

Pan American Health Organization

ADVISORY COMMITTEE ON MEDICAL RESEARCH

Seventh Meeting

Washington, D.C., 24-28 June 1968

Item 20 of the Agenda

PROGRESS REPORT ON RESEARCH IN LEPROSY  
AND OTHER TROPICAL DISEASES IN VENEZUELA

Ref: RES 7/7  
24 May 1968

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

Washington, D.C.

Table of Contents

	<u>Page</u>
Introduction	1
1. Immunological Reactions in Onchocerciasis	3
2. The Leishmanin Reaction and other Immunological Aspects of Leishmaniasis	10
3. Serological Reactions in Leprosy	17
4. Immunological Study of American Cutaneous Leishmaniasis through the Indirect Immuno-fluorescence Method	24
5. Identification of the Noncultivable Pathogenic Mycobacteria <u>M. Leprae</u> and <u>M. Lepraemurium</u>	31
6. Thalidomide Therapy in the Lepra Reaction	38
7. The Effect of Fresh Serum on the Leptomonads of Leishmania	49
8. The Methylene Blue Test	62
9. Some Ideas in Relation to a Project for Studying Susceptibility to Leprosy: Basis of the Transformation of the Infection into Disease, and Possible Ways of Preventing said Transformation	71
10. Behaviour of Patients with Lepromatous Leprosy and Diffuse Cutaneous Leishmaniasis in Relation to Skin Homografts	79
11. Inoculation of Human Leprosy in Laboratory Animals	88

PROGRESS REPORT ON RESEARCH IN LEPROSY  
AND OTHER TROPICAL DISEASES IN VENEZUELA\*

An effort has been made in Venezuela to integrate epidemiological, medical, teaching and research activities concerned with a group of diseases which constitute a public health problem, such as leprosy, leishmaniasis, onchocerciasis and other dermatological conditions.

With this purpose in mind, a closely knit coordination has been established between the Division of Sanitary Dermatology of the Ministry of Health and the Department of Dermatology of the Vargas Medical School and Vargas Hospital.

In relation to research, the development of the laboratories of biochemistry, immunology, cytochemistry, leprology, hystopathology, mycology and microbiology has made possible the following investigations some of which are on the planning stage, some are being carried out and some have already been completed.

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# IMMUNOLOGICAL REACTIONS IN ONCHOCERCIASIS

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The infection produced by Onchocerca volvulus constitutes a medical problem of importance in Venezuela. Epidemiological studies have revealed a total of almost 20,000 proven cases; undoubtedly a similar number remain undetected. The most serious long-term effect of this infection is ocular damage. Of some 5,000 cases investigated in Venezuela, 64% had lesions in the iris, cornea, and/or retina, sometimes producing partial blindness.

Except for skin-test reactions, the immunological aspects of this disease have not been extensively studied. Here in Venezuela, Maekelt has investigated the use of antigens prepared from adult filariae in skin tests, and Ciferri and co-workers (1965) and others have done similar studies in other countries.

In the present paper, we have studied the incidence of precipitating antibody and skin-sensitizing antibody in sera from patients with onchocerciasis, and have studied the response to microfilarial antigen in skin tests in patients with onchocerciasis as well as controls.

Initially, antisera from 50 patients under treatment for onchocerciasis were examined for the presence of precipitating antibody with specificity for adult filariae. The antigen used in this test was prepared by the saline extraction of filariae of Onchocerca volvulus dissected from excised nodules, and the sera were studied by the Ouchterlony method of double diffusion in agar. Blood samples were taken from patients which are grouped according to prominent clinical mani-

festations. (See Table 1). We were particularly interested in the correlation between the presence of antibodies and the presence or absence of ocular lesions. The group of patients with nodules is small and conclusions cannot be drawn with confidence; in the other groups, 55 to 67% of the sera gave at least one precipitin line, and in some cases as many as three. It is apparent that the incidence of antibody in patients with and without ocular lesions is not strikingly different.

Attempts were made to detect precipitating antibodies with specificity for microfilariae in these same 50 sera. Only three sera gave precipitin lines with a saline extract of microfilariae. It is not clear whether this reflects the actual scarcity of such antibody, or our inability to prepare an antigen of adequate strength. Testing of sera collected 15 days after the administration of Hetrazan (diethylcarbamazine) showed no increase in antibodies against either microfilariae or filariae, in spite of the fact that this drug kills large numbers of microfilariae which might be expected to act as a strong antigenic stimulus.

Nineteen of these same sera were tested for the presence of skin-sensitizing antibody by injecting 0.1 ml. of each serum intradermally into a normal individual, then challenging after 24 hours with an antigen prepared by extracting microfilariae of Onchocerca volvulus with saline. These reactions were all read 30 minutes after the injection of antigen. The positive reactions show very marked erythema and a slight wheal. Of the 19 sera tested, 15 gave strong immediate reactions. The presence or absence of skin-sensitizing antibody is not highly correlated with precipitating antibody, since each may be present in the absence of the other. One particular difficulty with

this type of test seems to be a considerable variability in the response of normal individuals to the test; of five individuals we studied, only two gave strong, reproducible results.

The presence of skin-sensitizing antibody in a relatively high proportion of the sera tested provides a rational basis for the usefulness of direct skin testing in the detection of infection with Onchocerca. We skin-tested 61 patients under treatment for onchocerciasis with an antigen prepared by the saline extraction of microfilariae of O. volvulus. Some time later, 31 individuals from an area non-endemic for this infection were skin-tested with the same antigen. Each individual received 0.1 ml of antigen intradermally in the left forearm and 0.1 ml. of saline in the right forearm. Erythema and size of the wheal were read 30 minutes after the injection. The results of these tests are shown in Table 2. Eighty-six per cent positive tests in the first group compares quite well with the 92-93 per cent positive one would expect using Hetrazan for diagnosis. Further investigation is necessary to determine whether the positive skin tests obtained in individuals from a non-endemic area are due to cross-reactivity with other parasites, a degree of toxicity of the antigen, or to the presence of individuals in this group who had been exposed to onchocerciasis in other locales.

#### Summary

Precipitating antibodies against Onchocerca volvulus were detected in 64 per cent of sera tested from onchocerciasis patients. The presence of this type of antibody was not highly correlated with the presence or absence of ocular lesions.

Skin-sensitizing antibody was demonstrated by passive transfer

to a responsive normal individual in 15 of 19 sera so tested.

Direct skin tests with microfilarial antigen were positive in 86 per cent of a group of onchocerciasis patients and 13 per cent of a control group, suggesting that further investigation of this type of test as an aid in diagnosis is warranted.

(This paper was presented at the national meeting of the Venezuelan Association for the Advancement of Science in 1966, and is to be published in more extensive form when additional material with Dirofilaria antigens is added).

TABLE 1

Incidence of precipitating antibody specific for filariae of *Onchocerca volvulus* in the sera of patients with onchocerciasis.

GROUP	TYPE OF LESION	PRECIPITATING ANTIBODY	
		N°. positive	
I	Without ocular lesions or nodules.	5/11	
II	Without ocular lesions with nodules.	5/5	
III	With ocular lesions without nodules.	11/18	
IV	With ocular lesions and nodules.	1/3	
V	With ocular lesions	6/6	
VI	Without ocular lesions	4/7	
SUMMARY	Without ocular lesions	Positive	
	With ocular lesions	(N°)	61.
	Without nodules	14/23	67.
	With nodules	18/27	55.



TABLE 2

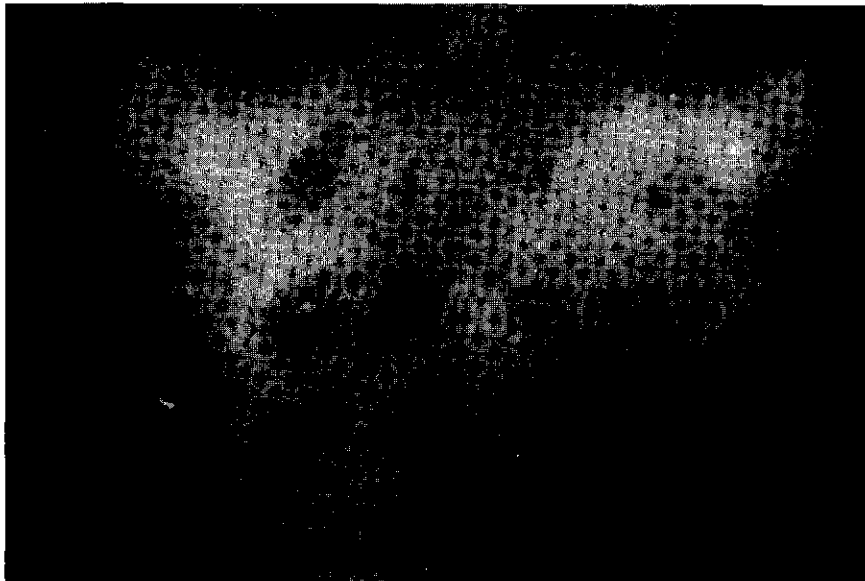
Immediate hypersensitivity to microfilarial antigen  
of Onchocerca volvulus

GROUP	WHEAL		ERYTHEMA		WHEAL AND/OR ERYTHEMA	
Patients with Onchocerciasis 61	Positive (a)		Positive (b)		Positive	
	N°	%	N°	%	N°	%
	43	70	42	69	33	86
Controls 31	4	13	3	10	4	13

(a) Reaction at least 4 mm. larger than the control.

(b) Average diameter of the positive reactions was 29 mm.

Figure 1. Photograph showing P-K tests with antisera from various patients with onchocerciasis. Three of the tests, in the top row, are negative. The injection in the lower corner is a saline control.



THE LEISHMANIN REACTION AND OTHER IMMUNOLOGICAL ASPECTS  
OF LEISHMANIASIS

The immunological aspects of leishmania infection are important not only from the viewpoint of basic knowledge, but also from two highly important practical aspects: 1) Development of an effective means of vaccination, and 2) The possible use not only of cutaneous skin reactions, but also of serological tests in diagnosis. Both of these problems remain largely unsolved with regard to tegumentary leishmaniasis of the New World, but some conclusions can be drawn from experiences here as well as from the more widely studied examples of kala azar and Leishmania tropica.

There are very few data concerning any natural immunity to infection with cutaneous leishmaniasis. Its occurrence in almost epidemic proportions under certain circumstances suggests that such immunity, if it exists, is probably minimal. Occupational factors play an important role in its incidence, which is highest in persons working outdoors in areas frequented by Phlebotomus, but there is no clear evidence of greater innate susceptibility purely on the basis of sex, age, or race.

Long-lasting, possibly life-long immunity is conferred by infection with cutaneous leishmaniasis—it becomes apparent even before the primary lesion is healed, as indicated by difficulty in super-infecting persons with primary lesions and the rapid evolution of secondary lesions when they can be established. This immunity to re-infection is accompanied by positivization of the Montenegro or leishmanin skin reaction. The immune status of persons with diffuse leishmaniasis, who are relatively anergic to the leishmanin test, has not been evaluated. Certainly

their disease does not proceed to spontaneous cure, as is usually the case in cutaneous leishmaniasis, but whether they are susceptible to super-infection with other strains of leishmaniae is not known.

The lasting immunity normally produced is apparently "sterile" and not dependent upon the continued presence of the parasite. During the course of the primary infection, however, a state of "premunition" undoubtedly exists, in which the presence of the parasite inhibits super infection.

The Montenegro reaction, described in 1926, is apparently a typical delayed hypersensitivity of the tuberculin type, characterized by cellular infiltration which reaches a maximum size at 24 to 48 hours. The reaction is elicited by injection of washed, phenolized leptomnads from in vitro cultures. Cross-reactions using leishmanins prepared from numerous species of leishmaniae are reported; similar antigens prepared from Trypanosoma cruzi also produce delayed reactions in persons with leishmaniasis. (Depieds, et al., 1959).

Diffuse leishmaniasis is characterized by relative anergy to the leishmanin test. Whether this reflects a species difference in the parasite, as has been assumed on the basis of clinical observations, differences in infectivity for laboratory animals, cultural differences, etc. (Medina and Romero, 1962), or whether this results from an immunological defect in the host is still not clear. Some information might be gained concerning this point by using culture forms of L. pifanoi in the preparation of a leishmanin antigen to be tested in persons with diffuse as well as localized leishmaniasis, as well as from the fluorescent antibody studies to be discussed separately.

The basic immunological nature of the leishmanin reaction has

never been clarified. In 1945, Dostrowsky and Sagher reported the successful transfer of the reaction to normal persons using serum from patients, using the technique of Prausnitz and Küstner. This is, of course, inconsistent with the immunological concept that delayed reactions of the tuberculin type are mediated not by serum factors, but rather by sensitized cells of the lymphoid series. Adler and Nelken (1965) used both whole-blood transfusions and leucocyte preparations in an attempt to transfer the reaction to normal persons and were not successful. In current experiments being carried out in the Laboratory of Experimental Microbiology, Dermatology Division of the Ministry of Health, we have attempted to transfer the leishmanin reaction induced in guinea pigs by sensitization with leishmanin in complete Freund's adjuvant to normal guinea pigs by means of lymphoid cells, serum, and a combination of the two, with minimal success. Serum alone and serum plus lymph node and spleen cell transfers gave reactions no larger than the controls; passive transfer of spleen and lymph node cells alone gave, in 8 cases, reactions about 4 mm. larger than control reactions. (See Table 1). Possibly the nature of the antigen itself accounts for some of the difficulty encountered—the leptomonads may be phagocytosed rapidly by macrophages and protected in the intracellular environment from effective contact with the passively transferred cells; undoubtedly there are other factors involved as well that differentiate the leishmanin reaction from the classical type of delayed hypersensitivity.

