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Biochemical analysis and immunogenicity of *Leishmania major* amastigote fractions in cutaneous leishmaniasis

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SUMMARY

Soluble *Leishmania* antigen (SLA) from both developmental stages of *L. major* (*L. major* MRHO/IR/75/ER) were prepared. Three and five subfractions of SLA from amastigote and promastigote were obtained by fast protein liquid chromatography (FPLC), respectively. Biochemical analyses and comparison of amastigote and promastigote SLA were done. The biochemical analyses revealed that the first fraction of *L. major* amastigote possesses a distinct band on its electrophoretic mobility pattern corresponding to a position of 24 kD, and it has enzymatic activity with characteristics of a cysteine proteinase. The isolated fractions of amastigote were tested for induction of proliferation, interferon-gamma (IFN-γ) and IL-4 production in cultures of peripheral blood mononuclear cells (PBMC) from individuals who had recovered and also chronic patients of cutaneous leishmaniasis caused by *L. major*. The cells of recovered individuals compared with chronic cases proliferated profoundly in response to the first fraction of amastigote SLA. In all recovered individuals, the IFN-γ, but not IL-4, was secreted in response to stimulation with the first fraction of amastigote SLA. In chronic cutaneous leishmaniasis, IFN-γ was infrequently observed in response to stimulation by all three fractions of amastigote SLA, but secretion of IL-4 was observed. These data indicate that first fraction of amastigote SLA is a strong inducer of primed human immune response to *L. major*, and may have a protective function.

Keywords cutaneous leishmaniasis biochemical analysis of amastigote soluble antigens interferon-gamma and IL-4 evaluation

INTRODUCTION

Infection with *Leishmania* causes a spectrum of diseases depending upon the host and on the *Leishmania* species involved [1–3]. In humans, the infection ranges from self healing lesions to a disseminated cutaneous disease, or a highly destructive mucosal disease, as well as ranging from an asymptomatic infection to a fatal visceral dissemination, causing one of the world's major health problems [4–6]. Studies in mice and humans strongly suggest that T cell-mediated immune responses play a central role in the outcome of the disease [7–9]. Differential activation of Th1 $CD4^+$ lymphocyte subsets in resistant strains of inbred mice, and Th2 subsets in susceptible strains, has been shown by many investigators [10–12]. However, in human cutaneous leishmaniasis this is less well defined [13–15]. Among several factors determining the balance between T cell subsets and the outcome of infection, are the characteristics of leishmanial antigens. It was recently reported that some T cell epitopes that are protective in the murine host do not elicit an immune response in humans [16], emphasizing the importance of testing leishmanial antigens against human cells. Defining the relevant antigen for protection should take into account that *Leishmania* are dimorphic protozoa. The flagellated promastigote stage of the parasite matures in the alimentary tract of the sandfly vector and is transmitted to the mammalian host during the vector's blood meal [17]. In the mammalian host, the parasite is readily phagocytosed by macrophages, and transforms into an amastigote. Adaptation by the parasite to the drastic changes in the external environment results in alteration of metabolism and biochemical composition and changes in gene expression.

Extensive studies have been carried out regarding the biochemical and immunological characteristics of promastigote antigens, but few studies of similar nature have been undertaken with amastigote antigens, due to difficulties in obtaining sufficient quantities of antigen [18]. Although initial infection of the mammalian host is induced by the promastigote form, almost 95% of the promastigotes will be lysed by the complement alternative pathway within minutes after infection, only a few of the inoculated

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promastigotes escaping this rapid destruction and gaining access to macrophages. Examining the cellular immune response of host to antigens unique for the amastigote is thus relevant. Indeed, it is the amastigotes that survive and multiply within the phagosome of macrophages and spread from one cell to another. The amastigote-infected macrophages are the target of protective $CD4^+$ T cells, the effectiveness of which could depend upon their specificity for amastigote-derived epitopes, possibly preferentially expressed in association with class II MHC molecules on the surface of infected macrophages.

In this study, we have isolated fractions of soluble *Leishmania* antigen (SLA) from both developmental stages of *L. major* (*L. major* MRHO/IR/75/ER) by fast protein liquid chromatography (FPLC), and partially characterized a distinct band of 24 kD in the first fraction of amastigote SLA. We have studied the immune response of human cells from naturally recovered individuals and non-healed patients with cutaneous leishmaniasis against promastigote SLA, amastigote SLA and its three subfractions. The discrepancy in the response emphasizes the importance of the first fraction of amastigote SLA as a strong inducer of an immune response to *L. major* in humans.

MATERIALS AND METHODS

Animals

Female Swiss nude and BALB/c mice 8–12 weeks old were obtained from an animal colony at the Swiss Institute for Cancer Research (ISREC; Epalinges, Switzerland).

Parasites

Leishmania major (MRHO/IR/75/ER) was used throughout the experiments. The parasites were provided by Dr E. Javadian (School of Public Health, University of Tehran). The parasites were kept virulent by continuous passage in BALB/c mice. Promastigotes isolated from skin lesions of infected mice were grown at 26°C in Dulbecco's modified Eagles' medium (DMEM) over a solid layer of rabbit blood-agar (NNN medium) until they reached a stationary phase of growth [19].

