Pan American Health Organization

ADVISORY COMMITTEE ON MEDICAL RESEARCH

Sixth Meeting

Washington, D.C., 12-16 June 1967

Item ___ of the Agenda

STUDY GROUP ON CHAGAS' DISEASE

Ref: RES 6/5
20 April 1967

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

Washington, D. C.

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

525 TWENTY-THIRD STREET, N.W., WASHINGTON, D.C. 20037, U.S.A.

CABLE ADDRESS: OFSANPAN

IN REPLY REFER TO:

TELEPHONE 223-4700

STUDY GROUP ON CHAGAS' DISEASE

SAN JUAN, PUERTO RICO

6-8 NOVEMBER 1966

MEETING

STUDY GROUP ON CHAGAS' DISEASE

San Juan, Puerto Rico

November 6-8,1966

M E M B E R S:

Dr. José O. de Almeida (Chairman)
Faculdade de Medicina
Universidade de São Paulo
Fazenda Monte Alegre
Caixa Postal, 301
Ribeirão Preto, São Paulo, Brazil

Dr. A. D'Alessandro
Tulane - Valle
International Center for
Medical Research and Training
Apartado 5390
Cali, Colombia

Dr. Fernando Beltrán
Dept. Microbiología y Parasitología
Facultad de Medicina
Ciudad Universitaria
México 20, D.F., México

Mr. Earl H. Fife, Jr.
Department of Serology
WRAIR
Walter Reed Army Medical Center
Washington, D.C.

Dr. Irving G. Kagan (Reporter)
Communicable Disease Center
Parasitology Unit
Atlanta, Georgia 30333

Dra. Feliza Knierim
Departamento de Parasitología
Facultad de Medicina
Universidad de Chile
Casilla, 9183
Santiago, Chile

Dr. G. A. Maekelt
Instituto de Medicina Tropical
Universidad Central de Venezuela
Ciudad Universitaria
Caracas, Venezuela

Dr. Franklin A. Neva
Dept. of Tropical Public Health
Harvard School of Public Health
25 Shattuck Street
Boston 15, Mass.

Prof.Aluizio Prata
Fundação Gonçalo Moniz
Rua Pedro Lessa
Caixa Postal, 701
Salvador, Bahia, Brazil

Col. Bryce C. Walton
Middle America Research Unit
Box 2011
Balboa Heights,Panama,Canal Zone

Secretariat:

Dr. Louis J. Olivier (Secretary)
Regional Advisor on Parasitic Diseases
Pan American Health Organization
525 23rd. Street
Washington, D.C. 20037

PREFACE

The Group met on November 6-8, 1966 in a room provided by the Dental School of the University of Puerto Rico through the kind offices of Dr. José Oliver-González. Appreciation is expressed for the hospitality and assistance provided.

The purpose of the meeting was to discuss serologic diagnosis of Chagas' Disease. It was intended that the complement fixation test should be given special attention. Those invited to the meeting, therefore were chosen for the contribution they could make to discussion of the specific subject to be dealt with.

INTRODUCTION

The Group recognized the large amount of work which has been published on the serodiagnosis of *T. cruzi* infection by many workers often under adverse conditions. It was noted that the complement fixation test is now the most widely used and best evaluated technique. However, it was also recognized that the complement fixation antigens and the methods for the test vary from one laboratory to another. It was agreed, therefore, that the test needs standardization, and that it is necessary to determine the relative value of the various antigen preparations in use today. This report will attempt to make progress toward these objectives.

To this end we are recommending an acceptable procedure for performing the complement fixation test and we propose a cooperative evaluation of several complement fixation antigens. In conjunction with such an evaluation, other diagnostic tests will be carried out on the same sera.

Finally, recommendations concerning the future course of work in serodiagnosis research and other applications are made.

Emphasis on the complement fixation test in this report is not intended to disparage other serodiagnostic procedures or to discourage the development of other techniques. On the contrary, there is a need for active study of other diagnostic methods. It may be pointed out in this connection that a standardized complement fixation test can form the basis for judging the value of other diagnostic techniques. Attention is called particularly to the hemagglutination, precipitation, and fluorescent antibody tests discussed elsewhere in this report.

