PARASITOLOGIC AND IMMUNOLOGIC DIAGNOSIS OF AMERICAN (MUCOCUTANEOUS) LEISHMANIASIS[1,2]

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The purpose of the work reported here is to evaluate a group of parasitologic and immunologic techniques used to diagnose American (mucocutaneous) leishmaniasis. One of the chief difficulties encountered is that of detecting parasites in the lesions. This makes it necessary to use serologic methods that are less than ideally sensitive and specific, a shortcoming confirmed by the findings of this study.

Introduction

A marked increase in the number of mucocutaneous leishmaniasis cases coming to the dermatology service of Brasília University's teaching hospital, together with study of an area endemic for this disease in the state of Bahia, led us to focus our attention on the problem of laboratory diagnosis of this protozoan disease.

For routine diagnosis, the usual procedure consists of checking the patient's lesions for *Leishmania* amastigotes and administering the Montenegro intradermal test *(27)*. Of the other diagnostic methods available, the indirect immunofluorescence test *(20,22)* has not been thoroughly evaluated, while the direct agglutination and immunodiffusion tests *(1,6,9)* are not used for routine diagnosis.

With regard to the etiologic agent of the different clinical forms of mucocutaneous leishmaniasis found in South America, researchers have become increasingly interested in isolating the responsible parasites—so as to investigate their biological *(3,23,24,25)*, biochemical *(8,17)*, and ultrastructural *(18)* characteristics for purposes of identification.

This article presents a number of observations concerning mucocutaneous leishmaniasis patients who received parasitologic and immunodiagnostic examinations, and discusses the sensitivity of the tests employed.

Materials and Methods

Selection of Patients

A total of 56 patients with mucocutaneous leishmaniasis were observed at the Brasília University teaching hospital's dermatology service and at various places in the municipalities of Cravolandia, Wenceslau Guimarães, and Ubaíra in the Brazilian state of Bahia. Each patient was given a physical examination; and, as a result, 29 were classified clinically as having an active cutaneous form of the disease, while the other 27 were classified as having an active mucosal form. A sample of venous blood was taken from each patient for serologic testing. The length of time that the lesions had been present ranged from 3 months to 26 years. The possibility that any patient might have Chagas' disease or kala-azar was ruled out.

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Diagnostic Laboratory Methods

Insofar as possible, nearly all the selected patients were given the same diagnostic tests in a parallel and systematic manner. Biopsy specimens taken with a skin punch were used to prepare contact smears and histological specimens, and to provide a source of triturated tissue for injection into hamsters. In addition, each patient was given the Montenegro intradermal test, and patient sera were tested for *Leishmania* antibody by indirect immunofluorescence and direct agglutination tests.

Parasitologic tests. Three contact smears were procured from each patient and were stained with Giemsa (pH 7.5) for 1 hour.

For purposes of histologic examination, part of each biopsy specimen was placed in Zenker-formol fixative for up to 6 hours and was then stored in 70 per cent alcohol to await processing. Subsequently, sections 5 microns thick were prepared and stained with hematoxylin-eosin for examination.

Triturates prepared from the biopsy specimens were inoculated into the snouts and hind feet of hamsters *(Mesocricetus auratus)* obtained from Brasília University's central vivarium. The animals were observed periodically so as to obtain a systematic record of the evolution of their infections. Material from infected hamsters was subsequently inoculated into other hamsters and Swiss 44 white mice. The isolated parasites were classified biologically according to the recommendations of Lainson and Shaw *(23,24,25)*. Cultures were produced in NNN and LIT[5] media as described by Camargo *(7)*, were maintained at 22°C, and were examined and transferred every 15 days.

Immunologic tests. All the patients were given the Montenegro intradermal test. That is, all were tested intradermally with antigen (leishmanin) made from promastigotes of *Leishmania mexicana amazonensis* (Josefa strain) isolated from a human case with a cutaneous lesion observed at the University of Brasília. These had been maintained in NNN and LIT culture media, with periodic reinoculation into hamsters. The protein nitrogen (N) content of approximately 5 x 10^6 flagellates per ml was determined in 30 μg N per ml, using the Goa microburet method *(19)*.

A tenth of a milliliter of antigen was inoculated intradermally into each patient's right forearm, and an equal volume of phenolated physiologic solution was inoculated into the left forearm as a control. The reading was made in millimeters of induration perceptible to the touch 72 hours after inoculation.

Antigen for the indirect immunofluorescence test was prepared from *L. mexicana amazonensis* promastigotes following the general guidelines of the method recommended by Guimarães et al. *(20)*. The reactivity of the 56 patients' sera was then tested using this antigen.

