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# EVALUATION OF CELLULAR IMMUNE RESPONSES TO AMASTIGOTE SOLUBLE *Leishmania major* ANTIGENS IN RECOVERED CASES OF CUTANEOUS LEISHMANIASIS

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**ABSTRACT**

This study was performed in order to define the cellular immune response of 12 recovered cutaneous leishmaniasis subjects to different soluble antigens of the amastigote form of *Leishmania major*. A soluble leishmanial antigen preparation (SLA) derived from highly-purified amastigotes from infected nude mice was fractionated by Mono Q column using Fast Protein Liquid Chromatography (FPLC) system. Three different fractions were obtained. The lymphoproliferative response, interferon-gamma (IFN-γ) and interleukin-4 (IL-4) to amastigote SLA and its three subfractions were measured. The highest proliferative response and specific IFN-γ production without synthesis of IL-4, was induced by the first fraction of amastigote SLA. These results showed that the individuals who had recovered from cutaneous leishmaniasis had expanded memory T- cell clones recognizing different antigens in the first fraction of amastigote SLA.

The main and most important point of this study was the identification of one fraction of an amastigote antigen which preferentially reacted with cells from recovered human cutaneous leishmaniasis cases.

**Keywords:** Cutaneous leishmaniasis, amastigote soluble *L. major* antigen, lymphocyte proliferation, cytokines.

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## INTRODUCTION

Leishmaniasis is an infection caused by intracellular protozoan parasites transmitted by various species of sandflies. These parasites e stagesoflife. They are flagellated extracellular promastigotes in the sandfly vector or *in vitro* culture, and aflagellar obligate intracellular amastigotes within mononuclear phagocytes of their vertebrate host.[1] They cause a spectrum of clinical diseases ranging from simple self-healing cutaneous lesions to disseminated cutaneous or destructive mucosal disease and from subclinical visceral infection to fatal visceral leishmaniasis. Research using human cells and both murine and hamster models has shown that resolution

of infection is dependent upon a complex set of events associated with cell-mediated immunity.[2-5] This includes T-cell responses to parasite antigens and the production of cytokines such as interferon gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor, resulting in macrophage activation and subsequently intracellular parasite destruction. The specific involvement of certain T-cell subsets in disease resolution or exacerbation has also been studied deeply. Th1 and Th2 cell subdivision, based upon differential cytokine profiles, is well understood in murine models.[6-8] Susceptibility to *L. major* is associated with the activation of Th2 cells secreting interleukin-4 (IL-4), IL-5, IL-6 and IL-10. In contrast, when the T-cell response to *L. major* is directed towards secretion of IL-2, gamma interferon (IFN-γ) and lymphotoxin (LT) by induction and expansion of Th1 subsets, infected animals are capable of controlling the infection and subsequently parasites will be eliminated.[4,5]

There is extensive epidemiological and limited human experimental data available about the nature of the parasite antigens involved in eliciting the cellular immune response in human infection. Moreover, the majority of studies have focused their investigation on immune responses against different antigens of promastigotes,[9-12] and a few reports evaluated the cellular immune response against amastigote antigens.[13] In this report, we demonstrate the importance of the first fraction of soluble *Leishmania major* antigen (SLA) of amastigotes as a strong stimulator of memory T-cells of recovered individuals with high production of IFN-γ without any synthesis of IL-4.

## MATERIALS AND METHODS

### Animals

Female Swiss nude and BALB/c mice of 8-12 weeks of age were obtained from the animal colony of the Swiss Institute for Cancer Research (ISREC), Epalinges, Switzerland.

### Parasites

*Leishmania major* (MRHO/IR/75/ER) which is presently used in Iran as a vaccine strain in field trials, was used throughout the experiments. The parasites were kept virulent by eontinous passage into BALB/c mice. Parasites isolated from skin lesions of infected mice were grown at 26°C in Dulbecco's modified Eagle's medium over a solid layer of rabbit blood agar until the stationary phase of growth was obtained.

