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THE USE OF DIFFERENT CONCENTRATIONS OF LEISHMANIAL ANTIGEN IN SKIN TESTING TO EVALUATE DELAYED HYPERSENSITIVITY IN AMERICAN CUTANEOUS LEISHMANIASIS

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Three concentrations of Leishmania mexicana amazonensis *sonicated whole promastigote antigen (30, 9.6 and 3 ug N in 0.1 ml) were prepared and 0.1 ml of each inoculated intradermally into patients who live in one endemic leishmaniasis region in Brazil. Patients were divided into groups with active cutaneous leishmaniasis (ACL), healed cutaneous leishmaniasis (HCL), mucosal leishmaniasis (ML), and controls (C). Skin reactions were recorded by measuring induration 48 hours after inoculation.*

Skin tests using 9.6 ug N/0.1 ml yielded the best diagnostic results since 97% of 30 patients with active lesions (cutaneous or mucosal) and 83% with HCL showed reactions of 5 mm or greater as compared with 4% controls. Tests using 30ug N/0.1 ml caused an unacceptable level of skin reactions with necrosis (10% of ACL patients tested and 17% of HCL, respectively). Tests using 3 ug N/0.1 ml were less sensitive since only 87% of patients with active lesions and 68% with HCL had reactions of 5 mm or greater.

The 3 ug N/0.1 ml dose was utilized to ask the questions whether skin delayed hypersensitivity decreased with time after the initial lesion and whether mucosal involvement is associated with enhaced hypersensitivity to leishmanial antigen. Decreased delayed hypersensitivity was noted only in those patients who had an initial lesion more than 30 years ago. The mean induration of the reaction in 10 patients with ML was 11.3 mm ± 7.15, in 41 patients with HCL, 9.27 mm ± 6.78 and in 20 patients with ACL 10.7 mm ± 6.10 mm. The percent of patients with 5 mm or greater induration was ML 80%, HCL 71%, ACL 90%. Thus, we could not confirm an association between enhanced delayed hypersensitivity and mucosal involvement in leishmaniasis.

Key Words: Skin test comparison. Antigen concentrations. Clinical forms. American mucocutaneous leishmaniasis.

A delayed hypersensitivity cutaneous reaction to leishmanin is present in most forms of cutaneous and mucocutaneous leishmaniasis with the exception of diffuse cutaneous leishmaniasis and some cases of post kala-azar dermal leishmaniasis[6]. The skin test was first proposed by Wagener in 1923[28], and was introduced as a diagnostic aid in American cutaneous leishmaniasis in man by Montenegro in 1926[18]. It is the most widely used skin test in parasitic disease and is a valuable ancillary investigation especially in mucosal disease where parasites are often difficult to find.

Shaw and Lainson 1975[27] observed that patients with mucosal leishmaniasis tended to have larger delayed hypersensitivity reactions than those with only cutaneous lesions. This suggested that mucosal disease could be associated with enhaced hypersensitivity response to leishmanial antigens.

This study was designed to compare a) three concentrations of antigen in three forms of American cutaneous leishmaniasis and b) to examine the hypothesis that mucosal disease is associated with more marked cutaneous hypersensitivity than cutaneous lesions.

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MATERIALS AND METHODS

1) *Patient population* – Ninety-nine patients were studied living in the area of Três Braços, Bahia, Brazil, were mucocutaneous leishmaniasis is endemic[4].

The following groups were established:

a) 20 patients with active cutaneous leishmaniasis (ACL) presenting as a skin ulcer.
b) 10 patients with mucosal lesions (ML) of the nose or oropharynx as well as a skin scar or active ulcer.
c) 41 patients with the old scars of cutaneous leishmaniasis (HCL) which are very typical in appearance (Figure 2)
d) 28 patients living in the same area without signs or a history of leishmaniasis.
e) 10 volunteers at the University of Brasília cons-

titued a control group without any history of leishmaniasis and living in a nonendemic area.