Isolation of amastigotes

Swiss nude mice were inoculated with 5×10^6 stationary phase promastigotes intramuscularly on each side of the hind leg. Approximately 2 months later, lesions were removed and amastigotes isolated from infected macrophages according to the methods described by Glaser *et al.* [20]. Briefly, lesions were suspended in isolation buffer (5·5 mM glucose, 2 mM EDTA, 10 mM KH_2PO_4/K_2HPO_4, 150 mM NaCl, pH 7·2) and gently forced through a wire mesh. The cell suspension was transferred to a glass Dounce homogenizer, and amastigotes were released from host cells with seven thrusts. After centrifugation (80 *g*, 5 min), the amastigote-containing supernatant was centrifuged (1300 *g*, 10 min). The amastigotes were then resuspended in 168 mM NH_4Cl in order to lyse erythrocytes. All steps were performed on ice except lysing the erythrocytes. Amastigotes were washed three times in isolation buffer, and sequentially filtered through polycarbonate filters of 8, 5 and 3 μm, respectively.

Preparation of SLA from promastigotes and amastigotes

SLA from promastigotes and from purified amastigotes were prepared according to methods described by Scott *et al.* [21], with some modifications. Briefly, purified amastigotes and promastigotes were washed four times in cold PBS and resuspended at 10^9 parasites/ml in 100 mM Tris–HCl, 1 mM EDTA (pH 8) with 50 μg/ml leupeptin, 50 μg/ml antipapain, 50 μg/ml aprotinin and 1·6 mM PMSF (all from Fluka). The suspensions were incubated 10 min on ice and sonicated at 4°C with two 20-s blasts. The amastigote and promastigote suspensions were then centrifuged (27 000 *g* for 20 min), and the supernatants collected and recentrifuged (100 000 *g*, 4 h).

Fractionation of L. major *amastigote and promastigote SLA by FPLC*

SLA from each parasite stage was dialysed against starting buffer (100 mM Tris–HCl, 1 mM EDTA pH 8) and then passed through a 0·22-μm filter. A total volume of 20 ml dialysed amastigote SLA (4·5 mg/ml) as well as 5 ml dialysed promastigote SLA (1 mg/ml) were loaded separately onto Mono Q column (Pharmacia HR5/5; Uppsala, Sweden). Bound molecules were eluted using a 20-ml linear NaCl gradient (0–1 M NaCl) at a flow rate of 0·75 ml/min. The protein content was monitored at 280 nm, and fractions were collected based upon the observed peaks. Each fraction was dialysed against three changes of PBS, and concentrated using a Centricon 3 (MW-Co 3000). The protein content of each fraction was determined using a BioRad protein assay (BioRad Labs, Richmond, CA). Each fraction was aliquoted and frozen at −70°C until use.

Biochemical analyses

Promastigote SLA and FPLC fractions of amastigotes were analysed by silver staining following separation by SDS–PAGE on a 5–20% gradient gel according to methods described by Laemmli [22]. Briefly, samples (2 μg) were heated to 100°C for 5 min in 1·25% SDS, 60 mM Tris–HCl pH 6·8, 1·3% 2-mercaptoethanol (2-ME) and 8% glycerol before loading gels.

In order to analyse the proteinase activity of amastigote and promastigote SLA, samples were loaded onto gelatin (0·2% w/v)-impregnated polyacrylamide gels. Samples were not heated or reduced before loading. The acrylamide concentration of separating gels was 12% (w/v). After electrophoresis, gels were immersed for 1 h in 1 *l* of 2·5% (v/v) Triton X-100 to remove the SDS and to allow proteinase to become active. Gels were immersed in incubation buffer (0·1 M sodium acetate buffer pH 5·5, plus 1 mM dithiothreitol (DTT)) for 4 h at 37°C to allow proteinase to digest the gelatin before staining the remaining gelatin with 0·05% (w/v) coomassie blue and destaining. The area of gelatin digestion became clear after coomassie staining.

To study the effect of inhibitors on the development of the proteinase band, each gel was cut into five strips and immersed in incubation buffer containing one of the following inhibitors: 20 μg/ml pepstatin, 20 μg/ml leupeptin, 1 mM EDTA, 1 mM 1,10-phenanthroline and 100 μg/ml E-64.

Donors and isolation of mononuclear cells

To study the cellular immune responses to promastigote SLA, amastigote SLA and its three subfractions, a matched study was designed using three groups as follows (Table 1): (i) recovered cases consisting of 12 individuals ranging from 13 to 38 years old, who were leishmanin skin test-positive (+; > 5 mm reaction) and possessed a scar; (ii) active cases of leishmaniasis consisting of 10 patients aged between 11 and 40 years, possessing chronic lesions, as well as a history of leishmaniasis of > 2 years. Diagnosis of leishmaniasis was confirmed by culturing tissue biopsy samples or

aspirates in biophasic medium (an agar slant containing 30% rabbit blood overlaid with complete DMEM). All patients were unresponsive to the leishmanin skin test antigen; (iii) five healthy individuals (25–45 years old) without a history of leishmaniasis were used as controls.

Approximately 20 ml of heparinized peripheral blood were obtained from each subject. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll–Hypaque gradients (Histopaque 1077; Sigma Chemical Co., St Louis, MO). Interfaces containing mononuclear cells were isolated and washed three times in RPMI 1640 (Gibco, Gaithersburg, MD). The cells were resuspended in 92% fetal calf serum (FCS) and 8% DMSO and then frozen in liquid nitrogen. When needed, cells were rapidly thawed, and washed three times in RPMI.