In addition, amastigote antigen was prepared from the same strain, following the procedure described by Shaw and Lainson *(31)*, and was used to test sera from 24 patients with parasitologically confirmed cases. This provided a means of comparing the relative sensitivity of the results when promastigotes and amastigotes were used as antigen.

Both indirect immunofluorescence methods employed human anti-gamma-globulin labeled with fluorescein isothiocyanate (F/P 0.64) from the Wellcome Research Laboratories, England (Lot K 0403); a Zeiss-Jena dark-field fluorescent microscope; a HBO-50 light source and ultraviolet lamp; and barrier filters and exciters 0G1 and BB 224, respectively. Interpretation of the findings obtained with our sera took into account the criteria adopted by Chiari *(10)*, Guimarães et al. *(20)*, and Shaw and Lainson *(31)*.

For the direct agglutination test, the methodology described by Allain and Kagan *(1)* was used both to prepare the antigen and for the test as a whole. The source of the antigen material was the forementioned strain of *L.*

[5]NNN = Novy, Nicolle, and McNeal medium; LIT = liver infusion tryptose medium.

mexicana amazonensis. A total of 46 patients were tested using this procedure.

Statistical Analysis

The data obtained were assessed by calculation of the reciprocals of geometric mean titers, tests of variance, and Student's t test of averages of the distribution frequency of the patients' serum titers. The levels of the tests were $p < 0.1$ and $p < 0.05$.

Results

Parasitologic Tests

The three parasitologic tests (involving contact smears, histologic examination, and hamster inoculation) revealed the presence of parasites in 24 of the 56 patients tested. Fourteen patients (25 per cent) yielded parasite-positive smears, and histologic examination of biopsy specimens from 15 of 55 patients (27.3 per cent) revealed amastigotes. Table 1 shows the clinical features of the 24 parasite-positive cases, as well as the results of these and other tests.

Of the 56 patients, 29 had the active cutaneous form of American leishmaniasis while 27 had the mucosal form of the disease. Sixteen cases (55.1 per cent) of the group with cutaneous lesions showed evidence of amastigotes in the lesions, but only 8 cases (29.6 per cent) in the group with mucosal lesions could be parasitologically confirmed. Of the 24 parasitologically confirmed cases (both cutaneous and mucosal), three (12.5 per cent) were confirmed on the basis of smears alone; one (4.1 per cent) was confirmed on the basis of histologic examination alone; and six (25 per cent) were confirmed on the basis of hamster inoculation alone. A relatively high density of amastigotes was found in another six patients with a long history of chronic lesions going back anywhere from 7 to 20 years, the parasites being detected in both smears and histologically examined biopsy specimens from the members of this group. The remaining eight patients all yielded positive results in at least two of the three parasitologic tests.

Four patients with recent ulcers (compatible with American leishmaniasis) only yielded positive parasitologic results through hamster inoculation—when the inoculated animals developed nodules containing numerous amastigotes. (The other two patients yielding parasitologically positive results only via hamster inoculation had chronic lesions.)

Of the 18 patients whose lesions yielded parasitologically positive results by other methods, 17 provided infected material that was inoculated into hamsters. Nine of these 17 animals became infected. Five showed no signs of cutaneous infection over periods of observation ranging between 5 and 9 months from the time of inoculation. Two were sacrificed because they were on the verge of dying but showed no signs of being infected 7 months after inoculation. One died unobserved. And one *Leishmania* strain (from patient 21) produced a visceral infection.

The isolates from patients 1, 2, and 3 were subsequently reinoculated into other hamsters between six and eight consecutive times. Although the incubation period was shorter for these latter passages—35-40 days, probably due to the fact that the inoculum became progressively richer in parasites—the evolution of the infection retained its basic characteristics (rapid growth of the lesion, early ulceration, intense cutaneous parasitization, and minimal inflammatory infiltrate). Cultures were easily obtained, and growth of the promastigote forms was exuberant. The three isolates adapted easily to Swiss 44 white mice; and the organisms from patient 3 produced metastasis in both hamsters and mice.

In contrast, isolates obtained from patients 17 and 20 evolved slowly, producing a barely perceptible hairless region at the inoculation site and a small parasitic nodule that showed no signs of ulceration even after many months of observation. Extremely few parasites were found, and the isolates' growth in culture media was very poor. A report concerning

Table 1. Clinical features, parasitologic findings, and immunologic test results of 24 cases of parasitologically confirmed American (mucocutaneous) leishmaniasis.