Limited tests of the Prausnitz-Küstner type have been carried out at the Hospital Vargas, using sera from 10 leishmanin-positive patients. These reactions were no larger than control sites in which only leishmanin was injected.

These problems concerning the nature of the leishmanin reaction do not obscure the fact that it is a useful indicator of immunity to re-infection, at least with the homologous strain of the parasite. The reaction is not, however, an absolute indicator of immunity. The leishmanin reaction becomes positive after immunization with killed or avirulent L. tropica, for example, but this type of immunization does not confer immunity to infection (Demina, 1967). The reaction may also become positive upon repeated retesting with leishmanin of normal individuals, but there is no evidence that this confers immunity. Positivation of the Montenegro reaction, then, does not necessarily indicate adequacy of vaccination methods in producing immunity, but in the case of natural infections it is a good indicator of immunity, at least to homologous strains of leishmaniae.

Reported attempts at vaccination against L. brasiliensis are very limited. In Venezuela, an attenuated strain is being tested by Pifano, but the results as yet have not been fully evaluated. Results with other species of leishmaniae are illustrative of the problems involved. Manson-Bahr (1962), using a strain of Leishmania isolated from ground squirrels, avirulent for human beings, reported success under laboratory conditions in preventing super-infection with L. donovani. When this vaccine was used in field trials in an area endemic for kala azar, however, the results were extremely disappointing (Heyneman, 1967). Experiments in Russia with L. tropica have given the following results: 1) Killed cultures or cultures of L. enriettii are ineffective in preventing infection. 2) Avirulent strains of L. tropica are ineffective, though they produce skin sensitization. 3) Strains of low virulence produce a weak immunity, not adequate for protection against highly virulent

strains. 4) Strains of high virulence produce a strong immunity, and are rather widely used in Russia (Demina, 1967).

The need for effective vaccination against L. brasiliensis, in which involvement of mucosal surfaces may lead to serious disfigurement, is obvious. Avirulent cultures can only be evaluated by extensive field trials. It would also seem to be of value to test virulent cultures of L. mexicana, since experiments with monkeys indicate that infection with L. mexicana confers immunity against subsequent infection with L. brasiliensis (Lainson and Bray, 1966). The former has the advantage of causing relatively small, rapidly-healing, non-metastasizing lesions which confer long-lasting immunity without the risk of involvement of mucous membranes.

Serological aspects of leishmaniasis and general conclusions will be considered in a separate paper.

TABLE 1

Material transferred	Skin reactions (mm.)	
	24 hours	48 hours
Spleen and lymph nodes	1. 10 x 1/8	6 x 1/8
	2. 9 x 1/4	9 x 1/4
	3. 9 x 1/4	7 x 1/8
	4. 9 x 1/8	10 x 1/8
	5. 9 x 1/8	-
	6. 8 x 1/8	9 x 1/8
	7. 8 x 1/8	7 x 1/8
	8. 7 x 1/8	-
	Av. 8.6 x 1/8	8 x 1/8
Peritoneal washings	1. 7 x 1/8	5 x 1/8
	2. 7 x 1/8	-
	3. 5 x 1/4	-
Spleen, lymph nodes, and serum.	6 x 1/8	5 x 1/8
Spleen, lymph nodes, and blood.	4 x 1/8	4 x 1/8
Spleen.	5 x 1/4	-
Lymph nodes.	5 x 1/4	-
Serum.	3 x 1/8	0
Controls	1. 5 x 1/8	5 x 1/8
	2. 5 x 1/8	5 x 1/8
	3. 5 x 1/8	-
	4. 4 x 1/8	3 x 1/8
	5. 3 x 1/8	-
	Av. 4.4 x 1/8	4.3 x 1/8



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SEROLOGICAL REACTIONS IN LEPROSY

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Serological reactions in leprosy infection have not been studied with as much interest as other aspects of the disease for various reasons: 1) Inability to cultivate the antigen has made the preparation of even a relatively pure antigen difficult. 2) Positive serological tests are not characterized by an absolute specificity for leprosy; no antigen specific for the leprosy bacillus has been isolated. 3) Serological reactions are of little use to the clinician, because of the lack of specificity mentioned and because the presence of antibodies is in no way correlated with the state of resistance of the patient.

In spite of these factors, there are some noteworthy observations concerning antibody production in leprosy. Reports by Rees, Sushida, and others indicate that antibodies reactive in precipitin tests with a wide variety of mycobacterial antigens are produced by most patients with lepromatous leprosy and many patients with borderline leprosy. In contrast, very few patients with tuberculoid leprosy have detectable circulating antibody against mycobacterial antigens. Reactional manifestations, such as erythema nodosum leprosum, are often accompanied by high levels of antibody. Most recently, a series of papers by Ouchterlony and co-workers have amplified the nature of the antigens involved and other aspects of the serological responses in leprosy.

The purpose of the present study has been to study a number of these serological features in some detail, using sera from patients with the more important clinical manifestations of leprosy. The aspects studied include

the following: 1) The incidence of precipitating anti-mycobacterial antibody in various forms of leprosy, using soluble antigens from ten strains of mycobacteria in precipitin tests. 2) Incidence of cryoprotein in various manifestations of leprosy. 3) Levels of circulating precipitins during reactional phases and subsequent periods of quiescence. 4) Limited characterization of the antibodies present in lepromatous sera.

We have studied the incidence of precipitating antibody in sera from patients with lepromatous, tuberculoid, borderline, and indeterminate leprosy, and in the reactional forms of the disease. The method of double diffusion in agar (Ouchterlony) was used to study the sera. Soluble antigens were prepared from ten strains of mycobacteria by means of the disintegration of washed bacilli by ultrasound. The protein content of each antigen, after concentration, varied from 4 to 12 mg. per ml. The strains used in this study include 7 cultivable mycobacteria - BCG, M. tuberculosis (H<sub>37</sub>Rv), M. tuberculosis bovis, a photochromogen, an atypical strain isolated from a pulmonary lesion, and two strains isolated from human leprosy. Three non-cultivable mycobacteria were used; M. lepraemurium, bacilli separated from human lepromas, and non-cultivable bacilli separated from lesions of hamsters injected with human leprosy material.

We have found antibodies against one or more of the ten strains of mycobacteria used in 75 per cent of a group of 76 sera from patients with lepromatous leprosy. Of these 76 sera, 41 were taken from patients with reactional symptoms. Thirty-three of these sera from reactional patients contained antibody. (See Table 1).

Of 8 sera from tuberculoid leprosy patients, only one contained antibody; this patient had had reactional symptoms. One serum from a group of 10 with indeterminate leprosy contained precipitating antibody, as did 4 from a group

of 14 with borderline leprosy. Thus the incidence of precipitating anti-mycobacterial antibody is very high in patients with lepromatous leprosy, intermediate in borderline leprosy, and low in the tuberculoid and indeterminate forms of the disease.

A number of these sera gave precipitin lines with 8, 9, or all 10 of the antigens used. The least reactive antigens included BCG, of the cultivable types, and bacillary antigen isolated from human lepromas. The studies of Estrada-Parra and others indicate that the active antigen in these precipitin tests with leprosy material is a polysaccharide. Concentration to a relatively uniform amount of protein may be quite unrelated, then, to the content of active antigen in each preparation.

Many of these sera gave two or rarely three precipitin lines with various of these antigens. We have not observed a specific line with the antigen prepared from lepromal bacilli, but rather lines of identity with several of the antigenic preparations, indicating the presence of antigens common to the group of mycobacteria.

We have used lepromin, prepared by the Mitsuda-Hayashi-Wade modification, as the antigen in precipitin tests, both unconcentrated and concentrated 4 to 6 times, with little success. Of the 108 sera tested, only 4 gave a precipitin line with this antigen. The sensitization of red blood cells with lepromin, using the method of Middlebrook and Dubos, also gave very few positive reactions, with low titers. Passive cutaneous anaphylactic reactions with sera from leprosy patients and various mycobacterial antigens were either negative or extremely tenuous.

Two methods used to characterize the antibodies in these sera--inactivation with 2-mercaptoethanol and separation on Sephadex G-200--indicate that antibody activity is found in both the macroglobulin and normal gamma globulin

fractions of the serum.

To study the role of antibodies in reactional manifestations of leprosy, we have taken serum samples before the initiation of treatment with thalidomide and afterwards, at intervals of one month, for 6 to 8 months. Although the reactional symptoms disappear soon after the initiation of treatment, there was no apparent diminution of the levels of circulating antibodies. This suggests that either precipitating antibodies play at best a minimal role in the pathogenesis of reactional manifestations, or that circulating antibodies do not provoke these reactions unless there is an excessive liberation of antigen, which does not occur during treatment with thalidomide (among other possibilities).

Finally, we have studied the incidence of cryoprotein in the sera of leprosy patients, according to the method of Trautman and Matthews. In 34 sera from patients with various clinical manifestations of leprosy, cryoprotein was encountered in 30, or 88 per cent. Auto-immune antibodies have been reported in this fraction of serum, but the washed precipitates do not contain precipitating anti-mycobacterial antibodies detectable by the method of Ouchterlony. We observed this high incidence of cryoprotein in sera taken from patients in a local leprosarium, whose infections were of long duration.

The incidence of positive tests for cryoproteins in sera taken from out patients of the Hospital Vargas, Caracas, Venezuela has been much lower, indicating that this abnormality of the serum undoubtedly develops only after prolonged infection.

These studies confirm the presence of precipitating antibodies in sera from leprosy patients; these antibodies react with antigens prepared not only from M. leprae, but from nine other strains of mycobacteria as well. The

incidence is high in lepromatous leprosy, and quite low in other forms. These studies have not revealed the presence of any antigen specific and characteristic of the human leprosy bacillus, but rather indicate the presence of one or various antigens shared with various mycobacteria.

Leprosy patients with disease of long duration show abnormalities such as the presence of cryoprotein in their sera, but this is not commonly observed in less severe or relatively new cases.

(This paper was presented in Spanish at the first National Meetings of Microbiology in Caracas, in 1967. An extended manuscript is being prepared for publication.)

Table 1INCIDENCE OF ANTI-MYCOBACTERIAL ANTIBODY IN LEPROSY SERA

Clinical form of leprosy	Number of sera tested	Number of positive sera
Lepromatous		
a. Reactional	41	33
b. Non-reactional	35	24
Tuberculoid	8	1
Indeterminate	10	1
Borderline		
a. Reactional	5	3
b. Non-reactional	9	1

Table 2

NUMBER OF SERA FROM EACH CLINICAL TYPE OF LEPROSY WHICH REACT WITH  
EACH OF THE TEN MYCOBACTERIAL ANTIGENS USED THE METHOD  
OF DOUBLE DIFFUSION AND PRECIPITATION IN AGAR

Strain of mycobacterium	Lepromatous leprosy	Tuberculoid leprosy	Borderline leprosy	Indeterminate leprosy
	(76 sera)	(8 sera)	(14 sera)	(10 sera)
Cultivable				
BCG	9			
H37Rv	22	1	2	1
<u>M. tuberculosis</u> bovis	24	1	2	1
440 (photochromogen)	39	1	3	
Valero (anonymous)	53	1	4	1
24 (human leproma)	49	1	4	1
129 (human leproma)	54	1	3	1
Non-cultivable				
<u>M. leprae</u>	11	1	1	
<u>M. lepraemurium</u>	35	1	3	1
Mycobacterium from a hamster injected with lepromatous material	37	1	4	1



IMMUNOLOGICAL STUDY OF AMERICAN CUTANEOUS LEISHMANIASIS  
THROUGH THE INDIRECT IMMUNOFLOUORESCENCE METHOD

Serological techniques have been widely used in the diagnosis of Kala-~~azar~~, but when they have been tried in cutaneous leishmaniasis, it has not been possible to demonstrate, in a constant manner, the presence of circulating antibodies.

There are reports in the literature of positive precipitin reactions obtained with cutaneous leishmaniasis sera (1, 2), but our preliminary results with this method, using the Ouchterlony technique of double diffusion in agar, have not been successful. Using concentrated culture filtrates and filtered lysates of leptomnads as antigens, we have obtained precipitating reactions both with sera from patients and from normal persons. The same tests done using L-D bodies as antigen gave the same, non-specific results.

Bray and Lainson, using immunofluorescent techniques, demonstrated circulating antibodies in the sera of patients against a great variety of Leishmania leptomnads and, in a lesser degree, against L-D bodies. Absorption with any of the strains of Leishmania removed not only homologous antigens, but also all activity against heterologous strains, indicating the existence of a group antigen common to at least five species of Leishmania. When these authors tried to use this technique for the identification of serum samples from infected and normal persons, the results were very disappointing, since the positive tests were almost as high in normal sera as in the sera from patients.

In our laboratory, indirect immunofluorescence has been the only method which has shown, in a constant and reproducible manner, the presence of circulating antibodies in a high percentage of the sera investigated.