The Group takes note that progress in the diagnosis of Chagas' disease is very important. The action of the Pan American Health Organization in convening the Study Group has provided a strong stimulus for future collaboration and future progress in research, diagnosis, and epidemiologic studies.

Complement Fixation Test

The serologic diagnosis of Chagas' disease is currently under study in many countries of South America and in a few laboratories in Central and North America. Review of the tests being carried out revealed that the complement fixation test (CF) is one of the procedures employed by most of the investigators. It is also the test employed by serum banks in Brazil and Venezuela for the detection of infected donors. The complement fixation test that is recommended for the diagnosis of Chagas' disease is a quantitative test based on the 50 per cent hemolytic unit patterned after the Wadsworth-Maltaner test (Wadsworth, Maltaner and Maltaner, 1931), as adapted for Chagas' disease by Freitas and Almeida (1949). The titer can be expressed either as a ratio of the amount of complement for 50% hemolysis in the presence of serum and antigen over the amount of complement required for 50 per cent hemolysis

with serum alone (Freitas and Almeida, 1949) or by the reciprocal of the serum dilution required for 50 per cent hemolysis in the presence of antigen and complement (LBCF monograph, 1965). The following micromodifications can be recommended for routine laboratory use by small diagnostic units: the microtiter CF test performed in plastic plates patterned after the LBCF tests (Casey, 1965) or the plate drop method developed by Almeida (1963) for blood banks in Brazil.

In these tests, complement (C') is titrated in order to determine the values of the von Krogh parameters: the amount of guinea pig serum required for 50 per cent hemolysis and the slope of the linear relationship obtained when the logits of degrees of hemolysis observed are plotted against the log of C' employed. A normal unit of complement to produce 50% hemolysis has an approximate value of 0.00120 ml of fresh guinea pig serum with a slope value of 0.2 + 0.02. The reproducibility of the CF test depends upon the use of complement of the same quality as determined by this method. The amount of complement to produce 50% hemolysis can be determined from any partial hemolysis obtained by use of tables calculated in accordance with von Krogh's formula.

An antigen employed by a reference laboratory should be carefully evaluated for sensitivity with sera from patients with parasitologically proven Chagas' disease and for absence of cross reactivity with sera from patients with other infections. The technique of the test employed by the reference laboratory must also be evaluated for reproducibility. To determine the reproducibility of a test, coded serum specimens should be titrated (no more than 8 specimens per day) over a period of time and the results subjected to analysis by the direct probability sequential method (Maltaner and Thompson, 1948). For Chagas' disease 299 split sera are tested in order to determine the expected frequency of defective observations. A defective observation is a pair of sera whose G values (difference in log values of the titers) is equal to or greater than 0.070 (Pangborn et al 1955).

The reproducibility of a test system must be determined before other antigens can be evaluated by the laboratory, (Freitas and Almeida,1949). The data from such an analysis in the Chagas' system showed that one may expect a lower frequency of defective observations than one expects in syphilis and leprosy systems (Almeida and Siqueira, 1960).

The isofixation curves (Almeida, 1956) with two dilutions of complement (3 and 6, 50% hemolytic units) provides a method for the quantitative evaluation of the relationship between complement and the serum, between antigen and complement, and the ratio between the antigen and antibody titers. With T. cruzi sera one obtains a type 1 isofixation curve and from this the amount of antigen to be used in the test can be calculated (Almeida, 1956).

The linear relationship between immune complexes (in terms of antigen or antibody) for different lots of antigen tested with a Chagas' serum are compared in order to detect differences in their reactivity.

Two lots of antigen can be evaluated by sequential probability analysis in the following manner. A total of 155 sera which are negative with the standard antigen are tested with the new lot of antigen. If no discrepancy is observed, the new lot of antigen is not rejected for being excessively non specific in its reactivity. When discrepant observations are noted additional sera must be tested in a number which can be determined from tables in Pangborn et al (1955). For maximum performance, no more than 8 sera should bet tested daily. If one defective observation is obtained when the first 4 sera are tested the antigen is rejected. If the antigen is not rejected, its reactivity is compared with the standard antigen by testing with serially diluted dilution of positive sera. From the degree of hemolysis obtained, values of G are calculated for each titration. Pairs of results are considered defective when differences in the values of G are equal to or higher than 0.070. When no defective result appears in the first 14 observations, the new lot of antigen is considered acceptable in specific reactivity with the standard antigen. If defective observations are detected, additional observation must be made in order to reach a decision with regard to the suitability of the antigen. The number of additional observations must be obtained from tables (Pangborn et al. 1955).