Patient No.	Clinical features			Parasitologic test results				Immunologic test results			
						Hamster Inoculation			Indirect immunofluorescence test		
	Clinical form of disease	No. of lesions	Duration of lesions	Direct smear	Hist. exam.	+/−	Months elapsed since inoculation	Montenegro intradermal test	Promastigote antigen titers	Amastigote antigen titers	Direct agglutination
1	cut.[a]	1	3 mos.	−	−	+	5 mos.	+	1:320	1:160	1:128
2	cut.	1	3 mos.	−	−	+	5 mos.	+	1:40	1:320	Not done
3	cut.	1	3 mos.	−	−	+	7 mos.	+	−	1:40	1:64
4	cut.	>1	3 mos.	−	+	−	8 mos.(no infection)	+	−	1:80	−
5	cut.	1	3 mos.	−	−	+	8 mos.	+	1:40	1:40	1:64
6	cut.	1	4 mos.	+	+	−	7 mos.	+	−	−	1:256
7	cut.	1	6 mos.	+	+	+	2.5 mos.	−[c]	1:320	1:40	−
8	cut.	>1	6 mos.	+	−	−	5 mos.(no infection)	+	−	1:320	1:64
9	cut.	1	10 mos.	−	+	+	3 mos.	+	1:40	1:40	−
10	cut.	1	1 year	+	−	Hamster died unmonitored		−	−	1:80	1:64
11	cut.	>1	2 years	+	+	−	9 mos.(no infection)	+	1:40	1:40	1:64
12	cut.	>1	2.6 years	+	+	+	2.5 mos.	+	1:160	1:160	1:128
13	cut.	>1	6 years	−	−	+	5 mos.	+	1:80	1:20	−
14	cut.	1	7 years	+	+	+	2.5 mos.	+	−	1:20	1:128
15	cut.	>1	9 years	+	+	Not inoculated		+	1:80	1:1,280	1:128
16	cut.	1	12 years	+	+	+	2.5 mos.	+	−	1:20	1:64
17	muc.[b]	>1	5 years	−	+	+	4 mos.	+	1:160	1:40	−
18	muc.	>1	12 years	+	+	+	2.5 mos.	−[c]	1:1,280	1:80	1:1,024
19	muc.	>1	5 mos.	+	+	+	4 mos.	+	1:1,280	1:40	1:128
20	muc.	>1	5 years	−	+	+	3 mos.	+	1:80	1:80	1:128
21	muc.	>1	9 years	−	−	+	5 mos.(visceral infection)	+	1:40	1:20	−
22	muc.	>1	13 years	+	+	−	7 mos.(sacrificed)	+	1:80	1:40	1:128
23	muc.	>1	16 years	+	+	−	7 mos.(sacrificed)	+	1:80	1:80	1:128
24	muc.	>1	20 years	+	Not done	−	5 mos.(no infection)	+	1:640	1:640	−
Total Positive				14	15	15		21 (87.5%)	17 (70.8%)[d]	23 (95.8%)[d]	16 (69.6%)

[a]Cutaneous lesion(s).
[b]Mucosal lesions.
[c]Patients giving no cutaneous response to PPD, candidin, or trichophytin.
[d]Student's t test indicates a statistically significant difference ($p < 0.1$) between these two figures.

biological and biochemical study of these and other isolates is now being prepared for publication. (Recently we were able to identify some of these strains isolated two years ago with the following results: *L. mexicana amazonensis* in patients 2 and 3 and *L. braziliensis braziliensis* in patient 20. We used: (a) biological parameters: morphologic growth in cultures, behavior in hamsters, and development in the digestive tract of *Lutzomyia longipalpis,* and (b) isoenzyme patterns—Cuba, C., in preparation.)

Immunologic Tests

Montenegro intradermal test. Of the 56 original patients, 53 (94.6 per cent) gave a positive response to the Montenegro intradermal test. Repeated tests of the other three patients at various times failed to produce a positive response. Two of these patients also failed to react when tested intradermally with such antigens as PPD tuberculin, candidin, and trichophytin, and also yielded a negative response to the DNCB[6] sensitization test, suggesting cellular immunodeficiency. All three of these patients were among the 24 yielding positive parasitologic results (see Table 1).

The indirect immunofluorescence test (promastigote antigen). Our records show that this test elicited positive titers for circulating fluorescent antibody from 35 (62.5 per cent) of the 56 patients. When the distribution of these positive titers among different types of patients was examined, the following results were obtained:

(a) With respect to the proven presence of parasites, 17 (70.8 per cent) of the 24 patients whose smears or tissues yielded amastigotes gave a positive response, with titers ranging from 1:40 to 1:1,280 and the geometric mean titer being 1:78. Most of the titers were distributed in the range 1:40-1:160. Somewhat fewer (18 of 32 or 59.4 per cent) of the patients without positive parasitologic findings yielded a positive response. Titers for these patients ranged from 1:40 to 1:160; the geometric mean titer was 1:43.

