

EVALUATION AND CHARACTERIZATION OF PARTIALLY PURIFIED SKIN TEST ANTIGENS PREPARED FROM *LEISHMANIA PANAMENSIS* PROMASTIGOTES

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Abstract: The present study was designed to evaluate skin test preparations prepared from *Leishmania panamensis* promastigotes in 30 active cutaneous leishmaniasis patients. The crude antigen preparation (CA) used was 10,000×g supernatant of the parasites-homogenate. The soluble extract was further resolved into 4 preparations (FA-1 to -4) with the aid of a Sephacryl S-200 gel filtration. There was no significant difference in the positive ratio and the average induration size between CA (10 μg protein/test) and Montenegro's antigen (MA; 5×10⁶ parasites/test). The reactivity of the delayed-type hypersensitivity to 10 μg dose of CA was shown with much the same intensity in the 25 μg dose of CA. In FAs (10 μg protein dose, except for 7.5 μg in FA-4), the positive ratio was as follows: 90.0% in FA-1, 77.8% in FA-2, 75.0% in FA-3 and 37.5% in FA-4. The positive ratio and the intensity of skin test response in FA-4 were remarkably low in comparison with those in CA or MA. Significant difference was found in the intensity of response between FA-3 and CA or MA. Based on these results, therefore, we concluded that 10 μg protein dose of CA of *L. panamensis* and same dose of the fractionated preparations, FA-1 and -2, were very suitable for the diagnosis of cutaneous leishmaniasis in endemic areas of the New World. Furthermore, it was estimated that at least some or all of the 5 proteins, approximately 66, 55, 45, 28, and 26 kD, were related to a specific delayed-type hypersensitivity in cutaneous leishmaniasis of the New World.

INTRODUCTION

The intradermal skin test is widely used for a presumptive diagnosis of visceral and cutaneous leishmaniasis in endemic areas of the Central and South America. Although the antigen commonly used is a suspension of whole parasites in phenolized saline (Buss, 1929),

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a little information on other preparations prepared from promastigote-extracts have been also reported (Furtado and Pellegrino, 1956; La Placa *et al.*, 1975; Shaw and Lainson, 1975; Reed *et al.*, 1986). Among them, Reed *et al.* (1986) have reported that a crude soluble extract prepared from ruptured *Leishmania chagasi* promastigotes was highly sensitive and specific for the diagnosis of American visceral leishmaniasis. We have also demonstrated that a similarly prepared soluble extract obtained from *L. braziliensis* complex was very useful for the screening of cutaneous leishmaniasis in endemic areas of Ecuador (Furuya *et al.*, 1989). In order to gain a better information on a standardization of skin test preparation and the antigen dose, we designed preliminary examinations to evaluate skin test preparations prepared from *L. panamensis* promastigotes. The present paper reveals that the soluble promastigotes extract and fractionated preparations of the soluble extract are highly sensitive for the diagnosis of active cutaneous leishmaniasis, and a characterization of protein components of the preparations will be discussed in this paper.

MATERIALS AND METHODS

Skin test preparations

Crude antigen preparation (CA): *L. panamensis* (MHOM/PA/71/LS94) obtained from Dr. P. Desjeux, PDP, WHO (formerly Instituto Boliviano de Biología de Altura, Bolivia) was cultured with the medium described by Pan (1984). After washing of parasites with a balanced salt solution, the harvested promastigotes were ruptured by a freeze-thawing procedure and centrifuged at $10,000 \times g$ for 30 min at 4°C (Furuya *et al.*, 1989). The supernatant was filtered through 0.45 μ sterile filter and lyophilized as CA.

Fractionated antigen preparation (FA): The above mentioned soluble extract was further resolved into 4 preparations, designated FA-1, -2, -3, and -4, with the aid of a Sephacryl S-200 (Pharmacia, Uppsala, Sweden) column being in equilibrium with 0.02 M phosphate buffered saline (pH 7.2). The solution of each peaks was condensed by ultrafilter (MW cut-out 10,000; Advantex Co., Japan), dialyzed against PBS, and then centrifuged by $10,000 \times g$ for 30 min at 4°C. After filtration, these FAs were adjusted 100 μ g protein concentration per ml, except for 75 μ g in FA-4.

Montenegro's antigen preparation (MA): 5×10^7 whole promastigotes per ml in sterile saline containing 0.5% phenol was used (Bray, 1985).

Skin test

Skin test was made on 30 patients with active cutaneous leishmanial lesions. The preparations were injected intradermally in 0.1 ml in flexor surface of the forearm. The skin test area was observed for erythema and induration at 48 hrs. Induration size of more than 5 mm (mean value of length and breadth) at the injection site was considered a positive reaction.