Lymphoproliferation and cytokine production

PBMC were cultured in complete culture medium containing RPMI 1640, 10% human serum type AB negative, 2 mM glutamine, 10 mM HEPES, 5×10^{-5} mM 2-ME, 100 U/ml pencillin and 100 μg/ml streptomycin. Cells were incubated in U-bottomed 96-well microtitre plates (Costar, Cambridge, MA) at a density of 2×10^5 cells/well in the presence of 50 μg/ml promastigote SLA, amastigote SLA, and three subfractions of amastigote SLA. Concanavalin A (Con A; 40 μg/ml) was used as mitogen. The concentrations of antigens and mitogens were optimized before each experiment.

PBMC were incubated for 5 days in a humidified atmosphere at 37°C and 5% CO_2. During the last 18 h of incubation, they were pulsed with 1·0 μCi/well ^{3}H-thymidine (Amersham, Aylesbury, UK). Cells were harvested onto glassfibre filters, and ^{3}H-thymidine incorporation was determined by liquid scintillation counting. All tests were performed in triplicate, and for each set of samples the median was recorded. Proliferative responses were expressed as their stimulation indices (SI), which represent the ratio of mean proliferation after stimulation to the mean proliferation of medium controls.

Secreted interferon-gamma (IFN-γ) and IL-4 were measured after stimulations and before thymidine addition. An aliquot (75 μl) from each well was collected, and assayed for IFN-γ and IL-4 production using a sandwich ELISA technique [23]. Briefly, a mouse anti-human IFN-γ MoAb was used as the capture antibody, and was bound to microtitre plates. Culture supernatants (100 μl) were then added to each well. Biotinylated mouse anti-IFN-γ was then added as the detecting antibody. After addition of the streptavidin–horseradish peroxidase (HRP) conjugate and peroxidase substrate, the optical density (OD) was read at 492 nm. The IFN-γ concentration was determined by comparison with a human recombinant IFN-γ standard curve. The assay was calibrated to detect IFN-γ within the range of 100–6400 pg/ml (all reagents were purchased from Medgenix Diagnostics, Belgium).

A sandwich ELISA technique was also used to measure IL-4 concentrations. A mouse anti-human IL-4 MoAb was used as capture antibody, and a biotinylated mouse antibody as detecting antibody. Human rIL-4 was used to delineate a standard curve ranging from 45 to 3000 pg/ml (all reagents were purchased from Medgenix Diagnostics GmbH).

Statistical analysis

The difference in the level of T cell proliferation and cytokine production was determined by analysing variance (ANOVA) and using the *t*-test. Differences were considered significant when $P < 0{\cdot}01$.

RESULTS

Fractionation of SLA by FPLC

SLA from both amastigote and promastigote stages were separated by FPLC on an anion exchange Mono Q column. The elution profile obtained using a linear NaCl gradient from 0 to 1 M is shown in Fig. 1. Three major peaks were observed for amastigote SLA (Fig. 1, star line). The first and the major peak accounted for 61% of the SLA protein, and was eluted before starting the NaCl gradient. Fractions 2 and 3 were eluted after starting the NaCl gradient and contained 24% and 15% of protein, respectively. The elution profile of promastigote SLA showed five major peaks. One peak was eluted before starting the NaCl gradient, whereas the others were eluted after starting the NaCl gradient (Fig. 1, solid line).

The three fractions of amastigote SLA were analysed by SDS–PAGE, followed by silver staining (Fig. 2a). The first fraction of amastigote SLA contained a predominant band of 24 000 D (Fig. 2a, lanes 5 and 6). This protein was eluted before commencement of the NaCl gradient on the anion exchange column. This protein is present in the soluble part (Fig. 2a, lanes 1 and 2) and to a lesser extent in the pellet (Fig. 2a, lanes 3 and 4). There are also other bands which are fraction 1-specific. According to the SDS–PAGE analysis (Fig. 2b), the 24-kD band is only detectable in amastigote SLA (lanes 1 and 2) and not in promastigote SLA (lanes 3 and 4). Since the promastigote and amastigote grew well at different temperatures, we analysed the influence of temperature on the expression of 24 kD band in promastigote. When promastigotes were grown at 37°C for 24 h, the 24-kD band appeared faintly in SDS–PAGE, as shown in Fig. 3 (lanes 2 and 3), compared with promastigotes grown at its ordinary temperature of 26°C (Fig. 3, lane 4).

