40	406
S.C. 0.3ml 1:100	71	28	99
S.C. 0.3ml 1:1000	24	5	29
S.C. 0.1ml 1:1000	58	2	60
Total	519	75	594

TABLE 2. LOCAL REACTIONS IN PRIMARY VACCINATIONS WITH CV-1

Method	No take or equivocal (%)	+ or ++ (%)	+++ (%)	++++ (%)	Total
M.P.	13	84	2	1	174
S.C. 0.3ml 1:100	61	37	2	0	67
S.C. 0.3ml 1:1000	59	41	0	0	24
S.C. 0.1ml 1:1000	69	31	0	0	58

TABLE 3. SYSTEMIC REACTIONS IN PRIMARY VACCINATIONS WITH CV-1

Method	None (%)	+ (%)	+++ (%)	Total
M.P.	84	15	1	175
S.C. 0.3ml 1:100	89	11	0	71
S.C. 0.3ml 1:1000	88	12	0	24
S.C. 0.1ml 1:1000	88	12	0	58

TABLE 4. PRIMARY VACCINATIONS WITH CV-1

Method	$TCID_{50}$	Geometric mean neut. titer	No. of persons
M.P.	Variable	52.06	101
S.C. 0.3ml 1:100	30,000	54.36	54
S.C. 0.3ml 1:1000	3,000	34.36	22
S.C. 0.1ml 1:1000	1,000	25.35	33

TABLE 5. NEUTRALIZING TITER (GEOMETRIC MEAN) ACCORDING TO FEVER

Method	98–100.9° F		101–102° F		103–105° F	
	G.M.	No.	G.M.	No.	G.M.	No.
M.P.	53.88	87	42.12	11	30	1
S.C. 0.3ml 1:100	58.29	40	51.44	10	33.47	3
S.C. 0.3ml 1:1000	31.83	17	44.60	5	—	–
S.C. 0.1ml 1:1000	25.42	33	25.20	3	24.49	2

marked modification with minimal lesions and no viral spread.

CV-1-72 would appear to be a suitable candidate strain for elective primary vaccination of children with eczema to lessen the chance of accidental vaccinia inoculation. Eventually it may become a substitute for standard vaccine in the primary vaccination of children in countries practicing routine infant vaccination.

CHAIRMAN STOKES: The next discussant is Dr. O. G. Andzaparidze of the Research Institute of Virus Preparations, Moscow, USSR.

DR. ANDZAPARIDZE: In his report Dr. Polak raised a very important question—that of comparative studies of vaccine virus strains.

I should like to tell you about experiments made at the Moscow Institute of Virus Preparations by Drs. S. Maremakova and U. Svel-Moldavski, who carried out several comparative studies on vaccine viruses. A study was made of various characteristics of these viruses: the genetic homogeneity of the population and its stability, its pathogenicity for rabbits when introduced intracerebrally and intradermally, and others. Dr. Svel-Moldavski also studied these strains on irradiated white mice. After irradiation with 400 Roentgen the mice were inoculated intravenously with an identical quantity of virus. All the strains except one were found to be pathogenic in mice in varying degrees and multiplied with different intensity in their lungs and brains. Only one vaccine virus strain, EM-63, did not show these characteristics, although there were other strains that were not pathogenic for rabbits after intracerebral inoculation.

It therefore appears that the test on irradiated rats produces a better response than the cerebral infection of rabbits and that the EM-63 strain is the least likely to produce reactions. However, this requires further study. This strain and the vaccine produced from it are genetically homogeneous and stable and are in line with requirements of the World Health Organization. Vaccine produced from the EM-63 strain, when administered to children, produces a mild vaccination process and shows a reaction in practically all cases.

CHAIRMAN STOKES: Our last discussant is Dr. K. Raska, World Health Organization, Geneva, Switzerland.

DR. RASKA: Several of us listening to Professor Stuart-Harris' excellent keynote address were not altogether happy with his skeptical general statement on the eradication concept. Of course, it is true that in recent years the word *eradication* has become too fashionable.

Some papers on the subject give the impression that the author either misunderstands the term or fails to realize the complexities of the problem, which must be considered from an ecological, technical, organizational, economic, and political point of view.

But smallpox, I think, is a disease which is generally regarded as extremely suitable for eradication. Global eradication of smallpox is technically feasible, and the unanimously accepted resolution* at the Eleventh World Health Assembly in 1958 seems to be fully justified.

Progress since then, however, has been far from satisfactory, mostly because the whole problem was underestimated and oversimplified.

Using the experience of the last six years, the World Health Organization has now prepared a ten-year plan for the global eradication of smallpox. Any prolongation of this ten-year period once the program has started would mean extra financial and material expense.

To the prerequisites for a successful smallpox eradication program enumerated by Dr. Millar I should like to add another—a practical but very important one. It is understood that intensified national efforts to eradicate smallpox are a contribution to international health. The entire world benefits from individual national campaigns, which are themselves an integral part of a worldwide eradication program. On the other hand smallpox is not the most important public health problem in most of the developing countries. It cannot, therefore, be expected that they will concentrate their limited financial and human resources on a problem not having high priority without substantial help from outside.

Highly developed countries that for several decades have been free from smallpox must vaccinate and revaccinate their populations, with a risk of fatal or injurious side effects. Moreover, the costs of doing this are so high that the additional cost of three years' routine vaccination would be sufficient to cover a ten-year program that would lead to the eradication of smallpox from the globe. To express it another way, an increased expenditure by developed countries on the smallpox eradication program would pay itself back within three years after the achievement of eradication. An end to smallpox and revaccination will mean considerable and permanent savings.

In view of the existing social, economic, and

* Resolution WHA11.54. *Off. Rec. Wld Hlth Org* **87**, 41–42, 1958.

health situation in many smallpox-endemic countries, WHO urgently needs more well-developed and smallpox-free countries to join and follow the generous example of the USSR, the United States, and a few European countries that are now giving substantial help to the ten-year plan for eradication of smallpox.

CHAIRMAN STOKES: Thank you, Dr. Raska. Are there any questions or further discussion from the main speakers?

DR. MILLAR: I should like to make one comment on the question of smallpox control in nonendemic countries.

First of all, let me say that I generally agree with Professor Downie's view that vaccination policies in nonendemic areas should be continually reviewed and the cost in human misery resulting from complications of smallpox vaccination weighed against the potential threat of smallpox introduction.

But I take issue with one of his points: reliance on epidemic-control activities as a major defense against imported smallpox. I think it has become abundantly clear in the last three years that to rely on epidemic-control activities is to rely on a fairly tenuous defense. For example, in the British outbreak of 1962, a fair number of cases developed before the matter came to the attention of public health authorities and epidemic control measures could be instituted. In Bradford, all cases were infected before the outbreak became known. Similarly, in 1963 in Stockholm the outbreak was already in its third or fourth generation before it came to light. This year in the United Kingdom over 60 cases of variola minor have occurred; by the time they were known, four generations of transmission had occurred.

While I think the principle is a good one, I think the human frailties involved make reliance on epidemic control measures a bit risky at this point.

DR. DOWNIE: I agree that the first introduced cases of smallpox have not always been detected in England. Sometimes the disease has only come to light in the second or third generation. But in spite of this, and in spite of the low general level of vaccination in Great Britain, our outbreaks have been no more extensive than those occurring in other countries in Europe where the vaccination rate is much higher and similar introductions have occurred.

Even though there have been more than 30 importations of variola major since the War into Great Britain, the number of cases resulting is not quite 300 altogether. During this same period, it is estimated, deaths from smallpox have been significantly fewer than the deaths from smallpox vaccination.

DR. POLAK: I should like to ask Dr. Andzaparidze a question about the selection of strains in rats. The strain selected in rats was also of low pathogenicity in children, but has he checked to see whether there is really a correlation between pathogenicity in rats and in children?

I mention this point because about 10 years ago several vaccinia strains were compared in Germany in rabbits and in eggs, and the conclusion was that the Bern strain seemed to be less pathogenic than other strains in this study. This was why we included the Bern strain in our study. But we did not find its pathogenicity very low—certainly not lower than that of the Copenhagen strain.

Furthermore, in infant mice the Ecuador strain is of very low pathogenicity, much lower than the Elstree strain, but we have found that in adults the Elstree strain gives less clinical illness.

I wonder, therefore, whether rabbits, eggs, or rats give a good idea of the degree of pathogenicity of vaccinia strains in human beings.

DR. ANDZAPARIDZE: I was very careful to mention that these were preliminary data, and I also said that we may, as the experiments proceed, find this characteristic of the strain to be connected with the reactions found in man. We are continuing the experiments and the collection of data.

DR. SOLOVIEV: I should like to revert to the question I asked concerning Dr. Meyer's report during Session V. As you remember, this report dealt with the question of combined vaccination against measles and smallpox.

At the time, I drew his attention to the fact that at present such a combination of two vaccines can hardly be considered well founded. In the first place, the evidence shown us is actually based on an increase of antibodies, that

is, on the serologic response of vaccinees. According to the data we have collected during quite a considerable experience in comparing antibodies after vaccination and the response to vaccination of those vaccinated, it must be said that antibody is not an index of immunity—or at least is only a relative index. It is essential, in studying the potency of smallpox and other vaccines, to determine response to vaccination and to take into account both serological data and reactivity for revaccination.

Secondly, it is still not clear whether such a combined vaccine will not produce more frequent complications—those connected with the central nervous system above all. Dr. Downie gave us an account of very important observations that confirmed once again that central nervous system complications after smallpox vaccination, though encountered rather seldom, are still a serious problem.

I therefore consider that before we make any recommendations about combining smallpox vaccine with other vaccines we must study these questions on a wider basis and in greater detail so that our data will be on a better footing and permit us to arrive at sound conclusions.

DR. KEMPE: I should like to ask Dr. Polak to outline briefly his views on the development of attenuated strains. He has remarked on the curious arrest in the development of such strains on the part of vaccine centers. Everyone in pediatrics certainly feels that, if universal vaccination is to continue in smallpox-free countries, something must be done about the virulence of current strains and the high and unnecessary morbidity and mortality. Most of our complications are in primary vaccination in infancy. In the United States, this first vaccination is not intended primarily to protect the child against exposure to smallpox; he is not exposed. It is intended to get him past primary vaccination, which we think is more dangerous than the second or third. Since it is these subsequent vaccinations, rather than the first one, that are expected to protect against possible adult exposure, it makes absolutely no difference whether a highly virulent or immunogenic product or an attenuated one is employed the first time.

Would Dr. Polak discuss the future of attenuated strains?

DR. POLAK: I am sorry to say that I cannot give a proper answer. If I could, I would have included it in my paper. At this moment it is quite possible to select strains with regard to pathogenicity in man, but I do not know how to test the immunogenicity unless by a field trial including several strains. That would of course be very difficult to do, but it has been done before. At this moment this is the only method available for comparing various strains with regard to protection against smallpox.

I do not know a laboratory method which could answer your question.

DR. DEINHARDT: I have a question for Dr. Kempe. Have the attenuated vaccinia strains you used in children with eczema also been used in children or adults with leukemia or lymphoma? Could they be used in such persons?

DR. KEMPE: The CV-1-72 strain has only been used in children with eczema. I would be loath to use even an attenuated live vaccine in patients who have major immunologic defects. We use vaccinia-immune gamma globulin and methisazone to protect these individuals when they go to smallpox-endemic regions.

DR. FENJE: I have two questions for the previous speakers. First, would vaccine lots that pass the NIH potency test also pass the WHO pock-count tests? Second, what is the correlation between the monkey cell tissue ID_{50} and the pock counts in the egg membranes?

DR. ESPMARK: In one of my tables I showed titers from tissue culture endpoint titrations in monkey kidney cultures and on the chorio-allantoic membrane; the difference on the average—in my hands, I wish to point out—was that the titers were 0.3 logs higher in the tissue cultures. Probably with an improved egg titration procedure the CAM titers would be higher.

SECTION B. RABIES

PRESENT CONCEPTS OF THE EPIDEMIOLOGY OF RABIES

J. FREDERICK BELL

U.S. Public Health Service, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory
Hamilton, Montana, U.S.A.

DR. BELL (*presenting the paper*): Anyone who has been even mildly interested in rabies will realize that this old disease is not declining. On the contrary, in some large areas of the world it occurs in highly epizootic incidence over long periods. Even in the United States, where we take so much pride in our ability to identify, pursue, and eradicate diseases, rabies continues to present new facets, to spread into new areas, and to increase or recur in old areas. The flexibility, ability to rebound, protean nature, and very unpleasant characteristics of this syndrome in man and lower animals have led to the development of a "rabies mystique." We accept mystiques in other aspects of our lives, but the attitude of mysticism is inappropriate and unbecoming when applied to any dangerous disease.

IATROGENIC RABIES

The recent occurrence (24) of a series of cases of rabies as a result of vaccination demonstrates again that fixed virus is not innocuous in man, in spite of a general and dangerous impression to the contrary (28). Rabies has also been produced in animals by vaccines ordinarily considered safe. Administration of Flury vaccine of low egg passage (LEP) into the subcutaneous neck tissues of bovines or into cats, skunks, or immature dogs has produced the disease. Whether LEP vaccine should ever be used is, in my opinion, open to question. Apparently its use is based on a vague but widely held belief that antigenicity is enhanced as a result of limited propagation in non-nervous tissue of the recipient. This concept is without scientific basis as far as I can learn. On the contrary, the weight of evidence points to the fact that propagation of live virus in the vaccine depends on access to the central nervous system, in which case disease may be produced. Available evidence indicates that immunogenicity depends on the intrinsic, unaltered antigen content of the dose administered. In this respect, LEP has no demonstrable advantage over HEP vaccine of equal antigen content and always carries the delicately poised threat of rabies infection.

Another popular belief is that unique antigenicity occurs in different strains of rabies virus (19). Undoubtedly one basis for this belief is the failure of vaccine to protect animals in certain epizootics. This concept is contrary to the well-established antigenic identity of isolates, regardless of geographic or animal source, and it has led to unnecessary work, time, and expense in producing special vaccines. The reason for repeated vaccine failures is a deficiency of antigen in the vaccine, either as produced or after storage.

RABIES IN DOGS AND CATS

In the United States as a whole there has been a gradual decline, both relative and absolute, in the importance of dogs as vectors of rabies. Sporadic cases and small outbreaks occur here and there, and in a few areas the disease is highly enzootic. Wherever rabies is known to be pres-

ent, the bite of an unapprehended dog poses a difficult question of treatment. Recent data (31) indicate that the saliva of dogs is not infectious more than three days before onset of illness.

Much of the credit for decreased incidence of rabies in dogs is rightly due to public health authorities on all levels, who have reacted to outbreaks by application of the logical and time-proven measures of quarantine, elimination of strays, and licensing, leashing, and vaccination of owned dogs. The efficacy of these actions, when thoroughly carried out, can hardly be questioned, and as a result they have been incorporated into a dogma that is sometimes applied unthinkingly because it is unquestioned. However, continuous presence of rabies in some areas with control measures and continuous absence of rabies in other large areas without control measures are evidence that all the answers are not be to found in those procedures. The essential absence of rabies in dogs in Montana, despite continuous presence of the infection in other species, gives rise to several interpretations, among them the belief that the dog population (as opposed to individual dogs) is not susceptible to rabies. It is well known that other vast areas—Hawaii, Australia, New Zealand, Iceland, Sweden, Norway, and the British Isles—are, and have been, essentially free of rabies. This freedom is usually credited to stringent quarantine of imported animals. This is a comforting thought, but it may not be completely true. In Argentina and Uruguay epizootic rabies is a disease of dogs in large urban areas, whereas the relatively small and scattered canine populations of ranches and towns, comparable to those in Montana, do not perpetuate the disease. This is not to cast any doubt, however, on the value of quarantine in preventing the entry of dangerous infected animals. The only question is whether such entry would initiate epizootic or enzootic disease.

A common recommendation by responsible public health experts, when asked about prevention of rabies in essentially rabies-free areas, is that dogs should be vaccinated. No doubt vaccination is usually harmless when properly done, and the recommendation protects the recommender whether or not it is followed, but it is questionable whether vaccination does any significant good in some situations. It can be argued that general vaccination is justified if it will prevent the occurrence of even one case of rabies, and that justification may be valid where both personnel and money are not needed for more rampant diseases.

In one large city in South America a campaign of dog poisoning has been carried out for eight years with a claimed rate of dog destruction of about 450 a day. During the eight years in question, rabies in man increased and then subsided somewhat. It seems apparent that the rate of dog destruction is nicely balanced with the rate of dog production and that steady employment of dog poisoners is assured. Probably if the same continuous expenditure of energy in dog destruction were employed after a crash program of dog elimination in that city it would be much more effective in controlling rabies.

I am fully aware of the sociological difficulties encountered in dog control by use of poison or the catcher's net, and it may be found that these tactics can be obviated by use of hormonal contraceptives that will prevent the constant recruitment of population without engendering individual or organized antagonism. This technique has been used with wild species (17).

The point I want to make here, if it is not yet clear, is that much of practical importance remains to be learned about the epizoology of canine rabies. The undoubted effectiveness of conventional measures for the control of conventional rabies has led to complacency and to confidence in the universal applicability of these procedures. The fact remains that there is no rabies in some areas where none of these measures has been applied and there continues to be canine rabies where they are applied with considerable effort and expense. I contend that studies of the ecology and population dynamics of urban and rural dog populations are long overdue. In proportion to their significance to man, scientific data on dog populations are extremely meager. It seems to me that no more fascinating subject of ecological study exists, yet ecologists have devoted much more time to obscure mammals or birds inhabiting remote desert, forest, and alpine areas. However, the fault does not lie with ecologists but with those who hold the purse that could hire the ecologist, and many politicians, at least, seem to take pride in being "practical" rather than intelli-

gent. Yet the practicality should be obvious when one considers how stray and owned dogs affect man directly or indirectly. A list of adverse relationships (for which I do not claim completeness) is given below:

A. Diseases and Parasites Transmitted to Man or Domestic Animals from Dogs:

1. Echinococcosis
2. Dipylidium
3. Ascarid Larva migrans
4. Dirofilaria
5. Ringworm
6. Mange
7. Ticks and fleas
8. Salmonellosis
9. Toxoplasmosis
10. Leishmaniasis
11. Chagas' disease
12. Leptospirosis
13. Tularemia
14. Rabies
15. Distemper and hepatitis
16. Pasteurellosis
17. Allergy

B. Nuisance or Damage to Property:

1. Livestock killing
2. Game animal destruction and harrying
3. Threats and anxiety
4. Wounds
5. Garbage scattering
6. Carcass disposal
7. Auto accidents

There is not sufficient time to document the severity of these threats, but I am sure everyone is aware of some of them. Surely they constitute sufficient reason for the most practical public health official to learn something about the dynamics of dog populations—for instance, rate of recruitment, source, home range, formation of packs and hierarchies, contact with other packs, and so on. Our ignorance in these respects is appalling.

Rabies in cats always seems to be ancillary to rabies in other species, although it may be common enough at times to constitute a special threat (18). Whether rabies ever exists in a cat population as an indefinitely self-perpetuating disease is uncertain, but it seems unlikely in view of the small numbers usually involved where dogs are frequently infected. Even casual knowledge of dog/cat intercourse would make one suspect the family dog rather than stray dogs as a source of infection of the family cat, but transmission in the opposite direction would not be so limited. The habits of cats also expose them to infection from sylvatic sources, and they have been found in contact with many rabid insectivorous bats (32). There is as yet no evidence that cats contract rabies from bites of insectivorous bats or from eating infected carcasses, although mice can be infected by ingestion of infected material. In any case, the ecology of domestic and feral cats is worthy of much more intensive study than it has received.

CHIROPTERAN RABIES

Bat rabies seems logically separable into two subheadings: vampire and insectivorous. If there is new information in the field of vampire rabies, I am not aware of it, but the beginning of a comprehensive investigation in northern Mexico promises some solid information in the future. Rabies of bat origin is the most serious disease of cattle in several large regions of Central and South America and in some areas nearly precludes the raising of cattle. I have been told that vampire bats are rabid in some areas, whereas in others, separated only by traversable jungle, colonies are quite free of the disease. Vampire bats may have become so cattle-dependent as to preclude their passage over jungle not inhabited by cattle, but Nehaul and Dyrting suspect migrating *Desmodus* as the vector of rabies in a jungle area of Guyana * that has no large livestock (22). Perhaps the bats were forced to take human blood under the circumstances. Epizootic rabies in cattle is referable to vampire bats, whereas isolated or sporadic cases may be contracted from dogs or wild animals other than bats. The chronic carrier state, known in vampire bats since the work of Pawan (27), is apparently sufficient to maintain the disease in that species.

Available evidence stresses separateness of vampire and canine rabies, although both may occur in one area. Dogs are considered relatively immune to vampire rabies by virtue of their ability to detect approach of the bats (3). Before vampire bats were identified as the source of epizootic cattle rabies in South America, the diagnosis of rabies was not considered seriously, since rabid dogs were not present in the areas (8).

* Formerly British Guiana.

I know of two instances, one in Egypt (N. R. Reid, personal communication, 1966) and one in India (26), in which horses rather than cattle were the species principally or solely involved, and in each case the vector remained unidentified in spite of careful investigation.

There are many aspects of rabies in hibernating insectivorous bats of the Temperate Zone that need clarification. Overwintering of the virus is not completely understood but Allen, Sims, and Sulkin (1, 2) have investigated the role of the brown fat as a reservoir of virus. At least two bats have been found rabid in northern latitudes in mid-winter (6), and we have had a bat survive 24 days after it inflicted an infective bite (unpublished data, 1966). In western Montana 11 of 13 known species of bats have been investigated and 8 of them have been found infected. The disease has been recognized in one or more bats every year for the past 13 years, and we must assume that we study only a very small fraction of the bats that are infected.

I suppose that everyone who studies rabies in bats is asked whether the disease has been newly introduced into the species. I have no worthwhile thoughts on the matter, nor am I aware of any.

The epizoology of rabies in bats in western Montana is of particular interest because compartmentation of the disease is seen so clearly there. With rather modest effort, 39 rabid bats have been identified in the area. We have attempted with equal vigilance to detect rabies in other species, but there have been no diagnoses of the disease in any of them. A lower incidence of rabies in bats has been found in eastern Montana. In the past few years, skunk rabies has been diagnosed increasingly there, but the latter occurrence is obviously an anticipated extension of skunk rabies from an adjoining state or province where it has been present for a long time. It seems clear that rabies is transmitted primarily from bat to bat, presumably by bite, because the disease is transmissible to adult mice and to man by the bite of naturally infected bats. There is no reason to doubt that bite transfer occurs also in the cave bats of the southwestern states, but the possibility of atmospheric spread has been demonstrated (9). The source of the miasma has not been identified. Urine has been found infected, and bats use urine for bathing (13). The fluttering of wings could create an aerosol. Nikolitsch (23) proposes that transmission of virus to screen-protected animals in cave experiments was by means of a gamasid mite that could penetrate the screen, but mites have not been found infected. Ectoparasites of other kinds have also been proposed as vectors of rabies (16). We were able to infect argasid and ixodid ticks by artificial means, but virus was retained only until ecdysis (5). If tick transmission occurs, it must be under very special and unusual conditions.

ARCTIC RABIES

Whether or not the subject of arctic rabies deserves special consideration is moot, but it has been accorded special names by both American and Russian workers (10, 15). The astonishing feature about the disease was the great delay in identifying the "arctic madness" that had been seen for many years, although rabies was recognized in Alaska in 1915 (11). Probably the occurrence of epizootics of distemper, hepatitis, and salmon poisoning, as well as rabies, clouded the picture and caused confusion. The recognized disease seems to be essentially circulated among arctic foxes, with common transmission to red foxes, sled dogs, wolves, and other species. The extent of serial transmission in other species is uncertain, but Johnson believes that smaller animals are the reservoir of infection (14). Man is surprisingly little involved, and the heavy clothing worn in the arctic is supposed to be responsible for resistance.

CHRONIC, ABORTIVE, AND CARRIER RABIES

Occurrence of these forms of rabies has been recorded so frequently, though with varying degrees of documentation, that there can no longer be serious doubt of their occurrence in vector species and in laboratory animals (20, 4). However, traditional belief is so strong that well-documented occurrences are considered exceptions because of species, virus strain, or circumstance, without invalidation of the rule of inexorable death. Whether common or rare, the

carrier state—or even for that matter recovery of an animal after it inflicts an infectious bite—greatly complicates decisions that must be made by physicians in enzootic and in epizootic areas. Only methodical investigation can determine how frequently nonfatal but infectious forms of rabies occur in dogs. The Pan American Zoonoses Center in Argentina is undertaking that study.

In Germany there is common belief and an official attitude that vaccination is conducive to the development of the rabies carrier status in dogs. This idea is not generally accepted outside of Germany.

SYLVATIC RABIES

Rabies in wild carnivores has occurred in recognized epizootics for hundreds of years (23). In the United States and in some areas of Europe sylvatic disease is the most common form and poses special problems of epizoology and control. Nikolitsch (23) thinks that a basic cycle of rabies in field mice may be responsible for outbreaks in foxes and other species. He and others have isolated the virus from small wild rodents, but the concept needs further substantiation before it can be accepted as a general explanation of carnivore epizootics. Bats have been postulated as the source of rabies in foxes in Tennessee on the circumstantial evidence of greater incidence of fox rabies in areas with many caves (12). Johnson's (14) intensive studies on wildlife rabies have convinced him that the *Viverridae* and *Mustelidae* constitute a permanent and essential reservoir, and he has adduced excellent evidence of the existence of mild rabies, which is nevertheless easily mutable to fatal rabies, in some mustelid species. Thus, the potential of a reservoir role for those species is demonstrated, but essentiality is not established. Some doubt is cast on that essentiality when one reviews the history of epizootics in various wildlife species in this country. The striking feature of those epizootics is the remarkable compartmentation that exists and persists over wide areas for years. In one area, skunks constitute a large proportion of diagnosed cases of rabies, whereas in another area foxes or raccoons are predominantly infected. In each case, infection occurs in many other species, but apparently it is not perpetuated serially in those other species. This finding only emphasizes the remarkable fact of susceptible individuals that comprise resistant populations within highly enzootic or epizootic areas. This phenomenon will remain part of the mystique until it is thoroughly investigated. Some advances in understanding it have already been made by the studies of Sikes (29) and Parker and Wilsnack (25), but much more needs to be done. It is possible and, indeed, it has been seen that an epizootic in one species will progress into an area where the disease is occurring in another species and this phenomenon of noncompartmentation or diffuse spread occurs.

Our limited understanding of factors involved in population susceptibility and resistance to rabies has led to unfortunate floundering and misdirection of efforts to control epizootics. The usual reaction to identified epizootic rabies is to exterminate wild carnivores of all kinds by poison and trapping. Sometimes these procedures are also used in nonepizootic zones to create sanitary cordons in the fond hope that invasion will not cross the unoccupied zone. These tactics create immense gratification in those responsible for them and in those who carry them out, because they appear to be so logical that they justify the existence of predator control services, and the results, in animal ears or carcasses obtained, can be converted into statistics that can be used to obtain more money. That money is needed in large quantities is attested by the fact that fox extermination has cost $26.00 per animal over a period of five years in New York and $207.00 per animal trapped in Tennessee (Annual Rabies Summary, CDC, May 1966). It should be obvious that if fox killing were very successful as a control measure it should not have had to be done over a five-year period.

Frequent claims are made for the success of extermination measures, but it is at least as well established that epizootics of rabies may be self-limited (33). These measures are not only distressingly inefficient; they also incur the risk of serious side effects.

Public health people should learn from ecologists that several results of carnivore extermination are completely predictable. Among these are the increase in prey species, occupancy of

the niche by other species, invasion from the outside by the same species, and greater reproductive potential of the depleted population. It is quite conceivable that the latter reaction could give rise to a more susceptible young population as replacement for a relatively resistant older population. Although I do not have access to data on foxes or skunks of various ages that would substantiate this statement, we have demonstrated quite clearly in mice that resistance to peripherally-inoculated virus increases markedly with age (Fig. 1). Support for this belief is available also from a study of epizootics in cats in which the incidence of infection was much higher in the young animals (18).

If this were a meeting of ecologists, I would dare to suggest quite seriously that trapped skunks and foxes should be vaccinated and released rather than killed, and that all hunting of the infected species be stopped. My attention has been directed to a similar recommendation made by Dean at a recent meeting in France. I do suggest that the value of killing such animals, whether in "buffer strips" or over large areas, is dubious at best.

I suppose that the relationship of rabies-like virus to some cases of multiple sclerosis in the USSR (7) must be mentioned, but after more than 10 years of study, the relationship is still not clear.

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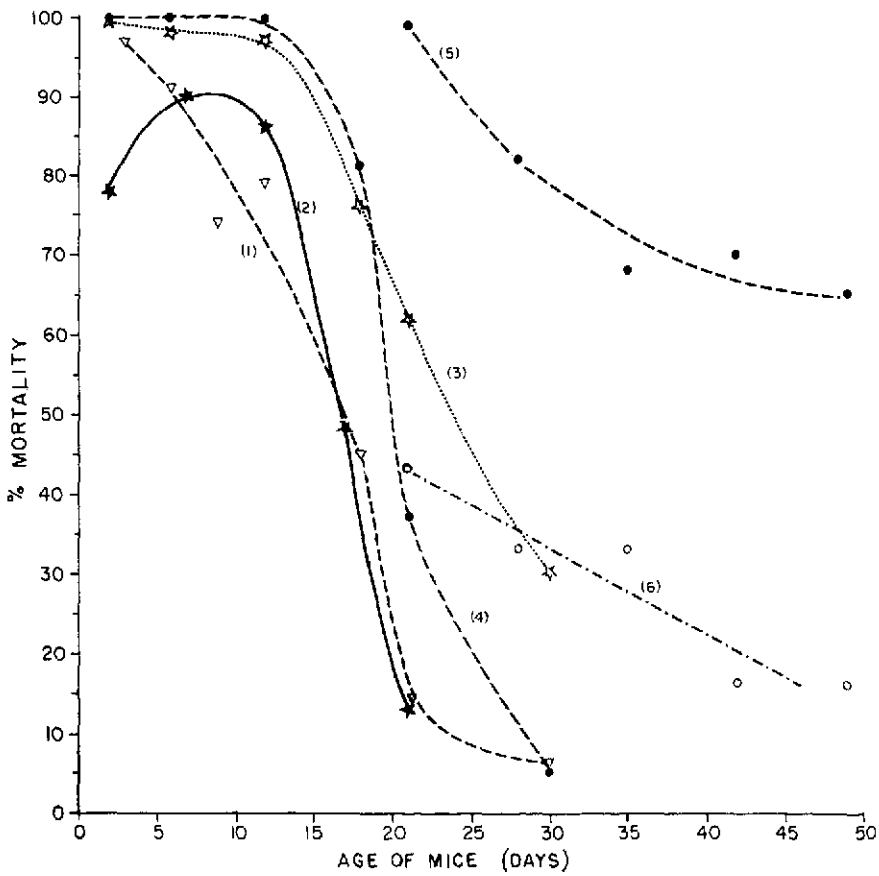

Fig. 1. Percentage of mortality in groups of mice of different ages after intraperitoneal inoculation of four low-passage rabies viruses (1-4) and two dilutions of fixed virus (5, 6).

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SECTION B. RABIES

VACCINES AGAINST RABIES: PRESENT AND FUTURE

H. KOPROWSKI *

The Wistar Institute
Philadelphia, Pennsylvania, U.S.A.

DR. KOPROWSKI (*presenting the paper*): On 3 August 1966 a 10-year-old boy from Bryant, South Dakota, sleeping in his back yard, was awakened by a striped skunk, which crawled into his sleeping bag and bit him on the thigh. While struggling to get away from the skunk, the boy was bitten also on the wrist, on the fingers of both hands, and behind the right ear.

The skunk escaped. What is believed to be the same animal was shot several hours later by the boy's father and was confirmed to be rabid by Seller's stain and by direct fluorescent microscopy.

A local physician cleansed the child's wounds with Phisohex and water and then painted them with tincture of merthiolate. A booster dose of tetanus toxoid was given at that time.

Eleven ml of antirabies serum were given within 18 hours after the exposure. Approximately one half the volume was infiltrated around the bite wounds; the other half was injected intramuscularly. The child was started on duck embryo origin rabies vaccine the same day and received a 1 ml dose daily for the next 21 days.

Twenty-four days after the exposure the boy developed a severe headache, within 48 hours he became irrational. After a short period of hyperexcitability, laryngeal spasm, and increased salivation he lapsed into a coma. He was hospitalized and remained comatose until his death on 5 September.

Impression smears from his brain, lungs, and salivary glands were negative for rabies on direct fluorescent microscopy; however, a positive virus isolation was made in mice, and the brains from the first mouse passage were shown to be positive by direct fluorescent microscopy.

This was the first human death attributed to rabies to be reported in the United States in 1966. As you can see, the patient received every treatment available recommended by the authorities. Even if the treatment failure could be attributed to the short incubation period in this particular instance, there is still the case of the child who died of rabies two years ago in Rabat, Morocco, three months after exposure and after receiving the recommended dose of immune serum and the complete course of duck embryo vaccine, followed by two booster injections.

It is perhaps unfair to cite only treatment failures and to forget that probably many more cases of rabies would be added to the hundreds recorded throughout the world every year if no treatment were available.

Treatment failures aside, it should be realized that postexposure treatment against rabies is still an archaic and dangerous procedure, and the currently available rabies vaccines are indisputably the crudest biological products injected under the human skin.

The antirabies vaccines currently being used for immunization of man are either of brain or duck embryo origin; brain-adapted fixed strain of rabies virus is being used as seed virus for both types of vaccine. In general, rabies-infected tissue is treated either with phenol,

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with phenol and heat, by UV, or with beta-propiolactone. Brain tissue vaccines containing completely inactivated virus are classified as Semple type; those treated only with phenol and containing infectious virus are known as Fermi type.

The long-accepted belief that the fixed virus is apathogenic for man has been challenged by the disastrous results that occurred in 1960 in Fortaleza, Brazil, when 66 human subjects were vaccinated with Fermi-type vaccine: 18 of these people died and rabies virus was isolated from their tissue. The sheep brain vaccine used contained 10^3 mouse LD_{50} and was released for use four days after addition of 0.5 per cent phenol. It received no other treatment. Thus, these deaths have to be attributed to the pathogenicity of fixed virus for man. Since this incident, attempts have been made to restrict the living virus contents of Fermi-type vaccine to a minimal level. I myself, however, would feel uneasy about injecting a patient with any preparation containing brain-tissue-adapted living fixed virus, which is pathogenic for many species of animals.

Circulating antibodies in human serum appear earlier and at higher concentration after injection of brain tissue vaccine than after exposure to duck embryo vaccine.

Although the important role of antirabies serum in severe bites should be stressed over and over again, the heterologous origin of this preparation raises the problem of acute allergic reactions and chronic serum sicknesses. The incidence of reaction varies from 16 to 34 per cent and is preponderant among children and adolescents. The logical solution to this problem is to replace heterologous serum with a homologous product of human origin. Such a product is not as yet available for general use, but in several laboratories men are being vaccinated with the virus in hopes of obtaining a potent human antirabies serum.

Postexposure treatment with immune serum may inhibit or delay the immune response to vaccination. It is therefore recommended that the complete course of vaccine injection be supplemented by repeated boosters.

Because rabies virus may persist for some time at the original site of exposure, local treatment of the wound is perhaps the most important aspect of postexposure treatment. Here some progress can be reported. Gone are the days of disfiguring burns with fuming mineral acids. After cleansing and debridement, a variety of virucidal agents may be applied topically with success—for example, soap solution, many quaternary ammonium compounds, and 50 per cent ethanol, including scotch, bourbon, gin, and brandy, but not iodine preparations. Topical application of antirabies serum was found to have a protective effect in guinea pigs infected with rabies through deep puncture wounds.

As you know, the main complication of treatment with vaccines of brain tissue origin is allergic encephalomyelitis. Since it has been shown experimentally that the allergen is a basic protein extracted from myelin, and since brain tissue of some species of newborn animals seems to contain very little myelin, rabies vaccines have been prepared in newborn tissue of mice, rats, and rabbits. Although it is too early to evaluate these vaccines completely, the incidence of neuroparalytic accidents should markedly decrease following their use.

Our goal is a preparation containing pure rabies antigen that would immunize successfully after one or a maximum of three injections and would be completely devoid of allergenic properties—namely, a tissue culture vaccine. But before discussing rabies virus in tissue culture, I will summarize in "capsule form" our present knowledge of rabies virus, particularly because this information is of recent origin and bears on the problem of protective treatment.

Physically, the rabies virus resembles a bullet, it has a beehive-like symmetrical structure. The nucleic acid core is surrounded by a double membrane. The infectious particles have a density of 1.20 g/ml and a sedimentation coefficient of 600S.

Chemically, rabies virus is a lipid containing ribonucleo-protein, which causes the formation in the cytoplasm of the infected cells of an inclusion body composed of protein and a small amount of ribonucleic acid.

It also contains specific antigenic material, as shown by immunofluorescence.

Rabies is the only RNA virus inhibited by one DNA inhibitor—arabinosyl cytosine (ara-C). Other DNA inhibitors such as Actimomycin D, mitomycin, and 5-Fluorodeoxyuridine (FUDR) enhance rabies virus (Table 1). The mechanism of action of ara-C is not yet elucidated, but

TABLE 1. EFFECT OF METABOLIC INHIBITORS ON THE GROWTH OF RABIES VIRUS IN HAMSTER BHK/21 CELLS

Inhibitor	Dose per 1 ml	Time of infection (Hrs.)	Cells containing rabies antigen* (%)	Infectivity of culture medium
Actinomycin	0.1 μg	+1	60	10†
Control			60	1
Mitomycin	2 μg	−3	55	
		+1	80	10
FUDR	10 μg	−3	90	10
Arabinosyl cytosine	50 μg	−3	10	0.01
Control			55	1

* At 24 hours after infection.
† Ten times greater than control medium.

probably it may be explained by inhibition of the formation of viral phospholipids. If this is true, rabies virus will have to be placed in the unique category of an agent inducing formation of phospholipids in the infected cell.

Although all mammalian tissue culture systems are susceptible to infection either with street or fixed virus, the degree of susceptibility varies from that of the highly susceptible rabbit kidney (RK/13) cultures and hamster fibroblasts (BHK/21 or Nil 2) to that of the highly resistant L or MK2 cells. Susceptibility of human embryonic fibroblasts falls somewhere in the middle. Great difficulties were encountered in serial propagation of rabies virus in a given tissue culture system, and usually, the virus disappeared after six or seven transfers. When it was found that cations enhanced adsorption and penetration of the virus, the susceptibility of any tissue culture to infection with rabies improved when the virus was adsorbed in the presence of, for example, DEAE-dextran at a concentration of 25-50 γ/ml (Table 2). The use of DEAE-dextran at each transfer permits indefinite propagation of the virus in any tissue culture system. The effect of the cation is mimicked by lymphocytic choriomeningitis virus (LCMV), which also specifically enhances adsorption and penetration of the rabies virus. In constrast to DEAE-dextran, the LCMV effect is specific only for certain tissue culture systems

TABLE 2. ENHANCING ACTION OF DEAE-D ON PENETRATION OF RABIES VIRUS IN BHK-21 CELLS

DEAE-d γ/ml	Rabies FA*	Mice LD_{50}†
200	100	4.4
50	100	4.6
–	20	3.6

*Percentage of fluorescing cells 24 hours after infection.
†Tissue culture medium 24 hours after infection.

such as the human diploid cell strain (HDCS) of rabbit endothelium (RE).

Conditions of cells in culture may alter their susceptibility to rabies. Cells that have been irradiated by ultraviolet rays are much more susceptible to rabies virus than are viable cells. From the safety standpoint, this method of virus growth, if adapted to commercial production, may have certain advantages.

Under ideal conditions of susceptibility, the virus penetrates the cell by pinocytosis within one to five minutes after exposure. Nine hours later a matrix-like formation is observed. After another three to four hours, several virus particles are formed within or around the matrix. Particles are rarely seen in association with the cell membrane or endoplasmic reticulum; for the most part they remain free within the cytoplasm. A cell filled with virus particles from 48 hours after exposure on does not show signs of degeneration.

In animals the virus travels from the periphery toward the central nervous system, always via the nerve trunk, without evidence of replication in either Schwann cells or perikaryons. It is one of the few viruses that infects only neurons of "choice location." The affected neurons do not show signs of degeneration and there is no accumulation of inflammatory cells. The same relationship may be observed on several tissue culture systems where rabies virus in an endosymbiotic state may be maintained for more than 300 cell generations without effect on its host cell.

To return to the vaccine problem, the ideal tissue culture vaccine should contain little or no extraneous material. This can be accomplished either by purification and concentration of virions or perhaps by isolation of antigen-containing inclusion bodies in the absence of infectious virus particles. Purification of rabies

virus particles has been accomplished by filtration through the Ecteola column and by centrifugation in sucrose and potassium tartrate gradients. Differential centrifugation or precipitation with zinc acetate facilitates concentration of the virus. Antigenicity of the purified preparation seems to be increased as compared to that of the crude product in preliminary tests.

Isolation of the antigen-containing inclusion bodies presents a more formidable task. Although their formation precedes the appearance of virus particles by three to four hours, the time interval is too short for effective separation of the two entities. It has recently been observed in preliminary experiments that treatment of tissue culture at the time of infection with arabinosyl cytosine and a protein inhibitor such as actidione will result in the reappearance of antigen-containing inclusions (Table 3), but the formation of infectious virus will still be inhibited. It is a long way from these basic laboratory observations to the isolation of an antigenic noninfectious material from infected cells that would be suitable for production of commercial vaccine. I feel, however, that this approach should be mentioned, for rabies virus may be one of the few nontumor viruses that can induce formation of a non-virion-associated antigen in the infected cells.

Many years ago Karl Habel showed that substrains derived from the original Pasteur strain differ considerably in their antigenic capacity. This observation led to the choice of the PM substrain for the production of Semple-type and duck-embryo vaccines. However, there was no way to isolate a pure virus clone from the heterogenous virus population and to test it for antigenicity in comparison with other genetically homogenous progeny of a single particle. Today plaques can be obtained on RK_{13} cells infected with rabies, and a plaque-purified progeny can be analyzed genetically and antigenically—a situation that presages almost unlimited possibilities for genetic characterization of a given viral population and for a scientifically sound approach to the choice of the most antigenic material for seed virus.

TABLE 3. STRANGE EFFECT OF ACTIDIONE ON INHIBITORY ACTION OF ARABINOSYL CYTOSINE (AC) ON RABIES VIRUS

AC present in culture medium	Actidione added at time of infection (hours)	Cells showing presence of rabies antigen† (%)
—	−3 through +4	90
+*	−3 through +4	70
+*	None	10
None	None	70

*From −3 through +24 hours.
†At 24 hours after infection.

These are some of the contributions of basic research to the ultimate goal of the production of a safe and effective vaccine for man.

The preliminary results obtained with tissue culture vaccines have come from work on HDCS as a substrate for production of living or inactivated vaccines. Although many strains have been used for vaccine production, we have concentrated our efforts on two: PM inactivated by beta-propiolactone and HEP Flury as living virus. In our seed lots we have always used virus adapted to HDCS. The HEP Flury, already highly attenuated after 200 passages in chick embryo, became further attenuated after passages in HDCS and completely lost its pathogenicity for intracerebrally inoculated hamsters, guinea pigs, and adult mice.

Preliminary evaluation of the two vaccine types in the mouse potency test revealed much greater immunogenic capacity of these vaccines than the standard Semple-type vaccine, duck-embryo vaccine, or chick-embryo vaccine infected with the HEP Flury strain. Nineteen dogs that received one injection of the HEP Flury vaccine were completely resistant to challenge with street virus.

Since these vaccines were designed for immunization of man, further evaluation of their antigenic potency was undertaken in primates. The results of the first trial were evaluated by the appearance of serum-neutralizing antibodies elicited by the two experimental vaccines in comparison with duck-embryo-inactivated vaccine and chick-embryo HEP living virus vaccine. The results (Table 4) indicate that the level of antibodies after one injection of HEP-Flury HDCS vaccine is much higher than after three injections of chick-embryo vaccine injected with the strain and that, moreover, a high level of antibodies is established within seven days after injection of the vaccine and persists for a long time.

TABLE 4. ANTIBODY RESPONSE IN MONKEYS IMMUNIZED WITH ATTENUATED RABIES VACCINE

Vaccination		Neutralizing titer of rabies-antibodies in sera of monkeys					
Type of vaccine	Number of doses	Days after vaccination					
		1	7	14	28	56	135
HEP/HDCS*	7	<10†	144	864	592	325	500
	3	<10	82	288	232	132	224
	1	<10	73	224	115	53	120
HEP/EE‡	3	<10	11	19	12	12	—

Source: Wiktor, T. J., and Koprowski, H. "Successful Immunization of Primates with Rabies Vaccine Prepared in Human Diploid Cell Strain WI-38." *Proc Soc Exper Biol Med* 118:1069–1073, 1965.
*Cells from culture series started from HEP-strain virus-infected human diploid-strain cells.
†Figures indicate average titers for each group of 10 animals.
‡Commercial egg-embryo vaccine infected with living HEP-virus.

Serum antibody levels elicited after three or after seven injections of PM-HDCS inactivated vaccine (Table 5) were lower than after use of living HEP vaccine but still higher than after seven injections of duck-embryo vaccine. Similarly, the antibody persisted for more than 135 days after vaccination.

In a more recent trial in vervet monkeys the levels of antirabies antibody elicited by vaccination with the PM-HDCS, HEP-Flury HDCS, and duck-embryo vaccines were related to the resistance against a massive challenge of 10,000,000 man infectious doses administered into the neck muscles of the vaccinated animals. An antibody titer of 1:80 or higher assured protection against the challenge. None of the sera of the monkeys vaccinated with duck-embryo vaccine examined so far show antibody levels such as those observed in animals immunized with the HDCS vaccines.

I feel that the way is now open for clinical trials in man. Immune response to one or maximum three doses of HDCS vaccines will be determined. The concentration, time of appearance, and persistence of antibodies will be carefully studied. A vaccination schedule may then be adopted for the general use of the vaccine not only for postexposure treatment but also for prophylactic vaccination.

Whether this event will take place in the near or more distant future will depend not only on the scientific merit of the case but also on the degree of resistance engendered by any new procedure in the field of vaccination of man. Hopefully, those who resist will do it solely for the reason which prompted Ninon de Lenclos,

TABLE 5. ANTIBODY RESPONSE IN MONKEYS IMMUNIZED WITH INACTIVATED RABIES VACCINE

Vaccination		Neutralizing titer of rabies-antibodies in sera of monkeys					
Type of vaccine	Number of doses	Days after vaccination					
		1	7	14	28	56	135
PM/BPL*	7	<10†	33	104	65	31	35
	3	<10	19	48	26	15	22
DE‡	7	<10	18	29	12	6	—

Source: Witkor, T. J., and Koprowski, H. "Successful Immunization of Primates with Rabies Vaccine Prepared in Human Diploid Cell Strain WI-38." *Proc Soc Exper Biol Med* 118:1069–1073, 1965.
*PM-strain virus-infected human diploid-strain cells preparation, after inactivation with *Beta* propiolactone.
†Figures indicate average titers for each group of 10 animals.
‡Commercial duck-embryo vaccine.

mistress of the great statesmen of 17th century France, to coin her famous statement "The resistance of a woman is not always a proof of her virtue, but more frequently of her experience."

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Results of experiments conducted at the Wistar Institute were obtained in collaboration with Drs. T. J. Wiktor, J. B. Campbell, M. M. Kaplan, and R. F. Maes.

Pathways of infection in experimental animals have been studied by Richard T. Johnson ("Experimental Rabies, Studies of Cellular Vulnerability and Pathogenesis Using Fluorescent Antibody Staining." *J Neuropath Exp Neurol* **24:** 662–674, 1965), and Tatsuya Uamamoto, Sugito Otani, and Hirotsugu Shiraki "A Study of the Evolution of Viral Infection in Experimental Herpes Simplex Encephalitis and Rabies by Means of Fluorescent Antibody." *Acta Neuropath* **5:**288–306, 1965).

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SECTION B. RABIES

DISCUSSION

CHAIRMAN STOKES: The first discussant will be Dr. Paul Fenje, Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, Canada.

DR. FENJE: I do not have sufficient first-hand information on the epidemiology and epizoology of rabies to be able to comment on Dr. Bell's paper. It seems that for many more years to come we will have to put up with the fact that rabies is around, which is one more reason to concentrate our efforts on the development of safer and more effective means of preventing the disease in humans and domestic animals. Maybe the time will come when eradication in wild animals by physical means will be supplemented or even superseded by biological methods.

Dr. Koprowski reported on the now very successful antirabies immunization trials in monkeys with both inactivated and attenuated vaccines produced in human diploid cells. Dr. Koprowski has a preference for the live vaccine, the development of which is certainly a greater challenge from the scientific point of view. On the other hand, from the point of view of acceptability a live vaccine creates more numerous problems than an inactivated one, and this is particularly true where rabies virus is concerned.

At the Connaught Laboratories we decided in favor of developing an inactivated vaccine for human use. It was produced in primary hamster kidney cells. At the International Rabies Symposium in 1965 I reported on successful trials in laboratory animals with such a vaccine, which appeared to merit clinical trials in humans.

Since that time we have immunized approximately 450 persons according to a pre-exposure vaccination schedule. All the vaccinated persons belong to the so-called high-risk group—veterinarians, veterinary students, laboratory workers, game wardens, and so on. Approximately 350 members of the group received a primary pre-exposure immunization treatment consisting mostly of three doses, and in about 100 persons who had been immunized previously with either Semple or duck-embryo vaccine, the booster effect of a single dose of the tissue culture vaccine was evaluated.

From all the vaccinees preimmunization and postvaccination blood samples were collected and have been or will be tested by the neutralization test in mice.

The following figures show the serological response in a group of 67 veterinary students who were submitted to primary pre-exposure immunization with three or two doses of our vaccine. The students were divided into three groups and immunized according to three different vaccination schedules: three doses two weeks apart, three doses at four-week intervals, and two doses eight weeks apart. Blood samples were taken after each vaccine dose. A statistically significant conversion rate could be demonstrated even after the first dose (Fig. 1). After the last dose, 64 out of the 67 students responded with a positive immune reaction—a conversion rate of 96 per cent. There was one nonresponder in each of the three groups.

A summary of the distribution of the 50 per cent endpoints before, during, and after immunization shows that while rabies antibody could not be found in any of the prevaccination samples, a gradual shift toward medium and high titers occurred during the treatment, and the final titers were in the high antibody group (Fig. 2).

If the medians are calculated separately for the three treatment groups, in both three-dose vaccination schedules the 50 per cent endpoints will be found at a serum dilution of about 1:50,

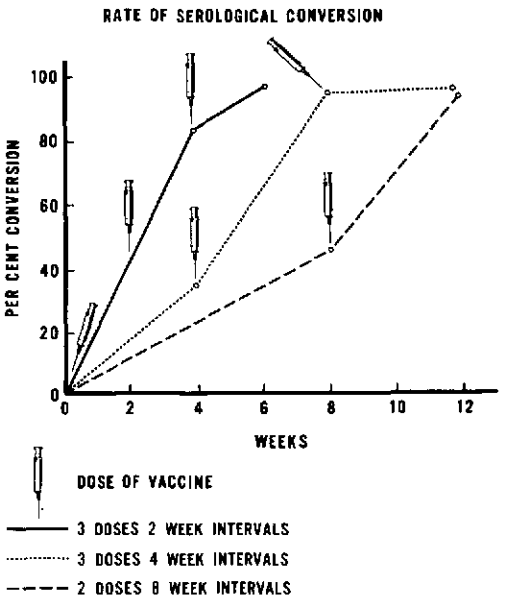

Fig. 1. Rabies vaccine of tissue culture origin in pre-exposure immunization of humans—Rate of serological conversion.

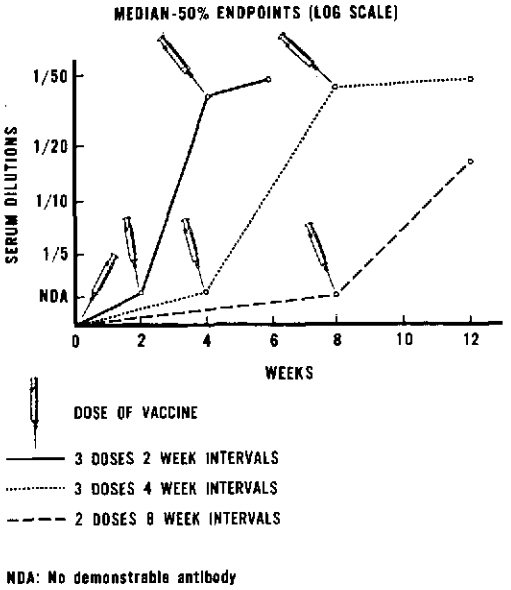

Fig. 3. Rabies vaccine of tissue origin in preexposure immunization of humans—Median-50 per cent endpoints log scale).

whereas in the two-dose vaccination group the median is only 1:20 (Fig. 3).

We certainly need more information before reaching a decision on the use of this type of vaccine in postexposure treatment of humans. We are currently planning to evaluate the vaccine in postexposure treatments of monkeys, and we hope that a combination of clinical and serological observations will help us to arrive at the right conclusion.

CHAIRMAN STOKES: Further discussion will now be presented by Dr. Martin Kaplan, Chief, Veterinary Public Health, World Health Organization, Geneva, Switzerland.

DR. KAPLAN: With reference to the subject of epidemiology, an ineradicable reservoir of bat rabies has now been reported in Thailand. The report, although still open to some question, adds some strength to the suspected presence of bat rabies in India. If the Thai and Indian

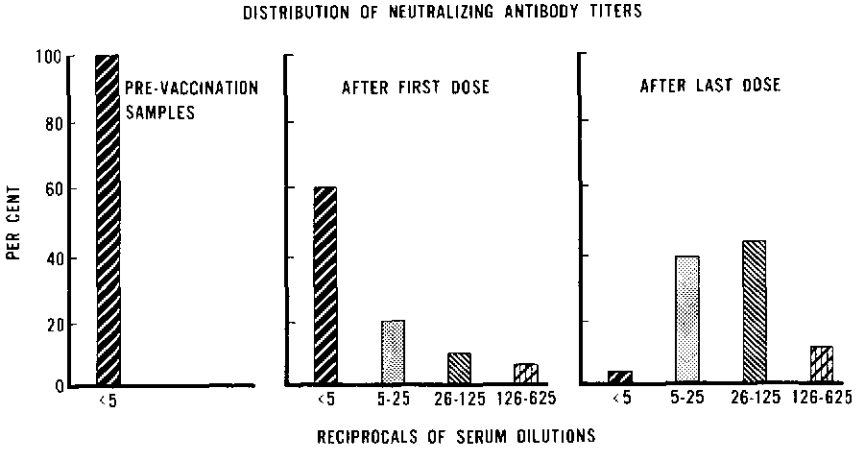

Fig. 2. Rabies vaccine of tissue culture origin in pre-exposure immunization of humans—Distribution of neutralizing antibody titers.

occurrences are confirmed, Africa will be left as one of the very few major land masses inhabited by bats where the virus has not been isolated from these animals—even though fairly intensive research has been under way in several African countries during the past few years.

It is clear, however, that despite the undoubted increase in wildlife rabies—notably in foxes in Europe, in foxes, skunks, and bats in the United States, and in vampire bats in Latin America—the dog remains the most important reservoir and transmitter of the disease for man. Therefore the dog should be the prime target of attack in control programs. The limited facilities of public health agencies found in most developing countries, should be concentrated on vaccination and stray-dog control in urban areas and not dissipated on the largely unrewarding task of attempting to control wildlife by presently known methods. We can *control* this disease to a variable and often large degree with the information we now have and with the capabilities existing in most countries, but we cannot *eradicate* it except in defined local geographical areas.

Although we should not ignore reported evidence that some dogs may be symptomless carriers and perhaps intermittent shedders of the virus, this occurrence would appear to be rare and certainly of little or no epidemiological significance in a control program that includes vaccination of dogs. Immune animals would smother the spread of such occasional transmitters. From the standpoint of general public health practice, there would appear to be no reason to alter the recommendations of the WHO Expert Committee on Rabies concerning biological prophylaxis in people exposed to dog bite when the animal remains healthy for ten days after the biting episode.

Can we derive any epidemiological inferences from the recent electron micrographic demonstrations that the rabies virus is morphologically and structurally similar, if not identical, to such strange bedfellows as the vesicular stomatitis virus, sigma virus of *Drosophila*, the arthropod-borne cocal virus, and the hemorrhagic septicemia virus (Egtved) of trout? I think not. I believe that the grouping together of these viruses, which so far have not demonstrated any serological relationships, illustrates a phenomenon that perhaps has not been taken sufficiently into account in the setting up of such classifications—namely, the finiteness of geometric patterns possible not only at the atomic level but also on the macromolecular level.

As for vaccines, I have two brief comments. I see little justification, except a commercial one, for continuing to spend time, resources, and money on the improvement of canine vaccines. Both phenolized nervous tissue vaccine and Flury strain vaccines are cheap and relatively easy to produce, and their worth has been proven beyond doubt. However, continued experiments on the duration of immunity with HEP vaccine might give us a single vaccine for use in cattle, cats, and young dogs and thus avoid the risks of LEP vaccine in these animals. HEP vaccine does not appear to be very effective in cattle, and for these animals we certainly require a better vaccine.

As for human vaccines, there is no doubt that intensive efforts are in order to develop a tissue-culture vaccine that could avoid the present long and unpleasant schedules of nervous-tissue and duck-embryo vaccines, which are sometimes ineffective and even dangerous—especially the nervous-tissue vaccines. The substrate tissue culture is of prime importance, and precautions should be taken to avoid the risks of virus passengers that may be present in primary tissue explants, not to mention potential oncogenic properties inherent in tissue culture lines that can be propagated indefinitely. Before any possible trial in humans at risk, tissue-culture vaccine candidates should be shown to be capable of protecting laboratory animals, especially nonhuman primates, at least as effectively as the present conventional vaccines do.

CHAIRMAN STOKES: The next discussion will be presented by Dr. Harald N. Johnson, California State Department of Public Health, Berkeley, California.

DR. JOHNSON: Dr. Bell has mentioned the need for studying arctic rabies. During the past 25 years we have witnessed large-scale epidemics of rabies in the arctic fox, but for many years prior to these outbreaks there was no evidence of the disease in the far north. The virus is able to exist in the arctic regions in some mammals, and the ermine (*Mustela er-*

minae) seems to be the best candidate for study as a reservoir host of the virus in this region. I believe that the far north is a good place to study the epidemiology of rabies because the total number of species found there is small. We can expect to find the reservoir host among the carnivores or the small mammals on which they feed. Rabies has not been identified in the small rodents, and bats are absent in most of the far north where rabies has been a problem in recent years.

Bat rabies has received much attention in past years in North America. The bat does not seem to play any role in the epidemiology of rabies in Europe, Africa, or Asia. In fact, large-scale surveys in these regions have failed to reveal evidence of infection in bats during the past few years. Bats are abundant in several countries that are free of rabies.

There are certain regions where rabies recurs at relatively frequent intervals without affecting dogs or related canine species. The occurrence of sporadic cases of skunk, weasel, and mongoose rabies without evidence of rabies among other carnivores suggests that these animals or other animals associated with them are the long-term reservoir hosts of the virus. The distribution and abundance of mustelids and viverrids fits the distribution of the endemic foci of rabies virus. The occurrence of sporadic cases of rabies in the mongoose in Puerto Rico and Grenada, where dog-to-dog transmission of rabies does not play any part in the maintenance of the disease, makes me believe that these islands are especially suited for the study of the role of the mongoose as a reservoir of rabies.

There are marked differences in the characteristics of strains of rabies virus isolated from naturally infected animals. Certain strains of the virus from spotted skunks and arctic wolves are characterized by a long incubation period in experimentally infected animals, absence of Negri bodies, tropism for lung and muscle tissue, and low pathogenicity for adult mice. Some of these strains would not have been recognized by the methods of isolation and identification used prior to the introduction of the FA test and the suckling mouse test.

The avianized Flury strain of rabies virus produced in chicken embryos is a very satisfactory vaccine for the immunization of dogs. That this is a safe and highly antigenic vaccine has been amply proved by experimental studies in dogs and by many years of experience in the field control of dog rabies. The use of other virus strains for the preparation of live virus vaccines for dogs seems ill-advised and unnecessary. In testing new vaccines there is the tendency to draw conclusions from experiments with small groups of animals and few controls. Preliminary trials of low-titer live virus vaccines may fail to reveal the danger of high-titer vaccines prepared from the same virus. This was the experience with the Umeno and Doi live virus vaccine for dogs. The safety of the Flury strain appears to be related to the selection of a virus population in the avian host system that has little pathogenicity for mammals. This characteristic is not fixed, since one or two intracerebral passages of the HEP variant makes this strain pathogenic for adult mice. This illustrates the danger of subpassage of this vaccine virus in mammals or even mammalian cell lines. We are familiar with the tendency of a vaccine virus to change its characteristics on serial passage and are thus aware of the need to check the antigenicity of the virus. By keeping a large bank of seed virus the passage level can be kept low and there is less chance of variation in virulence or antigenicity.

There is a tendency to belittle vaccines currently in use in order to push new vaccines. The Semple rabies vaccine prepared from rabbit brain has a great tradition and we do know what it will do in the immunization of man and animals against rabies. It is not in the interests of public health practice to speak of the terrible rabies vaccine treatment and the great hazard of paralysis from the Semple-type vaccine. Local and generalized reactions are not uncommon in vaccination with bacterial, rickettsial, and virus vaccines. The point to remember is that over the years WHO surveys have shown that the incidence of paralytic reactions from the nerve tissue rabies vaccine is less than 1 in 10,000 and that these reactions are seldom fatal.

We do want a vaccine with less nonviral antigen, but until we can grow a virus in the absence of cells we will have nonviral antigen in our vaccines. Purification of the virus antigen appears to offer the best line of study. The problem is to prove that the vaccine, in addition to stimulating certain antibodies that are demon-

strable by serological tests, actually produces immunity to rabies.

CHAIRMAN STOKES: The subject is now open for general discussion.

DR. LEPINE: The vast majority of human rabies vaccines currently in use throughout the world are phenolized preparations, whether the virus is completely inactivated as in the Semple-type vaccines or whether it still retains some active virus as in the Fermi-type vaccines. Besides being cheap and easy to prepare, such phenolized vaccines are of unquestionable efficacy.

However, whatever type of vaccine is considered, we are faced with the painful and shocking fact that vaccine failures occur from time to time—a striking example of which was given by Dr. Koprowski. These are unexpected failures that cannot be traced to the severity of wounds, the virulence of the street virus, or the delay in applying the treatment. We believe that such dramatic incidents could be avoided if the potency of the vaccine were increased.

Pending the production of a satisfactory tissue-culture vaccine, which is not yet available, Dr. Atanasiu, Dr. Gamet, and I have conducted experiments at the Pasteur Institute on a new type of vaccine, which comparative controlled tests in animals have shown to be markedly superior to the existing ones.

It consists of a partly purified sheep brain suspension, inactivated with beta-propiolactone and lyophilized. Although there is no trace of live virus left, its immunogenic potency is much higher than that of phenolized vaccines, and it shows no significant loss in potency after two years in storage or after 30 days at 37°C. Its value in human protection is now being assessed in African regions where rabies is highly prevalent.

Judging from the results so far obtained, this vaccine would have already replaced the Pasteur phenolized vaccine on the production line if it were not for the complicated procedures involved in introducing a new vaccine or substituting an already licensed one in our country.

Another point worthy of mention is the schedule of booster vaccine doses. The WHO Expert Committee on Rabies has shown that if hyperimmune serum has been administered prior to or currently with the primary vaccine treatment, booster injections are advisable in order to overcome the depressing effect of the serum on active immunization. The studies we have made on neutralizing antibody levels observed after the combined serum-vaccine primary treatment have led us to recommend three boosters, each of one vaccine injection, given at 30, 90, and 200 days after completion of the primary vaccine course.

Finally, in connection with the academic studies that were summarized by Dr. Koprowski, I should like to mention that rabies virus—street virus strains and fixed virus strains as well—has been adapted by Nadine Plus and Atanasiu to the fruit fly *Drosophila melanogaster*, and the result has been complete loss of neuropathogenicity for mammals, including the baby mouse. We do not yet know whether such transformed strains still retain their antigenicity and could be used for vaccines, but studies are in progress.

DR. FOX: I would like to speak on two points.

In the first place, Dr. Koprowski cited treatment failures and mentioned that they occurred despite the best available treatment. I think the fact that in both instances hyperimmune serum was given and followed by duck-embryo vaccine would suggest that the patients did not get the best available treatment.

It seems to be generally accepted that duck-embryo vaccine is not sufficiently potent to overcome the suppressive effect—that is, suppression of active response—inherent in administering hyperimmune serum, which is the reason for recommending the booster doses that Dr. Lepine has just emphasized. It would have been better if the patients had received a potent brain-tissue vaccine under these circumstances.

The other point I would like to stress is one that was alluded to by Dr. Koprowski and also by Dr. Fenje. I think we certainly have to face the fact that the risk of rabies is very high in many parts of the world and that there is an urgent need for a safe, acceptable method of pre-exposure immunization, much as we now use for tetanus in the form of tetanus toxoid. I believe the duck-embryo vaccine currently available best meets this need, but I am pleased to see that both Dr. Koprowski and Dr. Fenje, and perhaps others, are working toward an even

safer vaccine that can be widely used in this respect.

DR. KOPROWSKI: When I mentioned that in the cases of treatment failure the patients had received the best treatment available, I was referring to the fact that if there is a choice between duck-embryo and brain-tissue vaccine, duck-embryo is recommended because it incurs no risk of neuroparalytic sequelae.

The duck-embryo vaccine in the Moroccan case was used with booster injections. Thus the physicians were familiar with the interfering action of the serum. Even so, death occurred three months after treatment.

Undoubtedly several factors are involved. We have to consider whether the amount of immune serum given in the 1966 case was adequate and whether the potency of the duck-embryo vaccine at the time of immunization was as high as at its origin.

I agree with Dr. Fox that as far as persistence of potency is concerned, today the brain-tissue vaccine is still the preferred choice.

SECTION C. HEPATITIS

PRESENT KNOWLEDGE OF THE ETIOLOGY OF HEPATITIS

ROBERT W. MCCOLLUM

Department of Epidemiology and Public Health
Yale University School of Medicine
New Haven, Connecticut, U.S.A.

DR. MCCOLLUM (*presenting the paper*): The subject of hepatitis holds a position that is somewhat unique for this Conference. There is no hepatitis vaccine, nor is there any acceptable virus on which to base one. Infections due to a number of readily identifiable viruses occasionally produce signs, symptoms, and biochemical aberrations indicative of hepatic involvement in addition to their more characteristic clinical syndromes. Such viruses must account for no more than an extremely small fraction of the illnesses clinically diagnosed as viral hepatitis. While spectacular advances have occurred in other virus diseases during the past 20 years, from the virologists' viewpoint hepatitis has advanced little, if at all. Our knowledge of the etiologic agents is still based almost entirely on epidemiologic inferences and limited experimental human transmission studies. This is not to imply that hepatitis virology has been neglected. It is merely a reflection of the apparently insoluble problems that have plagued the study of the subject from the beginning.

The increasing clinical and public health importance of hepatitis over the past two decades has provided a continuing challenge to virologists seeking isolation and identification of the etiologic agents. Numerous reports of success have appeared. These agents, for lack of a better collective designation, have been referred to as "candidate" hepatitis viruses. To date no candidate has received adequate supporting documentation to gain general acceptance.

Some degree of confusion still exists in hepatitis nomenclature. The term viral hepatitis includes two similar clinical conditions with basically different but partially overlapping epidemiologic characteristics: infectious hepatitis (IH) and serum hepatitis (SH) (41). Evidence for immunologic distinctiveness was first provided by limited cross-challenge volunteer transmission studies some 20 years ago (19, 31). Recently Krugman and Giles, in studies soon to be reported, have added evidence in support of at least two varieties of hepatitis agent with differences in incubation periods and clinical manifestations. The question of single vs. multiple strains responsible for each type remains unanswered. The relative infrequency of multiple distinct episodes of hepatitis in a given individual would appear to speak against a multiplicity of unrelated antigenic types, but not necessarily so. The relationship between the agents of IH and SH is equally unclear. It is based at the moment on the similarities of their clinical manifestations and certain other shared characteristics, not the least of which is their resistance to laboratory adaptation.

More specific characterization of hepatitis agents is indeed limited. There may even be some basis for a lingering doubt that they represent viruses. One of the candidate SH agents [O'Malley's A-1 (33)] has now been identified as *Mycoplasma gallisepticum* (34) and an IH candidate [Chang's "lipovirus" (10)] may be an ameba of the Hartmanella genus (unpublished). However, the prevailing belief that the true etiologic agents of hepatitis are viruses appears to be well established. Evidence that they are small viruses rests on the results of a single filtration

study (27) in which an SH serum maintained infectivity after passage through a membrane of 52 mμ average pore diameter (APD), suggesting a particle size of 26 mμ or less. More recently, Krugman and Giles filtered a serum pool (Willowbrook No. 5), which contained both short- and long-incubation hepatitis viruses, and it retained its infectivity after passage through a 200 mμ APD membrane, the smallest APD included in their filtration series (unpublished results).

Both IH and SH viruses are generally considered to be unusually resistant to degrees of physical and chemical treatments that render most other viruses noninfective (21). The epidemiologic observations and experimental transmission studies on which these beliefs are based cannot be discounted. Nonetheless, rigid interpretation of such findings, particularly as they might be applied to viruses propagated under artificial circumstances, may be unwarranted. One would hope that the viruses as they exist in the original inoculum would display appropriate patterns of stability, but beyond this point physical and chemical resistance might deviate from the expected.

Human transmission studies conducted in the 1940's [summarized in (20)], and more recent ones as well (24), have indicated that IH virus is excreted in the feces during the latter half of the incubation period and probably not longer than a week after onset of jaundice—a total of three to four weeks. The same series of studies delineate a similar period of viremia. Although pharyngeal and urinary excretion have been suspected in a few epidemic situations, adequate supporting experimental evidence is lacking. SH virus transmission studies have been less extensive, but it appears that viremia is much more prolonged and may extend through most of the incubation period. It is usually stated that SH virus is not excreted and that transmission occurs only by a parenteral route. There are some who question the limited experimental evidence for this restriction (29).

In keeping with these patterns of demonstrated virus distribution, most of the candidate viruses have been derived from appropriate materials collected at appropriate times in the course of natural or experimental infection. Some investigators have been critical of the use of feces, particularly pooled specimens, from infants and young children as a source material for IH viruses because of the abundant viral flora often observed in normal populations of this age range. For this reason, agents isolated from individual blood, plasma, or serum specimens have tended to receive greater acceptance as candidates.

The IH candidate viruses reported during the past 10 years are by no means uniform in their physical, biologic, or immunologic properties (1–5, 7, 9–11, 13, 15, 16, 18, 23, 25, 26, 30, 33, 36, 38). Several are still insufficiently characterized to permit classification. Others are readily identifiable as specific members of recognized virus groups such as the adeno-, myxo-, herpes-, pox-, reo-, Coxsackie, and other enteroviruses. Such viruses lend themselves to epidemiologic studies by standard virologic and serologic methods, but the published results of such studies are for the most part insufficient to support broad etiologic significance.