Of the thirty patients with active cutaneous or mucosal lesions 23 (76.6%) had positive indirect fluorescent antibody titers (promastigote antigen) and in 20 patients, parasites were isolated from the lesions (Table 1). Eight stocks from these patients were identified as *Leishmania braziliensis braziliensis* by isoenzyme analysis[7].

2) *Antigens* – A stock *Leishmania mexicana amazonensis* (Josefa) isolated from a patient in Brasília was used to prepare the antigens for skin testing. This organism was maintained by serial passage in hamsters. It gave a rich growth of promastigotes in LIT medium (Liver Infusion Tryptose) and was harvested for these studies after eight days.

The organisms were washed twice in phosphate buffered saline (pH 7.2), centrifuged, resuspended in distilled water and sonicated (five periods of one minute each at 20 kh at 4° C). Specimens of the mixed suspension were used to determine total nitrogen concentration by the micro-Kjeldahl method[10]. The solution was adjusted to three concentrations of antigen namely 300μg Nitrogen per ml, 96μg N/ml and 30 ug N/ml using 0.5% phenol in saline. These antigens were kept at – 20° C until use. The antigens were tested to confirm their activity in hospitalised patients with known leishmanin skin reactions before use in this field trial.

Table 1 – *Clinical and parasitological findings in 30 active cases of Leishmaniasis (cutaneous and mucosal diseases) submitted to skin test.*

Patient n.º	*Clinical Form*	*Time of Evolution*	*N.º of Lesions*	*Age*	*Serology (IFAT)*	*Parasites*	*Identification (I = Isoenzymes)*
1	C *	2m ***	2	55	1:40	+	L.b.b. (I)
2	C	12y	1	40	–	+	
3	C	5m	1	42	1:80	–	
4	C	2,5m	2	60	1:40	+	L.b.b. (I)
5	C	8m	1	16	–	+	L.b.b. (I)
6	C	1m	1	19	1:40	+	L.b.b. (I)
7	C	2m	1	15	1:160	–	
8	C	3m	3	8	1:160	+	
9	C	4m	1	18	1:160	+	L.b.b. (I)
10	C	5m	4	4	1:80	+	
11	C	6m	1	3	–	–	
12	C	1.5m	1	18	1:40	+	
13	C	2m	1	53	1:40	+	L.b.b. (I)
14	C	1.5m	1	45	1:40	+	
15	C	3m	1	50	1:80	+	
16	C	5m	1	48	1:40	+	L.b.b. (I)
17	C	1y	2	66	1:40	+	
18	C	2.5m	1	65	1:20	–	
19	C	2m	1	28	–	+	
20	C	3m	1	30	–	+	
21	M **	2y	1	35	1:40	–	
22	M	3y	4	59	1:20	–	
23	M	3y	1	23	1:80	–	
24	M	5.5y	1	35	1:40	–	
25	M	4y	1	49	1:640	+	
26	M	1.5y	1	10	–	N.D	
27	M	2y	1	24	1:160	+	L.b.b. (I)
28	M	9y	1	37	1:2560	+	
29	M	2y	1	9	1:160	N.D	
30	M	2y	1	34	–	+	

* C: Cutaneous disease
** M: Mucosal disease
*** m: months
y: years
L.b.b.: *Leishmania braziliensis braziliensis*
N.D.: Not Done

3) *The cutaneous test* – The antigen suspensions were always mixed thoroughly before use. Each concentration was inoculated intradermally into the ventral surface of the forearm in a dose of 0.1 ml (Fig. 1). A control solution of the same volume of 0.5% phenol in saline was also inoculated at the same time. At 48 hours the size of induration at each inoculation site was measured with a millimeter calliper. An induration of 5 or more millimeters was regarded as a positive reaction but detailed measurements were taken. Figure 2 shows patients with the typical mucosal lesion and a cutaneous scar. The results of skin testing were analysed calculating the mean induration in millimeters and standard deviation for each group.

