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Immunosuppressive Activity of *Leishmania major* Culture Supernatant

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Abstract

BALB/c mice are highly susceptible to infection with the protozoan parasite *Leishmania major*. This susceptibility has been attributed in part to the expansion of Th2 cells and production of their cytokines, IL-4 and IL-10 and down-regulation of Th1 cytokine, INF-γ. The inability of susceptible hosts to mount the immune response necessary to activate macrophage and destroy the parasites can be due to the parasite-specific proteins that are able to modulate the immune system. In this report, we monitored the culture supernatant of the parasite in order to show the existence of immunosuppressive factor. The *L. major* parasites were isolated from lesion of infected BALB/c mice. Amastigotes were grown at 35 °C and promastigotes at 22-25°C in the presence of 10% FCS. Culture supernatants were harvested after 72 h of incubation and used for lymphocyte proliferation assay using lymph node and spleen cells stimulated with mitogens. We have shown that the excretory materials of promastigotes and axenic amastigotes have a potent immunosuppressive activity. Culture supernatants can suppress the proliferation of mitogen-stimulated lymph node lymphocytes (74%) or spleen cells (90%) in a dose-dependent manner. This result may show that parasite by excretory materials can influence INF-γ production by T cells and macrophage activation in order to survive within the macrophage and propagating inside the phagolysosome.

Keywords: Leishmania major, *Excretory factor, Immunosuppressive, Amastigote, Promastigote*

Introduction

Leishmania is a protozoan parasite that is distributed worldwide, being endemic in 88 countries. Leishmaniasis is a complex disease caused by infection with different species of *Leishmania* that 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 successfully. Each year, 1.5 million new cases of cutaneous leishmaniasis and 500,000 new cases of visceral leishmaniasis are estimated (1).

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. Subcutaneous injection of *L.major* into BALB/c mice results in a characteristic uncontrolled growth of the parasite at the site of inoculation which then spread to the local draining lymph nodes. Without intervention, the infection eventually visceralizes and causes the death of the animal. C57BL/6 mice, on the other hand, are able to mount an effective immune response against the *L.major* parasite and control the infection at an early stage. 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, is associated with extreme susceptibility in BALB/c mice (2).

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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 that multiplies in the phagolysosome vacuole of macrophages. Although most promastigotes (~80%) are destroyed 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, or parasite-specific proteins such as immuno-suppressive factors may also contribute to their ability to evade host recognition and thereby propagate within macrophage (4). Several studies have shown that promastigotes of *L.donovani* secrete or shed as many as 40 distinct glycol-proteins into the culture medium (5), however, the precise function of these antigens is not known.

In this study, we report the existence of immunosuppressive activity released into the culture supernatant by *L.major* promastigotes and axenic amastigotes.

Materials and Methods

Parasite and culture supernatant Lesion-derived amastigotes 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. Axenic *L.major* amastigotes were grown at 35 °C at pH 5 to 5.5 and promastigotes were grown at 22-25 °C in the presence of 10% FCS. Culture supernatants were harvested after 72 h of incubation by centrifugation and then filtered 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 penicillin, strep- tomycin and 10% heat-inactivated FCS. Cells were stimulated in the presence of different con- centration 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 d. 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. In addition, spleen cells of normal BALB/c mice were also removed and assayed the same way as lymph node cells.

Culture supernatants were concentrated by Ami- con membrane with a 10,000 M_r Cut-off and 5-150 µg of proteins were used for lymphocyte proliferation assay as mentioned above.

Results

We used culture supernatants of both axenic amastigotes and promastigotes of *L.major* parasites to study the effect of the secreted antigens on immune system modulation. Fig. 1 shows that amastigote culture supernatant can inhibit the proliferation of PHA or Con A stimulated lymphocytes of spleen cells of normal BALB/c mice by 83% and 65%, respectively. In addition, the excreted antigens of promastigotes can inhibit proliferation of PHA or Con A stimulated lymphocytes by 76% and 90%, respectively. Fig. 2 shows that when excreted antigens were concentrated and 5-150 µg was used for proliferation of lymph node lymphocytes, 74% and 45% suppression were obtained for amastigotes and promastigote antigens, respectively. When different concentration of FCS was used no suppressive activity was observed.