In some instances candidate viruses appear to have been definitely associated, though not necessarily etiologically so, with multiple cases of hepatitis occurring within a limited temporal and spatial setting. It is quite possible, particularly in relatively closed population groups, that viruses other than hepatitis viruses may be in active simultaneous circulation. Under such circumstances major difficulties arise in assigning significance to the comparative distribution of virus recovery and serologic test results among "infected" and "control" groups, a division complicated by the broad spectrum of response to IH infection as well as to infection from other viruses likely to be encountered. A candidate virus isolated from a single patient presents even greater difficulties in ascertainment of etiologic significance.

The confirmation of SH candidates would appear to offer less of a problem, since individuals exposed to certain types of transmission risks and matched unexposed controls should be readily identifiable. However, the prolonged and highly variable incubation period, the associated matter of long-term specimen collections, and the possibility of intercurrent exposure to additional unrecognized risks of infection combine to introduce difficulties of comparison not easily overcome. Recent serial biochemical and liver

biopsy studies of blood and plasma recipients offer evidence of unexpectedly high rates for anicteric and asymptomatic infections (17, 37). If these observations represent true SH infections, carrier rates among blood donors, and by implication among the general population, must be considered to be far higher than previously estimated. Such a high level of long- or short-term SH carriers as well as presumably noncarrier immune individuals could play havoc with the selection of study groups that would provide clear evidence in support of etiologic relationships.

Some of the candidates belong to categories of viruses (for example, herpes and adenoviruses) recognized for their capacity to persist in many individuals in a "latent" form for long periods, becoming apparent or reactivated from time to time as a result of varying stimuli. Perhaps hepatitis provides a suitable stimulus and the discovery of such viruses is merely fortuitous. However, the frequency with which various adenoviruses have been identified in association with hepatitis cannot be ignored. This relationship is deserving of further study. Even though results of serologic studies do not support an etiologic role for adenoviruses, these and some of the less common isolates (for example, myxoviruses and enteroviruses) may conceivably play some part in the pathogenesis and level of clinical manifestation of hepatitis infection without being involved as essential or specific components of an etiologic complex. This possibility also deserves further consideration.

Perhaps the most widely acclaimed hepatitis virus candidates are the three described in 1961 by Rightsel and co-workers (36). Two of the three, as high-level tissue culture (Detroit-6 cells) passages, have been reported to produce clinical and biochemical evidence of hepatitis in volunteers. In general, attempts to duplicate the tissue culture methods in other laboratories have failed. Two well-controlled independent studies in Australia are soon to be published (G. F. Cross and B. P. Marmion; A. A. Ferris and R. A. Cole). Their results negate the specificity previously ascribed to the cytopathic effects noted in Detroit-6 cells inoculated with sera from hepatitis cases, with "positive" tissue culture passage materials, or with an AR-17 prototype virus passage supplied by the original investigators.

Recently Hatch and Dye (presented at the 94th Annual Meeting of the American Public Health Association, San Francisco, 1966), reviewed the results of studies carried out during the past four years in the hepatitis laboratories of the Communicable Disease Center of the U. S. Public Health Service. These studies have involved both virologic and serologic evaluation of a number of candidate agents, some of which have never been reported in publications. The findings to date provide no support for etiologic relationships, but a few agents are still under study.

Hepatitis virus isolation attempts of recent years have been limited in large part to tissue culture systems, although a few reports of animal transmission have appeared (6, 35). The increasing list of observed cases of hepatitis among handlers of newly imported primates (22), particularly chimpanzees, has provided a strong stimulus to the further exploration of nonhuman primates as experimental animals for virus isolation attempts. Although several such studies have yielded occasional suggestive results, at the moment only one genus (*Saguinus*) appears highly promising as a feasible host system for hepatitis studies. Deinhardt and co-workers have demonstrated associated chemical and morphologic evidence of hepatic disease in marmosets inoculated with human acute-phase hepatitis serum or plasma (to be published). These effects are serially transmissible from marmoset to marmoset. The responsible agent has not been further identified or characterized, but appropriate studies, including neutralization tests, are in progress.

It is said that one learns from his own mistakes and those of others, but it is doubtful that any useful purpose would be served by further restating the long history of hepatitis virus isolation attempts and failures. This has been the subject of repeated reviews (12, 28, 39, 41). It is even doubtful that a more detailed appraisal of currently available information concerning those "candidate" viruses still "in the running" would prove useful. To the outside observer, and perhaps even more to some who deal more directly with the problem, it must seem that the duration of the candidacy status of many reported isolates has been exceedingly long, particularly in view of the techniques and facilities available for their study. This comment is not

intended as a blanket condemnation of all who have been so fortunate, or unfortunate, as the case may be, as to produce an agent from hepatitis case-related materials. No one will ever know how many such agents have been put quietly to rest without benefit of publication. However, I would venture to guess that several lifetimes of scientific effort have been spent by investigators following their own false leads as well as those engendered by what must often be regarded as the premature reports of others. Perhaps part of the problem and much of the confusion is related to an increasing emphasis and reliance on technology, a lack of sufficient regard for firm epidemiologic support in establishing etiologic relationships, and in some instances, a failure to recognize that the conditions that provide for the transmission of hepatitis may also foster the transmission of other agents. Many investigators have been careful to insert a strong note of caution in presenting their findings and their interpretations concerning possible etiologic associations. In spite of seemingly inevitable frustrations, the search for hepatitis agents continues to command wide attention and a degree of devotion, if not enthusiastic optimism.

It is quite apparent that our present knowledge of hepatitis etiology is far from complete. The final status of some candidate agents remains unsettled, and the information currently available provides little hope concerning their future. It is likely that when someone breaks the hepatitis barrier with a well-documented virus that can be distributed to and evaluated by others, there will be no need for a prolonged candidacy status. Only then can approaches to effective vaccines be considered.

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SECTION C. HEPATITIS

DISCUSSION

CHAIRMAN STOKES: The first discussant in this section will be Dr. I. William McLean, Jr., Division of Microbiological Research, Parke, Davis & Company, Detroit, Michigan.

DR. MCLEAN: It has been almost three years since we have reported on our work with hepatitis, so this is a welcome opportunity to tell what we have been trying to do. In our publications we have described our studies using the difficult, unstable Detroit-6 cell system and, more recently, our work with the more stable human diploid embryonic cell strains, particularly those of the lung. On either of these systems—least of all the diploid cells—is there a marked cytopathogenic effect (CPE). This is particularly true during early passages of a new isolate. Therefore, our current emphasis is on methods other than CPE for detecting the presence of virus in our cultures.

All the commonly accepted indicators are being tried—direct and indirect complement-fixation, hemagglutination and hemadsorption of a wide range of red cells, interference with known viruses, micro- and macro-precipitation techniques, and so forth—so far with negative results. The only interesting leads have been obtained with direct and indirect immunofluorescence using serum from human volunteer subjects taken three to five months after challenge with live virus or late convalescent serum from natural cases. The typical specific fluorescence we have observed is diffuse, cytoplasmic, and faint. These areas of the cytoplasm fluoresce red when stained with acridine orange. Immunofluorescence can be detected about 48 hours before CPE and can be seen in many infected cell cultures that never develop any evidence of specific CPE. Using this technique, we have identified the viruses isolated from two recent epidemics of IH—one in Florida and one in Michigan—as being of the same serotype as our AR-17. In contrast, virus isolated from hepatitis cases among the troops in Korea have invariably been of the WW-55 serotype. In summary, we must still admit that we do not have a stable, easily maintained culture system or any serological test that can be utilized for routine diagnosis.

The recent work conducted in cooperation with Dr. Boggs on human volunteers at Joliet, Illinois, has also been discouraging. In 1964 we summarized all of our results up to that time. The AR-17 isolate and related viruses produced infection, as evidenced by viremia and neutralizing antibody response, in nearly all of the presumably susceptible subjects given 10^7–10^8 TCID of virus, either intramuscularly or orally. In spite of this high infection rate, however, only 18 of 43 subjects became ill and had significantly abnormal liver function tests, 12 of them developing full-blown hepatitis with jaundice. Recently the 25th TC passage of the same AR-17 isolate was tested in 10 volunteers. According to the FA test, five of these men had AR-17 antibody and five were negative. None of the 10, however, showed any evidence of disease after challenge.

With the WW-55 plasma specimen (from a preicteric natural case of IH), the rates of frank clinical disease have been similar: 11 of 20 men at risk became ill and 9 of these were jaundiced. Illness was less frequent with the WW-55 TC isolates; of 20 men at risk, 9 of whom were injected intramuscularly and 11 of whom received the preparation orally, only one became ill with clinical hepatitis. More recently we have concentrated on materials from hepatitis cases in Korea. Of 35 volunteers inoculated with either acute-phase serum specimens from Korean cases or TC passage virus isolated therefrom, not a single man has developed hepatitis. We cannot

explain this apparent resistance of the Joliet volunteer subjects to the Korean specimens.

The original report of Bearcroft on the production of liver pathology in African monkeys by inoculation of human hepatitis material and the description by several authors of the association of human cases with exposure to primates lead us to become interested in the possibility of finding a useful laboratory animal among the available subhuman primates. In our first attempt, one of two carefully isolated cynomolgus monkeys became ill after an IV injection of AR-17 TC 15 virus. This animal had a definite increase in transaminase (five times base line), but unfortunately it died after the liver biopsy before other liver function tests had been performed. The liver showed changes suggestive of early hepatitis in stained sections, and electron microscopy indicated that these changes were similar to those described by Bearcroft and identical to those found in liver biopsy specimens from human hepatitis cases by Dr. Taylor in our laboratory. Since this experience, we have conducted two larger trials using several different virus preparations in 30 conditioned, isolated patas monkeys. There has been no significant evidence of hepatic disease in these trials. However, the positive results reported by Smetana in patas monkeys and by Holmes and Deinhardt in marmosets with our proven WW-55 plasma are most encouraging. Certainly, the determination of a susceptible laboratory animal would greatly facilitate continued hepatitis research.

CHAIRMAN STOKES: The next discussion will be presented by Dr. Saul Krugman, Chairman, Department of Pediatrics, New York University School of Medicine, New York.

DR. KRUGMAN: I should like to report on results of "Studies of Two Types of Infectious Hepatitis with Distinctive Clinical, Epidemiological, and Immunological Features,"* coauthored with Dr. Joan P. Giles.†

During the past 10 years more than 1,000 cases of infectious hepatitis with jaundice have been observed at the Willowbrook State School. Second attacks of hepatitis with jaundice have occurred in 5.5 per cent of this group. Recent studies have indicated that the two attacks of hepatitis may be caused by two immunologically distinct types of virus. The two types of virus have been designated MS-1 (derived from the serum of a patient during the first attack of hepatitis) and MS-2 (derived from the serum of the same patient six months later during the second attack).

The description of the endemic situation at Willowbrook and the justification for artificial transmission of the disease with informed consent have been described in detail in previous reports.‡ After a series of six separate trials the following distinctive characteristics of the two types of infectious hepatitis were observed:

1. *Incubation period:* 31–53 days for MS-1; 41–108 days for MS-2.

2. *Abnormal serum transaminase activity:* relatively short for MS-1 (3 to 15 days); relatively long for MS-2 (35–201 days).

3. *Thymol turbidity:* consistently elevated in MS-1 virus infection; frequently normal in MS-2 virus infection.

4. *Contagion:* the MS-1 infection was highly contagious for all six presumably susceptible contacts; MS-2 infection was less contagious, spreading to only two of five susceptible contacts.

5. *Infectivity by mouth:* both types of infectious hepatitis were infective by mouth. Oral administration of serum from a patient with MS-2 type of infection was highly infective; it produced anicteric hepatitis in 10 of 12 subjects after incubation periods of more than two months.

6. *Immunity:* patients who had a MS-1 infection were subsequently resistant to re-exposure

* These studies were sponsored by the Commission on Viral Infections, Armed Forces Epidemiological Board and were supported in part by a contract No. DA-49-193-MD-2331 from the U. S. Army Research and Development Command, Office of the Surgeon General, Department of the Army.

† From the Willowbrook State School, Staten Island, New York, and Career Scientist of the Health Research Council of the City of New York.

‡ Ward, R., Krugman, S., Giles, J. P., Jacobs, A. M., and Bodansky, O. "Infectious Hepatitis: Studies of its Natural History and Prevention." *New Eng J Med* **258**:407, 1958.

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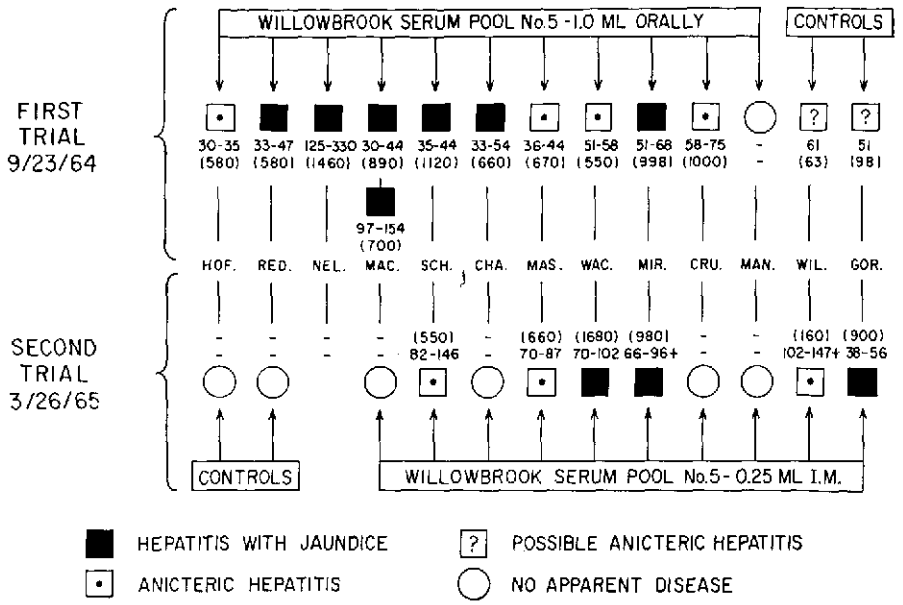


Fig. 1. Infectious hepatitis at Willowbrook—Results of first and second trials.

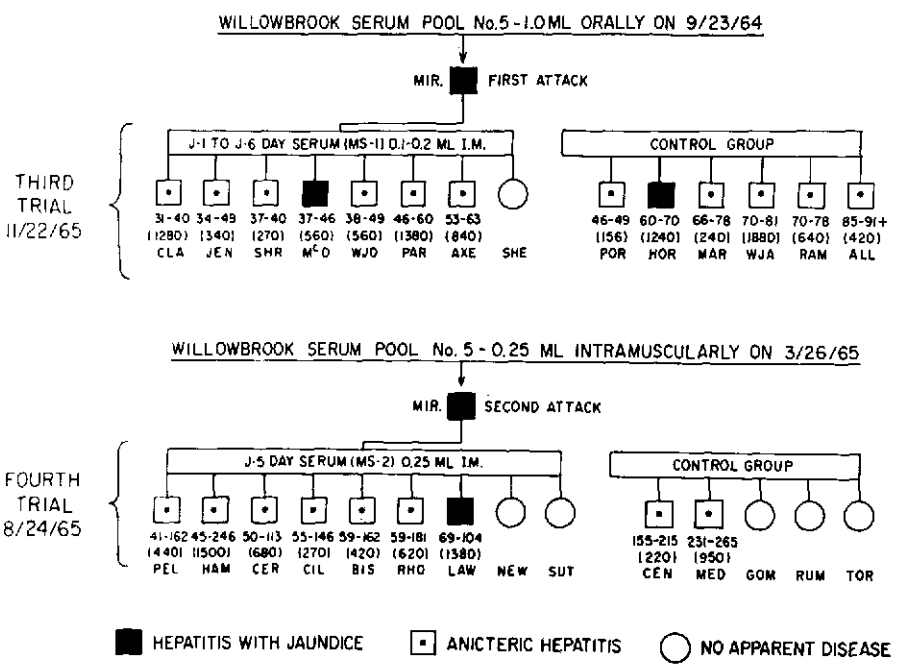


Fig. 2. Infectious hepatitis at Willowbrook--Results of third and fourth trials.

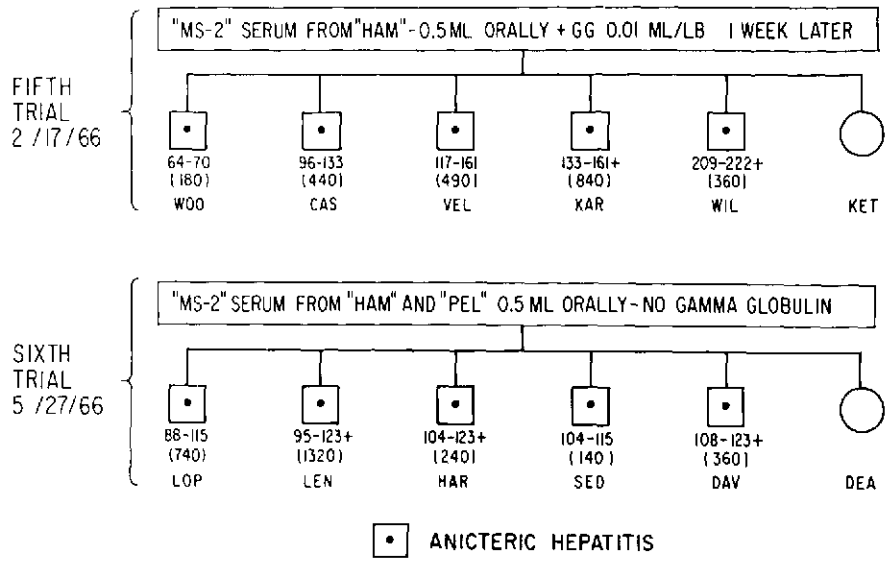
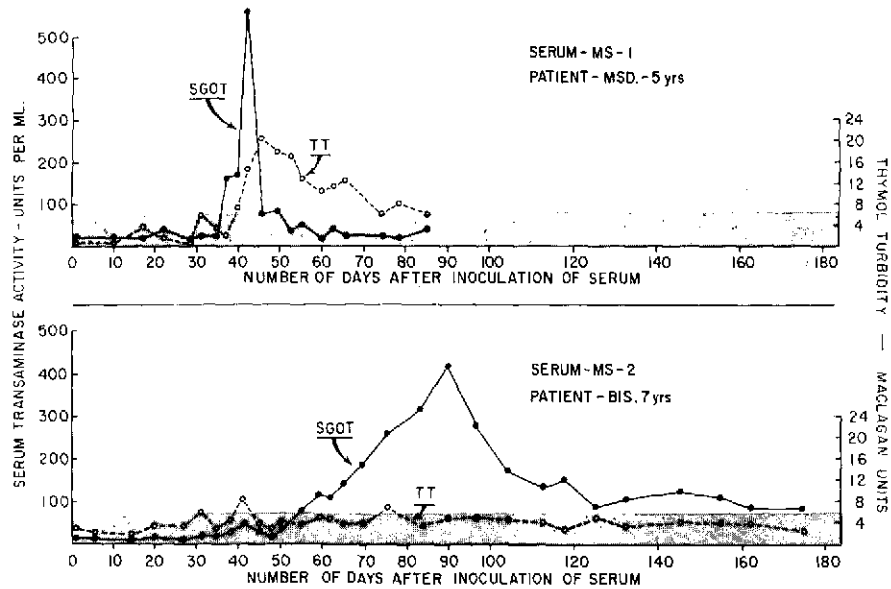


Fig. 3. Infectious hepatitis at Willowbrook—Infectivity of MS-2 serum by mouth, fifth and sixth trials.



Note: There was a spiking rise and fall of SGOT and abnormal TT activity following MS-1 infection. In contrast, there was a gradual rise and prolongation of SGOT activity and normal TT.

Fig. 4. Infectious hepatitis at Willowbrook—Pattern of serum transaminase (SGOT) and thymol turbidity (TT) response after inoculation of serum from first attack (MS-1) and second attack (MS-2).

to MS-1; limited data suggest that an MS-2 infection does not confer resistance to an MS-1 infection.

The data accumulated during the course of these studies are summarized in Figures 1, 2, 3, and 4.

First trial. Willowbrook Serum Pool No. 5 was prepared from specimens obtained from

27 Willowbrook patients three to seven days before onset of jaundice. After appropriate safety testing it was fed to 11 presumably susceptible subjects. Note occurrence of hepatitis in 10 subjects. The first number indicates the first day that the serum transaminase (SGOT) exceeded 100 units; the second number indicates the first day the SGOT declined to levels below 100 units. The figures in parentheses indicate the peak SGOT levels. In this group the incubation period ranged between 30 and 58 days. Note relatively short period of transaminase activity in all subjects except Nel.

Second trial. Willowbrook Serum Pool No. 5 was given to the same subjects six months later. Note second attacks of hepatitis in at least four subjects (Sch., Mas., Wac., and Mir.). Note longer incubation periods and more prolonged periods of transaminase activity.

Third trial. Serum from subject Mir. in the first trial during first attack of hepatitis (MS-1) was safety tested and administered to eight subjects by inoculation; six uninoculated subjects lived with them and served as controls. Note onset of hepatitis after a relatively short incubation period (31–53 days); relatively short period of transaminase activity (3–15 days); and high attack rate in control group, indicating that this disease is highly contagious.

Fourth trial. Serum from subject Mir. in the second trial during the second attack of hepatitis (MS-2) was safety tested and administered to nine subjects by inoculation; five uninoculated subjects served as controls. Note onset of hepatitis after a relatively long incubation period (41–69 days); long period of abnormal transaminase activity (35–201 days); and contact infection in two of five subjects.

Fifth and sixth trials. Oral administration of MS-2 serum. Note onset of anicteric hepatitis in 10 of 12 subjects after incubation periods ranging from 64 to 209 days. Also note prolonged period of SGOT activity in most patients.

CHAIRMAN STOKES: Further discussion will now be offered by Dr. Robert G. Ward, Chairman, Department of Pediatrics, Children's Hospital of Los Angeles, University of Southern California, Los Angeles, California.

DR. WARD: I hope it will be appropriate to say something at this time about efforts to mitigate the severity of post-transfusion hepatitis. Post-transfusion hepatitis is a big medical problem which, despite iatrogenic overtones, has recently been swept out from under the rug.

It is estimated that each year in the United States about 1.8 million persons are transfused, about 30,000 of them contract post-transfusion hepatitis, and approximately 3,500 of these patients die as a result of the conditions. The estimated case fatality rate is between 11 and 12 per cent.

The results of two studies suggest that 20 ml of gamma globulin may modify post-transfusion hepatitis by suppressing jaundice. The first study, by Grossman and co-workers, was carried out during World War II; the second study, by Mirick and co-workers, was published last year. In both, the effective dosage and schedule of gamma globulin was 10 ml within one week after transfusion, and another 10 ml one month later.

The use of gamma globulin has practical drawbacks. To give each transfused patient 20 ml of gamma globulin would require 36,000 liters a year—and this in the face of a total annual production of between 6,000 and 10,000 liters in the United States.

We have therefore been casting about for other approaches. We have asked the question: Could less gamma globulin added directly to blood in the bottle *before* transfusion promote neutralization of hepatitis viruses before they pass the portal of entry and thus limit the number of severe cases?

We are currently testing this hypothesis in collaboration with Dr. Ricardo Katz in a controlled study in Santiago, Chile. Since ordinary gamma globulin is not safe to give intravenously, we are using gamma globulin modified by the Swiss method (6 per cent solution incubated at pH 4, etc.), to render it safe for intravenous use. Ten ml of modified GG are added to each unit of blood, a dosage equivalent to one-fifth of that found to be effective by the intramuscular route in the two studies mentioned earlier. Although cases of hepatitis with jaundice have begun to appear in the groups under study, they are too few in number at this point to be meaningful and therefore will not be discussed here. I can report a significant finding, however, that concerns the safety of gamma globulin modified by the Swiss method: Dr. Katz has given it in

blood transfusions to over 700 patients without a single adverse reaction.

CHAIRMAN STOKES: I should like to call on Dr. Saul Krugman again, who will present a report on behalf of Dr. Joan P. Giles of the Willowbrook State School, New York, N.Y.

DR. KRUGMAN: I should like to report on the "Effect of Gamma Globulin on the Incidence of Infectious Hepatitis with Jaundice,"* of which I am a co-author.

Since 1960 gamma globulin has been used for the protection of nurses, hospital attendants, kitchen, and laundry personnel employed in the Willowbrook State School. From 1960 to 1962 the gamma globulin was offered to all new employees on an optional basis; it was accepted by 190 and refused by 177. Since 1962 it has been administered routinely to all new employees.

As indicated in Table 1, a single dose of gamma globulin (16 per cent solution, 4 ml), administered intramuscularly, approximately 0.03 ml per pound of body weight, provided significant protection. The administration of gamma globulin was followed by an 80 to 85 per cent reduction in the incidence of hepatitis with jaundice.

CHAIRMAN STOKES: The next discussant will be Dr. Friedrich W. Deinhardt, Chairman, Department of Microbiology, Presbyterian-St. Luke's Hospital, Chicago, Illinois.

DR. DEINHARDT: I have been asked to report briefly on attempts to transmit human viral hepatitis to marmosets. These studies, conducted in our laboratories by Dr. A. W. Holmes and me during the last two years, have already been mentioned earlier today by Dr. McCollum.

Two of five acute-phase sera obtained from human cases of viral hepatitis induced definite biochemical and histological evidence of hepatic disease in marmosets, and this disease was passed serially from marmoset to marmoset. Figure 1 shows the results of one of these passage series. In the first experiment, human viral hepatitis serum obtained on the third day of jaundice was inoculated intravenously into four marmosets. All inoculated animals developed abnormal hepatic tests 16 to 40 days later and liver biopsies also showed definite abnormalities. In the next experiment, serum obtained from one of the animals on the day that elevated serum enzyme activities were first observed was inoculated into a new group of four marmosets. Thereafter, five serial marmoset-to-marmoset passages were carried out with almost all the inoculated animals showing chemical evidence of disease with peak SGOT activities from 400 to 900 units and SICD activities as high as 12,000 units. Many animals had hyperbilirubinemia. Incubation periods varied, but they tended to average around 20 days, particularly in the later passages.

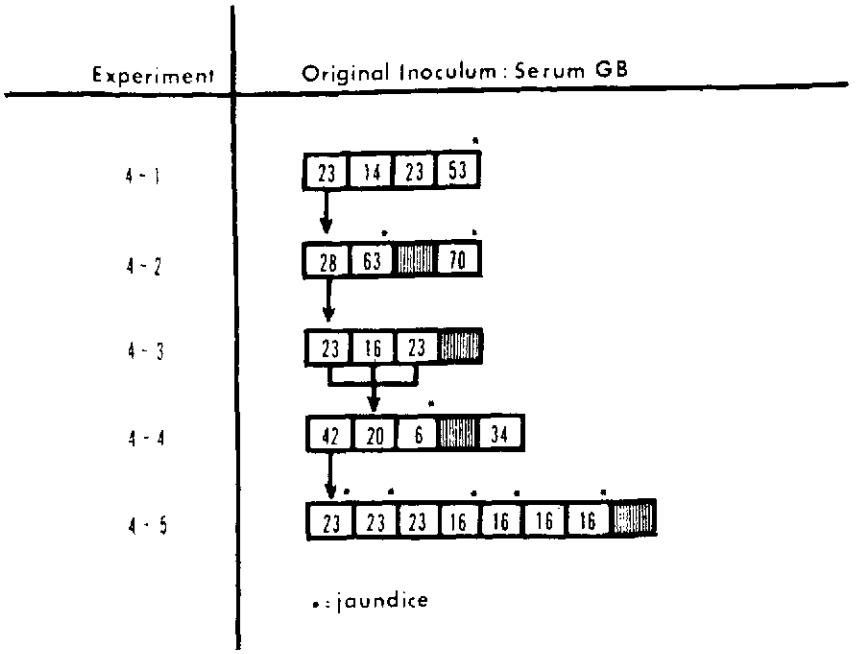
The results of a preliminary serum-neutralization test are shown in Table 1. The inoculum for all groups of animals in this experiment was acute phase marmoset serum obtained from animals of the last passage shown in Figure 1. As it can be seen, six out of six and five out of five animals inoculated with undiluted serum or serum diluted 1:1000 exhibited biochemical evidence of hepatitis, whereas partial neutralization was observed in the two groups of animals inoculated with undiluted serum mixed with human gamma globulin or late convalescent serum obtained from the same patient whose

* These studies were conducted under the sponsorship of the Commission on Viral Infections, Armed Forces Epidemiological Board, and were supported in part by the Office of the Surgeon General, Department of the Army.

TABLE 1. EFFECT OF GAMMA GLOBULIN ON THE INCIDENCE OF HEPATITIS WITH JAUNDICE IN WILLOWBROOK PERSONNEL DURING FIRST YEAR OF EMPLOYMENT

Year	Group	No. at risk	Hepatitis with jaundice		
			No.	Per cent	Reduction (%)
1960 to 1962	No GG	190	11	5.8	80
	GG, 4 ml	177	2	1.1	
1962 to 1965	GG, 4 ml	1,560	8	0.5	80–85*

*Estimated reduction of 80 to 85 per cent is based on a 4.0 to 4.5 per cent annual attack rate of hepatitis with jaundice in newly admitted Willowbrook patients during the period 1960–1965.



Note: Each row of blocks represents a single experiment, each block represents a single animal. Animals that showed no evidence of liver disease throughout the experiment are indicated by shaded blocks. Animals with biochemical evidence of hepatitis are indicated by open blocks, and the number inside the block is the number of days from inoculation to first abnormal hepatic tests. The vertical arrows originate from the blocks representing animals from which serum for passage was obtained. The asterisks indicate hyperbilirubinemia.

Fig. 1. Results of intravenous inoculation of human viral hepatitis serum in marmosets.

acute phase serum had initiated this passage series. No disease was produced in animals inoculated with the undiluted serum after it had been heated at 60°C for twelve hours and irradiated with 3 million roentgens.

TABLE 1. NEUTRALIZATION TEST WITH HUMAN γ-GLOBULIN AND CONVALESCENT HUMAN SERUM

Inoculum	No. of marmosets inoculated	Results		
		Hepatitis	Borderline	Negative
Undiluted*	6	6	0	0
10^{-3}*	5	5	0	0
10^{0} + γ − globulin	6	1	2	3
10^{0} + convalescent human serum†	6	2	2	2
Convalescent human serum†	4	0	0	4
Control marmoset serum	4	0	0	4

*10^{0} or 10^{-3} dilution of positive marmoset serum of the fifth marmoset passage, Barker series.
†Convalescent serum from Patient Barker.

These results indicate that marmosets may be regularly susceptible to human viral hepatitis, but they should not be regarded as proof that the disease observed in marmosets is indeed caused by the elusive agent of human viral hepatitis. The agent responsible for the disease in marmosets must be identified and its relation to the human disease must be established before any conclusions can be reached.

CHAIRMAN STOKES: Further discussion will be presented by Dr. Werner Henle, Professor of Virology, University of Pennsylvania School of Medicine, and Children's Hospital of Philadelphia, Philadelphia, Pennsylvania.

DR. HENLE: The search for the elusive human hepatitis viruses has been and continues to be severely handicapped by the general unavailability of well-documented specimens of known infectivity in sufficient quantity to permit broad and systematic exploration of new and old approaches. All too often nowadays only small samples of known or, more likely, of possibly

infectious materials are available to those who, undismayed by years of frustration, persist in their efforts toward isolation of these agents. Under such conditions, potentially fruitful ideas may go unrewarded simply because the starting materials were unsatisfactory.

There is a pressing need to organize under appropriate auspices a concerted effort to collect suitable specimens in quantity for distribution to interested laboratories. Of the many problems to be faced in such an effort, the one concerning "certification of infectivity" appears to be the most difficult to solve. Although in the past nothing short of inoculation of volunteers could have provided this information, the encouraging results obtained by Dr. Deinhardt in marmosets after inoculation of human hepatitis specimens may offer a means of selecting infectious materials. It would be gratifying indeed if today's discussion would lead to a rapid implementation of this plea.

CHAIRMAN STOKES: Now we will hear from Dr. Frederick O. MacCallum, Virus Laboratory, The Radcliffe Infirmary, Oxford, England.

DR. MACCALLUM: It has been left to me, a survivor of 30 years in this field, to bring up the rear.

It is depressing to see how little progress we have made in the last two decades. I do not wish to say anything of my own attempts to isolate a virus, all of which continue to be unsuccessful, but I should like to make a few comments on some of Dr. Krugman's results, which are of considerable interest. I am particularly impressed by the indication that his agent, which produced clinical hepatitis after a long incubation period when inoculated parenterally, produced very mild hepatitis detected only by sensitive biochemical tests when it was fed. As many of you know, no good evidence of hepatitis was obtained in any of the earlier adult volunteers who were fed virus B (SH), but the sensitive transaminase test was not then available.

The results of many of Dr. Krugman's other experiments in children have confirmed those obtained in adults more than 20 years ago. I thought it might be of interest to those of you not familiar with the history of the subject to see a table published in 1953, which was based on the results of experiments by Drs. Paul and Havens and co-workers, Drs. Stokes and Neefe and co-workers, and our group in Britain. You can see the possible reactions that may occur when you inoculate blood from a patient with suspected hepatitis into another human subject. Here is a possible explanation for the emergence of Dr. Krugman's two viruses. Other workers may have other explanations.

Whenever I collect specimens from suspected hepatitis patients for any new experiments I obtain feces as well as blood, and I prefer material from family contacts in community outbreaks rather than from patients in institutions. In the past 10 years I have only recovered one enterovirus from such stools when they were screened in a variety of cell cultures and in newborn mice before attempting nonconventional tests of one kind or another.

I also wish to state that I, like others, have failed to substantiate the claim of Bertok of Budapest that the human hepatitis virus can be transmitted to and cause disease in rats and guinea pigs stressed by the addition of ethionine in their diet.

TABLE 1. RESULTS THAT MAY THEORETICALLY BE OBTAINED BY INJECTION OF BLOOD FROM PATIENTS WITH HEPATITIS

Virus in donor's blood	Immune state of recipient	Result
A	O	Short incubation
A	B	Short incubation
A	A	No disease
B	O	Long incubation
B	A	Long incubation
B	B	No disease
AB*	O	Short + long incubation†
AB	A	Long incubation‡§
AB*	B	Short incubation§
AB*	AB	No disease

Source: MacCallum, F. O. *Brit Med Bull* 9: 221, 1953.
Notes: O = No immunity to hepatitis viruses A or B.
Number of healthy carriers of B unknown, but one estimated figure for one area in England during 1945–1948 was not less than 0.35 per cent. (Lehane, Kwanies, Upward & Thomson, 1949.)
*Considered to be A; carrier state with B was not recognizable.
†May be termed a relapse or even a second attack.
‡Suggested possible example to account for the results of MacCallum & Bradley (1944), which give wrong impressions as to incubation period.
§Cameron's mixed results (Cameron, 1943) could have been such examples.

In conclusion I wish to reiterate Dr. McCollum's plea that in the future professional virologists, as well as enthusiastic amateurs, refrain from publishing claims for new hepatitis viruses until they have checked their results with one or more reliable colleagues.

CHAIRMAN STOKES: Dr. Robert McCollum earlier I believe mentioned Dr. Marmion of Monash University, Melbourne, Australia. I do not know whether Dr. Marmion has any comment he would like to make or not.

DR. MARMION: Two reports have appeared from the Virus Research Laboratory at Fairfield Hospital, Melbourne, concerning the use of the special Parke-Davis clone of Detroit-6 cells for the isolation of agents from hepatitis cases. The first of these was by Cole,* and it described the isolation of cytopathic agents from the sera of hepatitis cases on the one hand and from healthy control persons on the other. There was quite a distinctive difference, and this looked encouraging. A second report by Cole, Danks, and Campbell† described the isolation of agents from cases of neonatal hepatitis.

I would just like to comment briefly on our subsequent experience with the Parke-Davis clone of Detroit-6 cells in Melbourne to put these reports in perspective.

Recently Dr. Ferris at Fairfield and my colleagues and I in the Monash Department have conducted blind trials in which coded specimens —acute-phase samples of serum from hepatitis and samples of serum from people with unrelated diseases such as bronchitis, pneumonia, degenerative vascular disease, and so on—have been put into the Detroit-6 cells by one set of observers and read by another.‡

When the final assessment of the changes in cell culture had been made by the observers, the code was broken, and it was found that there was no relation at all between the appearance of a cytopathic effect in the Detroit-6 cells and the clinical state of the donor of the serum.

The cytopathic effect observed in these cells is quite a real phenomenon. It can be transmitted to fresh cells using strong concentrations of the components of the tissue culture, but the effect rarely reaches titers beyond 10^{-2}.

Examination of flying cover slips from tissue culture inoculated with the material and showing a cytopathic effect, together with suitable controls, in immunofluorescence tests with convalescent-phase sera from the patients has not shown any significant positive results, nor have we been able to demonstrate antigen by any other method.

Examination of thin sections of cells inoculated with the cytopathic agent has not shown any virus particles. So we are left with only one marker, that of the cytopathic change in tissue culture, and this is not associated with the disease we are studying—hepatitis—but is distributed between cases and controls.

We feel therefore that the two previous reports issuing from the Fairfield Hospital Laboratories cannot now be regarded as substantiating the possibility of isolating virus from various types of hepatitis with the Parke-Davis clone of Detroit-6 cells.

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* Cole, R. A. "Viral Agents from Patients with Infective Hepatitis." *Aust J Exp Biol Med Sci* **43**: 497–504, 1965.

† Cole, R. A., Danks, D. M., and Campbell, P. E. "Hepatitis Virus in Neonatal Liver Disease." *Lancet* **1**:1368–1369, 1965.

‡ Ferris, A. A., and Cole, R. A. "Detroit-6 Cells and Infectious Hepatitis," and Cross, G. F., and Marmion, B. P. "Cell Culture and Infectious Hepatitis." *Med J Aust* (in press).

SESSION VIII

RICKETTSIAE, BEDSONIAE, AND ADJUVANTS

Thursday, 10 November 1966, at 2:00 p.m.

CHAIRMAN
DR. C. E. VAN ROOYEN

RAPPORTEUR
DR. J. P. FOX

Section A.
Rickettsiae

Presentation of Papers by:
Dr. Cornelius B. Philip
Dr. Charles L. Wisseman, Jr.
Dr. Paul Fiset

Discussants:
Dr. Imam Z. E. Imam
Dr. John P. Fox
Dr. Herald R. Cox
Dr. B. P. Marmion

Section B.
Bedsoniae

Presentation of Papers by:
Dr. Edward S. Murray
Dr. J. Thomas Grayston

Section C.
Adjuvants

Presentation of Paper by:
Dr. Geoffrey Edsall

Discussants (Sections B. and C.):
Dr. Roderick Murray
Dr. Frank T. Perkins
Dr. A. F. Woodhour
Dr. Richard Haas

SECTION A. RICKETTSIAE

NEW CONCEPTS ON THE EPIDEMIOLOGY OF TYPHUS FEVER

CORNELIUS B. PHILIP AND IMAM Z. E. IMAM *

DR. PHILIP (*presenting the paper*): Epidemic typhus has loomed large and persistently in the tragic annals of war, pestilence, and famine among mankind. The historical concept of the classic man-louse-man cycle (with recrudescences in man more recently recognized) has become thoroughly entrenched in our thinking about the natural maintenance of this infection. Growing evidence since 1957 that domestic animals may be involved in this cycle has been viewed with skepticism. Purely serological evidence, unsupported by isolations before and since that time, has rightly been discounted. Evidence accumulated since one of the present authors (C. B. P.) reviewed this subject before the PAHO Advisory Committee on Medical Research in April 1965 has increased interest, from both the civilian and the military standpoints, in the possible role of livestock in the reservoir mechanism of both murine and epidemic typhus fevers. A selected pertinent bibliography was attached to that report, which was issued under the title *Research Activities of PAHO in Selected Fields, 1964-1965*.†

The serological surveys begun in 1952 and recently summarized by Giroud and his colleagues (2) and the subsequent evidence, both serological and by strain isolation, of Reiss-Gutfreund in Ethiopia and Imam and co-workers in Egypt (references cited by Imam *et al.*, 5) have focused attention on potential extrahuman reservoir cycles of epidemic typhus in livestock in Africa. The modern refinement of rickettsial techniques has intensified these investigations.

Fragmentary evidence that livestock and/or their ticks are involved in typhus goes back three decades to the still-unconfirmed finding of *Rickettsia prowazekii* in the soft cattle tick, *Ornithodoros lahorensis*, in southern USSR by Klimentova and Perfilev (6) and their experimental infection of this tick. Other pertinent isolated reports, as in Turkey, have recently been reviewed by several authors (7). Experimental transmission by nymphs—but not by adults—of ixodid cattle ticks fed as larvae on typhus-infected rabbits was reported by Reiss-Gutfreund (13) in Ethiopia and also remains to be repeated. She likewise reported the first isolation of *R. prowazekii* from the blood of one goat and two sheep; its identity was later confirmed by Giroud (1). At that time, 17 per cent of cattle, sheep, and goat sera from the Addis Ababa abattoir had significant epidemic typhus antibodies by the Giroud microagglutination technique.

Weyer (17) has grown typhus organisms in ticks and other arthropods.

Present status of typhus fever investigations in livestock. Because of skepticism regarding some of her early Ethiopian studies, Reiss-Gutfreund performed two additional, separate studies (14, 15). She had originally isolated *R. prowazekii* from six lots of *Hyalomma* and *Amblyomma* ticks off cattle, which she augmented by two more from *Amblyomma* and another from *Hyalomma* in the final series. The identity of the last isolate (ZRS), which was accomplished in complete isolation from other typhus work, was amply confirmed at the Rocky Mountain Laboratory by both complement fixation (CF) and toxin neutralization (TN) (9). She also reported another isolate from a goat's blood in the second

* Dr. Philip (U. S. Public Health Service, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana); Dr. Imam (Virus Research Center, Production Laboratories, Cairo, U.A.R.).

† Document RES 4/2A.

series and found that 69 per cent of 247 abattoir animal samples had typhus agglutination titers of 1:128 or higher. In a limited survey in December 1963, surprisingly, Philip *et al.* (8) discovered no such reactors in the same abattoir, nor could they isolate typhus from either animal bloods or several species of ticks.

Meanwhile, Imam and Labib (3) and later Imam *et al.* (5), reported that 35 per cent of goat sera and 44 per cent of camel sera from Cairo abattoirs, and also 70 per cent of local donkeys, had CF antibodies in titers up to 1:128 against typhus-group (soluble) antigens. With washed specific antigens, 73 per cent of the positives were for the epidemic (E) agent and 27 for the murine (M); in this series none was reported with equivalent titers for both. In a village in Beni Swaif Province, 36 out of 103 inhabitants of all ages had E titers ranging from 1:32 to the maximum tested, 1:128; 25 out of 63 local sheep, goats, donkeys, and camels had E titers equivalent to those of the humans. Collections were subsequently shifted principally to animals in villages in 13 Nile Delta provinces where human typhus had formerly been reported. Table 1 reveals a comparative drop in the percentages of donkeys, camels, and goats reacting to typhus-group (soluble) antigens. Sera of some of the positive reactors, when tested with washed CF antigens, had a greater proportion of epidemic than of murine antibodies (Table 2). A seasonal increase in positive reactors was originally noted in abattoir samples in the fall and early winter months. Recent unpublished data of Imam suggest there may be a lesser rise in the summer and early fall and a major rise in winter and early spring (Fig. 1). The peak was observed between January and March.

TABLE 1. RESULTS OF TYPHUS GROUP CF TESTS ON SERA OF EGYPTIAN LIVESTOCK COLLECTED FROM JUNE 1965 THROUGH AUGUST 1966

Animals	Total	Positive*	Percentage
Donkeys	1,679	465	27.7
Camels	2,370	375	15.8
Goats	921	106	11.5
Pigs	1,887	121	6.9
Sheep	2,462	149	6.1
Buffaloes	151	3	1.9

*Titers of 1:8 and above considered positive with typhus group soluble antigen; a few were as high as 1:160.

TABLE 2. RESULTS OF CF TESTS ON A PORTION OF TYPHUS-POSITIVE SERA OF EGYPTIAN ANIMALS (TABLE 1) WITH WASHED SPECIFIC ANTIGENS

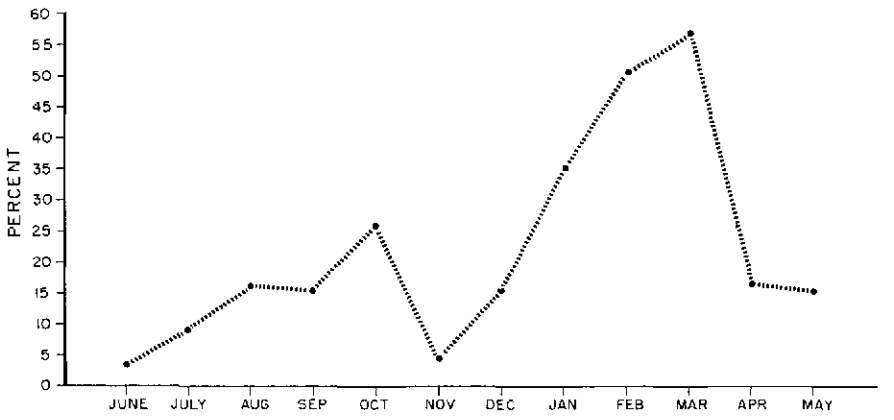
Animal	Total*	Epidemic (E) antigen	Murine (M) antigen	Indistinguishable E-M†
Camels	41	16	1	24
Donkeys	11	4	0	7
Goats	14	3	1	10

*Previously found to have titers of at least 1:8 against soluble antigen.
†Sera *not* showing at least an eightfold difference in specific titers.

It may have been coincidence that Imam did not accomplish the recent critical isolation of *R. prowazekii* until periodic sampling was begun of animals in provinces where human typhus had formerly been reported. At least two such isolates from donkey bloods, which were injected immediately into guinea pigs on the premises, have been passed serially in guinea pigs, with resultant characteristic syndromes, and in embryonated chicken eggs, which showed rich rickettsial growth in Gimenez-stained smears of yolk sacs. Convalescent guinea pig serums from both isolates also provided fourfold or greater differences (titers of 256 to 48 and 1024 to 64, respectively) in favor of epidemic over murine CF reactions with washed antigens at the Rocky Mountain Laboratory* and in Egypt. Studies are still in progress on these and other promising isolates from donkeys in different areas. The epidemic and murine antigens used by Imam were obtained from outside sources, and no strains of either agent had been maintained in his laboratories for over a year prior to the first isolation in October 1965. Protocols for these isolations will shortly be published by Imam and Labib.

Though the observations by Reiss-Gutfreund of infection in animals in Ethiopia are thus substantially confirmed in Egypt, there is still no explanation of the mechanism by which a significant proportion of livestock in these two countries becomes involved with either form of typhus, whose customary vectors to man are lice or fleas. Isolation from ectoparasites and ex-

* Data provided by Dr. L. A. Thomas. A similar difference was recorded by Dr. R. A. Ormsbee of RML by microagglutination technics.



Note: Combined data adjusted to assumed equivalent monthly collection of 100 each of five kinds of livestock.

Fig. 1. Seasonal curve of percentage of total animal reactors to typhus-group antigen in the Nile Delta, June 1965-August 1966 inclusive.

perimental transmission by ticks has not been accomplished as yet. Fleas are less host-specific and could conceivably transfer murine infection from rats to neighboring livestock, particularly pigs (in a few of which Imam has also found murine antibodies).

Reiss-Gutfreund reported the susceptibility of lambs and rabbits on which she infected immature ticks and showed transstadial passage from larvae to nymphs, but not to adults, as cited above; normal rabbits on which the nymphs were fed became infected.

Philip *et al.* (10) have tested the susceptibility of a few young western Montana livestock to the Ethiopian ZRS tick isolate of *R. prowazekii* mentioned above. A donkey, two twin kids, and two calves—the first six months old, the others younger—showed postinoculation rises in titer of both CF and TN antibodies (Table 3), which reached maxima in 20 to 30 days. This afforded evidence of infection, even though overt signs of disease were absent. While differentiation by CF between the two forms of typhus could not be made in our series, the epidemic form was clearly differentiated by significantly higher TN antibodies. Both types of antibodies persisted in the donkey in low titer for 452 days (CF titers of 128E and 32M; TN titers of 64E and 32M). The antibodies had practically disappeared from the calves and goats by or before 124 days. If reinforced by additional susceptibility tests, such data could influence judgment of the recency of infection in serologically positive animals such as are now being surveyed. Periodic transfers of blood from the test animals to guinea pigs failed to demonstrate rickettsemias, which is strange in view of the isolations reported above of *R. prowazekii* from bloods of sheep and goats in Ethiopia and from donkeys in Egypt.

A survey of native small Egyptian animals by Ormsbee (unpublished) has so far revealed no equivalent significant involvement.

Subsequent to the first report of Imam and Labib (3) of murine (*R. typhi*) reactors, a few more camels, donkeys, and pigs have been seropositive for this agent or were indistinguishable for either agent by means of washed antigens, but a considerable proportion of the recent animal samples that reacted to typhus group antigen remains to be checked specifically. Among 57 recently selected typhus-reacting sera tested with washed antigens at the Rocky Mountain Laboratory, most could not be differentiated, but three camels, six donkeys, one goat, and one pig had specific epidemic titers of 1:8 or higher; five donkeys, two sheep, and one pig were specific murine reactors. It is noteworthy that Reiss-Gutfreund recovered an isolate of *R. typhi* from *Hyalomma* ticks, also off Ethiopian cattle, in her third series of tests (1966), but no isolates of murine typhus have been accomplished during the animal work in Egypt.

Imam and Labib (4) discovered that dry ice (CO_2) treatment of some animal sera, mostly those with low titers of rickettsial antibodies, decreases or eliminates nonspecific factors. This

TABLE 3. DEVELOPMENT OF RICKETTSIAL ANTIBODIES IN LIVESTOCK INFECTED WITH *R. prowazekii* (ZRS STRAIN)

Day-agent / animal	20			30			60			90			124			278		
	E	M	S	E	M	S	E	M	S	E	M	S	E	M	S	E	M	S
Donkey	384 (256)	384 (16)	24	512 (512)	384 (64)	...	32 (256)	128 (64)	48	128 (256)	384 (32)	16	64 (128)	32 (32)	16	32 (64)	8 (16)	24
Goat 1	256	128	0	512	512	...	16	32	...	8	0	0	0	0	0			
Goat 2	96 (128)	128 (8)	8	192 (64)	128 (8)	...	16 (64)	32 (−)	...	8 (<8)	8 (−)	0	0 (<8)	0 (−)	0			
Calf 1	12 (16)	12 (−)	0	8 (16)	0 (−)	0	0 (8)	0 (−)	...				0	0	0			
Calf 2	12 (64)	48 (−)	0	32 (32)	24 (−)	0	8 (32)	0 (−)	...				0	0	0			

Note: Reciprocals of complement-fixing titers in plain figures; toxin neutralizing titers in parentheses.
E = Epidemic; M = Murine; S = Spotted fever; ... = Not done.

has been confirmed at Rocky Mountain (11). Nonspecific reactors have been observed, particularly among donkeys, not only in typhus foci in South America but in a certain number of donkeys from nonendemic western Montana. *Bona fide* typhus antibodies, on the other hand, resisted this treatment in sera of our infected donkey and also in those from convalescent guinea pigs and known human cases (Table 4). This test can therefore be of assistance in serological surveys of livestock for eliminating possibly confusing, though usually lower, cross-reacting spotted fever group antibodies.

Studies on typhus in livestock in the Western Hemisphere are still in a preliminary stage. While no *bona fide* typhus antibodies have been found in brief surveys of South American livestock (11, 12), Varela and Velasco recently reported (16) microagglutination of epidemic and murine typhus antigens by sera from some of the livestock samples, including donkeys, they collected in a Mexico City abattoir.

Indications suggest that domestic animals are originally contaminated with typhus by man, and so far no other hypothesis appears probable.

In closing, I am tempted "off the cuff" (so to speak), to invite the discussants with fresh viewpoints to speculate on how typhus is "spilling over" into the domestic animal population. There may be some clue emerge as serological surveys continue, in proportions of different animals that react, plus scrutiny of differences in their habits or intimacy of association with their owners. So far, donkeys are in the lead, but perhaps, if camels hump a little harder, they will catch up in proportion with typhus antibodies in future surveys.

It is known that human body lice will accept bloodmeals on camels without adverse effects. However, the considerable proportion of livestock reactors seems to discount this as a significant factor in consideration of reduction of human cases in Egypt and Ethiopia.

ACKNOWLEDGMENT

The indispensable assistance of Dr. Alfy Labib in the Egyptian studies discussed above is acknowledged; those studies were supported in part by Agreement 552509 between the National Institutes of Health and the Production Laboratories of the U.A.R. Ministry of Health.

CF data at the Rocky Mountain Laboratory were supplied by Drs. D. B. Lackman and L. A. Thomas, and TN tests were performed by Dr. E. J. Bell.

TABLE 4. RICKETTSIAL COMPLEMENT-FIXATION TESTS ON SELECTED SERA TREATED WITH CO_2 TO REMOVE SUBSTANCES THAT FIX COMPLEMENT NONSPECIFICALLY (THE IMAM TEST)

Sample nos.	Washed typhus antigens				Rocky Mt. spotted fever ant.		Saline-serum controls	
	Epidemic		Murine					
	A*	B†	A	B	A	B	A	B
Donkeys from South America								
76 (Mojo, Bolivia)	64	0	0	0	128	0	0	0
77	32	0	24	0	64	0	0	0
78	24	0	8	0	48	0	0	0
79	32	0	24	0	48	0	0	0
82	64	0	32	0	128	0	0	0
83	16	0	8	0	32	0	0	0
90 (Agra Pampa, R.A.)	16	0	0	0	64	0	0	0
95	128	0	32	0	96	0	0	0
102 (Yavi, R.A.)	256	0	64	0	96	0	0	0
107 (LaQuica, R.A.)	16	0	0	0	32	0	0	0
108	32	0	16	0	64	0	0	0
127	0	0	0	0	24	0	0	0
129	0	0	0	0	0	0	0	0
130	64	0	16	0	32	0	0	0
115 (Congrejillo, R.A.)	16	0	0	0	64	0	0	0
117	32	0	16	0	32	0	0	0
119	16	0	24	0	64	0	0	0
Donkey inoculated with R. prowazekii at RML								
D–1/20 days	384	320	256	320	24	0	0	0
D–1/32 days	512	640	384	240	—	0	0	0
D–1/92 days	128	80	384	15	16	0	0	0
Cows from Chile								
111 (Imperial)	24	0	8	0	48	0	0	0
167 (Temuco)	256	0	32	0	192	0	3+	0
Human cases of typhus								
66–183–Lab. infec.	128	80	64	20	64	0	0	0
84—M.P. (Chile)	256	80	64	80	—	0	0	0
85—L.P. (Chile)	64	80	16	60	—	0	0	0
18—LaQ. (Arg.)	64	40	32	20	0	0	0	0
36—LaQ. (Arg.)	32	10	16	5	24	0	0	0
Control guinea pigs								
Epid. typh. G. pig	512	320	64	80	0	0	0	0
End. typh. G. pig	128	60	1024	640	0	0	0	0
RMSF G. pig	0	0	0	0	256	320	0	0

*A = Reciprocal of titer before treatment.
†B = Reciprocal of titer after the following treatment: Serum diluted 1:10 with water and a small piece of dry ice added; precipitate removed by centrifugation at 2000 rpm; 0.1 ml of 8.5 per cent NaCl added to each 0.9 ml of supernate. The supernate was then inactivated at 56° C for one half hour and again tested by CF (11).

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SECTION A. RICKETTSIAE

THE PRESENT AND FUTURE OF IMMUNIZATION AGAINST THE TYPHUS FEVERS

CHARLES L. WISSEMAN, JR.

Department of Microbiology, University of Maryland School of Medicine, Baltimore, and Commission on Rickettsial Diseases, Armed Forces Epidemiological Board, U.S.A.

DR. WISSEMAN (*presenting the paper*): Whenever an assigned title includes the words *present* and *future*, some predictive capacity on the author's part is implied. Such an assumption is obviously fallacious, especially when it involves the attempt to foretell the course of human events. The story of epidemic typhus fever, and hence the need for vaccines, is closely dependent on the affairs of mankind. Nevertheless, some perspective may be attained by reviewing first the recent trends in epidemic or louse-borne typhus fever.

The last major occurrences of typhus fever were during and shortly after World War II. Since that time, the reported incidence of epidemic typhus in the parts of the world we know about has been declining; at present it appears to have retreated to a relatively few endemic foci. This may in part be the result of active control measures. More than likely, however, rising standards of living, with the attendant improvement in personal hygiene, and the more general availability of effective insecticides for personal use have contributed heavily to this decline.

Typhus is a disease that could die a natural death as living standards rise and the louse vector is eliminated. If active transmission were prevented, the threat of typhus could be expected to disappear within a generation or two as the existing human reservoirs live out their natural lives.

However, the human reservoir, even in places that are now essentially free of lice, is still very large and typhus could spring back as a major disease problem. Economic reverses, famine, wars, and civil unrest have notoriously disrupted the lives of countless people and have created the breeding grounds for typhus. Despite its current regression, the history of human events suggests that progress may be irregular, with major reverses possible from time to time. Hence, until typhus is finally eradicated from the earth, the possibility exists that conditions suitable for its spread may again arise. There is, and will be for at least the immediate future, a place for typhus vaccines and other control measures.

Should an outbreak occur today, it is most likely that the greatest reliance would be placed on vector control through the application of appropriate insecticides if this measure was operationally feasible. Resistance to insecticide among lice has been documented (1) and is of course a source of serious concern.

There is still much to be learned about the biology of typhus fever. We need to know with some precision where active transmission is still going on. Since it has been shown that man is the major interepidemic reservoir (2), it behooves us to learn the extent, nature, and distribution of the populations that remain as potential sources of infection, the factors causing recrudescence and how it can be avoided, its frequency, and so on. One study (3) suggested that serological recrudescence is much more

frequent than had previously been suspected. Accordingly, another study is currently under way in Israel to quantitate it (4). It is unknown whether stimulation by a vaccine might reduce the incidence of recrudescent infection. Moreover, the recent suggestion that extrahuman reservoirs of infection may exist (5) warrants critical appraisal.

Under the present circumstances, the role of typhus vaccine is distinctly limited—confined to situations in which vector control cannot be relied on. These include medical and laboratory personnel dealing with typhus cases and working with the organism, persons traveling to areas where typhus still occurs, and the military. Vaccine is also used as a component of typhus eradication programs, to render immune the susceptible segment of the population and hence reduce the spread of infection in lieu of totally effective vector control.

Two general kinds of typhus vaccine have received attention over the years: killed and live. The early history of typhus vaccines has been reviewed comprehensively by several authors (6–8). This paper is therefore confined to the vaccines currently in use and those under development.

Vaccines prepared from *Rickettsia prowazekii* grown in the yolk sac of embryonated hens' eggs, killed with formalin, and extracted with ethyl ether are apparently used almost universally today. This vaccine was developed under great pressure on a crash basis during World War II. The methods of production, safety testing, and potency assay have not changed much since that time. This is not because they constitute the best possible minimum requirements, but because there has been no great sense of urgency about typhus to create pressure for improvement. For over two decades we have been marking time, producing and using vaccines that have had little opportunity to demonstrate the kind of efficacy now generally demanded of new vaccines. It is time to reassess some of the premises on which this vaccine is based and to ascertain whether it is good enough.

Proof of the efficacy of the Cox-type killed typhus vaccine is very sparse. It depends on essentially nonquantitative epidemiological evidence derived from the World War II period (9–14) and on extremely limited direct challenge studies (15, 16).

The essence of the epidemiological studies may be summarized as follows: Satisfactory quantitative data are not at hand to indicate whether or not the incidence of typhus infection is significantly reduced as a consequence of immunization with killed vaccine. However, the severity of disease in vaccinated persons seems to have been reduced so that mortality was negligible. The numbers of rickettsiae in body lice feeding on vaccinated subjects suffering from epidemic typhus was markedly reduced, a fact of some epidemiological significance.

The results of challenge studies suggest that primary immunization modifies the severity of the disease and that primary immunization followed by a booster offers considerable protection against it. This protective effect has been demonstrated as long as 23 months after the booster dose.

Thus, while the limited evidence available suggests some protective effect, the information is not of the solid quantitative type that one would care to place great reliance on in case of a major onslaught of typhus.

Not only do we lack adequate information on capacity to prevent disease in man, but we also lack adequate means for testing the potency of typhus vaccines. Several empirical methods have been suggested or employed: capacity to protect guinea pigs against challenge (15–18), probably the soundest method on theoretical grounds; capacity to induce toxin-neutralizing antibodies in guinea pigs, the method currently employed in the United States (17); capacity to protect mice against toxic challenge (18); and the complement-fixing antigen content of the vaccine (19). For one or more reasons, none of these methods can be considered entirely satisfactory, and none has the degree of precision, reproducibility, or known correlation with protective capacity that might be desired.

The main reason for this state of affairs resides in our lack of basic information about immunity in typhus. While antibodies of various kinds have been measured in typhus fever, none have been clearly identified as being responsible for protection against disease. Nor, for that matter, has the possible participation of cellular immunity been explored to any great extent.

Hence, we have in effect no reliable laboratory means for determining the true immune status of an individual. Only the resistance to challenge can be accepted as a reliable index in our present state of knowledge. Likewise, we have only superficial knowledge of the antigenic structure of *R. prowazekii*. No really comprehensive antigenic analysis has been made with the immunological methods available today. We do not know which antigen or antigens are responsible for eliciting immunity in man. It follows then that we have no laboratory means for measuring directly the protective antigen content of a vaccine.

Without a practical means for measuring protective antigen content of a vaccine or for determining if, indeed, a vaccinated person has been rendered immune, we are thus faced with an intolerable situation from the standpoint of modern biologics control. This has meant that typhus vaccines must continue to be manufactured by the same old empirical methods, with little opportunity for innovation or improvement for fear that deviation from established procedure, by unknown effects, might result in the loss of what potency is known to exist. This clearly indicates that a new, concerted research effort must be made on the basic immunology of typhus fever if the present inadequacies are to be rectified. Nevertheless, some exploratory studies have been made to develop a partially purified, concentrated, and lyophilized typhus vaccine (20) in the hope of achieving a product that would retain its immunizing potency over prolonged periods of storage.

Only limited information is available on the most efficient immunization schedules with currently available killed vaccines. It is known that antibodies develop somewhat slowly after primary immunization. If the epidemiologic analysis of World War II data can be accepted, there is the suggestion that two doses of vaccine given three or more weeks before the onset of disease does modify the course of the infection. It is also known that a true secondary or booster response does not occur unless considerable time has passed since the primary dose —about six months for maximum response. The limited challenge studies reported by Fox *et al.* (21) suggest that immunity, as measured by resistance to the development of disease, is superior after a booster dose. Finally, it has been demonstrated that persons who have received one course of immunization will respond rapidly with a second antibody response as long as five to eight years later (22, 23). With these limited bits of information at hand, it is possible to visualize three types of situations, each differing in the regimen of killed vaccine administration necessary for optimal effect.

1. Preparation of a population to respond with rapid development of immunity at some time in the future, should the need arise. In this instance, a single orienting dose of a potent vaccine probably suffices, as has been demonstrated in the unpublished collaborative studies by the National Institutes of Health and the Navy under the aegis of the Commission on Rickettsial Diseases. The ultimate duration of the capacity to give a rapid, secondary type of response to a subsequent booster dose has not been established, but by inference might be expected to be a matter of several years.

2. Rapid development of some degree of effective immunity, as might be required in the face of an ongoing outbreak. Here, two or three doses of potent vaccine given at seven- to ten-day intervals, as is customarily recommended, may be partially effective early. But precise information on the optimum regimen is lacking.

3. The development and maintenance of a high level of immunity for a prolonged period. This would be desirable for persons who are intermittently exposed to risk of infection, such as laboratory workers or persons in endemic zones. On the basis of the information available, a one- or two-dose primary vaccination might be followed by a booster dose six months later. How often subsequent booster doses must be given to maintain effective immunity is unknown. On purely arbitrary grounds, recommendations have been made to repeat the doses at anywhere from three months to a year, depending on the urgency of the situation.

Thus, while it is possible to outline the rational uses of killed typhus vaccine to meet the needs of different situations, in each instance insufficient information is at hand to generate a high degree of confidence. The need for additional well-planned work is obvious.

The availability of effective chemotherapy has offset to some extent the urgency of developing

improved killed typhus vaccines. In areas where medical assistance is available, sporadic cases of typhus fever can be treated adequately with antibiotics without undue risk of loss of life. Under such circumstances, it is no longer a great triumph merely to lessen the severity of disease through prior vaccination. However, typhus still occurs in areas where medical assistance is not available. In such areas, vaccine might aid in reducing mortality and suppressing the spread of disease through systematic application as a part of a control campaign.

The other major class of vaccines is the living attenuated variety. While exploratory studies were made of living vaccines very early (6–8), none is in extensive use today. The only contender in this category at the moment is the E strain of *R. prowazekii*, first described by Clavero and Pérez Gallardo (24) and subsequently studied in considerable detail by Fox and his co-workers (21, 25). It must still be considered an experimental vaccine upon which additional work must be done, even though other groups explored its use subsequently (26, 27).

The work of Fox and his colleagues has clearly established the advantages of the living attenuated E strain: only a single dose is required; immunity is solid, develops rapidly (antibodies are detectable within about two weeks), and is long-lasting (subjects have resisted challenge as long as five years after vaccination).

Concern has been expressed by some over the possibility of reversion to virulence. No evidence has been obtained to indicate that this is likely. The strain has been passed serially about 270 times in eggs without significant change in properties. It has not yet been possible to infect lice by feeding upon vaccinated subjects. Moreover, it has been passaged serially in lice without alteration in properties.

The E strain immunizes by inducing a limited infection in man (26). Herein lie both the reason for its superiority as an immunizing agent and its greatest defects, because a small proportion of vaccinated subjects develop reactions of varying severity. Reactions have been of two varieties.

1. An early reaction, both local and systemic, may develop within about 24 hours after vaccination and may last two or three days. This reaction is dose-dependent and can be eliminated completely by administering a sufficiently low dose of vaccine. Adequate titration studies have not yet been performed to establish the minimum immunizing dose. The work of Fox *et al.* (25) suggested that a dose below 10^4 egg-infective doses would yield irregular antibody responses. Our own studies (26) suggest that, while the complement-fixation reaction was irregular at low doses, toxin-neutralizing antibodies appeared regularly with very small inocula. However, we have no observations on its persistence. Nevertheless, it does appear that the early reaction can be eliminated by the selection of proper dosage.

(2) A late reaction, not directly correlated with size of dose, has been observed in about 12 to 15 per cent of nonimmune subjects who received the vaccine. It occurs between about 9 and 18 days after vaccination and appears to be an expression of infection with the E strain. This late or delayed reaction is probably the most crucial point on which the acceptability of the vaccine hinges. Additional trials are in order to evaluate it further. Zdrodowski *et al.* (27) have attempted to minimize these late reactions by combining soluble typhus antigen with the E strain to stimulate the development of partial immunity prior to the onset of the late reactions. Such an approach warrants further study.

The E strain appears to be moderately stable on lyophilization. It can be administered rapidly to large numbers of people with the jet gun (26), which makes it particularly adaptable to large-scale use in remote areas.

One additional complication and its solution should be mentioned. Like most egg products, the E strain vaccine was found to be contaminated with the RIF agent. While no evidence has been obtained to indicate that this adventitious chicken virus causes any untoward effects in man, current concepts of biologics control demand that such passenger agents be eliminated from vaccines to be administered to man. A seed material of the E strain has now been prepared that appears to be free of such agents, and an experimental batch of vaccine has been produced. We look forward to further field-testing of the E strain with this new, "purified" vaccine.

To summarize, it appears that typhus vaccines will continue to be needed for some time to come. The killed vaccines in current use require additional study so that our knowledge about their standardization, optimal use, and efficacy may be brought to a state compatible with the modern trends in immunology. The potentially useful living attenuated E strain vaccine requires further evaluation of, and development of means to control, the late reactions that appear to be the greatest deterrent to its acceptability.

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SECTION A. RICKETTSIAE

VACCINATION AGAINST Q FEVER

PAUL FISET

Department of Microbiology, School of Medicine
University of Maryland, Baltimore, Maryland, U.S.A.

DR. FISET (*presenting the paper*): Most attempts to produce a vaccine that would protect against Q fever have met with an amazing degree of success. However, such success has not been without some drawbacks. The main problem with Q fever immunization in the past has been the high incidence of severe local reactions leading quite often to the formation of sterile abcesses. The incidence of such local reactions was usually higher in individuals who had previously been vaccinated (2, 11). The problem we are faced with at the moment resides not so much in the development of a potent vaccine as in the development of a potent vaccine that will induce a minimum of side effects and, ideally, that can be produced easily, economically, and safely.

As with many other infectious diseases, in Q fever immunoprophylaxis there are proponents of a killed vaccine and proponents of a live vaccine.

In this brief discussion I shall attempt to review some of the published works on these two types of vaccine and present, in a preliminary form, our own experience with vaccines of both types.

KILLED VACCINES

The introduction of the chick embryo as a highly susceptible host for the growth of *Coxiella burneti* (3) and the development of efficient purification procedures (10) have made it possible to obtain large quantities of organisms as a source either of diagnostic antigen or of vaccine. Usually such preparations were obtained after several consecutive passages in eggs. Such was the vaccine prepared with the Henzerling strain. This strain had been shown by Smadel *et al.* (8) to be highly immunogenic in guinea pigs and man. Until recently it has been used extensively as an experimental vaccine, at least in the United States (2). The Henzerling vaccine was prepared from the 22nd egg passage as an ether-extracted 10 per cent yolk-sac suspension. It was usually administered in three 1.0 ml inoculations at weekly intervals. With such a schedule, it was shown, approximately half of the vaccines developed detectable circulating antibodies and all resisted a respiratory challenge nine months after vaccination. As has been said, a high percentage of the vaccinees showed severe local reactions.

The observation that *C. burneti* undergoes a phase variation similar to the S-R variation of pneumococcus (9, 4) led to a new approach in vaccination against Q fever. In nature *C. burneti* exists in the "smooth" or Phase 1 form. On adaptation to chick embryos it changes readily to the "rough" or Phase 2 state. Phase 1 organisms possess a surface polysaccharide antigen that seems associated with virulence; Phase 2 organisms are devoid of this antigen and are considerably less virulent. Most vaccines, in the past, have been prepared from egg-adapted strains and for the most part were in Phase 2.

A few years ago it was suggested that a vaccine prepared from Phase 1 organisms might be considerably more efficient (5). Experimental proof of this hypothesis was provided by the work of Ormsbee *et al.* (7), who demonstrated that a Phase 1 vaccine was approximately 300

times more efficient than a Phase 2 vaccine in protecting guinea pigs.

Preliminary studies carried out in human volunteers by Lackman *et al.* (6) and Bell *et al.* (1) indicated that very small amounts of a Phase 1 vaccine were capable of inducing a serologic conversion and the development of a delayed hypersensitivity.

A study of a Phase 1 vaccine in human volunteers was undertaken under the aegis of the Commission on Rickettsial Diseases of the Armed Forces Epidemiological Board.* Sixty-four volunteers were given a single subcutaneous inoculation of 30 μgms of purified Henzerling Phase 1 organisms. This particular inoculum was selected on the basis of Ormsbee's results in guinea pigs (7) and was considered the best for inducing 100 per cent serologic conversions.