(b) With regard to the number of lesions present, Student's t test showed a significant statistical difference ($p < 0.05$) between the average of the serologic titers from patients with one lesion and that from those with multiple lesions (Table 2). This suggests that the number of lesions had an influence on circulating antibody levels.

(c) Regarding the duration of infection (the time of development of the initial lesion), 23 patients whose initial lesions apparently developed within the preceding year were compared with patients whose lesions were more than a year old. In general, the distribution of positive titers (between 1:40 and 1:160) was similar in the two groups, and no statistically significant differences were found.

(d) Regarding the two clinical forms of the disease, a statistically significant difference ($p < 0.1$) was found between the mean fluorescent antibody titers for patients with cutaneous and mucosal forms of the disease (Table

[6]DNCB = 2,4 dinitrochlorobenzene.

Table 2. Fluorescent antibody titers obtained with sera from 54 American (mucocutaneous) leishmaniasis patients with single or multiple lesions, using promastigote antigen.

No. of lesions	No. of sera yielding the reciprocal titers shown								No. of sera positive	No. of sera tested	% positive (reciprocal titers ≥ 40)	Geometric mean reciprocal titer
	≤ 20	40	80	160	320	640	1,280	2,560				
Single	12	8	3	4	1	—	—	—	16	28	57.1%	42[a]
Multiple	7	3	8	4	1	1	2	—	19	26	73.1%	80[a]

[a]Student's t test indicates a statistically significant difference ($p < 0.05$) between these two figures.

3), suggesting a relationship between the clinical form of the disease and these titers.

The direct agglutination test. Forty-six patient sera were tested by this method, field-work limitations precluding procurement of sera from the rest of the 56 patients studied. In all, 60.8 per cent of these sera yielded titers of 1:64 or greater that were considered positive for American leishmaniasis. The distribution of the positive responses was then analyzed in terms of the parasitology and indirect immunofluorescence test results, as well as the number of patient lesions, duration of the lesion(s), and the clinical form of the disease.

As Table 4 indicates, sera from parasitologically negative patients yielded a relatively low rate of positive response. On the other hand, 69.6 per cent of the patients with amastigotes present in their lesions produced serologic titers of 1:64 or greater.

Comparison of the geometric mean titers for fluorescent antibodies (1:78) and agglutinating antibodies (1:79) revealed no statistically significant difference in the two tests' results with sera from parasitologically positive patients. On the other hand, the geometric mean titers for fluorescent antibodies (1:43) and agglutinating antibodies (1:62) among parasitologically negative patients did show a statistically significant difference ($p < 0.1$). Overall, however, the two tests (indirect immunofluorescence and direct agglutination) showed virtually the same levels of diagnostic sensitivity when promastigotes were used as antigen in the indirect immunofluorescence test.

The number of lesions and the time it took the initial lesion to develop had no statistically significant effect ($p < 0.05$) on the titers of circulating agglutinating antibodies; nor did Student's t test reveal any significant difference ($p < 0.05$) between the titers of agglutinating antibodies obtained from the sera of patients with cutaneous forms of the disease and those with mucosal forms.

The 46 patients whose sera were tested for both fluorescent and agglutinating antibody yielded the following results:

1) No. responding positively to at least one test: 40 (87.0 per cent)
2) No. responding positively to the indirect immunofluorescence test: 29 (63.0 per cent)
3) No. responding positively to the direct agglutination test: 28 (60.9 per cent)

Table 3. Fluorescent antibody titers obtained with sera from 56 American (mucocutaneous) leishmaniasis patients with cutaneous or mucosal lesions, using promastigote antigen.

Clinical form of disease	No. of sera yielding the reciprocal titers shown								No. of sera positive	No. of sera tested	% positive (reciprocal titers ≥ 40)	Geometric mean reciprocal titer
	≤20	40	80	160	320	640	1,280	2,560				
Cutaneous	13	8	5	1	2	—	—	—	16	29	55.2%	40[a]
Mucosal	8	3	6	7	—	1	2	—	19	27	70.4%	78[a]

[a]Student's t test indicates a statistically significant difference ($p < 0.05$) between these two figures.

Table 4. Agglutinating antibody titers obtained with sera from 46 American (mucocutaneous) leishmaniasis patients with or without parasitologically confirmed cases. All the cases were diagnosed positive on the basis of clinical observations and the response to the Montenegro intradermal test.

