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ARTIGOS

HUMAN MUCOCUTANEOUS LEISHMANIASIS IN TRÊS BRAÇOS, BAHIA – BRAZIL. AN AREA OF *LEISHMANIA BRAZILIENSIS BRAZILIENSIS* TRANSMISSION. I. LABORATORY DIAGNOSIS

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Leishmanial parasites were detected in 71.2% of patients with cutaneous disease and 48% of patients with mucosal disease, using principally scanning of imprint smears and histological sections and hamster inoculation.

Parasites were more frequent in early cutaneous lesions ($p < 0.005$) of less than two month duration. Also they were more common in multiple than single mucosal lesions ($p < 0.02$) in spite of considerable prior glucantime therapy in the former group. 93% of cutaneous lesions had a positive leishmanin skin test and most of the negatives occurred in patients with lesions of less than one month duration. 97% of patients with single mucosal lesion and 79% with multiple mucosal lesions had a positive skin test. 86% of cutaneous disease and 90% of mucosal disease was associated with a positive indirect immunofluorescent antibody test at a $\geq 1/20$ dilution. In both groups multiple lesions were associated with higher titres and titres were significantly higher in patients with mucosal disease compared with cutaneous disease ($p < 0.01$).

Key words: *Leishmania braziliensis braziliensis*. Parasite isolation. Immunodiagnosis.

Três Braços is located in the forested littoral cacao growing region of the state of Bahia, Brazil (13° 40' latitude South and 39° 45' longitude West) 85 kilometers from the Atlantic coast. For more than twenty years it has been known to the Brazilian Ministry of Health as a focus of cutaneous leishmaniasis. It is one of the few areas where glucantime treatment has been administered by SUCAM personnel.

We established a field clinic in this community in 1975 and stimulated patients to seek investigation and treatment. Frequent field trips added further cases to this selected series collected from July 1976 – July 1982.

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We have presented evidence elsewhere[4] that in this area *Leishmania braziliensis braziliensis* (Lbb) is the parasite isolated from man in 96.7% of cases. We will describe in these papers the clinical presentation and initial evolution of this parasite in man. To date no animal model exists for Lbb and parasitological diagnosis in man is difficult[1]. Therefore in this first paper we discuss our diagnostic procedures in patients with cutaneous or mucosal disease considered in the two subsequent papers.

MATERIAL AND METHODS

Patients on first consultation were alloted an LTB number (Leishmaniasis Três Braços) used for all subsequent reference. A completed protocol included details of past and present residence for subsequent follow up and history of skin or mucosal lesions. Skin lesions were measured, their localisation recorded on a body map and their features described as regards morphology. A search for previous suggestive skin scars and evidence of mucosal disease was made.

Mucosal lesions were examined using a good light source, nasal speculum, tongue depressor and indirect laryngoscopy. Examination of the post nasal space was not done.

Two 4mm punch biopsies were taken from the

active edge of the suspected leishmanial skin lesion or with a cutting biopsy forceps from mucosal granuloma after local anesthetic (Lidocaine 1%). Imprint smears were made from the inferior surface of each biopsy, fixed in methyl alcohol, stained with Giemsa and searched for parasites. The first sample obtained by biopsy was triturated in normal saline and the suspension inoculated into the hind feet of a hamster. Hamsters were examined weekly for signs of cutaneous lesions or failure to thrive. Since our stocks frequently visceralise[4], animals were killed between six months and a year and the liver and spleen examined for parasites. Forty percent developed a subcutaneous nodule at the site of injection and this was aspirated and the material examined for amastigotes. Parasites recovered from these animals were maintained in Difco blood agar (biphasic 15% rabbit blood agar with saline overlay plus a mixture of penicillin 550 IU and streptomycin 100 mg/ml) and cryopreserved. The same medium was used for primary isolation attempts. The another skin biopsy was fixed in Zenker's solution for 2-3 hours and then preserved in 70% alcohol for histology. Sections cut after paraffin embedding were stained by haematoxylin and eosin. Details of antigen preparation and the methodologies used by us for leishmanin skin testing and estimation of circulating antibodies by indirect immunoflorescense (IFA) have already been described[3]. IFA titres of 20 or more are significant in our laboratory.