It has been shown that one feature of *L. mexicana* amastigote is its expression of very high levels of cysteine proteinases, most

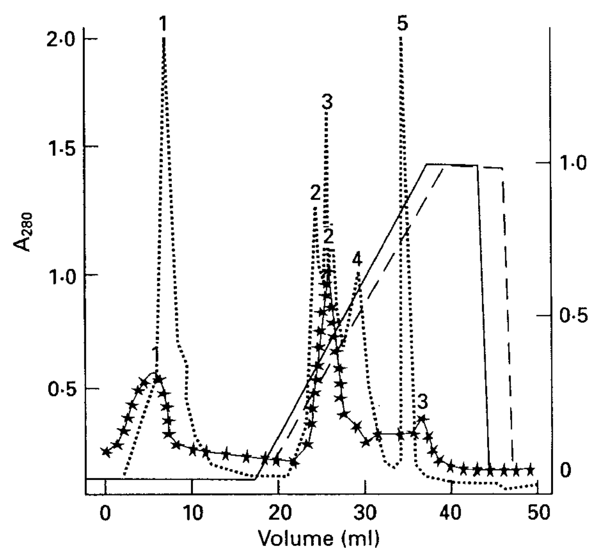
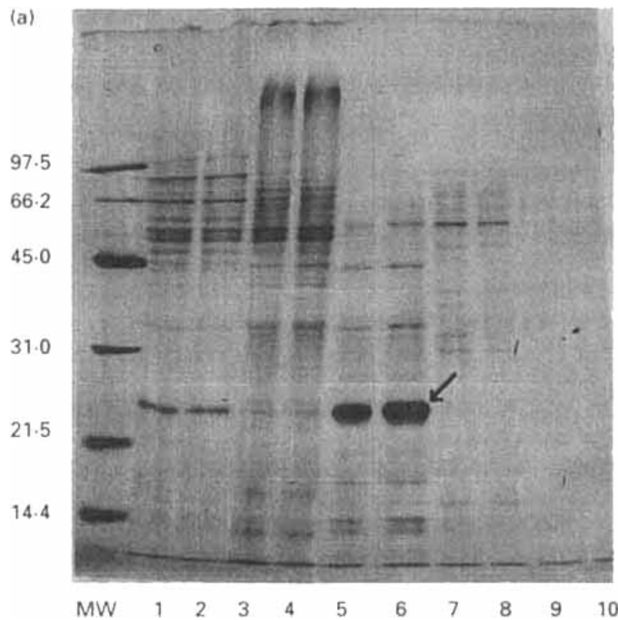


Fig. 1. Fractionation of amastigote (*) and promastigote SLA (solid line) by fast protein liquid chromatography (FPLC) anion-exchange chromatography. Conditions: samples, 4·5 mg of amastigote soluble *Leishmania* antigen (SLA) and 1·25 mg of promastigote SLA, in buffer A (100 mM Tris, 1 mM EDTA pH 8·0) were chromatographed on a Mono Q HR5/5 column. The column was developed using a NaCl gradient of 0–100% in buffer B (buffer A with 1 M NaCl) with flow rate of 0·75 ml/min and detection at 280 nm.



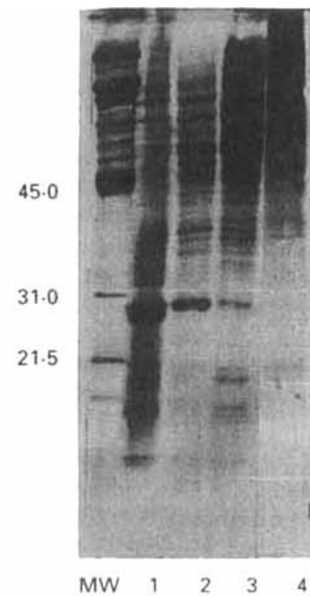


Fig. 3. Comparison of SDS–PAGE (12% acrylamide gels) protein profile of amastigote soluble *Leishmania* antigen (SLA) and promastigote grown at 37°C. Lane 1, Non-reduced and non-heated amastigote SLA; lane 2, reduced and heated amastigote SLA; lane 3, promastigote grown at 37°C for 24 h; lane 4, promastigotes grown at 26°C (both lanes 3 and 4 were reduced and heated). MW, Molecular weight markers.

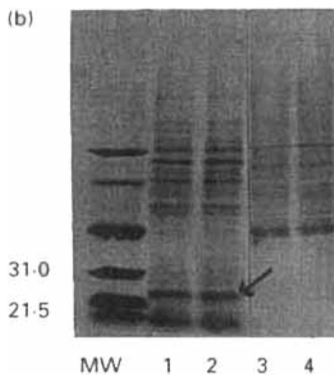


Fig. 2. (a) SDS–PAGE of *Leishmania major* amastigote soluble *Leishmania* antigen (SLA) and its three subfractions. Samples were run in duplicate on 12% acrylamide gels. Lanes 1, 2, amastigote SLA; lanes 3, 4, amastigote pellet (pellets were obtained after centrifugation at 27 000 ***g***); lanes 5, 6, first fraction of amastigote SLA; lanes 7, 8, second fraction of amastigote SLA; lanes 9, 10, third fraction of amastigote SLA. MW, Molecular weight markers. (b) SDS–PAGE of *L. major* amastigote and promastigote SLA. Samples were run in duplicate on gradient 5–12% acrylamide gels. Lanes 1, 2, amastigote SLA; lanes 3, 4, promastigote SLA; MW, Low molecular weight marker.