None of the vaccinees developed any serious local or systemic reactions. Approximately half developed some tenderness at the site of inoculation, which appeared within 24 hours and lasted between 24 and 48 hours. All developed circulating antibodies detectable either by complement-fixation or agglutination reactions. About 10 months after vaccination 13 of the volunteers were challenged by the respiratory route with 3,000 guinea pig ID_{50} of a Phase 1 strain. None of the vaccinees showed any signs of illness, whereas five out of the six controls developed clinical Q fever. Similar results were obtained in a parallel study carried out among military personnel at Fort Detrick (unpublished data; minutes of the Ad Hoc Committee on Q Fever Vaccine, Armed Forces Epidemiological Board).

LIVE VACCINES

A considerable amount of work has been published in the USSR on the development and testing of a live Q fever vaccine. This work has been reviewed recently by Zdrodowski *et al.* (12). In summary, an avirulent variant of the Grita strain of *C. burneti* was obtained by successive passages in chick embryo. By the 44th passage the strain had lost most of its virulence for guinea pigs and mice. This strain, now known as M-44, has been tested extensively in human volunteers. The authors report that side effects were minimal and that a large percentage of the vaccinees developed circulating antibodies. It is not stated whether the vaccinees were challenged at any time. Although the authors make no mention of it, in my opinion strain M-44 is a pure Phase 2.

In the course of comparing the infectivity titers of the Nine Mile Phase 1 and Phase 2 strains in guinea pigs, Dr. Lowenthal of the Walter Reed Army Institute of Research demonstrated that the Phase 2 variant was considerably less virulent than Phase 1. Whereas the two strains showed little difference in their ability to induce serologic conversion in guinea pigs, the Phase 2 variant was approximately four logs lower than the Phase 1 in its ability to induce a febrile response (Table 1).

These findings suggested that the Nine Mile Phase 2 might be a promising candidate for a live vaccine. It seemed to have a number of properties in common with the Russian strain M-44.

A small trial was set up to test the immunogenicity and safety of the live Nine Mile Phase 2 preparation. The study was carried out under the supervision of Dr. Hornick at the University of Maryland.

In a first cautious step two groups of six volunteers were inoculated subcutaneously with 1.0 ml of a 10^{-8} and a 10^{-6} dilution, respectively, of infected yolk sac. Only one of the vaccinees who received a 10^{-6} inoculation showed a mild local reaction. Subsequently, two more groups of six volunteers each were given 1.0 ml of a 10^{-4} and a 10^{-2} dilution, respectively, of infected yolk sac. Table 2 summarizes the results of this experiment.

Vaccinees in the 10^{-4} and 10^{-2} groups showed severe local reactions with fever. The reactions were more intense in the 10^{-2} group.

TABLE 1. TITRATIONS OF NINE MILE VARIANTS IN GUINEA PIGS

Strain	Febrile response	Serologic conversion
Nine Mile Phase 1	$1D_{50}=8.6$	$1D_{50}=10.3$
Nine Mile Phase 2	$I_5D_0=4.6$	$1D_{50}=9.6$

* The study was supervised by Dr. Richard B. Hornick and carried out at the University of Maryland. Details will be published elsewhere.

TABLE 2. VACCINATION OF HUMAN VOLUNTEERS WITH LIVE NINE MILE PHASE 2 (88TH EGG PASSAGE)*

Dilution	Reactions	Phase 2 response†	Phase 1 response‡	Resistance to challenge§
10^{-8}	0/6	0/6	0/6	0/1
10^{-6}	1/6 ‖	1/6	0/6	1/3
10^{-4}	6/6¶	3/6	0/6	3/3
10^{-2}	6/6¶	6/6	0/6	1/1

*Reproduced by permission of Dr. Richard B. Hornick, University of Maryland.
†Complement fixation test (R. B. Hornick *et al.*, unpublished).
‡Complement fixation, agglutination, radioisotope precipitation (6), and indirect immunofluorescence.
§Three thousand guinea pig ID_{50} by aerosol.
‖ Mild local reaction.
¶Severe local reactions with fever of three to five days duration.

Only one member of the 10^{-8} group developed detectable Phase 2 antibodies. He was also the only one in that group who had a local reaction and resisted challenge. It is our opinion that he had previously been exposed to Q fever.

In the 10^{-4} group three out of six developed Phase 2 antibodies. These were challenged approximately six months after vaccination and did not become ill. One of them had no detectable Phase 2 antibodies prior to challenge.

In the 10^{-2} group all developed Phase 2 antibodies. One was challenged and resisted.

None of these vaccinees ever developed Phase 1 antibodies except the three who became ill after challenge. It must also be stated that the Phase 2 titers in the vaccinees who showed a serologic response were usually quite low.

Although the number of volunteers in this study was quite small, the results seem to confirm those of the Russian authors. It is possible to immunize effectively against Q fever with a live strain of *C. burneti* in Phase 2. In the present study, however, there seemed to be a higher incidence of severe local reactions than was observed by the Russian workers.

Discussion

There seems to be very little to recommend a live vaccine for Q fever. Apart from the practical problems of production—safety-testing, potency-testing, storage, and so on—we feel that there are inherent dangers in such vaccines. Of course, whenever active egg material is inoculated into people the possibility exists that some of the fowl oncogenic agents, whose effects on man are at present unknown, may be transmitted. My concern at the moment is of a more specific nature and relates to *C. burneti* itself. We know that in animals it is possible to induce a variation from Phase 2 to Phase 1 with remarkable ease. We also know that Q fever in persons suffering from rheumatic carditis sometimes leads to a fatal rickettsial endocarditis in which Phase 1 organisms have been incriminated. I would therefore be very concerned about mass immunization programs where the possibility existed of inducing rickettsial endocarditis in some of the vaccinees.

As for the choice of a killed vaccine, the evidence obtained from animals and from small studies in human volunteers indicates that vaccines prepared from highly purified suspensions of Phase 1 organisms are considerably more efficient than the previously used Phase 2 preparation. The absence of severe local reactions with Phase 1 vaccines is another definite advantage. It is not that Phase 1 organisms are incapable of eliciting reactions of hypersensitivity, on the contrary, as was demonstrated by Bell *et al.* (1), minute amounts of Phase 1 material can give a positive skin reaction in sensitized individuals. We feel that the reason for the low incidence of local reactions is merely the small mass of foreign material inoculated.

We know that a single inoculation of 30 μgms of purified Phase 1 organisms is capable of inducing a solid immunity lasting up to ten months. We do not know how long it persists beyond this period. Nor do we know what kind of booster schedule should be established to maintain a high level of immunity in persons at risk. On this latter point I would venture a suggestion based on the observations of Bell *et al.* These investigators found that individuals who were skin tested with Phase 1 preparation containing as little as 0.5 μgms of organisms and showed a positive reaction also frequently showed a significant rise in their serum antibody level. It is therefore possible that a program of periodic skin-testing of persons at risk may also serve as a booster program.

Another point that may please any potential manufacturer of a Phase 1 vaccine is this: On the basis of published information we have

calculated that one yolk sac yields approximately 10 vaccine doses of the Henzerling Phase 2 used in the past. The yield of the Henzerling Phase 1, however, is of the order of 200 vaccine doses per yolk sac. It was also shown that a pure Phase 1 suspension could be autoclaved and retain its full immunogenicity for guinea pigs (Ormsbee, personal communication).

The important question at this point is, Who should be given this wonderful vaccine? I shall not attempt to answer this embarrassing question as I believe Dr. Marmion has some definite views on the subject.

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SECTION A. RICKETTSIAE

DISCUSSION

CHAIRMAN VAN ROOYEN: There has been a slight change in the sequence in which we will hear the discussants. It has been suggested that Dr. Imam Z. E. Imam be called on to speak at this time. Dr. Imam is with the Virus Research Center, Production Laboratories, Cairo, Egypt, U.A.R.

DR. IMAM: I should like to report on "Isolation of Two Strains of Epidemic Typhus from Donkeys in the U.A.R.," co-authored with Dr. A. Labib.*

The history of the two isolates of *R. prowazekii* mentioned in Dr. Philip's report is as follows: The first strain, designated El Hamra 1, was isolated on October 1965 from the blood of a donkey and was directly inoculated intraperitoneally into a guinea pig. On the 23rd day after inoculation, this guinea pig developed a febrile reaction of above 40°C, which continued for three days. On the third day of fever the guinea pig was sacrificed and its brain suspension was passed intraperitoneally into two guinea pigs. These developed a febrile reaction of above 40°C on the 11th to 13th day after inoculation. Their brains were harvested on the third day of febrile reaction and frozen.

In January 1966 a sample of the first guinea pig was passed into six-day-old embryonated chick eggs. On the second egg passage rickettsia-like organisms were seen in Gimenez-stained yolk sac smears.

An ether-extract antigen prepared from this egg passage gave a positive CF reaction in a titer of 1:6 to 1:8 in our laboratory and in NAMRU Laboratories against epidemic typhus serum only.

The adaptation to eggs was repeated again with the first brain material and also with second-brain-passage material. Antigens prepared from these egg adapted strains gave a positive CF reaction in a titer of 1:8 against epidemic typhus serum, but not with convalescent murine or Q fever sera.

The second passage of the egg-adapted strain was passed intraperitoneally into seven guinea pigs, which developed febrile reactions of above 40°C between the 10th and the 14th day after inoculation. Two guinea pigs were sacrificed, and the remaining five were left to convalesce.

Sera from these five guinea pigs taken three weeks after the last day of febrile reaction gave a titer of 1:128 with washed epidemic antigen and of 1:8 with washed murine antigen. In the Rocky Mountain Laboratories serum from one of these guinea pigs tested by Dr. Lackman gave a CF titer of 1:256 with washed epidemic antigen and 1:48 with washed murine antigen. Dr. Ormsbee confirmed these results by microagglutination test.

The second strain, designated El Hamra 2, was isolated in July 1966 by direct inoculation of the blood of another donkey intraperitoneally into a guinea pig. The guinea pig developed a febrile reaction of above 40°C on the 19th day after inoculation and was sacrificed on the third day. In turn, the brain and spleen suspensions were each passed in two guinea pigs, which developed febrile reactions of above 40°C between the 11th and the 14th day after inoculation. One guinea pig from each group was harvested and the other two were left to convalesce.

Sera from the convalescent guinea pigs gave a titer of 1:256 with washed epidemic typhus antigen and 1:16 with washed murine antigen. With Dr. Lackman they gave a titer of 1:1024

* These studies were supported in part by Agreement 552509 between the National Institutes of Health and the Production Laboratories of the U.A.R. Ministry of Health.

with washed epidemic typhus antigen and 1:64 with washed murine typhus antigen. These results were confirmed with the microagglutination test by Dr. Ormsbee.

The sera of the convalescent guinea pigs inoculated by El Hamra 1 and El Hamra 2 strains gave a positive CF reaction in a titer of 1:32 with antigen prepared from the egg adapted strain of El Hamra 1 strain. The El Hamra 2 strain has not yet been adapted to eggs, since we only started to work with eggs in November. Preliminary results by toxin neutralization carried out by Dr. John Bell on sera from these convalescent guinea pigs gave a titer of 1:256. Further studies are being carried out.

The sera of the two donkeys from which these isolates were made were serologically negative.

With these donkey isolates in the U.A.R. we have had the satisfaction of confirming Reiss-Gutfreund's previous reports of isolations from goats and sheep in Ethiopia.

CHAIRMAN VAN ROOYEN: I now call on Dr. John P. Fox, who is Professor of Preventive Medicine, University of Washington, Seattle, Washington.

DR. FOX: * Epidemic and murine typhus and Q fever were the only rickettsial diseases considered. Two main points of new information were presented: First, the surprising presence of *R. prowazekii* and *R. mooseri* in domestic livestock (cattle, donkeys, goats, camels, and swine) and in ticks in Ethiopia and Egypt was described, as were experiments confirming the susceptibility of several of these species to infection with *R. prowazekii*. Livestock infection may be seasonal and, at least in Egypt, centers about known typhus-infected villages. Second, Phase 1 Q fever rickettsiae have been found sufficiently more potent as inactivated antigens than those in Phase 2 that an antigenic mass too small to elicit severe local sensitivity reactions will induce solid immunity in man, equal to that following live Phase 2 vaccine, but with less apparent hazard.

Inactivated epidemic typhus vaccine has progressed little since World War II. Still needed are adequate potency tests, optimal inoculation schedules, and evaluation of protection against morbidity. Attenuated E strain (*R. prowazekii*) vaccine gives solid long-lasting immunity after a single dose, but a delayed reaction rate of 12 to 15 per cent is a serious disadvantage. Ways to minimize this are being explored.

Of the Bedsoniae, only trachoma was discussed. Recent evaluation indicates that mass chemotherapy (sulfonamides and/or antibiotics) has failed to control it. This may be due in part to the major contributions (not fully understood) of adverse living environment to the spread of infection and to both the earlier development and the greater severity of the disease.

Important steps essential to trachoma vaccine have been taken. Three antigenic types have been recognized; rich, relatively pure agent preparations have been made; possible methods for vaccine assay exist; and experimental vaccines, especially in mineral-oil adjuvants, have been tested in monkeys and man. Good protection against homologous challenge in monkeys and short-lived partial protection of children have been achieved. However, an increase in disease among one group of children given a monovalent vaccine and an increased severity of disease in monkeys given heterologous challenge suggest possible hypersensitization by a group antigen. Hence future vaccines for man should include all (one to three) prevalent antigenic types or should include only specific protective antigens, if they can be isolated.

The frequency of brief and low-level response to inactivated antigens underlines the need for ways to enhance and prolong the desired immunity. Adjuvants, especially water-in-oil emulsions, offer great promise for reaching this goal. A recently described metabolizable emulsion (peanut oil-Arlacel A-aluminum monostearate) may give the required enhancement without serious side effects. However, each vaccine-emulsion mixture must be safety-tested individually, since reactions may be determined in part by the antigen. Hope was expressed that full understanding of the mechanisms involved in the adjuvant effect may permit the preparation of antigens in a form maximally effective without the adjuvant.

CHAIRMAN VAN ROOYEN: I shall call next on Dr. Herald R. Cox of the Lederle Laboratories, Pearl River, New York, to discusss this subject further. Dr. Cox, as you all know, is a veteran in this field and knows many of the answers. I

* Some of these remarks refer to subsequent items on the program.

can think of no one better to answer some of the interesting questions that have arisen.

DR. COX: The data presented by Drs. Philip and Imam on the isolation of epidemic strains of typhus from domestic animals such as goats, sheep, and donkeys are certainly very stimulating and exciting. The only thing I would suggest and plead is that Dr. Imam and his co-workers take extra precautionary pains to be sure, in their isolation work, that no contaminating strains of typhus are allowed to enter the picture and that the strains of animals they are using are proved to be free from typhus and other rickettsia strains at all times during the work. As was discussed previously in connection with infectious hepatitis, it is necessary that when a claim is made it can be backed up.

I have heard that Dr. Imam is carrying out studies with sentinel animals, to determine how soon they become infected. If the Chairman will allow Dr. Imam to speak again, it would be interesting to know what he has learned in this respect.

I agree that we have never had a chance for a good field trial to demonstrate the efficacy of typhus vaccine, but we had a rather interesting experience at the Lederle Laboratories when we were producing the vaccine during the war years. You must remember that each yolk sac of eggs contains at least a billion typhus rickettsia per gram, so that there is enough material in two eggs to infect everybody in the world. We were handling 12,000 eggs a day, six days a week, over a period of three years, and of course during that time the laboratory became rather untidy. The decision was made to clean it up, and Tuesday, Wednesday, and Thursday were spent at this job by the 20 girls, using 5 per cent cresol solution. On Friday three painters came to paint the laboratory, and through an oversight by the medical department they were not vaccinated. They stayed in the laboratory only about a day, but two out of the three came down with typhus. We never had a single case of typhus fever in any of the girls who were working in the laboratory. They received three injections of vaccine, on days 1, 7, and 14, with a booster dose on the 28th day, and from then on they went to work. They did not even wear gloves in the laboratory. And we had a lot of broken eggs in the laboratory during those three years, I can tell you.

Now I would like to state along with Dr. Wisseman that we likewise have a strain of E typhus available, which has been cleaned up to free it from some of the Rous sarcoma agents that can be demonstrated in eggs. We know, however, that the RIF test cannot be used to demonstrate all leukosis strains. This should be kept in mind.

I might add that I do not believe that, if the time comes when it may be necessary to make a Q fever vaccine, there will be much difficulty in doing so. Lederle was licensed 12 or 14 years ago for Q fever vaccine, but we have never sold a single dose; it does not look, therefore, as if it ever will be a very big commercial item.

CHAIRMAN VAN ROOYEN: Thank you, Dr. Cox. I will next call on Dr. B. P. Marmion, Department of Microbiology, Monash University Medical School, Melbourne, Australia, who will present some further interesting data.

DR. MARMION: Although effective inactivated Q fever vaccines have been available for nearly 20 years, there have been two unresolved questions connected with them. First, what is the nature of the antigen stimulating immunity and protective antibody, and how may this be given in a potent and refined form? And, second, what is the nature of the antigen provoking the severe local reaction in some persons who are hypersensitive as a result of previous exposure to the living organism or to a vaccine?

As Dr. Fiset has mentioned, evidence from animal experiments and serological studies indicates that the Phase I antigen of the rickettsia is the major immunizing antigen. The promising results of the volunteer study he has just described fit well with this view of the importance of the Phase 1 antigen. To clinch the matter it would presumably be necessary to show that the inoculation of similar amounts of Phase 2 organisms does not confer immunity. It would also be of interest to know whether the small amount of Phase 1 vaccine used gives local reactions in known hypersensitive subjects and whether sensitivity is induced by repeated inoculation. As Dr. Fiset stated, there is some evidence from the skin-testing done by Bell and his

colleagues * that these would not be serious, but comparable tests are required.

Phase 1 antigen may be extracted by various chemical treatments, and there is evidence † that the hypersensitivity-inducing factor, probably a protein, can be separated from the serologically reactive Phase 1 antigen, which probably has carbohydrate determinants. Recent work by Ormsbee, Bell, and Lackman ‡ is particularly promising. They have extracted Phase 1 antigen with the organic solvent dimethylsulphoxide, and in animal tests vaccination with the extract stimulated immunity but did not provoke lesions in hypersensitive animals. I understand that the extracts contain lipid, carbohydrate, and a small amount of nitrogen. It would be of interest to know whether they are satisfactory immunogens in man.

There is one other question that deserves consideration in view of Dr. Fiset's and Dr. Cox's concluding remarks. If and when this "near-perfect" vaccine has been developed, is it going to have to look for a disease to prevent? Clinical Q fever appears to be on the decline in many areas, although sensitive antibody tests seem to suggest that contact with the rickettsia is not uncommon. Apart from the military deployed in areas with primitive animal husbandry, cover could reasonably be given to special-risk groups in laboratories, meat and fertilizer plants, and milk-processing plants and to veterinarians. More debatably, vaccination might be extended to patients with valvular heart disease in view of the rare but distressing complication of Q fever endocarditis. Lastly, and even more debatably, protection might be offered to persons at risk because they periodically visit farms and other foci of infection (such as land agents or agricultural machinery salesmen). But it would be interesting to have a short list of candidate groups for the vaccine from other participants in the conference.

CHAIRMAN VAN ROOYEN: There is some time available for a general discussion.

DR. BLAŠKOVIČ: Dr. P. Fiset clearly emphasized that inactivated *Coxiella burnetii* containing Phase 1 antigen is justified for vaccination purposes. I should like to summarize briefly some of the properties of this antigen as studied in the Rickettsial Laboratory of the Institute of Virology, Czechoslovak Academy of Sciences, Bratislava, by R. Brezina and his associates.

Phase 1 antigen was purified and concentrated § and was proved to be responsible for specific pyrogenic activity; the specificity means that it could be neutralized by corresponding antibodies.‖ Phase 1 *C. burnetii* was found not to be phagocytized if the serum of the organism does not contain specific antibodies against it. Both these properties of Phase 1 are of importance to the virulence of *C. burnetii.*¶ Phase 1 antigen may induce an early interferon production after being inoculated into white mice. Maximum interferon is released three hours after injection. However, this phenomenon is not limited to Phase 1 antigen. Two different strains of *C. burnetii*—a Phase 1 and a Phase 2—as well as *R. prowazekii* produced interferon after intravenous injection in white mice. The highest level of inhibiting activity of the interferon produced comes three to five hours after the inoculation of the strains.**

CHAIRMAN VAN ROOYEN: By popular request I call on Dr. Imam again. It has been suggested that we address some questions to him and give him an opportunity to answer.

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* Bell, J. F., Luoto, L., Casey, M., and Lackman, D. F. "Serologic and Skin-Test Response after Q Fever Vaccination by the Intracutaneous Route." *J Immun* **93**:403–408, 1964.

† Anacker, R. L., Haskins, W. T., Lackman, D. B., *et. al.* "Conversion of the Phase 1 Antigen of *Coxiella burnetii* to Hapten by Phenol Treatment." *J Bact* **85**:1165–1670, 1963.

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‡ Ormsbee, R. A., Bell, E. J., and Lackman, D. B. "The Influence of Phase 1 on the Protective Potency of Q Fever Vaccine." *J Immun* **92**:404–412, 1964.

§ Brezina, R. and Urvölgyij. "Study of the Antigenic Structure of *Coxiella burnetii.* I. Extraction of Phase 1 Antigenic Component by Means of Trichloracetic Acid." *Acta Virol (Praha)* **6**:87–88.

‖ Brezina, R., Schramek. S., and Urvölgyij. "Study of the Antigenic Structure of *Coxiella burnetii.* III Pyrogenic Effect of Phase 1 Antigen in Experimental Guinea Pigs." *Acta Virol (Praha)* **9**:180–185, 1962.

¶ Brezina, R., and Kazar, J. "Study of the Antigenic Structure of *Coxiella burnetii.* IV. Phagocytosis and Opsonization in Relation to the Phases of *C. burnetii.*" *Acta Virol (Praha)* **9**:268–274, 1965.

** Kazar, J. "Interferon-Like Inhibitor in Mouse Sera Induced by Rickettsiae." *Acta Virol (Praha)* **10**:277, 1966.

DR. IMAM: I should like to assure Dr. Cox, in reply to his question about laboratory contamination, that since the beginning of 1965 we have maintained no live strains of any kind of rickettsia in our laboratory. The guinea pigs are inoculated in the field directly from the animals and brought directly to our laboratory and kept two to a cage; a single thermometer is used for each cage and not used for another cage.

This thermometer is kept overnight in Lysol and the next day is used for the same cage. These guinea pigs used are inbred in our laboratory from a special farm supplying our laboratory. We have tested them randomly every now and then for the presence of antibodies to any kind of rickettsia and they are always negative.

DR. COX: And about the sentinel animals?

DR. IMAM: Last December we put 20 young goats and five donkeys, all negative to typhus, in one of the villages and left them to live with the other animals in this village. We followed them up monthly by taking blood samples and testing them for antibodies to typhus. After three months, during January, February, and March, about 50 per cent of these animals converted to positive CF reactors and we are still testing them all. We hope that this year we can introduce more animals experimentally, in more than one area, especially in the area from which we isolated this strain. This time we shall follow them by isolation and serology, to see whether or not we can isolate the strain from them.

CHAIRMAN VAN ROOYEN: I want to take the opportunity to ask one of our Russian colleagues to tell us something about the C strain of *R. prowazekii,* which was studied in Russia. Epidemic typhus has been primarily a disease of Central and European Russia, and it is logical that we should ask a Russian to tell us about it. Dr. Baroyan?

DR. BAROYAN: As I said before, the vaccine has been obtained in the laboratory of Professor Zdrodovski at the Gamaleya Institute in Moscow. It was prepared from the E strain and another that was more virulent. We have obtained a rather mild vaccine, which in volunteers produced a rather prolonged immunity. But we do not use this vaccine in our country. Most probably it would be necessary to use it should there be such social upheavals as have been mentioned here. In regard to animal reservoirs of typhus, we are trying through WHO to send an expedition to Ethiopia to study this question.

The Q fever vaccine has been sufficiently well described in the literature, with which you are familiar; it too was developed in Professor Zdrodovski's laboratory. It has been well tested. It has shown very good results. It is not infectious and it produces good immunogenicity. But I must repeat what Dr. Cox said: I do not see many people wanting to buy it. It is available, but nobody wants it—except that perhaps it could be used on farms where there may be contamination of an occupational character.

CHAIRMAN VAN ROOYEN: Thank you, Dr. Baroyan. We now pass to the next item of the program in Section B dealing with the Bedsoniae.

SECTION B. BEDSONIAE

EVALUATION OF CHEMOTHERAPY OF TRACHOMA *

EDWARD S. MURRAY AND ROGER L. NICHOLS †

DR. MURRAY (*presenting the paper*): Trachoma is one of the most important eye diseases of man. It is estimated that over 400 million people suffer from the disease and that 1 to 2 per cent of the cases result in moderate to severe visual disabilities. Thousands of children and adults are left blind or near blind from chronic effects of the disease (7). Treatment of trachoma with sulfonamides and antibiotics has been introduced in the past three decades. However, recent conflicting reports on the results of trials of various drugs have made it uncertain how effective chemotherapy is in the over-all control of trachoma.

Much of the difficulty in evaluating measures used to control trachoma is related to the characteristics of the disease (7, 11). Any clinicopathological description must perforce be schematic, for there is a wide divergence of opinion as to what the natural course of trachoma really is. It is well known, for example, that the age at onset, intensity, and duration of the disease, as well as the severity of sequelae vary from country to country. However, in general it is agreed that trachoma begins as a simple inflammation of the conjunctiva with the etiologic bedsonial agent growing intracellularly in the conjunctival epithelial cells. After an acute phase of several weeks' or months' duration, associated with hyperemia and follicular hypertrophy, the disease may take any one of a number of courses: it may progress toward healing; it may continue on as mild or subacute disease for months or years; or it can progress irregularly as a severe chronic disease toward corneal ulcers, pannus, and cicatricial lid deformities.

Many factors have been thought to affect the duration and severity of the disease. Geography, race, climate, and culture are traditionally cited. Superimposed secondary bacterial and viral infections are generally agreed to be important. Studies in Saudi Arabia, which will be discussed in detail, below, suggest that the socioeconomic factors of housing and hygiene may play a significant role.

Except for improvement of the general socioeconomic conditions affecting the course of trachoma, the means available for controlling the disease had been quite ineffective up until 1938 when the sulfonamides were introduced. The treatment of choice for two millenia had been copper sulfate, scarification, and expression of follicles—measures recommended by the Egyptians, Greeks, and Romans.

The sulfonamides were tried and immediately accepted as effective therapeutic agents. With the advent of antibiotics, the tetracyclines, which have been used most extensively, and later erythromycin were claimed also to be effective by topical application.

Theoretically the control of trachoma by chemotherapy is feasible. The *in ovo* and *in vivo* effects of tetracyclines and sulfonamides on the trachoma organism have been well documented. Using the embryonated egg as a laboratory model, a number of investigators have, with only minor disagreement, found trachoma

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† Dr. Murray (Harvard University School of Public Health, Department of Microbiology, Boston, Massachusetts); Dr. Nichols (Medical Department, Arabian American Oil Company, Dhahran, Saudi Arabia).

bedsoniae readily sensitive to sulfonamides, tetracyclines, and erythromycin (17, 6, 18). And while some trachoma strains do show more resistance than others to *in ovo* screening doses of various drugs, even a relatively resistant strain will be inhibited by a larger dose of the drug. Furthermore, no *in ovo* resistance of a strain has been confirmable by *in vivo* experiments (17). However, the best evidence that sulfonamides and antibiotics might be used to control trachoma comes from experiments with volunteers and persons contracting accidental laboratory infections.

Chemotherapy of Accidental and Volunteer Infections

There have been some 30 or more well-documented cases of trachoma that occurred as laboratory accidents or in volunteers and in which the treatment was begun either immediately or within a few weeks after onset of illness (10, 1, 13, 16, 3). These cases were caused by trachoma strains isolated from such diverse areas as Saudi Arabia, Taiwan, Gambia, Japan, South Africa, and China. Without exception, all reports indicated that these accidental and volunteer trachoma infections responded promptly to treatment with sulfonamides given orally or various tetracyclines administered topically. Clinical signs and symptoms disappeared within two or, at the most, three weeks after onset of treatment. A combination of a sulfonamide orally and a tetracycline topically was generally considered the best treatment.

In some accidental or experimental trachoma infections the disease was allowed to progress for from one to eight months or more before treatment was started, thus making it possible to study the pathogenesis of the inflammatory lesions on the conjunctivae and cornea. These infections of longer duration also responded to topical tetracyclines and oral sulfonamides; however, usually four or more weeks of treatment were required to effect a clinical cure. In some of the cases of treatment with topical tetracyclines relapses occurred. Retreatment of relapses with oral sulfonamide was always reported to effect a permanent cure.

Chemotherapy of Naturally Occurring Trachoma

We have discussed in detail the successful treatment of a relatively few accidental and volunteer cases because there is such a wide diversity of opinion as to whether chemotherapy is actually effective in the control of naturally occurring trachoma in large populations. Between 1938 and 1958 many reports of successful large-scale programs of treatment with oral sulfonamides and topical tetracyclines appeared. Cure rates of at least 50 per cent and frequently as high as 95 per cent have been claimed (2). For example, in 1944 the United States Public Health Service (4) reported a cure rate of 90 per cent in a group of 20,000 Indians treated with sulfonamides. In 1955 Mitsui (12) reported cures with topical tetracyclines in 76 per cent of his cases, and in 1959 Reinhards (15), in an extensive study sponsored by WHO, reported cures ranging between 56 and 80 per cent in a group of 6,757 Moroccan children. However, in these chemotherapy trials and in others reported up to 1959, placebo controls were not included and the examining ophthalmologists usually knew the patient's treatment status.

Workers engaged in treatment programs generally agree that while oral sulfonamides are at least as good as, if not better than, any other therapy, the danger of complications precludes their use in mass treatment campaigns. Therefore, the tetracyclines used topically in ointments or oil emulsions are the most feasible drug for community-wide treatment programs.

Various schedules of topical application of tetracyclines have given similar cure rates. Hence, most treatment is now carried out on what is called a standard WHO intermittent schedule, which is easily adapted to administration in schools. The schedule consists of two or three applications of ointment or oil emulsion each day for three or four days in one week of each month. This regimen is repeated for five or six months (15).

Evaluation of the effectiveness of trachoma therapy is fraught with many pitfalls, since there are so many subjective factors involved in the diagnosis of the disease. Signs of active disease shade imperceptibly into the classical signs of healing, and clinicians vary in their interpretation of vascular and epithelial changes. Treat-

ment failure is imperceptibly separated from recurrence of disease after a probable cure. Recurrences, moreover, can be due either to a lapse or to a reinfection from an outside source, and there is no easy method available to distinguish between the two events.

The reports of mass therapy would lead one to assume that chemotherapy is highly effective in the control of trachoma. However, not all workers have been so sanguine about the percentage of "cures." Some believe that the major effect of treatment is that it overcomes aggravating factors such as secondary bacterial infections and that the chemotherapeutic action on hyperplastic conjunctival lesions is more or less negligible. Others have questioned the effectiveness of chemotherapy by pointing to the incidence of trachoma over the years among the American Indians. Table 1 shows the percentage of active trachoma in American Indians reported by the United States Public Health Service from 1910 to 1963 (cited by Foster *et al.* [5]). It can be seen that the incidence of active trachoma remained at about 20 per cent between 1910 and 1937. With the initial interest and belief in the effectiveness of sulfonamides, the incidence dropped to 5 per cent in 1943. However, it was again about 19 per cent among Indian school children in 1959, and, in spite of active diagnostic and treatment programs between 1959 and 1963, it was still 17 per cent in the same Indian schools in 1963.

Moreover, there appear to be factors other than chemotherapy that affect the incidence of trachoma throughout the world. In 1910 it was a widespread and crippling disease. The cases of active disease numbered in the thousands and resulted in considerable blindness among white people in a broad belt extending from West Virginia through Kentucky and Tennessee to Arkansas (9). However, during the next 50 years it practically disappeared from these and other white communities in the United States.

Trachoma has also been disappearing steadily, but more slowly, from central and southern Europe, where it was endemic at the turn of the century. Those observers who are not impressed with the effectiveness of mass treatment programs have theorized that socioeconomic factors, especially those relating to improved housing and hygiene, have been more important than specific therapy in causing the disappearance of trachoma from European and American white populations.

TABLE 1. PREVALENCE OF ACTIVE CLINICAL TRACHOMA IN VARIOUS AMERICAN INDIAN GROUPS, 1910–1963

Year	Percentage active trachoma
1910	20
1912	22
1937	22
1943	5
1959	19
1963	17

Recent Chemotherapy Studies

In an effort to settle the question whether chemotherapy is effective in community treatment of trachoma, as was reported in the 1950's, several carefully controlled chemotherapeutic studies have been carried out in the past five years.

In the first of these studies Grayston and colleagues (19) treated school children in Taiwan (Table 2). In March 1963 Treatment 1, consisting of 1 per cent tetracycline ointment applied two times a day for six days a week for six consecutive weeks, was given to 202 children; 120 children served as controls. Treatment 2, a shorter course in which an oral sulfonamide was added to the topical treatment, was administered

TABLE 2. RESPONSE OF CLINICAL TRACHOMA TO TETRACYCLINE TREATMENT IN TAIWAN CHILDREN, 1963–1965

Treatment	Number of individuals	Percentage of active clinical trachoma			
		March 1963 Treatment 1*	October 1963 Treatment 2†	December 1963	January 1965
Tetracycline	202	92	—	64	76
No treatment	120	95	—	85	83

*Treatment 1: Tetracycline ointment 2 times a day for 6 days a week for 6 weeks.
†Treatment 2: Tetracycline ointment topically and triple sulfonamide orally 2 times a day for 6 days a week for 2½ weeks.

in October 1963. In December 1963, immediately after the second course of treatment, there was a slight difference in favor of the treated group. However, by the final examination in January 1965, a year after the last course of treatment, the difference between treated and control groups was negligible.

Foster and colleagues (5) also failed to demonstrate cure of trachoma using two treatment schedules in American Indian school children (Table 3). These investigators treated one group with long-acting sulfonamide and another with topical tetracycline, while a third group served as untreated controls. One year after the termination of treatment the percentage of active trachoma in treated and untreated groups was essentially the same.

Recently Jawetz and colleagues (8) treated a group of American Indian school children with topical tetracycline. They studied both clinical and microbiological responses. In a group of 82 Indian children, all of whom had active trachoma, 41 received tetracycline and 41 served as placebo controls (Table 4). In March 1966, four months after treatment, the incidence of active clinical trachoma had declined over 50 per cent in the tetracycline-treated group; however, the same percentage of decline also occurred in the placebo controls. By means of fluorescent antibody, specific inclusions were found in a similar proportion of conjunctival cells in both treated and control groups.

The results of these three recent chemotherapeutic studies conducted with controls are in sharp contradiction to the results of the mass campaigns in the 1950's in which cure rates of 60 to 95 per cent were claimed. A number of explanations for these recent treatment failures

TABLE 3. RESPONSE OF ACTIVE CLINICAL TRACHOMA TO TETRACYCLINE AND SULFONAMIDE IN AMERICAN INDIAN SCHOOL CHILDREN, 1963–1964

Treatment	No. of children	Percentage of active clinical trachoma one year after treatment
Topical sulfonamide	112	71
Oral tetracycline	106	61
Controls	107	64

TABLE 4. RESPONSE OF CLINICAL TRACHOMA TO TETRACYCLINE TREATMENT IN AMERICAN INDIAN SCHOOL CHILDREN, 1965–1966 (82 CHILDREN; 41 PER GROUP)

Date	Percentage of active clinical trachoma	
	Placebo group	Treated group*
October 1965	100	100
March 1966	44	45

*Treatment consisted of 1 per cent tetracycline HCl in oil 3 times a day for 6 weeks—27 October to 7 December 1965.

has been advanced. The question of whether the prevalence rate of active trachoma in a given population will increase, stay the same, or disappear is related to a number of interacting factors. Among these factors treatment may play a minor or a major role, and the socioeconomic circumstances of the individual, his family, and the community may be of great importance.

Effects of Housing and Hygiene on Trachoma Incidence and Severity

Since socioeconomic factors appear to have had considerable influence on the incidence of trachoma in the past, we would like to cite studies carried out in Saudia Arabia that suggest the effects of modern and substandard housing and hygiene on the clinical and microbiological character of trachoma.

Trachoma incidence is high in Saudi Arabia. Roughly nine out of every ten Saudi Arabians are affected with the disease at some time during their lives.

Since 1933 the Arabian American Oil Company (Aramco) has been engaged in oil production in Saudi Arabia. Up until the last few years, Aramco employees and their families have lived in oasis villages side by side with their neighbors who do not work for the company. The dwellings in these oasis villages are made of the traditional palm thatch, baked mud, or stone. In these homes electricity and running water are rarely found; windows and doors are unscreened; fly counts are high; garbage and sewage disposal is haphazard; and washing facilities are nonexistent or unsanitary (Fig. 1).

Since about 1956 Aramco has assisted in the

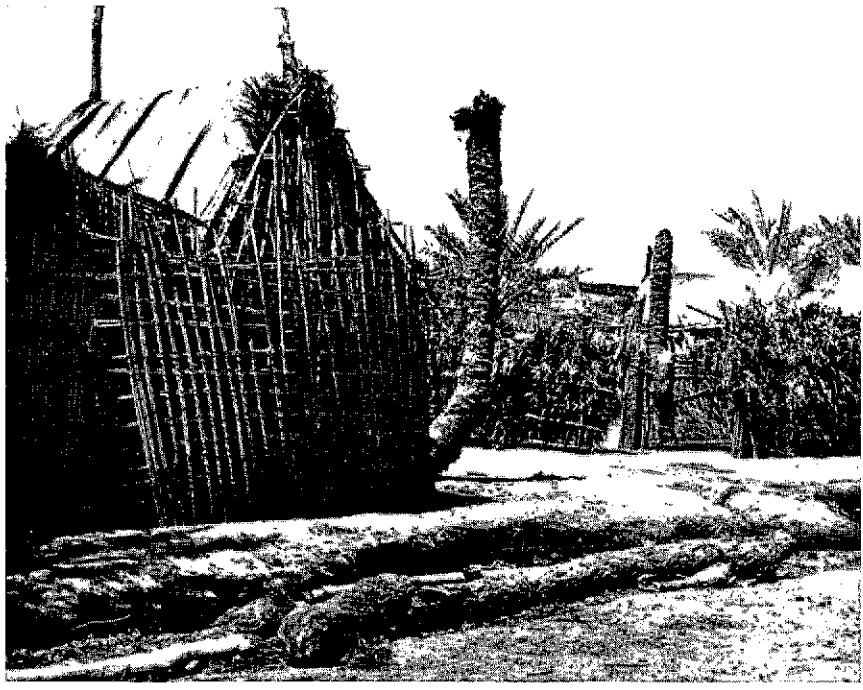

Fig. 1. Typical oasis village scene, Saudi Arabia.

building of modern housing units near their operation centers. These new townsites have modern conveniences such as electricity and running water, window screening, garbage and sewage disposal, and community washing facilities (Fig. 2). Over the past 10 years hundreds of Saudi Arabian families, company employees and others as well, have been leaving their oasis villages and moving into these attractive modern housing units.

In 1962, prior to launching a field trial of trachoma vaccines, a large-scale examination of Saudi Arabian children was undertaken (14). One of the major objectives was to see if there were differences in the character of trachoma as it evolved in a particular geographic area, among people with the same ethnic and cultural background but living under markedly different housing and hygienic conditions, such as those found in the townsites and oasis villages.

A total of 6,741 children from two modern housing townsites and six oasis villages were examined and observed over a two-year period. The children were examined both clinically and microbiologically. That is, in addition to turning up the eyelids to determine the presence or absence of clinically active trachoma, the investigators took scrapings of the conjunctival epithelium and examined them by the fluorescent antibody technique for the presence or absence of trachoma inclusion bodies. When the clinical data were arranged in oasis village and townsite groupings significant differences became apparent (Table 5). Among 4,560 townsite children examined, 55 per cent had clinically active trachoma, whereas among 2,181 oasis village children, 83 per cent had active disease.

When the microbiological data on the two groups were compared the differences became even more pronounced (Fig. 3.). Among the 1,802 oasis village children with active trachoma, 43 per cent had conjunctival smears that showed typical trachoma inclusion bodies when examined by the fluorescent antibody technique. By contrast, in only 3 per cent of 2,513 townsite children could inclusion bodies be demonstrated by FA.

Figure 4 shows both clinical and microbiological data from the townsites and oasis villages arranged according to age. In the upper part of the figure it is seen that in the oasis villages trachoma is rapidly acquired by

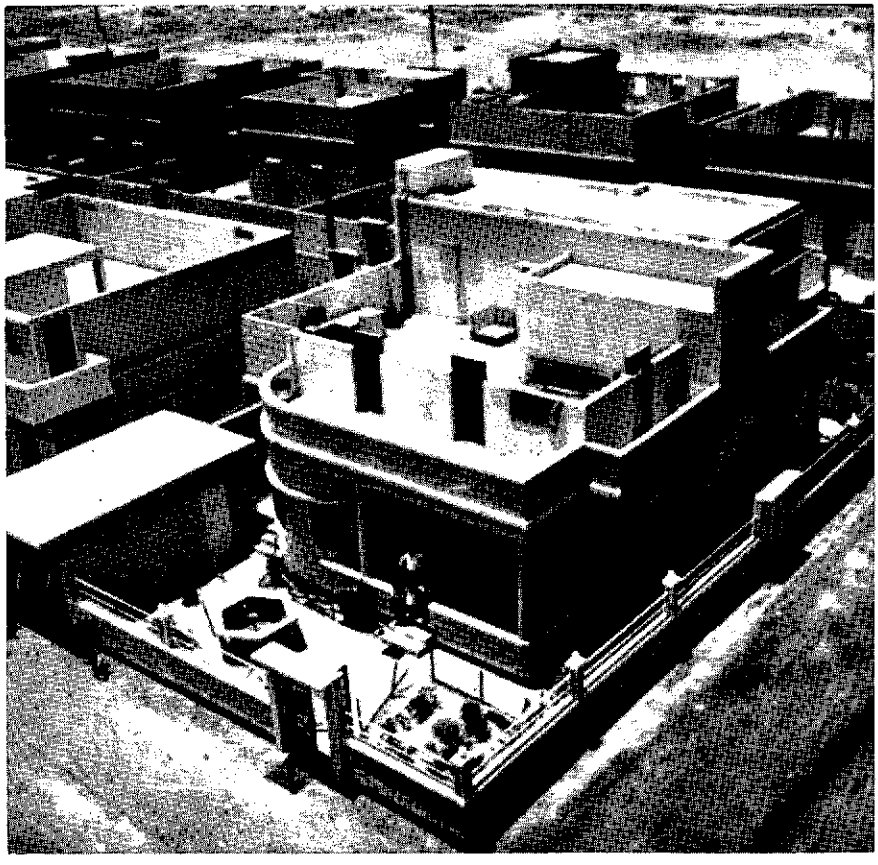

Fig. 2. Typical modern housing in townsite, Saudi Arabia.

newborn children. More than 40 per cent of them have clinical evidence of the disease by three months of age, and 90 per cent of them have contracted the infection by two years. In the socioeconomically favored townsites the disease is acquired more slowly, although by two to three years of age nearly 70 per cent of the children have it.

The sharp distinction in the microbiological character of the disease as it appears in the substandard and the modern living areas is well

TABLE 5. INCIDENCE OF ACTIVE CLINICAL TRACHOMA IN TOWNSITES AND OASIS VILLAGES, SAUDI ARABIA, 1962–1964

Townsites	2,513/4,560* = 55%
Oasis villages	1,802/2,181 = 83%

*Active clinical trachoma/total persons examined.

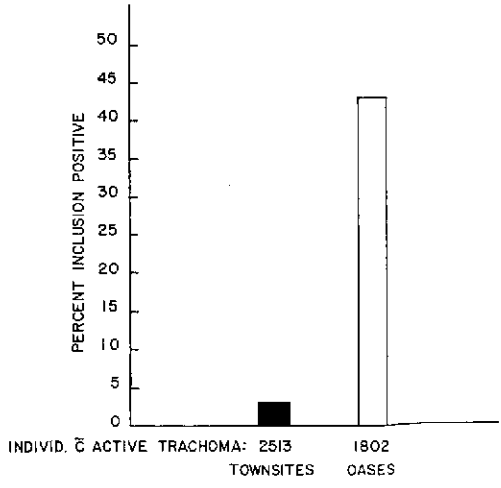

Fig. 3. Percentage of individuals with active clinical trachoma in whose conjunctival smears inclusion bodies were demonstrated by the fluorescent antibody technique, townsites and oasis villages, Saudi Arabia, 1962–1964.

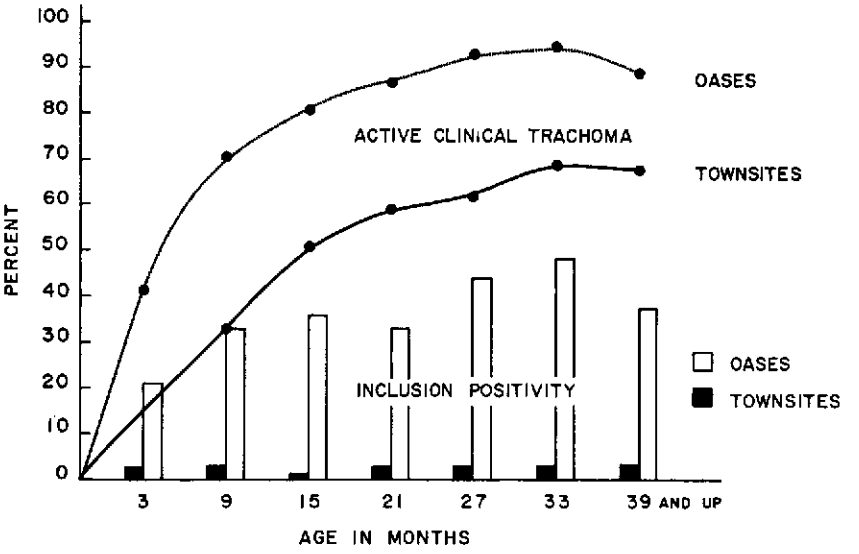

Fig. 4. Age-specific prevalence rates for active clinical trachoma and inclusion positivity in townsites and oasis villages, Saudi Arabia, 1962–1964.

illustrated in the lower part of Figure 4. Among the oasis village children with active trachoma, 20 per cent are inclusion positive by three months of age, and approximately 45 per cent by two to three years. However, in the townsites with modern housing it is the unusual child in whom inclusion bodies can be demonstrated by FA and there is no significant difference in inclusion positivity of children with active trachoma whether they are three months or three years old.

Trachoma is notoriously a disease that waxes and wanes. Its ups and downs are in all probability related to such factors as relapses, reinfections, and spontaneous cures. In Saudi Arabia the microbiological variation of the disease is very evident. FA staining of trachoma inclusions in smears of conjunctival cells was used to demonstrate conversions from microbiologic positivity to negativity and vice versa. Table 6 shows what happened to inclusion-positive children observed over a six-month period: 87 per cent of the inclusion-positive townsite children converted to negative, whereas only 43 per cent of oasis village positive children converted to negative. Conversion rates from inclusion negative to positive are shown in Table 7. Here there is an even greater difference between modern and substandard housing groups. While over 28 per cent of oasis village children who were originally inclusion negative converted to positive over the six-month observation period, only 2 per cent of the townsite children converted to microbiological positivity.

In a separate study (Nichols, unpublished observations) of children born in both the townsites and the oasis villages, those old enough to be viewed with the biomicroscope were examined for evidence of scarring and pannus. These disabling complications were observed frequently in the oasis village dwellers, but they were exceptionally rare in the townsite inhabitants.

Thus, by comparison with the oasis villagers, the townsite dwellers appeared to get the disease later, have a milder course with less evidence of pannus and lid deformities, recover micro-

TABLE 6. COHORT STUDY OF MICROBIOLOGICAL CONVERSION RATES FROM INCLUSION POSITIVE TO INCLUSION NEGATIVE OVER A SIX-MONTH INTERVAL, SAUDI ARABIA, 1962–1964

Place of residence	Number originally inclusion positive (+)	Percentage inclusion negative after 6 months (−)
Townsites	60	87
Oasis villages	440	43

TABLE 7. COHORT STUDY OF MICROBIOLOGICAL CONVERSION RATES FROM INCLUSION NEGATIVE TO INCLUSION POSITIVE OVER A SIX-MONTH INTERVAL, SAUDI ARABIA, 1962–1964

Place of residence	Number originally inclusion negative (−)	Percentage inclusion positive after 6 months (+)
Townsites	2,312	2
Oasis villages	721	28

biologically faster, have fewer relapses or reinfections, and show evidence by FA of far less infectious material in the eye.

These marked variations in the character of trachoma suggest that socioeconomic factors, especially those of housing and hygiene, can play a major role in determining the persistence and severity of the naturally occurring disease and hence may markedly influence the success or failure of community-wide treatment programs.

Summary

Oral sulfonamides and topical tetracyclines have been shown to be effective in the treatment of individual cases of acute trachoma. However, it is suggested that the course of subacute and chronic trachoma in a community is related to the interaction of a great many factors, among which treatment is only one. Age of onset, duration of disease, intensity of microbiological positivity in conjunctival cells, superimposed bacterial or viral infections, relapses, reinfection, and, finally, housing and hygiene are all factors that may play important roles in determining what course the disease will take.

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SECTION B. BEDSONIAE

IMMUNIZATION AGAINST TRACHOMA *

J. THOMAS GRAYSTON

Department of Preventive Medicine, School of Medicine
University of Washington, Seattle, Washington, U.S.A.

DR. GRAYSTON (*presenting the paper*): Although the trachoma organism has been available to study for less than 10 years, considerable progress has been made toward a better understanding of the immunology of the disease and toward development of prevention by immunization. The trachoma agent is a member of the Bedsonia group of organisms—intracellular parasites that have cell walls and methods of reproduction similar to those of bacteria (18). Successful methods of immunization against other Bedsonia have not been developed. Trachoma is a superficial disease that is chronic, recurrent, and long lasting. Except in cases of eventual spontaneous cure, there is little evidence of natural immunity. Despite these discouraging facts, our studies and those of other workers have shown that trachoma infection in humans and in primates can be prevented by immunization.

Collier (9) has recently reviewed all the published trachoma vaccine studies that have been done up to this year in various laboratories around the world. Thus, the present report will not review the literature; it will deal, rather, with some of the important advances in our knowledge of trachoma immunology, current developments, and problems of prevention by immunization. Examples will be drawn mostly from the published work of my colleagues and myself, and use will be made of new information presented at a recent WHO International Trachoma Symposium held in California in August 1966 (to be published as a supplement, *Am J Ophthal*, April 1967).

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GROWTH OF THE TRACHOMA ORGANISM

So far, high-titer trachoma agent has been obtained only by growth in the yolk sac of embryonated chicken eggs. The vaccines to be discussed have all been made from yolk sac starting material. Although trachoma strains can be grown in cell culture of heteroploid cells, high-titer material is not produced. There are no findings to suggest that cell cultures acceptable for the production of human vaccine will ever produce high-titer trachoma organisms. The best hope for a new material other than yolk sac for the growth of trachoma would appear to be a medium without living cells. Studies, particularly by Moulder and his associates (19), directed toward a better understanding of the metabolism of the Bedsonia may some day lead to the development of such a medium.

Assay of infectious titer of trachoma agents can be carried out with reasonable accuracy in the yolk sac of eggs. Egg assay with EID_{50} is preferable to ELD_{50} and shows good repeatability with 0.3 $\log_{10}$ variation (31).

PURIFICATION OF TRACHOMA ORGANISMS FROM YOLK SAC

A number of different methods for the purification of trachoma agent from the yolk sac have been employed in the preparation of vaccine. Collier (8) has used only differential high- and low-speed centrifugation. Bell and colleagues (4) have employed differential centrifugation, celite treatment, and ether extraction. We have used trypsin treatment of crude yolk sac and/or sonication, followed by differential centrifugation and further purification by four main methods (32). Our earliest vaccine preparations were treated with polymyxin (PEB), which precipitated some of the egg impurities and enhanced the complement-fixation (CF) antigen titer (37). Compared with later vaccines, these preparations were not of very high purity and were of relatively low antigenic mass. More recently we have employed DEAE Sephadex column chromatographic separation as a final purification step. Suspensions passed through such columns are highly purified, but they are of relatively low elementary body yield. The viability of the organism is not affected. Genetron, a fluorocarbon, has been used for purification, and highly purified elementary bodies have been prepared (Fig. 1). This method of purification is relatively easy and produces a high yield of organisms. It inactivates the organism, and its effect on antigenicity is the subject of experiments reported below. Finally, we have used

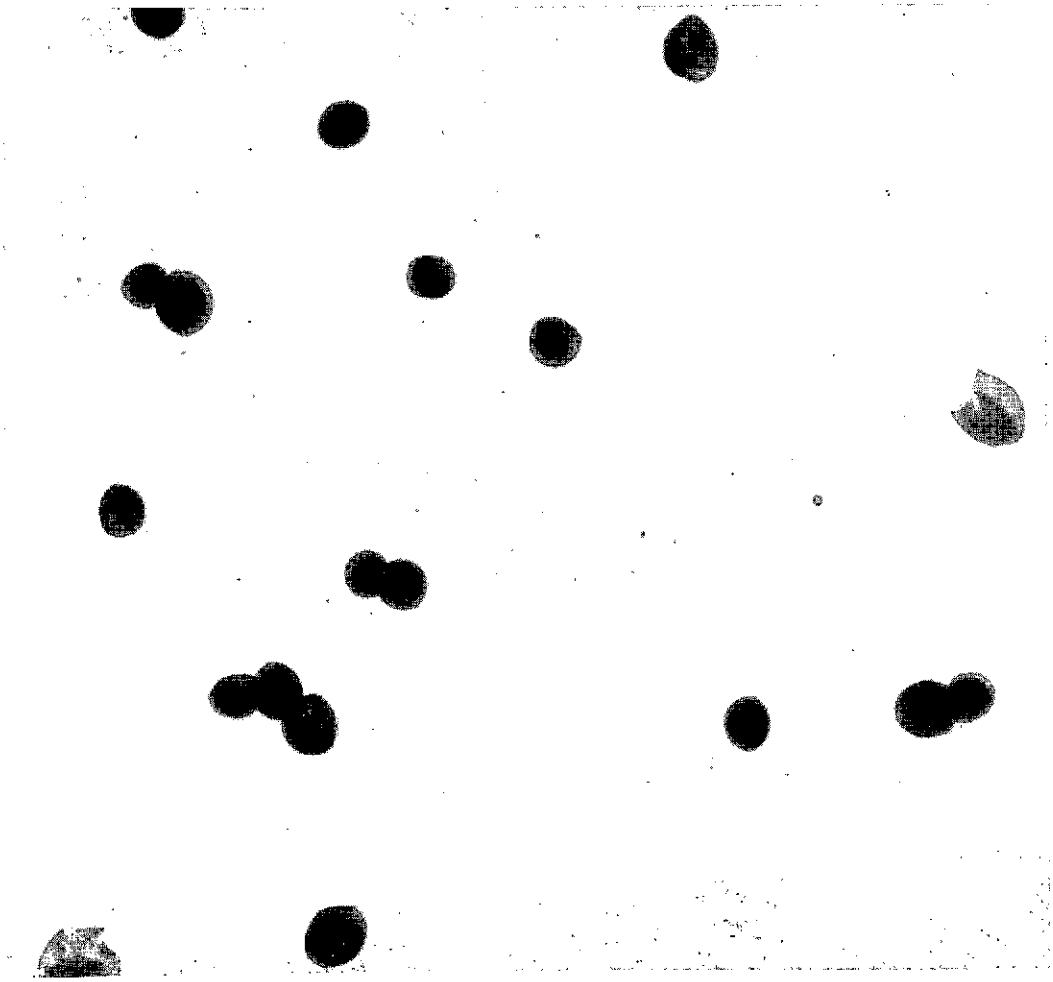


Fig. 1. Electron photomicrograph of genetron-purified suspension of trachoma elementary bodies (22,500 X).

sucrose-density gradient centrifugation in a 0 to 40 per cent continuous gradient. The organisms retain their viability in this method, which also produces a high yield. It is considerably more difficult to prepare vaccines in sucrose gradients than by genetron extraction.

Suspensions of purified trachoma organisms prepared by the numerous methods mentioned, have been used as trachoma vaccine in studies in humans and primates. Usually the suspensions have been inactivated with formalin, although Collier has employed live organisms in his studies (9). The vaccines have been aqueous suspensions, or they have been mixed with alum or emulsified with mineral-oil adjuvant.

MEASUREMENTS OF VACCINE POTENCY

In early studies the measurements of vaccine potency were crude and depended on concentration of the yolk sac by weight or egg titer before inactivation. The ability of a vaccine to elicit a CF antibody response has been shown to be poorly related to its protective capacity. More recently methods have been devised to count the number of elementary bodies in a suspension, either with the electron microscope (32) or by dark field microscopy (24). Such counts have been reproducible and appear to be related to potency (34).

We have developed a method for determining the potency of a vaccine by measuring its capacity to protect mice from toxic death (32). Mice die between 1 and 24 hours after intravenous inoculation of potent live Bedsonia suspensions. Such deaths can be prevented by prior immunization with homologous strains. The mice are given a 0.5 ml vaccine dilution intravenously two times, one week apart, and challenged one week later by the intravenous route with about 1.5 fifty per cent lethal doses (LD_{50}). Tenfold dilutions of the vaccine are employed beginning with a 10 per cent suspension based on the original yolk sac weight (1 gram per ml equals 100 per cent). In the example of the vaccine potency test shown in Table 1, the 50 per cent effective, or protective, dose (ED_{50}) was found to be a 0.246 per cent suspension. This dilution of the vaccine contained 1.6×10^5 particles per ml. ED_{50}s in the range of 10^5 to 10^6 elementary bodies have usually been found with vaccine preparations we have tested with each purification method. The results have been reproducible on repeated testings of the same vaccine.

ANTIGENIC TYPES OF TRIC ORGANISMS

Table 2 shows our antigenic classification of trachoma and inclusion conjunctivitis (TRIC)

TABLE 1. POTENCY TEST OF GENETRON-PURIFIED TW-3 TRACHOMA VACCINE IN THE MOUSE TOXICITY PREVENTION TEST

Vaccine dilution (%)	No. of mice	Death in hours after challenge										D/T	ED_{50}
10.0	10	ON										1/10	
1.0	10	ON	ON									2/10	
0.1	10	3	3	4	4	5½	ON					6/10	0.246%
0.01	10	3	4	4	5	5	5	5	ON	ON	ON	10/10	(1.6×10^5)
0.001	10	3	3	4	4	4½	5	5	7	7	ON	10/10	
Simultaneous titration of challenge dose used, TW-3:20%y.s.													
TW-3 y.s. 20.0	6	3	5	ON	ON	ON						5/6	
TW-3 y.s. 13.3	6	ON	ON									2/6	(LD_{50}:14.8%)
TW-3 y.s. 8.9	6	ON	ON									1/6	

Vaccine tested contained 1.3×10^9 particles per ml in 2000% suspension.
Challenge dose = 20%/14.8% = 1.35 LD_{50}

Note: Immunization, two 0.5 ml doses of vaccine dilution, is given intravenously one week apart, and challenge of 0.5 ml is given intravenously one week after the last dose of vaccine.
ON = Mouse died overnight.

TABLE 2. ANTIGENIC CLASSIFICATION OF TRIC STRAINS INTO SIX TYPES BASED ON CROSS-PROTECTION IN THE MOUSE TOXICITY PROTECTION TEST

A	B	C
5—OT—Middle East and Africa	13—OT—Taiwan 6—OT—Middle East and Africa 2—OT—Australia 2—OT—USA	22—OT—Taiwan 14—OT—India 1—OT—Canada
D	**E**	**F**
1—OT—Africa 1—NC—USA 1—Cx—USA 1—Ur—Great Britain	1—OT—USA 1—Ur—Great Britain 1—Cx—Great Britain 1—Cx—USA 2—Cx—Taiwan	1—NC—USA 1—Ur—Great Britain 1—PK—Great Britain 1—Cx—Great Britain 1—Cx—USA 3—Cx—Taiwan

OT = Ocular trachoma; NC = Neonatal conjunctivitis; Cx = Cervix; Ur = Urethra; PK = Punctate keratitis.

strains. This classification is based on cross-protection in the mouse toxicity prevention test (MTPT). Mice immunized by a homologous strain in the way just described are protected from death, whereas those vaccinated with strains of other types are not protected. Types A, B, and C contain only trachoma strains; 65 strains representing ocular trachoma strains from all over the world, have so far been classified into these three types (2, 30). This classification agrees closely with that proposed by Bell and his associates, who originally developed the MTPT for trachoma (4, 5) and are now employing a fluorescent antibody method for classification (3). Bell proposes two types, one with two major subtypes. Strains tested in both laboratories have been grouped identically.

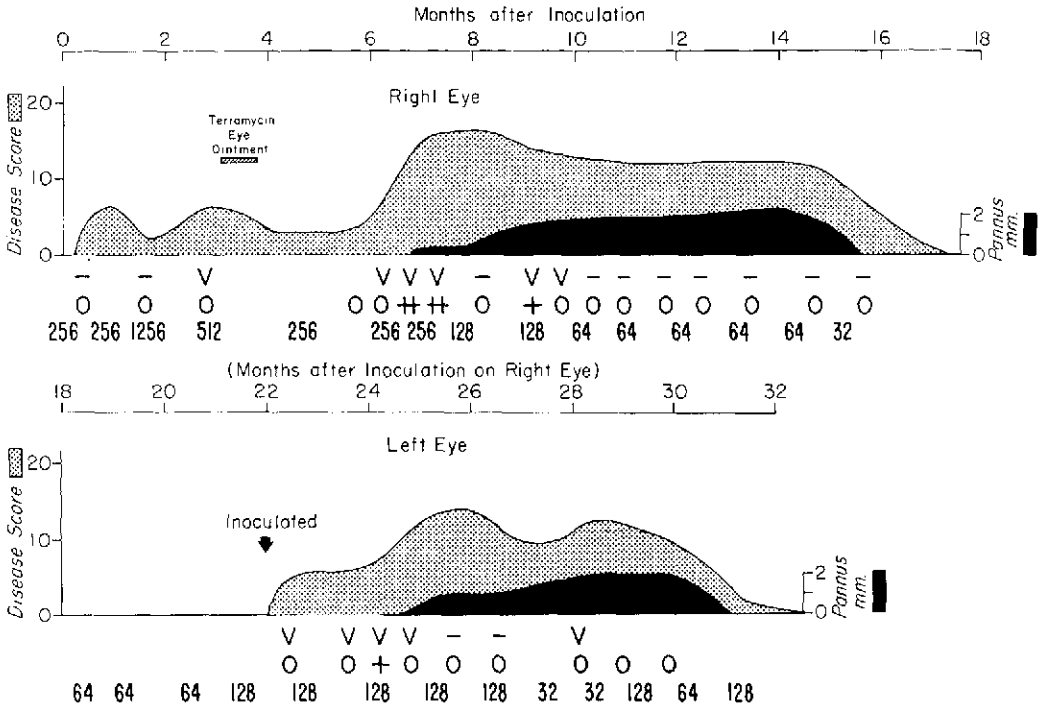
We have recently described three other types —Types D, E, and F. These are made up mostly of strains from the genital tract and from inclusion conjunctivitis of the newborn. Although at least some of these strains may cause the trachoma syndrome, they appear to have a different epidemiology. We have also found some biological differences in the laboratory. The Types D, E, and F strains cause more severe disease in the monkey eye and are more resistant to penicillin (26).

The fact that the findings of two laboratories using two different methods are in agreement suggests that a classification somewhat similar to the one shown on the table will eventually prove to encompass the trachoma and inclusion conjunctivitis agents.

TRACHOMA VACCINE EXPERIMENTS IN PRIMATES

Only primates develop conjunctival infection with TRIC agents. Collier has used baboons for a series of vaccine experiments (8, 10), and we have performed tests with the Formosan rock monkey (*Macaca cyclopsis*) (34). Most TRIC infections of the primate eye result in an acute follicular conjunctivitis that spontaneously disappears in a few months. However, we have occasionally seen a relapsing disease of increasing severity with pannus and conjunctival scars characteristic of human trachoma (33).

Figure 2 traces the course of an infection in a monkey challenged with the Taiwan ocular trachoma strain TW-1. Prior to the challenge, the monkey had been immunized with TW-1 strain vaccine and had demonstrated a CF antibody titer of 1:256. After challenge, an acute follicular conjunctivitis developed in the right eye. It started to wane six weeks later, but then there was a spontaneous relapse of the infection. After treatment with tetracycline eye ointment, which decreased the severity of the illness, there was another severe relapse at six months, followed by pannus formation with development of vessel ingrowth up to two millimeters. The disease healed spontaneously 17



V = TRIC agent isolated; − = Isolation negative; + to +++ = Typical inclusions seen; O = Inclusion negative. Figures represent CF antibody titer.

Fig. 2. The course and laboratory findings of trachoma infection with TW-1 strain in monkey #13, which had received TW-1 PEB alum vaccine.

months after inoculation. Five months later the left eye, previously uninvolved, was inoculated with the TW-1 strain and another unusually severe disease with pannus formation developed. The importance of the host response in the development of severe disease and pannus formation in both eyes of this monkey is emphasized by the fact that pannus formation was not observed in 100 other monkey eyes infected with the TW-1 strain.

A summary of the frequency with which we have observed pannus formation in monkey eye infection is given in Table 3. In a total of 617 monkey eye infections, pannus developed in 30. All cases of pannus occurred in monkeys who had previously been infected with TRIC agents and/or had received vaccine. There was no pannus in the 127 previously uninfected monkeys. Pannus developed much more frequently after infection with the Bour or IC-Cal-3 strains than after infection with the ocular trachoma strains TW-1 and TW-3. The Bour and IC-Cal-3 strains are Types E and F in the MTPT, the IC-Cal-3 strain being from neonatal conjunctivitis and the Bour strain from ocular trachoma in a California white male. It is suspected that the latter strain may be associated with a genital tract epidemiology.

Findings in the monkey model suggest that the chronic trachomatous changes of pannus

TABLE 3. FREQUENCY OF DEVELOPMENT OF PANNUS IN MONKEY TRACHOMA

TRIC strain	No previous infection, no vaccine	Previous TRIC infections and/or vaccine
TW-1	0/18*	2/84
TW-3	0/29	0/129
Bour	0/53	23/194
IC-Cal-3	0/15	5/59
Other	0/12	0/24
Total	0/127	30/490

*Number of monkey eye inoculations causing pannus/over number causing disease.

and scar are associated with a host response of hypersensitivity to the organism conditioned by previous infection or immunization with a homologous or heterologous strain (33).

We have utilized this monkey eye model for a series of experiments with vaccines. Experimental inoculation of the monkey eye, which is performed by rubbing a cotton-tipped swab soaked in a suspension of live organisms back and forth vigorously 20 times on the conjunctiva, has proven to be a strong challenge of immunity. Only highly potent vaccines have prevented infection. In our early experimentation with crude or polymyxin-treated aqueous suspensions, irregular results were obtained. The vaccinated monkeys sometimes had more frequent and more severe disease than did the controls (16).

Beginning with experiments in which mineral-oil adjuvants were used to potentiate the effect of the vaccine, consistent protection of at least a portion of the monkeys has been obtained (34). Crude suspensions of vaccine, both live and formalized, mixed with either complete or incomplete Freund-type oil adjuvants, have provided protection from disease.

Once we knew that protection could be obtained, we turned our attention to studies of purified vaccines, and in a series of experiments we demonstrated that vaccines highly purified by genetron, sucrose gradient, or DEAE Sephadex were as effective as crude suspensions in protecting the monkeys. The polymyxin-treated vaccine showed some protective effect when mixed with mineral-oil adjuvants, but less than that of other vaccine preparations. We also succeeded in evaluating cross-protection among different TRIC strains and the significance of types as determined in the MTPT.

In a series of experiments with the Bour and IC-Cal-3 strains, we demonstrated that highly purified vaccines were as effective as crude suspensions in protecting the monkeys and that there was no cross-protection between the two types. In the Bour challenge experiments shown in Table 4, a relatively light infection of the control monkeys was obtained in 16 of 27 showing disease. Good protection was found with the three types of Bour vaccine. The heterologous IC-Cal-3 vaccine failed to protect from infection, and the disease of the infected monkeys was more severe, as measured by the average disease score (29). The converse experiment with IC-Cal-3 challenge is shown in Table 5. Of 31 controls, 30 were infected. The protection afforded by the vaccine was less against this more severe challenge. Again, the heterologous vaccine provided no protection and the disease scores observed were worse. Although the breakdown is not shown, the IC-Cal-3 vaccine group included both crude and purified vaccines, which resulted in equal protection.

Table 6 shows a monkey vaccine experiment in which particle counts of elementary bodies in the TW-3 strain vaccines were available. Three injections of 0.25 ml of the oil-adjuvant vaccines were given at 0, 1, and 4 months. Challenge with the TW-3 strain was performed six weeks later. Four of seven control monkeys were

TABLE 4. MONKEY VACCINE EXPERIMENTS WITH BOUR AND IC-CAL-3 STRAINS, BOUR CHALLENGE

	Bour challenge		
	Infected/challenged		Average disease score
Control	16/27	57%	4.4
Bour vaccine			
Crude	3/15	20%	
Genetron	4/22	18%	
Gradient	3/11	27%	
Total	10/48	21%	5.7
IC-Cal-3 vaccine	4/6	67%	8.8

Note: The average disease score is calculated only on monkeys showing eye disease.

TABLE 5. MONKEY VACCINE EXPERIMENTS WITH BOUR AND IC-CAL-3 STRAINS, IC-CAL-3 CHALLENGE

	IC-Cal-3 challenge		
	Infected/challenged		Average disease score
Control	30/31	97%	4.3
IC-Cal-3 vaccine	13/21	62%	4.1
Bour vaccine	22/22	100%	6.0

Note: The average disease score is calculated only on monkeys showing eye disease.

TABLE 6. MONKEY VACCINE EXPERIMENTS WITH TW-3 STRAINS

Vaccine	Ten-week cumulative disease score*						
Control	17	17	13	12	0	0	0
Crude, live	0	0	0	0	0	0	
Crude, formalin	20	0	0	0	0	0	
DSP (1.6×10^8)	24	0	0	0	0	0	0
DSP (3.5×10^7)	62	61	47	39	33	0	0 0
DSP fluid (3.2×10^8)	20	7	0				

Note: All vaccines were with mineral oil adjuvant except the last one marked "fluid."
*Each number represents one monkey.

infected. All the monkeys were protected from infection by a live, crude vaccine. A similar vaccine to which formalin had been added protected all but one monkey. A vaccine prepared by purification in DEAE Sephadex columns and containing 10^8 elementary body particles was equally protective. However, a vaccine containing one log fewer particles failed to protect, and the disease scores observed were worse than those of the control monkeys. The 10^8 particle vaccine given as a fluid preparation without oil adjuvants failed to show protection in two of the three monkeys tested. We have recently used vaccine with 5×10^{10} particles both IM and IV without oil adjuvant in larger numbers of monkeys and have not succeeded in producing protection.

Table 7 shows an experiment with two Type B TW-5 strain vaccines, one prepared with sucrose gradient and the other by genetron extraction. The vaccines were formalized and combined with mineral-oil adjuvant. Both had been stored in the refrigerator for nearly one year before the test. Tenfold dilutions of the vaccines originally containing 4×10^9 particles were inoculated into a series of monkeys. Two 1 ml divided doses were given one month apart. The monkeys were then challenged six weeks later in the right eye with Type B TW-1 strain and in the left eye with Type C TW-3 strain. In all but one case, both eyes of the control monkeys were infected and showed relatively severe disease for these ocular trachoma strains. Both the vaccines with 10^9 particles provided good protection against the strong challenge but only against the homologous strain. The diluted gradient vaccine showed slight protection, but the diluted genetron vaccine did not. This correlates with the mouse potency tests, in which the gradient was stronger. There was no protection against TW-3 infection.