Presence of amastigotes parasitologically confirmed	No. of sera yielding the reciprocal titers shown							No. of sera positive	No. of sera tested	% positive (reciprocal titers ≥ 64)	Geometric mean reciprocal titer
	≤32	64	128	256	512	1,024	2,048				
Yes	7	6	8	1	—	1	—	16	23	69.6	79
No	11	7	2	2	—	1	—	12	23	52.2	62

In all, the indirect immunofluorescence test detected 12 cases not detected by the direct agglutination test, and the latter detected 11 cases not detected by the former. The remaining 17 cases responded positively to both tests. The individual responses of parasitologically positive patients to both tests are shown in Table 1. With regard to indirect immunofluorescence tests using amastigotes as antigen, the results showed this test to be very sensitive for diagnosing mucocutaneous leishmaniasis. That is, 23 (95.8 per cent) of the 24 sera tested by this method yielded a positive response, as compared to 70.8 per cent of those tested using promastigote antigen and 69.6 per cent tested for agglutinating antibody. Overall, it appears that the test using amastigote antigen is significantly more sensitive ($p < 0.1$) than the latter two. However, the distribution range of the titers in both fluorescent antibody tests was similar; the geometric mean titers were 1:136 (with amastigote antigen) and 1:80 (with promastigote antigen).

Discussion

It is not always easy to find the amastigote form of *Leishmania* in the characteristic ulcers of patients who have been clinically diagnosed as positive for mucocutaneous leishmaniasis. It is generally accepted that parasites are abundantly present in recent lesions and that amastigotes can be found with relative ease in smears from such lesions. However, when the lesions are chronic, whether mucosal or cutaneous, it is difficult and frequently impossible to find the parasite *(2,16)*. Zeledon and Ponce *(34)* have reported finding parasites in the lesions of 36 per cent of a group of 75 patients with the clinically diagnosed cutaneous form of leishmaniasis by using direct smears. In our study, contact smears from 14 of the 56 patients (25 per cent) yielded positive results, and the evidence points to the presence of parasites belonging to both the *L. braziliensis* and *L. mexicana* complexes. It is possible that the differences in the percentages of patients whose stained smears showed positive results could be partly due to the incidence of parasites belonging to the *L. braziliensis* complex.

Another matter that has not been fully resolved is how material to be examined should best be removed from the lesion or lesions of a parasitized individual and how the smear should be prepared. This question appears to be important, since there are a number of different criteria and procedures—such as scraping the ulcer's inner rim, excising a wedge-shaped fragment from the edge of the lesion, using a skin punch, and so forth. It should be noted that Aragão, Pessoa, and Pestana, who pioneered research on mucocutaneous leishmaniasis in Brazil, laid particular stress on the need for care and patience in obtaining and examining specimens for parasitologic diagnosis of the disease *(15)*.

Regarding microscopic examinations, it was generally difficult or almost impossible to identify parasitic forms of *Leishmania* in the histologic tissue specimens, even when the presence of such forms in the smears could be demonstrated easily. Overall, during the course of our study we observed amastigotes in tissue specimens from 27.3 per cent of the 55 patients whose specimens were subjected to histologic examination.

The use of hamsters in leishmaniasis research has resulted in noteworthy progress in the fields of pathology *(12,14)* and epidemiology *(23,24,25)* and has improved our understanding of *Leishmania* transmission *(11,26)*. In general, the authors whose works are cited have found the hamster very susceptible to *Leishmania* infection. To date we have not found a culture method that is satisfactory for primary isolation.

However, when we examine this rodent's contribution to diagnosis, it appears not to be an ideally susceptible animal permitting efficient isolation of the various subspecies of parasitic protozoans from the patients studied. We found that only a minority (35.8 per cent) of the animals developed the disease after being inoculated with material taken from our patients. Moreover, several hamsters inoculated with material from patients who showed

marked clinical and immunological evidence of active *Leishmania* infections—including the presence of parasites in smears and tissue specimens—developed no signs of parasitized lesions, despite prolonged periods of observation. Such negative results probably had to do with the density of the parasites in the patients' lesions, the viability of the parasite forms in the inoculum, or the absence of parasites in the injected inoculum, among other things. But it could also relate to the theoretical possibility that the hamster is not the animal of choice, or is not sufficiently sensitive to become infected with some strains of *Leishmania* capable of infecting man.

In any event, and despite these shortcomings, the hamster is the only laboratory animal we have at present for isolating *Leishmania* from mucocutaneous leishmaniasis patients. Obviously, inoculation of susceptible animals is of secondary importance in laboratory diagnosis of the disease *(28)*. Nevertheless, when one is attempting to confirm a diagnosis, establish a basis for treatment, or evaluate the action of a drug used in a patient, demonstration of the parasite's presence is of importance—and often this can only be done by using the hamster.

The usefulness of hamsters, despite their limitations, may be seen by examining the data on patients 1, 2, 3, 5, 13, and 20 that are shown in Table 1. All of these patients gave a positive response to the Montenegro intradermal test and also to two or all three of the serologic tests. Though neither smears nor histologic examinations revealed the parasites, hamsters inoculated with material from the patients' lesions developed parasite-infested nodules between 3 and 8 months after inoculation.