PREPARATION OF ANTIGENS

A variety of antigens is being employed for the serodiagnosis of Chagas' disease. As would be anticipated, independent investigations have led to the development of various methods for mass cultivation of T. cruzi for the production of antigen, the production and evaluation of a variety of serologically active antigen components, and use of these antigens for serodiagnosis. Since details of preparation of T. cruzi antigens have been published, the present report will present only the salient features of some of them and cite the appropriate references.

The antigens which are most commonly used for the diagnosis of Chagas' disease are the following:

1. Chloroform-jelly antigen (Freitas and Almeida, 1949)

T. cruzi, cultured in diphasis blood medium, are washed several times in saline until the supernatant is colorless and free of blood elements, frozen (-30ºC) and dried under vacuum over calcium chloride. The dried organisms are extracted with benzene in a glass homogenizer and redried. The material is extracted again in 9 volumes of distilled water plus 3 volumes of chloroform and shaken thoroughly with glass beads. The antigen is stored frozen.

2. Delipidized (benzene) antigen (Maekelt, 1960).

The organisms are grown on a sterile diphasic medium consisting of Difco brain heart infusion agar (37 g), Dextrose (10 g), Agar (20 g), and distilled water (1,000 ml) in 125 ml Erlenmeyer flasks. Stock cultures are also grown on the above medium but 2.5 ml of defibrinated rabbit blood

is added to each flask plus antibiotics. The organisms harvested from the medium without blood are disrupted by freezing and thawing in a dry ice and alcohol bath 10 times and then lyophilized.
The lyophilized organisms are extracted with benzene, dried under vacuum, extracted with distilled water, brought to isotonicity with 1.7% NaCl with merthiolate 1:5,000, and centrifuged. The opalescent supernatant is tubed in small amounts and stored frozen at -20ºC.

3. Delipidized (benzene) antigen (Maekelt, 1964)

This antigen is prepared from T. cruzi grown in cellulose sacs containing saline and suspended in a medium containing Bacto brain heart infusion (37 g), Dextrose (18 g), hemoglobin (200 ml) and distilled water (1,000 ml). After 21 days of incubation, with continuous agitation at 27ºC, the organisms are harvested, sonicated, and lyophilized. The lyophilized powder is extracted as in antigen Nº 2. The antigen is lyophilized and stored at -20ºC.

4. Crude water soluble antigen (Knierim and Saavedra, 1966).

The antigen is prepared from trypanosomes cultivated on a diphasic medium. Freshly harvested organisms are diluted with distilled water and subjected to 6 freeze-thaw treatments. After centrifugation the soluble product (antigen) is made isotonic with NaCl solution and stored at -20ºC.

5. Somatic protein antigen (Fife and Kent, 1960).

This is a protein fraction isolated from cultures cultivated in cellulose sacs, the dried blood clot component of the Little & Subbakow (1948) medium being placed outside the sacs. Lyophilized harvests are first treated with anhydrous ether and then extracted with buffered salt solution. The protein fraction obtained by chloroform-gel fractionation is used as antigen. The antigen is stored by lyophilization.

6. Exoantigen (Tarrant, Fife, and Anderson, 1965)

This antigen is a "culture filtrate" product obtained from T.cruzi cultivated in cellulose sacs in the medium utilized for antigen Nº 5 above. The trypanosomae are removed, the supernatant antigen dialyzed against saline, and certain non-antigen components removed by isoelectric precipitation at pH 4.6. The soluble components comprise the exoantigen and are preserved by lyophilization.

7. Methylic antigen (Batista and Santos, 1959)

This antigen is prepared from culture form T. cruzi grown on diphasic or liquid medium. The harvested organisms are dried and treated with benzene. The material is then extracted with pure anhydrous methanol in the autoclave for 15 minutes at 30 lbs. pressure (120ºC). The antigen is stable at warm temperatures.