These and other experiments have shown that vaccines with particle counts of 10^8 to 10^9 are required for protection against monkey eye challenge. Fluorocarbon-purified vaccines in which the elementary body particles are inactivated have repeatedly been at least as effective in preventing infection as sucrose-gradient or DEAE-Sephadex-purified preparations of equal particle count. So far we have established by cross-challenge experiments in monkeys the significance of four of the six antigenic types as determined in the MTPT—Types B, C, E, and F. In a recent unpublished monkey experiment we have shown that a trivalent vaccine containing

TABLE 7. MONKEY TRACHOMA VACCINE EXPERIMENT WITH TWO TYPE B STRAIN VACCINES AND SIMULTANEOUS TYPE B AND TYPE C CHALLENGE, ONE STRAIN IN EACH EYE

TW-5 vaccine		Ten-week cumulative disease score												
		TW-3 left eye							TW-1 right eye							
Control		54	40	49	43	35	16	15	51	46	42	32	31	8	0
Gradient vaccine	4×10^9	60	41	38	34	33	16	0	29	24	0	0	0	0	0
	4×10^8	49	46	46	42	34	34	16	35	33	12	10	0	0	0
	4×10^7	48	47	41	40	32	38	0	36	34	25	15	0	0	0
Genetron vaccine	4×10^9	58	51	44	39	26	24	6	28	0	0	0	0	0	0
	4×10^8	71	53	43	40	36	30	4	54	42	34	30	26	8	0
	4×10^7	60	45	45	42	30	26	0	44	37	33	18	7	0	0

ocular trachoma Types A, B, and C protects about equally against the three types given one in each eye in various combinations.

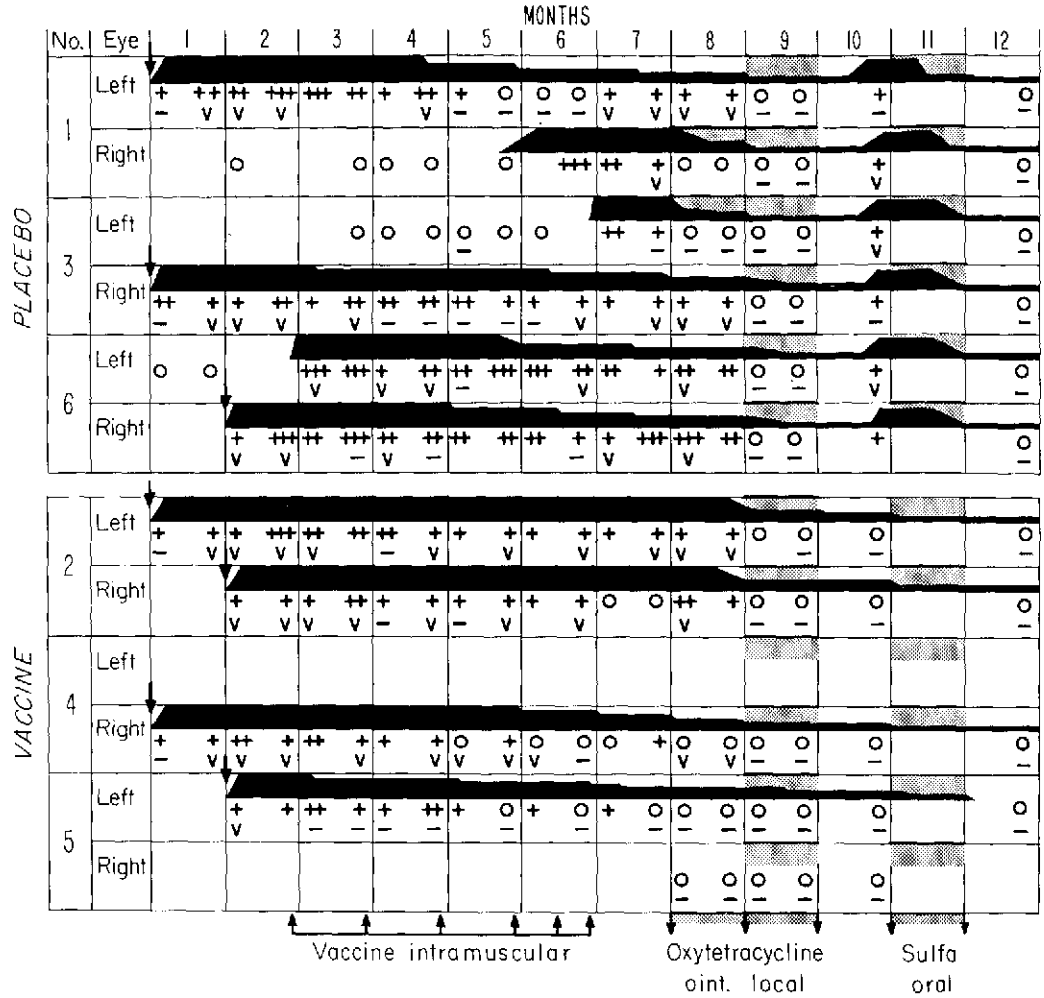
The vaccine experiments in monkeys have offered the opportunity for comparison of monkey eye protection with the potency determined in the mouse toxicity prevention test. Reasonably good correlation has been found with monkey protection, which requires approximately three logs more particles per millimeter than mouse protection. Field trials in humans have shown protection with vaccines less potent than those required to prevent experimental infection in monkeys.

Collier, in his vaccine studies with baboons (9) has concluded that live TRIC organisms injected by the intravenous route are the most effective protection against infection. Following formalin inactivation or the use of mineral-oil adjuvant he has had less effective protection and has occasionally enhanced disease. He has shown that, after parenteral injection of live organisms there is considerable growth in regional lymph nodes and in the spleen (10). Such growth of the live organisms after injection would justify increasing the potency of such vaccines.

TRACHOMA VACCINE STUDIES IN HUMANS

In 1958–1959 it was decided to infect humans with the newly isolated elementary body organisms to prove that these were the etiological agents of trachoma. The opportunity was taken to study the effect of polymyxin-treated vaccine on the course of the experimental illnesses (14). Figure 3 shows the course of eye disease in six blind volunteers who were infected with strain TW-1. All promptly developed disease in the inoculated eye. The height of the bar graph indicates the severity of the follicular conjunctivitis. Of the three volunteers given the placebo, No. 6 had a spread to the uninoculated eye one month after infection and No. 1 and No. 3 showed spread infection after four to six months. All six eyes were treated with tetracycline eye ointment during the ninth month after infection. The spread-infection eye was also treated during the eighth month in No. 1 and No. 3. After the treatment had resulted in virtual cure of the disease, we were surprised to find several weeks later an acute relapse in all six eyes. It was necessary to retreat with oral sulfa drugs to cure these volunteers. Among the other three volunteers, who some two months after their experimental infection were begun on a series of PEB vaccine injections, No. 2 was purposely infected in the second eye one month after the first infection. The other two never developed spread infection of the uninoculated eye. The most dramatic difference between these three vaccinated volunteers and the three who received the placebo was that after the month of tetracycline eye ointment treatment there was no relapse of the disease.

When studies in monkeys and human volunteers (15) had shown no adverse reactions to trachoma vaccine preparations, the first field trial for protective effectiveness of vaccine was undertaken in preschool children in a rural agricultural area near the seacoast of central Taiwan. The result of this early field trial, begun in 1959 and followed for six years, and also the results of a recent vaccine field trial in the New Delhi area of India are summarized in Table 8. The vaccine used in Taiwan was a relatively low-potency polymyxin-treated material (PEB) mixed with alum. Four injections were given during the first two years. The attack rate over six years was nearly 50 per cent. There was statistically significant protection after one and two years of the study, but the protection gradually decreased until at the end of six years there was essentially no difference between the children who had received the placebo and those who had received the vaccine (39). The Indian study was begun in 1965 and was carried out under the auspices of the Department of Ophthalmology, All-India Institute of Medical Sciences (13). High-particle-count gradient and genetron-purified vaccines were tested. Two injections were given three months apart. No adjuvant was used. Follow-up for one year showed particularly good protection with the genetron vaccine and also statistically significant protection with the gradient vaccine. The attack rate in the placebo group was expectedly high. In the mouse test the gradient vaccine had 600 potency units and the genetron had 300 units, thus paralleling the greater effectiveness of the gradient vaccine in the field trial.



Source: Grayston, J. T., Wang, S. P., Yang, W. F., and Woolridge, R. L., "The Effect of Trachoma Virus Vaccine on the Course of Experimental Trachoma Infection in Blind Human Volunteers." *J Exp Med* 115:1016, 1962.
Note: Bar graph represents severity of follicular reaction. Three volunteers were given trachoma vaccine; three were given placebo.

Fig. 3. Course of trachoma eye infection in six blind human volunteers infected experimentally with the TW-1 Taiwan trachoma strain.

We also carried out field trials with mineral-oil adjuvant vaccines (38). These studies were performed in first- and second-grade schoolchildren in central Taiwan. Table 9 shows two preventive trials, one with a bivalent and one with a monovalent vaccine. The bivalent TW-1 and TW-3 vaccine prepared in DEAE Sephadex was highly purified but contained a relatively small particle count. Each child received slightly less than 1×10^7 elementary bodies of each strain in two 0.25 ml injections. This compares with 2 to 4×10^9 particles received by each child in the New Delhi study. Although the attack rate was low, there was statistically significant protection from the vaccine over the three years of observation.

In the study using monovalent TW-3 vaccine prepared in the same manner, there was a higher attack rate among the children who received vaccine than among those who received the placebo. The attack rate in the placebo group was quite low—only 3 per cent over three

TABLE 8. TRACHOMA VACCINE FIELD TRIALS IN PRESCHOOL CHILDREN

Place and treatment	No. of subjects	Percentage of conversion to trachoma		
		1 year	2 years	6 years
Taiwan, Placebo	193	10	16	49
Vaccine, PEB	169	4	8	47
Effectiveness		66%	47%	
India, Placebo	87	37		
Vaccine, Genetron	90	19		
Vaccine, Gradient	92	10		
Effectiveness, Genetron		50%		
Gradient		73%		

years—whereas conversion to trachoma reached 8 per cent in the vaccinated group. Since we have shown that ocular trachoma Types B and C are about equally prevalent on Taiwan (30), the deleterious effect observed after use of the monovalent vaccine may be due to a phenomenon similar to the hypersensitivity effect of heterologous challenge observed in the monkey experiments reported above. No immediate or delayed reactions of any consequence were observed with these mineral-oil adjuvant vaccines.

TABLE 9. MINERAL-OIL ADJUVANT TRACHOMA VACCINE FIELD TRIALS IN TAIWAN SCHOOL CHILDREN

Vaccine	No. of subjects	Percentage of conversion to trachoma		
		1 year	2 years	3 years
Bivalent (TW-1 & TW-3)				
Placebo	354	4	7	9
Vaccine	351	2	4	5
Effectiveness		46%	45%	42%
Monovalent (TW-3)				
Placebo	299	2	3	3
Vaccine	299	5	7	8

In view of the repeated demonstration of the need for oil adjuvants in protecting monkeys, the lack of unfavorable side reactions, and the obvious need for a more potent and longer-lasting trachoma vaccine, we believe that further human trials should be carried out with a bivalent or trivalent oil-adjuvant vaccine containing a higher concentration of trachoma organisms.

In another approach to the use of vaccine, we carried out three field trials on the effect of vaccine plus drug therapy in first- and second-grade schoolchildren with active trachoma (36). In none of these studies was a curative effect of vaccine demonstrated. We were disappointed in each case by failure to cure more than a small percentage of trachomatous children with tetracycline eye ointment therapy alone or in combination with sulfa drugs given orally. In one study, after two intensive courses of treatment, we were able to temporarily cure 97 children who had received the placebo and 86 who had received bivalent oil-adjuvant vaccine. The vaccine was the same as that used in the preventive field trial of Table 9. In an additional two-year follow-up, it was demonstrated that 62 per cent of those who had received the placebo and 37 per cent of those who had received the vaccine showed reconversion to active trachoma. This is equivalent to 40 per cent vaccine effectiveness—the same as the rate found in the preventive field trial.

Results of field trials of vaccines carried out by the Harvard group in Saudi Arabia (9) and Portugal (25) and by Collier in Ghana (9) have shown only a small degree of protection, usually relatively short-lived. Both groups have used mineral-oil adjuvants with trachoma vaccine and have observed no untoward effects. The Harvard group produced sterile abscesses when typhoid vaccine was mixed with mineral-oil adjuvant for a control material (25). Italian workers have reported successful results from several field trials (6). One large trial followed over three years was considered difficult to interpret because only a small percentage of the original study group could be traced (9). They also used oil adjuvants and observed no reactions except for some long-lasting nodules in babies. In addition, one trial by the Harvard group showed a deleterious effect, with more disease

occurring in the vaccinated children (21). Interestingly, a tenfold dilution of the vaccine causing the increased number of cases provided short-lived protection in the same trial.

PRELIMINARY CHARACTERIZATION OF TRACHOMA ANTIGENS

Immunological studies of TRIC agents have been hampered by the paucity of good tests for antigens or antibodies. The mouse toxicity prevention test described above has been the most specific and useful one available. We have undertaken studies of the physical and chemical lability of the protective antigens of three trachoma strains—TW-3, TW-5, and ND-3 (35). The protective antigens are defined as those that actively immunize mice against toxic death. The most striking finding of the study was that the protective antigens were remarkably heat stable and retained antigenicity after boiling for one hour. Table 10 shows that antigenicity was also retained after the following treatments: five cycles of genetron extraction, ammonium sulphate precipitation, periodate oxidation (0.1 M sodium metaperiodate one hour at room temperature and overnight in refrigerator), and delipidization with acetone and ether (three cycles of five volumes of acetone followed by three cycles of four volumes of ethyl ether). The effect of these treatments on the particle count was negligible except with genetron and periodate, both of which caused a particle loss of approximately one log. Although there was some loss of particles with each genetron extraction, the major loss was after four and five treatments. The loss of protective effect against mouse toxicity paralleled the loss of particles. Therefore, as shown in Table 10, the ED_{50}s in terms of particle count were not reduced. Antigenic activity was destroyed without particle loss by treatment of purified preparations with crude trypsin, 0.5 per cent at 37°C for two hours, and partially lost after mild alkaline hydrolysis. Preliminary efforts to extract the protective antigens by trichoracetic acid precipitation or treatment with either cold or hot phenol failed. Further studies will be required before a reasonable estimate of the nature of the protecting antigen can be made.

In a preliminary report, Peters has indicated

TABLE 10. PRELIMINARY CHARACTERIZATION OF TRACHOMA ANTIGENS PROTECTIVE AGAINST MOUSE TOXICITY

	Effective $dose_{50}$ in particle count	
	Control	Treated
Heating, 100°C, 60 minutes	4.4×10^5	4.2×10^5
Five genetron extractions	2.2×10^5	2.6×10^5
Ammonium sulfate ppt	8.4×10^6	9.0×10^6
Periodate oxidation	3.8×10^6	1.9×10^6
Acetone and ether extraction	5.5×10^6	$<5.5\times10^6$
Trypsin, 0.5% 37° C, 2 hours	1.2×10^6	$>1.1\times10^9$
Alkaline hydrolysis	1.3×10^6	4.1×10^7

that treatment with neuraminidase enhances the immunogenic and antigenic properties of trachoma organisms (23). He immunized guinea pigs and measured the antibody response by CF, HA, and FA tests. He found that treatment with 2-mercaptoethanol and muramidase had similar enhancing effects, but not as pronounced as those with neuraminidase. A number of other treatments employed by him failed to enhance the antigens.

NATURAL HISTORY OF TRACHOMA

Information on the epidemiological pattern of trachoma is needed in order to know at what age and in what places vaccine may be used effectively. It is clear that the pattern varies from one geographic area to another. In villages near New Delhi, in the area used for the vaccine field trial, by two years of age over 80 per cent of the children had trachoma (13). In Taiwan the prevalence rates were much lower: 23 per cent of the first graders in the rural Taichung area used for the first vaccine field trial had trachoma, whereas 11 per cent had active disease at school entry in the other central Taiwan areas studied. Incidence rates based on six years of observation of the same children in the rural Taichung area and three years of observation in the other central Taiwan schools are shown in Table 11 (40). The annual conversion rates reflect the intensity of infection

TABLE 11. INCIDENCE RATES FOR TRACHOMA IN THE TAIWAN AREAS USED FOR VACCINE FIELD TRIALS

Area and period		Annual conversion rate for 6 years
Rural Taichung, 1960–1966	Preschool	#193—8.2 per year
	First grade	#877—7.9 per year
		Annual conversion rate for 3 years
Central Taiwan, 1961–1965	First grade	#3993—3.9 per year
	First grade	#653—2.1 per year

shown by the rates in incoming first graders. These incidence rates are of great interest because they show a higher annual conversion rate than can be accounted for on the basis of prevalence surveys, thus indicating considerable reversion to inactive disease or normal eye.

By repeatedly examining children both in vaccine field trials and in studies of the natural history of the disease we have frequently been able to demonstrate conversion and reversion in the same child. In a number of the individual children the clinical diagnosis of doubtful or initial trachoma without pannus was confirmed by laboratory evidence of infection. Many of these children reverted to normal eye, then later converted to trachoma, and eventually established chronic trachoma with pannus (40). Nichols, using fluorescent antibody for the determination of inclusion-positive conjunctival smears, has shown a very high percentage change from positive to negative and a high conversion to positive over six-month periods in Saudi Arabian children (20). These clinical and epidemiological findings, plus the fact that pannus occurred only in monkeys reinfected or given vaccine, leads us to conclude that the pathogenesis of trachoma depends on the immunological response of the host.

We believe that trachoma is a reinfection disease, not unlike tuberculosis, in which allergy to the infecting organism plays a role in the developing pathological picture. In an area like Taiwan, where the prevalence rate of trachoma increases every year up to the age of 20 and at that age involves about half of the population with clinical disease, we feel that in reality almost everyone is infected and that only after repeated reinfections or relapses does the characteristic clinical picture of chronic trachoma develop. In a study of Punjabi Indians in their environment and after immigration to British Columbia, Canada, my colleagues showed that continued activity of the disease and the development of serious sequelae was directly related to the length of residence in the endemic Punjab and inversely related to length of residence in Canada (12). These findings suggest that reinfection is more important than relapse in the pathogenesis of trachoma.

An argument advanced against the reinfection concept has been that pannus can occasionally be seen in children one to two months of age in hyperendemic countries. We felt that transplacental antibody might be involved in the pathogenesis of such early pannus. This would be similar to Chanock's suggestion that maternal RS virus antibody contributes to the pathology of lower respiratory infection early in life (7). Alexander and Chiang have recently shown that six-month-old monkeys on first inoculation developed severe trachoma infection with pannus if their mother's cervix was inoculated prior to birth with a homologous strain (1). The young monkeys were apparently sensitized without suffering any obvious disease, nor was there definite disease of the mother's cervix.

The reinfection concept of the pathogenesis of trachoma makes some of the hypersensitive reactions to vaccine more understandable. In addition to increased severity of disease after heterologous challenge, we have repeatedly seen more infection and more severe disease in monkeys that were given a vaccine of insufficient potency to provide protection. When we first observed this phenomenon several years ago, we were reassured to find that Cox had observed a similar phenomenon years before in studies of several viral and rickettsial vaccines (11). Although he presented no experimental results, he stated that preparations that did not contain enough antigen to produce a good immunogenic vaccine actually sensitized the vaccinated animals and made them more susceptible to challenge than the nonvaccinated controls.

In a *Mycoplasma pneumoniae* vaccine experiment in human volunteers, protection was afforded to those volunteers showing an antibody response to vaccine, but more severe disease

compared to controls was seen in those who did not show antibody response (28). Earlier in this conference, Parrott reported deleterious effects of killed RS virus vaccine (22) and Krugman presented examples of the severe allergic reactions to live measles vaccine in children who had previously received killed vaccine (17).

Hypersensitivity to vaccines would appear to be a general phenomonen that has not previously received much attention. This phenomenon may be more important in the development of viral and rickettsial vaccines, particularly killed vaccines, than tumor virus contamination, which is the subject of so much concern at present.

Conclusion

Under certain circumstances it is possible to protect primates, including humans, from clinical trachoma by immunization. There is a need for vaccines of higher potency and longer-lasting effects that can be prepared more easily. At the same time, it appears that methods must be found to reduce the hypersensitivity effects of immunization. The discovery of a subfraction of the total organism containing the protective antigen offers the best hope for more effective immunization. At present there is no commercially produced effective vaccine feasible for use in the developing countries where trachoma is a serious problem, nor is such a vaccine likely to become available in the very near future.

A great deal has been learned about the immunology of trachoma and the TRIC organisms since they were first isolated and grown in quantity less than 10 years ago. These intracellular organisms with complex cell walls cause diseases with interesting immunological and epidemiological characteristics. Further work directed toward the prevention of trachoma is justified both by the importance of this disease in the developing countries and by the broadly applicable immunological problems presented by this intermediate class or organisms.

Acknowledgment

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SECTION C. ADJUVANTS

PROBLEMS AND FUTURE OF IMMUNOLOGIC ADJUVANTS

GEOFFREY EDSALL

State Laboratory Institute, Massachusetts Department of Public Health and Harvard School of Public Health, Boston, Massachusetts, U.S.A.

DR. EDSALL (*presenting the paper*): As many speakers at this meeting have already pointed out, immunization, particularly immunization with inactivated antigens, is beset with numerous problems and limitations. Some vaccine preparations have proven to be ineffective despite all logical reasons to believe that they should have worked. In others, the effectiveness may be of an unacceptably low order of magnitude or only transient in its duration; the large amount of antigen required for an effective response may lead to reactions that preclude the general acceptance of the vaccine; or successful immunization may require more injections than it is practical to administer, especially as the number of desirable immunization procedures continues to increase.

In seeking to surmount these problems, investigators have naturally turned to adjuvant "substances which when mixed with antigens improve antibody production" (17). Before discussing adjuvants in detail, however, it is useful to consider the immune response in a somewhat broader context.

The first phase of the immune response, the so-called "lag period" or induction phase, may actually be the determinant phase in establishing the response, for clearly it is the point at which the number, and perhaps the kind, of cells committed to the desired response is decided.

The second recognized phase is signaled by the appearance and progressive rise in titer of specific protection, generally identifiable in the form of serum antibodies, against the infectious agent involved. This response reaches a peak anywhere from a few days to a month after the onset of immunization. It then declines, first relatively rapidly but then progressively, merging into an asymptotic phase, which ends in a prolonged and very slowly falling residual titer that may eventually sink below the level that current techniques can measure.

Subsequently, in most immunological systems, a secondary response can be induced after the first antigenic stimulus. The minimum effective interval for induction of a significant secondary response is probably at least two weeks, and the response improves as the interval is lengthened. This secondary immune response in most instances goes to much higher levels than the primary response, and although it often declines equally precipitously at first, it ends, like the first response, in an asymptotic residual level of protection, which is still generally much higher than the level seen after the first response. Usually this secondary type of response can be induced again and again, provided that a minimum interval is allowed between stimuli. The need for such an interval is probably based on the fact that a new generation of specifically responsive cells must be permitted to develop after the last previous stimulus.

There are many variations on this pattern. For example, there is often no true secondary response after the injection of polysaccharide antigens, perhaps because they contain their own built-in adjuvant and therefore achieve a maxi-

mal response from the beginning. Obviously, the pattern of response to living antigens is for the most part quite different from that observed with killed antigens. In general, the problems involved in the use of living antigens are not ameliorated by the use of adjuvants.

There are a number of factors that can markedly influence both the height and the duration of the immune response. One of obvious importance is the amount of antigen given. According to the information available on the relation of the antigen dose to the response obtained with different antigens the dose/response ratio varies considerably, but it generally appears to fall between 0.5 and 2 in logarithmic terms (30). Thus it is obvious that increasing the antigen dose will normally increase the response obtained. However, the use of this mechanism is limited, since for every immune response there appears to be a ceiling above which it is generally impossible to go. Also, larger amounts of antigen are more likely to give untoward reactions, if not with the first dose, then certainly with subsequent doses. On the other hand, too small a dose may produce only a transient response (29, 33, 34) without inducing lasting and effective immunity.

The physical state of the antigen has a major effect on its immunogenicity. Winebright and Fitch (10, 36), among others, showed the superior antigenicity of an aggregated antigen as compared with its "soluble" form. Indeed, several investigators (4, 6, 11) have shown that some antigens that can induce immunoparalysis if used in the soluble nonaggregated form may be highly immunogenic when the antigen molecule is aggregated. Furthermore, it has been shown that combination of antigen and antibody are more immunogenic than the antigen alone (32, 33). Indeed, this was the principle of the diphtheria toxin-antitoxin mixture that was widely used some years ago. The various effects of aggregation, together with some of the actions of adjuvants, are most readily explained by the assumption that phagocytosis is an essential first step in the immune response (9) and that aggregation facilitates this step (7, 11). Various studies over the years have suggested that antigen uptake is facilitated when the antigen is in a particulate rather than a soluble form. For example, the observations of Glenny *et al.* (14) over 30 years ago indicated that fluid diphtheria toxoid was rapidly eliminated from the host when injected, whereas alum-precipitated toxoid was readily taken up by the cells of the host. I emphasize this apparently obvious and widely accepted concept because I think that the role of adjuvants in immunization must be considered not only in terms of substances, but also in the broader context of the various possible mechanisms involved.

Adjuvants vary widely in their character, but in general they appear to have a number of factors in common. Thirty years ago Landsteiner (20) listed the factors that he thought were operative in the action of adjuvants as "delayed excretion of the injected material, engulfing of the particles by the cells producing antibodies, and the stimulation of cell activity"—an astute summary for its day. The early observations on aluminum salts suggested that they enhanced antibody production by prolonging the period of absorption of the antigen and delaying its elimination (14). However, adjuvants that are associated with depot effects, such as aluminum salts and water-in-oil emulsions, have likewise been shown (13, 35) to attract both antibody-forming cells and mononuclear phagocytes to the site of the depot, thus apparently assisting in the efficient uptake of the antigen and its utilization by the ultimate target cell. Along with this depot effect, however, it has been shown for both aluminum salt and water-in-oil emulsion adjuvants that the antigen-adjuvant complex is widely disseminated (13, 16). Thus antibody formation is initiated in a relatively short time at a number of sites, not simply at the depot. Indeed, for both types of adjuvants it has been demonstrated (12, 16) that early excision of the depot does not eliminate the resulting immune response.

Although a variety of adjuvant substances have been tried experimentally, interest in application of the principle to man has been focused primarily on the use of several forms of aluminum salt and on various types of water-in-oil emulsions. Although differences in the efficacy of different aluminum salts have been demonstrated, they have been shown quite clearly to be only minor (23).

Few valid comparisons have been made between the effectiveness of aluminum salts and

water-in-oil emulsions. However, one observation published recently does set forth these differences rather strikingly. MacLennan *et al.* (22), using a single batch of tetanus toxoid, administered in comparable amounts as a fluid preparation (three doses), as an aluminum salt preparation (two doses), and as a water-in-oil emulsion (one dose), demonstrated that the aluminum-phosphate-adsorbed toxoid induced approximately twice the average peak response as did the fluid preparation, whereas emulsified toxoid gave a peak response later, and about 10 times higher, than that of the fluid preparation. Furthermore, the antibody level observed after injection of the fluid preparation fell markedly within a year, whereas the levels maintained after injection of the alum-adsorbed or water-in-oil emulsified preparations remained fairly constant for a period of at least two years (28). Thus the use of either of these two familiar adjuvants led to a marked increase in the effectiveness of the immunizing process, and the water-in-oil preparation accomplished the best immunization with only a single injection. Furthermore, contrary to the general assumption, the initial response to the water-in-oil emulsified toxoid, as compared to the responses to the other preparations, did not appear to be significantly delayed.

In a detailed study of influenza vaccine in mice, in which the responses to water-in-oil and fluid immunization were compared, Berlin (2) showed that the rate of development of the primary response to the two agents was essentially identical; however, the antibody level observed after the injection of the water-in-oil emulsion continued to rise for a number of weeks after the response to the aqueous injection had reached its peak and begun to fall. Parallel observations, showing that the use of aluminum salt adjuvants not only did not result in any delay in the primary response, but instead slightly accelerated it, have been made in our laboratory (8, 21) and elsewhere. In fact, there is little sound evidence to justify the widespread teaching that for rapid immunization one must use fluid antigens. Indeed, there is considerable well-controlled data, including our own, to suggest that the opposite is true. In any case, the use of adjuvant preparations does not impair the onset of immunity, and with the preparations most widely used it greatly enhances the level and the duration of the response. However, it should be noted that both aluminum salts and water-in-oil emulsions, although highly effective with protein antigens, appear to have little if any enhancing effect on polysaccharide antigens.

The immunologic effectiveness of a single dose of antigen in water-in-oil emulsion has been demonstrated repeatedly, both in animals, with various antigens (13), and in man—with influenza vaccine (15), tetanus toxoid, and cholera vaccine (27). In contrast, a single dose of aluminum-adsorbed antigens, at least in the case of tetanus toxoid, has not produced a response effective enough to give lasting protection (26). Thus many hopes have been focused on the possible use of water-in-oil emulsions as a mechanism for establishing durable immunity with only a single inoculation.

However, in a number of individual studies the incidence of undesirable local reactions after the injection of water-in-oil emulsified antigens has been sufficiently high to discourage their general use. Specific examples are those reported by MacLennan *et al.* for tetanus toxoid (22), the Philippines Cholera Committee for cholera vaccine (27), and Snyder *et al.* for typhoid vaccine (29). The significance of these side reactions, as well as a possible explanation for them, needs to be further explored. In any case, a number of observations (5) have led to a feeling of caution with regard to the possible long-term side effects—autoimmunity, carcinogenesis, and so on—that may result from the injection of water-in-oil emulsion.

While none of these late side-effects has been shown to be important in man, the doubts they have posed have stimulated the further search for less reactive yet still effective adjuvants. A most promising example is the peanut oil-Arlacel A-aluminum stearate complex, generally known as "Adjuvant 65," which is based on the use of a metabolizable oil instead of one that remains unaltered in the tissues (37). Some of the results with the use of this adjuvant preparation for influenza vaccination have been striking, yielding peak and residual levels ranging from 4 to 10 times higher than those achieved by the aqueous vaccine (31). The final evaluation of any newly introduced adjuvant preparation, how-

ever, will depend on long-term observation, and it therefore seems too early to draw final conclusions regarding the general acceptability of this or any other new preparation.

Much interest has been aroused during the past 10 years by the evidence presented by Johnson *et al.* that "endotoxins" had an adjuvant effect on protein antigens (19). This observation has been generally thought to explain the adjuvant effect to typhoid or pertussis vaccines when mixed with toxoids—although it would be premature to assume that bacterial vaccines served as adjuvants only because of their endotoxin content. The action of endotoxins seems to be to accelerate and to enhance the early primary immune response (24), and it appears that this mechanism may be closely related to that described earlier by Jaroslow (18), in which the injection of breakdown products of nucleic acids enhanced or preserved immune responses.

Recent studies by Merritt and Johnson (25) and Braun and Nakano (3) suggest that oligonucleotides have a marked adjuvant effect, that endotoxins may exert their action through the release of such substances, and that the effect of these substances may be either to enlist the commitment of more antibody-forming cells at the onset of the response or to increase permeability so that the antigen introduced is more widely utilized. However, there is much work still to be done before the actual mechanism can be defined. In any case, the effect of this group of adjuvants appears to be almost entirely on the primary immune response, acting on the secondary immune response only to the extent that it elevates the number of committed cells on which the secondary response is based.

The role of endotoxic adjuvants in human immunization therefore may well be of relatively limited significance, apart from the fact that the use of such preparations is generally associated with undesirable reactions. The nonreactive so-called "endotoxoid" derivatives have not yet been shown to be significantly effective as adjuvants except in the mouse.

Time does not permit discussion of the great variety of other agents that have been considered as possible adjuvants, nor does it allow detailed consideration of many of the questions that have been raised concerning our knowledge of this subject. An example of the incompleteness of our knowledge deserves mention here, however, since it has recently been raised with new emphasis—namely, the lack of certainty as to whether or not aluminum salts have any significant adjuvant effect on the immunogenicity of bacterial vaccines (1). Indeed, our knowledge in this whole field is generally inadequate. Most of the work on adjuvants has been essentially empirical. In looking for something that might succeed, investigators have tried one adjuvant after another. Yet the fundamental immunological mechanisms that underlie the actions of adjuvants have not yet been elucidated. The matter will need far more attention than it has received so far.

Thus it appears that the major problems confronting long-term progress in the use of adjuvants for immunization can be solved only when sustained, first-rate, broadly diversified studies on the underlying mechanisms of the adjuvant phenomenon have been carried to the point where we can clearly describe these mechanisms, define the requirements for the ideal adjuvant mechanism, and then tailor our procedures so that these requirements are met. Indeed, it is reasonable to hope that with sufficient basic research it might prove possible to obviate the use of adjuvant substances as such by modifying the character of the essential antigen instead. Such a development might in turn reduce or eliminate the undesirable side effects that lurk in the background when more potent adjuvant substances are used. Certainly, it is only through basic research in this field that we will solve the problems which still beset immunization.

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SECTION B. BEDSONIAE AND SECTION C. ADJUVANTS

DISCUSSION

CHAIRMAN VAN ROOYEN: Our first discussant is Dr. Roderick Murray, National Institutes of Health, Bethesda, Maryland.

DR. MURRAY: Unquestionably, the use of adjuvant preparations of inactivated vaccines, toxoids, and other prophylactic agents results in considerable enhancement of the immunologic effect produced. As other speakers have mentioned, the result is manifested in two ways: in the level of antibody attained and in the persistence of desirable antibody levels. These results would be of great importance in immunization against diseases in which the protective antibody levels obtained with conventional preparations are not of a desirable height and tend to drop off rapidly with the passage of time.

Oil adjuvants have been used under experimental conditions for many years. Despite the apparent satisfactory experience with an oil-adjuvant influenza virus vaccine in military personnel, oil-adjuvant preparations are not in general use as yet in the United States, and their safety has been a subject of controversy.

A number of different adjuvants designed to be used with a variety of antigens are currently under study both in the laboratory and in the field. The antigens involved include vaccines and toxoids and also a variety of allergenic preparations, such as pollen extracts either pre-emulsified or emulsified in a "do-it-yourself" fashion immediately before administration. The problems of oil adjuvants are not confined to vaccines.

The most frequently used oil adjuvant preparation has been the mineral oil (Drakeol)-Arlacel preparation.

Except for the data obtained from military personnel, there is little information available on long-term follow-up and reporting. However, it is clear that local reactions, cysts, and draining sinuses do occur. Many of these have required surgical treatment. Although it would appear that these reactions may occur as an event of low frequency with many of the preparations studied, the fact that they have been observed with increased frequency in the case of certain preparations—an emulsified tetanus toxoid preparation, an oil-adjuvant cholera vaccine, and a typhoid oil-adjuvant preparation, to mention three—suggests that each emulsion should be studied separately. Until a given preparation has been subjected to clinical testing, it is not possible to generalize as to its safety. Nevertheless, a committee convened under the auspices of the Division of Biologics Standards and of the Food and Drug Administration, chaired by Dr. John Fox, did agree although not unanimously that oil-adjuvant influenza vaccine preparations could be used under certain circumstances, provided that long-term safety data in animals becomes available.

It is unfortunate that the collection of long-term data on the safety of such preparations and their components in animals was not undertaken earlier. This is being worked out now, however, and hopefully the information currently being derived from long-term animal tests will enable workers to define the conditions that reflect on safety not only with respect to local reactions but also with regard to the more elusive matter of potential oncogenicity. The rigid circumstances that apply to the clinical investigation of new drugs and biological products prior to licensing makes the acquisition of such information a matter of urgency.

CHAIRMAN VAN ROOYEN: Our next discussant is Dr. Frank T. Perkins, Division of Immunological Products Control, National Institute for Medical Research, London, England.

DR. PERKINS: In the first Medical Research Council trial the influenza vaccine incorporated in an oil emulsion gave a reaction rate of 17.5 per 10,000 inoculations. In the second trial, in which a more purified oil was used, the incidence of reactions dropped to 3.3 per 10,000 injections. The superior antigenicity of the emulsion vaccine was such that a decision was made to allow this product to be used especially for subjects at special risk of bronchial complications in the face of an influenza epidemic. During the winters of 1963 and 1964 about 1 million doses of mineral-oil emulsified vaccine were released. In all, some 40 reactions requiring surgical intervention were reported. A full investigation of these reactions was undertaken by the manufacturers and it was found that the incidence varied from as high as 1 in 21 to as low as 1 in several thousand. In many areas where the vaccine was used no reactions were observed. The reactions were not batch- or manufacturer-associated. Neither the individual idiosyncrasy of the reacting patients nor the local conditions under which the vaccine was given can be eliminated from our considerations. All vaccines passed the Berlin test before they were released for use. We have not withdrawn the mineral-oil emulsion influenza vaccine from the market, but understandably its use has decreased markedly.

Although we have done much work in the laboratory to determine what may have caused these reactions, we have been unable to simulate field conditions. I should be glad to hear of any similar experiences from others so that we may obtain a lead into what might have caused these reactions.

There is some indication that the purity of the antigen incorporated in the emulsion is as important as the quality of the oil and the emulsifying agent. We recall the adverse experiences in New Guinea using a particular batch of tetanus toxoid emulsified in mineral oil, and I now understand that there is evidence that the crude nature of the tetanus toxoid incorporated in the reactive vaccine was entirely responsible for these reactions.

There are data showing that the viscosity of the emulsion plays a part, since vaccines made with identical components increase in toxicity as they increase in viscosity. Indeed, it can be shown that a viscous and highly toxic vaccine, as measured by the Berlin test, when made into a fluid double emulsion (water in oil/oil in water), is nontoxic.

I hope that we shall continue to investigate the mineral oil emulsion vaccines because of the superior antibody response given.

CHAIRMAN VAN ROOYEN: The next discussant is Dr. A. F. Woodhour, Merck Institute for Therapeutic Research, West Point, Pennsylvania.

DR. WOODHOUR: I should like to report on "Response and Retention of Antibody Following Influenza Virus Vaccine in Adjuvant 65," co-authored with Drs. M. R. Hilleman, J. Stokes, Jr., and R. E. Weibel.*

A prime objective in the application of killed virus vaccines is to induce as high a level immunity of as long a duration as can be achieved using the least amount of antigen. This objective is especially applicable to influenza virus vaccine, in which the immunity induced by aqueous preparations does not last for more than one season.

As was mentioned earlier during this Conference (3), our clinical-laboratory team from the University of Pennsylvania and the Merck Institute for Therapeutic Research has been carrying out extensive studies (1–3, 5–7) to evaluate an emulsified peanut oil adjuvant that was developed by our laboratory at the Merck Institute (4, 8). The adjuvant vaccine consists of the aqueous antigen emulsified in peanut oil with Arlacel A as emulsifier and aluminum monostearate as stabilizer. All the components of the adjuvant have been used in man for many years, and all are readily metabolized in the body.

To date, Adjuvant 65 has been applied principally to influenza vaccine, and we have been especially interested in comparing the duration of the antibody response to the adjuvant vaccine with that of aqueous vaccine.

Figure 1 presents the initial and long-term hemagglutination-inhibiting antibody responses to the A2/Japan/305/57 and the B/Great Lakes/1739/54 components of a polyvalent vaccine, which contained the A1/AA/1/57 and A/PR8/34

* From the Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania, and Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.

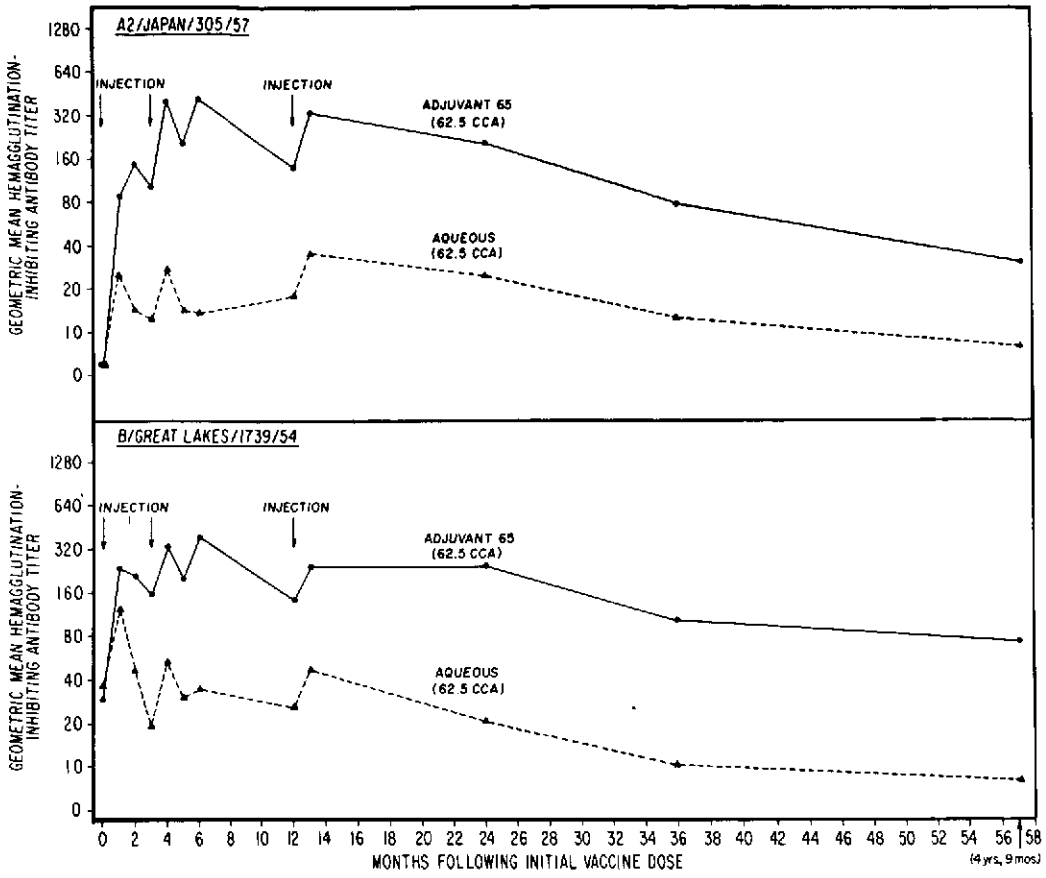

Fig. 1. Long-term antibody response in adults who received polyvalent influenza virus vaccine in Adjuvant 65, compared with controls who were given aqueous vaccine (Study 1B).

strains in addition to those shown. In both the aqueous and the adjuvant vaccines the content of Jap 305 antigen was 125 CCA units and the content of B/Great Lakes was 62.5 CCA units. The subjects were vaccinated initially on 15 January 1962, and were given a second dose of vaccine three months and again a year later. In each group there were 20 or 21 adults averaging 44 years of age. The initial antibody response to both vaccines was prompt. The level of responses to the adjuvant vaccine, however, was six to eight times higher. Further, whereas the antibody induced by the aqueous vaccine was rapidly lost, that induced by adjuvant vaccine was extremely durable with only minor decline by the end of four years and nine months.

A similar pattern of response was shown in a test in which the two vaccines contained the same viral antigens but the content of antigen in adjuvant was only one fourth that in the aqueous vaccine (Fig. 2). The subjects—17 to 20 per group—were children averaging 11 years of age. The first vaccine dose was given on 8 March 1963. The remarkable degree of immunologic enhancement and the durability of the high-level antibody are clearly shown. It is of special interest that between months 24 and 43 a significant increase in antibody against influenza B occurred among the recipients of aqueous vaccine and among the unvaccinated controls, but not in the adjuvant vaccine group. This coincided with the proved occurrence of influenza B in the institution during month 35 (February 1966). The natural infection appeared to cause antibody increases in the aqueous and nonvaccinated groups but no rises and hence protection in the adjuvant vaccine group.

Influenza vaccine in Adjuvant 65 has been

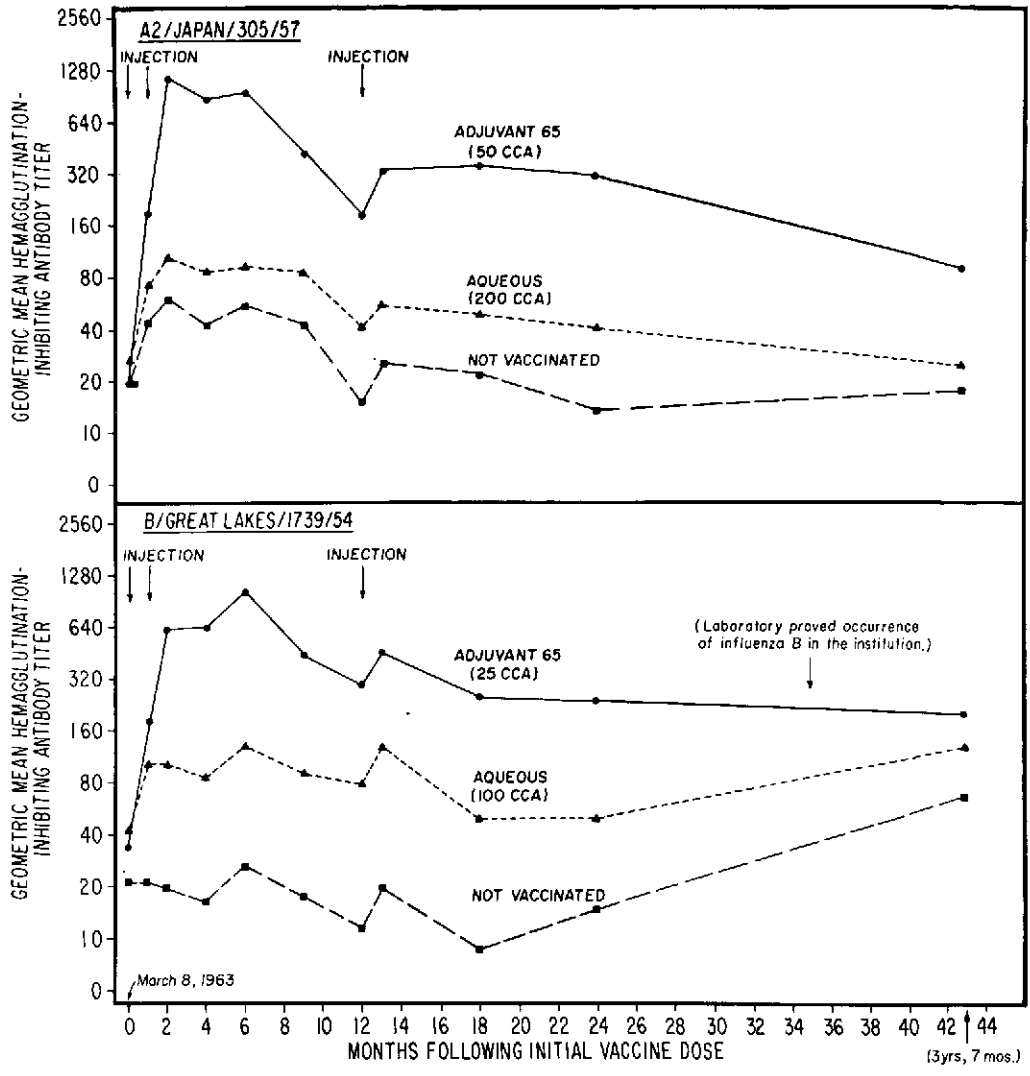

Fig. 2. Long-term retention of antibody against influenza virus following administration in immunologic Adjuvant 65 in children, compared with aqueous vaccine and unvaccinated controls (Study 2C).

given to roughly 15,000 persons to date without significant immediate or long-term clinical reaction. Short-term and long-term tests in animals have amply confirmed the safety of the product. Vaccine stored at 4°C for 9 to 41 months has been shown to have retained its full antigenic potency and to have remained nontoxic as measured in intradermal tests in guinea pigs and in intraperitoneal tests in mice.

In summary, the studies to date have shown that high-level long-term antibody levels can be achieved with complete safety through the use of Adjuvant 65. In tests to date it has been shown to work equally well with other viral antigens. The adjuvant holds promise for the development of long-term immunity in respiratory disease and in other illnesses of man in which killed virus vaccines are applied.

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(6) Weibel, R. E., Woodhour, A. F., Stokes, J., Jr., Metzgar, D. P., and Hilleman, M. R. "New Metabolizable Immunologic Adjuvant for Human Use. 5. Evaluation in Man of Highly Purified Influenza Virus Vaccine in Adjuvant 65." *New Eng J Med* **276**:78–84, 1967.

(7) Woodhour, A. F., Metzgar, D. P., Lampson, G. P., Machlowitz, R. A., Tytell, A. A., and Hilleman, M. R. "New Metabolizable Immunologic Adjuvant for Human Use. 4. Development of Highly Purified Influenza Virus Vaccine in Adjuvant 65." *Proc Soc Exp Biol Med* **123**:778–782, 1966.

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CHAIRMAN VAN ROOYEN: Our last formal discussant is Dr. Richard Haas, Institute for Hygiene and Medical Microbiology, University of Freiburg, Freiburg, Germany.

DR. HAAS: I should like to confine my discussion to one point: the quantitative aspect of the adjuvant effect, and this only with regard to aluminum hydroxide. This aspect seems to be of some interest in several respects, especially in connection with combined virus vaccines. Some time ago we started a study on the correlation between immunizing effect and adjuvant concentration with measles and poliovirus antigens. These experiments are still in the initial stages, so that only a few preliminary results can be mentioned here.

Figure 1 shows the distribution of mean neutralizing-antibody titers against poliovirus Type 2 in guinea pigs after one subcutaneous injection of inactivated polioviruses 16 days previously. The adjuvant effect was evident in three

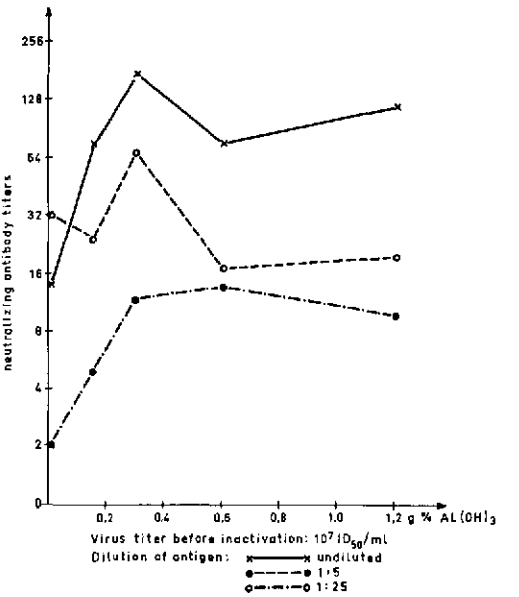

Fig. 1. Distribution of neutralizing antibody titers in sera of guinea pigs 16 days after a single dose of formaldehyde inactivated poliovirus Type 2.

different concentrations of antigen. The highest titers appear to have been reached with an aluminum hydroxide concentration of 0.3 g per cent. The few experiments carried out so far have not provided evidence of any substantial shift in the maximal concentration when the antigen concentration is altered. This point deserves further examination.

Figure 2 shows the adjuvant effect of aluminum hydroxide on measles antigen in guinea pigs given one intramuscular injection four weeks earlier. The antigen was prepared by ether-Tween-treatment according to Waterson and others. The increase in the mean titers was very significant and seemed in general to be higher than with poliovirus.

Figure 3 shows the distribution of mean hemagglutination-inhibition antibody titers in sera of rabbits roughly four and twelve weeks after one intramuscular injection of 20 MCA of the same antigen together with varying quantities of aluminum hydroxide. Maximal titers were found with an aluminum hydroxide concentration of 0.7 g per cent. This value may certainly not be regarded as entirely conclusive.

In Figure 4 I have tried in a very preliminary way to provide a scale for the concentration of

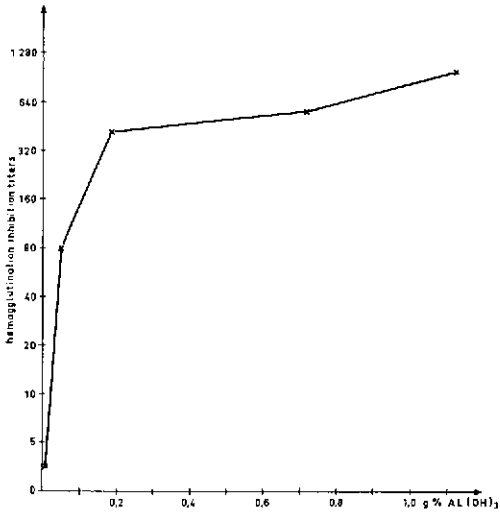

Fig. 2. Hemagglutination-inhibition antibody titers in sera of guinea pigs after a single dose of ether-Tween-treated measles virus (32 MCA/injection).

the maximal adjuvant effect of aluminum hydroxide for different antigens, with reference to our own work and that of others. This scale has many imperfections, because different species of animals, different parameters for the assessment of immunity, and different aluminum hydroxide preparations were employed in the experiments. A cautious estimate seems to reveal that the results so far would hardly justify the assumption that the adjuvant concentrations of maximal efficacy are identical for different antigens. This

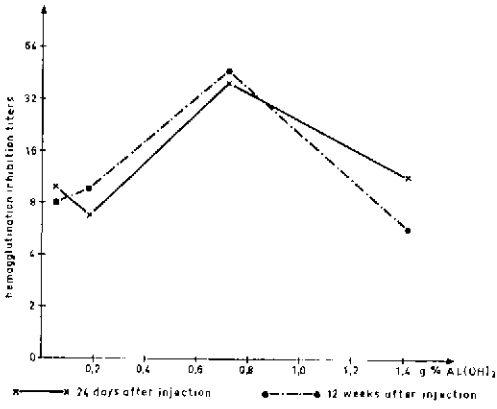

Fig. 3. Distribution of hemagglutination-inhibition antibody titers in sera of rabbits after a single dose of Tween-ether-treated measles virus (20 MCA/injection).

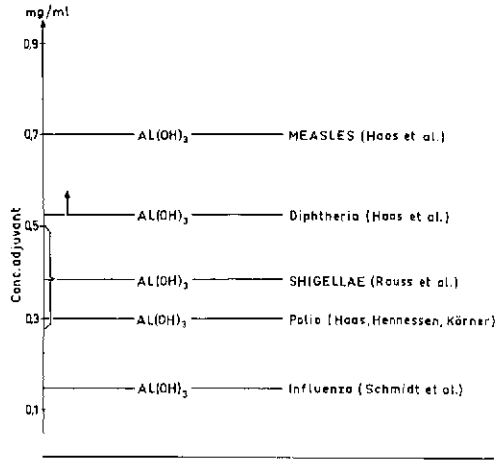

Fig. 4. Maximal activation of different antigens by $Al(OH)_3$.

does not necessarily mean that it would not be possible to find adjuvant concentrations that would have a nearly optimal effect on all the different antigens in a combined vaccine. The findings show that the adjuvant effect of $Al(OH)_3$ can be demonstrated with all virus antigens tested to date, at least so far as can be judged from relatively short observation periods and mostly from antibody titer. What will be observed in the long run remains to be determined.

Furthermore, some of our results seem to indicate that in some cases a certain increase in antibody titer may be achieved more easily by a relatively small increase in adjuvant concentration than by a comparable increase in the dose of antigen.

CHAIRMAN VAN ROOYEN: We will now have a short general discussion.

DR. LARIN: Dr. Grayston clearly indicated that a great deal of further study is needed before results will be achieved that are likely to produce a worthwhile vaccine against trachoma. I would like to mention the recent work that has been done in this field in England by a research group consisting of the Medical Research Council Trachoma Unit and the research departments of the Pfizer Group and Evans Medical.

This work has provided evidence that the TRIC agent can multiply in the skin and internal organs of several animal species, such as

guinea pigs, rabbits, and monkeys. Intracutaneous injection of TRIC agent in these animals induces an erythematous papule that disappears within approximately six days, depending on the agent dose. It is possible that several agent replication cycles occur in the skin, since the agent can be isolated from the skin of some animals up to three weeks after injection. The organism was isolated from regional lymph nodes, spleen, and liver of the animals that were inoculated in the skin.

Titrations of the tissues yielding the agent suggest that the agent has multiplied in these tissues. It is felt that these findings are of importance and that further studies of animal infection with TRIC agents may yield some significant information on the mechanism of immunity to infection with these agents.

DR. COX: I would like to mention that we have carried out studies to find out if trachoma strains would produce encephalitis in monkeys. We used two strains of trachoma—the Saudi Arabian 2 and the Egyptian 2—and we inoculated at least 10^8 elementary bodies, bilaterally, intracerebrally into monkeys, much as one would do with live poliovirus vaccine. We used cynomolgus, cercopithecus, and rhesus monkeys, five animals of each species against each strain of trachoma. We observed these animals for at least three months and then sacrificed them and did pathology studies comparable to those that would be done for poliovirus strains.

None of these animals showed any signs of illness during the observation period, and Dr. Jungherr, who was our pathologist at that time, could find no evidence of traumatic lesions in the brains of these monkeys.

DR. KLEIN: I would like to ask Dr. Murray whether there has been an increase in the resistance of trachoma agents.

DR. E. MURRAY: I have no evidence on that, but Dr. Grayston has. I wonder if he would be willing to comment on his trials of isolations performed in 1959 and again in 1963 after a four-year treatment period.

DR. GRAYSTON: We studied in the egg the antibiotic sensitivity of strains isolated in 1958 and 1959 in Taiwan compared to strains isolated in 1963 and 1964, and could find no evidence of change.*

DR. MURRAY: I might add that a number of the strains that have caused laboratory infections have come from areas where resistance to natural infection could be suspected. However, these strains in accidentally infected persons have all responded rapidly and satisfactorily to treatment.

DR. PHILIP: This is just to call attention to the term Bedsonia, which is a convenient group name. However, there is a group of workers—Dr. Page and his colleagues, who have come to the conclusion that climedia will be a good generic name to use.

CHAIRMAN VAN ROOYEN: We have further questions from three speakers, Drs. Davenport, Ungar, and Stuart-Harris.

DR. DAVENPORT: I should like to set the record straight on three items.

First, it should be mentioned that the local reactions observed after use of cholera vaccine in mineral oil were associated with the deposition of the vaccine subcutaneously, a route not recommended by us for influenza vaccine because of our concept that subcutaneous deposition of vaccine is associated with cyst formation; some of the experience obtained in England, I believe, confirms that this is a factor.

Second, Dr. R. Murray pointed out with regret that long-term safety studies were not initiated earlier in animals. I would agree with him. One of the impeding factors was that the rules keep changing, and what would have been acceptable in 1955 I am sure would not be acceptable today.

Lastly, with respect to influenza vaccine, I should like to emphasize that the highest rate of local reactions observed occurred in one year, 1952. The rate was 0.5 per cent. Studies by Salk and Bell showed that two factors were associated. One was age of the subject, the rate being higher in children; the other was color of skin—the rate of cyst formation being higher

* Shiao, L. C., Wang, S. P., and Grayston, J. T. "Sensitivity and Resistance of TRIC Agents to Penicillin, Tetracycline, and Sulfa Drugs." *Amer J Ophthal* (April 1967, in press).

in Negro recruits. Since the cleaning up of the Arlacel A, we have had no further trouble with subcutaneous nodules in the military. I know of only three instances since 1953, and there are mechanisms for permitting the Commission on Influenza to be acutely aware of their appearance.

At this session we have heard about good vaccines that we do not need. At this moment we are talking about a good vaccine that is not being used.

CHAIRMAN VAN ROOYEN: It is most unfortunate that we lack the time to amplify what you have had to say on adjuvants.

DR. UNGAR: I should like to make only a few comments on this complex problem.

As is shown in Table 1, in this investigation I used guinea pigs to demonstrate the importance of the correct choice of an adjuvanted antigen for optimal immunization effect.

Four groups of guinea pigs were immunized with five Lf of diphtheria toxoid, two doses at three-week intervals. The first group received two doses of the plain toxoid; the second group received two doses of aluminum hydroxide adsorbed toxoid; the third group received the plain toxoid as the first dose and the adsorbed toxoid as the second; and the fourth group received the adsorbed toxoid as the first dose and the plain toxoid as the second.

Marked diversity can be seen in the final response. The highest antitoxin titer was obtained in the group of guinea pigs injected with two doses, the first of which was adsorbed and the second plain. The second group that received the two adsorbed doses of the toxoid did not show any better effect as can be seen by the antibody titration.

Table 2 gives the results in a similar group of guinea pigs challenged with Schick toxin. The toxoids and the dosage scheme were exactly the same as in guinea pigs in Table 1. When these groups were challenged with 100 Schick toxin units, in the first group seven out of ten died; in the second group, five survived; and eight died in the third group that received the plain toxoid as a first dose. But in the fourth group, injected with the first adsorbed dose, two out of ten died. In the last column of the table you can see that when guinea pigs were challenged with 200 Schick toxin doses, only 2 out of 10 died, where in group 3 all 10 died when challenged with 200 Schick units.

The second comment I should like to make (Table 3) is on the type of antibodies we have observed in some of our guinea pigs. The experimental system involves antitoxin titration in groups of guinea pigs inoculated with three doses, and I emphasize three doses of diptheria toxoid. The first had toxoid diluted 1:4, the second received the plain undiluted toxoid, the third group received toxoid adsorbed with 2.5 mg/ml of aluminum hydroxide, the fourth group toxoid adsorbed with 5 mg/ml of aluminum hydroxide, and the last group received the toxoid in an oil emulsion we have used in the influenza vaccine. I should like to draw your attention to the difference in the titers and the type of antibodies in the blood of the animals 22 days after the first injection of the toxoid.

Only in the group of guinea pigs injected with the toxoid incorporated in the aluminum hy-

TABLE 1. RESULTS OF THE TITRATION OF SERUM ANTIBODY IN GUINEA PIGS

Group	Vaccine injection	Serum antibody level	
		Antitoxin*	Hemagglutinating antibody†
1	Plain-plain	Not done	776
2	Adsorbed-adsorbed	Not done	11,583
3	Plain-adsorbed	0.9899	1,351
4	Adsorbed-plain	2.7633	18,820

*Geometric mean, expressed as number of unit/ml serum.
†Geometric mean, expressed as the reciprocal of the final titer.

TABLE 2. RESULTS OF THE SCHICK TEST IN GUINEA PIGS EXPRESSED AS NO. POSITIVE/NO. TESTED

Group	10	20	50	100	200
1 (Plain/plain)	1/10	2/10	3/10	7/10	Not done
2 (Ads./ads.)	0/10	0/10	0/10	5/10	Not done
3 (Plain/ads.)	2/10	3/10	5/10	8/10	10/10
4 (Ads./plain)	0/10	0/10	0/10	2/10	2/10

TABLE 3. DIPHTHERIA ANTIBODIES

Preparation	Titers after days				
	12	22	40	56	77
Plain toxoid, diluted 1:4	<0.03	0.3	11.25	5.4	
Plain toxoid, undiluted	<0.03	0.52	15.2	8.6	
Toxoid with 2.5 mg Aluminum hydroxide/ml	<0.03	1.9 IgM	6.0	11	
Toxoid with 5.0 mg Aluminum hydroxide/ml	<0.03	2.3 IgM	12.4	11	
Toxoid Invirinal type	0.1	3.8 IgM	16.0	30	

Note: Immunization was carried out with three doses: first injection on day 0; second injection on day 22; and third injection on day 40. Bleeding and titers after 12, 22, 40, 56, and 77 days (I.E./ml in serum-pools).

droxide and in the oil emulsion were IgM antibodies observed, which I think is an indication of a delayed type of hypersensitivity. We did not see these antibodies in the group of guinea pigs inoculated with the plain toxoid.

CHAIRMAN VAN ROOYEN: Thank you. Dr. Stuart-Harris?

DR. STUART-HARRIS: If I could set the record straight, the first MRC trial with influenza vaccine containing adjuvants was made with Arlacel and Bayol F supplied by Dr. Salk. The second set of trials was done with Drakeol and Arlacel; so we did not use complete Freund's adjuvant in any trials. We have recently completed some trials of influenza vaccine using the peanut-oil adjuvant, both with material given to us by Dr. Hilleman and with some prepared in Britain. A definite adjuvant effect was seen. However, the antibody titers observed at one year were disappointing. They were slightly lower than those present one month after the peanut-oil vaccine and much lower than those found 12 months after the administration of a mineral-oil vaccine containing the same quantity of virus antigen.

DR. HILLEMAN: In reply to Dr. Stuart-Harris' remarks, I should like to state that the particular Adjuvant-65 lot of vaccine we furnished him had an emulsion stability problem relating to the presence of a chelating agent and an oil-in-water emulsifier that altered the quality of the adjuvant emulsion. It took time for this adverse effect to develop. The vaccine was used by Dr. Stuart-Harris about 10 months after preparation. Hence the findings obtained do not reflect the true performance potential of the peanut-oil adjuvant.

In all the studies with Adjuvant-65 influenza vaccine we have carried out to date, the antibody enhancement has been very great and this high antibody level has persisted for years.

I might add that we now have about a five-year follow-up in man after administration of multiple doses of Adjuvant-65 influenza virus vaccine. There have been no significant local or systemic clinical reactions either on a short- or long-term basis, which attests to the safety of the material. Similarly, all long-term animal data, which are by now quite extensive, show no untoward effect such as autoimmune disease, amyloidosis, cancer, systemic pathology, and the like.

One consideration of prime importance in relation to immunologic adjuvants is that concerning the degree and length of retention of the adjuvant components in the body and the metabolic fate of these components, since long-term retention of irritants might lead to untoward effects.

Based on pathology studies in rabbits given Adjuvant-65 into the compact sacrospinalis muscle, it has been shown that the adjuvant is almost completely removed from the site within 60 days. These components are metabolized quite rapidly and nearly all of the fatty acid portions of the components are eliminated as CO_2. As might be expected, there is minor utilization or recycling of the fatty acids, since these are normal body constituents. The mannide of the mannide monooleate is excreted as mannitol and the aluminum of the aluminum monostearate is excreted as aluminum. Thus, we

have an adjuvant whose components are readily metabolized and there is no long-term retention such as is obtained with mineral oil.

The antibody responses to the antigens in the adjuvants are rapid, just as rapid as with aqueous vaccine, and hence immunization is just as quickly achieved with adjuvant as with ordinary aqueous preparations.

I should like to say also that there are a number of proper conditions that must be met in order to obtain optimal adjuvant action. Most important, the emulsion must be proper and stable. Beyond this, there must be an adequate amount of antigen to provide the necessary loading dose. An amount of antigen adequate to give a low antibody response when given in aqueous form may not induce antibody at all in adjuvant. Given sufficient antigen, however, the adjuvant provides for very great response of antibody, both in amount and duration.

Finally, one of the principal added advantages of adjuvant use with influenza is the elimination of toxicity resulting from injection of the influenza virus antigen.

SESSION IX

VACCINATION PROBLEMS AND OTHER APPROACHES TO CONTROL OF VIRAL DISEASES

Friday, 11 November 1966, at 8:30 a.m.

CHAIRMAN
DR. K. RASKA

RAPPORTEUR
DR. J. H. DINGLE

Section A.
Problems of Vaccine Development

Presentation of Papers by:
Dr. Roderick Murray
Dr. Leonard Hayflick

Section B.
Chemotherapy

Presentation of Papers by:
Dr. D. J. Bauer
Dr. George G. Jackson
Dr. Herbert E. Kaufman

Discussants:
Dr. Frank T. Perkins
Dr. Frederick P. Nagler
Dr. C. Henry Kempe
Dr. Kenneth W. Cochran

Section C.
Interferon

Presentation of Papers by:
Dr. Robert R. Wagner
Dr. E. Frederick Wheelock
Dr. Monto Ho

Discussants:
Dr. P. De Somer
Dr. J. S. Youngner
Dr. John H. Dingle
Dr. D. Blaškovič
Dr. Samuel Baron
Dr. V. D. Soloviev

SECTION A. PROBLEMS OF VACCINE DEVELOPMENT

CONTEMPORARY PROBLEMS IN REGULATING THE POTENCY AND SAFETY OF VIRAL VACCINES

RODERICK MURRAY

Division of Biologics Standards, National Institutes of Health, U.S. Public Health Service, Bethesda, Maryland, U.S.A.

DR. MURRAY (*presenting the paper*): The title assigned to this presentation is a broad one that cannot be covered adequately in a short discussion. Hence, a number of brief topics have been selected to highlight some of the contemporary problems as seen from the point of view of those concerned with the national control of virus vaccines.

STANDARDS OF POTENCY

Once a vaccine becomes available, its potency is measured against that of some officially adopted standard or reference preparation. In the case of inactivated vaccines in particular, the potency of the standard or reference preparation may diminish with the passage of time. If this is not recognized early enough, and if steps are not taken to replace it, the vaccine "standardized" against such a preparation will not have adequate protective capacity.

These considerations are of great concern to national and international groups engaged in the standardization of biological products. To replace one standard with another by comparing the two in conventional laboratory tests is not sufficient. Given the built-in errors inherent in any biological comparison, a series of such replacements might end up with a "standard" that is no standard at all.

What is needed is some means of reproducing the conditions under which the original standard was adopted—usually field trials in which the vaccine's efficacy against disease in man is demonstrated and correlated with laboratory observations.

Thus, when new vaccines are being subjected to clinical evaluation it is important that the studies include sound information on measurable responses in man expressed in such terms as percentage of antibody conversion or average level of antibody, and that the methods used in these studies are sufficiently standardized so that when standard or reference vaccine preparations are renewed or replaced years later the results obtained in contemporary studies in man can be compared with the data that were used for validation of the vaccine's effectiveness in the first place. *The reference point should be what takes place in man.*

The control laboratory should obtain and make available stable materials such as serums or antibody preparations for general use in all the laboratory tests being carried out for the determination of antibody levels.

POTENCY TESTING OF LIVE AND INACTIVATED VIRUS VACCINES

Testing for the potency of live vaccines is in most instances a relatively simple matter of determining the virus titer. Once standard methods have been developed and can be applied in a reproducible way in testing laboratories, potency can be readily determined. Thus, if the bulk vaccine can be stored in a stable condition, it can be maintained for prolonged periods as long as the potency is regularly updated, and the vaccine can be made available for issue on rela-

tively short notice. The testing of multiple live virus vaccines is somewhat more complicated because of the necessity of neutralizing each of the components. This may emerge as one of the problems of potency testing if multiple live virus vaccines come into widespread use.

Potency testing of inactivated vaccines, on the other hand, is usually time-consuming, laborious, and expensive. In general it presents more problems of variation than does the testing of live vaccines, and it is not undertaken as readily. Although not appreciated by the scientific community outside the pharmaceutical industry and those whose job it is to be involved with the control of biological products, the potency testing of inactivated vaccines is a very real problem, particularly in the case of those whose use has become normalized through the heavy demands of mass vaccination programs.

MULTIPLE ANTIGENS

The simultaneous administration of several vaccines or other immunizing agents—for example, the inclusion of inactivated poliomyelitis vaccine and measles vaccine with the routine diphtheria, tetanus, and pertussis preparations used for children—is attractive both to those responsible for immunization programs and to those participating. Some of the advantages have been mentioned by other speakers on this program: reduction of the number of injections, simplification of immunization schedules, and the possibility of reaching persons who might not otherwise present themselves for immunization.