of which are stage-specific [24]. We decided to analyse the capabilities of the amastigote SLA in comparison with promastigote SLA for enzymatic digestion of gelatin incorporated in SDS–PAGE. This analysis revealed that amastigote SLA had a high activity on digestion of gelatin at pH 5·5 in comparison with promastigote SLA (Fig. 4a, lanes 1 and 2). This gelatin hydrolysis was observed over a molecular weight range of 21–66 kD for amastigote SLA, in comparison with the promastigote SLA mostly seen in the area of 50 kD (Fig. 4a, lanes 1 and 2). When freeze-thawed cell lysates of amastigote and promastigote were analysed using the same procedure, gelatin hydrolysis was observed over the molecular weight range 21–130 kD for whole amastigotes, but was not observed in whole promastigote cell lysates (gelatin digestion was observed over area 50 kD, Fig. 5). Furthermore, it was demonstrated that the predominant amastigote 24-kD band possessed an enzymatic activity on incorporated gelatin. In order to identify the type of enzymatic activity possessed by the amastigote SLA preparation, different proteinase inhibitors were used (Fig. 4b–f). Among the five different proteinase inhibitors, only leupeptin at an optimum concentration of 20 μg/ml could inhibit completely the action of gelatin digestion (Fig. 4e). The 24-kD band was also solely inhibited by leupeptin and E-64 (Fig. 4e,f).

Lymphoproliferative responses in recovered individuals

The proliferative response from PBMC of recovered individuals to amastigote SLA is shown in Fig. 6a. Cells from control individuals with no history of leishmaniasis (Table 1) did not show a proliferative response to SLA or its three subfractions. The pattern of lymphoproliferative responses to amastigote SLA among recovered subjects was heterogeneous, with mean SIs of $39·3 \pm 28·3$. The recovered patients, recently healed from a cutaneous leishmaniasis (Table 1, patients 7–11) had a higher proliferative response ($61·8 \pm 21·4$) compared with individuals (1–6 and 12) with a longer history of resolution ($16·8 \pm 21·4$). Figure 6b shows the production of IFN-γ produced by PBMC from recovered individuals. Their cells were readily stimulated to produce IFN-γ in the presence of amastigote SLA, suggesting that a protective memory T cell population was present. However, there was some variation in the concentration of IFN-γ among individuals, ranging from 150 to 2000 pg/ml (909 ± 700). PBMC from control groups did not produce IFN-γ in the presence of SLA.

All three subfractions of amastigote SLA were subsequently tested in proliferation assays using equal concentrations of protein (50 μg/ml). As shown in Fig. 6a, the first fraction of amastigote

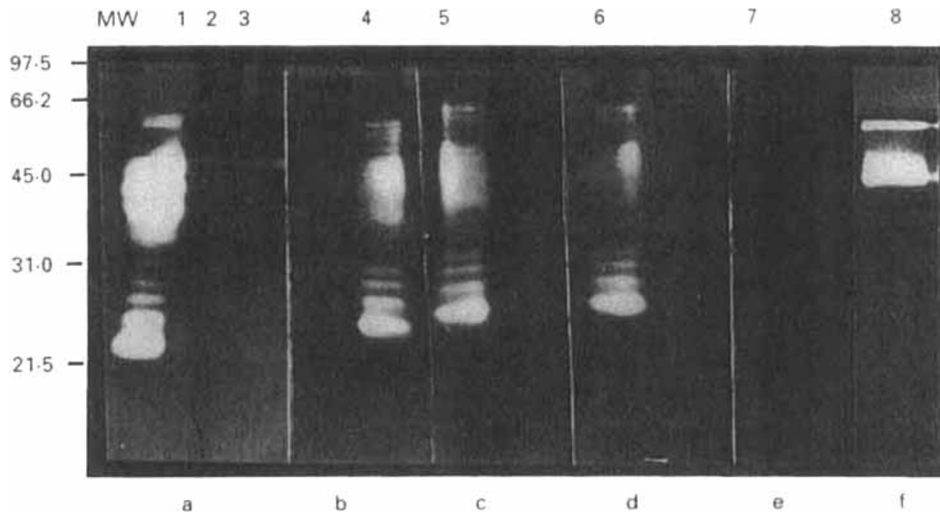


Fig. 4. Analysis of leishmanial protease activity on gelatine SDS–PAGE. (a) Lane 1, amastigote soluble *Leishmania* antigen (SLA); lanes 2, 3 promastigote SLA. (b) Lane 4, amastigote SLA in the presence of 20 μg/ml pepstatin. (c) Lane 5, amastigote SLA in the presence of 1 mM EDTA. (d) Lane 6, amastigote SLA in the presence of 1 mM 1,10-phenanthroline. (e) Lane 7, amastigote SLA in the presence of 20 μg/ml leupeptin. (f) Lane 8, amastigote SLA in the presence of 100 μg/ml E-64. MW, Molecular weight markers. Gels were treated with 2·5% Triton X-100 after electrophoresis as described in Materials and Methods, and were incubated for 4 h in 0·1 M sodium acetate buffer pH 5·5, 37°C in the absence (a) or presence of proteinase inhibitors (b–f).

SLA with mean SIs of 23·45 ± 17·52 was found to have the highest stimulatory effect compared with the second and the third fractions of the amastigote SLA. The mean SIs in the second and third fractions were 5·52 ± 4·97 and 10·20 ± 8·38, respectively. The mean SI on the first fraction was significantly different ($P<0·01$) from the second and third fractions. Figure 6b shows the concentration of IFN-γ in the culture supernatant of PBMC from recovered individuals cultured with three subfractions of amastigote SLA. The highest production of IFN-γ belonged to the first fraction of amastigote SLA, with mean production of 435 ± 352·17 pg/ml. The mean production of IFN-γ from the second and third fractions was 91·67 ± 97·31 and 197·5 ± 172·3, respectively. The production of IFN-γ by the first fraction was significantly different ($P<0·01$) in comparison with the second and third fractions. IL-4 was not detectable in recovered subjects after stimulation with SLA or its fractions (data not shown).