#### Material and Method.

We have used the indirect immunofluorescence technique with two different antigens: an antigen of Leishmania leishmaniasis, cultured in Nakamura medium, which allows the preparation of an antigen free of foreign proteins, and an antigen made with L-D bodies taken from lesions provoked in the hamster by the inoculation of human diffuse cutaneous leishmaniasis material.

The fluorescent equipment used is a Zeiss and has an illuminator with a high-pressure mercury lamp HBO 200, an exciter filter BG3 and a combination of barrier filters 41-53. The fluorescent anti-serum is goat anti-human globulin serum, made by Microbiological Associates; it was used diluted 1:5.

We tested 90 sera, 85 of which belonged to patients with american cutaneous leishmaniasis of the localized form and 5 to patients with diffuse cutaneous leishmaniasis.

As controls, we used 13 sera from normal persons from non-endemic areas and 9 from persons with no background of having been infected but from endemic leishmaniasis areas.

We felt that in this method the fixing of the antigen to the slide was very important, specially with a parasite as fragile as L-D bodies, and we tried several ways of doing it, such as formaldehyde vapors, formalin, heat during different lengths of time, acetone, etc.,

all of which were discarded as unsatisfactory. According to our experience, the only method which gave good, constant results, was fixing the slides with the antigen in a Coplin jar, with Methanol previously cooled to  $4^{\circ}\text{C}$ , during 15 minutes, at a temperature of  $4^{\circ}\text{C}$ .

### Results.

We are going to consider the results referring to each antigen separately.

#### I. Antigen of culture leptomonads.

We consider the results obtained with this antigen as non-specific, since the same kind of strongly positive fluorescency was found in the sera of patients and of normal persons. This result was the same for all the dilutions used.

#### II. L-D bodies antigen.

With this antigen, the results were absolutely specific, since all the sera from both control groups were negative from 1:5 dilution. Of the 85 sera from localized american cutaneous leishmaniasis, 54 were positive, with an intensity which varied from one plus up to a very strong three plus fluorescency, giving a percentage of positive sera of 63.5%. Of the five diffuse cutaneous leishmaniasis sera, all were strongly positive.

The titer of the positive sera was determined, finding that 22 of them were positive to a dilution of 1:10, 15 were positive to a dilution of 1:50 and 22 were positive to a dilution of 1:200 (Table 1)

The results of the immunofluorescency test and the results of the Montenegro test did not show any obvious relation to each other.

With the intention of absorbing these antibodies, we mixed three

of the strongly positive sera with culture leptomonads of L.brasiliensis. Our results were negative since the fluorescency persisted with the same intensity, showing that there had been no absorption of the antibody. These same sera were treated with L-D bodies, isolated from experimental lesions in hamsters; these parasites were fixed with Methanol at 4°C of unfixed. With both, the results were the same, the fluorescency was as intense as before, showing there had been no absorption.

Two of the intensely positive sera, one from diffuse cutaneous leishmaniasis and one from the localized form, were precipitated with saturated ammonium sulphate at concentrations of 33 and 50% in relation to the serum. The immunofluorescency made with the fractions obtained gave the following results: the precipitate from the 33% concentration was negative and the supernatant from that same concentration was positive, while the precipitate from the 50% concentration was positive and the supernatant was negative.

#### Observations.

We want to emphasize our observation that, in disagreement with reports from other authors, in our hands the indirect immunofluorescent technique in relation to leishmaniasis, is a specific test, from a 1:5 dilution, only when the antigen used is of L-D bodies. When culture leptomonads are used as antigen, the test loses its specificity, being positive both with sera from infected and normal persons.

The failure to absorb the antibodies producing fluorescency with L-D bodies, unfixed or fixed with Methanol at 4°C, may be due to an unstable antigen-antibody combination.

The results obtained with the fractioning with saturated ammonium sulphate seem to indicate that the antibody we are studying belongs to the macroglobulins.

The contrast presented by the positivity of the immunofluorescence test and the negativity of the Montenegro reaction, in the cases of diffuse cutaneous leishmaniasis, seems to be further proof of the lack of relationship between these two tests. It also shows that these patients are not as anergic as they seem to be, since they are able to produce circulating antibodies.

Our results indicate that the indirect immunofluorescence method is not applicable to the routine diagnosis of leishmaniasis, but we do believe that it is a good technique, with constant and reproducible results, for the immunological study of this disease.

#### Summary.

Eighty-five american cutaneous leishmaniasis sera and 5 of the diffuse form are investigated by the indirect immunofluorescence method, using as antigen L-D bodies isolated from experimental lesions in hamsters.

Sixty-three percent of the american cutaneous leishmaniasis sera and 100% of the diffuse form were positive. Of the positive sera, 37% were positive up to a dilution of 1:10, 26% to a dilution of 1:50 and 37% to a dilution of 1:200. The control sera were all negative.

There was no apparent relationship between the results of the immunofluorescence test and the Montenegro test.

The results given by the fractions obtained by precipitation with saturated ammonium sulphate seem to indicate that the antibody

belongs to the macroglobulins.

The indirect immunofluorescence test is not considered as applicable to the routine diagnosis of leishmaniasis, but as a good method for immunological studies of this disease.

TABLE 1SERA OF PATIENTS WITH AMERICAN CUTANEOUS  
LEISHMANIASIS

	MALES	FEMALES
1:10	15	9
Positives 1:50	9	6
1:200	15	7
Negatives	21	8
Total	60	30
<sup>4</sup> / <sub>5</sub> Positives	65	73

IDENTIFICATION OF THE NONCULTIVABLE PATHOGENIC  
MYCOBACTERIA M. LEPRAE AND M. LEPRAEMURIUM 1/, 2/

Imelda Campo-Aasen and Jacinto Convit 3/

In the experimental attempts at transmission of human lepromatous leprosy to laboratory animals it is of great importance to distinguish the noncultivable Mycobacterium leprae from the equally uncultivable M. lepraemurium, as well as from the strains that may be viable in the animal host with or without adaptive mutation in the new environment. Two methods are available at present for such studies, viz.:

1. Administration to a lepromatous patient of an antigen prepared from the mycobacterial strain in a manner similar to the Mitsuda-Hayashi method. As is well known, the antigen prepared from the bacilli found in lepromatous human lesions produces no reaction in the lepromatous patient. However, it is a long and indirect procedure, which will often depend on the presence, in the vicinity, of lepromatous cases as a source of bacilli.

2. The Prabhakaran method (2), which demonstrates that M. leprae oxidizes 3,4-dihydroxyphenylalanine after 15 to 30 minutes incubation. Because of the

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1/ Received for publication 20 September 1967.

2/ This investigation was carried out in the Histochemistry Laboratory, which is jointly sponsored by Vargas and Razetti Medical Schools of the Central University of Venezuela. The expenses of this investigation have been covered in part by Grant AI-04216 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014. Part of the equipment used was donated by the Pan American Health Organization (Regional Office of the World Health Organization).

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normal presence of dopa oxidase in the skin, splenic tissue is used, and by means of homogenization, differential centrifugation, and spectrophotometry, the usefulness of the method for M. leprae and its differentiation from M. lepraemurium is determined. This method, however, has been criticized with respect to its specificity (3). Furthermore, it involves difficulties in performance, and can be carried out only in a fully equipped laboratory.

The main purpose of the research here reported has been to develop a cytochemical method that will allow differentiation of the noncultivable mycobacteria among themselves, and specifically of M. leprae and M. lepraemurium.

#### MATERIALS AND METHODS

In our primary experiments with Baker's histochemical test, which is highly specific for phospholipids (1), we used 11 nodules from the skin of eight treated and three untreated lepromatous patients. The same number of peritoneal and dermic lepromas were secured from rats and mice inoculated with a strain of M. lepraemurium, which Dr. Y. T. Chang of the National Institutes of Health, Bethesda, Maryland, had kindly furnished us. In our investigation of the applicability of the test to mycobacteria we used two sets of preparations for each strain or species. One set was submitted directly to the method of the test, while the other was first submitted to the Baker process for the removal of phospholipids and thereafter to the test following the same steps as with the unextracted set. The materials studied were (1) sections from human lepromatous tissue and from leprous rat tissue, both cut to a thinness of 8-10 millimicrons, and (2) smears from homogenized preparations of the same tissues. Later on we also used simple lymph smears from human as well as animal sources of bacilli.

The experience gained in the primary experiments soon convinced us that

certain modifications of procedure and timing were necessary in order to obtain the best visualization of the bacilli in the smears. The Baker test for phospholipids, as modified by us for use with smears, is carried out as follows:

1. Dry the smears on cover slides at room temperature for 4-5 hours or in an oven for 1 hour at 37° C.
2. Fix the smears in Baker's formol-calcium for 5 hours.
3. Leave the cover slides overnight or for 16 hours in a 5% calcium dichromate solution.
4. Rinse with distilled water. Steps 3 and 4 are optional.
5. Place the cover slides in a mordant solution of calcium dichromate for 1 hour at 60° C.
6. Rinse with distilled water.
7. Stain in acid hematein at 60° C for 1 hour.
8. Rinse in distilled water.
9. Differentiate in boric ferricyanide for 1.5-2 hours.
10. Rinse in distilled water.
11. Blot dry.
12. Mount the cover slides with Permount on the unstained side of the slide and observe directly with oil immersion.

The Baker process for the removal of phospholipids, as adapted by us, is the following:

1. Fix in Bouin's fluid for 5-16 hours.
2. Place the cover slides in 70% alcohol for 5 minutes.
3. Place them in 50% ethyl alcohol for 5 minutes.
4. Rinse in tap water for 1-2 minutes.
5. Dehydrate in fresh pyridine for 2 hours.

6. Rinse in tap water for 5 minutes.

7. Continue with step N° 3 from Baker's method for smears as already outlined.

As a complementary procedure we used the PAS reaction (Tomassi-Schiff) on frozen sections, using the same formol-calcium as a fixative. A similar study was made with the use of cultivable mycobacteria, such as M. fortuitum, the bacillus Calmette-Guerin (BCG), M. chromogenum hominis, M. balnei, M. tuberculosis H37 Ra and M. butyricum, using the Baker as well as the PAS technic.

### RESULTS

Human lepromas and smears. Under low magnification the sections of human lepromatous tissue showed a granuloma of intense dark-blue color, which would indicate the presence of phospholipids or mucins or both, but as the blue color was not found in the extracted sections, it could definitely be attributed to phospholipids only. On higher magnification the blue masses were seen to be located in the cytoplasm of mononuclear cells or histiocytes. The immersion showed that the blue masses were made up of bacilli, some intact and some granular, the blue color indicating that they must have contained very large amounts of phospholipids. The unextracted smears prepared from homogenized lepromatous tissue, as well as the simple lymph smears, revealed isolated, intact or granular bacilli or globi stained a dark blue, but the blue coloration was absent in the extracted smears. Here again we had proof that the stain was retained exclusively by phospholipids in M. leprae.

Animal lepromas and smears. Our study of sections made from murine peritoneal lepromas revealed something remarkably different. The modified Baker test applied to those sections produced indistinct dark-blue granular o bacil-

lary forms in the cytoplasm. In the extracted sections the blue color not only persisted, but was actually intensified and better defined. Smears from homogenized murine lepromas and lymph smears from the same source showed blue-stained bacilli, in either the extracted and the unextracted slides. The PAS test was positive in the smears of the cultivable as well as the noncultivable mycobacterial species to which it was applied, such as M. leprae, M. lepraemurium, M. balnei, M. fortuitum, BCG, M. tuberculosis H37Ra, and M. chromogenum hominis.

#### DISCUSSION

The use of Baker's technic for phospholipids, with the modifications employed by us in smears, fills a need in the experimental field for differentiating M. leprae from M. lepraemurium. The studies carried out demonstrated that M. leprae is the only one of the noncultivable or cultivable mycobacteria used, that produces a clear and definite coloring in the Baker method for phospholipids.

The results obtained from tissue sections of murine leprosy demonstrate that the content of polysaccharides in these bacilli, and not the phospholipids, is responsible for the coloring obtained, since the blue color not only remained but was intensified upon extraction. We believe that the coloring of the phospholipids is an exclusive property of the M. leprae of human origin, which could be applied in the study of mycobacteria observed in experimental inoculation of human leprosy in laboratory animals or in a possibly successful culture. Another possible use of this method could be for the identification of M. leprae among the mycobacteria found in healthy persons originating from endemic areas of leprosy. It could also be applied to the study of the morphologic index of the M. leprae.

SUMMARY

A new and easily performed histochemical method for the identification and differentiation of two noncultivable mycobacteria, M. leprae and M. lepraemurium is presented.

Baker's method for phospholipids is used primarily and for their concomitant verifying extraction in tissue sections, as well as smears. In the latter our modified version of the Baker method was used.

The PAS technic, applied to the cultivable and noncultivable mycobacteria, gave positive results in all of our studies.

The importance of use of the method for its epidemiologic value in healthy carrier is stressed.

