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MUCO-CUTANEOUS LEISHMANIASIS: INTRADERMAL TEST WITH A PROMASTIGOTE SUSPENSION AND A CRUDE EXTRACT FROM LEISHMANIA BRAZILIENSIS

J. PELLEGRINO (1), Leógenes H. PEREIRA (2) and T. A. FURTADO (3)

SUMMARY

A comparative study between two different antigens (a promastigote suspension and a crude extract from **Leishmania braziliensis**) was carried out in 16 patients with muco-cutaneous leishmaniasis. Different parameters (diameters, area, and volume) were used for the reading of skin reactions. The statistical analysis showed a greater sensitivity of skin reaction to the crude extract ($p < 0.01$) over the promastigote suspension.

INTRODUCTION

The skin test with antigen from **Leishmania braziliensis** was firstly used for the immunological diagnosis of cutaneous leishmaniasis by MONTENEGRO [7], following the experiments of WAGENER [13] in laboratory animals with an alkaline extract of promastigotes of **Leishmania tropica.** A solution of sodium chloride and sodium carbonate was added to the **in vitro**-grown and saline-washed organisms and, after centrifugation, the supernatant fluid was used as antigen. Later, it was demonstrated that the activity of such extract was due to the presence of promastigotes in the liquid, as mentioned by CORRÊA & AMATO NETO [2]. Thus, suspensions of organisms from culture were employed for the intradermal test, with or without sonnication (CORRÊA & AMATO NETO [2]). The parameters used to standardize the antigens were the number of flagellates (**Leishmania braziliensis** or **Leishmania tropica**) (GOMES [4]; CORRÊA & AMATO NETO [2]; SERGIEV & SHUIKINA [10]; SHAW & LAINSON [11]) or the amount of nitrogen per ml of the solution (MELO et al. [6]). As a preservative, phenol (MONTENEGRO [7]; GOMES [4]; SERGIEV & SHUIKINA [10]; SHAW & LAINSON [11]) or thimerosal (CORRÊA & AMATO NETO [2]; MELO et al. [6]) were used. BARBOSA et al. [1] used promastigotes of **Leptomonas pessoai** as a substitute for **Leishmania braziliensis.**

Several extracts were also tested as substitutes for the promastigote suspensions. A crude from **L. braziliensis** was described by PELLEGRINO & FURTADO [9], as well as by SERGIEV & SHUIKINA [10] with **L. tropica.** A polysaccharide antigen was also reported by PELLEGRINO [8] and FURTADO & PELLEGRINO [3]. SHAW & LAINSON [12] used the supernatant of culture of promastigotes in dialysis membrane sacs for preparing an exo-antigen.

The quantitative readings of the skin reaction itself were usually described as "marked positive", "distinctive positive", "positive" "doubtful", or "negative" (4+, 3+, 2+, 1+, and 0, respectively) (GOMES [4]; CORRÊA & AMATO NETO [2]). A more accurate study was carried

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(1) Institute of Biological Sciences, Federal University of Minas Gerais and «Centro de Pesquisas René Rachou», FIOCRUZ, Belo Horizonte
(2) Institute of Biological Sciences. Department of Parasitology
(3) Department of Dermatology, Federal University of Minas Gerais
Address for reprints: C. Postal 1404, 30.000 Belo Horizonte, Brasil

out by MELO et al.[6] who established a positive correlation between the area of the skin reaction and the nitrogen concentration of the antigen.

The present studies have been started more than a decade ago, when attempts to standardize the reading of the intradermal test for the diagnosis of schistosomiasis on a quantitative basis were performed (see review by KAGAN & PELLEGRINO[5]). A brief summary of the results were presented at Ibero-Latin American Meeting on Dermatology, Lisbon, 1959. Owing to the interest of this subject it was decided to publish this and subsequent papers in full.

MATERIALS AND METHODS

A — **Culture medium** — The N.N.N. medium was used and the growth of **L. braziliensis** maintained for 10-12 days, and then the liquid phase with the flagellates was removed.

B — **Promastigote suspension** — The organisms from the culture medium were washed by centrifugation several times with saline and then suspended in Coca's solution with thimerosal 1/5,000 in order to get a final concentration of 1×10^7 flagellates per ml. The amount of nitrogen was 35 μgN/ml.

C — **Crude extract** — The washed promastigotes were lyophilized, broken in a mortar with fine sand and suspended in the same excipient to a concentration of 1/1,000 (37.5 μgN/ml), and stored at 4°C with occasional shaking for 5 days, then centrifuged at 800 g for 3 minutes. The supernatant was used as antigen.

D — **Patients** — The skin reactions were carried out in 16 patients with a clinical diagnosis of cutaneous leishmaniasis, all of them showing a positive Montenegro's test (promastigote suspension, 1×10^6 flagellates per ml, 0.5% phenol), with the exception of patient number 2, presenting a negative test but with a laboratory finding of the parasite in the lesion. The ages ranged from 23 to 53 years, case number 8 and 9 were women.

E — **Skin test** — Both antigens (0.1 ml) were injected intradermally in the flexor surface of the right or left forearm (with previous random choice). The readings of the tests were performed after 48 hours taking note of the following parameters (in cm):

D = larger diameter
d = minor diameter
h_o = skin thickness out the area of the reaction
h_1 = larger thickness of skin, in the area of the reaction.