CULTIVATION OF TRYPANOSOMA CRUZI IN CELLULOSE SACS

The cultivation of T. cruzi within sacs of cellulose dialysis tubing has been adopted by several laboratories for two reasons: (a) to obtain organisms free of solid culture medium components for use in making somatic antigens and (b) to concentrate and collect the antigenic products liberated by the organisms during their cultivation for the production of exoantigens. The basic principle involves the growth of the organisms within a loop of cellulose tubing the pore size of which allows passage of necessary nutrients, but retains the antigenic materials in the sac. Several minor modifications of this procedure give similar and acceptable results.

1. Walter Reed Army Institute of Research Method (Little and SubbaRow, 1943; Fife and Kent, 1960).

A sixteen inch length of tubing containing peptone broth is placed in a 1-liter flask containing peptone broth plus blood charcoal. Maximum growth is achieved in 30-35 days at 23-25ºC.

2. Maekelt Method (Maekelt, 1964)

A one-meter length of tubing containing saline is placed in a 3-liter flask containing brain-heart infusion broth-hemoglobin solution. Cultures are agitated by continuous shaking during incubation at ambient temperature (27ºC). Harvest is made after 21 days.

CULTIVATION OF T. CRUZI ON SOLID MEDIUM

There have been many modifications of diphasic media for culture of trypanosomes. The references cited deal with media from which trypanosomes have been used for preparation of antigens.

Kelser (1937) grew organisms on a diphasic medium consisting of beef extract, peptone, glucose, agar and blood as base. The overlay consisted of water of condensation. Davis (1943) modified this medium by adding the liquid medium without blood or agar as overlay.

Chang and Negherbon (1947) grew organisms on a base of liver extract and glucose in flasks with 50 ml of overlay. Freitas and Almeida (1949) grew organisms in a blood agar base and Chaffee et al. (1956) grew organisms in blood (Senekjie) base with an overlay of modified Locke's solution. Maekelt (1960) grew organisms in a blood agar base but for antigen preparation transferred the organisms to a brain heart infusion agar base without blood. This technique led to the harvesting of very clean organisms for antigen preparation. The method can only be used once since the organisms will not grow heavily after more than one transfer to an agar that does not contain blood.

CULTIVATION OF ORGANISMS IN MONOPHASIC MEDIUM

A simple protein-free medium was used by Zeledon (1959) for the selective growth of T. cruzi. Jadin and Pierreux (1960) grew trypanosomes in a solution of Hank's saline, calf serum, and extract of bovine red cells. Partially defined media have been prepared by Little and Oleson (1951), Citri and Grossowicz (1955), and Boné and Parent (1963). The medium of Boné and Parent (1963) does not contain serum. The cultivation of mammalian trypanosomes is reviewed by Tobie (1964).

Stability of Antigens

The crude extracts of T. cruzi (Kelser type) were relatively difficult to preserve, but Knierim (unpublished) reports they keep well when frozen at -20ºC. The Freitas-Almeida-benzenechloroform antigen is stable for at least 3 months when kept frozen. The Chaffee type antigen is stable as is the Maekelt type when lyophilized. The methylic antigen is stable at room temperature. The consensus is that the antigens in use today are stable. Antigen stability and antigenic decay require further study. Siqueira (1964) has recently shown, by direct probability sequential analysis that antigens 16 and 93 days old were similar in activity.

Antigen Purity

In selecting a strain of T. cruzi for antigen production, every effort must be made to assure that only T. cruzi is present in the cultures used in the preparation of the antigens. To detect a T. rangeli contaminant is difficult. T. rangeli is known to be present in many Chagas' endemic areas and knowledge of its distribution is still fragmentary.

Morphological studies of the flagellates in culture, in blood or in triatomid bugs, as well as the behavior of the infection in the vectors of T. rangeli used in xenodiagnosis (invasion of hemolymph and salivary glands), may prove a double infection. (To date Rhodnius prolixus is the vector for most of the known strains of T. rangeli, R. pallescens is the vector for the Panamanian strain and R. ecuadoriensis is the vector for T. rangeli in Peru.) Cloning may permit selection of pure T. cruzi strains. However, these procedures may fail to detect the presence of few T. rangeli or of forms hard to distinguish morphologically from T. cruzi. In order to overcome this difficulty, it is suggested that the strain in question be repeatedly inoculated into a culture medium which only supports the growth of T. cruzi. One medium known to have this property is brain-heart infusion (BH) with the addition of rabbit or human blood (Zeledon, 1959). The trypanosome strain under study must be adapted to the BH medium (especially newly isolated strains) by progressively adding it to the maintenance medium used in each laboratory. The strain is then transferred each 3 weeks for at least 6 to 8 times. The inoculum generally used is 2 to 5 mls for 10 ml of BH medium.

