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Partial Purification of a Potent Immunosuppressive Factor Excreted from *Leishmania major* Promastigote and Amastigote

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ABSTRACT

Recent scientific evidence indicates that distinct patterns of susceptibility in BALB/c mice to *Leishmania major* infection are attributable to the differential expansion of distinct $CD4^+$ T-cell subsets and their cytokines production. Production of the Th1 cytokine IFN-γ is associated with resistance, whereas production of the Th2 cytokines IL-4 and IL-10 are associated with extreme susceptibility. The major host immune defense mechanism against *Leishmania* is activation of macrophages by INF-γ derived from T cells. The inability of susceptible hosts to mount the immune response necessary to activate macrophage and destroy the parasites may be due to the parasite-specific proteins that are able to suppress the immune system. In the present study, we have semi-purified the excreted antigens of *Leishmania major* promastigote and amastigote by column chromatography. The isolated fraction showed a potent immunosuppressive activity on normal BALB/c mice lymphocytes stimulated with mitogens. Fifteen microgram of the isolated fraction caused 81% suppression of lymphocyte proliferation. These data may suggest that the parasite by secreting immunosuppressive factor down regulate the immune system and as a result survive in the body. *Iran. Biomed. J. 8 (2): 95-99, 2004*

Keywords: *Leishmania major*, Amastigote, Promastigote, Immunosuppressive Factor, Purification

INTRODUCTION

Leishmania is a protozoan parasite that is distributed world-wide, being endemic in 88 countries. Leishmaniasis is a complex disease caused by infection with different species of the leishmania which replicate inside the phagosomes of infected macrophages [1]. These diseases range from self-limiting cutaneous leishmaniasis (CL) that produce long-lasting ulcers and leaves scars upon healing to visceral leishmaniasis (VL) known as Kala-azar, which is a fatal infection if not treated efficiently. Among the many species of *Leishmania,* approximately 10 are important in human diseases. Each year, 1.5 million new cases of cutaneous leishmaniasis and 500,000 new cases of visceral leishmaniasis are estimated [1].

Leishmaniasis is difficult to treat and there is increasing resistance developing against the currently available drugs [2]. Due to the resistance of sandflies to insecticides and resistance of the parasite to the existing chemotherapy, new disease foci are identified every year. Therefore, there is an urgent need for vaccine development against leishmaniasis.

Infection of inbred strains of mice with *Leishmania major* has proven to be a valuable model system for studying host immune responses to the parasite. Recent scientific evidence indicates that these distinct patterns of susceptibility are attributable to the differential expansion of distinct $CD4^+$ T-cell subsets and their cytokines production. Production of the Th1 cytokine IFN-γ is associated with resistance to *L. major* infection (in C57BL/6 mice) whereas, production of the Th2 cytokine IL-4 and IL-10 is associated with extreme susceptibility in BALB/c mice.

Within the insect host, leishmania is present as flagellated promastigote form and upon infecting the mammalian host it differentiates into the smaller aflagellated round amastigote stage and multiplies in the phagolysosome vacuole of macrophages. Although most promastigotes (~ 80%) are destroyed

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after ingestion by resident macrophage [3], the remaining organisms convert to resident macrophage-resistant amastigotes and initiate replication within the phagolysosomes. This may be due to the inability of susceptible hosts to mount the immune response necessary to activate macrophage and destroy the amastigotes. Also, parasite-specific proteins such as immuno-suppressive factors may contribute to their ability to evade host recognition and thereby propagate within macrophage [4].

Macrophages infected by *Leishmania* amastigotes show changes in cell function and morphology including the down regulation of class II MHC expression and a lowered response to lymphokines. These alterations require communication between parasite and host cell which could be mediated by secreted amastigotes antigens [5]. In this study, we report purification of excreted antigens of promastigote and axcenic amastigote with potent immunosuppressive activity.

MATERIALS AND METHODS

Parasite and culture supernatant. Lesion-derived amastigotes (MRHO/IR/75/ER) were isolated from BALB/c infected 6-8 weeks before and cultured in NNN media (Novy-MacNal-Nicol) and later were transferred to the RPMI-1640 medium enriched with 20% FCS, 2 mM L-glutamine, 200 U/ml penicillin and 200 µg/ml streptomycin. Axcenic *L. major* amastigotes were grown at 35°C at pH 5 to 5.5 and promastigotes were grown at 22-25°C. Culture supernatants were harvested after 72 h of incubation by centrifugation and then filtered for lymphocyte proliferation assay.