Multiple antigens present complex problems in manufacture, testing, and control, since any difficulties relating to the safety or potency of one of the components will affect the entire preparation. Each of the components must be completely compatible with the others. The dating period will be governed by the most labile of the components, and, in the case of materials being held in reserve for future use, if one component loses its potency the entire lot or batch will probably have to be discarded because of the difficulty of readjusting and retesting such a mixture. An example of the complexity of this procedure was encountered not long ago when the pertussis component of a combined diphtheria, tetanus, pertussis, and poliomyelitis vaccine preparation demonstrated an unanticipated lack of stability.

Even when the components are compatible, the testing of a complex mixture is a formidable undertaking. Inactivated influenza vaccine could be cited as an example. With five or more component strains, we have what is virtually a series of separate potency tests. Difficulty with any one of these tests delays the availability of the vaccine. These difficulties may be due to actual deficiency of potency in the case of one or more of the components, or they may be due to test variation. The problems may arise in the hands of the manufacturer or in the hands of the National Control Laboratory. While in the case of a monovalent preparation readjustment of potency is a relatively simple matter, in the case of a multiple antigen it is a complex procedure that usually involves reassaying the potency of each of the components.

ADVENTITIOUS AGENTS

So many of the speakers at this Conference have alluded to the problems posed by adventitious agents in connection with the safety of virus vaccines that it hardly seems necessary to mention the subject again at this point in the program. Unquestionably, adventitious agents are a serious problem, particularly for live virus vaccines.

Adventitious viruses that are known and can be tested for can be eliminated from new vaccines if adequate controls are incorporated in the production and testing procedures.

However, the situation is more complex in the case of unknown agents whose presence in the production system and thus in the vaccine may be postulated but concerning which nothing else is known. This may appear to be a theoretical proposition, but it cannot be summarily dismissed, since we have the case of SV-40.

This simian virus, which was first reported in 1960, was present in a number of lots of inactivated poliomyelitis vaccine, but its presence was missed by the tests employed. Once the virus was recognized, however, it proved to be a relatively simple matter to free the seed viruses of the contaminant and to produce vaccine free of this adventitious agent. In the case of live

poliomyelitis vaccine, the problem was recognized in the United States in time to ensure that all lots of live poliomyelitis vaccine marketed were free of SV-40.

Inactivated adenovirus vaccine is another product involved with the SV-40 problem. Repeated attempts to free the seed viruses of SV-40 have failed, and attempts to produce adenovirus vaccine with other strains have been frustrated by low titers. The interaction of SV-40 and adenoviruses is now well known, and the problem of the safety of adenovirus vaccine is compounded still further by the inherent oncogenicity of Types 3 and 7, as demonstrated in baby hamsters. The release of adenovirus vaccine has been suspended until these problems can be resolved.

This continuing problem may eventually be solved by the use of substrates or cell growth techniques that give high assurance of freedom from adventitious agents. The validation of such methods will require substantial laboratory and clinical evaluation.

ONCOGENIC EFFECTS IN RELATION TO VACCINES

Demonstration of the oncogenic potential of such viruses as SV-40 and some of the adenoviruses in the baby hamster model, together with the discovery that the agent of fowl leukosis has been present in 17-D yellow fever vaccine presumably since it was originally produced a quarter of a century ago, has called attention to the need for a great deal of work on this aspect of virus vaccines. The problem is all the more complex because, while some of the adventitious agents encountered in the production and testing of virus vaccines may have oncogenic potential themselves, the same considerations apply to those agents that are only theoretically postulated. The resolution of these problems will call for a great deal of costly and time-consuming effort. The phenomena exhibited in connection with oncogenicity in baby hamsters may prove to have no connection whatsoever with the safety of virus vaccines for man, but in the absence of positive assurance each virus strain and production medium must be studied carefully.

The Division of Biologics Standards is already mounting programs designed to obtain information in these areas. In view of the problems, it is necessary to move carefully in the development of new virus vaccines and the use of new substrates. Until such time as information of the kind indicated is available, developers of new vaccines will have to make value judgments concerning the seriousness of the illness to be prevented in relation to the adverse properties—real or theoretical—of the vaccine. Under the circumstances, conservatism in the use of production systems seems to be the wisest course.

CLINICAL INVESTIGATIONS WITH VIRUS VACCINES

During recent years there has been a considerable tightening up in the regulation of clinical investigations in general, and investigations of new drugs in particular. In the United States the administration of this activity is the responsibility of the Food and Drug Administration; however, in the case of biological products, including virus vaccines, the task is delegated to the Division of Biologics Standards.

Basically, the system requires that each Investigative New Drug (IND) have a sponsor, who submits the following documentation: information concerning the manufacture and testing of the product, data from animal tests, the proposed plan of investigation, the names and qualifications of the clinical investigators involved, and complete references to the published literature on the subject. These submissions are studied by appropriate members of the staff of the Division of Biologics Standards.

Any conditions that reflect on the safety of the product or any proposal that obviously represents a poor experimental design or in any way would unduly involve the safety of the participants in the study is brought to the attention of the sponsor, who is thereupon forbidden to ship the material for clinical investigative purposes. The system might appear cumbersome and restrictive, but it works well. If the sponsor does not receive adverse comment, he may proceed with the study.

Obviously, it is impossible to invoke standards and requirements for a new product for which there is no prior experience. It is partly to obtain this very information that the clinical study is being conducted. Still, certain generalizations can be made. The safety testing

procedures should be analogous in nature and scope to studies conducted for similar products that have already been licensed. Administration to man should be made under carefully controlled conditions: the groups should be small at the start, with increments of about threefold or 0.5 $\log_{10}$ and adequate time between each study to complete the necessary observations. Periodic progress reports are required, and any adverse effects must be reported promptly.

The object of all such studies is, of course, to obtain sufficient information to permit ultimate licensing of the product so that it may be used freely. Before this can be accomplished, it is necessary to adopt regulations, standards, or requirements governing the safety and potency of the product. The information filed by sponsors in connection with the IND procedures greatly facilitates the development of realistic regulations and standards for new products.

PROBLEMS WITH PRODUCTS ONCE THEY HAVE BEEN LICENSED

Once a vaccine has been licensed and has come into general use, it is extremely difficult to obtain further data concerning its clinical safety and efficacy. For example, it would be almost impossible to carry out extensive clinical studies on polio vaccines in the United States today. First, it would be very difficult to locate groups of individuals to serve as suitable subjects, since so many people have been vaccinated, and second, any study involving controls who would remain unvaccinated for a period of time would be very difficult to justify in the face of availability of acceptable vaccines and a general endorsement of the principle of maximum coverage of the population.

The same principles can be applied to the concept of clinical safety. The problems encountered with inactivated poliomyelitis vaccine in the United States are still fresh in the minds of those who went through that episode. If nothing else, the experience produced a hardening of attitudes toward the safety of virus vaccines. In the case of live poliomyelitis vaccine, a number of so-called "associated cases" were uncovered under the system of poliomyelitis surveillance instituted for monitoring the use of these vaccines in the United States. It would have been comfortable to shrug these off as coincidental, but the fact was that, although it would be difficult if not impossible to attribute a causal relationship to the vaccine in any particular case, the vaccine was administered as separate monovalent preparations with association rates quite different for each of the types, as was emphasized by the report of the Advisory Committee to the Surgeon General of the U.S. Public Health Service in 1964.

Conclusions

Two main thoughts emerge from this discussion. First, many of the contemporary problems connected with virus vaccines have arisen because of the rapid acquisition of new knowledge in microbiology and its related fields, which foreshortens the time between discovery and practical application. Second, the time to obtain the information essential for validating the safety and potency of viral vaccines is prior to licensing.

There are some additional thoughts I would like to leave with the members of this Conference. In contemplating questions concerning the safety and potency of virus vaccines, we are confronted with a simple matter of definition. What is a vaccine? When does something become a vaccine? Etymologically, only smallpox vaccine could qualify for this name. It is my firm personal belief that the term "vaccine" should be applied only to products that have reached the stage of development at which they can be considered for licensing or its equivalent in the country of origin. If this usage were accepted, we would not be faced with the misunderstandings that arise when something that has been injected experimentally into a few mice is called a vaccine, or when results observed under strictly experimental conditions are interpreted as having direct bearing on the safety of virus vaccines already in general use. Perhaps a special name could be given to the preparation prior to licensing: "Potential Vaccine," "Experimental Vaccine," or even "Experimental Immunizing Agent."

Nothing has been said here about rickettsial vaccines, so perhaps it would be appropriate to note that a contemporary problem in this area is the scarcity of people who are actively working in the field of rickettsiology.

SECTION A. PROBLEMS OF VACCINE DEVELOPMENT

THE PRIMARY PROBLEM WITH VIRUS VACCINES *

LEONARD HAYFLICK

The Wistar Institute, Philadelphia, Pennsylvania, U.S.A.

DR. HAYFLICK (*presenting the paper*): If the past decade of research accomplishments in virology were to be put into its proper historical perspective, it could probably be described as the Golden Age of Virology. Indeed, the reports presented here at the Conference offer ample testimony to this observation. Surely the Golden Age of Virology is the product of the union of virus research methods and cell culture techniques. That this marriage was doubly blessed is evident in that it occurred simultaneously with the triumphs of antibiotic therapy. As a result, cell culture, which once bordered on being a "black art" because of its adherents' preoccupation with aseptic technique, has been brought within reach of even the most primitively equipped biology laboratory.

To carry the analogy one step further, it would be fair to observe that since the honeymoon one of the marriage partners has been neglected almost to a fault. I cannot help being impressed by the realization that there is such a large body of sophisticated research results on the viruses that compose modern vaccines and at the same time such an utter paucity of information about the cell substrates on which they are produced. The disproportion is perhaps even evidenced here at this Conference, where the subject of the host cells used in vaccine preparation has had a very subordinate role. Indeed, the proposed title of my paper, "The Diploid Cell and its Potential for Vaccine Production," is, I feel, symptomatic of the degree to which the biology of cell culture systems has been neglected by virologists concerned with the development of human virus vaccines.

One of the major principles of modern cytogenetics is that the cells composing a primary tissue culture precisely reflect the chromosomal constitution of the cells that form the tissue of origin. This principle, upon which rests the many significant developments made in the past few years in mammalian cytogenetics, is as well established as is for example, our expectation that the results of tests made on blood samples or tissue biopsies is a faithful representation of the biology of the tissue sampled. Consequently, it should be readily apparent that primary cell cultures, if they are made from normal tissue, are composed of diploid cells. Since all cell populations currently licensed for use as substrates for vaccine preparation must be primary, they are all, concomitantly, diploid. Thus, diploid cells per se cannot have attributes that would preclude them from being used as a substrate for human virus vaccines. On the contrary, this is a desirable property of any cell system used for virus vaccine preparation.

Since the use of diploid cells for vaccine production has been an accomplished fact for at least 10 years, there is little need to speak of its "potential." Nevertheless, I believe I can offer some insight into what I surmise was really intended in my assignment. The point at issue is, of course, not the question of cell ploidy, but rather the effect of serial propagation from culture vessel to culture vessel on the biology of cells that divide *in vitro*.

Primary cultures were chosen as the only

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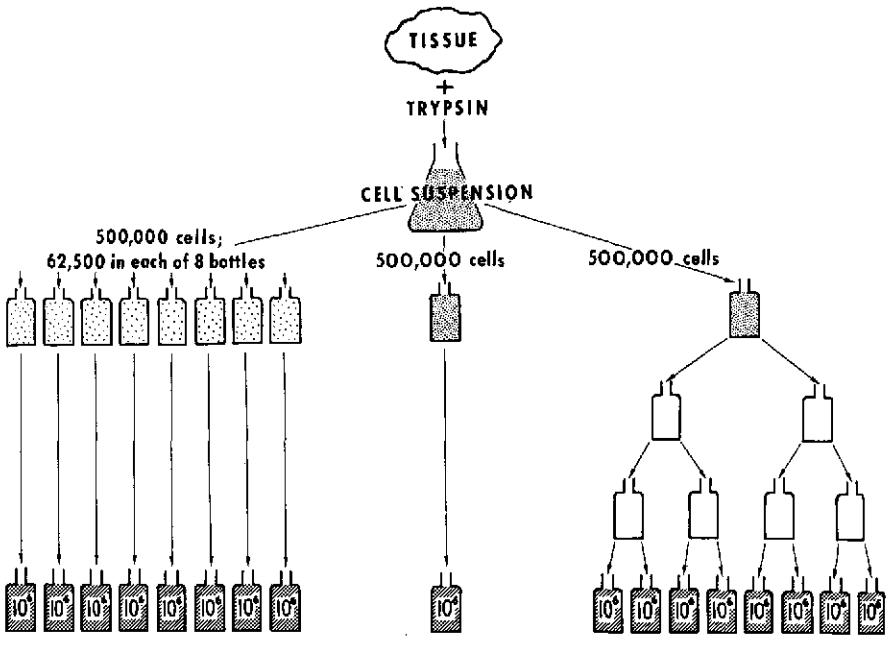
suitable substrate for vaccine preparation at a time when it was believed that the serial propagation of cell populations inevitably led to the acquisition of undesirable properties. In retrospect this decision was regrettable, for it was based on an erroneous extrapolation of experiences with mouse cells, which are uniquely unstable when cultivated *in vitro*. It is now well recognized that the cells of man and many other animal species can be serially propagated with full retention of the properties they had in the tissue of origin. Moreover, unlike mouse cells, the cells of man and of other animals do *not* inevitably acquire malignant or other abnormal properties. It is of the greatest importance, therefore, to appreciate that other than operationally, there are no important, measurable biological parameters that distinguish a serially passaged unaltered (untransformed) cell population from a corresponding primary cell culture. By generally accepted definition, a primary culture is a cell population derived from animal tissue that has been cultivated *in vitro* and has not undergone any subcultivations (27). Once subcultivated, unaltered cell populations derived from normal tissue can be referred to as cell strains and, like primary cultures they are diploid (11).

Also of immense importance is the concept that the cells in primary cultures may undergo many cell doublings prior to use as a vaccine substrate, depending on the density of the initial inoculum. A primary culture begun with a few cells will undergo many more cell doublings before becoming confluent than a similar culture initiated, for example, with one half of the total cell capacity of the culture vessel. Since primary cultures are acceptable as human virus vaccine substrates after many cell doublings, serially passaged cell populations should be equally acceptable. Trypsinization cannot be invoked as a procedure that would necessarily give rise to an unacceptable serially propagated cell population, since it has been universally employed in the production of the primary cultures now used for vaccine production. These concepts are illustrated diagrammatically in Figure 1.

In each of the three examples, 500,000 cells are used to initiate the cultures and the total capacity of any single culture vessel is assumed to be 1,000,000 cells. The example on the left supposes distribution of the 500,000 cells to each of eight vessels. The example in the center has the 500,000 cells introduced into a single vessel. Although both cultures are diploid and primary, 15 times more cells are produced in the multigeneration example on the left than in the central example. Even so, they both are primary cultures. In the example on the right the cell population has been subcultivated three times. Only in this operational aspect does it differ from the other two. In all three examples the cells are diploid and the populations have undergone at least one or more doubling. However, despite the arithmetical and biological identity of the three cell populations, only the left and central examples are the types currently acceptable as substrates for virus vaccine production.

If the most important single requirement for the acceptance of cells for human vaccine preparation is their identity with "normal" cells, then both primary and diploid cell strains satisfy this demand. Contrariwise, primary mixoploid cells or cells that have undergone a spontaneous or virus-induced alteration *in vitro* are unacceptable (7, 8, 12, 13). Thus, deviations from the normal karyotype should be sufficient cause to exclude any cell population from use in preparing human virus vaccines. If only for this reason, those cell populations with unlimited division potential defined by us previously as cell lines should be excluded from consideration (6, 11).

It should now be apparent that currently licensed primary cultures and cell strains are both diploid, and also that primary cell populations undergo numerous cell doublings prior to use just as do the multigeneration cell strains. What then are the differences between these two populations? Why is one currently used for vaccine production and the other not? The only difference between the two populations is that the cell strain, unlike the primary culture, has been subcultivated from one vessel to another one or more times. Yet, in spite of this, a cell strain may be found, with proper attention to arithmetic, to have undergone as many or even fewer *cell doublings* than a lightly seeded multigeneration primary culture. A multigeneration culture could be one that has undergone no subcultivations, one subcultivation, or many subcultivations. If it undergoes no subcultivations we call it primary. If it undergoes one or more



	PRIMARY	PRIMARY	CELL STRAIN
Ploidy	DIPLOID	DIPLOID	DIPLOID
Subcultivations	0	0	3
Cell doublings	4	1	4
Days to obtain net cell yield	7	3	9
Net cell yield	7,500,000	500,000	7,500,000

Fig. 1. Three possible ways in which cell cultures can be prepared as vaccine substrates.

subcultivations we call it a diploid cell strain. Yet both are multigeneration cultures. This, then, is the kernel of the problem. What significant biological event takes place when a primary culture is subcultivated to one or more new vessels? Certainly none that could distinguish a primary culture from a diploid cell strain. They simply cannot be distinguished.

Thus we find ourselves on the horns of a dilemma. We are incapable of distinguishing primary cultures from diploid cell strains, yet we find one to be acceptable as a vaccine substrate and the other not. I would challenge the inference that a distinction can be made between "continuous" and "primary" implied in the following passages from the United States Public Health Service Requirements: "Only primary cell tissue cultures may be used in the manufacture of poliovirus vaccine. Continuous line cells shall not be introduced or propagated in vaccine manufacturing areas," and "Measles Virus Vaccine, Inactivated, shall be produced only in primary cell tissue culture. Continuous line cells shall not be used and shall not be introduced into vaccine production areas" (29). Ironically, human virus vaccines are currently being licensed for distribution in this country after being produced in cells with the very properties that are thought to be expressly forbidden.

It would be reasonable to conclude, then, that human virus vaccines are currently being prepared on diploid primary cultures that have undergone as many total cell doublings as have some serially passaged diploid cell strains in which a number of trial vaccines have now been produced. The astute vaccine producer can double his yield of legal vaccine merely by halving his primary cell inoculation density.

If we are correct in our deduction that diploid cell strains are, like primary cultures, both diploid and multigenerational, then what advantages are to be gained if we employ cell strains in human virus vaccine production? In a word, the answer is "standardization." A tissue from a single donor has the potential of yielding great quantities of a diploid cell strain that can be preserved at low temperatures and then exhaustively examined for safety prior to use. This maneuver makes it possible to provide a uniform, fully characterized stock of seed cells, which are available for the production of a wide variety of viral vaccines.

Conceptually, this approach is similar to the "seed virus" system currently employed in vaccine production—the system in which a seed virus is fully characterized and then used to initiate all vaccine production, thereby assuring product uniformity. A "seed cell" system is possible with a diploid cell strain because specific tests can be introduced to ensure that the cells used for vaccine production do not differ in any way from the "seed cells" or even from primary cultures.

It would appear, then, that if the same principles for the control of the viruses used in vaccine manufacture were to be applied to the control of the cells used for their propagation many of the problems currently encountered with primary cell cultures could be circumvented. Paramount among these problems are the question of latent virus contamination of primary cultures and the potential hazard of the hybridization of the vaccine virus with an occult viral genome that may be present in primary cells. Furthermore, the risk of fatalities due to B virus infection of individuals working with monkey cells should not be minimized. These points have been considered in detail elsewhere (6–8, 13). Such latent virus infections are still capable of escaping detection by current safety tests simply because the cell populations used are not subcultivated until the termination of their finite *in vitro* lifetime and tested throughout this period.

Tests can be performed by many different laboratories on a human diploid cell strain throughout the course of its entire *in vitro* lifetime, and when the investigator is satisfied with the results it can then be used for vaccine production. Thus, from a single carefully examined cell population, large quantities of a number of vaccines could be prepared by several production facilities from a standard cell population derived from the tissue of a single donor. Modern cell culture techniques make it possible to derive such a standard diploid cell strain by cloning from a single cell, if necessary, to ensure the ultimate in uniformity (11). This is similar to the plaque procedures used for purifying virus populations.

The current practice, in which many new primary cell populations are continuously derived for vaccine production, repeatedly subjects the vaccines produced therein to the inherent risks of latent virus contamination. Similar risks of changed biological parameters have been circumvented by application of the "seed virus" concept to the viruses used in vaccine production. It is long overdue that such a concept should be applied to the other component of the system—namely, the cells.

In view of the fact that the primary cell populations currently acceptable for vaccine production are, like cell strains, both diploid and multigenerational, I am embarrassed to observe that I have offered you nothing conceptually unusual in advocating the use of diploid cell strains for human virus vaccine preparation. What would be new, however, would be for the diploid strain to be composed of human cells. Currently, human virus vaccines are largely produced in monkey kidney and chick embryo cells. These two animal species are examples par excellence of reservoirs for latent viruses, including the most serious kind—the oncogenic virus. Human diploid cell strains derived from embryonic lung, on the other hand, have not been found to harbor latent viruses.

Even if contamination should occasionally occur, a human cell strain has the great advantage that it can be exhaustively tested *before* being

used as a vaccine substrate. Numerous human diploid cell strains that have been examined have, however, not revealed latent viruses. The choice of embryonic human cells is based on the general belief that embryonic tissue is less likely to harbor such viruses than are the cells of adult animals. Embryonic human cells also have a much greater doubling potential than the cells of adult humans and thus a longer useful life span (9, 10).

The second advantage of using human embryonic fibroblasts is that spontaneous alterations or transformations are not known to occur (9, 11). Spontaneous alteration of monkey kidney cells is a frequent finding, and the number of potential cell doublings of chick cells is far too low to make them useful as a diploid cell strain.

The use of human cells for vaccine production has sometimes been criticized on the basis that any potential human cancer virus would be more likely to replicate in human cells than in the cells of other species. Thus it is concluded that a greater risk is entailed in using human cells than the cells of other species. However, Rous sarcoma virus, polyoma, SV_{40}, and the oncogenic adenoviruses are known to cross the species barrier and to replicate in the cells of animal species other than those to which the virus is indigenous. Indeed, the only known primate oncogenic viruses, SV_{40} and the oncogenic adenoviruses, have not been found to be oncogenic *in vitro* or *in vivo* to the cells of the species in which they are indigenous; rather, they express their oncogenicity in the cells of other species. One is therefore forced to the opposite conclusion—namely, that a vaccine prepared in cells homologous to those of the species being vaccinated is potentially the safest.

A third advantage of using human cells for vaccine production is that these cells have the broadest human virus spectrum of any culture system known. Those of us who are interested in potential rhinovirus vaccines are faced with the certain prospect of having to use human cells, since many rhinoviruses are uncultivable in the cells of other species.

In recent years, trial vaccines have been produced in the human diploid cell strain WI-38 to such virus diseases as those caused by poliomyelitis (1, 13, 15–17, 20–23), adenovirus Type 4 (2, 4, 24), rhinoviruses (14, 18), and rubella (25). Oral poliomyelitis vaccines produced in WI-38 were first made and administered in this country (13) and then in Europe (15–17, 22, 23) to a total of over 200,000 children. This vaccine has been found to be efficacious and to produce no untoward results (1, 13, 15–17, 20–23). An adenovirus Type 4 vaccine also produced in WI-38 and administered in enteric coated capsules has now been fed to well over 50,000 men during 1965 and 1966 in recruit training centers in this country. No untoward effects have been observed and a significant reduction in respiratory infection due to adenovirus Type 4 has been noted (2, 4, 24).* Plans are now completed for vaccinating well over 500,000 men with this vaccine during the winter of 1966–1967. There are also published reports dealing with the potential utilization of human diploid cells in the preparation of vaccines against rabies (31), vaccinia (28), varicella (26), measles (30), and arboviruses (5). Minimum requirements for the production of virus vaccines in human diploid cell strains have now been established by interested groups of investigators, and these have been adhered to in the production of the vaccines listed above (3, 19).

In conclusion, most if not all human virus vaccines can now be produced in a single cell strain derived from a single tissue from one donor. By employing modern methods of cell preservation at low temperatures in liquid nitrogen (9), it is probable that the most highly characterized and frequently used human diploid cell strain, WI-38, will be available for the production of millions of doses of a wide variety of human virus vaccines for many years to come. These achievements are possible thanks to the advances that have been made in cell preservation techniques, cytogenetics, and the solution

* Personal communication from E. L. Buescher, Col., MC, principal investigator, USA Med R&D Command Project 3A014501B71Q, Communicable Diseases and Immunology, Task 01, Work Unit 166, Viral Infections of Man. Harry M. Rose, M.D., collaborated in this work.

This vaccine field trial was conducted under the sponsorship of the Naval Medical Field Research Laboratory, Captain James H. Boyers, Commanding, and is the result of a collaborative effort between the Bureau of Medicine and Surgery, the United States Navy, and the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

of logistical problems associated with the finite doubling potential of human diploid cell strains *in vitro* (9–11).

Aside from reduced vaccine production costs and cessation of the slaughter of large numbers of monkeys, perhaps the greatest advantage of a human diploid cell strain is the fact that this uniform cell population can be standardized. The use of such cells means that the degree of reproducibility expected when employing a standard virus can now also be expected from the cells on which they are grown.

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SECTION B. CHEMOTHERAPY

CHEMOPROPHYLACTIC APPROACH TO THE CONTROL OF SMALLPOX

D. J. BAUER

Wellcome Laboratories of Tropical Medicine
Beckenham, Kent, England

DR. BAUER (*presenting the paper*): The use of thiosemicarbazones as prophylactic agents in smallpox stems from the original observation by Hamre, Bernstein, and Donovick (1) of the antiviral action of derivatives of benzaldehyde thiosemicarbazone in fertile eggs and mice infected with vaccinia virus. Antiviral activity was also found in other compounds of the same class by Thompson, Minton, Officer, and Hitchings (2). Attention then became concentrated on isatin 3-thiosemicarbazone, which was recognized to be an antiviral agent with an unusually high degree of activity (3). Bauer and Sadler (4), in examining its structure-activity relationships against vaccinia virus, found that the antiviral activity could be increased by alkylation in the 1-position. Thus, 1-methylisatin 3-thiosemicarbazone, with twice the activity of the parent compound, was selected for trial in man. This compound is now known as methisazone (Marboran). A parallel development of this work led to the discovery of 3-methyl-4-bromo-5-formy*liso*thiazole thiosemicarbazone (M and B 7714) by Buttimore, Jones, Slack, and Wooldridge (5). Both methisazone and M and B 7714 have been found to have a prophylactic effect in persons who have been in contact with smallpox infection.

Prophylaxis with methisazone

Variola major. The preliminary results of a methisazone trial conducted in Madras were published in 1963 (6). Subsequently the trial was extended for the purpose of evaluating the effect of different doses of methisazone. The final results have been briefly mentioned (7) and will now be presented in greater detail.

Visits were made to the homes of smallpox patients admitted to the Infectious Diseases Hospital in Madras. Persons who slept in the same room as the patient were taken as subjects for the trial. Records were made of their names, ages, and vaccination status, and they were offered treatment with methisazone in accordance with one of four dosage schedules. An approximately equal number of such contact persons were admitted into the trial but were not offered treatment. The contacts were revisited 14 to 16 days later and in some cases on other occasions as well. At the final visit the results of vaccination or revaccination performed after contact were recorded, and note was made of any cases of smallpox that had developed in the meantime.

The results are shown in Table 1. Among the 2,610 contacts who took methisazone, 18 (0.69 per cent) developed smallpox during the observation period and 4 died, whereas among the 2,665 contacts who were not offered treatment there were 105 cases of smallpox (3.94 per cent) with 18 deaths. The reduction in case incidence associated with treatment was highly significant ($p < 0.001$). Among the 2,287 contacts who took all the doses of methisazone provided, there were 6 cases (0.26 per cent) and 2 deaths. The difference in incidence in comparison with the untreated contacts was also highly significant ($p < 0.001$). However, the incidence among the 323 who failed to complete the course

TABLE 1. EFFECT OF METHISAZONE TREATMENT ON THE INCIDENCE OF CONTACT CASES OF SMALLPOX

Group	Treatment	Contacts	Cases	Deaths	Case incidence (%)
1	Treated, all dose levels...	2,287	6	2	0.26
2	Not completed.....	323	12	2	3.71
1 + 2	Total treated.....	2,610	18	4	0.69
3	Not taken.....	150	11	3	7.33
4	Not offered.....	2,665	105	18	3.94
3 + 4	Total untreated.....	2,815	116	21	4.12

of treatment—3.71 per cent (12 cases and 2 deaths)—was not much lower than that in the untreated group.

The data for the groups of treated and untreated contacts were analyzed statistically in order to detect imbalance in any factors other than methisazone treatment that might have contributed to a reduction in the incidence of smallpox. No differences were found in the sex or age distribution of the groups. Analysis of the distribution of categories of vaccination status showed that two of the four treated groups contained a higher proportion of unvaccinated persons and were therefore slightly biased against the success of treatment. However, since a negative bias can be ignored in deciding whether the drug is active or not, the investigators concluded that the reduction in incidence in the treated group was brought about by the treatment itself.

The incidence of contact cases in the four treated groups was as follows: no cases of smallpox among the 74 contacts who took 3 g twice daily for four days, one case among the 384 who took 1.5 g twice daily for four days, no cases among the 584 who took two doses of 3 g eight hours apart, and five cases among the 1,137 who took a single dose of 3 g (Table 2). The drug was administered orally; the doses quoted were those given to adults; children and infants received proportionately smaller doses. The number of contact cases was too low to permit comparison of the effectiveness of the different dosage schedules, but it may be inferred that a single dose of 3 g gives marked protection, which is increased by the administration of multiple doses.

In an outbreak of smallpox in Port Elizabeth, South Africa, Ferguson (8) observed four cases of smallpox among 43 contacts of incompletely specified vaccination status who were given methisazone, and he concluded that the results of chemoprophylaxis were disappointing. However, vomiting occurred after administration of the drug, and the proportion of the dose actually absorbed was therefore uncertain. Moreover, the final results of the Madras trial show that some cases of smallpox will still occur in spite of treatment.

Alastrim. These findings have been supported by the results of a methisazone trial against alastrim conducted in São Paulo, Brazil, by Ribeiro do Valle, Raposo de Melo, de Salles Gomes, and Morato Proença (9). There were 5 (2.6 per cent) cases of alastrim among the 190 contacts who took two doses of 3 g of methisazone and 3 (1.5 per cent) cases among 194 who took one dose of 3 g, compared with 48 (8.1 per cent) cases among 520 contacts who received no treatment (Table 3). The treatment and control groups were homogeneous with respect to age distribution. No case of alastrim occurred among contacts who had been vaccinated at some time before exposure (169 in the treated groups and 253 in the control group), and these persons were not considered in the further analysis. In 406 of the contacts, no primary vaccination was performed after exposure.

TABLE 3. TREATMENT AND VACCINATION STATUS OF CONTACTS *

Vaccination status		Dosage schedule (cases/contacts)				Untreated (cases/ contacts)
		8 × 3g	8 × 1½g	2 × 3g	1 × 3g	
Unvaccinated before contact	Vaccination not performed	0/2	0/8	0/8	2/14	14/26
	Vaccination failed	0/1	1/6	0/9	0/14	4/21
	Vaccination successful	—	0/26	0/54	1/65	26/131
Vaccinated before contact		0/71	0/344	0/513	2/1044	61/2258
Total		0/74	1/384	0/584	5/1137	105/2436

Among the 187 in the treated group, there were 7 cases (3.7 per cent); in contrast, there were 38 cases (19.4 per cent) in the control group. The reduction in incidence associated with treatment was highly significant (0.01 per cent), and in view of the absence of immunity in both groups it was ascribed to treatment with methisazone, which was the sole variable. Primary vaccination after exposure was carried out on 28 contacts in the treated group and on 48 in the control group; the first group had one case of alastrim and the second had four. When these figures were combined with those for the unvaccinated contacts, the incidence of alastrim was 8 cases (3.7 per cent) in the treatment group and 42 cases (15.7 per cent) in the control group. The reduction in incidence in the treatment group was again significant at the 0.01 per cent level.

The authors conclude that their results confirm the prophylactic effect of methisazone observed in the Madras trial. In commenting on the observations of Ferguson, they point out that the true incidence in the absence of treatment could not be ascertained because there was no control group.

Prophylaxis of Smallpox with M and B 7714

The results of a trial of M and B 7714 for the prophylaxis of smallpox carried out in

TABLE 3. TREATMENT AND VACCINATION STATUS OF CONTACTS *

—	No. of treated contacts			No. of untreated contacts	
	Dose of methisazone		Total treated	Refused treatment	Controls
	2 × 3g	3g			
A. *Not previously vaccinated*					
Primary vaccination not performed	92 (5)	95 (2)	187 (7)	37 (6)	219 (38)
Primary vaccination failed	9 (0)	11 (1)	20 (1)	2 (1)	30 (2)
Primary vaccination successful	4 (0)	4 (0)	8 (0)	0 (0)	18 (2)
Total A	105 (5)	110 (3)	215 (8)	39 (7)	267 (42)
B. *Previously vaccinated*					
Revaccination not performed	84 (0)	76 (0)	160 (0)	27 (0)	230 (0)
Revaccination failed	1 (0)	8 (0)	9 (0)	2 (0)	19 (0)
Revaccination successful	0 (0)	0 (0)	0 (0)	0 (0)	4 (0)
Total B	85 (0)	84 (0)	169 (0)	29 (0)	253 (0)
Total A + B	190 (5)	194 (3)	384 (8)	68 (7)	520 (42)

Figures in parentheses = Cases of alastrim.
*Source: Ribeiro do Valle L.A., Raposo de Melo, P., de Salles Gomes, L.F., and Morato Proença, L. "Methisazone in Prevention of Variola Minor among Contacts." *Lancet* 2: 976–978, 1965.

Madras have been described by Rao, McKendrick, Velayudhan, and Kamalakshi (10). The drug was given by mouth in a dose of 4 g on three successive days. The trial was restricted to persons who showed no signs of previous vaccination. Among 196 contacts who were treated with the drug, there were 40 cases of smallpox (20.4 per cent) and 7 deaths, compared to 60 (29.9 per cent) cases with 12 deaths among 201 contacts in the control group. The reduction in incidence associated with treatment was significant at the 5 per cent level. The authors concluded that the reduction in incidence was too slight to justify the use of M and B 7714 in the prophylaxis of smallpox. However, this conclusion only applies to the particular dosage schedule used, and it is possible that a greater effect could have been achieved by varying the dose and the frequency of administration.

Effect of Thiosemicarbazones on the Results of Smallpox Vaccination

The antiviral thiosemicarbazones inhibit the growth of vaccinia virus and may therefore be expected to have an effect on the results of smallpox vaccination. Some indication of this effect may be seen by studying the results of the Madras trial of methisazone as they relate to the vaccination status of the subjects (Table 4). When examining the contacts, only the presence or absence of a take was recorded and no measurements were made of the size of the lesions. The failure rate of primary vaccination was 13.82 per cent (21/152) in untreated contacts and 17.86 per cent (35/196) in treated contacts. Although treatment was associated with a higher failure rate, the difference was not significant ($p > 0.3$). Failure of revaccination occurred in 60.48 per cent (1143/1890) of the untreated contacts and in 65.67 per cent (1335/2033) of the treated contacts. The increase in failure rate associated with treatment was significant ($p < 0.001$). It therefore seems likely that methisazone has some slight inhibitory effect on vaccination, although it should be noted that treatment with methisazone is associated with a marked reduction in the incidence of smallpox in contacts in whom primary vaccination or revaccination failed. Thus it may be concluded that the use of methisazone is not detrimental from the public health point of view, as long as vaccination is repeated in contacts in whom it has failed.

The results of Ribeiro do Valle *et al.* show that treatment with methisazone was associated with an increased failure rate of primary vaccination—71.5 per cent (20/28), compared with 61.25 per cent (30/48)—and revaccination—100 per cent (9/9), compared with 82.6 per cent (19/23)—but the difference did not attain significance ($p < 0.25$).

The effect of methisazone on vaccination was subjected to a detailed study in human volunteers by Landsman and Crist (11). Revac-

TABLE 4. DISTRIBUTION OF VACCINATION STATUS

Vaccination status		Incidence of cases			
Before contact	After contact	Treated		Untreated	
Not previously vaccinated	Vaccination not performed	5/36	(13.89%)	14/26	(54.0%)
	Vaccination failed	3/35	(8.57%)	4/21	(19.0%)
	Vaccination successful	4/161	(2.48%)	26/131	(19.8%)
	Totals	12/232	(5.17%)	44/178	(24.7%)
Previously vaccinated	Revaccination not performed	1/230	(0.43%)	7/349	(2.01%)
	Revaccination failed	3/1355	(0.22%)	26/1153	(2.3%)
	Revaccination successful	2/678	(0.29%)	28/756	(3.7%)
	Totals	6/2263	(0.26%)	61/2258	(2.70%)
Records incomplete		0/115*	(—)	0/229*	(—)

*These contacts were mostly previously vaccinated adult males who were not seen at the time of the last visit.

cination was carried out on 32 subjects, and two days later 16 were treated with methisazone in a dose of 3 g twice daily for three days. The mean diameter of the lesions was 0.42 cm in the treated group and 0.59 cm in the untreated group. Treatment was also associated with a lower production of inhibiting antibody: fourfold or greater rises in titer occurred in 10 of the 16 untreated subjects but in only 4 of the 15 treated subjects.

Jaroszyńska-Weinberger and Mészáros (12) investigated the effect of methisazone on primary vaccination in 26 children in whom vaccination was contraindicated on account of skin disease, tuberculosis, or some other condition. The main object of their study was to determine whether primary vaccination was a safe procedure in such children if they were treated with methisazone. In view of the risk, they did not use a true control group; instead, they compared the results with those obtained in a comparable group of 29 children treated with antivaccinial gamma globulin. Treatment was begun on the fourth day after vaccination. The severity of the local lesion was significantly less in the methisazone group than in the group that received gamma globulin. Since gamma globulin has been shown to reduce the severity of the response to vaccination (13), it could be inferred that methisazone would have produced a significant reduction if a true control group had been used. Serial titrations of inhibiting antibody were taken on 19 children treated with methisazone and on 22 treated with gamma globulin. In all instances the titers rose to levels of 20 to 640. The geometric mean titers were 172.1 in the methisazone group and 193.3 in the group that received gamma globulin. The difference was not significant ($p > 0.5$).

Herrlich, Stickl, and Munz (14) found that methisazone had no effect on local lesions in infants and young children undergoing primary vaccination. Jaroszyńska-Weinberger and Mészáros point out, however, that Herrlich and coworkers gave methisazone too early in the incubation period for any effect to be expected.

In conclusion, it seems to be fairly well established that methisazone can reduce the extent of the local lesion, but that it has not yet been definitely shown to depress antibody formation.

Side Effects of the Antiviral Thiosemicarbazones

Vomiting frequently occurs during treatment with methisazone. In the Madras trial it occurred in 16.7 to 27.3 per cent of contacts receiving different dosage schedules. Ribeiro do Valle *et al.* observed nausea and vomiting in 66 per cent of the contacts in their study, Landsman and Grist reported severe vomiting in all their subjects, and Herrlich *et al.* observed the effect in 17 out of 33 children. Jaroszyńska-Weinberger and Mészáros noted vomiting in 10 out of 26 children but commented that it was in no way as severe as that seen by Landsman and Grist. Rao *et al.* observed vomiting in 74 per cent of the contacts treated with M and B 7714. They also noted a drug rash in 14 per cent of the subjects. The vomiting could not be prevented by the administration of antiemetic drugs.

Ferguson considered that the vomiting caused by methisazone could be reduced by previous administration of cyclizine. Ribeiro do Valle *et al.* found cyclizine ineffective. Chlorpromazine and cyclizine were found ineffective in the Madras trial.

Vomiting can be accepted as a side effect in the face of life-threatening infection, but it has the disadvantage of causing loss of some or all of the dose administered, thus diminishing the protective effect.

A Definition of Chemoprophylaxis

The results obtained with methisazone and M and B 7714 show that a virus disease can be prevented by administering a drug during the incubation period. This effect has been termed chemoprophylaxis. However, these drugs only act against virus that is actively multiplying; if the contact has escaped infection the drugs can do nothing. They are therefore being used against an existing infection that has not yet progressed far enough to become clinically manifest—that is to say, they are being used for treatment. However, from the public health point of view their activity is prophylactic, since they are used on apparently healthy persons and there is no way of knowing whether or not the contact is going to develop smallpox except by awaiting events. It is therefore convenient to

retain the term chemoprophylaxis, so long as one understands that it is used in a special sense.

Comparison of Chemoprophylaxis and Vaccination in the Prevention of Smallpox

Vaccination alone. Chemoprophylaxis is only effective when given during the incubation period of an infection that has already started. Vaccination is a standard practice in the same circumstances, but it is mainly used to give protection against future exposure. The results of the trials described above provide enough information to enable the effects of vaccination to be evaluated and compared with those of chemotherapy.

From Table 4 it can be seen that previous vaccination unaccompanied by chemoprophylaxis reduces the incidence of smallpox from 24.7 to 2.75 per cent—a 9.15-fold reduction; from Table 3, that it reduces the incidence of alastrim from 15.73 per cent to zero. Primary vaccination after exposure was performed on 152 contacts, 30 (19.74 per cent) of whom developed smallpox; in contrast, among 26 unvaccinated contacts, 14 (54.0 per cent) developed the disease. The reduction in incidence associated with primary vaccination *regardless of its result* is highly significant ($p < 0.001$). However, there is no difference in incidence between those in whom primary vaccination failed and those in whom it was successful. It therefore appears that the inoculation of dead antigen causes the production of a limited degree of immunity, which develops rapidly enough to confer some protection, whereas the extra amount of antigen produced by the multiplying virus appears too late to be of any value.

Such an effect was not noticed with revaccination, and the incidence figures show that revaccination, even when successful, conferred no protection against exposure that had already occurred.

Vaccination of contacts is the generally accepted procedure, but these figures show that revaccination is ineffective in conferring protection against recent exposure and further evidence would be desirable before it can be accepted that protection can be conferred by ineffective primary vaccination.

These findings only apply to the conditions prevailing in Madras, where exposure continues for several days as a result of late admission of the patient to the hospital. It is reasonable to expect that vaccination would be more effective in countries where it can be performed earlier in the incubation period. However, prolonged contact before detection of the index case is common in countries in which smallpox is endemic, and in these circumstances the effect of vaccination is limited to protection against future exposure.

Methisazone alone. In the trial against alastrim the majority of the contacts were not vaccinated after exposure, and the results therefore permit an assessment of the prophylactic effect of methisazone unaccompanied by vaccination. Among 187 unvaccinated contacts treated with methisazone (3.74 per cent) developed alastrim; in contrast, among 219 unvaccinated untreated contacts, 38 (17.35 per cent) contracted the disease. Treatment with methisazone alone therefore caused a 4.8-fold reduction in incidence. The same effect can be seen in the results of the Madras trial (Table 5). Treatment with methisazone alone caused a 3.9-fold reduction in unvaccinated contacts and a 4.7-fold reduction in previously vaccinated contacts who were not revaccinated.

Methisazone and vaccine combined. The re-

TABLE 5. EFFECT OF VACCINE ALONE AND IN COMBINATION WITH METHISAZONE ON THE INCIDENCE OF CONTACT CASES OF SMALLPOX

Vaccination after exposure	Vaccine alone	Vaccine and methisazone
	Incidence ratio Vaccinated/unvaccinated	Incidence ratio Untreated/treated
Not previously vaccinated		
Primary vaccination not performed	—	3.89
Primary vaccination failed	2.84	2.22
Primary vaccination successful	2.73	7.98
Previously vaccinated		
Revaccination not performed	—	4.67
Revaccination failed	0	10.46
Revaccination successful	0	12.76

sults obtained from the combined use of methisazone and vaccine in the Madras trial are summarized in Table 5. In contacts in whom primary vaccination failed, the use of methisazone secured a 2.2-fold reduction in incidence in addition to the 2.8-fold reduction due to the vaccination—an over-all reduction of 6.3-fold. In contacts with successful primary vaccination methisazone produced an additional reduction of 8.0-fold and an over-all reduction of 21.8-fold. In previously vaccinated controls revaccination produced no reduction, whereas when combined with methisazone reductions of 10.5- and 12.8-fold were obtained.

Suggested Procedure for the Treatment of Smallpox Contacts

Although vaccination is of doubtful value in protecting against previous exposure, it should be performed on all close contacts of smallpox to protect them against re-exposure in the immediate or more distant future. In nonendemic countries re-exposure occurs when there is a succession of cases in a closed community, such as a mental hospital, or sometimes in a family. In endemic countries with less well-developed medical services, the patient may remain at home as a continuing source of infection and other cases may occur in neighboring houses. However, the contacts should also be protected against the exposure that they have already incurred. Hitherto there has been no adequately effective means of doing this, but a course of methisazone treatment should now be given as a routine measure at the time of vaccination. This combined treatment should greatly reduce the incidence of contact cases and enable an outbreak to be brought to an end more rapidly.

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SECTION B. CHEMOTHERAPY

CHEMOPROPHYLAXIS OF VIRAL RESPIRATORY DISEASES *

GEORGE GEE JACKSON, EDITH D. STANLEY, AND ROBERT LEE MULDOON

Department of Medicine, University of Illinois
College of Medicine, Chicago, Illinois, U.S.A.

DR. STANLEY (*presenting the paper*): In recent years pessimism about the likelihood for successful treatment and prevention of viral infections has been replaced by optimistic expectations. Extensive experience with antibacterial chemotherapy, the development of tissue culture techniques, and a rapidly expanded knowledge of virology have led to practical methods for studying the ability of natural, biological, and synthetic compounds to inhibit a spectrum of viruses. The exhibition of antiviral activity has not been rare, but it has been relatively specific.

Successful clinical trials in man with N-methylisatin β-thiosemicarbazone for smallpox (2), 5-iodo 2′ deoxyuridine for herpes simplex keratitis (20), and amantadine hydrochloride for influenza (18) have heightened interest and activity in the search for clinically useful compounds and susceptible viral diseases.

Viral respiratory infections, because of their high frequency, are a major subject of interest in the search for effective chemoprophylactic and therapeutic agents. The identification of an increasing number of viruses that can cause respiratory illnesses in man and the demonstration of the specificity of immunity against the common cold (16) have made vaccine prophylaxis for upper respiratory infections seem logistically impossible. It is hoped, however, that the similarity of biologic properties within the different families of viruses may make for a somewhat uniform sensitivity of the strains to inhibition by drugs. If so, chemoprophylactic control of viral respiratory diseases is the method of most promise at this time.

A discussion of the subject may take into consideration the following aspects: description of the compounds—nature and spectrum of antiviral activity and toxic effects; evaluation of the use of a drug as a prophylactic and chemotherapeutic agent in man; and clinical recognition of etiologically specific infections for selective use of the drug.

MEASUREMENT OF ANTIVIRAL ACTIVITY

Recognition of antiviral activity is usually based on the effect of a drug on infected tissue cultures. Further *in vitro* and *in vivo* tests can establish the spectrum of activity and the relative virucidal and cytotoxic effects of the compound. The results of treatment of viral respiratory infections in animals are difficult to interpret. In the first place there is the problem of species differences, and in the second place there are seldom any valid indicators of nonlethal symptomatic disease in animals. In mice, death and mean survival time can be reproducible indicators of influenza. The infection must be lethal, however, and the effect of a drug can often be dramatic or nil depending on the number of lethal doses in the viral challenge. The precise dose that is the most meaningful with regard to natural infections in man is not known. A nonlethal infection of the turbinates

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of ferrets recently measured quantitatively by Haff (8) has some of the characteristics of a mild infection in man. Study of this model might be of assistance in the preliminary investigation of future drugs. To date, no methods have been developed for the quantitation of infection, symptoms, and lesions in available animals or *in vivo* systems with rhino-, entero-, parainfluenza, respiratory syncytial or adenoviruses. It is highly desirable that such animal systems be developed for the measurement of *in vivo* antiviral activity to justify and direct the clinical trials of potentially useful drugs. Such tests, however, neither exclude nor assure antiviral activity in man. Relatively few agents have been used for the chemoprophylaxis of viral respiratory infections in man. Some that have been tried are listed in Table 1 (3, 17, 22, 28, 18, 29, 19, 25).

MECHANISM OF ACTION

It is not the purpose of this paper to consider details of the mode of action of antiviral drugs, even those recommended for respiratory viruses. However, a brief consideration of the general ways in which antiviral activity is mediated will provide a better understanding of the results observed and give a basis for reasonable expectations from the use of a drug.

TABLE 1. SOME AGENTS TRIED IN MAN FOR CHEMOPROPHYLAXIS OF RESPIRATORY VIRAL INFECTIONS

Influenza
Cutizone (β-isopropylbenzaldehyde thiosemicarbazone)
ABOB (N′N′ anhydrobis (β hydroxy ethyl) biguanide HCl
Amantadine (1-amino adamantane)
Caffeic acid (3,4-dihydroxycinnamic acid)
Adenovirus
DNAase aerosol
Rhinovirus
Interferon (monkey kidney origin)
General Infections
Viractin (*Streptomyces griseus* origin)

The most prevalent type of antiviral activity demonstrated *in vitro* is the contact inactivation of viruses. Of the many substances capable of such activity, none of them so far has had clinical usefulness (10), although some have been helpful as tools for further laboratory research. Then there are the drugs that interfere with viral synthesis. The development of these agents has received considerable impetus from research in cancer chemotherapy. Analogues of the purine and pyrimidine base components of nucleic acids and various inhibitors of nucleic acid synthesis have often inhibited viruses, especially those of DNA type. Like IDU, however, their usefulness is generally restricted to those viral lesions that are available to topical or regional application, and usually their toxicity is too great to consider for use in viral respiratory infections except under dire circumstances. Finally, there are the antiviral drugs that block the synthesis of a viral-specific protein. Thiosemicarbazone may exert such an action on rabbit pox virus (1). Compounds of this type could be expected to have the greatest selectivity in the inhibition of viruses. So far, no agents have been found that act in this way against the respiratory viruses, but such compounds are being sought and can be expected.

Interferon has a cell-mediated effect that renders uninfected susceptible cells resistant to infection with viruses of various types. It has no direct antiviral action. Production of interferon in the respiratory tract can be stimulated by drugs as well as by infection or by inactivated viruses (15). Thus it offers a general mechanism for the chemoprophylaxis of viral respiratory infection. Another potential class of substances without antiviral effect but with a cell-protective effect are agents that could preserve cell integrity and function during viral infection. Although such a property is sometimes assigned to steroids, this approach to the chemoprophylaxis of viral respiratory infections is unexplored and unconfirmed.

AMANTADINE

Amantadine hydrochloride has introduced a new dimension to antiviral chemotherapy. The site of action is on the cell membrane (11, 16). As noted in Table 2, the drug does not inactivate the virus or its enzyme (neuraminidase). It does not induce resistance in susceptible cells after treatment. It does not affect adsorption of virus onto the cells, but its presence delays or prevents transmission of infectious virus into the cell. It possibly affects the early eclipse phase of virus replication, but within a few minutes after viral infection of the cell, viral synthesis and release are unaltered by treatment with the drug. The lingering of influenza virus on the exterior surface of the cell to which it is adsorbed in the presence of amantadine can be demonstrated by late hemadsorption on an infected tissue culture and by neutralization of the virus from the delayed application of antibody. This inhibition of cell membrane penetration was reported by Fletcher and colleagues (6) to be a characteristic of ammonium ions and various aliphatic amines. A similar effect was observed in tissue cultures using amonium chloride and several aliphatic amines containing from one to five carbon atoms. Electron microscopy showed decreased pinocytosis, and this presumably altered engulfment of the virus. The *in vivo* effectiveness of amantadine was believed to be related to its adamant structure, which is not degraded in the body but rather is excreted unchanged in the urine and in respiratory secretions.

The antiviral effect of amantadine is quite specific (7). Strains of influenza A_2 appear to be uniformly inhibited, as are most strains of influenza A, A_1, and C. There is no inhibitory effect on strains of influenza B. In some tissue culture systems activity can be shown against parainfluenza and rubella viruses.

TABLE 2. EFFECTS OF AMANTADINE ON INFLUENZA VIRUS IN TISSUE CULTURES

1. Does not inactivate virus on contact.
2. Does not inhibit action of viral neuraminidase.
3. Does not alter virus susceptibility of cells (no interferon produced).
4. Does not affect virus adsorption to cells.
5. Delays virus penetration of cell membrane.
6. Prevents cell pinocytosis.
7. Viral synthesis and release unaltered.
8. Effects are reversible by removal of drug.

CLINICAL EVALUATION

Clinical studies on the chemoprophylaxis of viral respiratory infections should include controlled observations to evaluate the specific antiviral effect of the drug. In naturally acquired disease, there should be surveillance to determine the specific etiology of the infections prevented or treated and simultaneous observation of the course of untreated persons. Study of a large population of relatively well but susceptible persons is necessary in order to accumulate sufficient data for conclusions, unless there is a high and abrupt incidence of a specific illness. More definitive results have been obtained using volunteers infected with an attenuated strain of virus. After a drug has been shown to have a beneficial effect in man, its effect on clinical illness can be tested against challenge with a less attenuated disease-producing strain of virus. Of the drugs used for chemoprophylaxis of respiratory infection in man, only amantadine hydrochloride in the prevention of Asian influenza can be tentatively assumed to be effective.

AMANTADINE CHEMOPROPHYLAXIS OF INFLUENZA IN VOLUNTEERS

The results of several investigations in which susceptible volunteers, usually with low levels of antibody, were given a virus challenge under controlled conditions of treatment with amantadine or a placebo capsule are summarized in Table 3 (18, 29, 13, 31, 14, 26, 27). The virus strain A_2 134 is an egg-grown attenuated live virus vaccine. The Bethesda 10/63, England, Scotland, and Rockville strains were less attenuated. The last was isolated and propagated only in human embryonic kidney. The other poorly attenuated strains were grown in monkey kidney or human respiratory epithelial cell cultures.

The index of infection in different studies was either a fourfold or greater serologic rise in antibody titer or a clinical influenzal illness. In two studies virus recovery from nasal or pharyn-

TABLE 3. INFLUENZA IN SUSCEPTIBLE VOLUNTEERS PRETREATED WITH AMANTADINE

Investigation	Virus challenge	Index of infection	No. obs.	Percentage positive Placebo	Percentage positive Treated
Jackson *et al.*, Urbana–1963	A_2 134 10^6 EID	Serology	98	70	38
Stanley *et al.*, Urbana–1964	A_2 134 10^6 EID	Serology	26	71	33
Hornick *et al.*, Baltimore–1964	A_2 Beth/63	Illness	47	33	4
Tyrrell *et al.*, Salisbury–1964 or	A_2 Eng/57	Serology	47	48	71
	A_2 Scot/57	Illness	53	26	37
	10^6	Virus recovery	27	15	21
Hornick *et al.*, Baltimore–1965	A_2 Rock–64	Serology	58	100	100
	64×10^3	Illness	58	62	28
Saliba *et al.*, Philadelphia–1965	A_2 Rock–64 64×10^3	Virus recovery	20	50	20
Schiff *et al.*, Cincinnati–1965	A_2 Rock–64 64×10^3	Serology	18	89	44
		Illness	18	45	0

geal washings was available to measure the drug effect. The trend of the results in all the investigations except the one reported from England (31), regardless of the index of infection used, was the same. Pretreatment with 100 mg of amantadine twice daily beginning one day before virus challenge caused a decrease in the incidence of infection by about one half. The average reduction for all of the studies calculated by using the most discriminatory index of each investigator was 57 per cent for the combined U.S. studies and nil for the English study. The reason for the paradoxical result of the latter investigation was not amantadine resistance of the strains of influenza virus used; the challenge dose of virus was 100 or more times greater than that used with the nonattenuated strains in the U.S. studies, and the higher dose might have negated the amantadine effect. The difference between the control and test groups is not statistically significant. In all but two of the other studies the difference in the incidence of influenza infection between the placebo- and amantadine-treated volunteers is statistically significant; in those two (29, 26), the trend was of the same direction and magnitude. In the second study by Hornick *et al.* (14), all of the volunteers had an antibody response, but the geometric mean increase in antibody titer was significantly less among treated volunteers than among the placebo controls. In other studies, amantadine has been shown not to interfere with the capacity of animals to form antibody against influenza virus, and therefore the lower incidence and titers of serologic response is a reflection of less infection (18, 29).

Table 4 shows a comparison of volunteers of equal susceptibility according to their antibody status and whether they were given pretreatment with amantadine or treatment beginning at the time of or approximately three hours after the virus challenge (29). The incidence of infection among the volunteers who received placebo in the low antibody group was nearly three times as great as it was among the placebo controls in the high antibody group. The reduction in infection produced by amantadine was similar in subjects with low and high antibody, but the incidence among the latter was too low to make the difference statistically significant. Delay in starting amantadine treatment until the time of virus challenge completely eliminated the drug's preventive effect on infection, even when treatment was continued for six days after the challenge. The data are conclusive in showing that the chemoprophylactic use of amantadine was of no benefit after infection was initiated, regardless of whether or not the patient had any degree of immunity from antibody.

TABLE 4. EFFECT OF SERUM ANTIBODY AND TIME OF TREATMENT WITH AMANTADINE ON PREVENTION OF INFLUENZA IN VOLUNTEERS

Antibody titer	Time of treatment	No. obs.	Infected (%)	Reduction vs placebo (%)
LOW (HI <20)	Preinfection	41	37	47 ($p = <.001$)
	Postinfection	64	72	0
	Placebo	85	70	—
HIGH (HI 20 or greater)	Preinfection	24	16	41 ($p = .15$)
	Postinfection	129	29	0
	Placebo	63	27	—

AMANTADINE CHEMOPROPHYLAXIS OF NATURALLY ACQUIRED INFLUENZA

Small, scattered, influenza epidemics occurred along the east coast of the United States beginning in January 1963. In several of these, influenza A_2 was identified as the causative agent. In early February, when a sudden increase of respiratory illness occurred among prisoners in the Philadelphia County Prison, Wendel and associates undertook the first test of the prophylactic effect of amantadine against natural disease (33). About three fourths of the prisoners, ranging in age from 17 to 54 years, participated. Under a double-blind code, a group of 794 men received either drug or placebo in the prophylactic trial. The dose of drug was 100 mg by mouth twice daily. The placebo was given in the form of identical capsules. In the group treated prophylactically, about 20 per cent were suceptible subjects, defined as having low serum influenza hemagglutinin-inhibiting antibody levels at the beginning of treatment. In the susceptible group pretreated with amantadine 27 per cent had a fourfold or greater rise in serum antibody, whereas among those who received the placebo 48 per cent had a comparable rise—a reduction from the placebo level of 46 per cent. A small number of illnesses occurred, predominantly in the placebo-treated group. Among the prisoners with initially high antibody levels, the reduction in the rate of infection as determined by antibody rise was only from 16 to 13 per cent in the drug-treated group. Even though a similar reduction occurred in the case incidence of influenza, the rate of illness was so low that it precluded any valid conclusions regarding the drug effect.

Fifty-five men with acute respiratory illness volunteered for a therapeutic test and were treated by random distribution with drug or placebo therapy, beginning some time after the onset of symptoms. Of the 32 tested, 13 had confirmed influenza. No beneficial drug effect was shown.

Since Wendel's initial work, a number of similar studies have been done on minor influenza epidemics occurring in various parts of the United States and in other parts of the world. Table 5 lists those from which complete data are available for review. In the two studies of children conducted by Quilligan (23) and Finklea (5), the former during February and March 1964 in Los Angeles and the latter during the following winter in Michigan, the prophylactic effect of amantadine was demonstrated both by the reduced rate of infection, as indicated by serologic rise, and by the reduced incidence of illness in the drug-treated groups. Harris obtained similar results from a study of prisoners in California (9)—a 61 per cent reduction in the infection rate and a smaller reduction in the number of clinical cases of influenza.

An analysis of the combined data reported from these studies gives observations on 1,806 subjects under controlled conditions (Table 6). The protective effect shown in subjects with initially low antibody is emphasized—a reduction of 57 per cent in the treated group as compared to the placebo group. In this entire population, regardless of the immune status at the time the treatment was begun, amantadine reduced the incidence of infection by 50 per cent. These results in the reduction of naturally acquired infection are almost identical to those observed

TABLE 5. AMANTADINE PROPHYLAXIS AGAINST NATURAL ACQUISITION OF INFLUENZA

Investigation	Antibody status	Index of infection	No. obs.	Percentage positive	
				Control	Treated
Wendel *et al.* (prisoners) Philadelphia, 1963	Low	Serology	144	48	27*
	High	Serology	556	16	13
	Random	Flu	700	2	<1
Quilligan *et al.* (children) Los Angeles, 1964	Random	Serology	169	17	10*
	Low	Flu	104	29	16
	High	Flu	65	33	0*
Finklea *et al.* (children) Michigan, 1965	Random	Flu	293	8	1*
Harris *et al.* (prisoners) California, 1966	Low	Serology	457	44	17*
	Low	Flu		8	6
	High	Serology	187	12	2*
	High	Flu		4	0

*p = <.05

in the volunteers. There was a low incidence of illness, but reduction by amantadine was 62 per cent and highly significant. No deaths from influenza were reported in any of these studies.

REACTIONS TO AMANTADINE

Ninety per cent of an oral dose of amantadine can be recovered in the urine, about half of it in the first 24 hours after ingestion. A single dose is excreted in from one to three days, with considerable variation in excretory rate occurring not only in different subjects but also from day to day in a single individual. At the recommended dose range, a cumulative effect may be seen in the first few days of treatment.

TABLE 6. AMANTADINE PROPHYLAXIS AGAINST NATURAL ACQUISITION OF INFLUENZA, CUMULATIVE DATA FROM FOUR INSTITUTIONS, 1963–1966

	No. obs.	Percentage positive		Percentage of reduction
		Control	Treated	
Serologic incidence of infection:				
Initial low antibody	601	46	20	57*
Initial high antibody	786	15	12	20
Subtotal random antibody	1,387	30	15	50*
Specific influenzal illness:				
Random antibody	1,806	6.3	2.4	62*
Ratio: Infections/illness	...	4.8	6.3	31

*p = <.001

Thousands of doses of amantadine have now been given, and serious toxic effects have been infrequent both in the studies we have reviewed and in our own trials, in which a dose of 200 mg per day was given. However, it cannot be claimed that the drug produced no deleterious effects or that the nature of the side effects is understood.

In our studies of healthy medical students we were able to show a clear relationship between the size of the daily dose and the occurrence of reactions (Fig. 1). When 400 mg a day—twice the recommended daily dose—was given, over 40 per cent of subjects experienced reactions. These reactions occurred with striking regularity about two to three hours after ingestion of the drug and usually lasted six hours if the medication was not repeated. The reactions observed were predominantly alterations of the emotional state and of cerebration. The students complained of depression and inability to concentrate. Several described sensations of depersonalization and altered body image. There was a remarkable degree of associated anxiety.

In reports from various clinical trials and other toxicity studies, insomnia, irritability, and

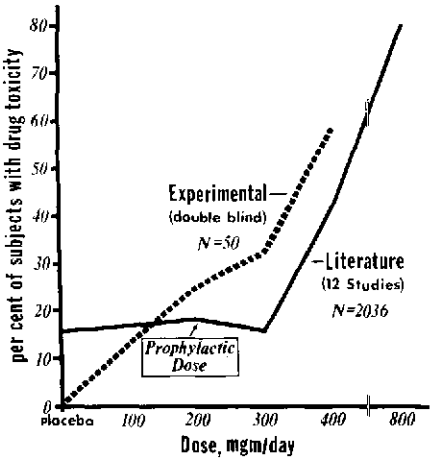

Fig. 1. Incidence of reactions to amantadine-HCl.

similar central nervous system effects have been noted (24). Gastrointestinal symptoms have also been reported, but the incidence of these as compared to the placebo-treated subjects was rarely significant. If the dose of drug was larger than 400 mg a day, the occurrence of toxic effects approached 100 per cent.

A trial of amantadine among patients, most of them elderly, in a chronic disease hospital was associated with an increase of 0.5 to 1.0 per cent per week in the death rate among drug-treated individuals. Of 147 persons taking 200 mg of amantadine daily for 11½ weeks, 26 died—an incidence of 1.5 per cent per week. For the four weeks prior to the study, the incidence of death among approximately 600 patients had been 1.1 per cent per week. During the test period, of 289 persons who received placebo 11 died, for an incidence of 0.3 per cent a week; of 60 who received neither drug nor placebo 8 died, for an incidence of 1.2 per cent. Thus the over-all weekly incidence of death among the control subjects was 0.5 per cent. The differences are significant, but the internal comparability of the groups is difficult to establish. Sera were examined for evidence of altered enzyme concentrations or altered liver or kidney function (SGOT, SGPT, alkaline phosphatase, and BUN), but no significant differences from the normal range were found. In two other studies, 124 elderly people received 200 mg of amantadine daily for 19 or 30 days. No deaths occurred. Until more information is available, the possible increased risk of amantadine among elderly people should be recognized.

CLINICAL USE OF AMANTADINE PROPHYLAXIS FOR INFLUENZA

Data on the usefulness of amantadine in the chemoprophylaxis of influenza and a comparison of different systems for demonstrating its effectiveness are shown in Figure 2. In each of the test systems shown, multiple oral doses of amantadine were given before the induced or natural exposure. The data accumulated for each set of bars is statistically significant. In the case of amantadine, each type of investigation—laboratory animals, volunteers given attenuated or virulent virus, and naturally acquired disease measured serologically or by clinical illness—accurately reflected the results obtained by the other methods. However, the difficulty of the studies increased progressively as the incidence of the positive index became smaller. If such observations are repeated with other anti-influenzal drugs, their clinical trial and the prediction of their usefulness will be simplified and expedited.

Amantadine has not been shown to have an effect on the prevention of viral respiratory infections other than Asian influenza. Its selective use, therefore, requires recognition of influenza as the etiology of respiratory illness. This cannot be done clinically, but influenza can be suggested on an epidemiologic basis and confirmed by isolation of the virus and by serology. The institution of prophylaxis among close contacts of clinical cases of influenza is indicated for the prevention of infection. The effectiveness of this regimen has not been shown. Asympto-

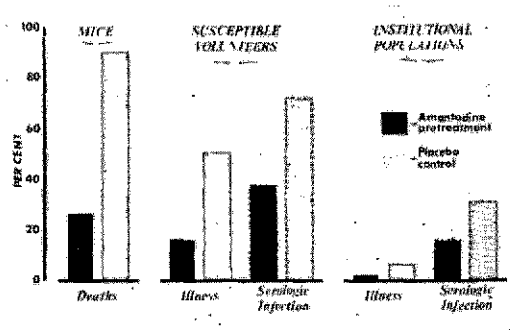

Fig. 2. Amantadine chemoprophylaxis of influenza.

matic contacts may already have become infected. It is known that influenza virus is shed from the respiratory tract before the onset of symptoms and that infection has already occurred one or two days before virus is shed (30). It must be expected, therefore, that the use of an index case as the indication for amantadine prophylaxis will give less favorable results than those given in the reports discussed here. Seasonal prophylaxis among high-risk groups could be given on a routine basis. The tolerance of different patients for amantadine and their reliability in taking the drug require further study before the effectiveness of a routine prophylactic regimen can be assessed. A preventive dose (200 mg a day) has been well tolerated, except possibly in the case of elderly invalids, who are among those for whom routine chemoprophylaxis might be indicated.

The amount of illness prevented by chemoprophylaxis will be related to the infectivity and virulence of the epidemic strains. At the present time, the attack rates for populations into which the introduction of influenza has been documented is about 30 per cent; clinical illness occurs in one out of every five persons who become infected (Table 6). At these rates, four clinical illnesses could be prevented for every 100 persons given prophylactic treatment and subsequently exposed to epidemic influenza. The benefit of such chemoprophylaxis for influenza must now be established by further patient and physician experience with the drug and by the future incidence of epidemic influenza.

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SECTION B. CHEMOTHERAPY

CHEMOTHERAPY OF HERPESVIRUS INFECTIONS *

HERBERT E. KAUFMAN

Department of Ophthalmology, University of Florida
College of Medicine, Gainesville, Florida, U.S.A.

DR. KAUFMAN (*presenting the paper*): Herpes simplex infection of the cornea is the most serious virus disease of the eye in the United States and Europe, and it is now the most important corneal disease leading to loss of vision in the United States. Although in the skin this disease is generally benign and self-limited, in the cornea and uveal tract it is serious and sometimes disabling because of chronicity and vision-impairing scar formation. Spontaneous clearing of herpetic keratitis within a reasonable period of time probably does not occur in more than 10 per cent of the cases, and in most cases permanent diminution of vision results (1). Moreover, since the advent of topical and systemic corticosteroid therapy for ocular disorders, herpes simplex keratitis seems to be more frequent and severe, and bilateral cases appear to be increasing in number (2).

THE STAGES OF HERPETIC KERATITIS

In order to discuss the therapy of this disease, it is necessary first to define the pathogenesis of its various stages.

(a) *Epithelial virus ulcers.* Acute infection results in a dendritic ulcer. Sometimes acute and primary infection also causes an epithelial keratitis, epithelial vacuoles, and a keratoconjunctivitis. As the dendritic ulcer progresses, it can widen, invade the stroma, and assume a geographic pattern. In these conditions the multiplication of virus causes tissue damage, and antiviral agents are useful.

(b) *Metaherpetic nonvirus ulcers.* Often after the dendritic phase a nondescript corneal ulcer occurs, usually with stromal edema. This lesion, called "metaherpetic" by Gunderson in 1936 (3), and "geographic" by Thygeson in 1953, will be referred to as "metaherpetic" in the present discussion. It does not contain virus, and a misunderstanding of its pathogenesis is responsible for much of the clinical frustration caused by this disease.

Experience with several hundred cases indicates that metaherpetic epithelial defects result from an edematous and unhealthy stroma and basement membrane to which the epithelium cannot adhere. Frequently patients with this syndrome complain of pain on arising, which becomes less during the day. Many of them respond to a bland ointment administered at night. In every way this syndrome is similar to the recurrent erosion of the corneal epithelium seen after trauma. The essential feature is damage to the stroma and Bowman's membrane, and it matters little whether this damage is caused by a foreign body, virus infection, or caustic chemicals. Since virus multiplication does not cause the condition, antivirals are of no benefit (4).

(c) *Stromal disease.* Stromal disease may be of three general types. In the first, iatrogenic stromal scars may result from the use of denaturing agents. In the second, virus may multiply in the stroma and cause localized, white, cheese-like lesions. These lesions, which may

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occur along with disciform lesions, appear to respond, though slowly, to antiviral agents. In the third, disciform keratitis begins as an edematous patch of cornea occurring over an area of folds in Descemet's membrane. It is usually accompanied by some iritis and appears to be caused by damage to the endothelium as a result of a hypersensitivity response to the virus.

In our laboratory we have documented a true delayed hypersensitivity to soluble herpes antigen. In the skin, it has the histologic manifestation of delayed hypersensitivity, it follows a consistent time course, and it is transferable by cells and not by serum (5). In the eye, the infection of sensitized animals results in a syndrome clinically similar to disciform keratitis, and the intracorneal injection of soluble antigen produces a similar picture (6, 7). Usually the involved area of the cornea is central, but it may be eccentric, or the whole cornea may be involved in a bullous keratitis. Neither this condition in its pure form nor herpetic iritis is helped by antiviral agents. Corticosteroids are dangerous if virus persists in the epithelium, since they can aggravate corneal ulcers and lead to perforation of the cornea and loss of the globe. They do, however, benefit the disciform lesion and the iritis.