RESULTS

A resident medical officer in the area has never been possible but the group is accustomed to making four visits a year for 10 days to a month to provide medical care. In 1976 we documented 19 patients with leishmaniasis. An increasing acceptance by the community has led to an increase in patients visiting the field unit and, in 1982, 82 patients were registered. The disease appears to be in constant sporadic transmission in the area. Of 288 patients records available, in 25 the initial clinical impression of leishmaniasis was not confirmed and in 24 the data available were too sparse to allow any firm conclusion. This left 239 patient records, 182 (76%) with cutaneous lesions and 57 (24%) with mucosal disease. Data were not always available for all individual cases for the type of analysis selected due to factors such as non availability of hamsters at the time, failure to read the Montenegro test 48 hours later, etc. For this reason the number of patients analysed in relation to a particular test is often less than the total number.

For clinical analysis we have chosen to classify both forms of mucocutaneous leishmaniasis (cutaneous and mucosal) according to whether single or multiple lesions were present. Details of such clinical presentation are given in subsequent papers.

Table 1 – *Rates of parasite demonstration in relation to duration of cutaneous disease (177 patients)*

Duration of disease in months	*Parasite demonstration**		
	Positive	*Total*	*% Positive*
1 or less	44	50	88.0 **
2	28	39	71.8
3	16	25	64.0***
4 – 5	13	23	56.5
6 or more	25	40	62.5
Total	126	177	71.2

* Demonstration of parasite by any one or more of four methods namely direct smears, histology, culture or hamster inoculation.

Comparing** with*** $p < 0.005$ (X^2 Test)

Table 2 – *Rates of parasite demonstration in relation to duration of mucosal disease with single or multiple lesions (50 patients)*

Duration of disease in months	*Parasite Demonstration** Single lesion*		*Multiple lesion*		*Total*		
	Positive	*Total*	*Positive*	*Total*	*Positive*	*Total*	*% Positive*
6 or less	3	8	1	2	4	10	40.0
7 – 12	2	5	4	5	6	10	60.0
13 – 24	3	7	1	2	4	9	44.0
25 – 60	1	6	2	3	3	9	33.9
61 or more	3	4	4	5	7	12	58.3
Total	12	33**	12	17**	24	50	48.0

* Demonstration of parasites as in Table 1

** Significant $p > 0.02$ (X^2 Test).

Tables 1 and 2 show the overall results of our attempts to detect parasites (by the methods described) in three groups of patients namely those with cutaneous lesions and those with single or multiple mucosal lesions. Although multiple cutaneous lesions were common (32%) these cases are not analysed separately since we always biopsied only the most recent lesion. In all five patients with cutaneous lesions of less than two months duration who had received 3-15 grams of glucantime before consultation we recovered parasites. Of five patients with lesions of more than six months duration who had been treated with more than 100 grams of glucantime parasites were found in four. A difference was noted in single and multiple mucosal lesions as shown in Table 2. Parasites were significantly more common in patients with multiple mucosal lesions ($p > 0.02$).

Mucosal disease is rarer and the duration of the active lesion is spread over a long time scale. In some patients biopsy was not possible for technical reasons.

Parasites were detected in 71.2% of patients with cutaneous disease and 48% of patients with mucosal disease. In cutaneous disease parasites were more frequently recovered early in the infection since the isolation rate in the first two months was significantly higher than in more longstanding infections ($p < 0.005$).

In seventy-one patients with cutaneous disease personally followed by one of us (EALC) parasites were found in 31.8% (22/69) of Giemsa stained smears, 48% (33/68) of histological sections, 33% (11/33) of cultures and 67.9% (36/53) of inoculated hamsters. The latter method was significantly more sensitive ($p < 0.01$). However, examining the overall data presented in Table 3, this procedure (hamster inoculation) does not differ substantially from smear.

Previous antimonial treatment could be shown to significantly diminish the chance of recovering parasites in single mucosal lesions (3/18) but not in multiple lesions (7/9) ($p < 0.05$). Since patients had often consulted several doctors previously the total amounts of glucantime used before we saw the patients were large being on average 75 ± 68 grams of drug for single lesions and 456 ± 328grams for multiple lesions. In spite of such a dose it is of note that parasites persisted in the latter group. One defect of our data on parasite isolation is that all four procedures namely smear, histology, culture and hamster inoculation were not available in all patients. Culture was the least used due to the lack of suitable medium and contamination problems.