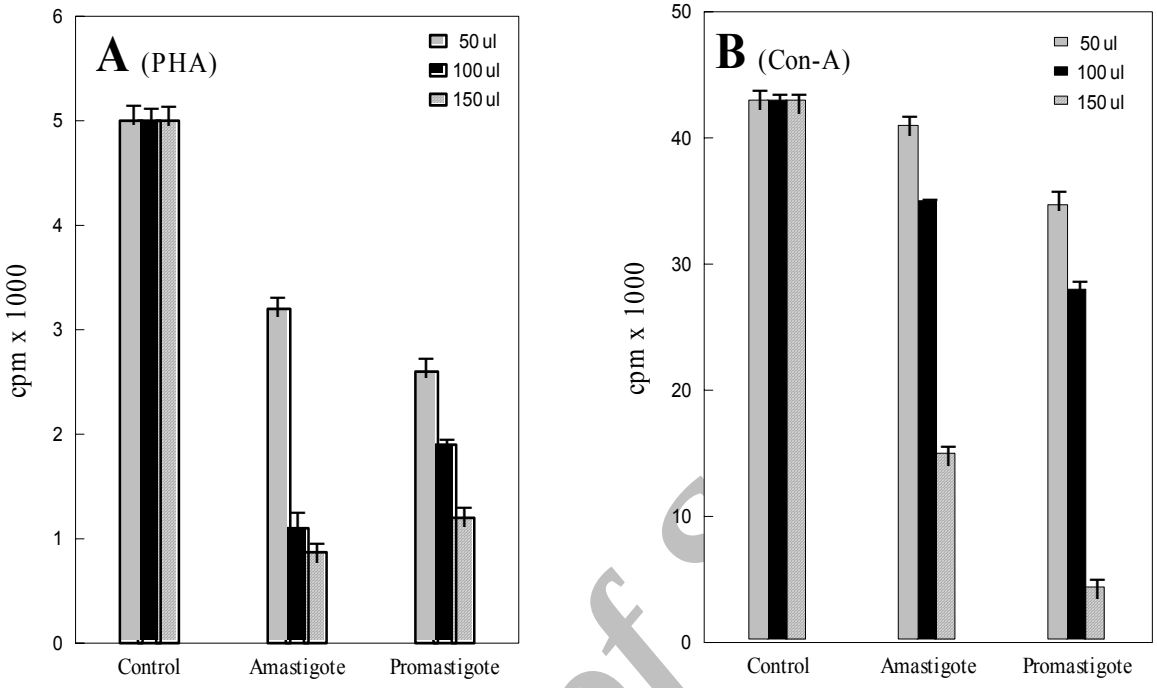

Fig. 1: Effect of different concentrations (50-150 µl) of excreted antigens of both promastigoes and amastigotes on proliferation of spleen cells after stimulation with (A) PHA or (B) Con A mitogens. Controls are mitogen-stimulated lymphocytes without excreted antigens. The results are the mean SD from three experiments.