Analysis of components of CA and FAs

CA and FAs were solubilized with 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. Samples were analyzed by SDS-PAGE as described by Laemmli (1970). After electrophoresis gel was stained by 0.25% Coomassie brilliant blue R250.

RESULTS

Skin test

Intradermal skin test using MA, CA, and FAs was carried out against 30 patients. The results were shown in Tables 1 and 2. Most of the patients had one or two active cutaneous lesions infected at 3 to 8 months ago. Parasites were confirmed from the lesions of 29 patients by smear specimens or culture method. Ten strains of *Leishmania* isolated from these patients were characterized by performing zymodeme, serodeme and schizodeme analysis (McMahon-Pratt *et al.*, 1982; Lopes *et al.*, 1984): 9 strains were identified as *L. panamensis*, and one strain was *L. braziliensis* (Table 1). The detailed characterization of these strains will be reported elsewhere.

Intensity of induration size of skin test using CA and MA was shown in Table 2. The CA at 25 μ g protein dose gave positive response in all 14 individuals, but, a superabundant response was also recognized in 2 subjects (Table 2). The 10 μ g protein dose of CA and MA gave 87.5% (14/16) and 93.8% (15/16) positivity, respectively. No response against CA and MA was observed in one patient. This patient had numerous lesions on the upper side of his right arm and had been treated with a corticosteroid for 3 months. There was no significant difference in the positive ratio and the average induration size between 25 and 10 μ g dose of CA. Moreover, the reactivity of the delayed-type hypersensitivity (DTH) to 10 μ g dose of CA was shown with much the same intensity in the MA.

As shown in Fig. 1, the soluble promastigotes extract was separated seven to eight peaks by Sephacryl S-200 gel filtration. An evaluation of skin test using FAs was made on 10

Table 1 Intradermal skin test responses to Montenegro's antigen (MA), crude antigen (CA), and fractionated antigens (FA) prepared from *Leishmania panamensis* in 10 individuals with active cutaneous leishmanial lesions

No.	Sex*	Age	Months after infection	Induration size (mm)						Parasite	
				MA	CA	FA-1	FA-2	FA-3	FA-4	Smear†	Species‡
1	M	28	3	9	8	6	7	6	4	+	L.p.
2	M	41	4	16	20	25	19	8	5	+	L.p.
3	M	23	3	12	10	16	18	16	9	+	L.b.
4	F	25	3	10	11	20	2	2	2	+	L.p.
5	M	19	3	6	4	5	5	5	4	+	L.p.
6	M	22	4	10	11	4	2	3	2	+	L.p.
7	M	21	4	16	17	10	16	7	3	+	L.p.
8	M	21	4	20	12	7	7	7	6	+	L.p.
9	M	38	12	12	15	22	N.D.	N.D.	N.D.	+	
10	M	40	?	13	18	10	10	N.D.	N.D.	-	
Positive ratio				100.0	90.0	90.0	77.8	75.0	37.5		
Average induration size (\pm SD)				12.4 (4.1)	12.6 (4.9)	12.5 (7.7)	9.6 (6.6)	6.8 (4.3)	4.4 (2.3)		

*M, male; F, female †+, positive; -, negative

‡L.p., *Leishmania panamensis*; L.b., *Leishmania braziliensis*

Table 2 Frequency distribution of induration size of leishmanial skin test using crude antigen (CA) and Montenegro's antigen (MA) in 30 patients with active cutaneous leishmanial lesions

Induration size (mm)	CA		MA
	25 (μg protein)	10	5×10^6 (parasites)
< 5		2	1
5 ~10		3	4
11~15	6	7	6
16~20	6	3	3
21~25		1	2
>26	2*		
Average induration size	20.1 ± 12.7	13.3 ± 7.2	13.1 ± 5.8

* Induration size of 2 examinees was 38×27 mm and 80×40 mm, respectively.

patients. The positive ratio against each FAs was as follows: 90.0% (9/10) in FA-1, 77.8% (7/9) in FA-2, 75.0% (6/8) in FA-3, and 37.5% (3/8) in FA-4. Among 8 subjects received all of the FAs, 3 patients reacted to all of the FAs, 2 patients reacted to FA-1, -2 and -3, and 1 patient only reacted to FA-1. In the last patient (No. 4), however, 6, 13, and 6 mm size of erythema was observed in the injection site of FA-2, -3, and -4, respectively. On the other hand, one patient (No. 6), showing positive reaction against MA and CA, did not react against all of the FAs. There was significant difference in positive ratio of skin test response between FA-4 and CA or MA ($p < 0.001$). Furthermore, significant difference was found in the average induration size between FA-3 and CA or MA ($p < 0.025$).