### Isolation of amastigote

Swiss nude mice were inoculated intramuscularly on both sides of the hind leg with $5\times10^6$ stationary phase promastigotes. Approximately two months later the lesions were removed and amastigotes were purified from infected

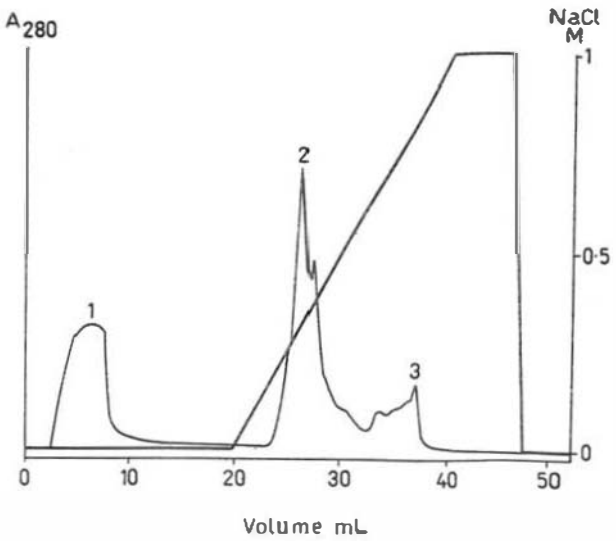


Fig. 1. Fractionation of amastigote SLA by FPLC anion-exchange chromatography. Condition: (sample) 4.5 mg of SLA in buffer A; (column) Mono Q HR 5/5; (buffer A) 100 mM Tris, 1mM EDTA, (pH 8.0); (buffer B) buffer A with 1M NaCl; (gradient) 0 to 100% buffer B in 20 mL; (flow rate) 0.75 mL/min; (detection) 280 nm.

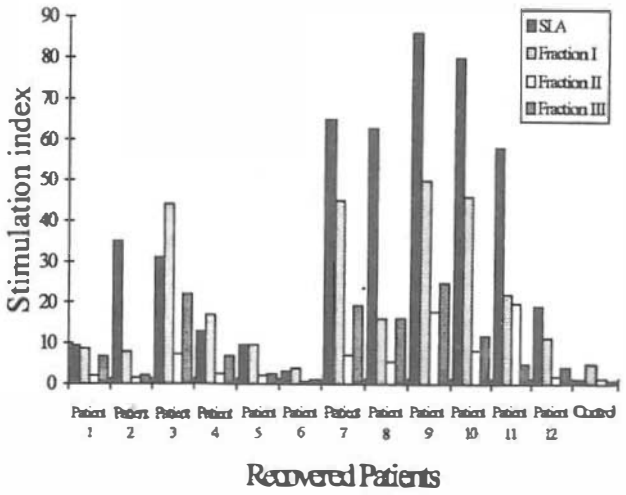


Fig. 2. Lymphocyte proliferative response in recovered patients with cuntaneous leishmaniasis after stimulation with soluble leishmania antigen of amastigote (SLA)■, first fraction of amastigote SLA□, second fraction of amastigote SLA□ and third fraction of amastigote SLA▨ (after 5 days of incubation with optimal concentration of all antigen 50 μg/mL).

macrophages according to Glaser's procedure.[14] Briefly, lesions were suspended in isolation buffer containing 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 the amastigotes were released from host cells with seven thrusts. After certrifugation at 80 g for 5 minutes, the supernatant which contained the amastigotes

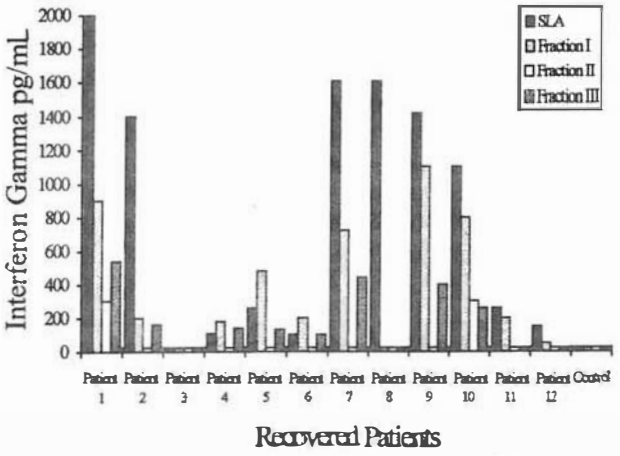


Fig. 3. IFN-γ production of recovered patients with cutaneous leishmaniasis. Data represent IFN-γ levels in supernatant of lymphocyte culture stimulated with SLA■, first fraction of amastigote SLA▤ second fraction of amastigote SLA□, and third fraction of amastigote SLA▩ after 5 days of incubation.