The proliferative response and IFN-γ production of recovered PBMC to promastigote SLA is shown in Table 2. The mean SI was 31·30 ± 21·98, which was not significantly lower than the amastigote SLA. The mean production of IFN-γ to promastigote SLA was 792·5 ± 624·28. The pattern of lymphoproliferative responses and IFN-γ production to promastigote SLA was heterogeneous, and similar to amastigote SLA.

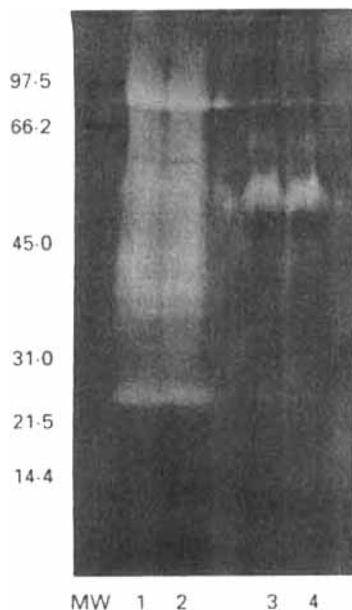


Fig. 5. Comparison of proteolytic activities in gelatin SDS–PAGE gels of *Leishmania major* promastigote and amastigote lysates (the lysates were prepared from × 10 freezing and thawing of parasite). Samples were run in duplicate. Lanes 1, 2, amastigote lysate; lanes 3, 4, promastigote lysate. MW, Molecular weight markers.

Lymphoproliferative response in non-healed patients

The evaluation of PBMC of normal controls and patients with active disease for their ability to proliferate in response to whole amastigote SLA and its subfractions is shown in Fig. 7a. PBMC from non-healed patients proliferated rather weakly in response to whole amastigote SLA, except for patients 3 and 7. The mean SIs were 18·43 ± 15·52. The SIs of non-healed patients were significantly different, and lower than that found for recovered patients ($P<0·01$). There was no production of IFN-γ in supernatant of cells stimulated with the whole amastigote SLA, except in patients 3 and 7 (Fig. 7b). Almost all patients produced IL-4, except patients 7, 8 and 9, in response to whole amastigote SLA. Mean IL-4 production was 59·68 ± 40·55 pg/ml, as shown in Fig. 7c. The SIs induced by amastigote SLA subfractions were similar to whole amastigote SLA, although patients 3, 4 and 7 compared with the other non-healed patients had higher SIs to amastigote SLA and its subfractions (Fig. 7a). Regarding specific IFN-γ production against each subfraction of amastigote SLA, none of the patients produced IFN-γ except patients 3 and 7 (Fig. 7b). Patients 1–4 produced IL-4 in response to all three subfractions of amastigote

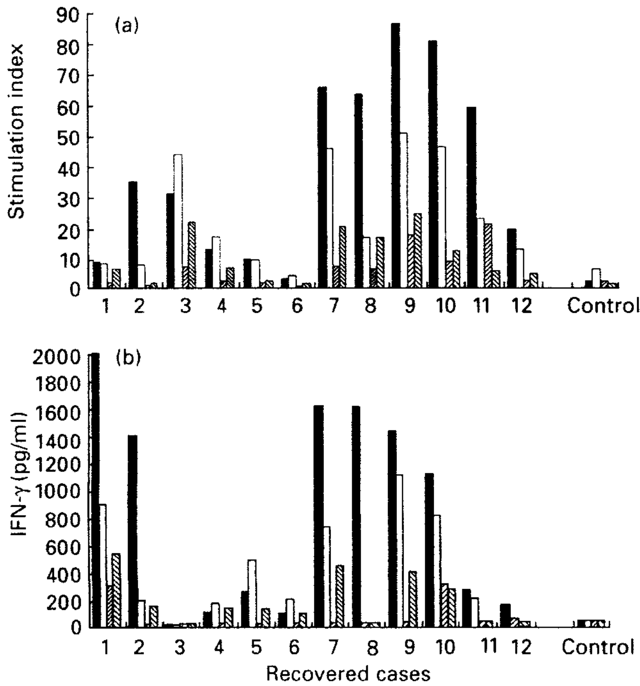


Fig. 6. Proliferation (a) and IFN-γ production (b) by peripheral blood mononuclear cells (PBMC) from recovered cutaneous leishmaniasis individuals in response to antigen stimulation: ■, Soluble *Leishmania* antigen of amastigote (SLA); □, first fraction of amastigote SLA; ▨, second fraction of amastigote SLA; ▧, third fraction of amastigote SLA. Of each antigen, 50 μg/ml were used.

SLA (Fig. 7c). Patient 7 was the only case that did not synthesise IL-4 against amastigote SLA and its subfractions, and patient 5 produced IL-4 only against whole SLA.

Surprisingly, the SIs induced by promastigote SLA were significantly lower than amastigote SLA (4·43 ± 4·37). All patients produced IL-4, with mean production of 65·5 ± 43·5 pg/ml, except patients 7, 8 and 9. In contrast, no IFN-γ production was detected in response to promastigote SLA, except in patients 3 and 7 (Table 3).