Purification of excreted antigens. Culture supernatants were concentrated by Amicon membrane with a 10,000-molecular weight cut-off, followed by fractionation by column chromato-graphy on a Sephacryl S-200 (Pharmacia) column (1 × 100 cm) in PBS buffer. Fractions were concentrated and after dialysis against RPMI 1640, filter sterilized and used for lymphocyte proliferation assay.

Lymphocyte proliferation assay. The draining popliteal lymph nodes of normal BALB/c mice were removed and lymphocyte proliferation assay was performed. Lymph node cells were plated in triplicate at 4×10^5 cells/well in 96-well flat-bottomed tissue culture plates in RPMI 1640 medium containing penicilin, streptomycin and 10% heat-inactivated FCS. Cells were stimulated in the presence of different concentration of culture supernatant of *L. major* amastigotes or promastigotes with PHA (50 µg/ml) or Con A (10 µg/ml). Cells were cultured in 5% CO_2 in air at 37°C for 3 days. During the last 18 h of culture, 0.5 µCi of tritiated thymidine per well was added. Cells were harvested onto a glass filter mat and radioactivity was measured in a beta-counter. Proliferation was measured as counts per minute.

Gel electrophoresis. The purity of protein profile was determined by 15% SDS-PAGE using Laemmli's method [6]. Gels were stained with silver reagent.

RESULTS AND DISCUSSION

In this report, we semi-purified the excerted antigens of *L. major* promastigotes and axcenic amastigotes by column chromatography. Figure 1 shows five fractions obtained from the column. The profile of amastigotes and promastigotes were the same. Each fraction obtained by column chromato-graphy was assayed for immunosuppressive activity. The maximum suppressive activity was seen in fraction V of both forms of parasite. This fraction showed dose-dependent antiproliferative activity and with 15 µg protein about 81% suppression of lymphocyte proliferation was obtained (Fig. 2). To determine the purity of the separated proteins, SDS-PAGE was performed on all fractions (Fig. 3). In fraction V, with maximum immunosuppressive activity only few protein bands (>20 kDa) were observed.

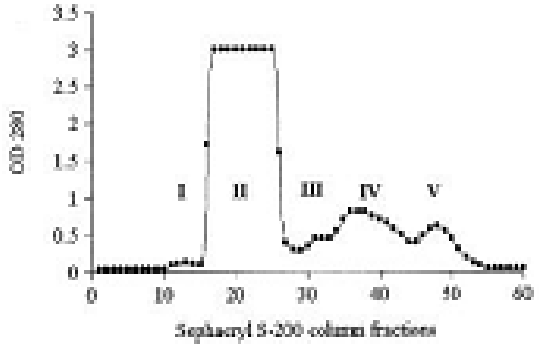

Fig. 1. Purification of *L. major* culture supernatant with immunosuppressive activity by Sephacryl S-200 column chromatography (100 × 2 cm). Fractions were collected and after concentration and filtration used for immunosuppressive activity.

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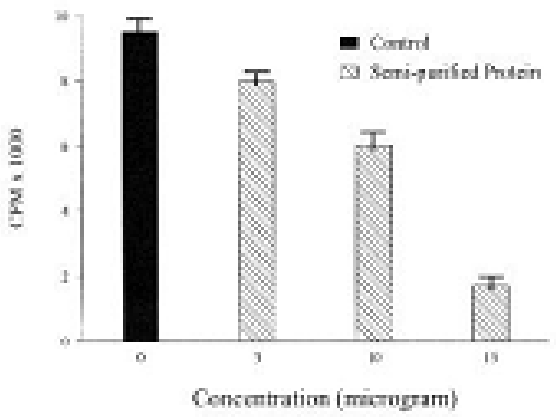

Fig. 2. Lymphocyte proliferation assay of fraction V obtained from Sephacryl S-200 column chromatography. Different concentrations of fraction were used on normal BALB/c mouse lymph node lymphocytes stimulated with PHA. With 10 and 15 microgram of protein almost 26 and 81% suppression of proliferation occurred, respectively.

Although the precise function of the secreted immunosuppressive factor(s) of *Leishmanai* is not known at this time, it may have survival advantage within the phago-lysosome by acting on activating macrophages or on the production of Th1 cytokines. There is also no direct evidence whether the suppressive protein is actively secreted or shed by promastigotes and amastigotes.