Forty-nine stocks from the 182 patients with cutaneous lesions and 11 stocks from the 57 patients with mucosal disease were identified by isoenzyme and/or monoclonal antibodies techniques as Lbb[4]. From two other patients not considered here parasites of the *Leishmania mexicana* complex were recovered.

All the patients described here had a histological picture suggestive of leishmaniasis. That is to say a granulomatous reaction rich in histiocytes, lymphocytes and plasma cells. More characteristic were areas of fibrinoid necrosis (35.2%) which often developed into disorganised granulomas (27.2%). A small proportion of these (5%) progressed to tuberculoid granulomas. Parasites were always difficult to visualise in all histological forms of the disease being present in very small numbers. They were usually absent in lesions with established granulomata.

Table 4 shows that 92.8% of the patients with cutaneous lesions had a induration 5mm or more in diameter 48 hours after intradermal inoculation of 0.1 ml of antigen (30 μg/protein/ml) in the forearm. The majority of negative patients had a lesion of less than one month duration but due to the small numbers this does not reach statistical significance. There was no association between the size of the skin test at 48 hours and the number or size of the lesions in this group.

Table 3 – *Results of parasitological examination using four different methods*

Methods / *Type of disease*	*Smear*			*Histology*			*Culture*			*Hamster inoculation*		
	Positive	*/ Total*	*(%)*	*Positive*	*/ Total*	*(%)*	*Positive*	*/ Total*	*(%)*	*Positive*	*/ Total*	*(%)*
Cutaneous	72	/ 174	(41.3)	49	/ 172	(28.4)	14	/ 91	(15.3)	64	/ 156	(41)
Mucosal	14	/ 51	(27.4)	8	/ 50	(16)	8	/ 26	(30.7)	13	/ 40	(32.5)
Total	86	/ 225	(38.2)	57	/ 222	(25.6)	22	/ 117	(18.8)	77	/ 196	(39.2)

Table 4 – *Rates of Montenegro skin test positivity in relation to duration of cutaneous disease and mucosal disease with single or multiple lesions*

Cutaneous disease				*Mucosal disease*							
				Single lesion				*Multiple lesions*			
Duration of disease in months	*Skin – test Positive*	*/ Total*	*% Positive*	*Duration of disease in months*	*Skin – test Positive*	*/ Total*	*% Positive*	*Skin – test Positive*	*/ Total*	*% Positive*
1 or less	40	/ 46	86.9	6 or less	6	/ 7	85.7	1	/ 2	50.0
2	37	/ 38	97.3	7 – 12	8	/ 8	100.0	6	/ 6	100.0
3	21	/ 21	100.0	13 – 24	8	/ 8	100.0	3	/ 3	100.0
4 – 5	25	/ 26	96.2	25 – 60	6	/ 6	100.0	1	/ 3	33.3
6 or more	32	/ 36	88.8	61 or more	7	/ 7	100.0	4	/ 5	80.0
Total	155	/ 167	92.8		35	/ 36	97.2	15	/ 19	78.9

Table 4 also indicates similar results for patients with single or multiple mucosal lesions. 91% of the group as a whole had a positive test. Four of the five patients with negative Montenegro skin tests had longstanding multiple lesions of the mucosae. There was no difference between the size of skin test induration in the two groups of mucosal patients. Significantly more necrosis occurred in the mucosal group when compared with the group with cutaneous disease ($p < 0.01$).

Table 5 – *Fluorescent antibody titres before treatment in sera of 128 patients with cutaneous leishmaniasis and 42 patients with mucosal disease related to the number of lesions*

Disease	*Number of lesions*	*Number of patients*	*Titres of IFA-IgG antibodies*									*% With positive titres*	*MGT p value (Student's t Test)*
			< 20	20	40	80	160	320	640	1280	2560		
	Single	85	14	13	19	5	19	5	–	–	–	83.7	50.1
Cutaneous	Multiple	42	4	5	9	9	12	3	–	–	–	90.5	54.5
	Total	128	18	18	28	25	31	8	–	–	–	85.9	54.4
	Single	29	5	3	8	5	7	–	1	–	–	85.7	34.11*
Mucosal	Multiple	13	–	1	1	2	4	2	2	–	1	100.0	187.75* $p < 0.001$
	Total	42	4	4	9	7	11	2	3	–	1	90.2	

MGT = mean geometric titre.