In this vein, Medina and Belfort *(29)* have recommended that material from suspected human lesions be inoculated into hamsters whose immune systems have been partly suppressed by administration of steroids. They have found this method invaluable for diagnosing mucocutaneous leishmaniasis. In addition, Hommel et al. *(21)* have succeeded in growing *Leishmania braziliensis braziliensis* in immunosuppressed hamsters.

Not long ago, Melo et al. *(30)* reported using the Montenegro test on patients who had been clinically and parasitologically diagnosed as having mucocutaneous leishmaniasis. Using antigens with varying concentrations of protein nitrogen (N) per ml, they demonstrated the existence of a linear relationship between the average areas covered by the skin reactions and the antigen concentrations used. On this basis, they concluded that a higher percentage of positive reactions was obtained when 40 μg N per ml was used. The antigen we used for our study contained 30 μg N per ml, and 94.6 per cent of our patients reacted positively; of those patients with parasitologically confirmed infections, 87.5 per cent reacted positively to the intradermal test.

Leishmaniasis serology has recently seen wider use of the indirect immunofluorescence technique for detection of circulating antibodies *(10,20,33)*. In our study, when this technique was applied using antigen prepared from promastigote leishmanias, the method detected only 62.8 per cent of the clinically diagnosed and Montenegro-positive cases, and only 70.8 per cent of the parasitologically confirmed cases. These results were obtained despite utilization of techniques known to be effective, and despite the fact that modifications which might interfere with the results were held to a minimum.

Aside from the acknowledged subjectivity involved in reading slides, various factors affecting the immunologic responses triggered by the infective agent lead to qualitative and quantitative variations in those responses as the disease runs its course. This is suggested by the information reported in Table 1, which shows that the levels of fluorescent antibody in sera from seven patients with active lesions containing parasites was so low as to elude detection when antigen prepared from promastigotes was used.

This picture contrasts sharply with the 95.6 per cent positive results of the same test when amastigote antigen was used. Despite the rel-

atively small number of positive cases tested, our findings show the test using amastigote antigen to be highly sensitive in diagnosing this disease—results agreeing with those of Shaw and Lainson *(31)*—and also show it to be significantly more sensitive than the same test when promastigote antigen is used. Thus, comparison of the antigenicity of the promastigote and amastigote forms of *Leishmania* shows that the intracellular (amastigote) forms apparently afford earlier and more efficient detection of circulating antibodies for purposes of diagnosing the disease. As Table 1 indicates, of eight patients with relatively recent lesions (3 to 12 months' development), sera from seven yielded positive titers when amastigote antigen was used, while only three yielded positive titers with promastigote antigen. Generally speaking, however, the average titers obtained with the amastigote antigen tended to be low, a finding previously reported by other authors *(5,13)*.

Our study failed to find any correlation between the fluorescent antibody titers obtained with amastigote antigen and the clinical manifestations of the disease, the duration of the lesions, or the number of lesions involved. The observed titers ranged from 1:40 to 1:1,280. Some of the relatively high ones were obtained with sera from patients with chronic forms (both cutaneous and mucosal) of the disease, despite a low incidence of very recent lesions in these patients. Before attempting to draw conclusions about this matter, we feel additional observations are needed.

Despite the relatively small number of cases tested, our findings suggest that mucosal involvement may have a marked influence on circulating antibody titers. This is contrary to the findings reported by Bittencourt et al. *(4)*, who feel there is no relation between the level of circulating antibodies and the clinical form of the disease.

The discovery that circulating fluorescent antibody titers are influenced by the number of the patient's lesions was reported earlier by Chiari *(10)*, after studying a group of mucocutaneous leishmaniasis patients with recent and exclusively cutaneous lesions. Chiari maintains, however, that the length of time involved in development of the initial lesion, parasitologic confirmation of the infection, and the patient's age have no significant effect on the circulating antibody titers.

In regard to the indirect immunofluorescence test, we feel there is every reason to use antigen prepared from amastigote forms of the parasite, to test such antigen's efficacy in epidemiologic surveys of the prevalence of infections, to confirm its usefulness in follow-up of patients under treatment, and to use it in assessing the efficacy of new therapeutic drugs *(32)*.

Our experience with the direct agglutination test showed it to be reasonably useful (its diagnostic performance was basically similar to the indirect immunofluorescence test using promastigote antigen), and we believe that its speed and simplicity would make it even more valuable for performing field tests. Its diagnostic sensitivity in our study failed to equal that obtained by Allain and Kagan *(1)*, but we have been unable thus far to determine why. It should be noted, however, that our experience showed simultaneous use of these two serologic tests can lead to substantially better immunodiagnostic results (86.9 per cent detection of positive cases) than use of either test alone.