Other Serologic Tests

1. Precipitin test (Muniz, 1947):

The precipitin test (PT) is a rapid serologic method for detecting antibodies in acute Chagas' disease. High sensitivity has been reported, but specificity requires further study. The antigen is a clear water-soluble polysaccharide fraction (Fuller formamide method) of culture forms of T. cruzi. For the test, micro-tubes of 2 mm diameter may be used to conserve antigen. For quantitative tests, serum dilutions must be made with undiluted negative serum. Capillary blood (heparinized or whole blood) can be drawn into the same micro-tubes in which the test will be performed. After centrifugation the polysaccharide antigen has to be added carefully, avoiding mixture of serum and antigen. The test is read after 15 minutes. Further studies have to be made to determine whether the test can be recommended as a rapid screening method for cases of early infection with T. cruzi.

2. Indirect hemagglutination test (Knierim and Saavedra, 1966; Kagan et al., 1966):

With the exception of CF tests, the indirect hemagglutination test (IHA) is one of the most evaluated serological methods for the diagnosis of chronic Chagas' disease.

There has been good agreement between the IHA and CF tests in sensitivity and specificity studies made on human diagnostic sera. (Cerisola, Chaben and Lazzari, 1902; Knierim and Saavedra, 1966). Further studies are necessary before the test can be recommended for routine use.

In the test, sheep or human tanned red blood cells are sensitized with delipidized or crude water-soluble culture-form antigens of T. cruzi. If sheep cells are used, previous absorption of heterophile antibodies is recommended for diagnostic purposes. Lyophilized T. cruzi antigen may also be used. Block titration of two-fold dilutions of antigen and antiserum must be made to determine the titer of the antigen, that is the highest antigen dilution, which permits the detection of the lowest amount of antibody in a test serum. The technique can be performed in tubes or plates and with a minimum total volume of 0.05-0.1 ml.

The titer of reactive sera expressed in two-fold dilutions is, in general, higher than the titers obtained in the CF test. In individuals infected with T. cruzi, the median titer ranges from 1:256-1:512.

The advantages of IHA in comparison with the CF test are: 1) Anticomplementary activity of sera does not influence the test; 2) the test is easier to perform because only a few elements are involved (red cells, antigen, serum); 3) the test can be performed more rapidly, 4) animal sera which do not lend themselves to the usual CF titration may be reactive in the IHA test.

It would be desirable to study early infections with T. cruzi (both asymptomatic and symptomatic) with the IHA test using non-tanned cells sensitized with polysaccharide fractions of T. cruzi and with tanned cells sensitized with exo and protein antigens. The specificity of the IHA test on sera of patients with disease states in which cross reactions might occur (with enphasis on leishmaniasis and infection with T. rangeli) should be made. The IHA technique has been applied with advantage for epidemiologic surveys of naturally infected animals.

3. Fluorescent antibody tests:

Serum antibodies to T. cruzi have been demonstrated with indirect fluorescent antibody tests (IFAT), using whole organisms as antigen, with culture forms (Fife and Muschel, 1959), blood forms (Sadun et al, 1963) and tissue forms (Biagi et al., 1964), and with soluble antigens (SAFA) using a fluorometer to read reactions (Tarrant et al., 1965). These tests have not yet been sufficiently evaluated to recommend their use in routine diagnosis, but there is some evidence that IFAT antibodies detected by trypanosomal form antigen appear earlier in acute infections than do complement fixing antibodies. On a theoretical basis, the use of trypanosomal and leishmanial forms might allow the detection of antigen-antibody systems not detected by use of antigens derived from culture forms. These tests might well prove to be valuable in providing additional parameters for the diagnosis and evaluation of Chagas' disease.