Previously we have shown that culture supernatants of *L. major* promastigotes and axcenic amastigotes have immunosuppressive activity (unpublished data). The secreted antigens prevented the proliferation of normal BALB/c lymphocytes that were stimulated with either Con A or PHA. Another group also characterized the immune responses elicited by *L. major* promastigote culture supernatant proteins [7]. However, these secreted proteins elicit strong *in vitro* proliferative responses from lymph node cells of *L. major* infected BALB/c mice and from leishmaniasis patient peripheral blood mononuclear cells. Immunization of BALB/c mice with these proteins could result in protection from lethal challenge of parasites. The discrepancy between our results and theirs are based on different factors. They used *L. major* Friedlin strain that was different from our strain (MRHO/IR/75/ER). For lymphocyte proliferation, they employed lymph node cells of *L. major* infected BALB/c mice, but we used lymphocytes of normal un-infected BALB/c mice.

In another report, crude antigenic fraction and subfractions of *Leishmania major* promastigotes by freeze thaw lysis showed inhibition of cytotoxicity of NK cells and IL-4 secretion, however, they induced IFN-γ secretion by human normal peripheral blood lymphocytes [8]. The molecular weight of fraction was between 29 kDa and 97.4 kDa. It seems the structural proteins of parasite has different functions than secretory proteins on immune system.

Previous studies have shown that promastigotes of *L. donovani* secrete or shed as many as 40 distinct glycoproteins into the culture medium [9], however, the only culture supernatant proteins that have been extensively characterized, cloned and sequenced are the secreted acid phosphatases [10, 11], certain members of the PSA-2 or GP46 family [12] and 22.1 kDa thiol-specific-antioxidant protein [7]. Unlike other species of *Leishmania*, promastigotes of *L. major* do not produce secreted acid phosphatases [13]. Therefore, our immuno-suppressive factor cannot be acid phosphatase. Other secreted proteins are a protein associated with lipophosphoglycan [14, 15] and proteo-phosphoglycan [16].

Several mechanisms have been proposed for parasite survival within phagolysosome. One survival mechanism may be the surface carbohydrates of the parasite that may decrease the accessibility to membrane proteins [17, 18]. The major host immune defense mechanism against *Leishmania* and the killing of intramacrophage parasites is macrophage activation by the release of T-cell factors especially IFN-γ, otherwise macrophage are unable to destroy the parasite [19]. Absence of IFN-γ production is responsible for the development of visceral leishmaniasis and diffuse cutaneous leishmaniasis [20]. Another mechanism

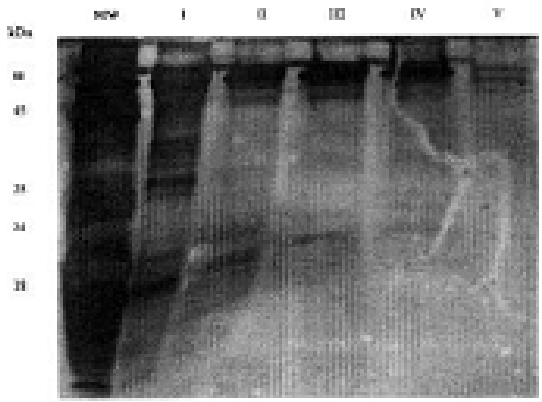

Fig. 3. SDS-PAGE (15%) analysis of *L. major* promastigotes culture supernatant fractions (lanes I-V) isolated from Sephacryl S-200 column chromatography. Fraction V had strong immunosuppressive activity.

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that prevents parasite killing by activated macrophages is the role of IL-10 that prevents macrophage activation and diminishes their production of IL-12 and TNF-α [21], also preventing the macrophages from responding to IFN-γ [22]. T cell responses during the early-phase of infection are down regulated by IL-10 and may facilitate parasite multiplication [23].

IFN-γ activated macrophages induce the intracellular destruction of amastigote forms through the inducible nitric oxide synthase (iNOS) [24]. TNF-α acts in synergy with IFN-γ for NO synthesis, whereas, the parasite upregulates the production of TGF-β which blocks IFN-γ-induced production of NO [25]. Recent studies show that macrophage-derived IFN-α and -β may play a central role in regulating the production of IFN-γ by macrophage (not by T, B and NK cells) as well as inducible NO synthase, and both are important for controlling vacuolar pathogens in macrophages [26]. As seen in mycobacteria, parasites remain in phagosomes without fusion with lysosomes and also avoid the generation of antigenic peptides in endosomal compartments [27].

It seems immunosuppressive factor(s) that is secreted by parasites may have different functions in modulating the immune system. By suppressing the lymphocytes proliferation, the production of IFN-γ may decrease and therefore prevents the macrophage activation. Other possibility may be due to the inactivation of cytotoxic T-cells or prevention of macrophages to produce NO for destruction of intracellular parasite. More data are needed to test these possibilities with purified immunosuppressive factor and to characterize its mechanism of action.

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