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## THALIDOMIDE THERAPY IN THE LEPRO REACTION 1/, 2/

Jacinto Convit, Jose M. Soto and J. Sheskin 3/

In earlier, initial investigations J. Sheskin of the Hadassah Hospital of Jerusalem reported that thalidomide (R-phthalimido-gludaside) was highly effective in the treatment of lepra reaction. More recently extensive trials by the double-blind method were carried out in Venezuela as a joint investigation by the Hebrew University of Jerusalem, the Division of Sanitary Dermatology of the Ministry of Health of Venezuela, and the Central University of Venezuela. In this investigation the results of the earlier work in Israel were confirmed, and a remarkable effectiveness of the drug in the treatment of the lepromatous lepra reaction was established (6).

The present report deals with the direct treatment of a group of lepromatous patients with thalidomide to counteract their lepra reactions, which, in the majority of the cases, were severe and of long duration.

MATERIAL AND METHODS

A group of 24 patients with lepromatous leprosy in a reactional stage were selected for the treatment. They presented polymorphous erythemo-nodose lesions, and the reaction was severe and of long duration in 70 per cent, as a result of months and even years of steady aggravation. Treatment with corti

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1/ Received for publication 24 march 1967.

2/ This investigation was supported in part by grants received from the U.S. National Institutes of Health, Grant Number AI 04216-05 and from the World Health Organization.

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costeroids had been frequent and of variable benefit. Secondary effects of those drugs had often been evident.

The patients were conventionally classified according to the intensity of their reactional state. By R3 we designated patients that showed profuse reactional eruptions of nodose or polymorphous erythema with intense dermalgia and edema of the extremities, pain in the joints and muscles, and severe headaches. In some patients, although not in all, there was acute neuritis. The general health of the patients was greatly lowered by fever, which rose frequently to above 39°C, vomiting, loss of appetite, and insomnia.

As R2 we classified patients with a less severe reaction, characterized by the symptoms of the R3 group, but in lesser degree, with fever not exceeding 38°C, but without prostration and with the ability to move about, although with some difficulty.

The R1 group included patients with erythemo-nodose or erythemo-polymorphous manifestations, with subfebrile temperature, with or without moderate pains, but without any notable lowering of their general health.

In the group of 24 patients selected, of whom 16 were males and eight females, 19 were classified as R3 or R2.5. In the group R3 we included two patients with acute polyneuritis who did not show the intensity of the reactional symptoms evident in the rest of the group. The age of the patients varied from 17 to 62 years, with 19 between the ages of 30 and 62. All of them were hospitalized under conditions of strict medical control.

The strictest vigilance was observed with the eight women in the group. They had been submitted to special control to eliminate all suspicion of pregnancy in view of the teratogenic properties of the drug.

Doses employed.- The drug was administered in daily doses of 400 mgm.



in portions of 100 mgm., every 6 hours. However, in the cases that had received treatment with corticosteroids during long periods the daily dose was 500 mgm. in the early stages of the experimental treatment.

In all patients a clinical observation of vital signs was made twice a day. Routine laboratory examinations were made of urine, feces, and blood, with determination of urea, glycemia, and transaminase, and repeated every two weeks.

A graphic record was kept for each patient, showing, day by day, all changes in his reactional state. When a regression was plainly evident, the dose of thalidomide was reduced to a maintenance level, which in some cases was as low as 50 mgm. daily without reappearance of the reactional syndrome.

Due to the uniform response to treatment of the patients, both of those which had taken corticosteroids for long periods of time and those who hadn't, a diagram was made of each group in order to show graphically the uniformity of the response.

### RESULTS

The observations justify us in considering the results separately in patients who had never been treated with corticosteroids in comparison with those who had been treated with those drugs for long periods previous to treatment with thalidomide. The initial reaction to the latter was quite different in the two groups.

1. Patients without previous treatment with corticosteroids. In this group the daily administration of 400 mgm. of thalidomide reduced the temperature from its previous febrile condition to normal in the course of 48 hours, and attenuated the reactional syndrome greatly during the same period. The

patients could sleep and eat, and get out of the bed in which they had been lying prostrate before. In the course of four to five days all symptoms of the reactional state had disappeared.

2. Patients treated with corticosteroids for long periods. When treatment with thalidomide was started, corticosteroid treatment was suspended. In these patients there was a marked aggravation of their reactional state during the first 48 hours of thalidomide treatment. Reactions that had been moderate previously would suddenly become intensified with the whole syndrome characteristic of the R3 degree. This, however, would regress in the course of the next seven days, but the patients required from 12-14 days of treatment before all symptoms of the reactional state disappeared. This became the rule in all patients previously treated with corticosteroids.

In contrast with the patients who had never received corticosteroids, this group required a total of 6-7 gms. of thalidomide before a complete remission of the reactional state was attained. As a matter of fact, they received daily doses of 500 mgm. of the drug during the early stage of the treatment.

In Figure 2 we have shown the typical evolution of the reaction of a patient previously treated with corticosteroids. Special mention must be made of three reactional cases. Of these, two were characterized by severe, acute polyneuritis. When thalidomide was administered, the two polyneuritic cases responded with remarkable lessening of the pain after the first 48 hours and complete remission of the reactional syndrome between the fourth and fifth day. By the end of two weeks the enlargement of the ulnar nerves had been reduced considerably. In the case of acute iritis the evolution was similar, with notable improvement after the first 48 hours, and complete

remission of symptoms in four to five days. In all three cases a maintenance dose of 50 mgm. daily was established gradually under complete control after the disappearance of the reactional syndrome.

In the study of laboratory data it was observed that the erythrocyte sedimentation rate was not significantly affected by the treatment. The contrary was the case with the leucocytosis that had been observed in a number of cases. In these the leucocyte count became normal after one to two weeks of treatment.

In the otherwise spectacular affect of thalidomide in counteracting the lepra reaction, there was one exception. It was a case of a lymphopathic reaction with high fever and with extensive, generalized adenopathy, which showed no improvement after two weeks, in spite of the fact that the patient had never been treated with corticosteroids.

#### DISCUSSION

The rapid effect of thalidomide in severe cases of reaction in lepromatous leprosy appears to be sufficient reason for us to give it foremost place among the antireactional drugs that have been used thus far. It appears very much better than the corticosteroids and it does not give the unfavorable side effects of the latter. Its greatest drawback is, naturally, its teratogenic activity, which makes it necessary to hospitalize the patients under treatment, and to keep the women of reproductive age under constant strict control. The possibility of using the drug in ambulatory patients should be restricted to males alone, and would depend on the eventual availability of an injectable "depot" preparation.

There is need for an investigation of the possibility of using thalidomide jointly with sulfone therapy in patients subject to recurrent lepra reactions from DDS.

We have already observed that patients who formerly did not tolerate even a minimal dose of DDS will tolerate up to 200 mgm. daily while receiving thalidomide. The increase in tolerance to DDS was observed even with the maintenance dose of 50 mgm. of thalidomide daily.

Another fact worth mentioning is the remarkable tolerance of patients to thalidomide. The only inconvenience that we have observed so far has been some constipation when the largest doses were given. It ceased when the daily dose was reduced to 200 mgm. In some cases there has been some edema of the distal parts of hands and feet, but that symptom also disappeared when the dose was reduced.

In five of our patients who had been treated continuously for five months we could find no symptoms of toxicity, clinically or by laboratory tests.

In some patients reactional manifestations have reappeared when the daily dose was lowered, but here again the syndrome disappeared spontaneously.

When treatment was discontinued after several months, some patients suffered a relapse, with symptoms as severe as those of their initial reaction, but they responded promptly to renewed treatment with thalidomide.

In view of the facts that thalidomide is split up into a dozen metabolites in the human organism (1, 2), and that some of these are teratogenic while others are not, it would be interesting to investigate the possibility that some of the non-teratogenic metabolites might have antireactional activity in leprosy.

There is need for an explanation of the longer time necessary for thalidomide to take effect in patients previously treated with corticosteroids, as compared with untreated reactional cases. Perhaps the corticosteroids suppress the activity of the adrenal cortex temporarily in one or more of its phases, and a certain time-lapse may be necessary before that activity is resumed and returns to normal.

SUMMARY

In a previous investigation by the double-blind method it was shown that thalidomide is highly effective in suppressing the lepra reaction. We have now used the drug as a regular therapeutic agent in the treatment of 24 lepromatous patients in reactional states of various degrees, although 70 per cent of them were cases of long duration in whom sulfone treatment had been suspended to prevent further aggravation of the reaction.

The administration of thalidomide in doses of 400 mgm. daily to patients who had not been treated previously with corticosteroids, restored body temperatures to normal within 48 hours and brought about a complete remission of the reactional syndrome in four to five days.

The time necessary for the drug to bring the reaction under control was much longer when it was administered to patients who had been treated with corticosteroids for long periods. When that treatment was suspended with the commencement of thalidomide therapy, there was a recrudescence of the reactional state and the treatment had to be continued with doses of up to 500 mgm. daily for two weeks before the symptoms subsided and disappeared.

Cases of acute polyneuritis incident to the reactional state were also controlled rapidly and completely under treatment with thalidomide, and the same was observed in a case of reactional iritis.

After the disappearance of the reactional syndrome the daily doses of the drug were reduced gradually to a maintenance dose of 50 mgm. The administration of the drug, even on a maintenance level, enabled resumption of DDS treatment in cases that were formerly intolerant to sulfones. To all appearances the problem of antileprosy therapy in cases subject to frequent reactions had been solved.

The secondary effects of the drug were slight. At the higher doses there

was some constipation which ceased when the dose was reduced to 200 mgm. daily. Under prolonged treatment edema of the distal extremities was also observed as a temporary side effect.

In view of the teratogenic activity of thalidomide patients should be hospitalized under strict control.

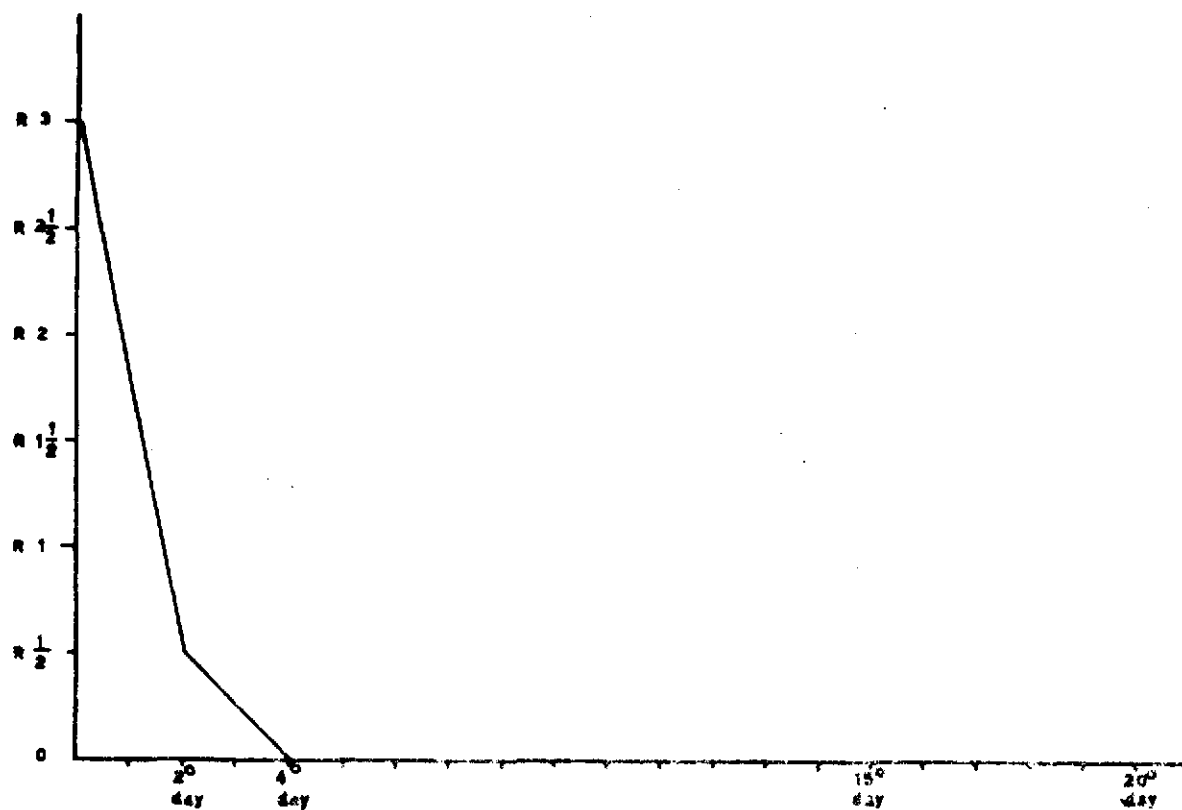
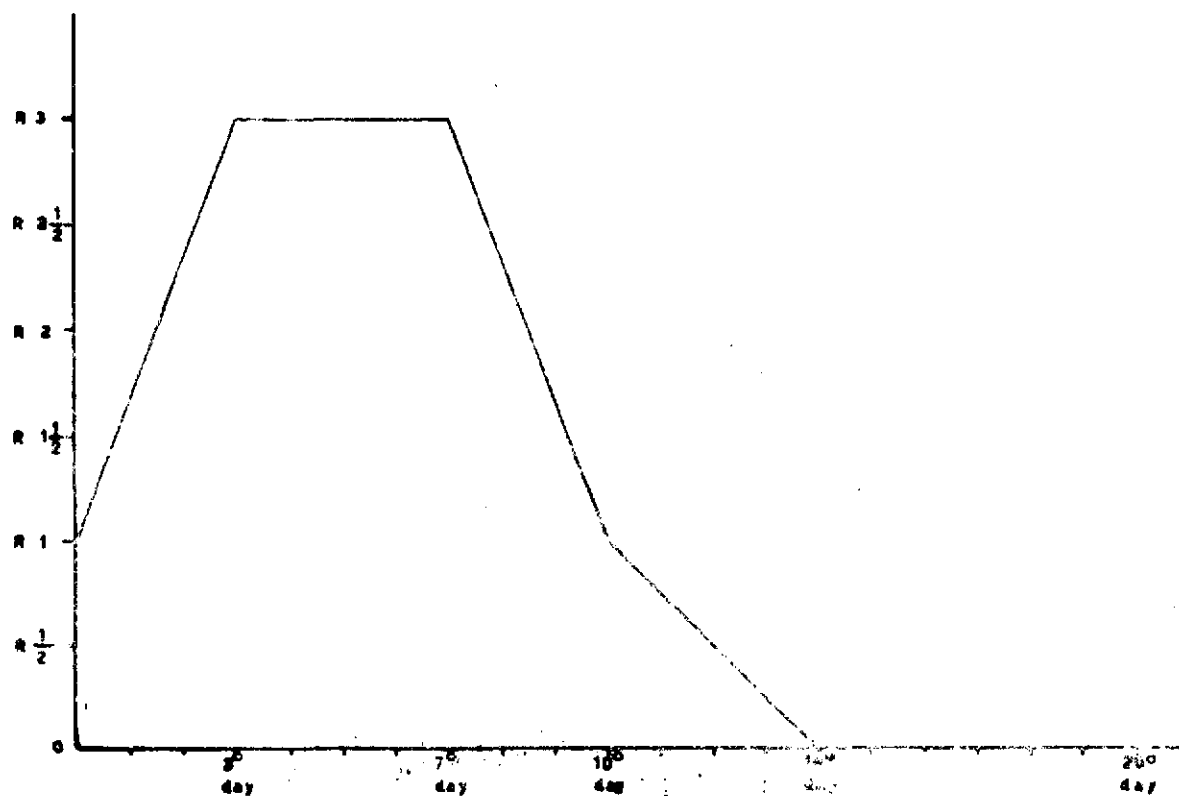
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Table 1: Treatment with thalidomide. Lepromatous patients in the reactional state