A sera dilution of one to twenty and above reacting to the immunofluorescent conjugate (anti-human IgG) in our laboratory is regarded as positive evidence of circulating antileishmanial antibody when tested with immunofluorescence antibody amastigotes antigen (IFAT). Table 5 and Figure 1 show that 86% of patients with cutaneous disease and 90% of patients with mucosal disease had such positive titres when first seen. There was no significant titre difference between mucosal and cutaneous disease but multiple mucosal lesions show significantly higher titres than other groups ($p < 0.001$). Significant associations could not be established between the level of the titre and such factors as the duration of the disease, age of the patient and success in the parasite detection.

DISCUSSION

Leishmania braziliensis braziliensis is so prevalent in Três Braços, Bahia that our diagnostic findings can be said to reflect human infection with this parasite. Our results confirm that it is a difficult parasite to visualise and isolate[1]. For this reason we have routinely used four methods.

As our results demonstrate, parasites are frequently found by one method and not by another. In simple visualisation procedures (smears or histology) amastigotes are scanty and the longer the specimen is examined the more chance there is of finding parasites[7]. The effect of formalin fixation causing

shrinkage and bad staining of amastigotes increases this difficulty in histological preparations. However our material shows a highly significant correlation for this method when compared with the other techniques used (Magalhães et al, in preparation).

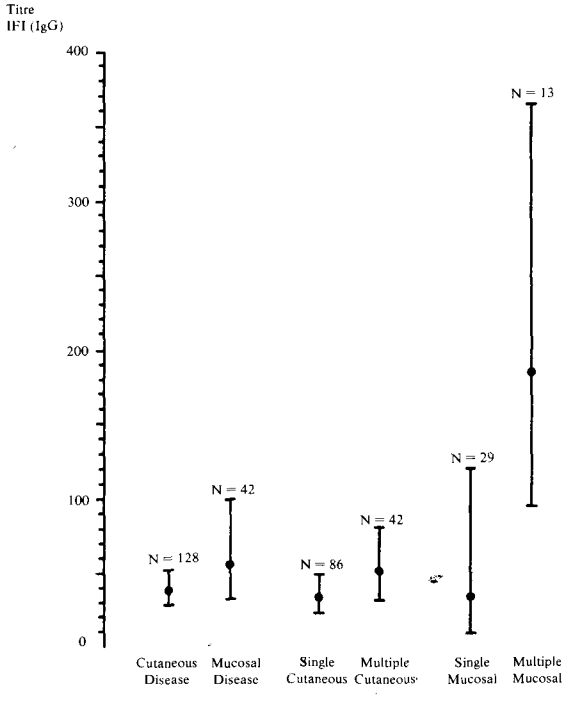

Figure 1 – Distribution of titres of IFAT (IgG) according to the clinical form of the disease and the number of lesions (the points on the vertical lines indicate the mean geometric titres: MGT X $\pm$ 1 interval of confidence.

We have used hamster inoculation as the main method in these early years to obtain isolates[7]. Hamsters survive well under field conditions but isolation is time consuming with Lbb since the parasite develops slowly and frequently visceralise. Animals have to be kept for a long time incurring further expense. The discrepancy in hamster isolation rates between the 71 personally observed cases and the overall results in Table 3 probably relates to the care with which hamsters were observed and maintained. In future we will probably employ hamsters more for isolation attempts from wild animals and phlebotomines where culture contamination is a problem.

Our results with culture have improved since this study was completed. Initially we had much contamination of needle aspirates or biopsies but recently we have reduced this to 10% of culture in cutaneous disease. Of the various media we have tried the biphasic blood agar medium using Difco agar has been most successful[7 15] and we have achieved 50% of positive cultures with this medium. Our results will be reported in full elsewhere.

Like other workers[6 11 14] we have shown that success in parasite detection is inversely proportional to the duration of the cutaneous lesion. We confirm that parasites are more difficult to isolate in mucosal disease[11]. The higher isolation rate in multiple mucosal lesions could be related to the adequacy of the biopsy specimen. Lesions high in the nose are difficult to biopsy and those of the larynx require hospitalisation and a general anaesthetic. On the other hand one cannot exclude the possibility that host factors favour parasite multiplication in disseminated mucosal lesions.

Previous treatment with glucantime will also influence parasite isolation as shown in our group with single mucosal lesions. In one study cultures rapidly turned negative after institution of glucantime therapy[9]. However as our results show past glucantime therapy does not necessarily interfer with parasite isolation which is always worth attempting even if the patient gives a history of prolonged antimonial therapy.