4. Additional tests:

A variety of serologic procedures has been reported in the literature. Techniques such as direct agglutination of living trypanosomes (Hauschka et al, 1950), indirect agglutination with Coombs test (Nussenzweig, 1955), conditioned hemolysis (Muniz, 1950), methylene blue dye test (Scorza et al., 1959), agar gel tests and immune adherence tests have not been adequately evaluated to be recommended for routine diagnostic or survey procedures. In addition, two unpublished tests (a rapid card test and a latex particle procedure) have been studied but require further evaluation.

COLLECTION, STORAGE AND SHIPMENT OF SERUM

Blood should be taken under aseptic conditions and should be processed in the laboratory within 24 hours of collection. If this is not possible, the serum should be separated from the clot aseptically in the field (to avoid excessive hemolysis), and preserved in sterile vials by the addition of merthiolate (1:5,000) or sodium azide (2 mg/ml). When sera are to be lyophilized, no preservative can be added since it will denature the antibody during drying. For special studies, blood, but preferably serum, can be collected on filter paper, dried, and shipped by regular mail. Blood on filter papers should be stored in the freezer.

Storage:

Sera should be stored in the frozen state at -20°C or lower in glass ampules or capped vials in small volumes. This facilitates use of the materials for titration by avoiding repeated freezing and thawing. Lyophilization of unpreserved serum is the best method of preservation. Dried sera can be kept at room temperature in sealed glass ampules under vacuum. A high frequency coil should be used to test the vacuum (of the ampules) after 24 hours of storage at room temperature.

Shipment of serum:

For evaluation studies, only lyophilized serum should be used. If this is not possible, serum should be shipped in the frozen state with dry ice.

RECOMMENDATIONS FOR COLLECTION OF STANDARD REFERENCE SERUM SAMPLES

Standard serum samples should be obtained from persons with proved *T. cruzi* infection (in the absence of *T. rangeli*), from non-infected individuals, and from patients with other infections likely to react in a nonspecific manner with *T. cruzi* antigens. These sera should be obtained in large enough quantities so that they will be available to participating reference laboratories. It is essential that information as outlined below be available concerning each donor from whom a standard serum sample is obtained.

It should be emphasized that the reference sera described herein do not refer to the routine positive and negative control sera which a serologic laboratory must have available for the day-to-day performance of tests. However, it is hoped that the criteria outlined below for reference standards will also be utilized as far as possible in selection of positive sera to be used for routine control purposes.

Sera from known T. cruzi infections:

Attempts should be made to obtain standard reference sera from different geographic areas of Central and South America. All "positive" sera should be from patients who have received no treatment with drugs commonly used against Chagas' disease. Information required for each positive serum should include the name, age, sex, and occupation of the patient; history of the patient's residence; data concerning duration of infection, clinical manifestations or other evidence of Chagas' disease and the date and type of parasitological confirmation of diagnosis; data concerning results of serologic tests including dates and titers; and clinical and laboratory data to rule out other diseases, especially leishmaniasis, tuberculosis, leprosy, treponematosis, malaria, brucellosis, and "collagen" diseases.

When possible the strain of T. cruzi should be recovered from the patient providing the serum and be maintained in culture, or preferably preserved in the frozen state.

Positive serum samples should be obtained from several different geographic areas. Preferably, the following should be represented: Mexico, Central America, Colombia, Venezuela, Chile, Argentina, Brazil (Bahia, Minas Gerais, Goias, São Paulo, and Rio Grande do Sul).

Negative sera:

The information and criteria required concerning each serum donor should include: Name, age, sex, occupation, and area of residence. The evidence ruling out Chagas' disease should include the following: history of no exposure to triatomid bugs; a normal electrocardiogram; no cardiomegaly by X-ray; a negative CF test for Chagas' disease; if the patient is from an endemic Chagas' area, a negative xenodiagnostic test; no evidence of other disease as judged by the usual physical and clinical laboratory examinations, with special reference to leishmaniasis, tuberculosis, trepanematosis, and leprosy.

Serum from patients with known disease:

It would be desirable to obtain a few reference sera from patients having disease states which might interfere with the tests. The following disease entities are suggested: Active pulmonary tuberculosis, syphilis (secondary period), leprosy (lepromatous and tuberculoid), leishmaniasis (integumentary and visceral), South American blastomycosis, disseminated lupus erythematosis and multiple myeloma.