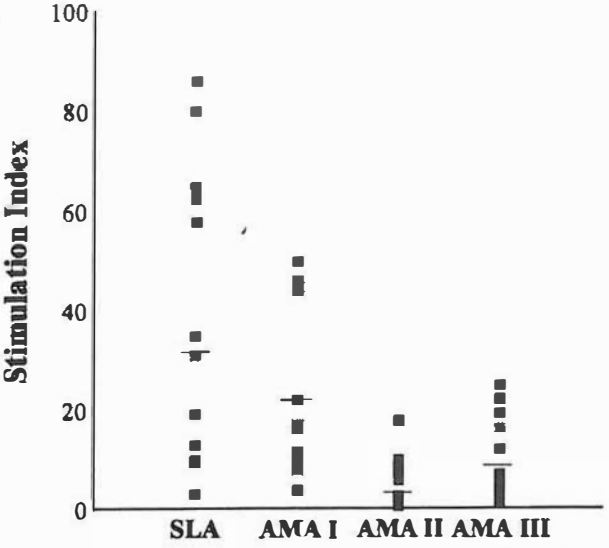


Fig. 4. Stimulation index of recovered cutaneous leishmaniasis subjects to amastigote soluble leishmania antigen (SLA), different subfractions of amastigote SLA: first fraction (AMA I); second fraction (AMA II); third fraction (AMA III); mean value indicated as —.

was centrifuged at 1300 g for 10 minutes. The pelleted amastigotes were resuspended in ammonium chloride (168 mM), in order to lyse red blood cells.

Amastigotes were washed three times in isolation buffer and sequentially filtered through polycarbonate filters with different pore sizes, 8, 5, and 3 μm respectively.

**Preparation of soluble leishmania antigen (SLA) from amastigotes**

SLA from purified amastigotes was prepared according to the protocol described by Scott,[15] with some modification. Briefly, purified amastigotes were washed four times in cold phosphate buffer saline (PBS) and resuspended at $10^9$ parasites per mL in 100 mM Tris-HCl, 1 mM EDTA (pH 8) with 50 μg/mL leupeptin, 50 μg/mL antipain, 50 μg/mL aprotinin and 1.6 mM phenylmethylsulfonyl fluoride, PMSF (all components from Fluka). The suspension was incubated for ten minutes in ice and sonicated at 4°C with two 20 second blasts. The amastigote suspension was then centrifuged at 27,000 g for 20 minutes, and the supernatant was collected and recentrifuged at 100,000 g for 4 hours.

**Fractionation of *L. major* amastigote SLA by Fast Protein Liquid Chromatography (FPLC)**

SLA was dialyzed against a starting buffer consisting of 100 mM Tris-HCl, 1 mM EDTA (pH 8) and passed through a 0.22 μm filter. A total volume of 20 mL with a concentration of 3 mg/mL from dialyzed SLA of amastigotes was loaded onto Mono Q column (Pharmacia HR5/5) and bound molecules were eluted by a 20 mL NaCl gradient of 0 to 1 M at a flow rate of 0.75 mL/min. Protein contents were measured at 280 nm and fractions were collected based upon the peaks observed. Each fraction was dialyzed against three changes of PBS and concentrated by centricon 3 (MW-Co 3000, Amicon). The protein content of each fraction was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Each fraction and SLA were aliquoted and frozen at -70°C until used.

**PBMC preparation and lymphocyte proliferation assay**

To study the cellular immune responses to amastigote SLA and three subfractions of SLA, a matched study was designed on recovered individuals and control subjects. Twelve recovered individuals ranging from 13 to 38 years old who were leishmanin skin test positive (> 5mm reaction) with scar were chosen. The control group consisting of 5 subjects with no history of leishmaniasis ranging from 25-45 years old were selected (Table 1).