Some of the difficulties we encountered in diagnosing mucocutaneous leishmaniasis show that we are still far from possessing parasitologic and immunodiagnostic techniques that are ideally sensitive and effective. There is an urgent need for techniques affording direct demonstration of the parasite or allowing it to be found through indirect methods (culture in appropriate media, inoculation into susceptible animals that develop evident lesions quickly, new histochemical techniques, etc.). It is also essential to refine and standardize parasite antigens—so as to substantially improve the potential accuracy of the serologic tests currently in use.

SUMMARY

This article reports on the sensitivity of various tests for detecting mucocutaneous (American) leishmaniasis, as indicated by the results obtained with 56 patients diagnosed as having the disease. Each patient received a physical examination and was classified as having either the cutaneous or the mucosal form of American leishmaniasis.

Biopsy specimens were then obtained for preparation of direct smears, slides for histologic examination, and triturated tissue for inoculation into hamsters. In the case of 24 patients, at least one of these procedures revealed the presence of *Leishmania* parasites. All three tests detected the parasite in six cases; two of the three yielded positive results in eight cases; and only one test provided parasitologic confirmation of the disease in 10 cases. Overall, each of the three methods detected parasites in about the same number of cases.

Also, patients' sera were used to perform indirect immunofluorescence and direct agglutination tests. Promastigote forms of *Leishmania mexicana amazonensis* were used in both tests, and amastigote forms were used in additional indirect immunofluorescence tests. When promastigotes were used, both tests obtained relatively low rates of positive response (63 and 61 per cent, respectively). However, when amastigote antigen was used in the indirect immunofluorescence test, a positive response was obtained from 95.6 per cent of the sera tested.

Among other things, these results reconfirm the desirability of using antigen prepared from the parasite's amastigote forms in the indirect immunofluorescence test. It would also appear appropriate to evaluate the efficacy of this procedure in epidemiologic surveys of the prevalence of leishmaniasis infections, to confirm its usefulness in patient follow-up, and to use it in seeking to determine the efficacy of new therapeutic drugs.

REFERENCES

(1) Allain, D. S., and I. G. Kagan. A direct agglutination test for leishmaniasis. *Am J Trop Med Hyg* 24(2):232-236, 1975.

(2) Azulay, R. D. Leishmaniose tegumentar. *Jornal Brasileiro de Medicina*, pp. 50-70, 1974.

(3) Barbosa, W., M. do C. Moreira de Souza, J. M. de Souza, D. M. Rassi, B. B. Gerais, and R. L. de Oliveira. Note on the classification of the *Leishmania* sp. responsible for cutaneous leishmaniasis in the East Central Region of Brazil. *Ann Trop Med Parasitol* 70(4):389-399, 1976.

(4) Bittencourt, A. L., A. Sodré, and Z. A. Andrade. Pesquisa de anticorpos circulantes pelo método de immunofluorescéncia na leishmaniose tegumentar. *Rev Inst Med Trop São Paulo* 10(4):247-252, 1968.

(5) Bray, R. S., and R. Lainson. The immunology and serology of leishmaniasis: I. The fluorescent antibody staining technique. *Trans R Soc Trop Med Hyg* 59(5):535-544, 1965.

(6) Bray, R. S. Immunodiagnosis of Leishmaniasis. In S. Cohen and E. Sadun (eds.). *Immunology of Parasitic Infections.* Blackwell Scientific Publications, London, 1976, pp. 65-76.

(7) Camargo, E. P. Growth and differentiation in *Trypanosoma cruzi:* I. Origin of metacyclic trypanosomes in liquid media. *Rev Inst Med Trop São Paulo* 6:93-100, 1964.

(8) Chance, M. L., W. Peters, and L. Shchory. Biochemical taxonomy of *Leishmania:* I. Observations on DNA. *Ann Trop Med Parasitol* 68:307-316, 1974.

(9) Chaves, J. Immuno-diffusion reactions among various samples of *Leishmania. Acta Científica Venezolana* 21:68-70, 1970.

(10) Chiari, C. A. Pesquisa de anticorpos circulantes na leishmaniose tegumentar americana pela reação de immunofluorescência indireta. Thesis. Federal University of Minas Gerais, Belo Horizonte, Brazil, 1971.

(11) Coelho, M. de V. Recent researches on the transmission of South American cutaneous Leishmaniasis. *Proceedings of the 7th International Congress on Tropical Medicine and Malaria* 2:320-323, 1964.

(12) Coelho, M. de V., and E. Coutinho-Abath. Experimental cutaneous leishmaniasis: I. Infection of albino mice and Syrian hamsters by *Leishmania mexicana. Rev Inst Med Trop São Paulo* 7:136-144, 1965.