Patient	Age	Sex	Intensity	Reaction of long duration	T r e a t m e n t	
					Previous steroid	Duration with thali domide months
J. R.	17	M	R3	yes	yes	1
M. M.	30	F	R3	no	yes	5
A. M.	55	M	R3	yes	yes	3.5
A. B.	32	M	R3	yes	yes	8
J. C.	43	M	R2.5	no	no	8
M. G.	36	F	R3	yes	yes	7.5
J. T.	43	F	R1	no	no	7.5
R. V.	57	M	R2.5	no	no	2
G. A.	33	M	R2	yes	yes	4
F. C.	23	M	R2.5	yes	yes	4
J. A.	46	M	R3	yes	yes	1.5
J. U.	45	M	R2.5	yes	yes	1.5
J. R.	30	M	R2.5	no	yes	3.5
D. CH.	62	M	R2	no	no	3.5
M. CH.	40	F	R3	yes	yes	2.5
E. R.	39	M	R3	yes	no	3.5
U. M.	18	F	R2.5	yes	yes	3
M. G.	30	F	R3	yes	yes	2
J. M.	52	M	R3	yes	yes	1
D. F.	47	M	R3	yes	yes	1
H. P.	53	F	R2	no	yes	1
R. P.	21	M	R2.5	yes	yes	2.5
M. O.	33	F	R1	no	no	1
J. B.	40	M	R3	yes	yes	1



NO 1. PATIENT WITHOUT PREVIOUS TREATMENT WITH CORTICOSTEROIDSNO 2. PATIENT WITH PREVIOUS TREATMENT WITH CORTICOSTEROIDS

THE EFFECT OF FRESH SERUM ON THE LEPTOMONADS OF LEISHMANIA

1. Preliminary Report

Marian Ulrich, Ph. D.,  
Dinorah Trujillo and  
Jacinto Convit, M.D.

Introduction.

The number of reports concerning the effect of normal serum against Leishmania in the leptomonad form is limited. In 1940, Adler reported the lytic effect of human serum against leptomonads of Leishmania donovani. In 1956, Taub extended this observation to a study of the activity of normal human serum against L. infantum and L. tropica, concluding that the absence of the lytic factor against L. infantum in the sera of young children might play a role in their susceptibility to infection with this parasite. In 1963, Lainson and Strangways-Dixon observed a similar lytic effect with L. mexicana, again using human serum.

In the present study, we have examined the fresh sera of a number of laboratory animals for the presence or absence of a factor causing rapid immobilization of leptomonads and the assumption of bizarre, rounded forms. Leptomonads of five species of Leishmania have been examined for susceptibility to this immobilizing factor. Preliminary studies were made to characterize the factor in terms of resistance to heat, dependence upon the presence of complement, and adsorbability with leptomonads.

Materials and Methods.

Cultures of leptomonads:

Leishmania brasiliensis, L. brasiliensis pifanoi, L. donovani, L. tropica, and L. enriettii.

Culture medium:

## Base-

Nutrient agar	23 g.
NaCl	6 g.
Glucose	10 g.
Distilled water	1.000 ml.

## Overlay-

NaCl	8.5 g.
Glucose	10 g.
Distilled water	1.000 ml.

The base was sterilized for 20 minutes at 121°C., then cooled to about 45°C. Defibrinated rabbit blood, which contained 2000 units of penicillin and 2 mg. of streptomycin per ml., was added in a concentration of 10 per cent. The medium was distributed in 6 ml. amounts in sterile screw-capped tubes, which were slanted until the medium solidified; then 4 ml. of the sterilized overlay (ten minutes at 121°C.) was added to each tube. The tubes were incubated at 37°C. for 24 hours to check for sterility, then refrigerated until use. Just before inoculation, a drop of penicillin (about 5000 units) was added to each tube.

## Test for immobilization:

Tubes of the medium described above were seeded with large inocula of leptomonads, so that growth was abundant in 3 or 4 days (at least 10,000 leptomonads per mm<sup>3</sup> of medium). One-tenth ml. of the serum to be tested was added to 0.5 ml. of the culture medium containing leptomonads. After 15 and 60 minutes at room temperature, drops of the mixture were placed on slides, covered with cover slips, and examined with the light microscope, using the 40 x lens. The percentage of immobile, rounded leptomonads was carefully estimated, viewing a large number of microscopic fields.

## Collection of serum:

Samples of blood were allowed to clot about 15 minutes at room temperature and then centrifuged. The serum was used immediately frozen for use within 48 hours. The lability of the immobilizing factor at room tempera-

ture and in the freezer (-20°C.) has not been studied, but the assumption was made for these studies that the time between removal of the blood and the use of the serum (or storage in the freezer) should be as brief as was reasonably practicable. Sera included in this report were taken from adult rabbits, rats, guinea pigs, hamsters, mice and gerbils.

### Results.

The effects of the sera studied are reported in terms of immobilization and the loss of all flagellar movement, since this was the first and most obvious result seen when leptomonads were treated with active sera. This was accompanied by rounding of the leptomonads, but complete lysis during the one-hour period was not extensive.

Table 1 presents the results when 0.1 ml. of fresh serum from various adult laboratory animals was added to cultures of L. brasiliensis and L. brasiliensis pifanoi. These animals fall into three groups on the basis of the presence or absence of the immobilizing factor in their sera. Sera from rabbits, guinea pigs, and rats all immobilize the two strains of leptomonads used. Sera from mice and gerbils are essentially non-reactive in this test. The figures given in the table for these animals are somewhat misleading, since we never saw more than 10 per cent of inactive forms with any of these sera. The most irregular results occurred with hamster sera, which showed broad variation from highly active to non-reactive. Cultures of leptomonads that were more than a week old were slightly more resistant to the effect of reactive sera than younger cultures and already contained a number of inactive leptomonads, so they were not used for subsequent studies.

When sera from several of these species of laboratory animals were tested with nine other strains of Leishmania which are maintained in this

laboratory, the results shown in Table 2 were obtained. It is apparent that the immobilizing factor is not of particularly limited specificity, but rather manifests itself against a number of species of Leishmania.

To amplify this point, fresh rabbit serum was absorbed twice with washed leptomonads of L. brasiliensis pifanoi, during four hours and then overnight in the refrigerator. The absorbed serum was then tested for immobilizing activity against the 11 strains of leishmaniae; activity against all 11 strains was completely eliminated by the absorption. A control serum subjected to the same manipulations but without adding leptomonads was not inactivated by this procedure.

A number of experiments were performed to determine if the immobilizing factor was a naturally-occurring antibody, an antibody-complement system analogous to the bactericidal system active against many Gram-negative bacilli, or some other substance in the sera of certain species of animals. These experiments are presented in Table 3.

Reactive sera became inactive after absorption with leptomonads or heating at 56°C. for 30 minutes. The heat-inactivated sera were inactive when combined with naturally inactive sera, but the absorbed sera gave an active combination. Either absorption or treatment with heat of the sera of mice and gerbils eliminated their capacity to form an active combination with absorbed reactive sera. These data indicate that two factors are required for immobilization; that both are heat labile; one is specifically adsorbed by leptomonads and the other is not. Sera from gerbils and mice are inactive because of the absence (or presence in very small amounts) of the nonabsorbable factor.

The results of treatment of guinea pig serum for 30 minutes at various temperatures are shown in Table 4. At temperatures of 50°C. or below, inactivation was incomplete in 30 minutes; at 55°C. or higher, agglutination

was often pronounced but all flagellar movement was apparently normal and most of the parasites maintained their normal elongated form. As standard procedure for inactivation by heat, we used treatment at 56°C. for 30 minutes.

The possibility that mouse and gerbil sera contained an inhibitor, capable of inactivating active serum from another species, was studied by mixing equal amounts of one or the other of these sera with reactive sera from guinea pigs and rabbits. These mixtures were as active as the guinea pig or rabbit sera alone.

We considered the possibility that the culture medium itself, which contains rabbit blood, might provide certain factors which interact with the various sera added. This was at least partially eliminated by the preparation of media in which mouse blood was substituted for rabbit blood. The reactions using cultures of leptomonads grown in the medium prepared with mouse blood were essentially identical to those obtained with the original medium. Washed leptomonads were also used in a few experiments and reacted as did the original cultures.

Two experiments were performed which we hoped might be indirectly indicative of the role of this system in natural infections.

The immobilization test was performed at 37°C., using cultures of L. brasiliensis and L. brasiliensis pifanoi and fresh guinea pig serum. At this temperature, immobilization of the leptomonads in control tubes without serum was extensive within one hour, but rather limited during the first 30 minutes. Cultures mixed with fresh guinea pig serum were inactivated in less than five minutes at 37°C.

We also tested a serum from a patient with diffuse cutaneous leishmaniasis for immobilizing factor against both L. brasiliensis and L. brasiliensis pifanoi, since the immunological response of these patients is

abnormal both with regard to their failure to limit the infection and in their relative anergy to the leishmanin test. The serum of this patient was as active against these strains of leishmaniae as normal human serum.

### Discussion.

Earlier reports have referred to the presence in serum from normal persons of a factor which causes immobilization and lysis of several species of Leishmania, but it has not been characterized nor reported in the sera of laboratory animals. In the present study, we have found what is presumably the same factor (though rapid lysis is not a characteristic of the system studied here) in the fresh sera of a number of laboratory animals, and its apparent absence or presence in very small amounts in the sera of gerbils and mice. Addition of mouse serum in concentrations as high as 50 per cent did not cause immobilization of cultures of leptomonads, while sera from guinea pigs were active at a concentration of 9 per cent.

The observations reported here indicate that at least two heat-labile factors are responsible for immobilization of leptomonads. One component is removed by adsorption of sera with leptomonads; the other factor remains active in the serum after absorption. The absence of the latter factor in the sera of mice and gerbils apparently explains their inactivity. This system seems analogous to the bactericidal system, involving complement and antibody, but it has not been conclusively demonstrated that the non-absorbable factor is indeed one or more of the components of complement. Sera from mice are devoid of bactericidal activity because of a defective complement system, but serum from gerbils contains measurable bactericidal activity.

What role this system may play in natural infections is unknown. As Lainson and Strangways-Dixon have pointed out, infections may be esta-

blished in spite of the presence of the lytic factor in human serum because the leptomonads penetrate host cells rapidly after inoculation, where they are protected from damage. Possibly the leptomonads inoculated by insect bites are much less susceptible than forms from culture media to this factor, which would in part explain the observation that relatively enormous numbers of culture forms are often necessary to initiate infections.

The absence of the immobilizing factor in the sera of mice and gerbils may have implications concerning the role of small rodents as natural reservoirs of Leishmania. High temperature and other factors undoubtedly inhibit free circulation of leptomonads in these animals, but the absence of an inhibitory factor in the serum may provide a somewhat less hostile environment for their initial infection and subsequent development than in animals which contain the immobilizing factor.