Approximately 20 mL of heparinized peripheral blood was obtained from each subject. PBMC were isolated by centrifugation over Ficoll-hypaque gradients (Histopaque 1077, Sigma), the interface cells were washed three times in RPMI 1640 (Gibco) and stored frozen in liquid nitrogen in 92% fetal calf serum and 8% DMSO. Before using, the cells were rapidly thawed, washed three times in RPMI and resuspended in complete culture medium containing RPMI 1640 with 10% human serum type AB negative, 2mM glutamine, 10 mM Hepes, $5\times10^{-5}$ mM β-mercaptoethanol, 100 U/mL penicillin and 100 μg/mL streptomycin. PBMC were cultured in U-bottom 96-well microtiter plates (co-star) at density of $2\times10^5$ cells per well in the presence of 50 μg/mL of amastigote SLA and three subfractions of amastigote SLA. Concavalin A was used as a mitogen at 40 μg/mL. Optimal concentrations of antigen and mitogen

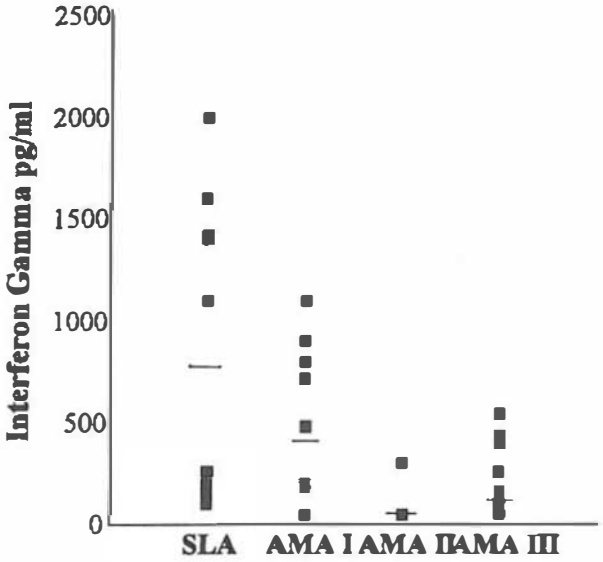


Fig. 5. IFN-γ production of recovered cutaneous leishmaniasis subjects stimulated with amastigote soluble leishmania antigen (SLA) and different subfractions of amastigate SLA: First fraction (AMA I); Second fraction (AMA II); Third fraction (AMA III); mean value indicated as —.

were determined in kinetic experiments performed prior to the present study.

PBMC were incubated for 5 days in a humidified atmosphereat 37°C and 5% $CO_2$ and pulsed during the last 18 hours with 1.0 μCi {$^3$H} thymidine per well (Amersham, United Kingdom). Cells were harvested onto glass fiber filters, and {$^3$H} thymidine incorporation was determined by liquid scintillation counting. All tests were performed in triplicate. Proliferative responses were expressed as the stimulation indices (SI), which represent the ratio of mean proliferation after stimulation to the mean proliferation of medium controls.

### *In vitro* cytokine production and determination

Supernatant from antigen stimulated cells were collected just before adding thymidine. 75 μL per well were collected and each triplicate was pooled. Culture supernatants were assayed for IFN-γ and IL-4 production using sandwich ELISA technique.[16] Briefly mouse anti-human IFN-γ monoclonal antibody (mAb) as capture Ab, was bound to microtiter plates, followed by addition of 100 μl. culture supernatant. Then, mouse biotinylated anti-IFN-γ as detecting Ab was added. After addition of streptoavidine horse-radish peroxidase conjugate and peroxidase substrate, the optical density (OD) was read at 492 nm. The IFN-γ concentration was determined by comparison to 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 GmbH, Belgium).

For IL-4 assay, like IFN-γ assay, sandwich ELISA technique was used. Mouse anti-human IL-4 mAb as capture antibody and a mouse biotinylated antibody as detecting agent were utilized. Human rIL-4 was used to delineate a standard curve ranging from 45 pg/mL to 3000 pg/mL (all reagents were purchased from Medgenix Diagnostics, GmbH).

### Statistical analysis

The cytokine production and lymphocyte proliferation due to SLA and its subfractions were compared and analyzed by Student's t-test. P-value of $<0.05$ was considered significant.

## RESULTS

### Liquid chromatographic fractionation of amastigote SLA

In order to separate different proteins of the amastigote SLA by liquid chromatography, SLA was fractionated on an anion exchange Mono Q column. The elution profile obtained with a linearly
shown in Fig. 1. The results obtained showed 3 major peaks in amastigote SLA referred to as AMA I, AMA II, and AMA III, respectively. The first and major peak which accounts for 61% of the protein in SLA was eluted before starting the NaCl gradient, whereas AMA II and AMA III contained 24% and 15% respectively and were eluted after starting the NaCl gradient.