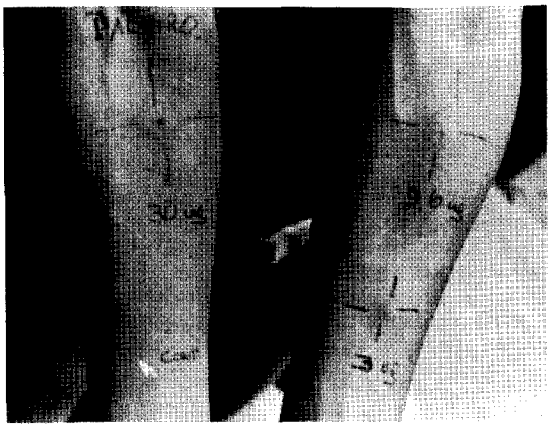

Figure 1. Results of delayed hypersensitivity skin testing using three doses of antigen and control read at 48 hours. The graded dose response is seen.

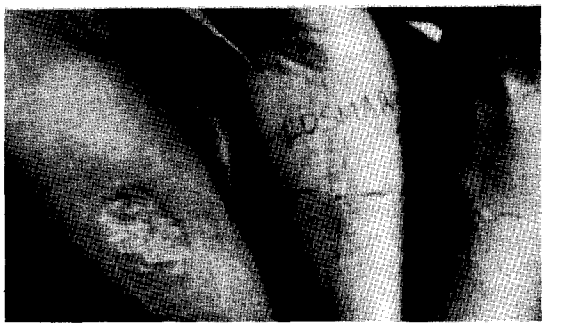

Figure 2. A 7 year old boy with total absence of nasal septum from mucosal leishmanisasis and a 13 year old girl with positive graded skin tests on the left forearm and a characteristic scar on the right forearm. Such scars resemble burns being slightly depressed, hypopigmented and fibrotic.

RESULTS

Table 2 shows the results of the skin test using the three antigen concentrations in patients with active cutaneous lesions (ACL) and mucosal lesions (ML), scars, and controls from the endemic area. The frequency of positive reactions in the controls from the endemic region is very low and present only at high antigen concentrations (4% using a dose of 9.6 μgN). In patients with active disease or a scar, skin reactivity increased with the antigen concentration. There was no difference between these two groups in the number of positive skin reactions at the highest concentration. The highest concentration (30 μg/0.1 ml) however was unaceptable since it occasionally caused necrosis at

Table 2 – *Delayed hypersensitivity responses to three concentrations of leismanin in three patient groups.*

Patient Groups	*Number of Patients Studied*	*Number of Patients with Reaction of ≥ 5mm Using the Concentrations of Antigen (μg N/0.1 ml) Given Below.*		
		3	9.6	30
a – b Active Lesions (ACL and ML)	30	26 (87%)	29 (97%)	30 * (100%)
c Scars	41	27 (68%)	34 (83%)	40 ** (98%)
d Control	28	0	1 (4%)	2 (7%)

* 3(10%) and ** 7(17%) of patients with necrotic cutaneous reaction

the inoculation site and axillary lymphadenopathy (10% and 17% of patients with active or healed disease, respectively). 9.6 μg/0.1 ml was the most satisfactory dose for routine diagnostic work since it was very sensitive but induced a degree of skin reaction which was only of temporary inconvenience. The 10 volunteers from the University of Brasília were negative to all three antigen concentrations.

Reaction size in relation to antigen dose is expressed diagramatically in Figure 3 for patients with active lesions. This clearly shows the marked difference that concentration of antigen makes in skin test reactivity in this disease. 3 μg/0.1 ml yielded a scatter of skin reaction in patients with active disease, whereas 30 μg always yielded reaction greater than 10 mm. In Table 3, the three antigen concentrations are related to lesions duration in eight recently acquired cases of *L.b.brazilensis* infections. Positive reactions to all three antigens were obtained in one month after onset of the active lesions. The mean skin test reactivity to 3 μg compared to time after initial lesion was 10 mm for less than 5 years, 9 mm for 6 to 10 years, 14 mm for 11 to 20 years in both groups with healed scars and mucosal disease. The intensity of the skin test could not be related to the age of the scar in the inactive group except that three patients with scars more than 30 years in the past yielded reactions to 3 μgN of 5,4 and 0 mm induration consistent with a slight age related decrease in Montenegro skin test reactivity.