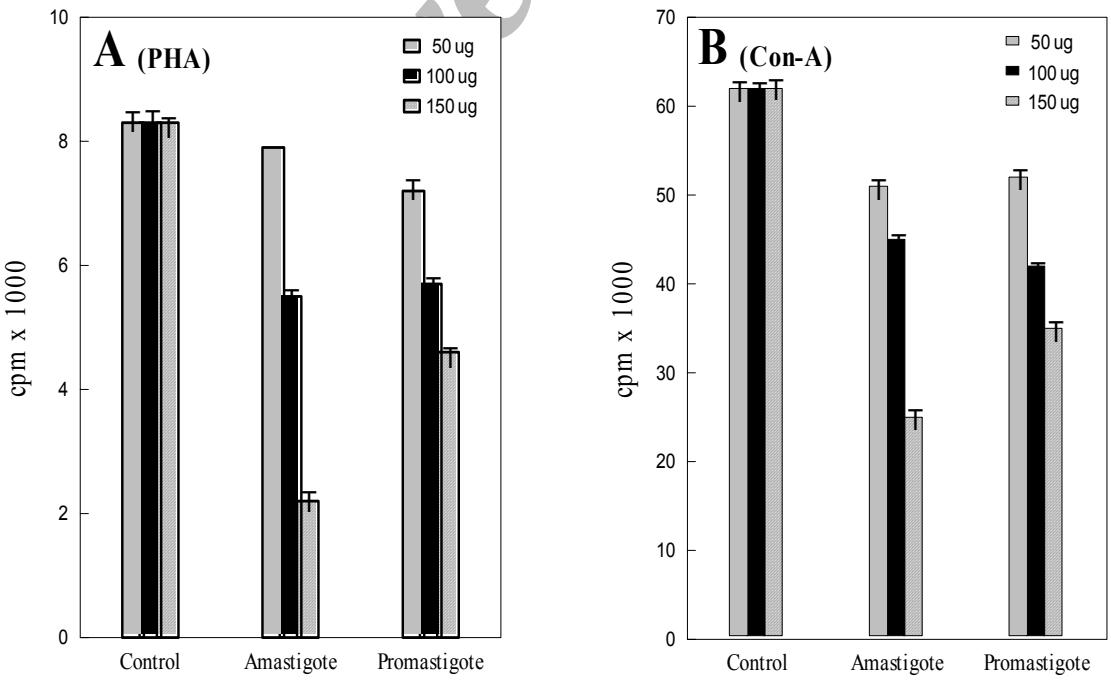

Fig. 2: Effect of different concentrations (50-150 µg) of excreted antigens of both promastigoes and amastigotes on proliferation of lymph node cells after stimulation with (A) PHA or (B) Con A mitogens. Culture supernatants were concentrated

with Amicon filter with 10 kDa MW cut off. Controls are mitogen-stimulated lymphocytes without excreted antigens. The results are the mean SD from three experiments.

Discussion

In this report, we have shown that culture supernatants of promastigotes and axenic amastigotes forms of *L.major* parasite have immunosuppressive activity. The secreted antigens prevent the proliferation of normal BALB/c lymphocytes that were stimulated with either Con A or PHA. Another investigator (5) also charac- terized the immune responses elicited by *L.major* promastigote culture supernatant proteins. 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 than our strain (MRHO/IR/75/ER). For lymphocyte proliferation, they employed lymph node cells of *L.major* infected BALB/c mice, but we used lym-phocytes of normal un-infected BALB/c mice.

Several studies have shown that promastigotes of *L.donovani* secrete or shed as many as 40 distinct glycoproteins into the culture medium (6), however, the only culture supernatant proteins that have been extensively characterized, cloned and sequenced are the secreted acid phosphatases (7, 8), certain members of the PSA-2 or GP46 family (9) and 22.1 kDa TSA protein (5). Unlike other species of *Leishmania*, promastigotes of *L.major* do not produce secreted acid phosphatases (10). Other secreted proteins are a protein associated with lipophosphoglycan (11, 12) and proteophosphoglycan (13). These investigators showed that the secreted antigens have protective capabilities and can enhance the proliferation of peripheral blood mononuclear cells.

The suppressive activity is not due to the FCS concentration, since different concentration of FCS did not have antiproliferative activity. Although the precise function of the secreted immunosuppressive factor (s) of *Leishmania* is not known now, it may have survival advantage within the phagolysosome by acting on activating macrophages or on production of Th1 cytokines. There is also no direct evidence whether the suppressive protein is actively secreted or shed by promastigotes and amastigotes.

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 (14, 15).

The major host immune defense mechanism against *Leishmania* and the killing of intramacrophage parasites is macrophage activation by the release of T-cell factors specially IFN-γ, otherwise macrophage are unable to destroy the parasite (16). Absence of IFN-γ production is res-ponsible for the development of visceral leishma-niasis and diffuse cutaneous leishmaniasis (17). Another mechanism that prevents parasite killing by activated macrophages is the role of IL-10. IL-10 prevents macrophage activation and down regulates the production of IL-12 and TNF-α by macrophage (18), as a result, macrophage can not respond to IFN-γ to eliminate the intracellular parasites (19). T cell responses during early-phase infection are down-regulated by IL-10 and may facilitate parasite multiplication (20).

Several investigators have focused on the nitric oxide-mediated cytostatic or cytostasis activities of activated macrophages on intracellular (21-23) and extracellular parasites. IFN-γ activated macrophages induce the intracellular destruction of amastigote forms through the inducible NO synthase (iNOS). Several cytokines, such as TNF-α and TGF-β, are involved in the regulation of NO-mediated killing of intracellular parasites. TNF-α acts in synergy with IFN-γ for NO synthesis, whereas, the para-

site up-regulates the production of TGF-β which blocks IFN-γ-induced production of NO (24).

It seems immunosuppressive factor (s) secreted by parasites may have different functions in modulating the immune system. By suppressing the lymphocytes proliferation, the production of IFN-γ decreases 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 the mechanism of action.

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