Sufficient clinical and laboratory evidence must be provided to support the diagnosis in each case.

RECOMMENDATIONS

1. The Group recommends that a comparison be made among established laboratories active in the diagnosis of Chagas' disease to determine the reproducibility of tests being made and to test the sensitivity, specificity and reactivity of diagnostic antigens. To accomplish this end, small numbers of well-documented positive sera should be collected from several endemic areas. In addition, normal sera should be collected from individuals living in non-endemic areas. The sera should be divided into numerous small portions and lyophilized. Two coded specimens of each serum should then be sent to each participating laboratory for evaluation. The sera should be tested by the procedures being employed for routine diagnosis in that laboratory. In addition, the antigens being used by each participating laboratory should be sent to all other participating laboratories so that each laboratory will have an opportunity to test each standard serum with all the antigens. The data obtained from all the laboratories can then be analyzed by direct probability sequential analysis to determine the reproducibility of the tests. Moreover, the sensitivity, specificity, and reactivity of the various antigens will be rigorously tested and their suitability for use as reference antigens will be determined. It is anticipated that five laboratories might participate in this study using Dr. Almeida's laboratory as the coordination center. Details of the evaluation procedure will be formulated later by the collaborating laboratories. It is expected that this evaluation may be accomplished by the cooperating laboratories with little outside assistance. However, financial aid may be needed to facilitate procurement of the large standard serum specimens and to defray costs of shipment of the sera and antigens.

2. The Group recommends that research in the microchemical analysis of Trypanosoma cruzi antigens employing the techniques of agar-gel analysis, immunoelectrophoresis, column chromatography, and other methods be made. The type of immunoglobulin detected by each diagnostic test and the role of each in the pathogenesis of infection should be explored. Definitive studies should be made on antigenic differences of T. cruzi strains, on the basis for differences in virulence between strains, and on the role of life cycle stages on the antigenic mosaic of the trypanosome. The degree of cross reactivity obtained with sera of man and animals infected with T. rangeli must be studied. The cross reactivity obtained with different strains of Leishmania in the Western Hemisphere is not known and requires further study. It is further recommended that research on the improvement of culture methods be made in order to obtain various life cycle stages for antigenic analysis.

3. It is recommended that PAHO consider setting up a procedure for the collection and distribution of literature references on Chagas' disease and closely related subjects to interested research and public health workers in the Americas. Annotated citations would be desirable. Consideration should be given to cooperation with the WHO program for dissemination of such information on African trypanosomiasis.

4. Consideration should be given to the collection of a series of both positive and negative sera for use as standards to evaluate antigens and to judge the quality of serologic test performance. The sera should be in large volume and should be accompanied by supporting data relating to history of the donors and to its reactions in other serologic tests.

5. A Test for Chagas' disease should be made on all sera from endemic areas submitted for routine syphilis serology.

6. Following the procedure often employed with other serologic methods in many countries, it is recommended that local laboratories routinely send samples of tested sera to be checked by a reference laboratory.

7. It is recommended that the Pan American Health Organization sponsor a training course for laboratory technicians responsible for serologic tests for diagnosis of Chagas' disease. Such a course is needed to train technicians of public health laboratories and blood banks.

8. It is suggested that the PAHO convene a study group in Chagas' disease diagnostic techniques when work on the development of standard procedures, as recommended here, has been sufficient to justify evaluation of the program.

9. Although the following recommendations do not relate directly to the development of standardized serologic procedures and antigens, we feel that they are worthy of consideration:

a. All blood banks in countries where Chagas' disease is endemic should perform serologic tests to exclude donors with antibody titers.

b. During prenatal care of pregnant women in endemic areas, serologic tests should be performed. Babies born of mothers with positive tests should be followed carefully for signs of infection.

c. When autopsy reveals the deceased had chronic myocardiopathy serologic tests should be made to aid in determination of the cause of death. Serum, pericardial exudate, or spinal fluid may be tested.

d. All persons in endemic areas with chronic cardiomyopathy mega-esophagus and megacolon should be tested for Chagas' antibody.

e. Children in endemic areas with primary lesions following Triatoma bites (Chagomas,ophthalmic ganglionar complex); periods of fever; or enlargement of spleen, liver and lymph nodes should be tested.

f. Repeated serologic tests should be made on infected individuals to study:

a) Fluctuation of titers during the course of the disease or of the infection.
b) The relation of clinical symptoms to serologic titers.
c) The effect of therapy on serologic titers.