It has been found in animals and confirmed in man that there is a competitive antagonism between antiviral agents, which benefit the herpetic keratitis, and steroids, which make it worse (8, 9). By combining steroids and antiviral agents, one can induce any severity of keratitis desired. With steroids alone, keratitis is extremely severe; with antiviral agents alone, the disease may be cured; with the two in combination, intermediate grades of severity can be obtained. Because of the competitive antagonism that is set up when antiviral agents are added to corticosteroid therapy, corticosteroids may be used with greater safety in conditions that are secondary to virus infection but in which the virus multiplication may be exacerbated by the corticosteroids. Double-blind studies in man in England showed that when steroids were used with placebo on deep keratitis 68 per cent of the patients had recurrence of epithelial disease, whereas when IDU was added only 8 per cent had such recurrences (10).

CLINICAL TRIALS IN MAN

Initial studies of topical IDU in man indicated that it was completely nontoxic and highly effective. Unfortunately, these first trials are not comparable to later studies, since the most dramatic event that occurs after IDU therapy is usually a breaking up of the ulcer and a rapid initial healing.

Although the early clinical trials in man defined a therapeutic regimen that appeared to be effective and identified the types of corneal disease in which this type of medication might be expected to be of aid, only double-blind studies could confirm the clinical impression of therapeutic effectiveness. To date, six double-blind studies on the efficacy of IDU in treating acute herpetic keratitis have been reported (11–16). From the results so far, there can be no question but that IDU is effective in man when the acute disease caused by virus multiplication is treated (Table 1).

IDU appears to be virtually nontoxic. Excellent studies on wound healing indicate that even when the epithelium is completely removed a dose of IDU that can completely inhibit virus DNA synthesis does not inhibit epithelial wound healing (17). Similar experiments by Hanna show that tritiated thymidine uptake by the epithelium is not decreased even though the epi-

TABLE 1. DOUBLE-BLIND HUMAN STUDIES—ACUTE DENDRITIC KERATITIS

Study	Results	IDU	Placebo
Paterson *et al.*	Healed,	74%	7%
	5 days Rx	24/33	2/29
Burns	Good and	65%	33%
	excellent	15/23	5/15
Laibson and	Good,	68%	27%
Leopold	8 days Rx	15/22	7/26
Jepson	Improved,	92%	58%
	7 days Rx	11/12	7/12
Hart *et al*	Healed,	73.7%	15.4%
	7 days Rx	14/19	2/13
Davidson and	No Stain,	41.4%	21.4%
Evans	7 days Rx (Positive virus culture).	7/17	3/14
Total cases studied.....		126	109

Source: 11–16.

thelium is exposed to effective antiviral concentrations of IDU (18). When the stroma is wounded or when large areas of the stroma are destroyed so that the maximal division of stromal cells is stimulated, there is a decrease in the uptake of tritiated thymidine and in the strength of stromal wounds after exposure to large doses of IDU. Except for occasional cases of allergy, therefore, IDU can be said to be nontoxic and to cause only a modest retardation in stromal wound healing.

Although the toxicity of IDU may be minimal, the other deficiencies of this agent are serious. We find that in experimental viral keratitis it is possible to apply standard pharmacological techniques and to plot *in vivo* dose-response curves (19). The severity of ulcers may be graded, let us say, on the basis of zero to four, zero representing complete healing of the ulcers and four representing involvement of the full cornea. If enough animals are used and grading is done on a double-blind basis, a comparison of the severity of ulcers in the treated eyes and in the control eyes permits the estimation of therapeutic efficacy and the plotting of a standard dose-response curve. When this is done, the concentration of IDU in drops that gives 50 per cent improvement is 0.06 per cent, whereas the maximal concentration used clinically is 0.1 per cent. The final concentration, therefore, is close enough to ED_{50} so that the margin in excess appears to be very small indeed. This fact becomes especially important when the competitive antagonism of steroid effect is considered. IDU competitively antagonizes the deleterious effects of corticosteroids on the eye, but for maximal safety IDU must be used with great frequency and corticosteroids as seldom as possible. An agent with greater solubility or potency would be expected to have considerably greater effect; however, the steroid antagonism by IDU is sufficient for this combination to be clinically useful in the treatment of disciform keratitis and herpetic iritis.

A drug that decreases the amount of virus might be expected to decrease the tendency to latency and the subsequent rate of recurrences. On the other hand, the suppression of multiplication might favor the development of latency. In actual fact, extensive clinical studies by Carrol indicate that the recurrence rates after IDU therapy, placebo therapy, and cautery are virtually identical (20).

Drugs such as IDU can inhibit virus multiplication at several sites. IDU is chemically similar to thymidine, the naturally occurring component of DNA, and its activity is due to the competition in metabolism between the normal nucleotide and the drug. IDU inhibits the phosphorylation of thymidine and the polymerization of thymidine into DNA. It also can be incorporated into the viral DNA resulting in a false DNA that cannot replicate and that may be sufficiently different from the normal virus DNA so that it cannot be assembled with the protein and lipid coat or capsid into complete virus.

In tissue culture, IDU-resistant virus is easily produced by passing normal virus in progressively higher concentrations of the drug. This resistance is genetically stable, but its mechanism is not clear. Cancer cells resistant to IDU and bromodeoxyuridine (BDU) acquire this resistance by loss of the enzyme thymidine kinase (TK). This enzyme is required for the initial phosphorylation of the IDU and BDU and the passage of these drugs into the cell; the loss of the enzyme prohibits their entering the metabolic pathways. Infection by the viruses of herpes simplex and vaccinia induce the cell to produce large amounts of thymidine kinase, and it has been postulated that loss of the ability to induce this enzyme might be responsible for virus resistance to IDU. If this were true, it would be difficult to understand the mechanism by which trifluorothymidine could be effective against IDU-resistant virus, since it too must be phosphorylated, presumably by the same enzyme. This problem has been directly studied (21). Centifanto investigated the treatment of normal virus, IDU-resistant virus, and a mutant strain of virus that was deficient in the ability to induce thymidine kinase (TK− virus). She found that the TK− virus was susceptible to IDU despite its genetic inability to cause cells to produce large amounts of this enzyme, although slightly larger amounts of IDU might be required for therapeutic activity. Similarly, IDU-resistant virus induced cells to make normal amounts of thymidine kinase. Thus, the mechanism of virus resistance to IDU must be different from that by which cancer cells become resistant to this drug.

The clinical importance of IDU resistance is

not completely clear, since there are several factors that may be responsible for a lack of therapeutic efficacy of the drug. One of the most common difficulties with therapy is the treatment of the wrong type of lesion. The only lesions against which antiviral drugs are effective are those caused by multiplying virus. Occasionally post-infectious (or metaherpetic) ulcers are treated by these agents. Since these ulcers are not caused by multiplying virus, the drugs are ineffective. Similarly, iritis and disciform keratitis are not caused by multiplying virus. The second possible cause of therapeutic ineffectiveness is insufficient concentration of the drug. Concentrations of IDU are limited by its poor solubility and penetration. The experimental results previously cited indicate that the concentrations of IDU obtainable are just slightly above those necessary for therapeutic efficacy. It seems likely that some more virulent strains, or strains that affect the conjunctiva and lids—tissues that are less susceptible to IDU therapy—may be clinically resistant to the drug.

Whether true IDU resistance, in the sense of genetically determined enzymatic resistance to this agent, is becoming a progressively significant problem is not clear. The studies of Maxwell (22), however, indicate that resistant cases are not becoming more frequent as the use of IDU is continued. They also show that newer agents are effective in treating IDU-resistant cases.

Cytosine arabinoside (CA) is an antiviral agent that in some ways is superior to IDU (23, 24). One major advantage of CA is that there is no cross-resistance between this agent and IDU, so that virus resistant to IDU is sensitive to CA (19). In addition, resistance does not develop to this agent as rapidly as it does to IDU (25). From the point of view of virus resistance, it may be clearly superior. In addition to its effect on resistant virus, it is very soluble and can be highly concentrated. This permits less frequent therapy, and a much more efficient antagonism of the steroid effect. The only property of this agent that renders it less than ideal is its toxicity. When given to patients, it produces small, glittering, vacuole-like areas in the lower layers of the corneal epithelium (26). If therapy with high concentrations of CA is continued, small staining areas may appear on the corneal epithelium and ocular inflammation may develop. It is therefore much less selective than IDU in inhibiting virus rather than host DNA. This toxicity is a sufficiently serious problem to limit the clinical usefulness of this drug.

MADU (Methylaminodeoxyuridine) is also effective in antagonizing herpes simplex keratitis. It is soluble and it has the great advantage of being only slightly incorporated into DNA (27). Unfortunately, its potency is so low that it will probably not be clinically useful (28).

Heidelberger (29) synthesized 5-Trifluoromethyl-2′-deoxyuridine (F_3TdR), which is the most potent agent to date for treating experimental herpes simplex keratitis and results in the most rapid and complete healing of the ulcers. Sufficient concentrations can be obtained with this agent to permit optimal steroid antagonism and effective therapy with infrequent use. Moreover, even when F_3TdR is administered to man every two hours during the day and night in a concentration approximately 100 times the therapeutic concentrations of IDU, it appears to cause no corneal toxicity (28, 30). It does not share resistance with IDU, so that IDU-resistant virus remains sensitive to trifluorothymidine. It is possible that with the ability to clinically employ high concentrations of this drug local adenovirus infections may also be treatable. Double-blind clinical trials are in progress to investigate these problems.

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SECTION A. PROBLEMS OF VACCINE DEVELOPMENT AND SECTION B. CHEMOTHERAPY

DISCUSSION

CHAIRMAN RASKA: We will now proceed with the discussion. I call on Dr. Frank T. Perkins, National Institute for Medical Research, London, England.

DR. PERKINS: Much attention has been paid to the characteristics and purity of the seed virus from which a vaccine is made. It is now time for us to show similar concern for the quality of the substrate used for growth of the virus.

Ever since the SV-40 virus was found to be a contaminant of some batches of rhesus monkey kidney tissue, there has been a trend to avoid the use of this tissue and to replace it with a cell system free from contaminant viruses. The production of live attenuated measles vaccine is a good example. The manufacturers have established large flocks of chickens free from fowl leukosis viruses, and far less material has been rejected because of contaminant viruses than was the case with poliomyelitis vaccine production in monkey kidney tissue. At this Conference we have heard that there are no fewer than five attenuated rubella viruses under investigation and each one is grown on a different cell system. Had the rubella virus been isolated 10 years ago, it is doubtful whether anything but monkey kidney tissue would have been considered.

The recent discovery of the presence of oncogenic genomes having the ability to combine with the viruses grown for vaccine production emphasizes the need to avoid any tissue in which there may be the slightest suspicion of contamination. Thus the move away from monkey kidney to substrates derived from healthy animals known to be free from contaminating viruses is an important step in the right direction. It is still not the ideal solution, however. Both Dr. Ikić and Dr. Hayflick have mentioned the possibility of establishing a cell seed system, which is as important to our future needs in virus vaccine production as the virus seed system is today. A diploid cell population that can be frozen indefinitely until it has been extensively examined and standardized before use in vaccine production has enormous advantages. As you have heard, some vaccines are already being produced in such a system of human diploid cell cultures, and confidence in the use of such tissue is rapidly growing. All objections to the use of such a cell system are theoretical, having no practical foundation, and the theoretical objections apply equally well to the substrates being used today.

The Permanent Section on Microbiological Standardization of the International Association of Microbiological Societies regards the need to move away from the use of "unacceptable" substrates as an urgent problem and has formed a committee on cell cultures to investigate the possibility of establishing standards for "acceptable" tissues. This committee collects, evaluates, and disseminates information concerned with any *in vitro* substrate suitable for virus vaccine production. The most important requirements are that the substrate be free from contaminants, remain normal throughout its life, and be capable of being established as a cell seed system. The characteristics gaining the most attention by the committee are (a) biochemical tests that may be used as an index of normal tissue, and (b) the acceptable limits of karyology that all candidate tissues should satisfy. The committee has made available the report of their third meeting, and, although the revised standards for

karyology are not included, we will circulate them to all those who indicate their interest in this subject.

Our virus vaccines will certainly be much safer when we use a cell seed system, and we should move toward this ideal situation.

CHAIRMAN RASKA: Thank you, Dr. Perkins. Our next discussant is Dr. Frederick P. Nagler, Virus Laboratories, Department of National Health and Welfare, Ottawa, Ontario, Canada.

DR. NAGLER: The problems concerning the control of viral vaccines may be somewhat different in countries that are dependent on the importation of these products.

The introduction of international requirements for biological substances, including viral vaccines, by the World Health Organization has greatly assisted these countries in selecting vaccines that have been duly prepared and tested, and it is gratifying to know that most manufacturers take the necessary steps to see that their products meet these requirements.

However, some problems still remain to be solved by the health authorities in the importing countries. In some cases the prospective recipients of the vaccines have susceptibilities and clinical reactions to the vaccine viruses that differ from those of the persons tested in the original trials. It is then the responsibility of the government authorities in these countries to ascertain the safety and potency of the products in question.

In Canada, for instance, before using one of the live measles vaccines on the Eskimo population of the Northern Territories, the Federal Government carried out a limited field trial under close medical supervision in one of these regions. It was found that the clinical reactions in the Eskimos were somewhat more severe than those in the white population of the same area, and, as a result, it was decided to vaccinate Eskimos only under very strict medical supervision.

Although the regulations and requirements for the production and control of viral vaccines are highly specified, the detailed design of the field trials for testing their safety and efficacy in the human population has often been left in the hands of the manufacturers. This may lead to quite a variation in results, particularly if the clinical follow-up is done by nonprofessional personnel and if no accurate diagnostic procedures are employed in cases of adverse reactions.

However, it should be stressed that even under the most stringent conditions certain variations in the results of such trials will have to be expected. This was clearly demonstrated during a series of well-designed field trials on attenuated measles vaccines carried out by a number of independent research teams in different countries under the auspices of the World Health Organization. The experience gained from this study emphasizes again the need for well-planned and well-controlled field trials. It would be advisable to have the detailed plans of such trials submitted to the government authorities by the manufacturers prior to their commencement and to have "on site" inspections carried out by these authorities during the trials.

In general, it has become more and more the responsibility of government authorities to check periodically the safety and potency of commercially produced viral vaccines once they have been licensed and released for general use. This is usually accomplished by retesting the vaccines in government laboratories and by introducing a reporting system on adverse reactions encountered. In Canada a permanent advisory committee on immunizing agents, established by the Federal Government several years ago, screens all reports on adverse reactions submitted to the Food and Drug Directorate by medical health officers and practicing physicians throughout the country and advises the Government on any steps to be taken to avoid future complications.

CHAIRMAN RASKA: Thank you, Dr. Nagler. I now call on Dr. C. Henry Kempe, Department of Pediatrics, University of Colorado Medical Center, Denver, Colorado.

DR. KEMPE: Sixteen children and adults suffering from *Vaccinia gangrenosa* or very grave progressive *Eczema vaccinatum* who had failed to improve after adequate therapy with vaccinia-immune gamma globulin were recently treated with methisazone (1-methylisatin 3-thiosemicarbazone).

Three children and four adults with life-threatening progressive *Eczema vaccinatum* who

continued to show extension of old lesions and development of new ones after four to six days of vaccinia-immune gamma globulin therapy were given methisazone orally in an initial dose of 200 mg/kg, followed by 50 mg/kg every six hours for three days. In all seven patients the clinical response was prompt and seemingly specific.

Of nine patients (six children and three adults) suffering from prolonged and relentlessly progressive *Vaccinia gangrenosa* unresponsive to antibody therapy, five (two children and three adults) showed early reappearance of vaccinia-delayed hypersensitivity, prompt clinical response, and virologic cure after treatment with methisazone—although complications of their underlying disease led to the ultimate death of four of these five patients. The other four patients in the group failed to show any clinical or virologic response despite the fact that *in vitro* testing showed strain sensitivity of one strain tested to date. In three of these four failure cases a single high blood level was obtained after the loading dose, but absorption as judged by serum levels was minimal thereafter. Possible explanations for this marked failure to obtain adequate serum levels might be drug binding by the large vaccinal mass or the presence of an inducer enzyme in these patients. Vomiting did not account for these findings.

The treatment of serious *Eczema vaccinatum* can be expected to yield prompt results with the combined use of vaccinia-immune gamma globulin and methisazone. In the case of *Vaccinia gangrenosa*, however, the great immunologic variability underlying the clinical syndrome of this disease makes very thorough immunologic studies necessary before any treatment is undertaken. Results to date suggest that patients with acquired immunologic defects (lymphoma, leukemia) and those who have single defects (Bruton-type agammaglobulinemia) do better than those with multiple defects (Swiss type).

When conventional gamma globulin or methisazone therapy fail, multiple exchange transfusions with high-titer blood may be life-saving if the patient has an intact lymphoid system and is not immunologically tolerant. However, in the absence of small lymphocytes and of delayed hypersensitivity to various antigens (*Monilia*, etc.) in the patient, the use of competent white blood cells in even small transfusions of whole blood has led to acute and fatal graft-versus-host disease.

CHAIRMAN RASKA: Thank you, Dr. Kempe. Our next discussant is Dr. Kenneth W. Cochran, School of Public Health, University of Michigan, Ann Arbor, Michigan.

DR. COCHRAN: The three principal speakers in this section represent the three pioneer clinically effective antiviral drugs—methisazone, amantadine, and idoxuridine. From their presentations certain facts stand out. First, successful clinical experience has been reported and therefore chemical modification of viral disease is now more than an act of faith performed in the laboratory. This is particularly significant in view of past pessimism about the prospects of antiviral drugs. Secondly, there are *three* pioneer drugs, which should allay the suspicion that the first one was totally accidental or was somehow unique. Whether future control of viral disease relies on drugs, vaccines, or both is a matter for the tribunal of time, but now, at least, the flexibility of choice is in prospect. It is important, therefore, to be sure that the trials are fair so that the basis of choice is valid and known. One should, for instance, emphasize what two of the principal speakers conveyed in their titles—namely, that of the three pioneer compounds two, methisazone and amantadine, are effective prophylactically. Whereas the use of vaccines for *immuno*prophylaxis is commonly accepted, there may be a tendency, perhaps even indicated in the naming of this section of the program, to associate drugs with treatment rather than prevention—chemo*therapy* rather than chemo*prophylaxis*.

As antiviral drugs leave the protection of their early investigators and emerge into the clinical arena, proper long-term evaluation and general acceptance will require their proper use. Proper use, especially prophylactically, may have additional importance now that viral resistance to each of the three pioneer drugs has been described, though only experimentally for two of them (1–4).

In comparing *chemo*prophylaxis and *immuno*prophylaxis, one may note that in general the chemical approach is disadvantageous in regard

to frequency and duration of treatment but offers advantages in rapidity of onset and breadth of spectrum. Amantadine, for instance, appears to have activity against all A subtypes of influenza virus and several other myxoviruses (3, 5). Also, in relation to spectrum some apparently minor changes in structure can lead to major changes in activity, such as the gaining of antipoliovirus activity with the addition of butyl groups to methisazone, a shift in spectrum from large DNA virus to include small RNA virus (6), and the engendering of hope that other molecular changes can lead to further broadening of antiviral spectra.

Two of the principal speakers mentioned other antiviral substances in addition to the three pioneer compounds. To that list may be added α-methyl-1-adamantane-methylamine hydrochloride (Exp 126), which in early laboratory studies appears to have some advantages over amantadine in treatment of viral respiratory disease (7, 8). The development of additional substances emphasizes the problem of testing raised by one of the speakers. No matter what type of test is used for discovery, some sort of demonstration of activity *in vivo* would seem to be necessary before clinical testing. Again referring to the three prototype compounds, it is of interest that each was introduced into clinical testing with data derived for the most part from simple tests in single species in common laboratory rodents—rabbits for idoxuridine (9) and mice for methisazone (10) and amantadine (5, 11).

Some promising new sources for other antiviral agents have also been introduced by our speakers. Phagacin, reported by Dr. Kaufman, may presage a variety of antiviral natural products derived not only from common antibiotic sources but also from other bacteria, the examples being too many to cite here, and from higher organisms, such as shellfish (12), mushrooms, and miscellaneous plants (13–18). Some of the last-named substances occur as foods or as drugs of folklore and may therefore already be making an inapparent contribution to the control of viral disease. Thus, natural product sources of antivirals may be worthy targets for investigation of the ecology of disease as well as for development of new drugs.

It has been said that to some extent the language of infectious disease is the language of warfare—the pharmacologist, for example, revels in his armamentarium of drugs to attack various conditions. In the militaristic language of our present missile age, one could say that there has been a drug gap between man and his pathogenic viruses but that the antiviral drugs mentioned here represent the first and second generation weapons in a race in which man is still behind but is gaining on the viruses.

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CHAIRMAN RASKA: Thank you, Dr. Cochran. Dr. Togo would like to have a few minutes to comment on the Jackson-Stanley paper.

DR. TOGO: In connection with Dr. Jackson's paper, I would like to present our data on double-blind volunteeer studies conducted to assess the prophylactic efficacy of amantadine (1-adamatanamine hydrochloride) and its analogue Exp 126 (alpha-methyl-1-adamantanemethylamine HCl).

Male inmate volunteers were carefully screened for serum-neutralizing antibodies. Subjects whose prechallenge serum contained none or a low level of antibody for A_2 influenza, Rockville/1/65 strain, were enrolled. The drug, amantadine in 100 mg capsule or Exp 126 in 200 mg tablet, was given every 12 hours for about 10 days. A total of 300 mg of amantadine or 600 mg of Exp 126 was given prior to viral challenge. Viral challenge materials were prepared with the Rockville/1/65 strain in primary human embryonic kidney cell culture, and an inoculum containing 64,000 $TCID_{50}$ was administered nasopharyngeally.

A classical flu syndrome of two to three days' duration was induced beginning within 36 hours after the challenge. In the amantadine study, severe illness was observed in 6 of the 29 placebo-treated controls but not in any of the 29 subjects who received the drug. In the Exp 126 study, however, 15 of the 28 controls and 1 of the drug-treated subjects had severe illness. There was a striking difference in the over-all incidence of clinical illness between the control and drug-treated subjects. In the amantadine study, 18 cases were observed in the placebo-treated group, whereas 8 cases were seen in the drug-treated group. In the Exp 126 study, clinical illness was induced in 24 of the placebo-treated subjects and in only 7 of the drug-treated subjects. The total number of subjects with febrile illness was also significantly lower among the drug-treated subjects. The illness observed in drug-treated subjects was considerably less severe than the typical influenza infection observed in control subjects. Antibody acquisition was almost 100 per cent in both studies, although the geometric mean neutralizing antibody titers in postchallenge sera were fourfold lower in the drug-treated groups.

Thus amantadine and its analogue Exp 126 significantly diminished the severity and occurrence of experimentally induced Asian influenza infection yet allowed for the formation of antibodies.

CHAIRMAN RASKA: Thank you. Dr. Beale from Glaxo Laboratories would like to speak.

DR. BEALE: In connection with Dr. Kaufman's demonstration of the value of the rabbit's eye for assessing chemotherapeutic agents against herpes simplex, I should like to point out that Professor Barrie Jones and Dr. Wise have developed a quantitative technique for titrating viruses in the rabbit's cornea.

Figure 1 shows a titration of vaccinia virus in a rabbit's eye by this technique. Serial dilutions of virus are inoculated into the cornea by means of capillary pipettes. On each eye a total of 16 insertions can be made. The same technique can be used to titrate herpes simplex virus, but the lesions are smaller and if readings are delayed more than 48 hours secondary lesions occur. The technique allows a quantitative assessment of the effect of antiviral agents.

Figure 2 shows the effectiveness against vaccinia virus of an unknown substance present in antibiotic fermentations being screened for antiviral properties.

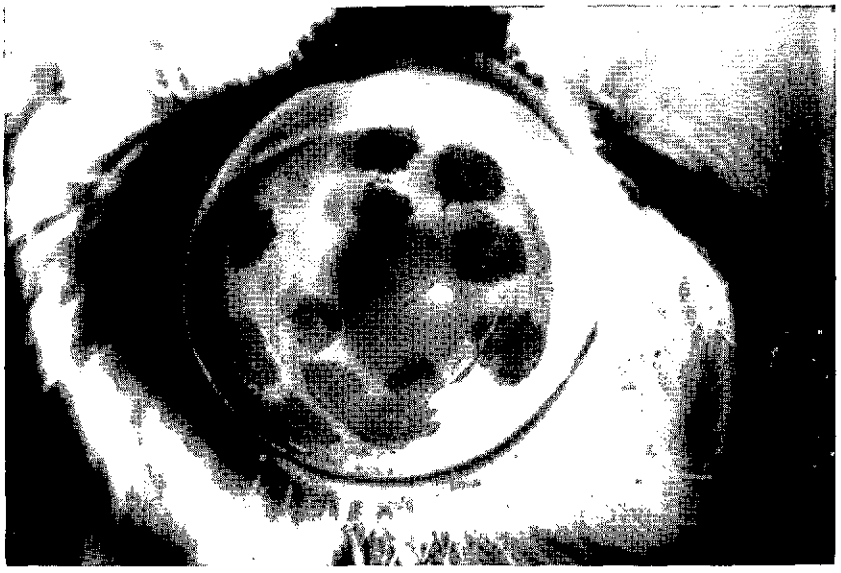

Fig. 1. Titration of vaccinia virus in a rabbit's eye.

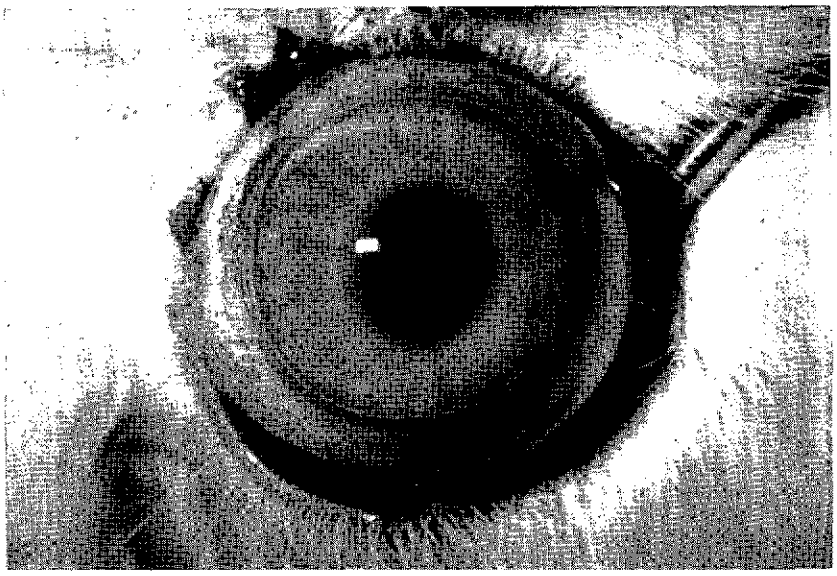

Fig. 2. Effectiveness against vaccinia virus of an unknown substance.

CHAIRMAN RASKA: Thank you, Dr. Beale. Is there anyone else who would like to make additional comments?

DR. UNGAR: I have a question for Dr. Bauer. It is rather an obvious one. Could these unpleasant reactions be avoided by a different method of administration or by an improved form of presentation, such as capsules?

DR. BAUER: Unfortunately, methisazone can only be given by mouth. Even so, absorption is no more than 10 per cent of the dose administered. When it is given intramuscularly or subcutaneously it can be picked up reasonably well. Small animals, the mouse, for example, can take up a dose of 1 mg quite easily. In larger animals and in man, however, doses of 100 or 200 mg are necessary, and the surface/volume

ratio is so unfavorable that almost none is taken up. We are therefore compelled to administer the drug by mouth.

It is possible, of course, that if it were given in enteric coated capsules or some similar form some of the gastric irritation now associated with it might be eliminated.

Another possible approach would be through derivatives of methisazone that are not metabolized to such an extent, or that are picked up to a much greater degree from the gut, so that small doses can be given.

At the moment, however, I think we are stuck with a drug that must be given by mouth, and, although this method of administration causes vomiting in a fair proportion of cases, we cannot do very much about it.

DR. TYRRELL: I should like to add a comment about some work being done at Salisbury on a different substance. Fucidin, which was originally derived as an antibacterial agent, has been found in some laboratories to inhibit the multiplication of Coxsackievirus A_{21}, previously known as Coevirus, in tissue cultures.

Doggett and others administered this virus to volunteers who had been treated with Fucidin and who had sufficient amounts of the drug in their blood to be highly inhibitory as measured *in vitro*. However, the volunteers apparently were not protected. The viruses recovered from them were still sensitive to the drug. The apparent explanation for this phenomenon was that the concentrations of the compound in the nasal secretion were far lower than they were in the blood.

This is one of the difficulties I think we shall encounter in moving from laboratory tests—either with test tubes or with animals—to tests in man. The answers we find may be useful to other workers as well.

SECTION C. INTERFERON

THE INTERFERONS: SOME UNSOLVED PROBLEMS OF ACTION AND BIOSYNTHESIS *

ROBERT R. WAGNER AND THOMAS J. SMITH †

DR. WAGNER (*presenting the paper*): Progress in interferon research has been truly encouraging during the brief decade since Isaacs and Lindenmann (1) made their original observations. After considerable early skepticism, even our colleagues in biochemical virology have begun to recognize the potentialities of interferons as biologic probes for investigating cellular factors that regulate the expressions of viral nucleic acids. Of equal importance is the insight gained from such studies into potentialities for chemotherapy and chemoprophylaxis of viral infections. This is not to say that successful application of this information is a simple engineering problem. Quite the contrary: with all significant scientific discoveries, more questions have been generated as each problem has been partly solved. In fact, we now appear to be fast approaching the stage at which the questions are proliferating far more rapidly than the answers. The danger, of course, is that the ascending curve of our knowledge may be reaching a premature plateau. To avoid such an eventuality, it is necessary to raise our sights above the limitations of our methodology. There can be little virtue in playing the same biologic theme with minor variations.

The lesson seems clear that we must continue to direct our efforts to solving the central problems of the chemical nature, action, and biosynthesis of interferons. Some of the techniques are available, and others soon will be, to approach these problems directly rather than grope in the dark with a methodology that has just about outlived its usefulness. Our purpose in this report is to indicate two areas of interferon research that promise to be highly rewarding. No attempt will be made to cover the field, which has been adequately done in recent reviews (2, 3).

ACTION

The prospects seem bright for coming to grips with key problems that relate to the action of interferons. The purification procedures, although still far from perfect, should now enable investigators to test interferon effects under rigidly controlled conditions. Many of us were led astray in the early days by premature attempts to localize the cellular sites of interferon action. With the wisdom of hindsight we now know that most of the effects observed were due to impurities in the preparations we were blithely calling interferons. Unfortunately, the tendency still persists to some extent.

It might be appropriate at this time to make a plea for international standards to be used as references for all workers engaged in biochemical studies of interferon action. It is no longer adequate simply to describe the preparations in common use as having been "partially purified" by precipitation, chromatography, or both. Since it is unreasonable to expect every laboratory to cope with the formidable engineering problems concerned with production, concentration, and purification, the only realistic solution is to ask a few large industrial laboratories to

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† Dr. Wagner (Department of Microbiology, Johns Hopkins University School of Medicine, Baltimore, Maryland); Dr. Smith (Walter Reed Army Institute of Research, Washington, D. C.).

undertake this job on a contractual basis. The World Health Organization would seem to be the logical agency for supervising such a program because of the international implications of this research. Such an undertaking is not merely an academic exercise. If interferons are to realize their potentials as chemotherapeutic agents, we must make a concerted effort to solve the fundamental problems of their mechanisms of action before the knowledge can be intelligently applied.

The direction of this research is coming into clearer focus. A great deal of the impetus came from the ingenious experiment of Joyce Taylor (4), who proved that the action of at least one interferon is cell-mediated and dependent in its early phase on intact capacity to transcribe cellular RNA. She showed that when cells were first exposed to actinomycin and then to interferon, viral RNA synthesis proceeded more or less normally after infection. However, pretreatment of cells with interferon resulted in marked inhibition of viral RNA synthesis despite the later addition of actinomycin.

It is a pity that no one has satisfactorily pursued this lead to study the kinetics of the reaction in greater detail, but the implications of her data, subsequently confirmed by others, seem clear. It can be assumed that interferon is not the active antiviral agent but merely serves to induce the transcription of a specific messenger RNA that codes for a new protein. This protein (or conceivably another product that it in turn induces) seems to be the true inhibitor of viral RNA synthesis. Other investigators have found that interferon action also has a puromycin-susceptible (5) and fluorophenylalanine-susceptible (6) phase, thus suggesting the requirement for protein translation of the hypothetical, newly transcribed mRNA.

Logic dictates that a concerted effort be made to find the Taylor antiviral protein, if it exists, by studying its presumed interaction with viral RNA. Unfortunately, it seems unlikely that it can be identified by simply adding extracts of interferon-treated cells to virus-infected cells. If it were that easy, several of us would have stumbled on the active principle long ago (7). It seems prudent to assume that the Taylor protein is not readily transported from cell to cell, as interferon itself appears to be. Therefore, the most reasonable approach would seem to be to test for the presence of the interferon-induced Taylor protein in cell-free polypeptide-synthesizing systems of the Nirenberg type.

Recently, Marcus and Salb (8) have brilliantly exploited such a system to demonstrate that an interferon (presumably acting by the induction of a Taylor protein) can cause a translational defect in the capacity of viral polyribosomes to synthesize polypeptide directly. Phenol-extracted RNA of Sindbis virions binds to normal (not treated with interferon) chick ribosomes, and the resultant polysomes incorporate amino acids in a complete energy-generating system and then dissociate by the end of 30 minutes at 37°. In contrast, Sindbis viral RNA associates somewhat less well with ribosomes from interferon-treated cells, but the complexes do not break down and fail to incorporate amino acids into acid-precipitable polypeptides. Moreover, interferon does not convert ribosomes to the resistant state if actinomycin is present in the cultures. This effect of interferon on ribosomes appears to be highly selective for viral RNA; the coding efficiency of polyuridilic acid and pulse-labeled cellular mRNA remains intact after being complexed with ribosomes from interferon-treated cells. Marcus and Salb (8) interpret their data as indicating that "interferon acts by inducing a translational inhibitory protein which binds to ribosomes and prevents translation of viral RNA while permitting normal readout of cellular mRNA." Extending this interpretation also leads to the hypothesis that an inhibition of the synthesis of viral replicase is the primary lesion caused by interferon, and thus results in inhibiting viral RNA synthesis. Under these conditions the infection would abort.

If these experiments can be confirmed and extended to other virus-cell systems, the implications for further research on interferon action would be far-reaching. Among other things, a means would be provided of searching for the translational inhibitory (Taylor?) protein—that is, for the true chemotherapeutic agent in interferon systems. Such an experimental approach would also enable investigators to compare the action of the numerous varieties of interferons that are being uncovered. Another dividend might be the insight gained into the selective inhibition of viral RNA synthesis with-

out impairment of cellular RNA synthesis, which would provide impetus for a rational search for simpler and more selective antiviral compounds.

BIOSYNTHESIS

The problems that arise in the investigation of interferon biosynthesis appear to be much more formidable than those concerned with interferon action. The basic difficulty is the necessity of relying on rather crude techniques for producing, characterizing, and quantitating interferons. Our dilemma is not unlike that of all other biologists who find that they can assemble only trace amounts of an elusively active chemical. The difficulty is compounded by the staggering multiplicity and the apparent molecular inhomogeneity of the interferons that are constantly being uncovered. Instead of having to deal with only a single interferon, which might permit a neat unitarian theory of mechanism of production, we are confronted with many interferons even in preparations that originate from a single animal species or a presumed single cell type. It might help, parenthetically, if we paid more attention to cloning techniques to ensure more uniform cell populations, but even this would probably not resolve the present problems.

Nothing daunted, many investigators have attempted to approach problems related to interferon biosynthesis by rather crude biologic methods and have come up with a number of very interesting leads. Only a limited number can be recounted here.

1. Several molecular species of interferon can be identified in sera of animals infected with a single viral or nonviral inducing agent (9).

2. The molecular weight of virus-induced interferon may differ considerably from that of endotoxin-induced interferon (10).

3. Unlike interferon synthesis by cultured cells, interferons that appear in sera of intact animals in response to the intravenous injection of bacterial endotoxin or statolon are not suppressed by puromycin or cycloheximide (11). This finding has led to the hypothesis that endotoxin or other nonviral inducers cause the release of preformed interferon, a reaction that presumably should not require an intact capacity of the host to transcribe RNA or synthesize protein *de novo* (11, 12).

These experiments seem to indicate the existence of multiple genetic sites and of at least two different mechanisms for the production of interferons.

We have made an attempt to examine the general applicability of these hypotheses by resorting to *in vitro* systems that it was hoped might permit somewhat greater control of experimental variables than the intact animal does. The models chosen were primary cultures of rabbit cells, because of their capacity to produce interferons in large quantities and at extremely rapid rates. One important lead came from the findings of Glasgow (13) and Wheelock (14), who have shown conclusively that leukocytes of mouse and man are efficient producers of interferon in response to viral or nonviral inducers. In studies to be reported in detail elsewhere (15, 16) we have found that cultures of peritoneal macrophages obtained from rabbits with sterile peritonitis synthesize various molecular species of interferon.

Kinetics of Virus-Induced Interferon Synthesis

Representative data are summarized in Table 1. When monolayer cultures of $\sim 8 \times 10^7$ peritoneal macrophages were exposed to Newcastle disease virus (NDV) at an input multiplicity of $\sim$4 PFU/cell, interferon appeared in the medium within two hours. The titers rose to a peak of 10,000–20,000 PDD_{50}/ml at four to six hours, as measured by plaque reduction of vesicular stomatitis virus (VSV) plated on monolayers of rabbit kidney (RK) cells. The RK cells themselves showed a similar response to NDV infection, and the final yields were roughly proportional to those from macrophage cultures. Intravenous injection of the same amount of NDV caused a somewhat more rapid appearance of circulating interferon, which reached equivalent peak titers by the end of two hours. Therefore, the kinetics of interferon synthesis differs only slightly and probably not significantly in these three systems.

We also tried to distinguish between the interferon-synthesizing capacity of RK cells and macrophages by testing their susceptibility to actinomycin. Cells in separate cultures were

exposed to actinomycin, 10 μg/ml, for one hour at various intervals before or after infection with NDV. Media collected six hours after virus induction were dialyzed free of residual actinomycin and tested for interferon content (Table 1). No detectable interferon was synthesized by either cell type exposed to actinomycin before or at the time of NDV infection. However, complete resistance to this action of actinomycin was noted after one hour in macrophage cultures and two hours in RK cell cultures. It appears from this experiment that the hypothetical interferon-specific mRNA is transcribed somewhat sooner in macrophage cultures. Even so, this observation probably does not signify an appreciable difference in the mechanism by which virus-induced interferon is synthesized by the two cell types.

TABLE 2. INTERFERON PRODUCTION BY CELL CULTURES IN THE ABSENCE OF VIRAL INFECTION

Predominant cell type		Maximum interferon yields*			
		4°	37°	4° to 37°	With endotoxin†
Peritoneum	granulocyte		±5		
Peritoneum	macrophage	<5	80	160	640
Spleen	mononuclear		<5		
Lung	macrophage				30
Kidney	epithelial		<5		

*Media collected 6 to 24 hours after incubation of cultures were tested for inhibition of VSV plaque formation on RK cells. Yields expressed as PDD_{50}/ml.
†*E. coli* lipopolysaccharide, 10 μg/ml, added to cultures at time 0 and incubated at 37° for 6 hours.

Interferon Synthesis by Uninfected Macrophages

To serve as controls for the foregoing studies of virus-induced interferon synthesis, media were removed from uninfected cultures of peritoneal macrophages and assayed for interferon content. Much to our surprise, appreciable amounts of a viral inhibitor appeared in these uninfected cultures. Moreover, the exposure of macrophage cultures to *Escherichia coli* endotoxin, 10 or 100 μg/ml, augmented the yield of this viral inhibitor about four- to fivefold. Both the "spontaneously" produced and the endotoxin-induced inhibitors released from macrophages fulfilled the criteria for classification as interferons, on the following grounds: (a) they were nonsedimentable at 100,000×g; (b) they were nondialyzable; (c) their antiviral activity was completely or almost completely destroyed by crystalline trypsin; (d) there was no direct antiviral activity against VSV; (e) they actively inhibited encephalomyocarditis virus as well as VSV; and (f) they were specific with regard to animal species. The non-virus-induced interferons were somewhat more labile to heat and acid than the virus-induced interferons, but not enough to make it possible to distinguish between them.

Table 2 shows some of the conditions under which cultured macrophages produce interferons in the absence of infection. As may be noted, interferon appears in the cultures incubated at 37° but not in those incubated at 4°. Slightly but consistently greater yields were obtained when the macrophages were kept at 4° for a day or two and then warmed to 37°. No inter-

TABLE 1. VIRUS-INDUCED RABBIT INTERFERONS

Source	Interferon titers after NVD induction*				% 6-hour IF yield† when actinomycin added at		
	1 hr	2 hr	4 hr	6 hr	0 hr	1 hr	2 hr
RK cells.....	<10	80	2,560	3,840	<0.1	8	88
Macrophages....	<10	1,280	10,240	15,360	<0.1	100	100
Serum.....	160	10,240	10,240	5,120			

*One ml NDV (3.2×10^8 PFU) was inoculated on rabbit kidney and macrophage cultures or injected intravenously. Culture media or sera were assayed for interferon by inhibition of VSV plaques on RK cells. Titers are expressed as 50 per cent plaque-depressing dose (PDD_{50}).
†Cell cultures were infected with NDV, exposed to actinomycin D (10 μg/ml) for 1 hour at time of infection (6 hour) or 1 and 2 hours later, and media assayed for interferon (IF) content at 6 hours after infection. Results are expressed as per cent of control yields.

feron could be recovered from ultrasonically disrupted macrophages at 4° or on further incubation at 37°. The addition of endotoxin to the culture media resulted in augmented yields. In fact, the possibility could not be excluded that minor contamination of the media with pyrogenic endotoxin was responsible for the "spontaneous" production of interferon by macrophages. No interferon activity could be detected in cultures of peritoneal granulocytes, renal epithelium, or explanted spleen cells, but alveolar macrophages produced small amounts after endotoxin induction.

Although the interferon yields from uninfected macrophages were only 1 per cent or less of those from virus-infected macrophages, the conditions for production as recounted above seemed similar in both systems. These observations led to the postulate that macrophages synthesize interferon *de novo* in response to endotoxin induction, unlike intact animals, which have been considered to release preformed interferon after an intravenous injection of endotoxin (11, 12). Our hypothesis was considerably strengthened when we found that the kinetics of interferon synthesis by macrophages is indistinguishable from that induced by viral infection. Interferon appeared in the medium within two hours after macrophage cultures were warmed to 37°, with or without added endotoxin. The titers then rapidly rose to a peak at four to six hours and thereafter remained relatively stationary or declined. Studies with actinomycin provided even more conclusive evidence of a requirement of active cellular macromolecular synthesis for interferon production by uninfected macrophages. Interferon synthesis was completely inhibited by actinomycin added to these cultures within the first hour after induction, but not thereafter. In contrast, puromycin blocked ongoing interferon synthesis at any stage.

It seems permissible to conclude from these data that the "spontaneous" or endotoxin-induced formation of interferon depends on intact transcriptive and translational functions of macrophages. Of course, the results do not provide any basis for determining whether virus and endotoxin induce the expression of the same interferon gene or, for that matter, whether the same cells in these obviously heterogeneous populations are capable of synthesizing both kinds of interferon. We did learn, however, that "spontaneous" production of interferon by a macrophage culture did not impair its capacity to synthesize interferon on subsequent viral induction.

Molecular Weight Estimates of Several Rabbit Interferons

None of the foregoing data provided any real basis for distinguishing among rabbit interferons produced by different cells in response to different inducers. In an attempt to estimate their molecular weights, various interferons were subjected to gel filtration through Sephadex G-100. For this purpose we used a long column with high resolving power, which was calibrated with a series of marker proteins of known molecular weight (16). Each of the following four types of interferon preparations was studied: serum of rabbits injected intravenously with NDV, media from RK cells infected with NDV, media from macrophage cultures infected with NDV, and media from macrophage cultures exposed to *E. coli* endotoxin. Effluents from the column were assayed for interferon by VSV plaque inhibition on replicate plates of RK cells. The assay system seemed quite reliable and reproducible. The molecular weights were estimated according to the method of Andrews (17) by comparing the elution volume of peak interferon activity with that of marker protein peaks.

The pertinent results of a series of experiments are summarized in Table 3. The interferons studied showed a surprising degree of molecular heterogeneity, at least so far as their diffusional properties are concerned. All the preparations appeared to contain at least two molecular species, and endotoxin-induced macrophage interferon may contain as many as five. It is impossible, of course, to determine how many of these different interferons represent polymers or biologically active subunits. In particular, the presence of trace amounts of activity at M.W. $\simeq$ 134,000 in virus-induced RK and macrophage interferons may simply reflect the binding of the active component to a large protein constituent that is biologically inert.

Despite obvious difficulties in interpreting

TABLE 3. MOLECULAR SPECIES OF RABBIT INTERFERONS IDENTIFIED BY PEAK ACTIVITY OF FRACTIONS ELUDED FROM SEPHADEX G-100

Source	Inducer	Major molecular weight classes, ~ values*			
		>134,000	51,000	45,000	37,000
Kidney cells	NDV	+	0	++++	0
Macrophages	NDV	+	0	++	+++
Serum	NDV	++	++++	±	0
Macrophages	Endotoxin†	++	0	±	++

*Comparative amounts of interferon present in each peak fraction are shown on a scale of 0 to ++++, where + signifies barely but definitely detectable and ± signifies variable slight activity on different runs.
†In some preparations of endotoxin-induced macrophage interferon two additional but variable peaks were detected equivalent in molecular weight to 77,000 and 30,000.

these results of gel diffusion studies, several basic points stand out:

1. Virus-induced kidney interferon is predominantly a single homogeneous molecular species of M.W. $\simeq$ 45,000.

2. Virus-induced macrophage interferon also contains a similar component, in addition to another distinct species of M.W. $\simeq$ 37,000.

3. Virus-induced serum interferon may also contain a component of M.W. $\simeq$ 45,000, but the predominant activity is greatest at a molecular weight equivalent to 51,000, and about 10 per cent is excluded from the gel (M.W.$\geqq$134,000). This finding appears to rule out macrophages as the primary source of the interferon synthesized in response to intravenous injection of NDV.

4. Endotoxin-induced macrophage interferon is polydisperse, but one component, which is consistently present, resembles the interferon of M.W. $\simeq$ 37,000 found in media of macrophage cultures infected with NDV.

These experiments leave little doubt that several different interferons can be synthesized by cells of a single animal species. In fact, the possibility cannot be excluded that a single cell may contain the genetic information for synthesis of more than one interferon. The many problems raised by these studies and earlier ones by other investigators (9) can only be resolved by more refined techniques and by the use of different parameters for comparing the physical and chemical properties of interferons. Particular attention should be directed to evaluating the significance of interferon production by macrophages, which have long been suspected of playing a key role in tissue defenses against viral infection.

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SECTION C. INTERFERON

THE ROLE OF INTERFERON IN HUMAN VIRAL INFECTIONS

E. FREDERICK WHEELOCK

Departments of Preventive Medicine and Medicine
Western Reserve University School of Medicine and
University Hospitals, Cleveland, Ohio, U.S.A.

DR. WHEELOCK (*presenting the paper*): The self-limiting course of most human viral infections suggests that host defense mechanisms play a vital role in the recovery process. Substantial evidence has accumulated in recent years to indicate that factors other than antiviral antibodies are concerned in recovery from the primary exposure and infection with a pathogenic virus (2). One potentially important nonimmune mechanism involves the production of substances that inhibit the replication of viruses within infected cells and thereby limit the spread of infection in the host. One such substance is interferon, a small protein produced in cells in response to viral infection. Interferon has strong inhibitory properties *in vitro* against a wide variety of viruses and has been demonstrated to have marked antiviral effects in animal hosts when applied before or at the same time as virus inoculation. Although the importance of interferon in recovery from viral infections in man cannot be fully determined at present, its production in a variety of clinical infections has been well documented. Since this virus-inhibitory protein is present at the site of virus replication it is probable, *a priori*, that it plays an active role in suppressing the infection. The importance of this role will be the subject of the present review.

Interferon Production in Human Viral Infections

Interferon has been detected in a variety of viral infections of man. Two general types of infections have been studied: (a) naturally occurring clinical viral infections and (b) infections produced by the inoculation of active virus vaccines.

The vaccine studies have yielded more meaningful data because of the high degree of control implicit in the experimental design. The identity of the virus and the time of the inoculation are known, the size of the inoculum is constant in all subjects, and subclinical as well as clinical infections can be studied. It is therefore possible to establish temporal and quantitative relationships between interferon production and such other factors of infection as time of onset, type of virus producing the infection, nature and severity of the illness, virus multiplication, and antibody response.

Finally, the development of new active virus vaccines permits studies of the role of interferon in a wide variety of viral infections.

Vaccinia Virus

Interferon has been detected in a variety of cell culture and animal systems infected with vaccinia virus. It was not unexpected, therefore, to find interferon in extracts of dermal crusts from vaccinia virus vaccinations in man (30). The production of interferon at the site of vaccination could either be a nonfunctional response to vaccinia virus replication or serve to suppress virus replication until host immune mechanisms are mobilized. The latter possibility is supported by the demonstration that the vaccinia virus dermal reaction can be completely suppressed in man by the prior sub-

cutaneous administration of interferon (27). Furthermore, recovery from vaccinia virus infection (as measured by titers of virus at the site of inoculation in the skin) in guinea pigs with suppressed antibody responses could be directly related to interferon production at the dermal site (9).

The detection of interferon in dermal crusts of vaccinia virus vaccinations raises the question whether this virus inhibitor might also be present in the crusts of smallpox lesions together with variola virus. The presence of interferon in dermal crusts might inhibit initial virus replication at the site of variolation and lead to the relatively benign disease often seen with this procedure.

Chickenpox

Extracts of dermal crusts from patients with chickenpox were found to contain interferon (Wheelock, unpublished). This finding is of some importance in explaining a report of a seven-year-old boy who developed zones of inhibition of measles rash surrounding chickenpox lesions that had appeared a week before the measles rash (20). The inhibition of measles virus replication by interferon has not been reported, but Enders (6) has suggested that the attenuation of strains of measles virus may be due to the ability of these strains to induce and in turn be inhibited by interferon (see section on Measles below).

17 D Strain of Yellow Fever Virus

The 17 D vaccine strain of yellow fever virus has been shown both to induce interferon production and to be inhibited by it (Wheelock, unpublished results). In a study of young adults, vaccination resulted in viremia in 14 of the 15 volunteers, and a virus inhibitor with properties identical to those attributed to virus-induced interferon was detected in the serums of 10 of the 14 with peak titers between the fifth and seventh days; the other 4 produced no detectable interferon, and the serum of the remaining vaccinee contained neither virus nor interferon (32). The 10 subjects with circulating interferon had viremia on the same day as interferon or on preceding days. Peak interferon production occurred 24 hours after maximum viremia at a time when the mean antibody titer was below 1:10. However, the presence of antibodies against 17 D virus in a majority of the subjects within 24 hours after interferon was detected prevented an assessment of the relative importance of these factors in the cessation of viremia.

Measles

A virus-inhibitory factor with the properties of interferon was detected in the sera of 90 per cent of children who had received the active Edmonston measles vaccine without gamma globulin (23). This interferon appeared in the circulation six to eleven days after vaccination and seemed to parallel the febrile response. No attempts to isolate virus were made and therefore no correlation between viremia and interferon production could be drawn. The time of the appearance of interferon after vaccination suggests that this virus inhibitor might be responsible for the amelioration of clinical measles when the vaccine is administered within three days after exposure to the wild virus. However, no studies on the sensitivity of measles virus replication to inhibition by interferon have as yet been reported. Therefore the possibility of direct virus-virus interference cannot be excluded as an explanation for the amelioration of clinical measles by vaccination.

In a subsequent study (24) it was demonstrated that vaccination with measles virus could inhibit the dermal response to vaccinia virus administered 10 days later. The fact that the highest titers of interferon were present on the tenth day after measles virus vaccination suggests that the inhibition of vaccinia virus replication was caused by interferon. This possibility is supported by the aforementioned ability of interferon to suppress the dermal response to vaccinia virus when administered previously at the same site but prior to the virus (27). The demonstration that endogenously induced circulating interferon may have an *in vivo* inhibitory effect on an interferon-sensitive virus provides a basis for its potential use in the treatment of clinical viral infections.

Influenza Virus

The well established ability of myxoviruses to induce the production of interferon in infected cells made it likely that this substance would be

produced in clinical influenza virus infections in man. A virus-inhibitory substance with properties of interferon has been reported to be present in the acute phase throat washings (14) and sera (Ray, Gravelle, and Chin, to be published) of patients with influenza.

In order to establish temporal and quantitative relationships between interferon production and such other indices of infection as virus shedding, clinical illness, and antibody production, a study of volunteers inoculated intranasally with the Bethesda 10/63 strain of Asian influenza virus was undertaken by Jao, Wheelock, and Jackson (manuscript in preparation). Thirty-nine young adult prisoners were selected and, in a double-blind fashion, 30 received virus and 9 received control preparations. Virus shedding and interferon levels were determined in daily throat washes, interferon and antibody levels were measured in frequent serum samples, and each patient was scored for illness on a 14-point scale including symptoms of upper and lower respiratory tract infections, malaise, and fever.

Figure 1 presents the individual profiles of the 30 volunteers who were given virus. Fifteen were positive in at least one of the categories listed on the ordinate. Two had a noninfluenzal illness and the other 13 are listed below for they failed to produce antibody, virus or interferon or to become ill. None of the 9 controls shed virus, became ill or produced interferon, but 1 developed antibody to influenza presumably from secondary infection.

Fourteen of the 30 developed a rise in antibody, and 10 of these shed virus. One vaccinee shed virus but produced no antibody. Thus an antibody rise was the most sensitive index of infection.

Six of the volunteers in Figure 1 produced interferon in both nasal washings and sera (closed circles); all 6 shed virus, became ill and had an antibody rise. Two others produced

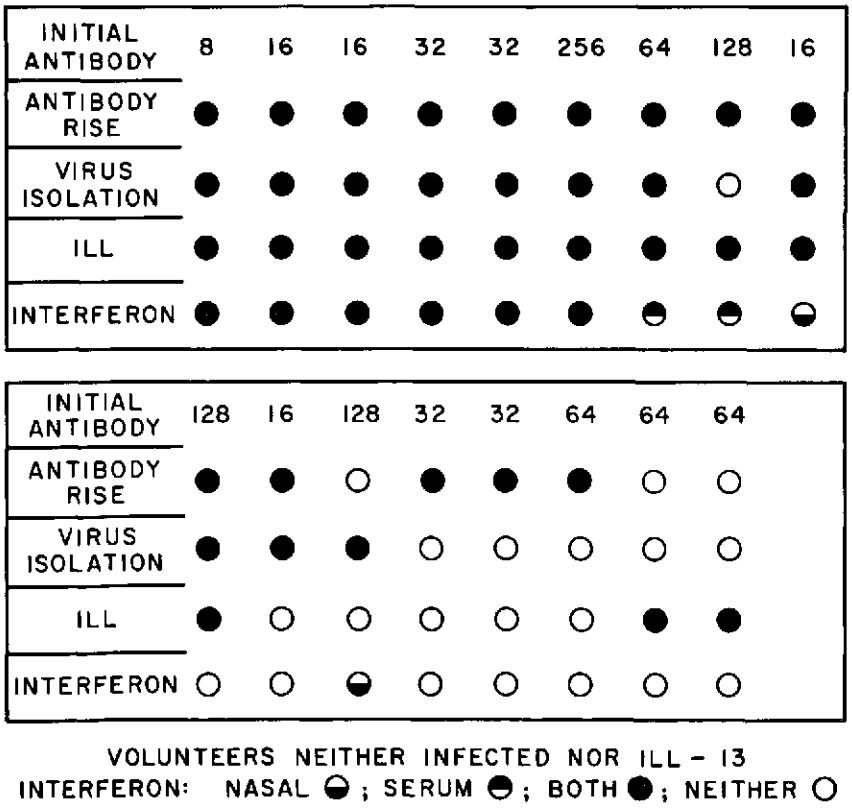


Fig. 1. Profiles of virus, interferon, antibody production, and clinical illness in 30 volunteers vaccinated with active influenza virus.

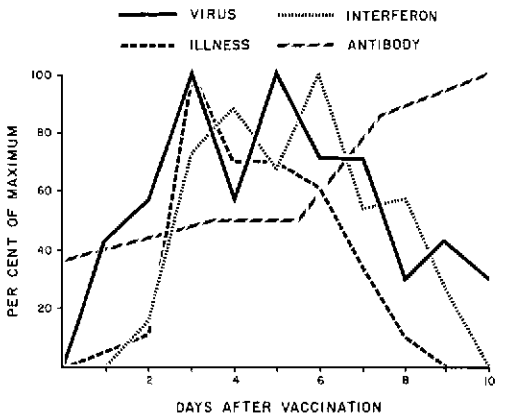
interferon in the nasal washings only (upper half of circle closed); one of these was positive in all other categories; the other was ill but virus was not recovered. Two volunteers produced circulating interferon only (lower half of circle closed); one of these shed virus and was ill, the other shed virus but was not ill.

Table 1 summarizes the interferon results in the 30 volunteers who were inoculated with virus. Of the 10 who produced interferon, 8 were ill, shed virus and had an antibody rise, 1 was ill, shed no virus, and had an antibody rise and one was not ill, shed virus, but had no antibody rise. Of 11 virus shedders, 9 produced interferon, whereas only 1 of 19 nonshedders produced interferon.

Figure 2 shows the daily averages of virus, illness, interferon, and neutralizing antibody production in the 8 volunteers who produced nasal interferon, with each plotted as a percentage of the 10-day maximum. Virus is plotted as a percentage occurrence on each day, since no titers are available. Illness is plotted as the arithmetic mean symptom score, and interferon and neutralizing antibodies are plotted as geometric means.

This figure shows that virus was produced early, rising to a probable maximum in three days and then gradually subsiding. Illness appeared in a sudden burst between the second and third days and subsided completely by the ninth day. Interferon followed the virus curve by about one day. In this plot, interferon and illness went together in the early stages, but illness subsided earlier. Neutralizing antibodies were produced during the first 10 days, reaching a mean titer of 12 on the tenth day.

Although the temporal relationship between interferon production and virus shedding suggests that interferon may be involved in recovery from infection, the production of neutralizing antibodies, even in low titers, during the first 10 days prevents a clear assessment of the role of each of these two antiviral agents in the recovery process.



Note: The eight volunteers under study produced detectable nasal interferon in response to vaccination with influenza virus. Interferon and antibody production were plotted as geometric means; virus shedding as per cent occurrence, and clinical illness on a symptom score basis.

Fig. 2. Temporal and quantitative relationships in virus, interferon, antibody production, and clinical illness in the eight volunteers.

Insight into the role of interferon in influenza virus infection in man can be gained from a study of mice. Isaacs and Hitchcock (17) reported that a virus inhibitor with the properties of interferon appeared in the lungs of infected mice, with the highest titers of interferon coinciding with the decrease in the virus content of the lungs. The protective effect of interferon when administered prior to influenza virus infection in mice was demonstrated by Takano *et al.* (28). Pollikoff *et al.* (25) reported that interferon levels in influenza virus-infected mouse

TABLE 1. INTERFERON PRODUCTION IN 30 VOLUNTEERS VACCINATED WITH ACTIVE ASIAN INFLUENZA VIRUS

	Antibody rise				No antibody rise			
	Virus shedding		No virus shedding		Virus shedding		No virus shedding	
	Ill	Not ill	Ill	Not ill	Ill	Not ill	Ill	Not ill
Total number of vaccinees	9	1	2	7	0	1	1	9
Interferon positive	8	0	1	0	0	1	0	0

lungs decline during the later stages of virus replication, thereby confirming similar observations in other virus-host systems. Immune mice produced less interferon than control mice as a result of the action of antibody in resisting reinfection.

Ermolieva *et al.* (7) induced interferon production in mice by the intranasal inoculation of ultraviolet light (UV)-irradiated virus and thereby protected mice challenged with the active virus. The application of this idea to man was carried out by Balezina *et al.* (Information Exchange Group #6, Memo #120, 1965), who observed that the administration of UV-irradiated influenza virus to adults during an influenza outbreak decreased the incidence of the disease by four- to fivefold. No interferon studies were performed, but on the basis of the mouse experiments reported by Ermolieva *et al.* (7) Balezina and his co-workers suggested that the reduction of influenza incidence in the group of persons treated with UV virus might be due to the production of endogenous interferon.

Mumps Virus

Interferon was detected in the cerebrospinal fluids of 8 out of 17 patients with documented mumps virus infections (Larke, to be published). All 17 patients were febrile and had a leukocytic response in the spinal fluid. The mean geometric titer of interferon in the positive fluids was 33 units, the highest titers being found on the second day of illness, when the febrile response was greatest. There was no consistent relationship between the interferon levels and the presence of virus or leukocyte counts in the spinal fluids. However, only single samples were collected from 15 of the 17 patients, and the possible interrelationships between leukocyte, virus, and interferon levels in the spinal fluids during the course of the illness could not be studied. The interferon detected in these spinal fluids was similar to virus-induced interferon except for a partial lability at pH 2 and at 56°C.

Enterovirus

The cerebrospinal fluids of 17 patients with various types of enterovirus infections were studied, and low titers of interferon were detected in 12 of the 17 specimens (Larke, to be published). No association between interferon levels, presence of virus and leukocyte counts in the CSF could be made. However, there was a direct correlation between interferon levels and fever: the highest levels of both were found on the second day after the onset of illness.

Respiratory Syncytial Virus

Low titers of circulating interferon were found in 4 out of 17 children with RS infections by Ray, Cravelle, and Chin (to be published). The interferon-positive serums were collected during the first three days of illness while the patients were still febrile. No difference in severity of illness between the interferon-positive and the interferon-negative cases could be noted.

Rhinovirus

Interferon has been detected in the nasal washings from volunteers infected with Coxsackie A-21 and less frequently with rhinovirus NIH 1734 (T.R. Cate, personal communication). No correlations could be made between interferon titers and severity of illness or virus shedding. The nasal interferon appeared early during the period of illness.

Infectious Hepatitis

The presence of interferon was sought in the peripheral blood of patients with infective hepatitis by Wheelock, Schenker, and Combs (manuscript in preparation). Multiple serum samples from 34 subjects in all stages of acute and chronic hepatitis were collected during a six-month interval and assayed for interferon. All the patients were hyperbilirubinemic on admission and showed a considerable elevation of serum SGOT, but the course was quite variable—hospitalization ranged from 1 to 45 days (mean 16.6 days) and there were two deaths. No virus-inhibitory activity was detected in any of the serum specimens. The broad sampling of sera from these patients may establish an important negative in the pathogenesis of this probably virus-caused disease.

VIRUS DISEASES OF UNKNOWN ETIOLOGY

Respiratory Infections

Interferon was detected in the acute sera from 9 out of 51 patients with clinical viral infections predominantly respiratory in nature (31). From

the 9 patients, 5 convalescent sera were available and contained no virus inhibitory activity. All the acute serum samples were collected during the first nine days of illness. The presence of interferon only in the acute serums indicates that the interferon-dependent resistance to viral infections does not persist longer than a week or two.

Meningitis

The cerebrospinal fluids from 23 out of 58 patients with probable viral meningitis contained low titers of virus inhibitor with all the properties of interferon except for a lability at pH 2 (15). There was a correlation between leukocyte concentration in the CSF and presence of interferon, which suggests that the leukocyte may have been the site of production.

Table 2 presents briefly the number of human viral infections in which interferon has been detected and the site of detection.

ROLE OF INTERFERON

The bulk of the evidence for the role of interferon in viral infections in man is indirect, obtained from studies with experimental animals.

TABLE 2. INTERFERON PRODUCTION IN HUMAN VIRAL INFECTIONS

Disease	Interferon found in
Naturally occurring	
Chickenpox	Dermal crusts
Influenza	Pharyngeal washings and serum.
Mumps	Cerebrospinal fluid
Enterovirus	Cerebrospinal fluid
Respiratory syncytial	Serum
Aseptic meningitis	Cerebrospinal fluid
Vaccine inoculated	
Vaccinia	Dermal crusts
Yellow fever, 17-D strain	Serum
Measles	Serum
Influenza	Pharyngeal washings and serum
Virus inoculated	
Rhinovirus	Nasal washings

Exogenous interferon has been demonstrated to have striking antiviral effects when administered to animals prior to virus inoculation (1, 4, 8, 17, 18, 21, 22, Information Exchange Group #6 Memo #88). Interferon that has been actively induced in animals by a variety of viral and nonviral agents has also been effective in inhibiting replication and disease production by subsequently inoculated viruses (3). The latter studies are important for two reasons: first, the induction of endogenous interferon results in the production of titers far exceeding those that can be passively injected, and, second, the production of interferon in the same animal when it is challenged with a pathogenic virus more closely corresponds to what happens in naturally occurring viral infections. These experiments, however, differ from natural infections in that in the former the interferon is inoculated or synthesized at a site distant from the viral infection to be inhibited. This leads to a rapid dilution of interferon in the entire body with low interferon/cell concentrations. With natural infections, in contrast, the highest concentration of the interferon produced at the site of virus replication will be in the uninfected cells contiguous to the focus of infection. This is an important point since the antiviral activity of interferon depends on its concentration. Thus, experiments with passively injected or endogenously induced interferon, while successful in demonstrating the antiviral activity of interferon *in vivo*, still yield results that probably do not fully reflect the actual virus-inhibitory potential of interferon.

In natural infections the critical interferon concentrations are those at the borders of expanding virus-infected foci and in the circulation, through which the virus may spread to new sites in the body. Virus replication in circulating leukocytes would be directly affected by plasma interferon levels. Furthermore, circulating interferon would diffuse through to protect potential host cells at sites distant from the primary infection. Interferon may act at these sites in any of the multiple cycles of virus replication in the host, and thus limit the spread of the infection. The production of interferon at the site of infection, it has been further demonstrated, is enhanced by factors associated with inflammation, such as increased temperature and

lowered pH and the leukocytic response. Ruiz-Gómez and Isaacs (26) reported that in *in vitro* systems the highest titers of interferon were produced at temperatures above the optimal temperature for virus replication. DeMaeyer and DeSomer (5) reported increased interferon production in virus-infected cells incubated at an acid pH. It would be well to consider separately at this time the role of the human leukocyte in viral infections, for these cells appear in large numbers at the site of virus infection and have been shown to be one of the richest sources of interferon.

LEUKOCYTES AND INTERFERON

The role of the leukocyte in viral infections of man has not been fully defined. A wide variety of viruses have been demonstrated to replicate in the human leukocyte *in vitro;* theoretically, therefore, the leukocyte *in vivo* may be an important factor in the dissemination of viruses throughout the host.

In recent years human leukocytes have been studied for their interferon producing potential, and myxoviruses (Strander and Cantell, Information Exchange Group #6, Memo #169, 1966), measles (13), and the 17-D strain of yellow-fever virus (Wheelock, unpublished) have all been shown to elicit interferon in leukocyte cultures *in vitro*. The titers of interferon produced in cultures in response to inoculation with each of these viruses vary considerably, but several workers have found that the parainfluenza group of viruses—Newcastle disease and Sendai virus—induce the highest titers of interferon in leukocytes.

Since all the studies on interferon production in human cells have been conducted *in vitro*, it is important to consider how relevant they are to the *in vivo* state. Figure 3 illustrates the effect of incubating leukocyte cultures at 37° on interferon production induced by Newcastle disease virus (33). The cultures were prepared from the buffy coat of peripheral blood and incubated at 37°C. Immediately after preparation and at intervals thereafter, different sets of cultures were inoculated with Newcastle disease virus. As is seen in Figure 3, the maximum titers of interferon were produced in cultures inoculated immediately after their preparation.

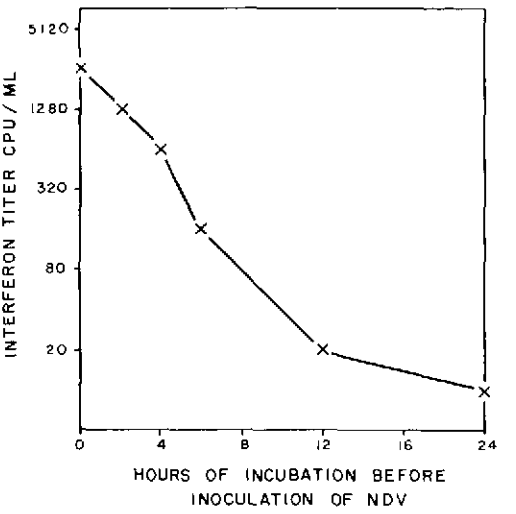


Fig. 3. Effect on interferon production of incubation of human leukocyte cultures at 37° prior to Newcastle disease virus inoculation.

Incubation of cultures at 37°C for as little as four hours before virus inoculation significantly reduced interferon production. The ability of the leukocytes to support virus replication when incubated 24 hours prior to virus inoculation attested to their viability. In these experiments it was found that maximum titers of interferon were produced in cultures inoculated with a virus/cell multiplicity of 1. Furthermore, the amount of interferon produced was directly related to the concentration of cells in the culture. A cell concentration of 8×10^6 per ml (similar to that in normal blood) produced 20,148 units of interferon in response to NDV inoculation.

It can be concluded from these studies that leukocytes are capable of producing extremely high titers of interferon *in vitro*. However, the deleterious effect of incubation *in vitro* on their interferon-producing capabilities suggests that studies on the role of the leukocyte in viral infections in man should be conducted *in vivo* whenever possible, and that *in vitro* studies should be interpreted with caution.

In *in vivo* experiments in mice (10, 11), the peritoneal macrophage has been shown to contribute to the host defense against virus infection presumably through interferon production. In these experiments it was demonstrated that peritoneal macrophages produced interferon in response to vaccinia virus and that this interferon was involved in protecting the mice

against a subsequent intracerebral inoculation of vesicular stomatitis virus. It was further shown (12) that on second exposure to a virus the peritoneal macrophages in immune animals produced more interferon than on the primary exposure. This enhanced interferon response of leukocytes in immune animals may be a major factor in the resistance to virus reinfection.

INTERFERON AS A PROPHYLACTIC AND THERAPEUTIC AGENT IN HUMAN VIRAL INFECTIONS

Monkey kidney interferon has been shown to inhibit the dermal response to vaccinia virus in man when previously inoculated at the same site but prior to the virus (27). The success of this experiment led to an attempt to protect the nasal mucosa and pharynx against respiratory viruses prophylactically by the prior administration of interferon (29).

In these experiments it was found that interferon made in monkey kidney cells failed to protect human volunteers against infection with a number of respiratory viruses. This may, however, be explained by the fact that the titers of the interferon preparations employed were low.

Topically applied interferon has been demonstrated to have a specific antiviral effect on the epithelial stages of human vaccinial keratitis although no effect was observed on the stromal stages of the disease (19).

The administration of high titers of human interferon to patients with a variety of viral or suspected viral diseases has been recently reported by Falcoff *et al.* (Information Exchange Group #6, Memo #211, 1966). The interferon was prepared in human leukocytes inoculated with Sendai virus and then concentrated by a partial purification technique using CM Sephadex. The administration of the interferon was well tolerated by these patients, and the effects on the course of the diseases are at present under study.

The importance of interferon in the recovery process depends on the *in vivo* dose needed for inhibiting virus replication. On the basis of a study of the chick embryo cell culture-eastern equine encephalomyelitis virus system, Hilleman (16) has estimated that the required virus-inhibitory dose of interferon for man, equivalent to cell cultures, would be 40 mg. Attempts to produce such large quantities of human interferon *in vitro* have to date been unsuccessful, although the human leukocyte appears now to provide a rich *in vitro* source of production (33).