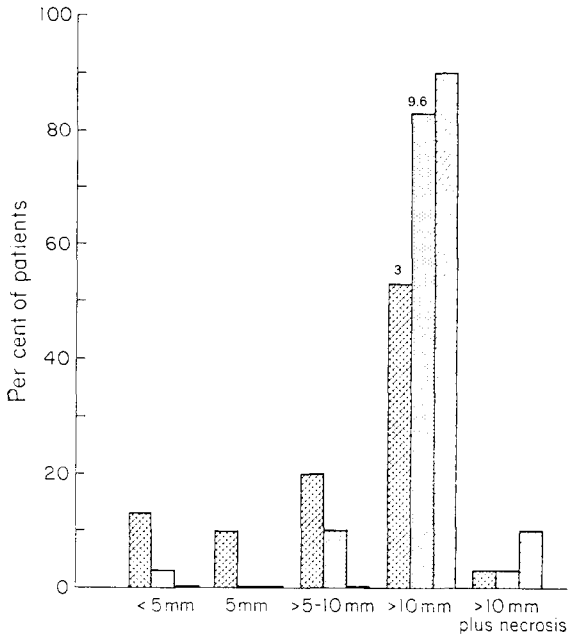

Figure 3. *Comparison of the magnitude of the skin test reaction to three concentrations of leishmanial antigen in 30 patients with active lesions.*

The dose of 3 μgN/0.1 ml was selected to examine the relationship between mucosal disease and degree of skin reactivity. Table 4 lists the induration

Table 3 – *Results of the three doses of skin test antigen in eight patients with proven active* L. braziliensis braziliensis *infection of less than 5 months in duration.*

Patient Number	*Duration of Disease (months)*	*Magnitude of the Reaction in Millimeters **		
		3 μg N/0.1 ml	9.6 μg N/0.1 ml	30 μg N/0.1 ml
1	1	7.5	11.6	15.9
2	2	5.0	13.2	14.2
3	2	5.5	8.0	13.0
4	2.5	13.1	18.7	21.0
5	2	7.0	12.0	19.0
6	3	20.5	18.3	21.7
7	4	15.0	14.0	23.8
8	5	7.5	10.8	10.0

(*) Mean induration size for the three groups are 10, 13.3 and 17.3 mm respectively. Standard deviation. (S_x) 5.50; 3.66; 4.80.

Table 4 – *Skin testing in the various forms of Leishmaniasis. Mean diameter of induration using the 3 μg N/0.1 ml*

Clinical form of Leishmaniasis	*Number of Patients Studied*	*Mean Induration in Millimeters ± 1 SD*
Active cutaneous	20	10.7 ± 6.10 *
Mucosal	10	11.3 ± 7.15 *
Scar	41	9.3 ± 6.78

(*) Not significant ($p < 0.05$) compared to scars only

diameter in three groups of patients. No significant difference between mucosal and cutaneous lesions in size of the delayed hypersensitivity reaction was noted. By comparing the percent of patients with reactions at 5 mm or greater, no differences were detected (ML 80%, HCL 71%, ACL 90%).

DISCUSSION

Our study confirms that the Montenegro skin test is a useful indicator of active or previous cutaneous leishmaniasis, but that the dose of antigen used determines the sensitivity of the test. 9.6 μgN per dose was found to be the minimum concentration which identified most patients. We confirmed that the skin test was always positive by one month after onset of an active lesion, and persisted with the same degree of reactivity for at least 20 years. Thereafter, a slight decrease in skin test reactivity was noted. We found no correlation between the occurrence of mucosal disease due to leishmaniasis and enhanced skin test reactivity.

Skin testing with leishmania antigen to document the prevalence of cutaneous leishmaniasis has been used for many years. It has been considered a test with good specificity[19] an observation confirmed in our Brazilian control group, but few careful studies have been done[13]. Furtado[9] has summarised what is known in Brazil regarding cross reactions with other diseases. Patients with leprosy and previous visceral leishmaniasis may react to leishmanin. Both these infections are very rare in our study area.