REFERENCES CITED

Almeida, J.O. (1956) J. Immunol. 76, 259

Almeida, J.O. (1963) Bol.Of.San.Pan. 50, 133

Almeida, J.O. and Siqueira, A.F. (1960) Rev.Inst.Med.Trop. S.Paulo 2, 204

Batista, S.M. and Santos, U.M. (1959) O Hospital 55, 343

Biagi, F.F., Tay, J., and Martinez-Murray, R. (1964) Bol.Of.San.Pan. 57, 237

Boné, G.J. and Parent, G. (1963) J.Gen.Microbiol. 31, 261

Casey, H. (1965) Public Health Monograph Nº 74, 31

Cerisola, J.A., Fatala Chaben, M., and Lazzari, J.O. (1962) Prensa Med. Argen. 49, 1761

Chaffee, E.F., Fife, E.H., and Kent, J.F. (1956) Amer.Jour.Trop.Med.Hyg. 5, 763

Chang, S.L., and Negherbon, W.O. (1947) J.Inf.Dis. 81, 209

Citri, N. and Grossowicz, N. (1955) J.Gen. Microbiol. 13, 273

Davis, D.J. (1943) Public Health Rep. (U.S.) 58, 775

Fife, E.H. and Kent, J.F. (1960) Amer.Jour.Trop.Med.Hyg. 9, 512

Fife, E.H. and Muschel, L.H. (1959) Proc.Soc.Exp.Biol.Med. 101, 540

Freitas, J.L.P. and Almeida, J.O. (1949) O Hospital 35, 787

Hauschka, T.S., Goodwin, M.B., Palmquist, J., and Brown, E. (1960) Amer.Jour.Trop.Med. 30, 1

Jadin J. and Pierreux, G. (1960) J. Parasit. 33, 85

Kagan, I.G., Norman, L., and Allain, D.S. (1966) Rev.Biol.Trop. (Costa Rica) 14, 55

Kelser, P.A. (1936) Amer. Jour.Trop.Med. 16, 405

Knierim, F., and Saavedra, P. (1966) Bol.Chil. Parasit. 21, 39

LBCF Monograph (1965) Public Health Monograph Nº 74, 34

Little, P.A. and SubbaRow, Y. (1943) J.Bact. 50, 57

Little, P.A. and Oleson, J.J. (1951) J.Bact. 61, 709

Maekelt, G.A. (1960) Zschr. Trop. Parasitol. 11, 152

Maekelt, G.A. (1964) Rev. Venez.Sanid.Asist.Soc. 29, 1

Maltaner, E. and Thompson, W.R. Research Report, May 11, 1945
Div. of Lab. & Res., New York State. Lab. of Health, 1948

Muniz, J. (1947) Brasil Med. 29, 261

Muniz, J. (1950) O Hospital 38, 685

Nussenzweig, V. (1955) O Hospital 47, 701

Pangborn, M.C., Maltaner, F., Tompkins, V.N., Beecher, T., Thompson, W.R. and Flynn, M.R. (1951) WHO Monogr.Ser. Nº 6

Sadun, E.H., Duxbury, R.E., Williams, J.S., and Anderson, R.I. (1963) Jour.Parasit. 49, 385

Scorza, J.V. and Alvarez, Argentis & Ramos, Italia & Dagert, C. and Díaz Vázquez A. and Torrealba, J.F. (1959) Arch.Venez. de Med.Trop. y Parasitol. Med. 3, 121

Siqueira, A.F. (1964) Rev.Inst.Med.Trop. São Paulo 6, 268

Tarrant, C.F., Fife, E.H., and Anderson, R.I. (1965) Jour.Parasit. 51, 277

Tobie, E.J. (1964) J. Protozool. 11, 418

Wadsworth, A.B., Maltaner, F., and Maltaner, E. (1938) Jour.Immnol. 35, 217

Zeledon, R. (1959) Jour. Parasitol. 45, 652