SDS-PAGE profiles of CA and FAs

Lane 1 in Fig. 2 shows the Coomassie brilliant blue R250-stained profile of the CA, and lanes 2 to 5 show the pattern of FAs. About 35 bands were recognized in lane 1 (CA). Seven bands were weakly stained in lane 2 (FA-1), 25 bands were recognized in lane 3 (FA-2), 18 bands were observed in lane 4 (FA-3), and 9 bands in lane 5 (FA-4). Among these bands, 4 bands migrating in the region approximately 66, 55, 45, and 26 kilodalton (kD) were common to all of the FAs. SDS-PAGE proved that most of protein components of the soluble promastigotes extract of *L. panamensis* was recovered in FA-2 area by Sephacryl S-200 gel filtration.

DISCUSSION

Until recently, no exact information has been available on species or subspecies level characterization of the genus *Leishmania* of cutaneous leishmaniasis in this country. Three strains of *Leishmania* isolated from active cutaneous leishmaniasis patients have been first characterized as *L. panamensis* by isoenzyme electrophoresis and monoclonal antibodies

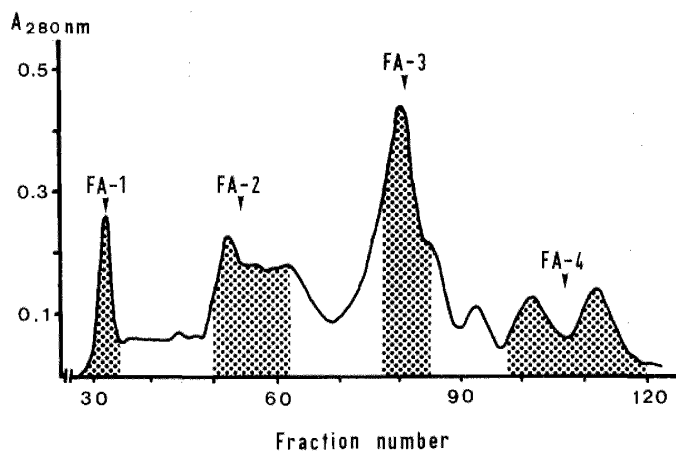


Figure 1 Effluent pattern of soluble protein extract of *L. panamensis* by Sephacryl S-200 gel filtration. Each of the shaded area of figure was collected and concentrated by ultrafilter as a FA preparation of skin test. column, 2.5×60 cm; buffer, 0.02 M phosphate buffered saline (pH 7.2); sample, 2.5 ml of soluble protein extract of *L. panamensis*; flow rate, 18 ml/hr; fraction, 3 ml/tube.

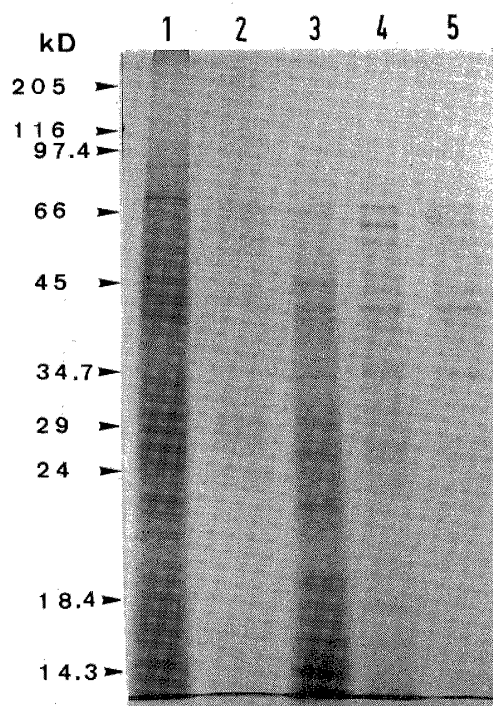


Figure 2 SDS-PAGE profile of soluble extract preparations prepared from *L. panamensis*-promastigotes. Lanes: 1, crude antigen preparation (CA; 45 μ g protein); 2, fractionated antigen preparation FA-1 (14 μ g protein); 3, FA-2 (48 μ g protein); 4, FA-3 (28 μ g protein); 5, FA-4 (17 μ g protein). The electrophoresis was done at 6.5 mA for 12 hrs. The positions of molecular size are indicated. kD, kilodaltons. 12% gel.

(Mimori *et al.*, 1989). Of the 26 strains newly isolated in this country, 23 were identified as *L. braziliensis* complex (12 *L. panamensis*, 7 *L. guyanensis* and 4 *L. braziliensis*) by enzyme electrophoresis (Armijos *et al.*, 1990). In the present studies, therefore, we used *L. panamensis* promastigotes for preparing of the present skin test preparations.