When tests have been done in regions endemic for cutaneous leishmaniasis wide variations in skin test reactivity in patients without a history of disease or a scar have been reported. In our area we have found, in other studies[4], that 6% of such patients yield a positive test. In the state of São Paulo 25.6% of people with positive skin reactions had no visible sign of leishmaniasis[23] in Minas Gerais 13.4%[12] in the Xingu National Park for Indians in Mato Grosso 62.5%[1], in Amazonia 32.9%[8] and in Rio de Janeiro state figures vary from 3.7 – 24% depending on the community studied[17 26]. While different conditions of the test may account for some of this variation it is likely that subclinical infections occur without leaving a recongnisable scar.

Increased sensitivity of the test by use of high antigen concentration was first reported by Rotberg[24] who showed that with a concentration of 1 x 10^7 promastigotes per milliliter 92% of patients with cutaneous leishmaniasis had positive reactions. Melo et al[16], utilizing a concentration of 40 μg of protein nitrogen per milliliter had a 96% positivity rate and suggested that an antigen of this strenght was desirable. Our results support their findings but high strength antigens do produce undesirable skin reaction. On the other hand, we found that when the weakest antigen concentration was used 32% of the 41 patients with scars were non reactors. This is important since leishmanin skin test surveys are frequently used to assess the incidence and prevalence of cutaneous leishmaniasis using similar antigen concentrations.

Our studies also confirm that antigens prepared from a different organism, *L. m. amazonensis*, induce satisfactory skin reactions in patients with proven lesions due to *L. braziliensis braziliensis*. This is not surprising since heterologous antigens outside the leishmania genus, such as *T. cruzi*[21 22] and *Leptomonas pessoai*[3] yield positive reactions in cutaneous leishmaniasis.

The time of onset of skin test reactivity and its duration after the initial lesion has been of interest. We have noted, as have others[2 21], that the test may be negative early in infection but, by one month, nearly all are positive.

Recently three groups of workers have studied leishmanin reactivity some years after an initial test and reported a decline in incidence of positive reactors[14 15 20]. This is a variance with the view that, once established, skin hypersensitivity persists indefinitely. Analysis of our data does not suggest that the time elapsed between the healing and the skin testing influences the intensity of the reaction except when lesions have been healed for more than 20 years[19].

The destructive effects of espundia have been attributed to a hypersensitivity phenomenon[5] or autoimmune reaction[11]. It has been suggested that the basic mechanisms of cutaneous and mucosal disease depend on the toxicity and immunogenicity of the parasites[25]. To test the hypothesis that mucosal disease is associated with a greater degree of skin hypersensitivity to leishmanin it was necessary to select the results using the lowest concentration of antigen in view of our data in Table 2. We detected no significant difference in skin test reactivity between mucosal and active cutaneous cases or mucosal and healed cutaneous cases. This is at variance with the observations made by Shaw and Lainson 1975[27], in a different region of Brazil.

Our data confirm the necessity to standardise the procedure of the Montenegro both in the antigen used and the methodology of the test reading. Ideally, one carefully standardized antigen should be distributed by an international agency. This standardization should include careful taxonomy of the strain used for antigen production, concentration of promastigotes per milliliter, preparation method for preserving the antigens and total nitrogen determination. Each lot is best standardized biologically in infected man against a stock of antigen of known activity. An exoantigen has been reported to yield an immediate hypersensitivity reaction in 74.4% of 41 patients[27]. This observation is of practical importance and should be further investigated since return after two days to read the reaction is often difficult under field conditions.

ADDENDUM

Recently Castes M, Agnelli A, Verde O & Rondón AJ. (Characterization of the cellular immune response in American cutaneous leishmaniasis. Clinical

Immunology and Immunopathology 27: 176-186, 1983), have shown that the skin test response (as mean induration in mm.) was significantly greater in mucocutaneous leishmaniasis (MCL, 11 patients) than in 32 patients with localized cutaneous leishmaniasis (LCL).

This difference from our results might be explained by the small groups examined in both series and the fact that some of our patients with mucosal disease had secondary malnutrition which would depress skin responses.

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