Table 1. Characterization of recovered and active cases

Subject no.	Age (years)	Sex	Skin test	Duration (years)
Recovered individuals				After suffering from cutaneous Leishmaniasis
1	29	F	+	8
2	38	M	+	12
3	13	F	+	4
4	16	F	+	5
5	32	F	+	10
6	34	F	+	7
7	35	F	+	3
8	14	F	+	2
9	20	M	+	2
10	26	M	+	2
11	21	M	+	3
12	22	M	+	16
Active cases				Duration of lesion still active
1	20	M	–	4
2	11	F	–	3
3	25	F	–	4
4	21	F	–	5
5	21	F	–	6
6	35	M	–	14
7	25	M	–	15
8	25	M	–	6
9	30	F	–	10
10	40	M	–	12
Control individuals				
1	30	F	ND	
2	25	M	ND	
3	45	M	ND	
4	32	M	ND	
5	36	F	ND	

DISCUSSION

Soluble extracts of *L. major* promastigotes and amastigotes (SLA) were fractionated by anion-exchange chromatography, giving rise to two different elution profiles. A predominant protein of 24 kD was eluted in the first fraction of amastigote SLA, which was absent in the other fractions. This protein was not present in promastigote preparations, but it was induced after incubation of promastigote at 37°C for 24 h. This result indicates that the expression of this protein may be temperature-dependent or inducible at 37°C. The *Leishmania* parasite has two developmental stages, which proliferate under different conditions of temperature and pH, and it is likely that there are some stage-specific proteins that are expressed only at certain temperatures or pH [25]. This result confirms other studies, in that a sudden increase in the ambient temperature is one of the most obvious environmental changes during the transit of *Leishmania* parasites from insect vectors to mammalian host [26], and could play an important role in the differentiation and expression of different antigens in *Leishmania* and should be taken into consideration for the identification of vaccines based on amastigote-specific antigen.

Table 2. Stimulation indices (SI) and levels of IFN-γ in the supernatants of recovered cutaneous leishmaniasis to promastigote soluble *Leishmania* antigen (SLA)

Recovered cases	SI	IFN-γ (pg/ml)
1	12·9	1300
2	25·6	1200
3	25·1	200
4	7·9	150
5	12·3	280
6	10·1	150
7	40·4	1200
8	61·1	1500
9	71·2	1800
10	48·1	980
11	50·1	260
12	12·3	150
Mean ± s.d.	31·30 ± 21·98	762·5 ± 624·28
Controls	1·3 ± 0·6	Under detection

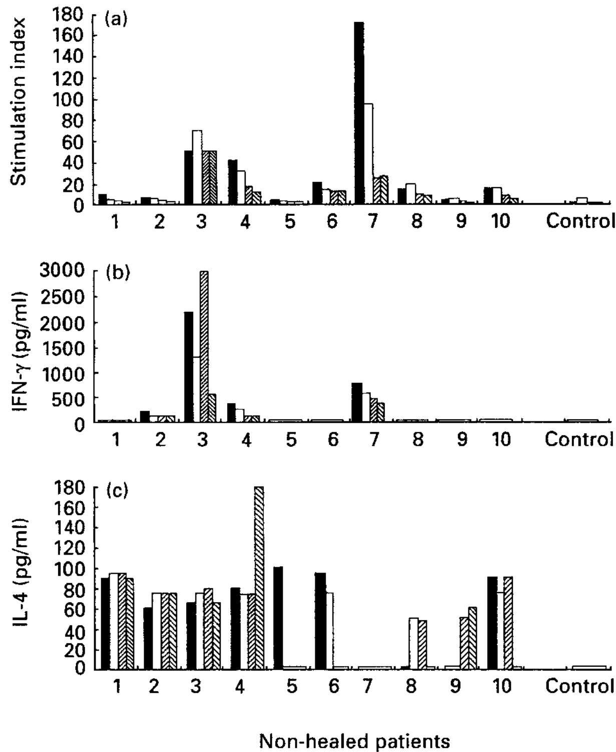


Fig. 7. Proliferation (a), IFN-γ (b), and IL-4 (c) production by peripheral blood mononuclear cells in non-healed patients with cutaneous leishmaniasis in response to antigens. ■, Soluble *Leishmania* antigen of amastigote (SLA); □, first fraction of amastigote SLA; ▨, second fraction of amastigote SLA; ▧, third fraction of amastigote SLA. Of each antigen, 50 μg/ml were used.

Table 3. Stimulation indices (SI), levels of IFN-γ and IL-4 in the supernatants of chronic cases of cutaneous leishmaniasis to promastigote soluble *Leishmania* antigen (SLA)

Patient	SI	IFN-γ (pg/ml)	IL-4 (pg/ml)
1	0·9	25	110
2	6·17	25	70
3	15·2	1500	80
4	3·25	110	75
5	0·97	25	90
6	1·8	25	110
7	8·9	250	UD
8	1·3	25	UD
9	1·3	25	UD
10	4·5	25	110
Mean ± s.d.	4·43 ± 4·37	203 ± 437·5	65 ± 43·5
Control	1·3 ± 0·6	UD	UD

UD, Under detection.