#### Summary.

Fresh sera from normal adult rabbits, guinea pigs, and rats contain a factor which causes rapid flagellar immobilization and rounding of the leptomonad forms of Leishmania. The factor is absent or present in very small amounts in the sera of mice and gerbils, and variably absent or present in the sera of hamsters.

The factor was found to be active against all of the species and strains of leishmaniae tested: L. brasiliensis, L. brasiliensis pifanoi, L. donovani, L. tropica, and L. enriettii. Absorption with L. brasiliensis pifanoi removed the activity against all the other leptomonads tested.

Preliminary experiments indicate that two heat-labile components are required for the immobilization of leptomonads; one is removed by absorption with leptomonads and the other is unaffected by this procedure. By



analogy with other immunological systems, these factors may be naturally-occurring antibody and one or more components of the complement system. The non-reactive sera from gerbils and mice do not contain the non-absorbable factor.

#### Acknowledgments.

This work was in part supported by Grant N°. 5R01. AIO4216 from the Public Health Service, National Institutes of Health, USA, made to the Venezuelan Association for Dermatologic Investigation.

The technical assistance of Mrs. Brunilda de Salas is gratefully acknowledged.

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Note: Added after submission of the paper:

Our attention has been drawn to a paper published by M.S. Ben Rachid (Archives de l'Institut Pasteur de Tunis 44, 155-161, 1967) concerning primarily the lytic effect of normal human serum against L. infantum. The author mentions the activity of serum against various species of leishmaniae and trypanosomes, and has examined the sera of laboratory animals for lytic activity. All sera tested, including those of gerbils and mice, were lytic. The activity is attributed to a non-specific, properdin-like factor which is complement-dependent. Somewhat different proportions of serum and culture were used by Dr. Ben Rachid, but this alone does not explain the reported lytic effect with serum from mice, since we observed no such effect, nor immobilization, when we repeated an experiment using those proportions. As mentioned previously, lysis was a rare occurrence

in the experiments reported by us; indeed, rounded, ~~imm~~obile parasites regain their normal morphology and capacity to reproduce when inoculated into fresh medium. Such factors as age of the culture, medium, proportions of reactants used, and species differences may all be important in interpreting these results, and will be considered in more detail in a future publication.-

TABLE 1

IMMOBILIZATION OF LEPTOMONADS WITH FRESH SERUM OF  
LABORATORY ANIMALS

Animal	Number of sera tested and percentage of immobilization					
	L. brasiliensis			L. Brasiliensis pifanoi		
	> 95%	5-95%	< 5%	> 95%	5-95%	< 5%
Guinea pigs	11	2		11		
Rabbits	9			10		
Rats	7			7		
Hamsters	14	12	5	30	3	3
Mice			13		1	16
Gerbils		2	10		3	10

TABLE 2

ACTIVITY OF SERA FROM LABORATORY ANIMALS AGAINST  
VARIOUS SPECIES OF LEISHMANIA

Strain of <u>Leishmania</u>	S e r u m			
	Rabbit	Mouse	Gerbil	Guinea Pig
<u>L. donovani</u> , LE	Pos	Neg	Neg	Pos
<u>L. donovani</u> , M	Pos	Neg	Neg	Pos
<u>L. donovani</u> , Sc	Pos	Neg	Neg	Pos
<u>L. tropica</u>	Pos	Neg	Neg	Pos
<u>L. enrietti</u>	Pos	Neg	Neg	Pos
<u>L. brasiliensis</u> , Br	Pos	Neg	Neg	Pos
<u>L. brasiliensis</u> , I	Pos	Neg	Neg	Pos
<u>L. brasiliensis</u> , II	Pos	Neg	Neg	Pos
<u>L. brasiliensis</u> , III	Pos	Neg	Neg	Pos

TABLE 3

REACTIVITY OF NEGATIVE SERA WITH ABSORBED AND INACTIVATED  
COMPONENTS OF REACTIVE SERA

Combinations of factors tested <sup>a</sup>	R e s u l t
1. Heat-inactivated rabbit or guinea pig serum Fresh gerbil or mouse serum	No immobilization
2. Absorbed rabbit serum Fresh gerbil or mouse serum	Immobilization
3. Absorbed rabbit serum Absorbed gerbil or mouse serum	No immobilization
4. Absorbed rabbit serum Heat-inactivated gerbil or mouse serum	No immobilization

<sup>a</sup>These factors are all inactive against leptomonads when tested individually.

TABLE 4

INACTIVATION OF THE IMMOBILIZING ACTIVITY OF NORMAL  
GUINEA PIG SERUM BY HEAT

Temperature °C.	Degree of immobilization
40	100%
45	95%
50	15%
55	0%
60	0%
65	0%

THE METHYLENE BLUE TEST

1. In Murine Leprosy and in Lesions Induced in Hamsters After Inoculation with Materials from Cases of Borderline and Lepromatous Leprosy 1/, 2/

José Luis Avila and Jacinto Convit 3/

It has been known for many years that the lesions of lepromatous leprosy patients retain methylene blue after injection of a 1 per cent solution of the dye. In 1956 Convit et al. (6) found that patients with the borderline type of leprosy retained the blue pigment in the lepromatous parts of their lesions, but not in those with a tuberculoid structure (4). Later Convit and Góihman (3) found that the methylene blue test was also positive in xanthomatosis, while skin diseases such as deep mycoses, leishmaniasis and trepanomatosis gave negative results.

Apart from the foregoing observations Convit et al. (5) succeeded in 1962 in producing granulomas in hamsters at the site of the inoculation of material from human borderline lesions. The hamster granulomas contained an abundance of acid-fast bacilli, in sharp contrast with the human lesions, but the pathogen resisted all attempts at cultivation in media used currently for mycobacteriaceae. It was likewise found possible to produce granulomas in hamsters by inoculating bacilli from lepromatous human lesions, but the bacilli that developed in the hamster from such precursors were found to be cultivable on numerous occasions (5).

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1/ Received for publication 24 March 1967.

2/ This investigation was supported in part by grants received from the U.S. National Institutes of Health, Grant Number AI 04216-05

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It has seemed important to us to obtain data on the behavior of leprotic lesions in animals in the presence of methylene blue. For this purpose we have carried out experiments with laboratory animals inoculated with bacilli from borderline as well as from lepromatous human lesions and also with animals inoculated with murine leprosy bacilli. The experiments with the latter were made in the laboratories of the Department of Dermatology of the Vargas Hospital in Caracas and those with the former took place in the Laboratory of Experimental Bacteriology of the Division of Sanitary Dermatology of the Ministry of Health, also in Caracas.

#### MATERIALS AND METHODS

The animals used in this study were the following:

1. Mice inoculated with Mycobacterium lepraemurium of a strain furnished us by Dr. Y.T. Chang of the National Institutes of Health, Bethesda, Maryland, U.S.A., from peritoneal mouse lesions of six months' development.
2. Uninoculated and apparently healthy control mice.
3. Rats with lesions of six months' development from inoculations with a strain of M. lepraemurium furnished us many years ago by the late Dr. J.D. Aronson of the Henry Phipps Institute of the University of Pennsylvania. These rats showed either ulcerating skin lesions about 1 cm. in diameter or nodules about 0.3 cm. in diameter.
4. Uninoculated and apparently healthy control rats.
5. Golden hamsters (Cricetus auratus) inoculated intradermally on the back of their ears with bacilli of human borderline leprosy origin, obtained from lesions produced in hamster to hamster passages.
6. Golden and albino hamsters inoculated behind their ears with bacilli of human lepromatous origin, obtained from lesions produced in hamster to hamster passages. The experimental lesions on the ears of the hamsters inoculated with bacilli of borderline origin, as well as those from lepromatous precursors, were of six months'



development, as in the case of the lesions in mice and rats from M. lepraemurium.

7. Uninoculated and apparently healthy hamsters, forming a control group.

The doses of methylene blue used and the manner of administering the dye are explained in Table 1. At the end of all the experiments the animals were killed and autopsied immediately. The autopsy included a careful examination of all lesions, whether reatrouicular, peritoneal, or visceral. In all cases the lesions were stained for acid- and alcohol-fast bacilli by the Ziehl-Neelsen method and examined microscopically.

### RESULTS

In the animals inoculated with bacilli of borderline or lepromatous origin, and in those inoculated with M. lepraemurium, the dermal and peritoneal lesions retained the methylene blue with an intensity proportional to the dose administered (Figs. 1 and 2.) The blue color became visible as soon as the peritoneal lesions were opened or the nodules of the ears were sliced. It was intensified as the tissues were exposed to the atmosphere.

A generalized, although somewhat faint blue color became visible in the viscera of the animals in the course of their exposure to the air. This color was presumably due to prior formation of the leucoderivative in all the tissues and subsequently reconversion by atmospheric oxidation into the original blue compound.

In the mice inoculated with murine leprosy it was found, as soon as the abdominal cavity was opened, that many lymphoid structures of the omentum were intensely blue. The presence of acid-alcohol-fast bacilli in these structures, and their absence in lymph glands that did not retain the dye, proved their infiltration from peritoneal lesions.

In the autopsies made of the control animals no visceral tissues were found to be stained blue, but the presence of the leucoderivative was indicated by the blue tinge they assumed after being exposed to the atmosphere. As the animals had been killed a few

hours after the administration of the last dose of the dye, evidently they had not had time to eliminate the leucoderivative formed.

In the cutaneous lesions of the animals inoculated with murine leprosy, as well as in those produced by inoculation with bacilli of lepromatous or borderline origin, the autopsies revealed only a faint blue tinge in spite of repeated doses of the dye and the presence of acid- and alcohol-fast bacilli.

### DISCUSSION

It is evident from the results of our experiments that the lesions that developed in the hamster after inoculation with bacilli of lepromatous or borderline origin, fix methylene blue, as do also the lesions of murine leprosy.

In previous experiments (4, 6) it was shown that the only skin lesions of human patients so far known that reacted positively in the methylene blue test were those of lepromatous and borderline leprosy (in the latter the lepromatous components only) and xanthomas (3). These three diseases have one histopathologic feature in common, viz., the presence of "foamy cells" characterized by a high lipid content (8).

On the other hand, the experimental lesions produced in laboratory animals by inoculation with lepromatous, borderline (5), or murine leprosy material (9), also show a "foamy" structure. The fact that such structures are found in all leprotic lesions that give a positive methylene blue test gives much strength to the hypothesis that the lipids characteristic of the "foamy" cells are somehow involved in the retention of the dye. At the same time, the fact that direct and prolonged exposure to the atmospheric oxygen produces a blue coloration in the viscera of healthy animals injected with methylene blue, might well indicate that all tissues are capable of retaining the dye and transforming it into its leucoderivative. Once so transformed, the leucoderivative might be temporarily combined with a lipid, but on exposure to air it is partly oxidized with regeneration of the blue color.

In human lepromatous and borderline leprosy and in the lesions produced in the hamster by their pathogens, as well as in murine le-

prosy and in xanthomatosis it is possible that:

1. there is a decrease in the activity of an oxidation-reduction system of the lepra cell, which prevents the reduction of the dye to its leucoderivative, and/or

2. the leucoderivative is irreversibly reoxidized to the blue form and chemically or colloiddally combined with a cytoplasmic lipid.

There is an interesting observation that may have to do with the phenomenon of methylene blue retention. When tissue of laboratory animals that had been infected with M. lepraemurium and subsequently injected with the dye is homogenized in normal saline at 2°C the pigment is not dissolved in that medium, in which ordinarily it is highly soluble. This would indicate that it is firmly bound to some cytoplasmic substance present in infected but absent in healthy tissue.

The increase in intensity of the blue tinge on exposure to air would indicate the presence, jointly with the dye, of a leucoderivative labile to photooxidation with or without the intervention of a dehydrogenating enzyme.

The negativity of the methylene blue test in tuberculoid and indeterminate leprosy, as well as in deep mycoses, leishmaniasis and trypanosomiasis, together with the weak positivity in animals with murine leprosy, may well indicate that one of the two factors that have been postulated as responsible for the retention of the dye is lacking in those lesions. In another investigation (2) we have found that there are lipids in normal tissues capable of fixing it strongly. We may suppose that wherever the dye is retained in the tissues there is a great modification of the oxidation-reduction activities in favor of oxidation. If that is so, it would explain the retention of the dye in lepromatous and borderline leprosy, although not necessarily its chemical or colloidal fixation to a lipid factor. Nevertheless, further investigations may reveal that the phenomenon of fixation is also connected with an aberrant oxidation process, although the exact aberration of the enzymatic functions may have to await determination. We can say with certainty, however, that le-

promatous leprosy, as well as rat leprosy, and xanthomatosis also, have in common an enzymatic aberration that is intimately connected with the positive reaction in the methylene blue test.

Studies are under way to determine the affinity of various lipids for methylene blue.

#### SUMMARY

The methylene blue test has been studied in laboratory animals with lesions produced experimentally by inoculation with material - derived originally from lepromatous and borderline leprosy. A similar study has been made in rats with cutaneous lesions from M. lepraemurium and in mice with visceral lesions from the same pathogen. In all cases the study was made in comparison with control groups.

In all the inoculated animals a blue coloration appeared in the infiltrated tissue, but in the control groups no such retention of dye could be observed. The intensity of the blue tinge was proportional to the doses of dye injected.

The cutaneous lesions of murine leprosy (ulcers and nodules) gave weaker positive reactions than peritoneal lesions of the same origin or dermal lesions resulting from the experimental inoculation of leprotic material of human origin.