### Proliferative and cytokine responses of recovered PBMC to amastigote SLA and its fractions

The proliferative response of PBMC from recovered individuals to SLA of a inastigote and its fractions are shown in Fig. 2. Cells from control individuals with no history of leishmaniasis showed no proliferative response to SLA. The pattern of lymphoproliferative responses to amastigote SLA among recovered subjects was heterogenous with stimulation indices ranging from 3 to 86 (39.3±28.3). The recovered patients who recently had recovered from cutaneous leishmaniasis (patients 7 to 11) have a higher proliferative response (61.8±21.4) compared to individuals with a longer history of recovery (16.8±21.4). The variability in time after resolution may influence the specific T-cell response to different antigens in the amastigote SLA. As shown in Fig. 2, the first fraction of amastigote SLA with a mean stimulation index of 23±17.52 was found to have the highest stimulatory effect compared to the second and third fractions of SLA, with mean stimulation indices of 5.52±4.97 and 10.20±8.38, respectively.

The difference between stimulation indices of the first fraction and the second or third were significant, with

$p<0.05$. Therefore, the first fraction of amastigote SLA had the most potent stimulatory effect on PBMC of recovered cutaneous leishmaniasis individuals and it could stimulate different clones of memory T-cells to respond to different antigens which included the first fraction of amastigote SLA.

IFN-γ production by lymphocytes proliferating in response to amastigote SLA and its fractions was measured in supernatants collected from corresponding wells (Fig. 3). It shows that PBMC from recovered patients were readily stimulated to produce IFN-γ in response to amastigote SLA, suggesting the presence of a protective memory T-cell population. But there was some variation between individuals ranging from 150 to 2000 pg/mL. PBMC from the control group showed essentially no production of IFN-γ. The IFN-γ response to AMA I with a concentration of 435±352.1 pg/mL was the highest. Interferon-gamma production due to AMA II and AMA III was 91.67±97.31 and 197.5±172.3 pg/mL, respectively. Although the lymphoproliferative response to AMA III was low, the quantity of IFN-γ production was rather high (half of AMA I).

The production of IL-4 in recovered subjects with amastigote SLA or any of its fractions was undetectable.

The overall view of lymphoproliferative response and IFN-γ production due to amastigote SLA and its three subfractions of 12 recovered subjects is summarized and shown in Figs. 4 and 5, respectively.

## DISCUSSION

In this study, a soluble extract of *L. major* amastigotes was fractionated by anion-exchange chromatography, leading to isolation of three different fractions based upon charge characteristics.

We analyzed proliferation and production of IFN-γ and IL-4 by PBMC of 12 recovered cutaneous leishmaniasis individuals who were all leishmanin skin test-positive, to amastigote SLA and its three different subfractions. Data obtained in this work demonstrated that PBMC from recovered cases proliferate to SLA very strongly, with high production of IFN-γ.

Many of the recovered patients' responses appeared to have variation either in lymphoproliferation or production of IFN-γ. The heterogeneity in the pattern of response may be due to differences in the level of intensity (extent and duration) of infection, or genetic restriction of the host anti-leishmanial T-cell responses.[10] Therefore, in subclinical or very self-limited cutaneous leishmaniasis, T-cells are sensitized by a fewer number of antigens and the responses to these antigens may be sufficient to confer immunity.

The results of this study emphasize the importance of the first fraction of amastigote SLA as a strong inducer of human immune responses to *L. major*. There have been many efforts to search for proteins responsible for protection in leishmaniasis. Applications of a purified fraction instead of whole parasites is an improvement for vaccine production, both in terms of standardization and reducing unwanted side effects. It seems that by working further on analysis and properties of the first fraction of amastigote, probably new approaches will be opened in producing an effective and purified vaccine in the future. The main and important result of this study was the use of antigens from the amastigote stage of the life cycle. Although initial infection of the mammalian host occurs by the promastigote form, almost 95% of promastigotes will be lysed by complement alternative pathway[14] in initiation of infection, and only a few parasites escape the immune response and gain access to macrophages. It has been shown that partial proteolysis of amastigotes occurs and eventually these peptides bind to MHC class II molecules and are expressed on the surface of macrophages, to be recognized by $CD4^+$ T-lymphocytes.[18,19] Therefore, search for the specific cellular immune response of the host against amastigote antigens is an important subject, because it is the amastigotes that multiply inside macrophages and spread from one cell to another and survive easily within the phagolysosome of macrophages.

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