Since the parasite is so difficult to isolate it would be quite wrong to discuss parasitologically positive patients in these papers. We seek three lines of indirect evidence of leishmania infection to establish a probable diagnosis, namely histology, skin test reaction to leishmanin and the presence of circulating antibodies.

The histological appearance is helpful confirmatory evidence: even although the chronic skin granuloma is not diagnostic unless parasites are seen, it is suggestive. Also other diagnoses such as *Paracoccidiodes brasiliensis* infection and carcinoma can be excluded. The presence of necrosis is more suggestive of Lbb infection than other skin granulomas due to fungi, tuberculosis, sarcoid or leprosy. Unfortunately our early hopes[12] that some sort of prognostic classification could be established using histological processes such as necrosis and granuloma formation have not been confirmed to date. Fine details of histological interpretation vary with pathologists and polar forms such as frank necrosis or tubercle formation have a variable prognosis. These aspects will be fully discussed elsewhere (Magalhães et al, in preparation).

The value of the leishmanin skin test and the indirect immunofluorescent antibody test as ancillary

aids to diagnosis confirm our previous findings[2,3]. Why the Montenegro test is negative in few cases later in cutaneous disease is not clear. This observation was first made by Montenegro in his original paper[10]. The negative leishmanin skin tests observed in late multiple mucosal disease is possibly associated with secondary malnutrition. We have already reported a fatal case in which such malnutrition occurred and in which we have evidence of reversal of the skin test[8]. Deaths have occurred in these patients from Três Braços and we believe a negative leishmanin skin test in advanced mucosal disease to be a bad prognostic sign. The more frequent necrosis encountered in patient with mucosal disease suggests that a more pronounced hypersensitivity response is associated with this form of the disease[13]. However in terms of induration size a significant difference could not be detected between cutaneous and mucosal lesions. In another study we were unable to demonstrate increased hypersensitivity in mucosal disease using 3 different doses of leishmanin (Cuba et al, unpublished data). More studies are needed on this matter.

Early serological diagnosis is useful in mucocutaneous leishmaniasis and fluorescent antibody titres are relevant in our area where Chagas' disease and visceral leishmaniasis have not been encountered in nine years of clinical practice. Also triatomines have never been detected in houses. The higher titres found in patients with mucosal multiple lesions could be explained by a greater antigenic stimulation from a larger parasite mass. Since fluorescent antibody titres in infections with Lbb are low, we have recently evaluated the sensitivity of Elisa test[5] compared to the IFAT in 74 patients from whom parasites were recovered.

Significant antibody titres were present in the Elisa test in 95% of patients compared with 82% positivity with the indirect fluorescent antibody test. Although both tests are valuable the Elisa IgG antibody test would appear to be more sensitive and will be employed in our diagnostic and seroepidemiological studies in the future.

RESUMO

O emprego de esfregaços por aposição (imprint), cortes histológicos e inóculo em hamster foram, em conjunto, capazes de detectar *Leishmania* em 71,2% de pacientes com acometimento cutâneo e 48% com acometimento da mucosa.

Os parasitos eram mais freqüentes em lesões cutâneas recentes ($p. < 0,005$) do que após dois meses de duração. Também eram mais freqüentes em lesões múltiplas da mucosa do que naquelas isoladas ($p < 0,02$) embora o primeiro grupo tivesse recebido anteriormente terapia por glucantime. 93% dos pacientes com lesões cutâneas tiveram o teste de leishmania positivo sendo que a maioria dos casos negativos ocorreu em pacientes cujas lesões tinham menos de um mês de duração. 97% dos pacientes com uma única lesão da mucosa e 79% daqueles com múltiplas lesões foram positivos para o teste de leishmanina.

O teste de imunofluorescência indireta apresentou positividade em diluições de 1:20 em 86% dos casos de acometimento cutâneo e 90% daqueles de acometimento da mucosa. Nos dois grupos as lesões múltiplas estavam associadas com os títulos mais altos e estes eram significativamente mais altos nos pacientes com acometimentos da mucosa quando comparados com os casos de comprometimento cutâneo ($p < 0,01$).

Palavras chaves: *Leishmania braziliensis braziliensis*. Isolamento de parasito. Imunodiagnóstico.

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