It is suggested that one or both of two aberrant functions may be instrumental in bringing about the positive methylene blue test, viz., (a) an aberration in the oxidation-reduction function favoring oxidation and inhibiting reduction, and/or (b) a combination of the dye with a lipid peculiar to lepromatous leprosy and xanthomatosis.

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FIG. 1. Left side: nodule in hamster inoculated with material from borderline leprosy.

Right side: nodule similarly sectioned after repeated injection of hamster with methylene blue, showing localization of the dye.

FIG. 2. Localization of methylene blue in the lepromatous mass in the peritoneal cavity of a mouse infected with M. lepraemurium, after injection of the dye.-

Table 1. Doses, routes of administration and results of methylene blue test in animals with experimental leprotic lesions and with murine leprosy.

Type of disease in source material	Animals	Route	Dose in mgm./100 gm. body weight	Type of lesion	Results
Lepromatous	Hamster	Intraperitoneal	20 40	Dermal	0 ++++
Borderline	Hamster	"	20 30 50	"	0 ++ +++
Murine Leprosy	Rat	"	25 50 75	"	0 0 +
Murine Leprosy	Mouse	Subcutaneous Intravenous	0 50	Visceral	0 ++ +++

SOME IDEAS IN RELATION TO A PROJECT FOR STUDYING SUSCEPTIBILITY TO LEPROSY:  
BASIS OF THE TRANSFORMATION OF THE INFECTION INTO DISEASE, AND  
POSSIBLE WAYS OF PREVENTING SAID TRANSFORMATION

This proposed research project covers the following points:

- I. An epidemiological survey of hiperendemic focci of leprosy, such as Apure State and the Island of Guara. This survey would consist of clinical examination and bacteriological investigation for mycobacteria, of each of the persons living within those areas; approximately 10,000 persons per year.
- II. An immunological study, by the Mitsuda test and determination of circulating antibodies, of persons found to be bacteriologically positive, even while not showing clinical manifestations of the disease.

An experimental study, in laboratory animals, of the possibility of transferring resistance to leprosy; a study of this same possibility in human beings, using the early reaction to lepromin (the Fernandez test) and the late Mitsuda reaction as means of evaluation.

The utilization of drugs and chemical substances which develop cellular immunological responses, as a means of stopping the leprosy infection from developing into the disease.

- III. A cytochemical study of the persons who have been classified as susceptible, as a means of determining possible enzymatic aberrations.
- IV. A biochemical study of those individuals, with the same purpose in mind.

PROPOSAL:

In the animal world, in general the individual members show adequate responses to the millions of antigens with which they come in contact during their evolution.

Referring to *Mycobacterium leprae* as the antigen and to man as the only organism capable of developing leprosy, it is observed that this disease manifests itself in a gamout of diverse responses from the serious extreme of progressive lepromatous infection to the benign tuberculoid form which may undergo spontaneous involution. Between these two extremes there is a multitude



of forms of reaction, from the indeterminate form to the borderline responses with their clinical polymorphism, indicative of diversified immunological responses.

In the Eighth International Congress of Leprology, convened in Rio de Janeiro in 1963, the Immunology Committee considered that the individuals in any population exposed to infection with *M. leprae* fall into one of the three following categories:

1) Group of normal reactors, capable of response, who are or would be resistant to the infection. 2) Slow reactors, susceptible to the development of non-lepromatous forms of the disease. 3) Poor reactors, in a small minority of whom the disease is persistent and progressive, resulting in the malignant lepromatous form of infection.

Our working hypothesis will be that leprosy infection would be transformed into a serious disease in those persons who, having a lysosomal enzymatic defect would be incapable of destroying *M. leprae* or who, having an immunological incompetence of their reticulo-endothelial system, are incapable of manufacturing transfer factor. This possibility is suggested by the fact of their not showing delayed hypersensitivity, a reaction that is being studied as an immune response associated with a heightened resistance to intracellular microbial infections. These persons would belong to the group of "poor reactors" already mentioned.

The study of these possibilities could be made in the lepromatous patient, but this would have the disadvantage that one might argue that the mentioned defects might be a consequence of the disease, caused by the great modifications that the macrophages undergo in lepromatous leprosy, and not a defect pre-existing to the disease.

For this reason, we think the study should be done in apparently healthy individuals who live in a focus of leprosy.

During the last year, we have made a series of epidemiological studies in hyper-endemic zones in the state of Apure, a zone of plains in the southern part of Venezuela that is connected with Caracas by a good 250-mile highway. In two leprosy foci in Apure, we have made a thorough census in a search for cases of leprosy. When the entire population had been examined (one of 250 inhabitants), the apparently healthy persons (i.e., those without clinical signs of leprosy) were submitted to a bacteriological study. From each person, six slides were prepared from material taken from the ear lobes, elbows, and knees. The slides were stained by the method of Ziehl-Nielsen and examined carefully for acid-alcohol resistant bacilli. As a result of these examinations, acid-alcohol resistant bacilli were found in the slides of seventeen (17) persons; some with as many as 14 bacilli in a single preparation. Some had one the preparations positive; others two, three, or more positive preparations (of the six slides taken).

These persons, who continued living in the mentioned foci of leprosy, were observed periodically. In the last examination, performed in May, 1967, three of these individuals had clinical manifestations of very recent appearance of the disease. One has the tuberculoid form, the other has a lepromatous macule with numerous bacilli, the third was an indeterminate leprosy case.

These facts led us to believe that these persons were infected with leprosy bacilli, the disease being in incubation in some of them. Afterward we gave the Mitsuda test to this group, classifying the individuals as Mitsuda negative or positive. Since the majority of the group are adults, it seems to us that those who gave a negative lepromin reaction are those who in the future

may present lepromatous leprosy.

The proposed investigation consists of the study of this last group, comparing it with those persons who had acid-alcohol resistant bacilli in the skin but gave a positive Mitsuda reaction, and with completely normal Mitsuda-positive persons.

This bacteriological examination would be extended, during a five-year period, to 50,000 persons living in the leprogenic areas of Apure and Island of Guara. A biopsy would be performed on a certain number of the people with mycobacteria in their skin, for histopathological purposes and for inoculating in media for mycobacteria.

We propose the hospitalization of small groups of the three mentioned categories in the Hospital Vargas, where the Service of Dermatology has an adequate number of beds available for investigation.

As we have said, the investigation will be realized from the epidemiological, immunological, histochemical, and biochemical points of view.

Epidemiologically, each case will be carefully studied to see what relation exists with infective cases of leprosy. A more complete bacteriological study will be done, in order to have an idea of the size of the population of acid-alcohol resistant bacilli that these persons harbor. Each person will be minutely examined from the clinical point of view, in order to discard the possibility that he is already overtly infected, with very discrete manifestations, difficult to observe.

In the immunological aspect, as we have said, each person will be classified with regards to negativity or positivity, and degree of the latter, in relation to the skin test made with the standard Mitsuda (lepromin) antigen ( $160 \times 10^6$  bacilli per cc). We will also do a study of the bacillar digestion capacity

of their macrophages, comparing its results with the data obtained with the Mitsuda test.

Efforts will be made to induce a positive Mitsuda reaction in that group of carriers with negative reactions by injections of lepromin or other mycobacterial preparations at regular intervals.

Serum antibody levels against mycobacterial antigens will be studied by the method of Ouchterlony and other more sensitive techniques in the three groups of patients.

The following histochemical investigations will be done:

I Enzymes

1) Acid phosphatase

a) Sodium beta glycerophosphate

2) Beta glucuronidase

a) 6-bromo-2-naphthyl-B.D. glucuronide

3) Esterases

a) Alpha naphthyl acetate

b) Chlorobromo-indoxyl-acetate

c) Naphthol AS-D chloroacetate

4) Cholinesterase

a) Iodated acetylthiocholine

5) Lipases

a) Naphthol AS-nonanoate

6) Proteases

a) Aminopeptidases

(L-leucine-methoxy-2-naphthylamide)

7) Respiratory enzymes

a) Adenosine triphosphotase (A.T.P.)

b) Diaphorases NADH and NATH (malic acid and glucose-6-phosphate)

- c) Ubiquinones or coenzyme
- d) Lactic and succinic dehydrogenase
- e) Cytochrome oxidase

## II Electron Microscope studies

- 1) In normal control skin
- 2) In the skin of healthy carriers, Mitsuda positive and negative.

### Enzymes

- 1) Acid phosphatase (Gomori)
- 2) ATP-ases (Essner, Novikoff)
- 3) Cholinesterases (Lehrer and Ornstein).

These histochemical features will be studied not only in skin biopsies, but also in material taken from induced foreign-body reactions.

In the biochemical aspect the cells will be macerated and the lysosomes separated from the homogenate by methods of subcellular fractionation. The physical characteristics of these particles which control their activity in a centrifugal field will be measured; i.e. size, density, and osmotic activity. The enzymatic content of purified lysosomes will be determined, as well as their identity by electron microscopy, using the method of Gomori for acid phosphatase as well as techniques for identification of esterases and various glucosidases.

Parallel to these investigations, we will study the following aspects.

An experimental investigation consisting of sensitizing guinea-pigs with *M. leprae*, injecting their transfer factor to normal recipients and testing these recipients with killed *M. leprae* as antigen, to see whether the 48-hour Fernandez reaction develops.

The use chemical substances such as DDS, whose mechanism of action might be of enzymatic interference, to treat preventively, persons with signs of

infection. This group would be compared with another, statistically balanced, group of people in the same conditions of infection, but untreated.

The administration to infected persons of substances which, as proposed by MacKays, unchain favorable immunological responses in cells of the reticulo-endothelial system.

The injection of transfer factor obtained from tuberculoid patients and normal Mitsuda positive people to Mitsuda negative people, to see whether the positive reaction can be induced in them.

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BEHAVIOUR OF PATIENTS WITH LEPROMATOUS LEPROSY AND DIFFUSE  
CUTANEOUS LEISHMANIASIS IN RELATION TO SKIN HOMOGRAFTS

Lepromatous leprosy and diffuse cutaneous leishmaniasis are two diseases characterized by an incapacity of the host to respond to the infecting agent, manifested by the suppression of delayed hypersensitivity responses to this same agent. There also seems to be a general depression of this type of responses to other agents and, in the case of lepromatous leprosy, apparently there is an anergy to normal skin used as antigen. In view of this, we chose these two diseases as models to study the behaviour of skin tissue in relation to homografts, from normal donors or from patients with similar complaints.

In these diseases, the disturbance of the immunological mechanism associated with delayed hypersensitivity responses could be explained from two different view points. One of them considers the disturbance as being pre-existent in the host and specific, to M. leprae in people who develop lepromatous leprosy, and to L. brasiliensis in people who develop diffuse cutaneous leishmaniasis. Once the pathological state is established, since both are systemic diseases, with an important invasion of the reticulo-endothelial system, this would produce secondarily a general disturbance of delayed hypersensitivity responses (1, 2, 3, 4).

The other possibility considers the disease itself as producing an immunological paralysis, characterized by a disturbance of delayed hypersensitivity responses. It is based on the proposition that the treated patient, when well on the way to recovery, may also recover this type of responses.

MATERIAL AND METHODS.-

We selected for this investigation five lepromatous leprosy patients, one with tuberculoid leprosy and one with diffuse cutaneous leishmaniasis.



Each patient received a skin homograft on the volar surface of the forearm. This graft was obtained in some cases from a patient with a similar disease and in others from a normal donor. The size of the graft was never larger than 3 cm along the transversal axis.

A day by day control was kept, photographs were taken each week and a biopsy done a month after the operation. This biopsy consisted not only of the graft, but also surrounding host tissue.

Periodic observation showed that, in the lepromatous leprosy and diffuse cutaneous leishmaniasis patients, the graft remained in normal conditions and adhered to host tissue until the 20th day, after this it underwent a progressive mummification process, but still remaining adhered to host tissue. Later, it was seen that the skin graft was progressively substituted by host tissue, but the usual rejection phenomenon never occurred.

In the tuberculoid leprosy patient, the rejection was identical to that of a healthy person.

There was no difference in the rejection phenomenon when the graft came from a normal person or from a patient with a similar disease.

The biopsy taken 30 days after the operation was included in paraffin and the sections stained with hematoxilin-eosin and Fite-Faraco.

From the hystopathological view point, the biopsy showed that the graft was formed by two layers: one superficial layer of hyalinized collagen with abundant cellular infiltration of spindle-type cells. In the deeper part, there was another layer where the hyalinization was less intense, with cellular infiltration. This layer was limited towards its inferior portion by infiltration tissue formed by vacuolated cells, with pale nuclei. Towards the periphery of the graft an epidermal flap could be seen, which penetrated

partially under the very hyalinized portion of the graft. Between those two layers there was an intermediate zone, with cellular infiltration of round cells which showed important kariolitic phenomena.

The Fite-Faraco stain showed that the granulomatous reaction provoked by the graft simulated a lepromatous granuloma, without bacilli.

Two of the lepromatous leprosy patients and the diffuse cutaneous leishmaniasis patient received a second graft six weeks after the first one. The three patients showed a typical rejection, two weeks later.