Future studies on the role of interferon in viral infections in man may therefore consist either of inoculations of high-titered interferon preparations or of attempts to stimulate the body to produce its own interferon by employing nonviral inducers of interferon synthesis. In this way nonspecific host defense mechanisms may be used as a therapeutic approach to speed recovery from viral infection.

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SECTION C. INTERFERON

PROSPECTS FOR APPLYING INTERFERON TO MAN *

MONTO HO AND BOSKO POSTIC

Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh
Pittsburgh, Pennsylvania, U.S.A.

DR. HO (*presenting the paper*): Since the discovery of interferon in 1957 (42), hope has been entertained that it may prove to be a clinically useful antiviral agent (21). In this regard, interferon has many theoretical advantages. It is a stable, "natural," nontoxic substance derived from cells that acts by increasing cellular resistance to viruses and not directly on viruses. Its continued presence is not essential after it has acted on the cells. It does not appear to be very antigenic, which suggests that it might be used repeatedly. It has been purified and has been shown to possess very high specific biological activity (53). No mutants resistant to interferon have been developed (91), although variants with different degrees of sensitivity to it have been found (97). Most important of all, it is effectively active against a wide spectrum of RNA and DNA cytocidal and oncogenic viruses. However, despite these many theoretical advantages, its promise as a prophylactic or therapeutic drug is as yet unfulfilled (30). The crucial unsolved problem is that a human interferon of adequate potency is not yet available. It is also said that interferon can only be applied prophylactically before the onset of a viral infection (30); but this need not be an overriding disadvantage, since it is known that potent enough preparations are effective if administered after an infection (96).

There is a second way in which interferon may be applied: by inducing its formation in the patient. An "inducer" may thus be used to bolster a natural defense mechanism against viruses. We shall attempt to document the fact that at this time very much more interferon can be induced than inoculated. If appropriately developed, the induction of interferon could be an alternative or a supplement to the more conventional specific immune prophylaxis.

We shall review here the literature and also some unpublished observations from the standpoint of these two potential applications of interferon. Important problems to be solved will be highlighted and future prospects will be discussed. The general aspects of what is known about interferon have already been reviewed recently (21), and no pretense of covering the literature exhaustively is made.

THE ADMINISTRATION OF INTERFERON

Local Use of Interferon

That interferon administered locally or topically can check a virus infection at local sites is well documented in experimental animals and to some extent in man (32, 21). Isaacs and Westwood protected rabbits against vaccinial skin lesions with interferon obtained from rab-

* Unpublished work from the authors' laboratory was supported by N.I.H. grant AI-02953. As a reference for the general aspects of the interferons, the reader is referred to "Interferons," edited by N. B. Finter (21). Most of the private communications cited in this paper were obtained through Information Exchange Group #6, sponsored by the National Institutes of Health (U.S.A.). The term "units" of interferon is used throughout this paper to mean the minimal amount needed to produce observable antiviral effect. Units vary with different systems of titration, which are not strictly comparable.

bit kidney cell cultures inoculated with UV-irradiated influenza virus (43). Nagano and Kojima did the same with interferon obtained from rabbit skin or testes inoculated with active vaccinia virus (60). Andrews also used interferon to protect cynomolgus monkey skin against vaccinia infection (2). Cantell and Tommila protected rabbit eyes against vaccinial lesions (10). The instillation of interferon intranasally was shown by Takano *et al.* to reduce deaths from a challenge with influenza virus in mice (89).

With respect to studies in humans, the British Scientific Committee on Interferon showed that a skin site preinoculated with an interferon prepared from rhesus-monkey kidney cell cultures depressed the vaccination reaction in 38 volunteers, who reacted normally to vaccination at an untreated site at the same time (81). Jones and Galbraith effectively used a similar interferon to treat vaccinial keratitis in man (46).

The British Scientific Committee on Interferon also reported recently on an attempt to prevent the common cold with interferon in human volunteers (82). The same type of moderately potent interferon as in 1962 was used; it inhibited the replication of M rhinovirus strain in monkey kidney cells at a dilution of about 200. The viruses chosen— parainfluenza virus Type 1, M rhinovirus, and Coxsackie A-21 or Coevirus —were capable of causing colds and were first shown to be sensitive to interferon. The interferon was given as nasal drops or sprayed into the nose the day before and the same day as the virus inoculation. Neither by clinical observation nor by testing for virus replication was there any evidence of protection. The reason for the failure may have been that the interferon was removed too rapidly by the nasal epithelium or that the preparation used was not potent enough.

In contrast, Soviet workers have reported on the successful use of human interferon for the prevention of influenza virus replication in 157 volunteers (86). The interferon, prepared from human leukocytes, titered 1:64 to 1:128. It was administered by nasal spray before an intranasal challenge of 10^6 egg infectious doses of live attenuated influenza virus. The volunteers were divided into three groups approximately equal in size. The first received human interferon, the second chicken interferon, and the third medium 199. Throat washings were cultured for virus and sera obtained before infection and 14 days after inoculation were assayed for hemagglutination-inhibiting antibody. The group of volunteers that received human interferon yielded two and a half times fewer virus isolates than the other two groups, which were in that respect indistinguishable. Antibody rises were found in only 38 per cent of the group treated with human interferon, whereas 61 and 51 per cent of the other two showed rises. Almost no vaccination reactions were noted in the group receiving human interferon. It appears from this study that interferon of only moderate potency produced some effect in decreasing influenza virus replication. This is difficult to understand in view of the failure of the British study. One possibility may be that the attenuated influenza virus used in this study was unusually sensitive to interferon. The British experience makes it seem that consistent results will have to await the availability of more potent preparations.

Systemic Use of Interferon

Favorable effects following the systemic administration of interferon have been reported, although in general they are less pronounced than those resulting from local application (96, 24, 21). More recently, Finter reported interesting data on the quantities of interferon required for effective antiviral prophylaxis by systemic administration (20). He used a concentrated preparation containing about 40,000 units per ml obtained from mouse brain infected with West Nile virus. The challenge infection consisted of an intraperitoneal inoculation of encephalomyocarditis (EMC) virus or Semliki Forest virus. A single dose of 15,000 units of interferon, given intramuscularly to mice 20 hours before infection was protective against 80 LD_{50} of Semliki Forest virus; a 5,000-unit dose was not. The protection could be overcome by larger doses of virus, a phenomenon supported by data obtained in cell cultures (33). Intramuscular inoculations were superior to subcutaneous ones. Maximum protection was provided by the administration of interferon 7 to 24 hours before challenge; no protection was observed if it was given 48 hours before chal-

lenge. Interferon given after challenge was also much less effective: 12,400 units given three hours after infection afforded hardly any protection. No additional protection was provided if more interferon was injected after the challenge virus if a sufficient prophylactic dose at the right time had already been given.

Somewhat more favorable results were obtained by Baron *et al.* (5, 6). A single dose of as little as 200 units given intravenously to mice provided a small degree of protection against an intracerebral challenge of EMC virus. Eight doses totaling 1,600 units provided complete protection against a 40 per cent paralytic and lethal dose of EMC virus. Incomplete protection was provided against a 95 per cent lethal dose of VSV inoculated intracerebrally, which suggests that the degree of protection depends on the agent and the virus dose inoculated.

Gresser and co-workers (private communication) also administered concentrated mouse brain interferon of the Finter type to treat disease caused by the Friend virus. Without treatment, this virus produced marked splenomegaly one month after it was inoculated. Suggestive protection, as measured by decreased splenomegaly, was observed when mice were submitted to a schedule of treatment with an interferon titering 1:6,000 to 1:12,000, consisting of two 0.2 ml intraperitoneal doses before challenge and one dose daily for 30 days thereafter. Marked inhibition of splenomegaly was observed only when more potent interferon, titering 1:64,000, was given according to a more intensive schedule consisting of three doses given prior to challenge and two doses daily for 30 days thereafter. Pretreatment with interferon for only three days was ineffective; continuous therapy was shown to be necessary. When intravenous inoculations were substituted for intraperitoneal ones, the schedule was found to be ineffective. Nor were the intravenous administrations as effective for maintaining sustained serum interferon levels (see the next section). Friend virus infection is an example of a chronic virus infection and is basically different from the models of acute encephalitis used by Finter and Baron *et al.*

These three works taken together show that, for an evaluation of the effect of interferon, many factors must be considered. These include the dose and the potency of the preparation, the route of administration, the organ sites reached, and the natural history of the virus infection in question. But it is quite clear that to attain significant tissue levels by systemic administration, preparations of high potency are of prime importance. From Finter's data, a total dosage of about 10,000 units per 20 gm mouse is required. Extrapolated to the 70 kg man, this would amount to 35 million units. Similarly, Hilleman has estimated that to protect all the cells of the human body, and assuming equal distribution of interferon, 40 mg of pure interferon is required (30). This is equivalent to 10 million units, assuming that 1 unit is equivalent to 0.004 μg (53). These rough estimates will have to be adjusted up or down as we learn more about the body distribution of interferon (discussed below) and the effect desired. But it is reasonable to conclude that a preparation of human interferon titering around 10 million units per ml is desirable before systemic use in man can be seriously entertained. Since we have nothing that approaches such potency at this time, the systemic use of interferon in man has been held in abeyance.

Falcoff *et al.* (private communication) reported on the systemic administration of large volumes of crude interferon in man. It was prepared from human leukocyte suspensions infected with Sendai virus. The preparations, freed of infectivity by acidification, were injected intravenously into 24 patients suffering from various serious diseases of suspected or recognized viral etiology. There were 18 patients with acute leukemia, 2 with herpes zoster complicating lymphoma, 3 neonates with cytomegalovirus infection, and 1 with herpes simplex infection. The patients with cytomegalovirus disease survived after treatment, but it is questionable whether this was due to the therapy, since the related mouse virus is insensitive to the action of interferon (63). The neonate suffering from generalized herpes died after apparent clinical improvement following the injection of interferon. No effect was observed in the cases of cancer, although a "favorable" impression of the treatment was recorded. Although therapeutic efficacy was not clearly demonstrated in this study, it provided important information on the amount of impure leukocyte interferon given intraven-

ously that can be tolerated by man. Newborn babies received daily, with no untoward reaction, as much as 40 ml titering about 2,048 units per ml. Most of the adults, however, developed chills and malaise 30 minutes after an administration. While this did not prevent the continuation of the treatments, such febrile reactions are undesirable and are to be expected in view of possible leukocyte incompatibility in man (103). No side effects were noted when interferon prepared from amnion cells was used. The preparations were inoculated for as long as 400 days according to a schedule of daily or biweekly injections of 10 to 40 ml. One patient received in this manner a total of 3,620 ml containing an equivalent of 7.4 million units. No antibodies against interferon were found even after repeated inoculations. While the tolerance of humans to such impure preparations of interferon is encouraging, it is still doubtful that the amount inoculated would have been sufficient for effective antiviral prophylaxis or therapy.

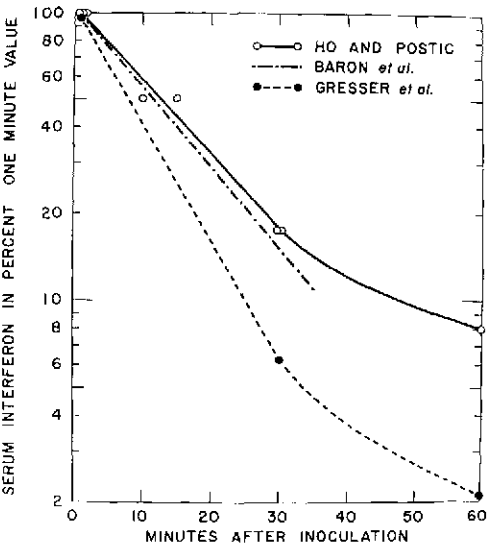
Distribution of Inoculated Interferon

It is surprising that despite the intensity of research activities in various aspects of interferon (21), there is relatively little information on its pharmacology, distribution, metabolism, and excretion. Such information is indispensable to an evaluation of any agent for prophylactic or therapeutic purposes. No studies on the metabolic fate of interferon have been reported. We do not know to what extent it is degraded and to what extent it is excreted or fixed by the tissues.

Baron *et al.* followed the decrease of serum concentrations in mice after an intravenous inoculation of interferon (5, 6). They found that these decreased at the rate of about thirtyfold per hour, which is equivalent to 96.6 per cent clearance per hour. Subrahmanyan and Mims (1966) also studied the fate of interferon in mice after an intravenous inoculation of 340 units. Only very small amounts were detected in the blood, liver, kidney, and lungs 5 to 13 minutes after inoculation, and none was detected in the spleen and brain. None was found in the bloodstream or in any organ six hours after it had been inoculated. Clearly, the disappearance of interferon from the circulation was too rapid for this relatively small amount to be followed for any length of time.

Gresser and his associates (private communication) measured the distribution of interferon after intravenous and intraperitoneal inoculations in mice. After an intravenous inoculation of 0.2 ml interferon titering 1:64,000 per ml, the concentration in the serum was 3,840 units one minute later. This decreased to 80 by the end of an hour; that is, the titer was reduced by over 99 per cent in one hour (Fig. 1). In a second test, the reduction in titer was from 480 to 40 in ½ hour, which is a reduction of 92 per cent, and calculates out again to a disappearance rate of over 99 per cent an hour, assuming an exponential rate of disappearance.

We have also conducted studies on the clearance of inoculated interferon from the bloodstream of rabbits. Interferon was inoculated in the form of serum obtained from rabbits four hours after an inoculation of 10^8 PFU Newcastle disease virus. The amount inoculated and the one-minute serum concentrations are shown in Table 1. Periodic concentrations at later intervals are plotted in Figure 1. According to this figure, the half time in rabbits is 11 minutes; for mice, as calculated from Baron's and Gresser's data, it is 10.7 and 7 minutes. There is a tailing effect in our disappearance curve and in



Note: The curves drawn from data of Baron, *et al.* (5, 6) and Gresser, *et al.* (private communication) are for mice, and the one from Ho and Postic is for the rabbit.

Fig. 1. Clearance of interferon from the circulation.

TABLE 1. DISTRIBUTION OF INTERFERON ONE MINUTE AFTER INTRAVENOUS ADMINISTRATION

Animal	Wt. (kg)	Units inoculated	Serum concen.	Amount in plasma	Percentage in plasma	Percentage in tissues	Volume of distribution*
	1.3	119,500	256	14,967	12.5	87.5	36
Rabbit†	1.0	163,840	256	11,264	6.9	93.1	65
	1.3	119,500	256	14,976	12.5	87.5	36
Mouse‡	0.02	12,800	3,840	3,840	30	70	17
	0.02	3,200	480	480	15	85	33

*In per cent body weight (ml/100 gms).
†Ho and Postic.
‡Calculated from Gresser *et al.* (private communication).

Gresser's. This may be due to varying disappearance rates of different molecular species of interferon found in rabbit serum (48). An alternative explanation is that there is a re-entry of interferon into the bloodstream from the tissues.

Some idea about the distribution of interferon can also be gathered from our data and from Gresser's (Table 1). After interferon is inoculated, clearly most of it disappears immediately from the circulation. Assuming equal distribution, the volume into which it is distributed is 17 to 65 per cent of body weight. Most of the values are over 33 per cent. Since the plasma and the extracellular and intracellular spaces are about 5, 20, and 50 per cent, respectively, of body weight (1), it may be assumed that the distribution of interferon extends definitely to the entire extracellular space and may probably cross cell barriers into the intracellular space. It is, of course, unknown what concentration gradient the cell membrane provides, and further studies are clearly needed.

With respect to the excretion of interferon, Gresser *et al.* found significant levels in the urine, but the amount excreted was not quantitated. We have approached this problem by studying the renal clearance of induced interferon (see "The Inducer," (p. 638). Gresser *et al.* also found that after an intraperitoneal inoculation of interferon, the serum levels could be maintained much longer. This suggests alternative routes for administering interferon, but the possibility that crude mouse brain interferon may have had a nonspecific effect on the permeability of the peritoneal cavity in this case must first be considered.

Human Interferon

From the above considerations, interferon must titer millions of units per ml to be clinically useful. The preparations must also be of human or primate origin, because interferon is species specific (21). There is as yet no readily available preparation that satisfies these requirements.

Ho and Enders (37) first used human cells of amnion and kidney origin to make interferon, but these preparations were too low in potency to be clinically useful. The cells used cannot be supplied in the quantities needed for further development. One type of readily available human cells is diploid cell strains (26). However, no practical large-scale method of making interferon from such cells has yet been described. Interferon titering up to 3,072 units per ml was obtained by Gresser using human leukocytes infected with Sendai virus (23). More recently, Merigan *et al.* concentrated human interferon by lyophilization and obtained titers up to 1:90,000 per 4 ml (58).

Several investigators have described methods of obtaining large amounts of human interferon from leukocyte cultures. Strander and Cantell (private communication) considered making interferon from cultures of white cells obtained through a blood transfusion service. One unit of blood yielded more than 10^9 leukocytes, which is equivalent to the number of cells in 100 Roux bottle cultures. The leukocytes could be preserved for one day at 4° without affecting their interferon-producing capability. They were clumped by EDTA, the red cells were eliminated after lysis by NH_4Cl, and the leukocytes were incubated in a spinner culture. Live NDV and

Sendai virus were shown to be the best inducers. The maximal activity of the preparation obtained was 2,000 units per ml as assayed on a line of human amnion cells with VSV as the challenge virus. Similar results were obtained by Falcoff *et al.* (private communication).

Wheelock (private communication) also induced interferon in cultures of human leukocytes from healthy adults. Under optimal conditions, NDV-induced interferon titered 10,240 units per ml as assayed in diploid cells of human fetal lung challenged with Sindbis virus. The author found that the infection of freshly suspended leukocytes with NDV was essential for efficient induction. Induction by phytohemagglutinin in leukocytic cultures was also previously reported by Wheelock (99). But it was of fairly low activity—10 to 80 units per ml. Older leukocytes, even after a few hours of incubation, rapidly became ineffective as producers of interferon on exposure to NDV, despite sustained viability. Lymphocytes were found to be the main source of interferon.

It thus appears that the preparation of interferon from leukocyte cultures is feasible on a large scale. But the potency of the preparations and the side reactions they produce still leave much to be desired. The development of a simple purification and concentration method for such preparations is desirable.

INDUCTION OF ENDOGENOUS INTERFERON

The application of exogenous interferon is beset by the theoretical and practical problems outlined above. An approach of greater potential usefulness is to induce endogenous interferon (21, 32, 30). The primary advantage of induction is that more interferon can be induced than can be inoculated. A second advantage, which is less well defined, is that some types of induction procedures may impart protective effects beyond the interferon produced.

There are no precise estimates of the amount of interferon produced in the host after induction. Baron *et al.* (5, 6) estimated that in a 14 gm mouse, 100,000 units of circulating interferon could be induced during viremia following peripheral virus infection. We estimated the amount of interferon produced from the amount excreted. First, we ascertained the proportion of inoculated interferon recoverable in the urine: 0.5 to 2 per cent. Next we measured the amount excreted during a 30-hour period following induction: 100,000 to 300,000 units. By applying the proportion we could then estimate the total renal load, which should approximate the amount produced. Using this method, we found that after the inoculation of NDV, 5 to 60 million units were produced in a 1 kg rabbit. This estimate is probably on the low side, because interferon fixed by tissues or otherwise not available for excretion is not included (see also "Distribution of Induced Interferon," p. 644, for renal clearance of interferon). But if humans could produce interferon at this rate, clearly many billions of units of interferon might be induced.

The induction of interferon for viral prophylaxis has at least two distant progenitors in addition to recent studies on interferon (21). First, it is related to measures practiced from time immemorial to enhance, actually or figuratively, so-called nonspecific resistance. The use of nonspecific vaccine therapy in infectious and noninfectious diseases was commonplace before the advent of antibiotics and steroid therapy, and is still used to some extent for diseases of the eye (66). Microbial substances used include killed vaccines of gram-positive (e.g., staphylococcus) and gram-negative (e.g., Salmonella bacteria), "autogenous" vaccines prepared from organisms obtained from foci of bacterial infection in patients, and tuberculin. The other measures used included "nonspecific protein therapy," which consisted of an injection of such substances as boiled milk. These anachronistic measures might well be reconsidered in the light of the possibility that some of them may induce interferon. Second, the use of live or inactivated viruses to induce interferon rests on the groundwork provided by earlier work on viral interference (78, 29). These studies provide some information on the status of the animal inoculated with the interfering virus and the duration of its effect.

Hoskins described the interference between two strains of yellow fever viruses in monkeys (39). Several investigators reported that viral interference could take place between antigenically unrelated viruses (14, 13, 95). Schlesinger *et al.* distinguished clearly between specific immunity and interference (79, 80). Guinea pigs

were made resistant to an otherwise lethal dose of EEE virus by prior inoculation with killed WEE virus vaccine and live WEE virus. It is likely that the interference noted was at least partly due to induced interferon in view of more recent findings that interferon can be isolated from the brains of mice inoculated with arboviruses (31, 92, 93, 69). Burnet and Fraser (9) noted that an established allantoic infection by one influenza A virus (strain MEL) protected chicken embryos against hemorrhagic brain lesions due to an intravenous injection of another strain (NWS). This was not direct interference of one virus with another at the same site but was an example of interference "at a distant site." Ten years later, Crossberg *et al.* (24) presented evidence suggesting that interference at a distant site could be explained by the production of interferon in the infected allantoic cells and its diffusion into a distant organ such as the brain.

The protective effect of an intracerebral injection of influenza A virus in mice against challenge by WEE virus lasted for at least 15 days (95). It began to wane after two weeks. An elevated resistance after similar treatment was found to range from 10 to 21 days in other studies (14, 80, 16).

The problem of whether or not resistance imparted by an infective virus can be entirely explained by the interferon produced is not entirely settled. A distinction has been made between "infection interference" and "inhibitor interference" (28). Along similar lines, we can distinguish between nonspecific cell immunity and interferon effect. The two phenomena are not necessarily synonomous, but may overlap. Ho (34) compared the resistance produced in chick embryos by two different strains of influenza A viruses, of which one was a potent interferon producer (WS strain), and the other was not (PR-8 strain). Both strains, when preinoculated, produced the same degree of resistance to a lethal challenge of WEE virus. From this study it was considered unlikely that the nonspecific cellular immunity brought about by a virus infection was entirely mediated by interferon.

The Inducer

Assuming that induced interferon is more effective than administered interferon in protecting the host against virus infections, what inducer can be applied to man? A list of inducers identified to date is given in Table 2. It will undoubtedly be lengthened and modified in time.

To be considered for human use, an inducer should be effective, nonpathogenic, nontoxic in its acute and long-term effects, and, preferably, nonantigenic. A casual perusal of the table convinces one that most of the agents listed do not satisfy all or even most of these conditions.

The viruses have been studied most thoroughly for their interferon-inducing effect. The important work of Baron and Buckler (4), and a great deal of subsequent work (21), demonstrated that intravenous inoculations of large doses of infective and in many instances nonreplicating viruses, especially those belonging to the myxo- or arbovirus groups, induced high titers of circulation interferon. The crucial point here was that the dose of virus inoculated had to be high. For example, the minimal effective dose of NDV required to induce interferon in a mouse was 10^6 PFU (4). Consistent interferon induction in the 1 kg rabbit required 10^8 PFU or more Sindbis virus or NDV (Kono,

TABLE 2. INDUCERS OF INTERFERON

Microorganisms

1. Viruses: both DNA and RNA cytocidal and oncogenic viruses; active and inactivated
2. TRIC agent (Trachoma-inclusion conjunctivitis) agent
3. Rickettsia
4. Bacteria (especially gram-negative), living and killed
5. Mycoplasma
6. Protozoa (*Toxoplasma gondii*)

Products of microorganisms

1. Bacterial products (bacterial endotoxins)
2. Fungal products (statolon, helenine)

Others

1. Nucleic acids (animal and plant)
2. Phytohemagglutinin
3. Pyran copolymer
4. Cycloheximide

*These inducers have been described in detail earlier (21) except for the following agents described in private communication: TRIC agent (Merigan and Hanna); toxoplasma (Rytel and Jones, Merigan *et al.*); mycoplasma (Youngner *et al.*); and, pyran copolymer (Regelson). The effects of cycloheximide on interferon in mice were reported by Youngner *et al.* (106).

Postic, and Ho, unpublished data). If these figures are extrapolated to man, about 10^{10} PFU of these or similar viruses would be required to induce interferon systemically. It is doubtful whether inoculating such large doses of living virus would be justified as a prophylactic or therapeutic measure except in the case of a life-threatening disease against which no other measure was available.

An alternative would be to create an infection in which enough virus replication occurs for interferon to be induced. Baron *et al.* (5, 6) emphasized the importance of producing viremia, although intradermal vaccinial inoculations were followed by the production of interferon detectable in dermal crusts (100). Whether or not the ingestion of live polio vaccine of the Sabin type induces interferon is not known. The formation of circulating interferon following the introduction of live viral vaccines that replicate systemically has been demonstrated. Wheelock and Sibley (102) found circulating interferon in 10 out of 15 patients after administering the 17-D strain of live yellow fever vaccine. All 10 subjects had viremia, with peak titers on the fifth day. The highest interferon titers were found one day later. Petralli *et al.* (67) found interferon in 17 out of 18 patients six to eleven days after live measles virus immunization. The highest titers were found on the tenth day.

However, it is doubtful whether the induction of interferon by live vaccines can be generally used for viral prophylaxis. It is only moderately effective (see "Application of Induced Interferon," below) and it is a one-shot procedure. The development of specific immunity effectively precludes a repeat application of the same agent.

The use of an inactivated virus preparation would be less objectionable, but inactivated viruses are less effective as inducers. While active Sindbis and NDV were excellent inducers of interferon in the rabbit, UV-irradiated Sindbis virus was ineffective (52, and unpublished data). Youngner *et al.* reported that UV-inactivated NDV was an effective inducer in the mouse, but not in chick cell cultures (105). It would be desirable to investigate systematically whether viral subunits could induce interferon. W. Schäfer (private communication) attempted without success to induce interferon with the H-antigen of fowl plague virus. Curiously, little work on the ability of viral nucleic acid itself to induce interferon has been published. Isaacs suggested that it is the nucleic acid of viruses that is necessary for the induction of interferon (40). While viral nucleic acid is not the common denominator of interferon inducers (Table 3), it may still be an unusually potent inducer.

The use of other living or dead microorganisms to induce interferon in humans would be as objectionable as using viruses, if not more so. Bacterial endotoxins are undesirable because of their toxic effects. R. H. Michaels (unpublished data) attempted to induce interferon in man with typhoid vaccine. He followed the schedule of inducing fever prescribed by Sutton and Dodge for the treatment of chorea (88). In three children with leukemia refractory to conventional chemotherapy, he inoculated intravenously one to three doses ranging from 0.05 ml (15 million organisms) to 0.15 ml per dose. In each case, fever of 104° to 106°F was produced. No circulating interferon could be detected in any of these children 2 to 24 hours after the administration of the vaccine. It is very likely that despite the marked pyrogenic response, the amount of bacteria inoculated was insufficient to induce interferon. This opinion is based on the fact that in a 1 kg rabbit, 0.2 mg of dried killed *E. coli* was required to induce endotoxin (36). Borecky and Lackovich (8) used 2.8×10^9 killed *B. pertussis* to induce interferon in mice. It is doubtful whether any of these substances are

TABLE 3. INDUCTION OF INTERFERON BY NDV IN LEUKOCYTE CULTURES FROM TOLERANT RABBITS*

Treatment of rabbits	Leukocytes/ml	Interferon titer† after	
		6 hours	24 hours
None	3.0×10^7	80	80
Pertussis vaccine	1.5×10^7	113	640
None	1.1×10^7	11	320
None	1.3×10^7	320	640
Endotoxin	6.2×10^6	113	320
Endotoxin	9.0×10^6	320	640

*White cells were obtained from rabbits made tolerant with 2.0 ml of pertussis vaccine (10^{11} cells) administered 72 hours before bleeding or with 10 μg of endotoxin 24 hours before bleeding.

†White cells were incubated at 37°C following inoculation of NDV at the input multiplicity of 10 and assayed for interferon after 6 and 24 hours (52).

satisfactory candidates for the systemic induction of interferon in man. One approach may be to separate out the interferon-inducing factor in these complex substances from their toxic properties. Whether any simple "factor" exists is of course unknown.

A possible candidate for a relatively nontoxic inducer of interferon is the fungal product statolon, which has been shown to inhibit virus infections in mice (71) and in monkeys (12), and helenine (77). Kleinschmidt and Murphy (50, 51) showed that statolon was an effective inducer of interferon. After an optimal dose of statolon (175 μg per mouse), the serum interferon levels reached their peak (5,000 units) at about 12 hours and then decreased to a constant level, which was maintained at about 100 units per 2.5 ml for at least seven days. This persistence of circulating interferon is quite different from observations with viruses (5, 6). In the case of rabbit, we found for example that the interferon levels in the blood were largely gone 24 hours after the inoculation of Sindbis or Newcastle disease viruses (52, 70). If interferon levels can be made to persist, this would represent a distinct advantage for statolon as an inducer.

Kleinschmidt and Murphy further report that the protection against M virus infection in mice provided by one intraperitoneal injection of statolon lasted from two weeks to 30 days (51), which compares favorably with the duration of interference after an inoculation of virus. They also reported that a subcutaneous injection of statolon in monkeys induced interferon formation that reached its peak (1,000 units) at 24 hours. The serum levels had largely disappeared by 48 to 72 hours.

Statolon appears to be about as effective an interferon-inducer as viruses. If it was proved to be nontoxic in its acute and long-term effects in man, it could be considered as a potential prophylactic agent against virus infections. No published data on its pharmacology are as yet available. It is a complex, high-molecular-weight substance that is still largely unpurified. The chances that it would be entirely nontoxic do not appear good.

Another fungal product, helenine, derived from *Penicillium funiculosum*, was shown to be comparable to statolon in its antiviral and interferon-inducing capacity both in cell culture and in mice (83, 84, 77). Helenine protected mice effectively against a lethal challenge of Semliki Forest virus when it was administered 12 to 48 hours before infection. It had little or no protective effect if given more than three days before or shortly after the virus. When helenine-treated survivors were rechallenged after two weeks with the same agent, they all succumbed to infection (84). Repeated injections of helenine at two- or three-day intervals exhausted the capacity of the host to be protected. This interesting finding could be explained on the basis of tolerance to interferon induction (see "Tolerance," below).

A class of viral inhibitors that act in part by inducing interferon is chemically defined polyanions such as pyran copolymer, which is a vinyl ethyl ether meleic anhydride copolymer (Regelson, private communication). This substance has been shown to inhibit the effects of Friend leukemia virus in mice and to produce interferon in these animals. Unfortunately, it produces thrombocytopenia and cytoplasmic inclusions in the leukocytes, and its long-term effects are largely unknown. Studies to determine whether it forms interferon in man are under way (Regelson, private communication). Attempts to identify a nontoxic inducer of interferon of defined chemical structure are clearly indicated.

Since the suggestion of Isaacs (40) that the stimulus for induction of interferons may be "foreign" nucleic acid, several reports have appeared purporting to demonstrate this in cell cultures (72, 45). Intranasal instillation of nucleic acid from various animal and plant sources reduced mortality in mice from an intranasal inoculation of influenza virus (90). Yeast nucleic acid had to be given in daily doses of 0.25 to 2 mg for one to four days before virus challenge. Other routes of administration were ineffective. The same workers also reported that protamine sulfate administered intranasally before or together with the nucleic acid potentiated its effect (98). The decrease in mortality was associated with a reduction in the virus content of the lungs of treated animals, although in a later study the authors found nucleic acid treatment to produce no effect on virus titers (private communication). A virus inhibitor resembling interferon was obtained from the lungs of nucleic treated animals. Interestingly, this inhibi-

tor did not show species specificity, since the authors were able to titer it on chick embryo cells (90). Nucleic acid also protected against the pneumonitis and fatal meningo-encephalitis caused by an intranasal inoculation of EMC virus. The protection was not associated with increased interferon concentrations in the lung.

The authors did not account for the protective effect of nucleic acid. While induced interferon may have played a role, they suggested that the uptake of large amounts of nucleic acid by the nasopharyngeal and pulmonary cells may have impaired their capacity to take up "viral RNA." The efficacy of nucleic acid is an example of how the inoculation of complex substances may inhibit virus infection by means other than the interferon mechanism. Its prophylactic effect warrants further exploration. At the moment, the large doses required and the marginal effect produced appear to militate against widespread application to humans.

Another point brought out by this work is that a potential interferon inducer may be effective locally but not systemically. Whether nonviral inducers of interferon can actually induce interferon locally without having to stimulate the reticulo-endothelial system is not known (52). If they could, many systemically toxic agents might find some use locally.

Problems and Conditions of Induction

General conditions that favor the production of interferon have been identified in studies using cell cultures and the intact host (21). Only those of importance in considering the induction of interferon in man will be discussed here.

There are reports that immature animals or tissues are less effective producers of interferon. Isaacs and Baron reported that chorioallantoic membranes taken from 11-day-old chick embryos produced "20 times as much interferon as 6-day-old membranes" (41). Heineberg *et al.* (27) inoculated mice of various ages with Coxsackie B_1 virus. They found that suckling mice died in two days with high tissue levels of virus without producing any appreciable amount of interferon. In contrast, six-week-old mice produced high titers of interferon in the liver, spleen, brain, muscle, and kidney. It is therefore possible that induction measures will be less effective in immature animals. But these two interesting studies may not indicate an intrinsic inability of younger animals to form interferon. A virus may induce less interferon in the younger animals because less virus replicates than in adults and cells are not exposed to as much virus. Alternatively, the infection may be so virulent in the young that there is insufficient time for the production of interferon. Furthermore, these studies refer only to virus-induced interferon, and the relationship of age to induction by nonviral stimuli remains to be explored. In this respect, the report that germ-free rats produce interferon after the inoculation of endotoxin is of interest (DeSomer, private communication).

The effect of temperature on interferon formation is also profound. It has been suggested that interferon production is a property of attenuated viruses (18), which generally have a lower optimal temperature for growth than virulent ones (56). In general, the optimum temperature for interferon production is higher than that for virus replication (73, 74). Interferon production was higher in chick embryos held at 36°C to 40°C (3) and in mice kept at 25°C than in those at 4°C (75). The effect of ambient and body temperatures on interferon formation after an intravenous inoculation of Newcastle disease virus or of endotoxin was also studied in rabbits (70). As previous studies had showed, the amount of interferon induced by virus was elevated by increasing the ambient temperature to 35°C, which also raised the body temperature. Cooling the animals at 4°C resulted in lower interferon levels. Similar findings in mice have recently been reported by Soloviev *et al.* (85). However, neither heating at 35°C nor cooling affected endotoxin-induced interferon. In addition, a significant increase in the production of endotoxin-induced interferon was found in shorn rabbits whose body temperatures were lower by 1° to 1.5°F than those of intact animals (70). If induced interferon prophylaxis develops to the point where several agents are available, the presence or absence of fever or hypothermia in a patient may be one of the determining factors in the choice of the inducing agent.

The effect of hormones on interferon production may also be of clinical importance. It has been well established that the administration

of cortisol will inhibit the formation of virus-induced interferon (49, 57, 76). The doses administered are mostly in the pharmacological range. In the rabbit, Postic *et al.* (unpublished data) found that multiple doses of 250 mg cortisol were necessary to inhibit virus-induced interferon, but 1 to 5 mg were sufficient to inhibit endotoxin-induced interferon. More interestingly, adrenalectomy had a profound potentiating effect on endotoxin-induced interferon. It appears that normal physiologically circulating cortisol levels are sufficient to depress interferon induction by endotoxin. Clinically, the possibility arises that the induction of interferon may be more effective in certain disease states—Addison's disease, for example.

Tolerance

An important consideration in evaluating the prophylactic value of induced interferon production is the well demonstrated fact that tolerance or refractoriness to induction occurs (38, 104). Thus, after a first inoculation of either a virus or a bacterial endotoxin, the animal is partly or wholly refractory to a second inoculation for up to about six days. There is "crossed" tolerance, in that one inoculation of endotoxin will also render the animal tolerant to the induction of interferon by a virus. Similarly, an inoculation of virus will also render the mouse hyporeactive to endotoxin (104). This latter sequence of inoculations did not result in hyporeactivity in the rabbit (38) but has been confirmed in the rat (15).

If tolerance occurs in man—and there is no reason to believe that it does not—it will prevent the repeated administration of an inducer at short intervals. It may also account for the absence of continued synthesis or release of interferon by cells in the presence of the inducer. The explanation of this interesting phenomenon is unknown, but several possibilities will be considered here.

The first hypothesis is that tolerance is mediated by a diffusible humoral factor. The simplest explanation is that interferon itself inhibits further interferon production. That this can occur has been demonstrated in cell cultures (94, 64, 55, 22). Paucker and Boxaca (65) found on closer analysis that the factor repressing responsiveness was not interferon itself but was only associated with it. Using L cells in suspension and UV-irradiated NDV as an inducer, they found that, after liberating interferon, cells remained refractory indefinitely if they did not divide, but for only one or two divisions if they did. All the substances that induced interferon also produced refractoriness: a nonviral substance such as statolon induced interferon and the refractory state, while endotoxin did neither. Crude preparations of interferon rendered cells refractory, but the "repressor" could be dissociated from the interferon upon purification. The relationship of this factor to the "blocker" of Isaacs *et al.* (44) and the virus enhancing factor of Kato *et al.* (47) is not fully defined, but it probably is not the stimulon of Chany and Brailovsky (11), since it does not act by blocking interferon action. There is no reason to believe that the "repressor" may not also operate *in vivo*. The only problem is whether it is in fact the operative mechanism for tolerance. An important objection, we find, is that cells from tolerant animals do not have a reduced capacity for forming interferon (Table 3).

The second candidate for a humoral mediator of tolerance is a factor that inactivates the inducer. Ho *et al.* described a serum substance found in the rabbit made tolerant by endotoxin that could inactivate small amounts of endotoxin (38). However, it is difficult to see how this factor could explain tolerance to a virus inducer, since "tolerant" serum was not shown to be active against viruses (38). In general, the humoral theory of tolerance is made less likely by the fact that tolerance could not be transferred by passive transfer of tolerant serum (38, 104).

The second general hypothesis to explain tolerance is a cellular one. Interferon release has many characteristics of a "one-shot" affair (35), and therefore a period of exhaustion after interferon release may be what we observe as tolerance. However, this is a rather vague proposition and there are at least several possible formulations of it, which need not be mutually exclusive. If we consider that 1 kg rabbits may be made tolerant with small amounts of endotoxin (0.1 to 0.01 μg), which are insufficient to induce any detectable interferon formation (38), it is difficult to see how the cells can have been exhausted by releasing interferon. It would be

more reasonable to assume that another rate-limiting parameter necessary for the production of interferon is exhausted. This parameter is as yet unknown, but it may be that the reticulo-endothelial system is affected in such a way that inducers (e.g., endotoxins) do not reach the target cells but are more effectively cleared (7, 54). Other possibilities are the destruction or displacement of interferon-forming cells (8) or the exhaustion of an enzyme necessary for interferon release. The evidence for the former is incomplete, and there is no evidence for the latter.

An experiment in our laboratory may illustrate some of the vagaries of the tolerance phenomenon (Table 4). Rabbits preinoculated intravenously with pertussis vaccine similar to that used by Borecky and Lackovic (8) produced tolerance to endotoxin and NDV, but not to Sindbis virus. The duration of tolerance to endotoxin induction was about 7 to 12 days, and to NDV was even longer. The pertussis vaccine itself induced no detectable circulating interferon at all in our hands. It is suggested that these two viruses are cleared differently by the reticulo-endothelial system in the rabbit. It is more difficult to produce tolerance to Sindbis virus, a virus that persists longer in the bloodstream and may replicate to some extent (52).

TABLE 4. TOLERANCE TO INDUCTION OF INTERFERON AFTER PERTUSSIS VACCINE *

Day vaccine given before inducer	Type of inducer	Interferon titer
None	Endotoxin	52
1	Endotoxin	3
7	Endotoxin	2
12	Endotoxin	25
None	NDV	723
1	NDV	2
2	NDV	6
12	NDV	84
None	Sindbis	111
2	Sindbis	256

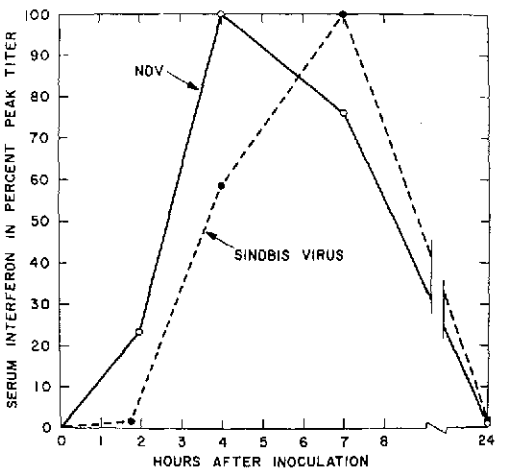
*About 12 antigenic units or 10^{11} cells, administered intravenously before 10 μg of endotoxin or about 10^9 PFU of virus. "None" signifies that no vaccine was given. Each titer represents the mean obtained from 2 to 6 animals bled 2 hours after endotoxin or 4 hours after virus.

NDV, on the other hand, shows no evidence of replication in mice and rabbits and is usually undetectable a few hours after inoculation (3, 70; Postic and Ho, unpublished data). The two viruses also induce peak serum interferon titers at different times (Fig. 2): the interferon induced by Sindbis virus reaches a peak at seven hours, and that induced by NDV, at four hours. These findings are compatible with the suggestion that the two viruses behave differently with respect to the clearance and interferon-forming functions of the reticulo-endothelial system.

Considering these various findings, we tentatively conclude as follows:

1. One explanation for tolerance is a diffusible repressor associated with interferon (65). This would explain the acquired refractoriness in cell cultures, and might conceivably explain certain cases of tolerance *in vivo* in which large amounts of interferon and the associated repressor are produced. However, it does not explain tolerance in cases where no interferon is produced, where cells from tolerant animals are found to be reactive, and where tolerance cannot be transferred.

2. Tolerance *in vivo* is based on changes in the reticulo-endothelial system. It is not an "all-or-none" phenomenon. "Crossed" tolerance varies with the agent and species used. It is



Note: These values are calculated from mean titers obtained from large numbers of rabbits as described by Ho and Kono (*Proc Nat Acad Sci* **53**:220, 1955) and Postic, *et al.* (70).

Fig. 2. Serum interferon levels after inoculation of Sindbis and NDV in the rabbit.

most efficiently produced by agents that alter the function of the reticulo-endothelial system. Endotoxin is a better agent in this respect than viruses. The precise change in the reticulo-endothelial system is not known, but it may be a physiological change rather than one that can be detected in alteration of cellular function at the present stage of understanding.

From all this, it would appear that, after further investigation, tolerance in man need not absolutely vitiate the usefulness of induction measures. Agents may be found that do not produce much tolerance. Repeat administrations of inducers may be possible with agents that do not produce crossed tolerance.

Distribution of Induced Interferon

We have already discussed the distribution of interferon after its inoculation in the host. Subrahmanyan and Mims (87) found that after ectromelia virus was inoculated in the footpads of mice, interferon was found, in decreasing order of concentration, in the spleen, lymph nodes, liver, and blood. The intracerebral inoculation of a neurotropic West Nile virus induced large amounts of interferon in the brain, where the virus multiplied, and none in the lung, kidney, spleen, where there was only a small amount of virus. It is likely that the presence of interferon in the organs was associated with the replication of the virus at local sites.

The formation of interferon after a large intravenous dose of virus seems to follow to a large extent the virus clearing system, which is the reticulo-endothelial system (59), although not much information is available on the exact sites of viral clearance. After a large intravenous dose of Sindbis virus in the rabbit, the highest concentrations of interferon were found in the spleen, liver, and serum and negligible amounts were found in the other tissues. In general, the amount of interferon found corresponded to the amount of virus present (Kono and Ho, unpublished data). There may be certain exceptions to this observation. Subrahmanyan and Mims observed that in order for any interferon to be detected in liver tissue, the samples had to be quick-frozen within five minutes after collection; if they were left at room temperature for 30 minutes, no interferon was found. The reason for this may be that the liver possesses a capacity to break down interferon, perhaps via proteolytic enzymes. The liver may be the organ that degrades interferon.

There are relatively few quantitative data on the distribution of interferon in other tissues following induction, although very likely it distributes itself widely in the extravascular body compartments (Table 1). Oh and Gill (62) found that after the injection of endotoxin in the rabbit, interferon was found in the aqueous humor of the eye. We shall present below some of our studies on the distribution of induced interferon in the excretory system and in fetal tissues.

Interferon has been found in the urine of mice and rabbits after the inoculation of interferon or its induction with endotoxin (Gresser *et al.*; Oh, private communication). We presented above some data on the amount excreted in the rabbit after induction. With respect to the rate of such excretion, we calculated the renal clearance of interferon in 1 kg rabbits, by determining the amount excreted in timed urine samples and mean serum concentrations. On the basis of six clearance studies, 30.6 ml of plasma per hour was cleared of virus-induced interferon. Assuming the plasma volume to be 45 ml (1), this meant that 68 per cent of the plasma volume was cleared in an hour. There is thus a substantial excretion of interferon as long as plasma levels are maintained. Some of the highest interferon titers we have observed in this laboratory were in urine samples. In contrast, the renal clearance of endotoxin-induced interferon was only 1.5 ml of plasma an hour, or 3.3 per cent of the plasma volume. One explanation for this difference could be a decrease in glomerular filtration or renal plasma flow following the injection of endotoxin, but we did not detect any difference from normals in the creatinine clearances of animals injected with either virus or endotoxin. These results suggest that different interferons (25, 48) are excreted differently and that the excretion pattern is an important consideration in maintaining effective tissue interferon levels after the induction or inoculation of interferon.

Knowledge of the transplacental distribution of interferon would be a prerequisite for admin-

istering an inducer to a pregnant patient for antiviral prophylaxis aimed at the fetus. We studied the diffusion of interferon across the placenta by inoculating 10^{10} PFU of NDV into 4 to 5 kg pregnant rabbits about a week before term. The levels of interferon in various material and fetal tissues are presented in Table 5. It is clear that interferon induced in the mother diffuses into the fetus and that there is a gradient in interferon levels between maternal and fetal tissue which suggests that interferon in the latter is derived from the mother. The interferon disappears rapidly from the fetal circulation. There is no evidence for the induction of interferon in the fetus, but this point requires further study. These data suggest that it is at least possible to protect the fetus by inducing interferon in the mother.

Application of Induced Interferon

Baron *et al.* (5, 6), studying the effect of induced interferon in mice, observed maximum protection (65 per cent) when 10^8 egg infectious doses of NDV were injected intravenously 24 hours before an intracerebral challenge with EMC virus. The protection decreased by 15 to 30 per cent as the interval shortened, and no significant protection except for some delay in deaths was observed when the inducer was given 24 hours later. A similar degree of protection was observed against an intraperitoneal dose of Germiston virus, which caused death by spreading to the central nervous system by viremia. The amount of interferon available at the target organs, and the duration of protection were not studied.

TABLE 5. TRANSPLACENTAL DISTRIBUTION OF VIRUS-INDUCED INTERFERON

Tissue	Interferon titer	
	4 hours*	7 hours
Maternal serum	8,192	512
Placenta	905	30
Embryonic blood	256	16
Embryo skin and muscle	80	<16
Embryo-viscera	40	<16
Amniotic fluid	64	16

*After inoculation of 10^{10} PFU NDV. Fluids titered on basis of 0.3 ml, tissue 0.3 gm wet weight.

Oh and Gill present some interesting data on the prevention of corneal lesions due to NDV by injecting endotoxin locally or intravenously in the rabbit (61, 62). They showed that induced circulating interferon was the protective agent, since the protection could be transferred. However, they point out that the corneal lesion was not associated with viral replication. A question may be raised as to what precisely the effect of the serum factor was.

Petralli *et al.* attempted to determine the efficacy of interferon stimulated by live measles vaccine (68). They vaccinated 131 healthy, nonimmune children, ranging in age from 11 to 36 months, after the inoculation of measles vaccine. Smallpox vaccination takes were completely blocked when the vaccine was administered 9 to 15 days after measles immunization. Children vaccinated at the time of measles immunization or 20 days later responded like the controls. The period of peak resistance corresponded to the time of measles virus replication and the onset of circulating interferon (tenth day). The resistance persisted only up to the fifteenth day. By then there was already much less resistance, and it could be overcome by a stronger vaccine. Six children without detectable circulating interferon levels were also protected, which suggests that protection may be present without detectable interferon levels.

It has been postulated that the induction of interferon following immunization with live vaccines may account for resistance to measles infection three days after measles immunization (68) and for the benignity of smallpox vaccination (100). Conversely, the induction of interferon shortly after immunization with multiple living agents may adversely affect successful takes (102), although the evidence for this is slight (17).

One of the few reports on the use of induced interferon therapy in man to treat a disease of suspected viral etiology is that of Wheelock and Dingle (101). A 26-year-old male with acute myelogenous leukemia received large doses of six different viruses (Sendai, NDV, PR8, and Lee strains of influenza virus, Semliki Forest, and Sindbis viruses) at various times throughout a four-and-a-half-month period. Two out of 33 serum samples collected during therapy showed detectable levels of interferon. It was

also found post-mortem in the spleen, the bone marrow, and some lymph nodes. After the administration of each of these viruses, there was marked clinical and cytological improvement. It is, of course, unknown whether the therapeutic effect was related to interferon. Even if leukemia is a virus disease, it is questionable whether it could be affected by interferon at this late stage. What effect other nonspecific reactions elicited by the virus injections may have had is unknown. There was, for example, a period of pyrexia after each inoculation of virus.

Balezina and co-workers in the USSR (personal communication) reported on the prophylactic effect of an intranasal administration of UV-inactivated swine influenza virus during an epidemic outbreak of influenza. The irradiated virus was given to 265 construction workers living in hostels, and a comparable number served as controls. In the 10 days following these administrations, the incidence of influenza was 9 per 1,000 in the treated group and 45 per 1,000 in the untreated controls. No direct measurements for interferon were made, but the effect was assumed, on the basis of work in mice (19), to be due to the development of endogenous interferon by the irradiated virus. The mouse studies do not explain why mouse "interferon" induced by irradiated virus could be assayed effectively on chicken fibroblasts. This brings up the possibility that the effect of the irradiated virus in man was due to another type of virus interference. Otherwise, these works taken together provide one of the few reports to date if not the only one, of successful application of induced interferon prophylaxis against a known virus disease in man. It also shows that perhaps induced interferon, just like administered interferon, should first be tried locally rather than systemically.

Prospects

If an effective and nontoxic inducer can be found, the induction of interferon may provide quick-acting prophylaxis against a wide range of viral infections. At the moment, the duration of its effect would be fairly short—weeks rather than months. With these considerations in mind, some types of viral diseases against which it may be effective can be identified.

1. Induced interferon therapy would be most suitable against virus diseases for which specific immunization is unavailable or may be impractical to develop. Local infections may be easier to attack by local induction measures than generalized infections. The upper respiratory viral infections, especially those caused by viruses of the common cold, would seem to fall into this category, since the number of agents in this group may be too large to be handled effectively by specific immunization (30).

2. Induced interferon may be used as an accessory measure in epidemics or during the incubation period of an infection when quick prophylaxis is required before specific immunization can take effect. It may be used when specific immunization is risky, as is the vaccination of eczematous children, for example. It may be used in lieu of specific immunization in patients who are unusually susceptible to a large number of viral agents—for example, to prevent respiratory infections in cardiac patients and the chronically ill. It may be considered when active or passive immunization is not available or is ineffective, as for the prevention of fetal damage due to rubella in nonimmune gravida where exposure is intense.

3. Induced interferon may also be considered to prevent the extension of an already acquired viral infection. Some viral infections remain latent and may become chronic or recurrent. Recurrent herpes simplex, cytomegalovirus, and herpes zoster infections are examples. But if the leukemias and lymphomas are eventually found to be caused by viruses, and if continued cell-to-cell infection is important in their pathogenesis, the use of induced interferon may be considered.

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SECTION C. INTERFERON

DISCUSSION

CHAIRMAN RASKA: We shall now proceed with the discussion. Instead of Dr. De Somer, the discussant will be Dr. A. Billiau from the Rega Institute for Medical Research, University of Louvain, Louvain, Belgium.

DR. BILLIAU: I should like to report on "Urinary Excretion of Interferon in Rabbits," by Dr. P. De Somer, and co-authored with Drs. E. DeClercq and E. Schonne, of the Rega Institute for Medical Research.

Introduction. Among the problems to be solved before interferon can be used clinically are the diffusion of this substance in the tissues, its uptake by different organs, the metabolism of its destruction, and its excretion. The kinetics of *in vivo* interferon induction by either virus or endotoxin have been studied in different animal species. The literature on this subject has recently been reviewed by Finter (2). From these studies and also from a report of Subrahmanyan and Mims (6) it appears that interferon is rapidly eliminated from the circulation. Yet it is not clear in which organ it is preferentially taken up or excreted. Two recent studies reported the presence of interferon in the urine: Gresser and co-workers recovered a considerable amount in the urine of mice to which the inhibitor had been passively administered (3), and Oh found it in the urine of rabbits after the intravenous injection of endotoxin (4). The preliminary results reported in this paper deal with the urinary excretion of interferon in rabbits after the intravenous injection of either Sindbis virus or endotoxin.

Methodology. Albino rabbits weighing about 1.8 kg were used in this study. The Sindbis virus was propagated and titrated as described elsewhere (1). The rabbits received intravenously about 10^9 to 10^{10} PFU of the virus. The endotoxin was prepared from *Proteus rettgeri* following the method of Roberts (5). The interferon determinations were carried out with a plaque-inhibition technique using primary rabbit kidney tissue cultures in 60 mm plastic petri-dishes and vesicular stomatitis virus for the challenge (1).

Results. Five rabbits were intravenously injected with Sindbis virus; eight hours later the sera and spleens of the animals were taken. Urine was collected during the last four hours of the experiments. The samples were dialyzed against Sörensen's glycine buffer at pH 2, in order to kill residual Sindbis virus. The pH was restored to neutral by dialysis against phosphate-buffered saline. The results of interferon titrations are summarized in Figure 1 (part B). The figures for rabbits Nos. 3, 4, and 5 indicate that interferon was about five times more concentrated in the urine than in the serum. In rabbits Nos. 6 and 7 higher serum and spleen interferon levels were found. The urine samples of these rabbits were not acidified but titrated in the presence of an antiserum against Sindbis virus. Interferon titers of 54,000 and 88,000 units were obtained.

The excreted inhibitor was examined more thoroughly and was found to be fairly stable at pH 2, not dialyzable, trypsin-sensitive, and species-specific. Furthermore, it was not encountered in control urine samples (part A of the figure), so that it can be identified with high probability as an interferon.

In a second series of experiments we tried to demonstrate the presence of interferon in the urine of endotoxin-treated rabbits. Four animals received 2 mg of endotoxin intravenously; they were sacrificed after two, three, four, and five hours, at which times serum samples and spleens were taken. The urine was collected

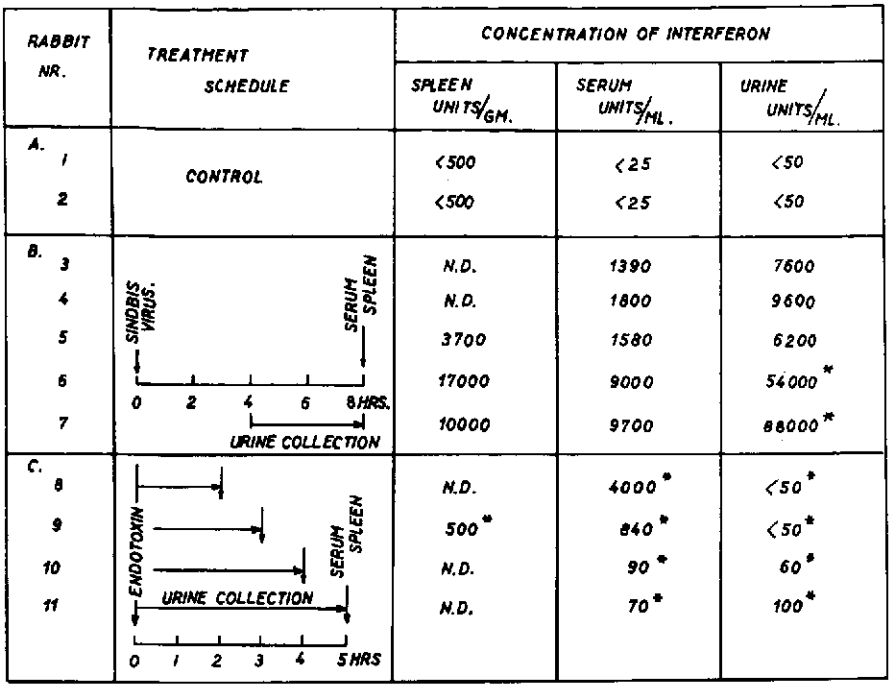

Fig. 1. Urinary excretions of interferon in rabbits.

over the entire period of the experiments. The results of interferon determinations are shown in part C of the figure. The highest concentration of interferon in the serum was found two hours after the injection of endotoxin; thereafter the titer rapidly declined. In contrast, in Sindbis virus-treated animals the urine contained only a minute amount of the inhibitor, which appeared two or three hours after the peak value in the serum.

Discussion. The presence of interferon in the urine of Sindbis-virus-treated rabbits indicates that the kidney either synthesizes it or excretes serum interferon. The fact that Gresser and coworkers recovered interferon from the urine of mice to which the inhibitor had been passively administered tends to argue in favor of the second possibility. According to this hypothesis, the failure of endotoxin-induced interferon to be excreted in the urine could be explained by its different physico-chemical properties, first of all its higher molecular weight (7).

From a practical point of view it is interesting to note that the kidney seems to actively concentrate virus-induced interferon. This observation might indeed open a new approach to the preparation of pure interferons. Considering recent developments in interferon induction by relatively harmless drugs, it is even conceivable that human interferon will in the future be obtained from urine.

Summary. After the intravenous administration of Sindbis virus in rabbits, a virus inhibitor having the physical and biological characteristics of an interferon was demonstrated in the urine. The concentration of interferon in the urine was up to five times that in the serum. In contrast, when a *Proteus rettgeri* endotoxin was used as the interferon inducer, only minute amounts of inhibitor appeared in the urine, despite a good yield of serum interferon.

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CHAIRMAN RASKA: Thank you, Dr. Billiau. May I ask Dr. Julius S. Youngner, Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, to continue.

DR. YOUNGNER: The subject of my discussion is "Modification of Interferon Response in Animals."*

Introduction. The potential use of endogenous interferon for the prevention and treatment of virus diseases in man raises many problems. This short discussion deals with one of these problems: the alteration of the interferon response of the host by exposure to various viral and nonviral stimuli. The studies summarized have been carried out in the mouse; obviously, the applicability of the phenomena described to other species of animals, including man, remains a subject for conjecture and future experimentation.

In mice it is possible to identify at least two separate and distinct patterns of interferon production stimulated by different viral and nonviral materials. One of these can be termed the "live virus" response. Intravenous injection of infective Newcastle disease virus (NDV) or live *Brucella abortus* results in the appearance of interferon that is newly synthesized (8) and reaches peak titers six to twelve hours after injection. The molecular weight of the interferon produced by this response is variable (28,000 to 77,000) and depends on the stimulus employed and the time of bleeding (2, and unpublished results).

The other clearly definable interferon response can be termed the "endotoxin-type" response and is characterized by the appearance in the blood of a heavy molecular weight (85,000 to 90,000) interferon that is released from a preformed state and reaches maximum levels about two hours after the intravenous injection of the stimulus (2). The stimulus in this case can be certain intact gram-negative bacilli or lipopolysaccharides prepared from the cell walls of these organisms.

In addition to the studies described above, procedures known to alter the reactivity of animals to the lethal or pyrogenic effects of endotoxin were investigated for their influence on the appearance of interferon in the circulation of mice injected with the different stimuli. The results to be described below deal with one aspect of this problem: the production of a state of reduced interferon response (hyporeactivity) in animals pretreated with different viral and nonviral stimuli.

Experimental work. Methods for the inoculation and bleeding of mice and for the assay of interferon in L-cells using the plaque-reduction method with vesicular stomatitis virus have been described in detail previously (6, 8).

Studies have been reported (7) that showed that mice injected intravenously with endotoxin exhibited the usual pattern of peak interferon levels in the plasma two hours later but that a second dose 48 hours later failed to produce circulating interferon. The data presented in Figure 1 show (a) the responses of control mice to inoculation with endotoxin and Newcastle disease virus (NDV) and (b) the responses of mice to these inocula when challenged 48 hours after a single dose of endotoxin. The results demonstrate that prior treatment with endotoxin completely eliminated the characteristic appearance of interferon that followed the inoculation of endotoxin in untreated mice. With NDV challenge, mice treated 48 hours previously with endotoxin showed a markedly depressed

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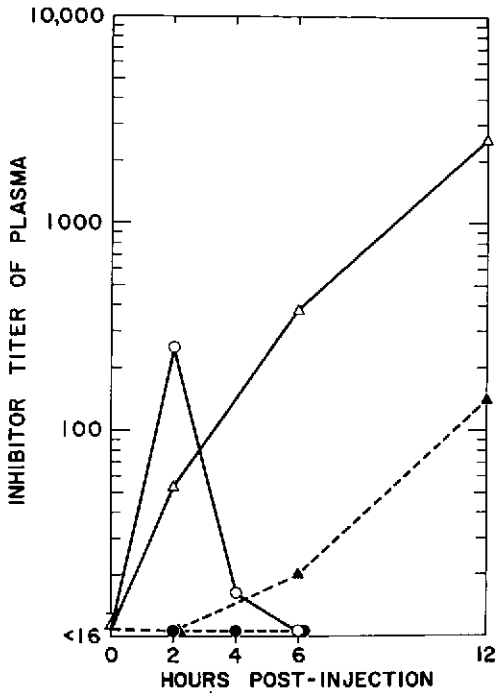


Note: Groups were injected as follows: ○ = No prior treatment, 125 μg endotoxin; ● = Pretreatment with 125 μg endotoxin 48 hrs. before, 125 μg endotoxin; △ = No prior treatment, 5×10^7 PFU of NDV; ▲ = pretreatment with 125 μg endotoxin 48 hrs. before, 5×10^7 PFU of NDV (Data from ref. 7).

Fig. 1. Effect of prior injection with endotoxin on interferon titers of plasma pools from mice at different times after intravenous injection of *E. coli* endotoxin or NDV.

interferon titer compared to the untreated controls.

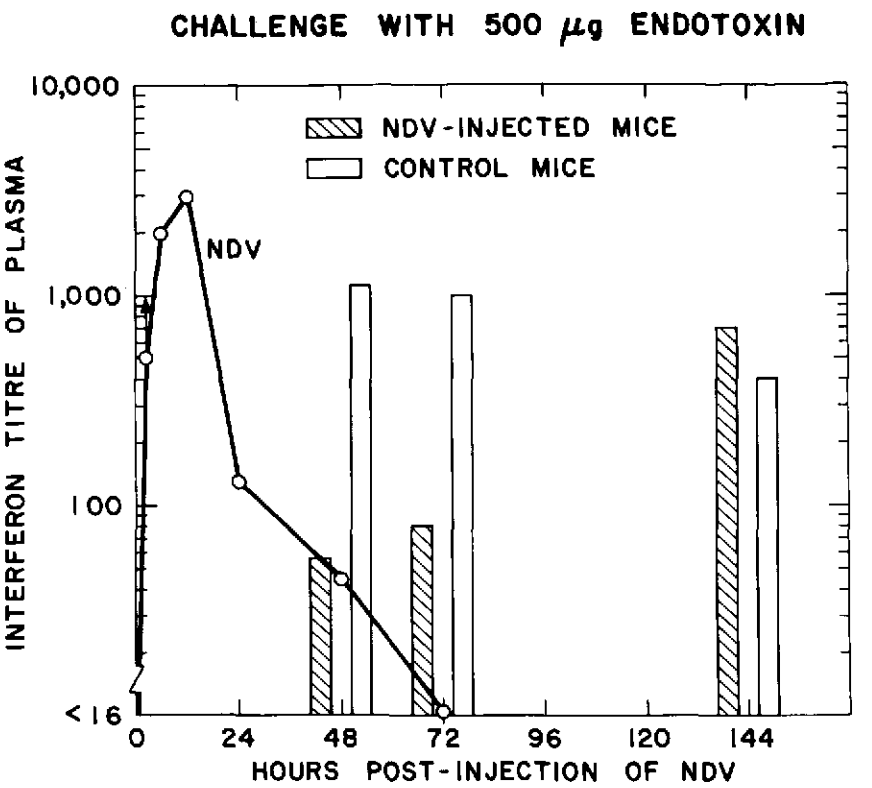
Data were also obtained that showed that pretreatment of mice with NDV decreased the interferon response to endotoxin and that this decreased response did not disappear until about six days after the NDV injections (see Fig. 2). This experiment also demonstrated that a decreased appearance of interferon in hyporeactive animals is not limited to the materials used to produce this state. Additional information was obtained that ruled out a humoral factor as the cause of the hyporeactive state (7). A humoral factor also seems unlikely in hyporeactivity in rats (1). In contrast, other workers have suggested that a humoral factor is involved in hyporeactivity of rabbits (3).

The experiment summarized in Table 1 was designed to study the phenomenon of heterologous hyporeactivity when NDV was given to mice intravenously and interferon production was stimulated 48 hours later with different nonviral materials. The mice were injected intravenously with 2×10^8 PFU of NDV; the usual pattern of interferon production was obtained (see legend, Table 1). Forty-eight hours later, mice that had received a single injection of NDV were injected intravenously with *E. coli* endotoxin, live *Brucella abortus* organisms, or statolon, an anionic polysaccharide from *Penicil-*

TABLE 1. APPEARANCE OF INTERFERON FOLLOWING INJECTION OF NDV, E. COLI ENDOTOXIN, BRUCELLA ABORTUS, OR STATOLON INTO MICE INJECTED 48 HOURS PREVIOUSLY WITH NDV

Stimulus (i.v.)	Mice pretreated 48 hrs previously with	Interferon titer of plasma at		
		2 hours	6 hours	10 hours
NDV (Herts) (2×10^8 PFU)...	Saline.....	520*	8,000	15,000
	NDV (2×10^8 PFU)†....	130	800	760
E. coli endotoxin (250 μg).....	Saline.....	1,350	—	—
	NDV.....	240	—	—
B. abortus (1.7×10^8).....	Saline.....	78	2,100	1,400
	NDV.....	<16	330	270
Statolon (1,000 μg).....	Saline.....	400	600	1,300
	NDV.....	25	500	150

* Pooled plasmas from 10 mice.
†Interferon titers of plasma after first injection of NDV: 2 hours, 900; 6 hours, 6,000; 10 hours, 15,000; 24 hours, 1,100.



Note: Interferon titers following injection of 2×10^8 PFU of NDV (○); a challenge dose of 500 μg of endotoxin was given at 48, 72, and 144 hours and plasmas were collected 2 hours later (see bars). (Data from ref. 7).

Fig. 2. Influence of prior injection of NDV on interferon response of mice to *E. coli* endotoxin.

lium stoloniferum (4, 5). After this second inoculation, interferon titers were determined at the times that had been found appropriate with the different stimuli. As controls, mice that had received saline intravenously were inoculated with the same stimuli used in the animals pretreated with NDV. The results showed a significant reduction in interferon production by the different stimuli in mice pretreated with NDV.

Table 2 summarizes the results of another experiment, in which mice were challenged with various viral and nonviral stimuli 48 hours after pretreatment with these materials. The patterns of homologous and heterologous hyporeactivity showed that in mice pretreated with NDV reduced interferon titers resulted from the second injection of NDV, endotoxin, and statolon. Pretreatment with endotoxin or statolon produced homologous hyporeactivity and a markedly depressed interferon response to NDV. In contrast, endotoxin and statolon did not produce reciprocal hyporeactivity (9), which points to the probable differences in the cell populations affected by these substances and again empha-

TABLE 2. PATTERNS OF HOMOLOGOUS AND HETEROLOGOUS HYPOREACTIVITY IN MICE PRETREATED AND CHALLENGED WITH DIFFERENT STIMULI OF INTERFERON

Mice pretreated* with	Percentage inhibition of interferon titer in plasma after challenge 48 hours later with		
	Endotoxin	NDV	Statolon
Endotoxin.....	100	95	0
NDV.....	100	98	94
Statolon.....	0	75	84

*For dosages employed, see Table 1.

sizes the cellular (rather than humoral) basis of this reduced responsiveness.

Summary and conclusions. It has been demonstrated that interferon in mice can be depleted or exhausted by certain stimuli so that subsequent stimulation with the same or different materials elicits markedly reduced amounts of interferon in the circulation. The cellular nature of this state of hyporeactivity has been proposed. Any consideration of the use of endogenous interferon for the prevention or treatment of virus infections in man must take this phenomenon into account.

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CHAIRMAN RASKA: Thank you, Dr. Youngner. We will now hear from Dr. John H. Dingle, School of Medicine, Western Reserve University, Cleveland, Ohio.

DR. DINGLE: Major attention at this Conference has been focused on the prevention and control of viral and rickettsial diseases by the use of specific vaccines prepared from the etiologic agents of these infections. Such emphasis is unquestionably deserved, particularly in view of the striking success that has been achieved with some of the vaccines. Moreover, we know many of the factors involved in specific immunity, though by no means all of them. But the specific approach of artificial immunization presents, for a certain major group of diseases, seemingly insurmountable difficulties, as has already been indicated by several of the speakers and discussants.

It seems to me that more thought and effort should be given to other approaches. Certainly, chemoprophylaxis and chemotherapy constitute one of them. Another is the matter of nonspecific resistance or "nonspecific immunity" and the factors involved in such resistance. We have striking examples showing that such resistance does exist. One is the resistance and susceptibility of various animal species to a given virus. Another is the fact that even the most virulent of viruses will not kill all the members of a population they infect; in fact, they will not infect detectably all who are exposed.

Are there ways in which this nonspecific resistance can be supported and strengthened? There must be, and the actual methods for doing so may not be too difficult. Interferons and interferon-like substances are now in the spotlight, but such subjects as complement, properdin, and phagocytic activities of cells should not be forgotten. This area of investigation seems to offer tremendous possibilities with respect both to therapy and to prevention.

CHAIRMAN RASKA: Thank you, Dr. Dingle. I now call on Dr. D. Blaškovič from the Institute of Virology, Czechoslovak Academy of Sciences, Bratislava, Czechoslovakia.

DR. BLAŠKOVIČ: It has been shown that virus inhibitors strongly resembling the classical interferon could be induced in animals with various microbial products such as lipopolysaccha-

rides,* statolon,† and helenine.‡ Recently Šikl *et al.*§ obtained a highly branched water-soluble mannan of about 5,500–8,000 M.W. from *Candida albicans* with specific rotation $[\alpha]_D^{20} = +56°$. In mice injected intravenously with 100 μg of this mannan, an interferon-like substance has been detected in the serum as soon as two hours after injection. Its properties are as follows: not sedimented at 104,000 G for 1 hour; partly inactivated at pH 2 for 24 hours; partly inactivated by trypsin; completely inactivated at 65°C for 30 minutes; showed species specificity but not virus specificity; showed "early" appearance in circulation of mice. By 24 hours after one intravenous dose of mannan the interferon had disappeared from the circulation (see Table 1). With a dose range of 10 to 300 μg of mannan per mouse, no interferon was demonstrated in the spleen.