Skin thickness was measured with the aid of a pachymeter up to 0.1 mm.

The area was estimated by the formula

$$A = \frac{\pi \times D \times d}{4}$$ and the volume by

$$V = \frac{(h_1 - h_o)}{2} \times A.$$

RESULTS

Quantitative findings are summarized in Table I. As can be seen, more intense reactions were observed with the crude extract (CE) in comparison with the promastigote suspension (PS), when evaluated by the different parameters (diameters, area, or volume). A statistical significant difference ($p < 0.01$) was found between CE and PS reactions with the above measurements (Student's test for paired observations). Twenty control individuals reacted negatively to both antigens.

DISCUSSION

At least six different antigens were described for Montenegro's skin test: alkaline extract from fresh saline-washed promastigotes, crude extract (also alkaline) from dried and mortar-broken organisms, suspension of flagellates without sonnication, sonnicated suspension of the parasite, exo-antigen from the supernatant of cultures, and polysaccharide antigen.

When promastigote suspension (PS) and crude extract (CE) were compared for the skin test in the same patient, the data obtained showed a marked increased sensitivity of the CE over PS. There is no question about the superiority of the PS over the early alkaline

T A B L E I

Cutaneous leishmaniasis: skin reaction

Comparative study between a promastigote suspension (1 x 10^7 organisms per ml) and a crude extract (1/1,000)

Patient number	Promastigote suspension						Crude extract					
	D	d	h_o	h_1	Area (cm^2)	Volume (cm^3)	D	d	h_o	h_1	Area (cm^2)	Volume (cm^3)
1	0.6	0.6	0.20	0.35	0.3	0.0	2.0	1.5	0.28	0.45	2.4	0.3
2	0.0	0.0	0.20	0.20	0.0	0.0	0.0	0.0	0.20	0.20	0.0	0.0
3	1.8	1.6	0.18	0.40	2.3	0.3	1.5	1.5	0.16	0.48	1.8	0.3
4	1.3	1.3	0.25	0.64	1.3	0.3	3.5	2.6	0.25	1.0	7.1	2.7
5	1.0	1.0	0.23	0.42	0.8	0.1	3.0	2.0	0.20	0.55	4.7	0.8
6	1.7	1.5	0.26	0.60	2.0	0.3	4.0	3.2	0.23	0.80	10.1	2.9
7	1.7	1.5	0.20	0.65	2.0	0.5	3.5	2.0	0.20	0.67	5.5	1.3
8	4.5	3.0	0.20	1.00	10.6	4.2	4.5	2.0	0.19	0.85	7.1	2.3
9	4.0	2.5	0.25	1.00	7.9	3.0	4.7	3.5	0.28	1.00	12.9	4.7
10	1.3	1.1	0.20	0.60	1.1	0.2	1.7	1.7	0.20	0.70	2.3	0.6
11	1.4	1.0	0.30	0.60	1.1	0.2	2.3	2.3	0.30	0.90	4.2	1.3
12	0.8	0.8	0.20	0.70	0.5	0.1	1.5	1.5	0.20	0.80	1.8	0.6
13	1.6	1.6	0.20	0.90	2.0	0.7	3.5	3.2	0.20	1.10	8.8	4.0
14	2.7	2.0	0.30	0.86	4.2	1.2	3.5	2.4	0.25	1.30	6.6	3.5
15	3.5	3.0	0.28	0.70	8.2	1.7	6.5	4.5	0.29	1.16	23.0	10.0
16	1.2	1.2	0.25	0.38	1.1	0.1	3.5	2.0	0.23	0.66	5.5	1.2
Mean and standard error	1.82 ± 0.46	1.48 ± 0.37	0.23 ± 0.06	0.63 ± 0.16	2.84 ± 0.71	0.80 ± 0.20	3.08 ± 0.77	2.24 ± 0.56	0.22 ± 0.06	0.79 ± 0.20	6.47 ± 1.62	2.26 ± 0.57

extracts, as described by WAGENER [13] and MONTENEGRO [7], but CE is prepared with dried and mortar-broken organisms instead of from fresh promastigotes and certainly it differs, at least quantitatively, from those.

It seems that alkaline extractions from entire promastigotes gives relatively poor results. On the other hand, an extract of particulated organisms, probably showing quantitatively more substances in solution, would present also unsolved particles in suspension, which could explain the good results shown by CE. The increased sensitivity of skin reaction to CE over PS (both antigens with the same amount of nitrogen per ml) probably is due to the solubilization and fragmentation of the antigenic material in the former, instead of whole organism suspension.

RESUMO

Leishmaniose cutâneo mucosa: Intradermorreação com suspensão de promastigotas e um extrato bruto de Leishmania braziliensis

Um estudo comparativo entre dois diferentes antígenos (suspensão de promastigotas e extrato bruto de **L. braziliensis**) foi realizado em 16 pacientes com leishmaniose tegumentar. Diversos parâmetros (diâmetros, área

e volume) foram usados para a leitura das intradermorreações. A análise estatística dos dados demonstrou maior sensibilidade das reações ao extrato bruto ($p < 0,01$), no mesmo paciente, que as observadas com o outro antígeno.

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