Although a standerdization of antigen concentration in intradermal skin test for visceral and cutaneous leishmaniasis had not yet been done, 50 to 25 μg protein dose is used normally (Kerdel-Vegas, 1982; Bray, 1985; Reed *et al.*, 1986). In the present study, it was definitely shown that 10 μg dose of CA was able to stand comparison with a relatively large number of promastigotes of MA in detecting DTH in patients with active cutaneous lesions. There was no significant difference in the positive ratio and the average induration size between 25 μg and 10 μg antigen dose of CA. In the test with 25 μg protein dose of CA, superabundant intradermal response was observed in a few active and some cured cutaneous leishmaniasis individuals (data not shown). From these results, 10 μg protein dose of soluble promastigotes extracts (CA) of *L. panamensis* will be suitable for the diagnosis using intradermal skin test against cutaneous leishmaniasis in the New World.

For a characterization of skin test antigens, it has been recently reported that two defined glycoconjugates purified from *L. amazonensis* was able to induce a specific DTH response to infected susceptible and resistant mice strains, and that one of the glycoconjugates was a degradation product of a 17 kD antigen present in promastigotes and amastigotes (Rodrigues *et al.*, 1986a, b). Partially purified antigens containing 94 to 64 kD proteins, derived from *L. infantum* or *L. major* promastigotes and isolated under reducing conditions with SDS-PAGE, were also able to induce specific DTH reactions in mice (Frommel *et al.*, 1988). In the present studies using partially purified preparations, no significant difference was observed in the positive ratio and the average induration size between FA-1 and -2 and CA or MA. By SDS-PAGE analysis of CA and FAs, 5 bands migrating the region approximately 66, 55, 45, 28 and 26 kD were common to the both FAs. From these results, it is assumed that at least some or all of these 5 antigens of *L. panamensis* may be related to a specific DTH response in active cutaneous leishmaniasis patients infected with *L. braziliensis* complex.

Cross-reactivity at the skin test level between different leishmanial species has been demonstrated in humans and experimental animals (Manson-Bahr, 1961; Adler and Gunders, 1964; Bryceson *et al.*, 1970), although the reactions to heterologous organisms appeared to be of lesser magnitude (Weissberger *et al.*, 1973; Neal and Miles, 1976). Recently, Reed *et al.* (1986) also reported that a crude soluble promastigotes extract prepared from a heterologous parasite, *L. amazonensis*, was clearly less effective than a crude extract prepared from a homologous parasite, *L. chagasi*, in detecting DTH in cured American visceral leishmaniasis patients. In the present examinations, it was found that the present preparation was highly sensitive in the intradermal skin test against active cutaneous leishmaniasis patients suffering from heterologous organisms, *L. braziliensis*. There was no appreciable difference in the intensity of responses in patients caused by homologous and heterologous organismus. The results suggest that the present CA and FAs contain some common antigens, may be highly antigenic components, to *L. braziliensis*. It was concluded that the soluble extracts of *L. panamensis* would be very useful for diagnosis of active or cured cutaneous leishmaniasis caused by *L. braziliensis* complex in the endemic areas of the New World.

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Leishmania panamensis promastigote 型原虫から精製した
皮内反应用抗原の効果判定とその特性

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Leishmania panamensis promastigote 型原虫から作成した種々皮内反应用抗原の効果をも30人の皮膚型リーシュマニア症患者で判定した。原虫ホモジネートの10,000×g遠心上清を粗抗原(CA)とし、更に Sephacryl S-200 ゲルを用いて4画分(FA-1からFA-4)を得た。CA(10 μgタンパク量/テスト)による皮内反応の陽性率、および硬結径は Montenegro 抗原(MA; 5×10⁶原虫/テスト)でのそれらと比較して優位差がなかった。更に、10 μgタンパク量のCAによって誘発される遅延型皮内反応の反応の強さは、同抗原液を25 μgタンパク量で用いた場合と同等であった。分画抗原(FA-4は7.5 μgタンパク量、他は10 μgタンパク量)での皮内反応陽性率はFA-1が90.0%、FA-2が77.8%、FA-3が75.0%、FA-4が37.5%であった。これら4抗原のうち、FA-4は陽性率および反応の強さの両面で、又FA-3は反応の強さの点でCAやMAでのそれらと比較して著しく劣っていることが判明した。以上の結果から、*L. panamensis* 原虫から作成した皮内反应用抗原のうち、10 μgタンパク量のCAおよび同タンパク量のFA-1、FA-2分画抗原が新大陸での皮膚型リーシュマニア症の診断に適していることが結論づけられた。更に、これらの抗原液を構成しているタンパク質のうち、少なくとも66, 55, 45, 28, 26 kDタンパクの全て、又は一部が新大陸での皮膚型リーシュマニア症の遅延型皮内反応惹起に関与している可能性が示唆された。

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