(13) Convit, J., and M. E. Pinardi. Applying the indirect immunofluorescence test to the study of American cutaneous leishmaniasis. *Derm Int* 8:17-20, 1969.

(14) Coutinho-Abath, E., and M. de V. Coelho. Experimental cutaneous leishmaniasis: II. The pathology of leishmaniasis by *Leishmania mexicana. Rev Inst Med Trop São Paulo* 7:145-155, 1965.

(15) Coutinho, E. *Tratado de Clínica das Doenças Infectuosas, Parasitárias e Peçonhentas* (sixth edition). Rio de Janeiro, Guanabara Koogan, 1957.

(16) Furtado, T. A. Diagnóstico laboratorial de leishmaniose tegumentar americana. *Anais Brasileiras de Dermatologia* 47:211-218, 1972.

(17) Gardener, P. J., M. L. Chance, and W. Peters. Biochemical taxonomy of *Leishmania:* II Electrophoretic variations of malate dehydrogenase.*Ann Trop Med Parasitol* 68:317-325, 1974.

(18) Gardener, P. J., L. Shchory, and M. L. Chance. Species differentiation in the genus *Leishmania* by morphometric studies with the electron microscope. *Ann Trop Med Parasitol* 71(2):147-155, 1977.

(19) Goa, J. A micro buret method for protein determination; determination of total protein in cerebrospinal fluid. *Scand J Clin Lab Invest* 5:218-222, 1953.

(20) Guimarães, M. C. S., V. L. Giovannini, and M. E. Camargo. Antigenic standardization for mucocutaneous leishmaniasis immunofluorescence test. *Rev Inst Med Trop São Paulo* 16:145-148, 1974.

(21) Hommel, M. W., W. Peters, and M. L. Chance. *Leishmania braziliensis braziliensis* in immunosuppressed hamsters and nude mice. *Trans R Soc Trop Med Hyg* 69(1):9-10, 1975.

(22) Kagan, I. G. Advances in the immunodiagnosis of parasitic infections. *Z Parasitenkd* 45:163-195, 1974.

(23) Lainson, R., and J. J. Shaw. Leishmaniasis in Brazil: V. Studies on the epidemiology of cutaneous leishmaniasis in Mato Grosso State and Observations on two distinct strains of *Leishmania* isolated from man and forest animals. *Trans R Soc Trop Med Hyg* 64(5):654-667, 1970.

(24) Lainson, R., and J. J. Shaw. Epidemiological Considerations of the Leishmanias with Particular Reference to the New World. In A. Fallis (ed.). *Symposium on Ecology and Physiology of Parasites.* University of Toronto, 1971.

(25) Lainson, R., and J. J. Shaw. Leishmaniasis of the New World: Taxonomic problems. *Br Med Bull* 28:44-48, 1972.

(26) Lainson, R., R. D. Ward, and J. J. Shaw. Experimental transmission of *Leishmania chagasi,* causative agent of neotropical visceral leishmaniasis, by the sandfly *Lutzomya longipalpis. Nature* 266:628-630, 1977.

(27) Marsden, P. D. Leishmaniasis. *N Engl J Med* 300:350-352, 1979.

(28) Marsden, P. D., and R. R. Nonata. Mucocutaneous leishmaniasis: A review of clinical aspects. *Revista Sociedade Brasileira Medicina Tropical* 6:309-326, 1975.

(29) Medina, R., and E. Belfort. Leishmaniasis de la mucosa naso-bucal. *Archivos Venezolanos de Medicina Tropical y Parasitología Médica* 1:307-316, 1973.

(30) Melo, M. N., W. Mayrink, C. A. da Costa, P. A. Magalhães, M. Dias, P. Williams, F. G. Araujo, M. de V. Coelho, and S. M. Batista. Padronização do antígeno de Montenegro. *Rev Inst Med Trop São Paulo* 19:161-164, 1977.

(31) Shaw, J. J., and R. Lainson. A simply prepared amastigote leishmanial antigen for use in the indirect fluorescent antibody test for leishmaniasis. *J Parasitol* 63:384-385, 1977.

(32) Walton, B. C. The indirect fluorescent antibody test for evaluation of effectiveness of chemotherapy in American leishmaniasis. *J Parasitol* 56 (section II): 480-481, 1970.

(33) Walton, B. C., W. H. Brooks, and I. Arjona. Serodiagnosis of American leishmaniasis by indirect fluorescent antibody test. *Am J Trop Med Hyg* 21:296-299, 1972.

(34) Zeledon, R., and C. Ponce. Parasitological and immunological diagnosis of cutaneous leishmaniasis in the New World. *Proceedings of the Third International Congress of Parasitology* 1:239-240, 1974.