#### COMMENTS

In the primary skin homografts done in patients with lepromatous leprosy and diffuse cutaneous leishmaniasis, the grafts kept their normal appearance until the end of the third week, remaining well adhered to the tissues of the host; this means that the rejection phenomenon took twice as long to appear as that of a normal person. From this moment on, the tissues of the host started a slow penetration of the graft until they substituted it completely, while the graft itself became mummified. The hystopathologic structure showed the characteristics of an isomorphic reaction, since it had the appearance of a lepromatous granuloma, without any bacilli.

With the second graft, the rejection time was also longer than normal, being twice as long as that of a healthy person.

Due to all these facts, we consider that the rejection mechanism of homografts, as a manifestation of delayed hypersensitivity, is evidently altered in the diseases studied.

#### SUMMARY

Based on data referring to disturbances of the mechanism of delayed hypersensitivity reactions in lepromatous leprosy and diffuse cutaneous leishmaniasis,

we studied the behaviour of a small number of patients with these diseases in relation to primary and secondary skin homografts.

In the primary graft, the rejection took place in a period of time much longer than normal and it had special characteristics, such as a progressive substitution of the graft by host tissue. In the second graft, the rejection was typical, but also retarded.

The hystopathological response of the tissues of the host had the characteristics of an isomorphic granuloma.

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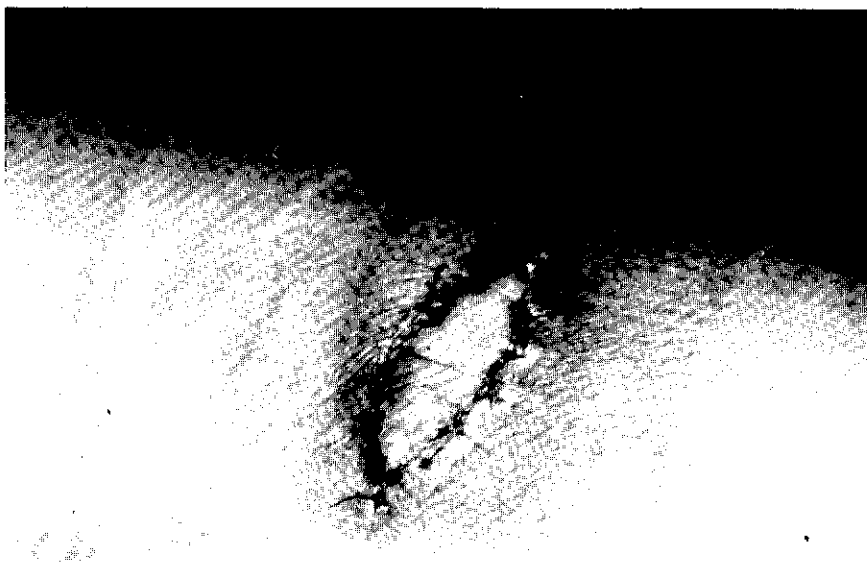
Photograph 1. Appearance of the homograft in the lepromatous leprosy patient 24 hours after the operation.



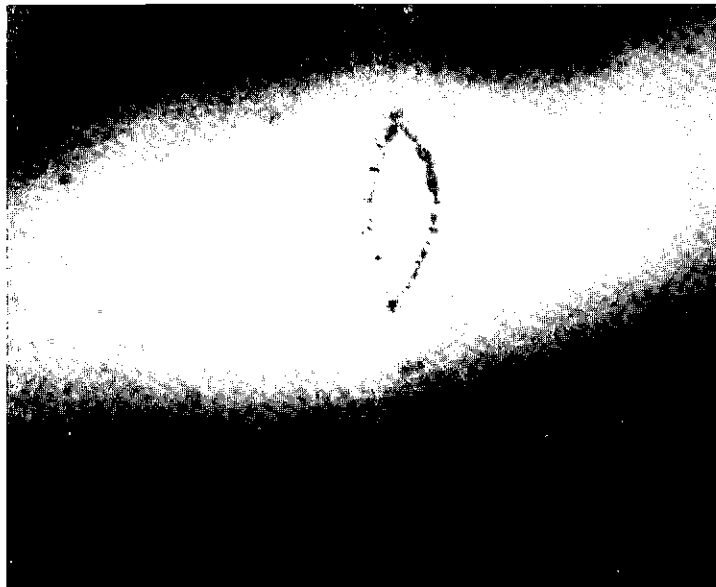
Photograph 2. Appearance of the homograft in the lepromatous leprosy patient 20 days after the operation with a beginning of mummification.



Photograph 3. Appearance of the homograft in the lepromatous leprosy patient 32 days after the operation, totally mummified.



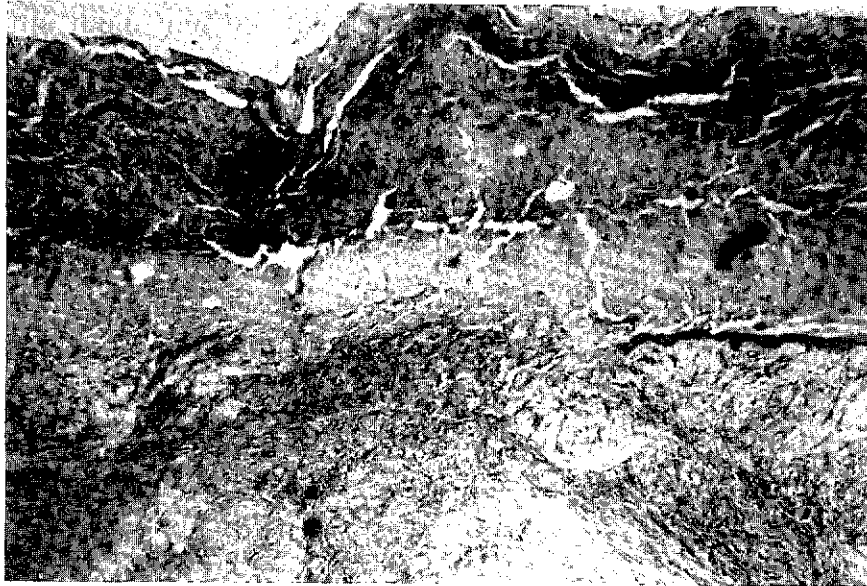
Photograph 4. Appearance of the homograft in the diffuse cutaneous leishmaniasis patient 24 hours after operation.



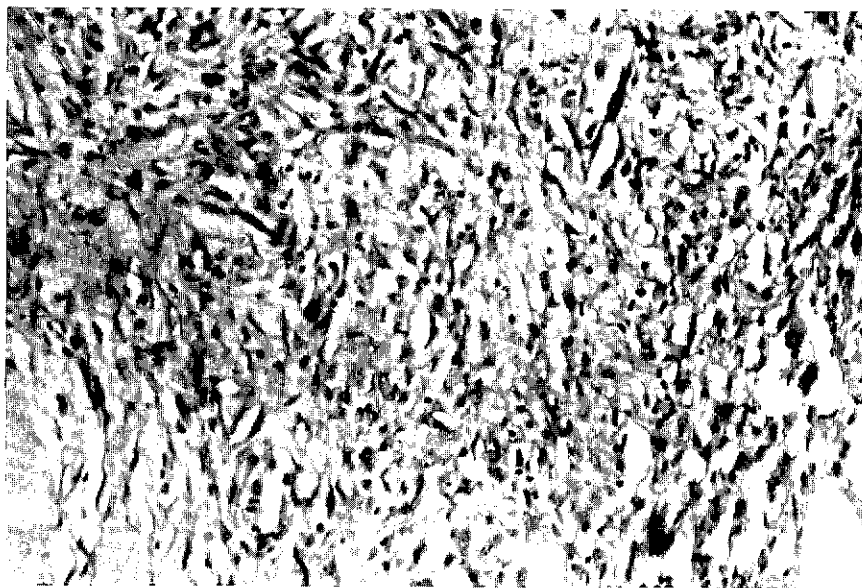
Photograph 5. Appearance of the homograft in the diffuse cutaneous leishmaniasis patient 20 days after the operation.



Photograph 6. Appearance of the homograft in the diffuse cutaneous leishmaniasis patient, totally mummified.



Photograph 7. Biopsy taken 30 days after the homograft was implanted. Lepromatous leprosy patient. Magnification 6x. It shows the graft still adhered, the epidermal flap penetrating under the right side of the graft and the cellular reaction.



Photograph 8. Biopsy taken 30 days after the homograft was implanted. Lepromatous leprosy patient. Magnification 16x. Shows the isomorphic granuloma under the graft.



INOCULATION OF HUMAN LEPROSY IN LABORATORY ANIMALS

There have been numerous attempts to inoculate human leprosy in laboratory animals. Of the investigations which have been done in the last few years, the following should be mentioned because of their importance. All these investigators used as inoculum material taken from human lepromatous leprosy and, therefore, rich in bacilli. Adler (1) based his work on the possibility of lowering the natural defenses of the animal by splenectomy, thus making it more it more susceptible to acquire the disease after inoculation.

Binford (2) thought that the inoculation, to be successful, should be made in regions of the animal with the lowest temperatures, such as the ear, testicles or foot-pads. He used hamsters for his experiments.

Chatterjee (3) based his research on the election of a highly susceptible animal, using black hybrid mice.

Bergel (4) induces important alterations in the animal by submitting it to a deficient diet, which he calls pro-oxidative, with which he provokes an aberrant metabolism.

Shepard (5, 6) bases his research on the inoculation of bacilli obtained from lepromatous leprosy into the foot-pads of mice. He emphasizes the importance of the conditions of the bacillus itself, especially in relation to the number of bacilli which stain uniformly with Ziehl-Nielsen stain. He uses small inocula, about  $5 \times 10^3$  bacilli. He also states that environmental temperature is important and he keeps his inoculated animals at around 18°C.

Since we began our work on the inoculation of leprosy in the golden hamster, our orientation has been the following:

1°) We selected hamsters because they are Mitsuda negative (6) and, therefore, the bacilli might have a better chance of survival.

2°) We gave great importance to the characteristics of the inoculum, feeling that the bacilli to be inoculated should have the best conditions possible to survive and multiply within the tissues of the animal inoculated. From this last point of view, we considered that in human lepromatous leprosy, the interaction between *M. leprae* and the host cell had produced a situation of extreme adaptation of the bacteria to the intracellular environment, which allows an enormous multiplication.

This situation evokes a metabolic symbiosis, which would cause the selection of a stable mutant of the bacteria (7, 8, 9, 10). We thought this would make it difficult for the bacteria to survive once they had been taken out of the human intracellular environment and inoculated into an animal of another species.

Furthermore, we considered the fact that electron microscopy studies of lepromatous granulomas indicated that a high percentage of bacilli showed important degenerative phenomena and only a small number had the appearance of intact bacilli.

We considered that bacilli obtained from borderline material had more chance of survival since the immunological conditions of the patient have not permitted the adaptation of *M. leprae* seen in the lepromatous patient. It is this type of leprosy, especially in cases of short evolution, where we have found the highest percentages of solid staining bacilli; therefore, viable. This fact has been corroborated by electron microscopy studies of this type of leprosy.

Our results have been the following.

The primary inoculations from human to animal have been done with inocula varying between  $5 \times 10^4$  and  $19.4 \times 10^6$ , bacilli, depending from the kind of patient the material was obtained. In a certain percentage of the cases, we obtained slow evolving, nodular granulomatous lesions, after a period of 8 to 12 months. These initial lesions were located at the inoculation site and their size was of a few millimeters. After a few passes from animal to animal, the lesions became larger and extremely rich in bacilli which were also found in the viscera of the animal, especially the spleen.

A summary of our data shows that up to now, from 52 groups of animals inoculated with borderline material, 16 showed macroscopic lesions at the site of inoculation and 13 showed an increase of the number of bacilli inoculated. These last 13 groups showed macroscopic lesions after a few passes from animal to animal. Of the other 23 groups, some were negative and the rest are still under observation.

Of the 63 groups of hamsters inoculated with lepromatous material, only 7 developed macroscopic lesions at the site of inoculation and in 9 we observed multiplication of the bacilli inoculated.

We have also considered important the fact that the bacteria found in the lesions induced in the hamster by inoculation of lepromatous and borderline material, have grown on Lowenstein-Jensen medium only in a few cases. Although the material from every passage from animal to animal is routinely inoculated in this medium, negative cultures are found much more frequently than positive ones.

We prepared an antigen by suspending in saline the bacilli obtained from lesions induced in hamsters by the inoculation of borderline material. When we injected this antigen in lepromatous patients,

it produced a nodular erythematous reaction which evolved to central ne crosis after three weeks. It is worth mentioning that a high percentage of these patients showed a positivization of the Mitsuda reaction when it was done on the other forearm.

Since our inoculations of leprosy material in the mouse footpad, following Shepard's technique (5), had produced very irregular results, one of our co-workers visited Dr. Shepard's laboratory, in Atlan ta, to learn all the technical details.

Since her return, we have started a series of parallel experiments comparing inoculations in hamsters with the technique developed by us with the inoculation of mice done with Shepard's technique.

We plan to prepare an antigen from the bacilli obtained from mice inoculated by the Shepard method, and inoculate it in lepromatous patients to compare its activity with standard lepromin.

In view of the results obtained, we feel that it would be very interesting to send material obtained from lesions developed in our hams ters to other investigators, to be studied and compared with their own results.

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