Interferon formation has also been observed *in vitro* when mouse peritoneal cells were treated with mannan from *Candida albicans*. As expected, in tissue culture medium 199 (with 20 per cent calf serum and antibiotics) the activity of interferon persisted longer than in the circulating blood of mice (see table). However, the interferon-releasing ability of the peritoneal cells quickly disappeared: when they were grown *in vitro* for 48 hours or more no further production of interferon could be detected.

Similarly, we failed to induce interferon formation in continuous L mouse fibroblasts or in primary chick embryo cells.

These experiments seem to indicate that purified homopolysaccharides devoid of detectable lipids or proteins can induce interferon-like substances in mice; that most probably the cells

Table 1. INTERFERON INDUCTION BY MANNAN FROM CANDIDA ALBICANS

In mice 100 μg i.v.:	
serum	+
spleen	−
Time of maximal interferon titers in serum	2 to 6 hours
Time of complete disappearance of interferon from serum	approx. 24 hours
In vitro:	
Mouse peritoneal cells 100 μg/2.5 x 10^6 cells	
24 hours after explantation	+
48 hours after explantation	−
Time of maximal interferon titer in growth medium	6 to 12 hours
Time of complete disappearance of interferon from growth medium	approx. 48 hours
Minimal interferon inducing amount of mannan	approx. 10 μg/2.5 x 10^6 cells
Mouse L fibroblasts (48 hours old)	−
Chick embryo cells (24 hours old)	−

of the reticulo-endothelial system are responsible for interferon release, since *in vitro* only the mouse peritoneal cells were active in this respect; that *in vitro* these cells preserve their interferon-releasing ability for only a limited time; and that properties other than the polyanionic character of a molecule (Kleinschmidt *et al.*) must be responsible for interferon induction in the case of mannan from *Candida albicans*.

CHAIRMAN RASKA: Thank you, Dr. Blaščovič. I now call on Dr. Samuel Baron, Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

DR. BARON: I should like to report on the "Role of Interferon during Rubella Virus Infection of Green Monkey Kidney Cell Cultures," co-authored with Dr. Ken Wong.‖

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‖ Department of Virus Research, Microbiological Associates, Bethesda, Maryland.

A number of studies have demonstrated that cell cultures and human embryos may become persistently infected with rubella virus. These and other studies have shown that cultures infected with rubella virus are resistant to superinfection with heterologous virus and that small to moderate amounts of interferon are produced. However, there is insufficient evidence for a final determination of whether interferon plays a role in maintaining the persistent infections, in inducing resistance to superinfection, or in the development of vaccine strains of rubella virus. A study was undertaken to obtain more information on the effect of interferon during rubella virus infection of primary African green monkey kidney cell cultures (GMK). The results indicate that much of the inhibition of rubella virus and heterologous virus could be accounted for by the amount of interferon produced, the degree of resistance induced (Fig. 1), and the high sensitivity of rubella virus to the interferon system (Fig. 2). Furthermore, it was observed that the final yield of rubella virus was proportionate to the multiplicity of infection (Fig. 3). From the dynamics of the interferon response it seems

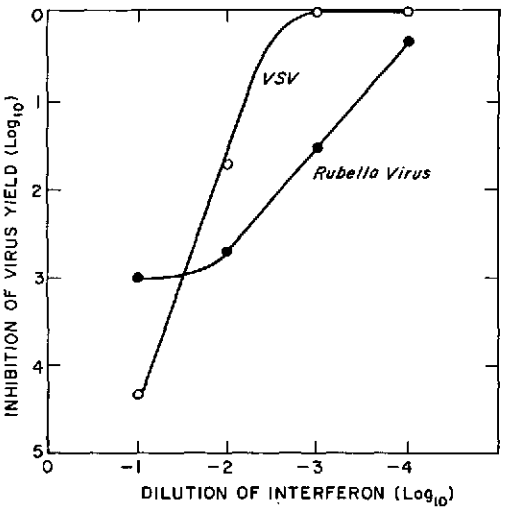

Fig. 2. Comparative inhibition by interferon of vesicular stomatitis virus (VSV) and rubella virus in GMK.

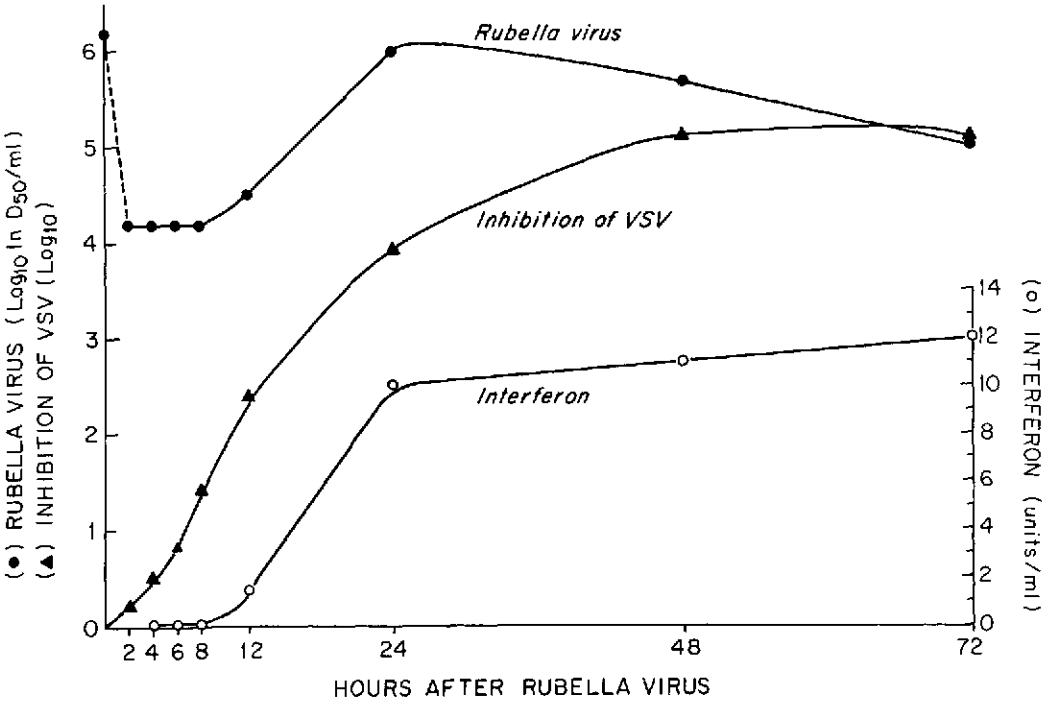

Fig. 1. Rubella virus, interferon and challenge virus yields in GMK infected with rubella virus.

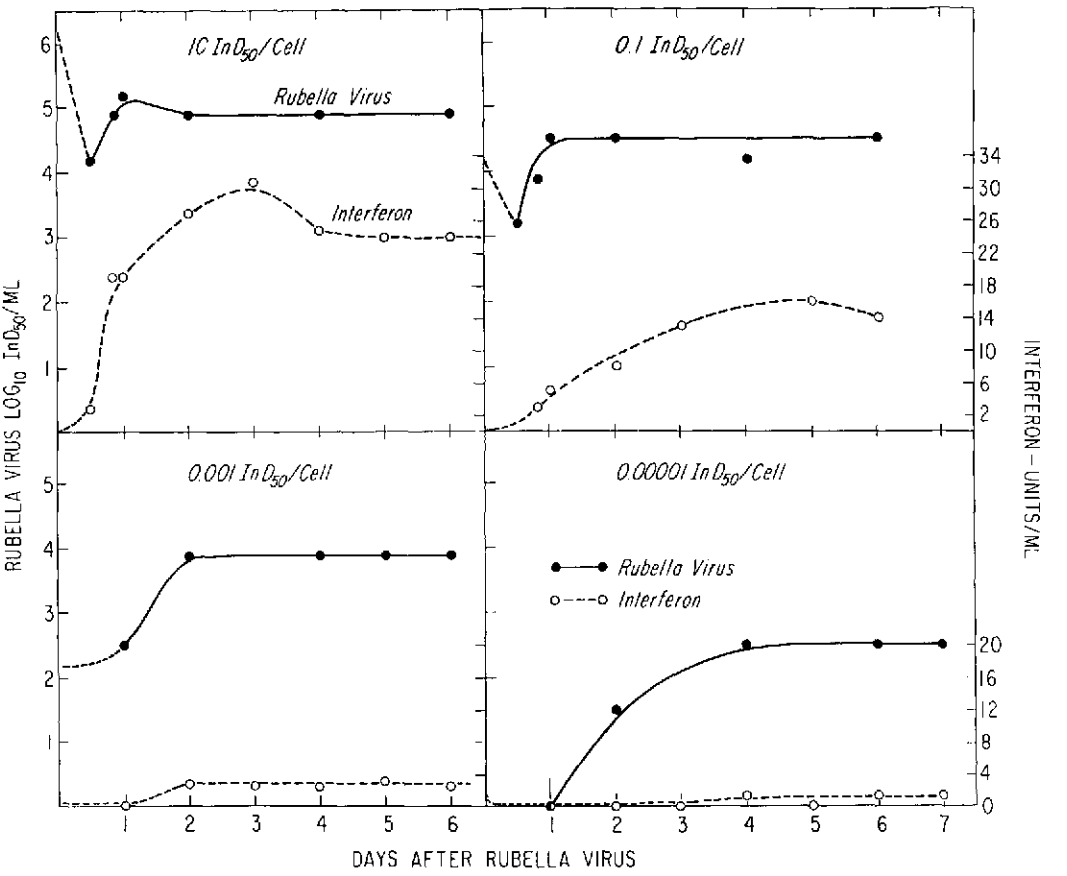

Fig. 3. Effect of infecting dose of rubella virus on virus and interferon yields in GMK.

likely that interferon is an important cause of the dose response to rubella virus in GMK.

CHAIRMAN RASKA: Thank you, Dr. Baron. Our next discussant is Dr. V. D. Soloviev, Gamaleya Institute for Epidemiology and Microbiology, Academy of Medical Sciences, Moscow, USSR.

DR. SOLOVIEV: Interferon is undoubtedly a factor of importance to viral infections in general and to influenza in particular. Various aspects of the problem are being studied at our laboratory.

The susceptibility of human beings to influenza virus at different periods of the year is being determined. This is immediately related to the elucidation of the factors responsible for increased rates of respiratory diseases in cold seasons. Observations on volunteers infected with vaccine strains of influenza virus have shown that their susceptibility decreases sharply in summer and rises in winter. This correlates with the levels of interferon produced by volunteers in different seasons: in summer the level is three to four times higher than in winter.

We prepare exogenous human interferon by using suspensions of leukocytes obtained from donor blood. A comparison was carried out in 157 volunteers, who received intranasally either human-leukocyte-produced or chick-embryo-produced interferons, or a placebo. Those who were given the leukocyte-produced interferon proved almost three times as resistant as the controls to infection with vaccine strains of influenza virus. The chick-embryo-produced interferon proved to be insignificant in effect.

An investigation of endogenous interferon was carried out by administering inactivated in-

fluenza viruses, vaccinia virus, or a placebo. Observations on 182 volunteers revealed that the most effective stimulant tested for evaluating resistance to subsequent infection was the influenza virus inactivated by ultraviolet irradiation.

Considering the results of the observations on exogenous (leukocyte-produced) and endogenous interferons, we believe the advantage to lie with the former. Human-leukocyte-produced interferon is harmless, and with further purification and concentration it may prove not only more effective for local application but also of potential importance by pararespiratory and parenteral routes of administration. For the stimulation of endogenous interferon formation it seems necessary, in spite of the satisfactory results observed, to seek inducing agents more acceptable for human use than the virus-containing tissue suspensions.

CHAIRMAN RASKA: There are a few moments left for free discussion. Dr. Tyrrell.

DR. TYRRELL: We have studied specimens from patients with common colds in organ cultures of human nasal and tracheal epithelium. Among these specimens were some that contained viruses that could be propagated in organ cultures and could not be adapted to tissue cultures. They were detected only by producing colds in volunteers. Two of these viruses have now been found by Dr. Hoan to stop ciliary activity of organ cultures and to have the basic properties of rhinoviruses. Organ cultures infected with other strains have been examined by negative contrast electron microscopy by Mrs. J. Almeida. One contains virus particles indistinguishable from avian infectious bronchitis; similar particles were also found in organ cultures infected with the 229 virus of Hamre *et al.* Cultures inoculated with these specimens contained material indistinguishable from the internal component of parainfluenza and related viruses. These viruses still cannot be detected in organ culture but all but one can now be propagated in this way using a somewhat modified technique.

SESSION X

GENERAL DISCUSSION

Friday, 11 November 1966, at 2:00 p.m.

CHAIRMAN
DR. ALBERT B. SABIN

RAPPORTEUR
DR. ANDREW J. RHODES

Summary of the Conference

Dr. Sven Gard

Discussion with special emphasis on future needs for research in laboratory, clinic, field, etc.

Closing Remarks

Dr. Abraham Horwitz

GENERAL DISCUSSION

CHAIRMAN SABIN: We will start this afternoon's session with a short communication by Dr. A. Fabiyi, National Institutes of Health, Bethesda, Maryland, on the effect of heterologous antibodies on the serological conversion rate observed after 17D yellow fever vaccination.

DR. FABIYI: I should like to thank the chairman for allowing me a few minutes to report on the effect of heterologous antibodies on the serological conversion rate after 17D yellow fever vaccination.

There is evidence to support the contention that the presence of antibodies to viruses serologically related to yellow fever interfere with antibody response to successful yellow fever vaccination with the 17D strain vaccine adapted to mouse brain. Vaccination was performed using the scarification method.

The results showed 96.2 per cent conversion among 157 persons without antibodies to yellow fever-related viruses, as compared with 84.5 per cent conversion among persons with group B virus antibodies. The results were shown to be statistically significant. In contrast, no correlation was found between prior infection with Ilesha virus—an arbovirus serologically unrelated to yellow fever or group B arboviruses—and antibody formation after 17D yellow fever vaccination.

These studies suggest that (a) persons who live in an area in which group B viruses are endemic and yellow fever does not exist should ascertain their immune status to yellow fever after vaccination before traveling to yellow fever endemic areas; (b) further studies should be conducted to find a method of vaccination against yellow fever that yields maximum conversion rates regardless of the epidemiologic status of the geographic area where the vaccine is given; and (c) there are many more problems relating to yellow fever and vaccination against the disease of which we are still ignorant.

CHAIRMAN SABIN: I will now call on Dr. Sven Gard, who will present a summary of the deliberations held during the Conference.

DR. GARD: In his introductory address, Dr. Stuart-Harris ably summarized this Conference before the fact; he presented a list of present achievements, failures, and remaining pressing problems; and he discussed the ways and means by which the desired goals could be reached. After these five days of reports and discussions, I can only express my admiration for his succinct statements. There is little I can do to improve on them, but I shall try, with the aid of the rapporteurs, to underline some of the important facts that have been brought to our notice.

SMALLPOX

It was generally felt that the necessary conditions for an effective control of smallpox have now been clarified and that the laboratory and technical basis for such an endeavor has been consolidated. Whether the associated financial, sociological, and administrative problems can be solved in the foreseeable future seems still uncertain, however.

Concerning the policy to be adopted by developed countries free of endemic smallpox, different opinions were presented. The mass use of smallpox vaccine is undeniably associated with risks of such a magnitude that, with the safety standards now generally adopted, the product would probably not be accepted, should the question of licensing come up for reconsideration. The need for more attenuated vaccine strains with maintained immunogenic capacity and for more well-defined products than those now being used is obvious. Progress in this was reported, justifying a moderate optimism.

ENTEROVIRUSES

The feasibility of practically complete control of poliomyelitis and of substantial reduction, if not eradication, of the flora of wild virus strains is now well documented. It would seem that this aim might be achieved with the aid of either live or inactivated vaccines, provided the latter are of sufficient potency. Irrespective of the type of vaccine used, a sufficient coverage of the population at risk is a prerequisite for success and a satisfactory level of general immunity must be maintained, which includes systematic continued vaccination of the infant population and which may call for periodic serologic surveys and possibly revaccination.

It was pointed out that poliomyelitis is beginning to emerge as a recognized medical problem also in tropical areas. The abundance of other enteroviruses circulating in the tropics might be expected by interference to present special problems in connection with attempted application of live poliovirus vaccine in such regions.

The medical importance of nonpoliomyelitis enteroviruses was discussed, and some, such as Coxsackievirus Types A7, B2, and B5 and echovirus Type 9, were mentioned as being sufficiently important to justify development and application of specific vaccines.

MEASLES

Live measles virus vaccines, both the original Edmonston B and later developed more attenuated strains, have been used on a large scale in the United States, the Soviet Union, West Africa, Chile, and the United Kingdom. These vaccines have not caused serious reactions, although pyrexia was observed in a proportion of vaccinees and convulsions in considerably less than 1 per cent. They have provided solid and presumably long-lasting immunity.

Inactivated vaccines have not been so widely employed. Two are available as whole virus vaccines and one prepared from the hemagglutinin of disintegrated virus has been tested experimentally. With sufficiently potent products and an immunization schedule, including a booster injection after a suitable interval, very high serum titers and apparently good protection have been obtained. Primary immunization with inactivated virus may include a state of hypersensitivity, in turn resulting in unexpected local and general reactions on subsequent exposure to attenuated or wild measles virus.

Preliminary studies of measles vaccine combined with smallpox and other vaccines have given promising results.

RUBELLA

Rubella occurs in epidemics in intervals of about seven years (in the United States). The majority of cases are found in children under 15 years of age, but infections in adolescents and young adults are not infrequent. Age distribution of serologic immunity, when compared to that against measles, shows a displacement of about six years toward higher age groups. The disease in itself is mild but assumes medical importance through the fact that infection in the first trimester of pregnancy might be transmitted to the fetus, causing abortion, stillbirths, or various congenital defects in a considerable proportion of cases.

Surveys in the United States and Japan have shown that 10 to 30 per cent of women of child-bearing age are susceptible to disease. The recent development of a hemagglutination-inhibition test has considerably facilitated serologic surveys and further information on the ecology of rubella virus can be expected to be available in the near future.

There is certain although not conclusive evidence that γ-globulin treatment may reduce the incidence of clinical disease and that the incidence of congenital defects may be similarly reduced.

Several attenuated variants of rubella virus have been obtained after serial passage in tissue culture and have been tested in monkeys and in children. Such strains have been found to produce subclinical infections, with little or no shedding of virus but with serologic conversion and apparent protection against the natural disease.

MUMPS

Mumps, which occasionally is the cause of serious complications in adults, would seem to

call for a safe and effective vaccine providing long-lasting protection. Tests with inactivated vaccines have so far not been encouraging, since the immunity obtained has been of short duration. Live attenuated vaccine has been developed and tested for several years in the USSR and now also in the United States. In both cases it has been found that serial passage in tissue cultures is associated with a continued gradual attenuation which may result in loss of infectivity for man and consequently also in loss of immunogenic capacity. At a suitable level of attenuation these strains have proved capable of inducing subclinical infection without shedding of virus, but with production of serologic immunity and protection against natural disease. The question of the duration of immunity would, however, seem to need further elucidation.

The results so far reported might be considered as definitely successful or highly promising. In the field of *respiratory tract diseases* the records are more equivocal.

ADENOVIRUSES

Of the many serotypes hitherto identified, relatively few appear to cause significant illnesses in man, but some of them tend to give rise to epidemics of sometimes considerable proportions. Inactivated, polyvalent as well as live, monovalent vaccines have been developed and tested, especially in military personnel, and have appeared to be safe and to be capable of reducing specifically the attack rates of respiratory tract diseases. The discovery of the oncogenic capacity in certain experimental animals of several human adenovirus serotypes and of the phenomenon of their "hybridization" with other agents of known oncogenic capacity has, however, introduced a totally new aspect in the problem of active immunization. Although there is as yet no evidence that any of these viruses is oncogenic to man, use of inactivated vaccine to be administered parenterally has, for the sake of safety, been suspended. Trials with live virus of types which have so far not shown any oncogenic capacity, given in enteric-coated capsules, continue, however, and have yielded encouraging results.

INFLUENZA

The problems besetting the control of influenza are of a different nature. Inactivated vaccines used on a large scale over the last two decades have proved to have high protective value in military populations while the results of mass vaccination of civilian populations have often been less convincing. The reasons for this are not clearly understood, but it might well be that more emphasis should be placed on vaccination of young children as a means of reducing the rate of spread of infection.

The difficulties encountered in attempts to develop effective live influenza virus vaccines derive mainly from the fact that the virus during passage in the laboratory tends to become overattenuated and thus lose its immunogenicity, as well as from the fact that lack of reliable markers precludes estimation of the suitability of different candidate strains.

The biggest problem is still the antigenic instability of the A group viruses. The question of the nature of and the basis for the antigenic variation, whether it represents a fundamentally cyclic phenomenon or a progressive evolution, is still open. The increasing number of group A viruses found in animals such as birds, swine, horses, and possibly other species are in this connection of great interest. Although evidence of transmission of animal strains to man is lacking, the possibility of animal reservoirs of human influenza must be seriously considered as well as the possibility that new strains may emerge as a result of hybridization in nature between human and animal strains. It might be of significance that the B group viruses, which apparently are much less well represented among animals (although a possible B swine strain has now been isolated), display considerably less antigenic variation.

PARAINFLUENZA

Inactivated parainfluenza virus vaccines have elicited antibody responses in young children, but data on the protective effect are not yet available. Combined vaccines of parainfluenza virus and other agents thought to be of etiological significance in shipping fever of cattle have been reported to be effective.

RESPIRATORY SYNCYTIAL (RS) VIRUS

Present results of attempts to immunize against RS virus infection are not particularly encouraging. In this case the nature of the antibodies produced may be of importance. Presence of maternal antibodies does not protect the infant against bronchiolitis or pneumonia, and the placenta-passing antibodies alone can therefore obviously not afford effective protection.

RHINOVIRUSES

Rhinoviruses found to be associated with common colds are known to fall into many different serological types with no or little antigenic overlapping. Inactivated virus vaccines have been tested experimentally in volunteers. In challenge tests they have been found to afford protection, but only against viruses of the same serotypes as those used in the vaccine. Serial studies have shown that any one person might undergo infections with a sequence of viruses of many different serotypes during a relatively short period of time. Thus, prospects for control of common colds by means of active immunization seem at present to be gloomy.

On account of the complex etiology of human respiratory tract diseases, the use of monovalent vaccines (with the exception of influenza) can hardly be expected to cause significant reduction of morbidity rates except under very special conditions. It is possible, however, that polyvalent preparations of carefully selected components might be of some aid in attempts to reduce the number of severe illnesses, particularly those of the lower respiratory tract. Some such experimental vaccines have been tested with encouraging results.

MYCOPLASMA

Although neither virus nor rickettsia, the *Mycoplasma* species (*M. pneumoniae*) associated with atypical pneumonia was also discussed. Vaccine preparations have been tested and found to afford protection against disease. The possible use of purified extracts of the causative microorganisms was foreshadowed.

ARBOVIRUSES

The same confusion that characterizes the discussion of control of respiratory tract diseases seems to reign in the arbovirus field, although for slightly different reasons. Arbovirus infections might be characterized as zoonoses, and by definition an arbovirus should multiply both in the vertebrate host and in an arthropod vector involved in the natural cycle of transmission. More than 200 such viruses are known, which are most usefully classified immunologically. The homogeneity of the arbovirus group is, however, questionable.

About 70 arboviruses are known to produce a wide variety of diseases in man, ranging from grippe-like fever through meningo-encephalitis to hepatitis with hemorrhagic manifestations and hemorrhagic fevers. Fatality rates may amount to 20 per cent but most infections are probably symptomless. In recent years several major epidemics have been reported, their most disturbing feature being the appearance of previously unknown viruses or of previously unobserved clinical manifestations.

It was pointed out that the control of arbovirus infections posed many problems and that immunoprophylaxis, with a few notable exceptions (yellow fever), would not seem to be an urgent one. Since man usually is not a natural host of these viruses, becomes involved more or less accidentally, and usually does not serve as a source of further human infections, morbidity rates are generally low, the appearance and distribution of cases is unpredictable, and a rational basis for mass prophylaxis is seldom established. Even if vaccination of special groups, such as laboratory workers and certain local populations, is justified, commercial interest is insufficient to stimulate the long and costly process of developing the corresponding vaccines. Besides the live yellow fever vaccine, two inactivated mouse brain vaccines against RSSE and Japanese B are available, however, and others (against VEE and dengue) are under study.

By and large, control of these diseases must rest on vector control and other means by which the natural cycle of transmission might be interrupted.

Since 1953 severe hemorrhagic fevers have appeared in Argentina and Bolivia. The causative

viruses were recovered from wild rodents and one of them also from ectoparasites, but transmission was most probably due to close, direct human-rodent association. Rodent control has, indeed, proved effective in an urban outbreak.

RABIES

Recent electron microscopical identification of the rabies virus has provided a previously unsuspected morphological link between this agent and certain arthropod-borne and other viruses. The actual significance of the morphological similarities observed cannot yet be assessed, however. Biochemical studies indicate that rabies virus may occupy a hitherto unique position, in so far as phospholipid synthesis seems to be essential for production of virus-specific antigen and infective virus.

Dogs continue to be the main source of infection for man, and greater efforts are necessary to extend vaccination of pet dogs and eradication of stray dogs. The HEP Flury vaccine is a satisfactory prophylactic agent for dogs.

Wild life rabies, although prevalent in vast regions of the world, seems not to pose serious problems in relation to man, but continuous observation is necessary. Some wild life hosts are, however, a source of cattle rabies. A satisfactory live virus vaccine for cattle is available.

Pre-exposure vaccination of humans is justified only for very special groups. The present vaccines of the Semple or duck-embryo type need to be improved. Production in tissue culture of antigens of satisfactory potency now appears feasible. For postexposure treatment the urgency of cleaning the wound with suitable antiviral agents must be stressed. Production of human immunoglobulin to replace the horse serum now available was recommended as was the development of more potent products than the duck-embryo vaccine.

HERPESVIRUSES

In the herpesvirus group the recurrent herpes simplex assumes definite medical importance. Since many different factors seem to affect this phenomenon in various ways, the possible effects of immunization or "desensitization" should be judged with utmost caution.

The need for an effective vaccine against simian herpesviruses for protection of laboratory workers and others handling monkeys (including Latin American species) was stressed. An inactivated B virus vaccine has been prepared but is not yet licensed. Similar vaccines against pseudorabies have also been produced.

The possibility of prevention of B virus infection by local application of γ-globulin was mentioned and attention was directed to the fact that the protective capacity resides mainly in the IgA fraction of the serum.

Varicella-zoster and cytomegaloviruses were discussed and the seriousness of the latter infection in infants was stressed. Both these viruses exhibit the phenomenon of latency, and recent observations on the consequences of immunosuppressive therapy suggest that the adult carrier rate of cytomegalovirus may be high. No immunoprophylaxis against these viruses can be expected in the near future.

HEPATITIS

Concerning hepatitis it was stated that no virus has as yet been isolated that is generally acceptable as the cause of either infectious or serum hepatitis. It is desirable that sources of well-documented specimens of blood and feces should be maintained in some central laboratory and that acute- and convalescent-stage sera from a variety of situations should be collected and preserved for future study.

Meanwhile, further attention should be given to the risk of transfusion hepatitis. The use of a new method of preparation of γ-globulin to render it suitable for direct intravenous injection was described, a procedure which seems to make γ-globulin protection of blood recipients feasible.

RICKETTSIAE

Of rickettsial diseases only epidemic and murine typhus and Q fever were discussed. The surprising discoveries of apparent *R. prowazekii* and *R. typhi* infections in domestic animals in Ethiopia and Egypt were described. Inactivated typhus vaccine of essentially the same type as that developed during World War II is available but seems not to be used. Q fever rickettsiae in phase 1 have been found the most suitable source

for production of a vaccine that will not elicit local sensitivity reactions.

TRACHOMA

Trachoma infection seems to produce little or no natural immunity. Although both active immunization and chemotherapeutic measures may have a beneficial effect in individual cases, prevention of reinfection seems to be the only means by which any durable results of community programs can be achieved. The best and perhaps only way of accomplishing this is by improvment of housing and general sanitary conditions.

ADJUVANTS

A problem of central importance in active immunoprophylaxis is production of sufficiently high levels of protective antibody of sufficient duration. In this respect adjuvants properly used can be of great value. It is evident that the molecular or particle size of an antigen may determine its immunogenic effect, which in turn indicates that phagocytosis might play a decisive part in the initiation of an immune response. The dipolar nature of such adjuvants as aluminum hydroxide or water in oil preparations may act both by aggregating the antigen and attracting the phagocytes. By further studies elucidating the nature of the adjuvant effect a more rational basis for development of effective products may be found.

It was emphasized that adjuvants in vaccines for use in humans should preferably be metabolizable and capable of being eliminated by the organism. Since the nature of the antigen may determine the reactions to an adjuvant-containing preparation, each such vaccine must be tested individually.

VACCINE DEVELOPMENT

Problems concerned with vaccine production were discussed. It was emphasized that systems of safety and potency control (in man as well as in laboratory tests) should be developed before general use. Final evaluation of effectiveness must be made in man.

Diploid cells of man and other species can be propagated serially with maintenance of the properties of the tissues of origin. Such cell strains can be tested exhaustively for safety (i.e., absence of indications of oncogenic characteristics and of latent viruses) providing uniform, standardized cultures for vaccine production.

CHEMOTHERAPY

Methisazone has been effective in preventing the development of smallpox and alastrim in persons exposed to these diseases.

Antiviral chemoprophylaxis against common respiratory infections has not yet been satisfactorily demonstrated. Amantadine hydrochloride may have some prophylactic effect against strains of influenza A2 virus but no demonstrable therapeutic effect.

Ocular virus diseases have been successfully treated with idoxuridine. Of new drugs, trifluorothymidine holds promise for the treatment of adenovirus, herpes simplex, and vaccinia infections. Phagicin, a polypeptide produced by bacteriophage-infected *Escherichia coli* may be useful for systemic therapy of virus diseases.

INTERFERON

Purification procedures, cell-free test systems, and other new techniques now offer possibilities of defining the mechanism of action of interferons. Elucidation of the biosynthesis of interferons presents much more difficult problems because of the multiplicity and heterogeneity of the substances induced by different agents in various animal species or even in single cell types.

The detection of interferon in a variety of viral infections, naturally occurring as well as after inoculation of live vaccines, suggests that interferon plays an active role in limiting the spread of infection. It might be used as a therapeutic or prophylactic agent against virus disease by administration of an exogenous product or by induction of its endogenous formation. In the former case the application of sufficient concentrations at the right site may present particular problems. Induction of endogenous interferon in man has not yet been extensively studied.

CHAIRMAN SABIN: Thank you very much, Dr. Gard.

For the past three days we have had the privilege of hearing documented presentations on the present status of almost all vaccines and of some other procedures that are now being used or are being contemplated for the control of diseases produced by viruses or rickettsiae. You have just heard a summary of the subject matter that has been discussed.

For the most part, the papers that were presented were written before the people came here. Even the brief discussions, with certain exceptions, were written ahead of time.

Since I had to be hospitalized during the course of the Conference, I spent three days in bed reading some of these papers. I found that it is probably even more informative to read the papers in bed than to sit and listen to them at a conference.

Now, then, what is the difference between a conference and reading prepared papers in bed or in any other comfortable attitude? The difference, hopefully, is that in the presence of so many very competent people who have spent their lives in studies on these problems it might be possible to exchange views on some of the outstanding gaps in our knowledge, to discuss some of the important questions that remain unresolved, and to put on record unreconciled differences in judgment or opinion.

Accordingly, it was the decision of the Program Committee that we should spend our time not in a disorganized discussion of almost every topic that has been presented here but rather in the deliberation of a few selected questions that were regarded as having special importance.

It was decided to spend a great deal of our time on viral respiratory disease, and we shall begin with that. We will deal with it under three headings: (1) influenza, (2) viruses of major importance in respiratory disease during the first two years of life, and (3) viral respiratory disease other than influenza in older children and adults.

Influenza remains uncontrolled despite the availability for the past 25 years of vaccines with varying degrees of effectiveness.

What kind of research is needed for the development of a type of vaccine or vaccination procedure that could be used on a mass basis for the elimination of influenza as a frequently recurring epidemic disease? One of the questions for discussion here is whether a new approach might be possible as a result of the experiments of Dr. Florence Lief that were reported by Dr. Henle. These studies indicated that by sequential *infection* of guinea pigs with a small number of different antigenic types of influenza A virus it was possible to elicit a very broad antibody response for antigens that were not even demonstrable in the strains used—a result that was not obtained with killed virus vaccine. Conceivably, if such a result could be achieved by sequential infection of suitable attenuated viruses, we might have a new approach for vaccinating the child population.

I would like to ask whether there is anyone in the audience who has had personal experience with experiments on sequential infection with different antigenic types of influenza A virus. Dr. Henle and Dr. Lief are no longer here, but we have already seen their data.

DR. HENNESSEN: We were able to reproduce Dr. Henle's results, but we also found that with a potent adjuvant we could obtain similar results with only one or two injections. When a Type A_1 influenza virus is injected with a potent adjuvant, antibodies against PR-8 as well as against $A_2/57$ will be obtained.

CHAIRMAN SABIN: Dr. Hennessen, did you work with killed influenza vaccine? Dr. Henle made the point that this sequential effect was obtained only when live influenza viruses were used.

DR. HENNESSEN: We used formalin-inactivated virus.

CHAIRMAN SABIN: Then we may say that your results are different from the ones reported by Dr. Henle in that you were able to obtain the same effect with killed virus preparations.

DR. PEREIRA: I think the question of one versus multiple infections and also the question of only one infection versus hyperimmunization may be considered also in connection with influenza B. We have found repeatedly that when we compare influenza B strains using postinfection sera—that is, sera from ferrets infected intranasally—we get a much more specific reaction than when we compare these same strains

using hyperimmune sera from rabbits, in which the virus does not multiply.

DR. STANLEY: We are currently conducting a study in which we are revaccinating young adults with a single strain of live attenuated A_2 virus at monthly intervals. I can only tell you that the early results seem to indicate that we are obtaining a broad antibody response to several influenza A viruses.

DR. MORRIS: There is no doubt that the findings reported by Dr. Henle are reproducible. Broadened antibody responses after repeated exposures to the same strain of influenza virus occur not only in animals but also in man. However, it is doubtful whether this broadened antibody response is protective against virus strains other than those closely related antigenically to the strain that stimulated the formation of the antibody. Our work in volunteers shows clearly that a broadened antibody response is detected in the blood of man after repeated exposure to the same strain of virus. However, the broadened antibody response is only protective against the inducing strain and other strains closely related to it antigenically. The findings in this work have important bearing on the strain composition of influenza virus vaccine and suggest the need for prompt incorporation of each new variant of influenza virus into the vaccine.

DR. HILLEMAN: In using immunologic adjuvants such as Adjuvant-65, we find a considerable broadening of the antibody response within the subgroups of influenza but no significant broadening between subgroups. Thus, for example, there is no significant response to A or A_1 resulting from vaccination with A_2.

CHAIRMAN SABIN: Now I would like to get some judgment on the question of the potential good results to be obtained from sequential infection with live virus based not on experimental data but on the natural history of influenza virus infections. Is there anything in the natural history of influenza infection and immunity in man to suggest that if we could immunize children with several different strains sequentially we might achieve an immunity that could be long-lasting and cover a broad range of different types? I should like to ask Sir Christopher Andrewes to comment on this.

SIR CHRISTOPHER ANDREWES: I should like to believe that this is possible. Maybe it is, but I do not think we have any positive evidence at this time. So far, when new strains of influenza have appeared they have always taken us by surprise. We just do not know whether when the next one comes along, which might be in the next year or two, we shall be taken by surprise again.

I should like to suggest that from an unorthodox point of view the extermination of influenza is perhaps not impossible to achieve. After all, nature has done the job for us in exterminating the A_0 and A_1 strains, which we no longer have with us. We do not know whether the change from A_0 to A_1 to A_2 is a question of mutation or whether it is a question of introduction into the human population of a new strain of influenza from some animal species. I myself believe there is more hope of abolishing influenza by understanding how this happens than by any work on vaccines.

DR. DAVENPORT: I think there are at least two reasons for being cautious about the practicability of inducing a broad composite antibody in children by use of attenuated strains.

One, I think it should be recognized that guinea pigs react entirely differently than humans to the antigens of influenza A or B, and the serologic reactions of guinea pigs, if they are taken as a model, can be misleading.

Two, we already know that the natural disease in humans does what Dr. Henle found the infections to do in his guinea pigs in a somewhat modified form. That is to say, young children in their first infection will react in a highly specific manner, but with repeated infections the antibody response broadens. It has been known for some time, for example, that young children infected with an A prime strain will show antibody increase to swine. They will also show antibody increase to PR-8. But of course their major response is to the infecting agent. Yet immunity, even to natural infection, is transitory, and composite antibody in humans is only built up after decades of natural exposure. I suspect a formidable number of vaccinations with attenuated strains would be required.

The failure of the 1957 live vaccine trials using A prime strains also speaks against the immediate operational likelihood of our being able to achieve composite antibody with attenuated strains. It is an extremely interesting idea, but I am not very hopeful at present.

CHAIRMAN SABIN: Before going on to the next question, I would like, for the record, to give the impression I have gained from this discussion.

First, it would appear that there is no agreement that only sequential experimental infection with several different strains of live influenza virus will achieve a broad antigenic response to antigens not represented by the strains actually used. It seems to me it would be very important in the near future to establish whether or not this is so.

Second, if it should turn out that the human body can do something very special when sequential infections are experienced—and we must get confirmation of this so it does not hang on as an unresolved question for another 20 years—then I think it would become very important to determine what new approaches can be made to the development of attenuated influenza vaccines that would be technologically and otherwise more applicable. The data on attenuated influenza virus presented by the people here have indicated that there is some deficiency. Apparently what we want are attenuated influenza strains that would be asymptomatic in children, antigenically effective, and controllable by a biologic standards laboratory.

Is there anyone here who would like to give a general indication of the direction in which such work should proceed in the future?

DR. SOLOVIEV: We know quite well what influenza is, and we know even better the nature of the virus that produces influenza. However, further experiments should include, in addition to work of the type reported by the speakers who took the floor before me, studies in other directions. We must not only find out why people get sick, we must also find out why they do not get sick.

During the last epidemic outbreak in Moscow, which I had occasion to observe, it became quite obvious, as it was in the Asian influenza epidemic, that a great number of persons did not get sick. We could even say that more people did not get sick than did. If we were to try to study the reason for this we might find the solution to the problem as a whole.

CHAIRMAN SABIN: You did not tell me how you are going to get those good attenuated influenza strains. Do you have any plans?

DR. SOLOVIEV: Yes, sir.

CHAIRMAN SABIN: Have you got any good ideas that you think other people should be working on, too, in this direction?

DR. SOLOVIEV: At present we are trying to study the genetics of influenza virus. Although considerable efforts of research workers have been channeled in a number of different directions in the study of influenza viruses, very little work has been done on the genetic properties and genetic characteristics of these viruses, and it is quite clear that this course will probably be the best one.

DR. SMORODINTSEV: Vaccination of children is no doubt the most useful task accomplished with attenuated live viruses. Our experience, as I have said before, has shown that the immunogenic activity of live vaccines is much greater. We have observed the children and we have seen that it is much more difficult to obtain comparable immunization in adults.

Vaccine strains can be obtained rather easily, but our difficulty is that sometimes they are hyperattenuated rather than sufficiently virulent. We can rather rapidly obtain highly immunogenic strains for children if we use certain methods—for example, cultivation of strains that have been attenuated on human tissues at high temperature so that very soon they will become more virulent. As far as we can see, these strains are not clinically dangerous, since they do not produce any dangerous sicknesses.

I think that tissue cultures should be used in preference to volunteers in laboratory studies for increasing the virulence of the strains.

Highly immunogenic virus strains we can get very easily, but the prolonged immunization of children into adulthood is what may protect us against new antigenic variations.

In this respect we have had an interesting experience in Leningrad. We have obtained immune gamma globulin against influenza from many thousands of voluntcers. We consider that this is very useful in treating certain cases, and we use the serum of volunteers that have been vaccinated 12 or 15 times. We usually vaccinate them with live vaccine every three months in order to keep the antibodies at a very high level. We also vaccinated them in 1965, when we got the new variant, and we observed that their reactions to this new variant were no different from those in the general population. Even though vaccination was much more intensive than that on the guinea pigs, we were not able to obtain by hemagglutination-inhibition tests any idea of the reaction to the new strains that we have found.

CHAIRMAN SABIN: Thank you very much. The next question has to do with chemoprophylaxis, especially during influenza epidemics.

This morning we had presented to us data on amantadine hydrochloride. Although there are indeed some very hopeful things going on in this field, to some of us it appeared as if there was still very much work in progress. Yet, if I am informed correctly, the Food and Drug Administration of the United States, which is supposed to use as rigorous criteria for granting permission to sell drugs as the Division of Biologics Standards uses for licensing vaccines, three weeks ago gave permission for the commercial sale of this drug. In view of this recent development, I think it is particularly important to get some expression of opinion from this group here now. On the basis of the data reported by Jackson and Stanley this morning and all the other data that they summarized, I should like to ask if this is a drug that deserves more extensive research to permit a yes or no answer regarding its usefulness as an antiepidemic tool. If so—if it does deserve further investigation—then I should like to ask what is being done to get the necessary missing information regarding the optimum dosage, the minimum and maximum times for treatment, and, especially, the toxicity of effective dosages.

I would very much like to have an expression of opinion on this subject—not at the moment from Dr. Stanley, who has already spoken this morning, but first from other people who have had experience. I would like information particularly on these questions: Is this a drug that should receive more extensive research? What should be done to get the neccssary missing information regarding all these things I have mentioned?

DR. DAVENPORT: In answer to your question, my opinion is that it needs further investigation. A study plan has been submitted to the Bureau of Medicine of the U.S. Navy to conduct a trial this fall of tolerance to the drug at the U.S. Naval Training Center, Great Lakes, Illinois. If the information gained from this pilot study warrants use of the drug in a military setting, then we will move on to a large field trial that has been planned.

Hopefully, in the face of an epidemic challenge matters such as administrative difficulties in dispensing the drug, problems in getting the people to take it, and questions concerning the duration of treatment needed can be worked out.

This will be a long job. It cannot be done in one year.

DR. STANLEY: We would agree that more study is needed. At present our impression is that the drug might be a useful addition at the time of an epidemic, should an epidemic occur during the interim period between the time of vaccination and the development of adequate antibody response.

CHAIRMAN SABIN: I should like to get an expression of opinion on what more needs to be done to get the necessary missing information regarding optimum dosage, minimum and maximum times for treatment, and toxicity of effective dosages when used in more than a small number of volunteers, particularly since your data show that a dose that is twice the effective dose is not tolerated even by small numbers of individuals.

DR. STANLEY: What Dr. Davenport has just said may add enough information if the data are accumulated for adequate numbers. I do not think there is any doubt that the drug is toxic at the levels we showed this morning, and this can only be evaluated in human experiments.

CHAIRMAN SABIN: I wonder whether anyone here would give a defense for the use of this drug at the present time not as a tool for experimental study but for general use and prescription by physicians.

I do not want to appear frivolous, because I think this is a very serious and important approach—not only for the control of influenza, until we learn more about effective vaccination, but also for the control of many other respiratory virus infections. But I think we must proceed with more coordinated and more cooperative effort. I dare say that this drug has been investigated for a number of years, and the questions being asked might have been answered long ago if investigators had got together.

In the monograph on this same subject—viral and rickettsial vaccines—that was recently distributed by WHO, another drug was mentioned: ABOB.

Can anyone here tell us the present status of tests against influenza with ABOB or any other potential drugs that are now being investigated?

DR. DAVENPORT: Dr. Meiklejohn, who was here earlier in the Conference, conducted a field trial of ABOB—which we called abob—at Lowry Air Force Base. It was carried out in a classic double-blind fashion. The drug was shown to be totally ineffective, and our interest in further tests of it ceased abruptly.

CHAIRMAN SABIN: We shall next go on to viruses of major importance in respiratory disease during the first two years of life. The question on which I should like to have some views expressed is concerned with the most promising direction for future research, particularly in the light of data that were presented here by Drs. Chanock and Parrott: that the serum antibody produced by killed RS vaccine and, if I am not mistaken, to a certain extent also by parainfluenza vaccine proved not to be protective on direct challenge and that there was a very important role for local respiratory tract antibody and perhaps other local immunity factors resulting from infection—and perhaps also, ultimately, from the use of attenuated viruses. Therefore, should the main effort now be directed toward the development of attenuated RS and parainfluenza vaccines that could be used early enough in life to protect infants during this very critical period? Would Dr. Chanock say something about this, please?

DR. CHANOCK: In our presentation on the first day Dr. Parrott and I indicated that serum antibody contributed by the mother to the infant or stimulated in the infant by an injection of inactivated vaccine did not protect against lower respiratory tract illness caused by RS virus. From the age distribution of RS infection and illness early in life, there was also some suggestion that maternally transmitted serum antibody might play a role in the pathogenesis of serious illness in young infants.

On the basis of these findings we concluded that the main thrust of our effort in the future should be in the direction of live attenuated vaccines. As we indicated in our presentation, we are currently evaluating a low-temperature-adapted RS strain.

Another reason for choosing this course was the finding that antibody in nasal secretions was more effective in conferring resistance to parainfluenza Type 1 virus infections in adults than was antibody found in serum.

The finding that vaccine-induced serum antibody in young infants was not protective also contributed to our interest in accelerating the effort to develop attenuated strains. A number of questions remain to be answered.

First, do infants respond to infection as adults do, with the development of antibody in nasal secretions? Studies are currently under way, but we will not have the answer for another few weeks.

Second, will it be possible to develop strains of RS and parainfluenza virus that will infect and not produce illness in infants? This is of some importance, since infants are the primary hosts for whom such vaccines are most urgently required. We have preliminary evidence that an RS strain adapted to growth at low temperature will produce a silent infection in adults, but we are a long way from being able to test its safety and efficacy in young infants.

We plan to carry out a series of investigations in which individuals of progressively lower age will be tested in sequence. The information from each test will be used to plan the test for the next younger age group. I estimate that it will be at least another year before the prime group, the young infants, will be tested.

CHAIRMAN SABIN: In your judgment, would attenuated candidate strains of RS and parainfluenza viruses ultimately have to be tested in very young babies before it is known whether or not they are useful or applicable?

DR. CHANOCK: Yes, I think that goes without saying.

CHAIRMAN SABIN: In the light of what you have just said, do you believe that spending a great deal of effort on the development of more potent killed RS and parainfluenza vaccines is warranted? If so, why?

DR. CHANOCK: I think we must study inactivated vaccines at the same time we evaluate attenuated virus vaccines. There has to be a concurrent effort. Dr. Francis showed a number of years ago, with a potent inactivated influenza vaccine given parenterally, that it was possible to stimulate the development of antibody to influenza virus in nasal secretions.

We may never develop attenuated paramyxovirus strains suitable for use in young infants. For this reason we should continue the effort to obtain more potent inactivated vaccines. The enhancing effect we have seen with low-potency vaccines can probably be overcome if high enough levels of antibody can be stimulated and if such antibody spills over into the nasal secretions.

CHAIRMAN SABIN: I should now like to ask Dr. Hilleman for his opinion.

DR. HILLEMAN: We have been much encouraged by our findings with killed virus vaccines. First of all, we find that with properly concentrated, purified, killed parainfluenza virus vaccines we can get just as good antibody responses as with killed influenza vaccine. And we know that killed influenza vaccine can be most effective.

Matters are a bit more difficult with RS virus because the yield of virus in cell culture is rather poor, but we find we can get a good antibody response if we put in enough antigen.

A long time ago Dr. Francis' group showed that there is a direct relationship between the level of circulating antibody and immunity to influenza. I believe that this is going to apply equally well to RS and parainfluenza. We have some evidence that in the field trials the RS and parainfluenza vaccines were actually affording protection in terms of lower cumulative attack rates in children given monovalent RS or trivalent parainfluenza 1, 2, and 3 vaccine than in the controls.

As I said in my paper and will emphasize once again, I think we need more RS antigen and, most important, we need to put these vaccines into adjuvants. However, we will not use adjuvants until we have made the vaccines completely safe from the standpoint of possible autoimmunization to the monkey kidney component.

DR. CHANOCK: I think the evidence is quite clear that high levels of serum antibody correlate with protection in the case of influenza, of parainfluenza, and of RS.

However, there is also another correlation that has been known for a number of years, since the early work of Dr. Smorodinstev and Dr. Francis: after natural infection there is a correlation between the level of antibody in serum and the level of antibody in nasal secretions. To base one's whole effort on the induction of antibody in serum is really to work with antibody that is less effective in providing protection. Furthermore, inactivated vaccines given parenterally tend more to stimulate serum antibody. For this reason I think it is worthwhile to increase efforts to induce antibody where it is most protective—in the respiratory tract secretions.

DR. HILLEMAN: Dr. Fazekas-St. Groth many years ago talked about pathotopic potentiation. This referred to getting the circulating antibody out into the respiratory secretions—causing the respiratory tract to weep, as it were, to permit the antibodies to reach the proper site. This may be very important for the future.

One other point I failed to mention is that with very young infants it might perhaps be more important to apply vaccine to the mother than to try to immunize the newborn. In this way the maternal antibody might be raised to a high enough level to protect the baby during the first few months of life.

CHAIRMAN SABIN: My next question is this: Are adenoviruses of sufficient importance in

serious—I emphasize *serious*—illness in this age group, the first two years of life, to warrant the extensive effort that would be required to develop acceptable vaccines for use in children during that period? Let me call first on Dr. Fox.

DR. FOX: You stress serious illness. Everyone, I think, realizes the difficulty that has been experienced by our own group and by others in measuring the illness potential of adenovirus infections in civilians, and particularly in young children. The kinds of observations that we have been making serve to provide an upper limit of the pathogenic potential. This is higher in the younger age group than in older children. It would approach, perhaps, 50 per cent.

Now, this is starting with infections that were picked up, not because of illness, but in the course of routine searching for infection and then looking to see what illness is related.

These illnesses that are related to adenoviruses at least temporally are more severe over all, if fever is used as a criterion, than the respiratory illnesses found with other virus associations or without any association that we can establish.

If an illness with a frequency of 50 per cent, half of it febrile and some of it fairly serious, is considered to be worth preventing, then adenovirus illness may be worth preventing.

CHAIRMAN SABIN: What do you mean by 50 per cent? In the data on the relative role of adenoviruses in bronchiolitis, tracheobronchitis, and so forth, that have been published by Dr. Chanock, I was impressed by the fact that the frequency of adenoviruses associated with these illnesses, as compared with their frequency in control populations, was quite low. Dr. Chanock may have additional data on other surveys; I think Dr. Cockburn suggested, during our discussion of this, that there might be some.

DR. FOX: Our approach is quite different from that of Dr. Parrott and Dr. Chanock, who are looking at children who come to medical attention. I am talking about infection and illness found among children living in their own families. Some of them ultimately do come to medical attention.

This 50 per cent is all the illness that is temporally associated with the true onset of adenovirus infections. Some of this is probably not due to adenoviruses; I do not know how much of it is, but I feel that the proportion is fairly high, and I think it may well be worth trying to prevent.

DR. CHANOCK: I hope that Dr. Bell will comment on the longitudinal studies at Junior Village. But first I think it might be worthwhile to mention that in the studies at Children's Hospital adenoviruses were found to account for about 5 per cent of all respiratory tract illnesses, when the occurrence of infection in the control group was subtracted from that in the illness group. Although this is a small percentage, it nevertheless represents a considerable number of severe respiratory tract illnesses.

DR. BELL: For many years we studied rather intensively the illness and microbial experiences of nursery children at Junior Village. This is a Washington, D.C. welfare institution for homeless but otherwise normal children. The size of the study group varied from 40 to 100; there were several new admissions and discharges each week. All the study children were six months to two years of age. The illness observations included constant medical surveillance, with daily rectal temperatures, of all children. The microbial observations included the study of routine serologic specimens and of throat and anal specimens collected at least once a week from each child.

Epidemiologically, the group resembled a tremendous family with a large number of susceptibles. It was infected with every virus that commonly occurred in the community. These infections spread so readily within the group that there was an average of more than one new virus infection per child per month.

Despite the abnormal frequency of infection, the study permitted an assessment of the relative importance of illnesses induced by different viruses when the whole range of severity of illness following a natural infection could be observed. This is somewhat different from hospital studies of the relative importance of such illnesses among children ill enough to seek medical attention and otherwise selected.

In Junior Village we found that rubeola virus generally caused the most severe illnesses and that adenoviruses were second as the cause of severe illness during the first two years of life.

CHAIRMAN SABIN: I take it, then, that the sense of all these statements is that there is enough serious illness that a suitable adenovirus vaccine would be a real addition to the total armamentarium for the prevention of respiratory disease during the first two years of life.

Along this same line, without going into the natural history of *Mycoplasma pneumoniae*, is a vaccine needed for this age group? Dr. Chanock.

DR. CHANOCK: I think it is quite clear from the published studies and from our experience that in the first two years of life illness is generally very mild. After that, *Mycoplasma pneumoniae* does assume importance as a cause of lower tract illness and should be considered for inclusion in any multivalent vaccine.

CHAIRMAN SABIN: If I understand you correctly, this is not so much for the protection during the first two years as for what will come later.

Now we will move on to the next category—viral respiratory disease other than influenza in older children and adults. Let us carry on from where we left off. Is a *Mycoplasma pneumoniae* vaccine indicated for all older children and adults in the general population? And if not for the general population, then for whom? As Dr. Stuart-Harris said in his keynote address, in considering any immunization procedure or the development of any immunizing agent we must begin by defining its objective.

Let us leave military groups completely out of consideration at this time; that is not our specific interest here. If a good mycoplasma vaccine were available, would it be advisable for everybody in the population? Dr. Chanock.

DR. CHANOCK: In a series of excellent epidemiological studies, Dr. Grayston and his group at Seattle have shown that for all ages *Mycoplasma pneumoniae* pneumonia morbidity is approximately 1 per 1,000 persons per year. This is obviously a gross underestimate, since it is based on patients diagnosed by their group health physicians as having atypical pneumonia. It is quite clear that many people have such illness without the diagnosis. For special age groups—the group from five to thirty—the morbidity is about two or three times higher than for the total population. If a correction figure is added for unrecognized cases it is quite clear that *M. pneumoniae* is an important cause of lower respiratory tract disease. In other studies carried out in England and in Finland it has been estimated that *M. pneumoniae* is responsible for between 30 and 50 per cent of the pneumonias that bring young adults into the hospital. A number of years ago Dr. Evans showed that about a quarter of all lower tract illnesses that brought students to the University of Wisconsin infirmary could be ascribed to *M. pneumoniae* infection.

CHAIRMAN SABIN: If it should be found that multiple inoculations and frequent revaccinations were necessary, would you still recommend a mycoplasma vaccine for the entire population?

DR. CHANOCK: No. I think I would reconsider under those circumstances.

DR. FOX: I should like to expand a little on the work of Dr. Grayston and his group. Dr. Chanock pointed out the contribution of *M. pneumoniae* to hospitalized atypical pneumonias or nonbacterial pneumonias as they occurred in the group health clinic. Dr. Grayston's group has also looked at the families of patients with pneumonia caused by mycoplasma. In most of those families, infection occurred in many of the other members, and a high proportion of these cases were associated with significant morbidity. Often they were found radiologically to have pneumonitis, although this had not been suspected on ordinary clinical grounds. Thus, over and beyond the hospitalized cases there is a large amount of disease occurring in persons more than five years of age that is of importance and conceivably could be prevented.

CHAIRMAN SABIN: Let me repeat my question to Dr. Chanock. If multiple inoculations and revaccinations are necessary to maintain a protective effect, do you still think that a mycoplasma vaccine would be indicated for the whole population instead of just special groups?

DR. CHANOCK: Until the nature of the vaccine and the duration of immunity are known, it will not be possible to answer that question.

DR. STOKES: I believe most pediatricians will agree that in the hospitals *M. pneumoniae* is seen to be replacing the pneumococcus, to some extent, as the chief cause of pneumonia. I believe the value of the vaccine against it has been so well determined that, although much more study is required, it appears very likely that in the future such a vaccine will be useful, particularly if an adjuvant is added to maintain sufficient antibody.

DR. FELDMAN: I want to insert a word of caution about mycoplasma. Before we get into the question of the general use of a vaccine for *M. pneumoniae,* we ought to know more about its epidemiology. In the family studies that we have been conducting for nine years now, we have not found a single person under the age of 16 who has acquired antibodies for this organism. All our results are obtained with the direct HI test that we have described.

In the American military recruit population we surveyed in 1962, about 56 per cent had antibodies; but among Brazilian recruits, only 14 per cent were positive. On the other hand, about 70 per cent of Point Barrow Eskimos under the age of four had such antibodies.

The experience in Seattle is certainly different from ours. I believe that this difference is very real. What we seem to be dealing with is a very spotty disease that occurs with different frequencies in different parts of the country.

In our University Hospital, we are still seeing much more pneumococcal pneumonia in children than that caused by mycoplasma. Thus, this picture, too, appears to be quite different in different places.

CHAIRMAN SABIN: I think we must also remember, of course, that *Mycoplasma pneumoniae* lends itself to chemotherapy.

DR. GRIST: I agree with the last speaker. In studies over about 15 years in Glasgow, Scotland, in adults and children with pneumonia in hospitals, we found fewer than 1 per cent with serological evidence of infection with this organism. The prevalence is spotty. There was a small outbreak recently in a village in Scotland with illnesses of a wide range of severity, but this still has not changed our experience in Glasgow.

CHAIRMAN SABIN: Because our time is running out, I shall merely mention my next question, which is this: What additional information is needed before a *Mycoplasma pneumoniae* vaccine can be recommended for general use? I think that probably we need both laboratory and technical information as well as epidemiologic information. Let us go on.

Now, excluding military groups, is there a real need for adenovirus vaccines for older children and adults? Who will say yes? Nobody.

The next question is: What research is most urgently needed to establish the possible usefulness of endogenous interferon in the control of the particular category of viral respiratory diseases that we have been talking about—those in older children and adults of which even now most cannot be associated with known viral agents? In the light of this morning's presentation I am particularly interested in having an expression of opinion. We heard this morning, for example, that Dr. Soloviev was able, with interferon made from human leukocytes, to diminish infection with attenuated influenza virus. What specifically needs to be done to determine whether or not exogenous interferon is something to pursue extensively?

DR. HILLEMAN: My own feeling is that the quantity of interferon that theoretically would be needed to protect the body's estimated 10^{13} cells would be phenomenal, considering yields and availability. For instance, it takes 0.004 gamma of purified chick-embryo interferon to protect 100,000 chick embryo cells against Eastern equine encephalomyelitis virus challenge. Translated to man, this is 40 mg—a tremendous amount. At present there is no acceptable, practical means of producing this amount of material in any cell system.

There are other problems as well, such as how to get the interferon distributed to the right cells and how to avoid its destruction by proteases while it is in transit in the blood. So far as I am concerned, the efforts in this area should go to interferon inducers and not to the utilization of interferon *per se.*

CHAIRMAN SABIN: My next question has to do with endogenously-induced interferon or other nonspecific host resistance. Would you like to express yourself on that?

DR. HILLEMAN: We know that there are several means for inducing interferon but we have no inducers useful in the practical sense at the present time. I would re-emphasize that the practical approach seems to be that of getting the patient to make his own interferon. In addition, we might use drugs to cut down the urinary loss of the interferon he makes.

CHAIRMAN SABIN: This is a very important subject. Perhaps the ultimate control of respiratory disease in older children and adults may come from research in this field. Dr. Soloviev, what do you think about this question?

DR. SOLOVIEV: I think that we must make a distinction between exogenous interferon for general action and for local action. I agree that for general action a rather large quantity is needed, but, as I mentioned in my statement, I think that for local action smaller doses can be used. These have been sufficiently effective to encourage us to continue our research work.

CHAIRMAN SABIN: The next question to which we want to address ourselves concerns steps for accelerating the availability of vaccines that are now on the horizon. What criteria should be used to select candidate strains for an intensive cooperative effort of testing under a variety of conditions, in increasing numbers of persons, as a prerequisite for licensure? The hope is to avoid putting a great deal of effort into the testing of strains that for one reason or another will not be acceptable and to concentrate, rather, on those that will be acceptable *a priori*. For rubella vaccine the criteria we might discuss here include presence of virus in the throat, tissue for cultivation, and effect on human embryonic tissue.

In other words, is it possible at this stage to decide that a vaccine strain that does not appear in the throat or appears to a lesser extent should immediately be given priority over one that does appear, if it otherwise fulfills the desirable criteria? Or that a vaccine strain that is grown in the egg—with which a great deal of experience has already accumulated—would be preferable to one grown in monkey kidney or in a human diploid line? And should the criteria include tests on various human embryonic tissues so that a strain with no deleterious effect in tissue culture, whatever significance that may have, would be regarded as preferable to one that does have such an effect?

DR. KRUGMAN: First, may I set the record straight about my forecast? If this schedule is a reliable forecast, a licensed product for vaccination against rubella should be available by 1971, approximately four years from the present time. Well-controlled trials designed to demonstrate lack of communicability of the vaccine virus will be time-consuming. These studies must be conducted with many small groups of susceptible persons under conditions which promote close contact.

A failure to detect virus in the pharynx does not rule out its presence. A susceptible person is more sensitive than the tissue culture system. In an earlier study a 10^2 dilution of rubella serum was infectious for susceptible children; it was not infectious for African green monkey kidney tissue culture. Therefore, the failure of the vaccine virus to spread is more important than its presence or absence in the throat.

As a pediatrician I am keenly aware of the hazards associated with prematurity as well as postmaturity.

CHAIRMAN SABIN: The 1971 prediction may also be premature if certain activities are not better coordinated. But if you had a choice, Dr. Krugman, between a vaccine strain that appears in the oropharynx by the available techniques and one that is not demonstrable in the oropharynx, both giving no evidence of spread, which would you choose?

DR. KRUGMAN: I would choose the one that was not demonstrably present in the pharynx—if it were immunogenic.

CHAIRMAN SABIN: Do you believe that tests on human embryonic tissue should be a requirement for candidate strains?

DR. KRUGMAN: What kind of tests?

CHAIRMAN SABIN: Tests of whether or not the virus prevents the cells from multiplying seriatim or has other effects that can be measured.

DR. KRUGMAN: I do not know whether these tests would have any significance.

CHAIRMAN SABIN: I bring this up because it seems to me that—while a virus may not have the same effect *in vivo* as on tissues in culture—a strain that had no demonstrable deleterious effect on various human embryonic tissues, and in other ways was satisfactory, would be preferable to one that, for example, prevented those cells from multiplying.

DR. PLOTKIN: Although I would like to agree with you, I do not feel that such a test would be of much use. I think that we should work further on *in vitro* tests of fetal damage; but, at least with the strains we have tested, I see no relationship between their effect on human cells *in vitro* and their apparent attenuation as judged by the currently available tests in man.

I might add one other point: Although it would obviously be highly desirable to test any vaccine to determine whether virus passes the placenta to the fetus, it seems to me that eventually we will be using a rubella vaccine in girls before the childbearing age, and that even if the results of such testing were positive it might still be possible to use a rubella vaccine in children alone.

DR. HILLEMAN: I agree with Dr. Plotkin. This vaccine is not for pregnant women, and I should think that whether the virus did or did not grow in embryonic tissue is academic since the aim of the nonexcreting vaccine is to prevent the chance of fetal infection.

In the practical sense, when it is a matter of trying to get out a rubella vaccine quickly, a vaccine will be approved much sooner if it is shown that virus is not excreted from the recipient. We believe that such a virus can be developed and that it will still be immunogenic. We have shown this already for mumps, where we had the same problem. We must, of course, await the event, but I think it possible for rubella also in the foreseeable future.

CHAIRMAN SABIN: I think we should remember that in the general use of rubella vaccine some women getting the vaccine might be pregnant without knowing it.

DR. MEYER: There is one aspect of the problem that I do not think has been brought out clearly here. I only repeat what a number of us agreed upon earlier, in private discussion.

Given a vaccine attenuated strain that is not excreted, the question is: Would this be the desirable strain? Dr. Krugman answered this earlier. We have answered it in the same way. There are two concepts here: safety and efficacy. If the assumption can be made that material that is not excreted is just as efficacious as material that is, obviously anyone would take the material that is not excreted.

Unfortunately, the real question has not been answered and will not be for some time: How much attenuation do we want, how much should we strive for? This is the experimental basis for many studies being done on the various attenuated strains.

Candidate rubella strains can be chosen at several levels of attenuation. With the lower levels of attenuation high antibody titers, a mild rash, and perhaps even virus spread to contacts are observed. With the higher-passage strains there is evidence of greater attenuation—no rash, no communicability, less and less virus in the pharynx, but also a considerably reduced antibody response. This seems to be a basic characteristic of the virus. What we are trying to determine is how much antibody is needed and how much virus shedding is acceptable. I do not believe anyone is trying to claim that any strain currently available is ideal.

With our HPV-77 strain at its current level of attenuation the GMT hemagglutination-inhibiting antibody titer of vaccinees is approximately sixteenfold below that seen in ordinary virulent rubella. Is this important or not? This is only partially answered. We also know that this strain has not spread to contacts of vaccinees and has other desirable characteristics.

CHAIRMAN SABIN: What I personally would plead for is that, however the criteria may be arrived at by a competent body, they be set up at an early stage. Since we are already faced with four or five potential candidates, the effort should not be diluted. If certain strains failed to meet definite criteria already set, work would not be wasted on them to the exclusion of a greater effort on the optimal candidates.

There is another question on rubella that I should like to put forward here. In view of the somewhat different results that have been re-

ported on gamma globulin, should specially prepared antirubella gamma globulin of very high potency be tested for its protective capacity?

Until there is a vaccine for widespread use, and even after one is available, there will be a need for a protective agent for women already known to be pregnant.

DR. STOKES: I should like to speak on this problem because rubella is only one of a number of diseases against which protection by concentrated antibodies in gamma globulin may be possible. Since gamma globulin of unknown antibody titer has clearly been protective in certain well-conducted studies, a gamma globulin of known high titer should be even more effective. Even if an adequate attenuated rubella virus vaccine becomes generally available, public apathy will inevitably limit mass protection; it would therefore appear that passive protection with a worth-while product should be available for the emergencies of exposure during early pregnancy. The new feasibility of intravenous use of gamma globulin unquestionably increases the usefulness of high-titer preparations. Furthermore, plasmapheresis of high-titer donors can greatly increase the availability of such globulins. The same points apply to other diseases that have been discussed this week—rabies, mumps, hepatitis, and perhaps specially prepared IgA antibodies to aid in protection against B virus and herpes simplex virus.

DR. KRUGMAN: As you have indicated, Dr. Sabin, the efficacy of gamma globulin for the prevention of rubella infection is certainly a controversial subject. In my opinion even convalescent gamma globulin would be of limited value. Studies on the natural history of rubella have indicated that virus is shed from the pharynx as long as a week before the onset of rash. In the usual type of contact, a pregnant woman exposed to her own child with rubella may be infected long before the disease is recognized clinically. Under the most ideal circumstances she would receive gamma globulin about four to six days after exposure, and by that time it is likely that infection would have occurred. We know from experience that the casual type of contact is usually not associated with a high attack rate of rubella. In my opinion, too much time, effort, and money would be wasted in a program devoted to preparing antirubella gamma globulin. I do not think it will solve the problem.

CHAIRMAN SABIN: We now have two different points of view. The issue is whether or not high-potency gamma globulin is needed, *not* for the solution of the rubella problem, because nobody is thinking of it as such, but for the need that will still remain—that of exposed pregnant women, whose exposure may not always be along the lines Dr. Krugman has just described and for whom something must be done. The issue is whether or not, in the light of the theoretical concepts Dr. Krugman has pointed out, we now have enough information to say, "No, this is not necessary."

DR. KRUGMAN: The availability of the specific hemagglutination inhibition test will be helpful. It will then be possible to screen all pregnant women exposed to rubella. The large number of immune persons (approximately 55 per cent) could be reassured. If gamma globulin were given to exposed susceptible pregnant women, it would be important to obtain a convalescent sample of serum to test for the occurrence of subclinical infection.

DR. PLOTKIN: Dr. Krugman has expressed most of my own views. I should like to point out that there is also a social problem involved. To be blunt, there is another way of dealing with a pregnancy in which rubella has intervened, as determined by laboratory tests. For this reason, and those Dr. Krugman has already so effectively stated, I do not feel that the effort involved in producing such a product would be justified at this time, with the present hopes for other means of prevention.

CHAIRMAN SABIN: I do not want to have the last word, but I am always reminded of the fact that even with the best of vaccines there will be millions of people who do not get it. Thousands of women will become pregnant and be exposed to rubella before it is eradicated, and some immediate action will be required for them.

I should like to thank you all for the discussion of these questions, which bring certain conflicting points of view into focus, and I want to thank the Program Committee of the Pan American Health Organization and the World

Health Organization for giving us the opportunity to meet so many old friends and exchange opinions and judgments.

I now call on Dr. Horwitz to give his concluding remarks to this Conference.

DR. HORWITZ: I sincerely believe that the Conference has more than fulfilled our expectations, particularly with regard to the content of the material presented and analyzed, as well as the kindness of the participants in subjecting themselves to a very tight schedule. I want to reiterate our gratitude in the name of the Pan American Health Organization and the World Health Organization for your presentations and for the spirit in which you have contributed your efforts.

I am certain that I also reflect the feeling of all who could not attend this important gathering, and particularly of institutions and individuals that will benefit from the publication of the Proceedings. The rapidity of change in the field of viral and rickettsial diseases explains our urgency in planning to publish the Proceedings within the next few months and distribute them immediately thereafter throughout the world.

Although many unknowns have been elicited during the discussions, thus indicating new leads to research, fundamental facts have been reaffirmed or established so that they should be taught to and learned by the students. There is no question of the value of publishing the Proceedings of this Conference—in a form similar to that of the two volumes on live attenuated polio vaccines—for the medical schools and scientific institutions of the developing countries. One needs only to consider how empty of material in this field the libraries of these countries usually are, to understand the significance of a *mise au point* in a series of fundamental subjects.

Beyond the facts that we have at hand today there is the ecological approach with which many of the problems have been analyzed. We should like this approach to be extended over medical education as a whole. It is obvious that the causation of diseases needs a comprehensive review to establish the real chain of events, which quite often is more than the apparent etiology. Payne has advanced, in this regard, very interesting proposals referring to both acute and chronic diseases. I believe that during the Conference this way of thinking became evident in the discussion of several of the subjects.

I should like, in all fairness, to make a few remarks regarding the value of the Conference for the participants themselves. One of you was kind enough to tell me that he felt during this week as if he had been reading a very modern book on viral and rickettsial diseases. I hope that equally important as what was said during the plenaries was what was discussed privately by you. You have left with us so much that we should like to feel that you have obtained something too.

We have developed some experience in group dynamics through the organization of several meetings of this nature during the year throughout the Hemisphere. We want to know, as a sort of evaluation, what the participants think and, particularly, how you believe this type of meeting might be improved. We should like to have your frank views, for this will be of great value if we are to continue in this periodic analysis of the stage of knowledge in viral and rickettsial diseases. We would appreciate it if you would write to us on the matter. I, for one, should like to see more time for free discussion in each subject.

We hope that you will forgive us for the mechanical system which sometimes interferes with your thoughts and works. It is one of those devices that, we are told, differentiates civilized from uncivilized societies.

Once again, our deep gratitude to all of you and our best wishes for your safe return to your countries. It has been a pleasure to have had this illustrious group in our house.

INDEX

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