Leishmania possess relatively high levels of proteinase activity, and some functions have been proposed for the proteolytic enzymes responsible [27–29]. The proteinases are subject to differential control during the life cycles. Here, we have shown that amastigotes are significantly rich in enzymatic activity. This gelatin hydrolysis pattern was specifically observed in a molecular weight ranging between 21 and 66 kD. The highest activity of hydrolysis was seen in the mol. wt range of 31–50 kD. Using freeze-thawed amastigote cell lysate, we observed a wider range of gelatin hydrolysis pattern in the 21–130 kD mol. wt range. However, the promastigote SLA preparation demonstrated a lower enzyme activity, mostly in the area of 50 kD. When promastigote cell lysates were used, considerable gelatin hydrolysis was observed for proteins of 50 kD, as well as faint digestion in the area of 63–66 kD. Thus there is considerable overall difference in proteinase activity between amastigotes and promastigotes. As is known, there are major differences between amastigote and promastigote survival conditions [30,31]. Amastigotes can live in the acidic and hostile environment of the phagolysosome of macrophages, and their division and optimum metabolism occur at pH 4·5–5·5, at 37°C. In contrast, promastigotes survive best at a neutral pH and acidification of the medium to pH 5·5 drastically reduces transportation of necessary amino acids [32].

Leupeptin was the only inhibitor to obstruct gelatin hydrolysis, but when a high concentration of specific cysteine proteinase inhibitor, E-64, was used, gelatin hydrolysis was completely inhibited in the area of 21–45 kD. This result strongly suggests that the amastigote-specific 24-kD band could be a cysteine proteinase. In *L. mexicana* it has been shown that two distinct groups of cysteine proteinases are present [33–35]. The first group consists of enzymes ranging in size from 60 to 130 kD, which is common to all stages of the parasite's life cycle. However, the activity is higher in amastigotes compared with promastigotes [36]. The second group of proteinases consists of low mol. wt (16–36 kD) enzymes, which are specific to amastigotes, and are absent in mid-log phase of promastigotes [37–39]. In *L. donovani* the low mol. wt proteinases are absent. It has been shown by Coombs *et al.* [40] that amastigote forms of *L. major* possess proteinases with a mol. wt of 63–130 kD only. However, our results demonstrate that both amastigote SLA and amastigote cell lysates have proteinase activity at a wider range of 21–130 kD. This difference may be due to the different strains used in each study.

Biochemical composition differences in this strain prompted us to test the response of peripheral T cells from individuals with a history of cutaneous leishmaniasis and non-healed patients with active lesions to promastigote SLA, amastigote SLA with its three subfractions. The PBMC from recovered cases proliferated strongly to both forms of SLA, associated with high production of IFN-γ, although the responses due to amastigote SLA were higher. Since SLA is a crude preparation of *L. major* soluble antigens of parasite, it contains a mixture of different antigens, therefore it could stimulate different clones of memory T cells. Among the amastigote SLA subfractions, the first fraction resulted in a higher proliferative response and IFN-γ production by T cells in comparison with other fractions. The heterogeneity in the pattern of responses in recovered cases may have been contributed to by the extent and duration of infection or the genetic restriction of the host anti-leishmanial T cells [41]. Overall, the proliferative response of T cells of non-healed patients was not as high as in recovered cases. Furthermore, the level of IFN-γ production was heterogeneous, and IL-4 production was detectable. These patients had shown significant differences in their proliferative responses to amastigote and promastigote SLA. Although their lower response to promastigote SLA is a matter of speculation, it is clear that initial infection of mammalian host occurred in promastigote form,

but it is the amastigote form which persisted and presented to the immune system. Their T cells were able to respond better to the amastigote antigen than to the promastigote antigen. The result of this stimulation is the activation of the Th2 pathway, with considerable production of IL-4 but not IFN-γ. The heterogeneity that is seen among non-healed cases may be due to the extent of chronic infection or the degree of immune response suppression that could occur among non-healed patients. It should be emphasized that both patients 7 and 3 presented peculiar cases of cutaneous leishmaniasis. In patient 7, the right eyelid was inoculated with parasites, and the lesion disseminated to the eye mucosa. The patient had been treated with 300 doses of glucantim, yet remained uncured. Cells from this patient produced IFN-γ in response to stimulation with SLA and its subfractions, but did not produce IL-4. The response of patient 3, who had a very large lesion with several nodules on her nose, demonstrated a different response to stimulation with amastigote SLA and its subfractions. Both cytokines IFN-γ and IL-4 were produced in this case. It has been shown that even within a group of individuals with cutaneous leishmaniasis, the balance between IFN-γ and IL-4 production by peripheral blood T cells in response to *Leishmania* antigens reflects the severity of the disease [42]. Thus, there is strong evidence for correlation between differential activation of T cell subsets and the severity of disease in human leishmaniasis. Although polarization of the human T cell response into Th1 or Th2 is not as well defined as in the experimental animal system, there is some evidence that the balance of cytokines at the site of primary activation of *Leishmania* may be of major importance for the development of Th1 or Th2 response [43].

Most studies have focused on the immune response against different antigens of promastigotes, and few reports have presented the cellular immune responses against amastigote antigens. The result of this study emphasizes the importance of the first fraction of amastigote SLA as a strong inducer of human immune response to *L. major*. It could be possible that further studies on the analyses and immunogenic properties of the first fraction of amastigote-derived SLA will have relevance to the production of defined molecules that could be tested for their vaccine properties.

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