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Comparison of Th1 and Th2 Responses in Non-Healing and Healing Patients with Cutaneous Leishmaniasis

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Abstract

Background: Cutaneous leishmaniasis is an endemic disease in many regions of Iran, including the city of Mashhad. In recent years, some cases have not responded to Glucantime, the usual treatment for this disease. The cellular immune response caused by T-helper type 1 (Th1) cells has an important role in protection against leishmaniasis, and activation of the T-helper type 2 (Th2) response causes progression of the disease. By analyzing these responses we hope to find a more effective treatment than that currently in use for leishmaniasis patients.

Methods: The cellular immune responses in 60 cases of non-healing and healing cutaneous leishmaniasis, and individuals in a control group, were analyzed by measuring cytokines released by peripheral blood mononuclear cells (PBMCs) when stimulated with *Leishmania major* antigens by Enzyme Linked Immuno Sorbent Assay (ELISA).

Results: Subjects from the healing group secreted more interleukin-12 (IL-12) and interferon gamma (IFN-γ) ($p<0.05$) and less interleukins -4, -5, -10 (IL-4, IL-5, and IL-10) ($p<0.005$) and -18 (IL-18) ($p=0.003$) than the non-healing group.

Conclusions: The results demonstrate that secretion of cytokines that activate Th2 response including IL-4, IL-5 and IL-10 in non-healing subjects was higher than healing subjects and secretion of cytokines that activate Th1 response including IL-12 and IFN-γ in healing subjects was higher relative to the non-healing subjects. In this study it has been shown that the level of IL-18 progresses disease in non-healing patients when the level of IL-12 gets decreased.

Keywords: Cutaneous leishmaniasis, Cytokines, Glucantime

Introduction

Leishmaniasis represents a multifarious of diseases with a clinical and epidemiological diversity (1). An estimated 12 million humans are infected, with an incidence of approximately 1.5 million cases of the cutaneous type of the disease (2). Cutaneous leishmaniasis (CL) is an endemic disease in many locations of Iran, including the city of Mashhad. The clinical ending of *Leishmania* infection in humans, ranging from relatively mild to severely life-threatening disease, depends on host- and parasite-associated factors (3). The pentavalent antimonials have been used for over 60 years to treat leishmaniasis, but in the recent years, not all cases have responded to these drugs. One drug in this class with reduced efficacy is Glucantime. Successful treatment of

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leishmaniasis requires not only a decrease in parasite number but also the increase of an effective cell-mediated immune response (2). Th1-type cellular immune responses play an important role in defense against infection with *Leishmania* parasites, whereas activation of Th2-type cells results in progressive disease (4, 5). In the present study, we investigated cell-mediated immunity in non-healing CL subjects by measuring production of the cytokines interleukins -4, -5, -10, -12, and -18 (IL-4, IL-5, IL-10, IL-12, and IL-18) and interferon gamma (IFN-γ). The responses were compared with those of non-healing CL subjects and healthy controls.

Materials and Methods

Study groups

In this study 60 subjects were selected from number 1 (Abobargh, Vila) clinic and Ghaem Hospital (Mashhad, Iran) between December 2006 and March 2007. The study groups were as follows: the first group consisted of 20 subjects who had recovered after one period of treatment from CL (healing group); the second group consisted of 20 subjects with chronic CL lesions (non-healing group) who had not recovered after three periods of treatment (group 2); the third group consisted of 20 subjects with no history of leishmaniasis who were leishmanin skin test negative (control group). All subjects signed a written consent and the ethical guidelines. All subjects were confirmed negative for human immunodeficiency virus (HIV), human T-cell lymphtropic virus (HTLV-1), and intestinal helminthes that promote Th2-type cell production, and fasting blood sugars (FBS) were determined.

Isolation of mononuclear cells

Blood was collected from healing CL, non-healing CL, and control subjects and treated with ethylenediaminetetraacetic acid (EDTA). PBMCs were isolated by Ficoll (Biogene) density centrifugation at 3000 rpm. The cells were washed twice with phosphate-buffered saline (PBS). Cell viability was ascertained by trypan blue exclusion.

Antigen and mitogen

A clone of *Leishmania major* (*L. major*) (MRHO/IR/75/ER, a gift from Dr. H. Nahrevanian, Department of Parasitology, Pasteur Institute, Iran) was used for this study. The parasites were cultured to stationary phase in RPMI 1640 (Gibco) with 17% heat-inactivated fetal calf serum (FCS) (Gibco). The promastigotes were harvested and adjusted to 4×10^7 parasites/ml followed by rapidly freezing and thawing the parasites 5 times and a final ultrasonication for 5 minutes. This method prepared crude antigen (Ag) of *L. major* to stimulate PBMCs. The samples were stored at -70 ºC until use.
Phytohemagglutinin (PHA) (Gibco) was used at a concentration of 10 µg/ml.

PBMC culture

PBMCs were cultured in RPMI 1640 (Uroclone) enriched with 10% heat-inactivated FCS and 1% penicillin-streptomycin solution (Biosera). The cells were incubated with crude *L. major* Ag (41.5µg/ml) and mitogen (PHA) (10 µg/ml) at 1×10^6 cells/ml in volumes of 1 ml in 12-well round-bottom microculture plates (Nunc, Denmark). The cultures were incubated for two days at 37 °C under 5% CO2. The supernatants were stored at -70ºC until use.

Cytokine measurements

Cytokines in supernatants were measured by an enzyme-linked immunosorbent assay and the biotin-avidin system according to the manufacturer's guidelines. The assays were calibrated to detect IL-4, IL-5, IL-10, IL-12 and IFN-γ (Biosource Kit) and IL-18 (Bender MedSystems Kit) within the ranges considered in the protocol.

Statistical analysis

Cytokine levels of subjects in each group were analyzed by the analysis of variance (ANOVA) and Kruskal-Wallis in the SPSS software. $P<0.05$ was considered significant.

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Results

Of the 60 subjects evaluated in this study, 20 were identified as non-healing, 20 as healing, and 20 as controls. Clinical findings for the study groups are summarized in Table 1.

Table 1. Major characteristics of healing, non-healing, and control groups.

Characteristic	Study group		
	Healing	**Non-healing**	**Control**
No.	**20**	**20**	**20**
Mean Age year (range)	**23 (15-35)**	**27 (15-50)**	**39 (20-54)**
M/F ratio	**8/12**	**8/12**	**4/16**
Mean no. of lesions (range)	**2.35 (1-9)**	**3.45 (1-5)**	**–**
Treatment type (systemic/local)	**10/10**	**10/10**	**-**

Cytokine production

PBMCs from subjects were incubated with Ag and mitogen and the levels of each cytokine were determined in the supernatants. IFN-γ was significantly higher in the healing (P=0.033) and control (P<0.0001) groups than in the non-healing group, while no significant difference was observed between the healing and control groups (Fig. 1). IL-4 was significantly higher in the healing than in the non-healing group (P<0.0001), and higher in the control than in the healing group (P=0.033), while no significant difference in was observed between the non-healing and control groups (Fig. 2). IL-5 was significantly higher in the non-healing than in the healing group (P<0.001) and significantly higher in the control than in the healing group (P=0.029), while no significant difference was observed between the non-healing and control groups (Fig. 3).

IL-10 was significantly lower in the healing than in the non-healing group (P<0.0001) and significantly higher in the control than in the healing group (P=0.001), while no significant difference was observed between the non-healing and control groups (Fig. 4).

IL-12 was significantly higher in the healing (P=0.031) and control (P=0.04) groups than in the non-healing group, while no significant difference was observed between the healing and control groups (Fig. 5).

IL-18 was significantly higher in the non-healing than in the healing (P=0.003) and control (P=0.004) groups, while no significant difference was observed between the control and healing groups (Fig. 6).

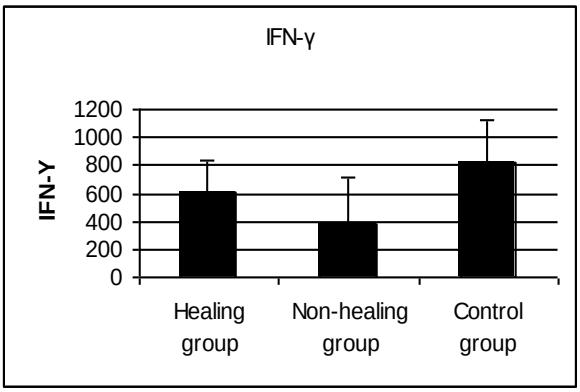

Fig. 1. Concentrations of IFN-γ in culture supernatants of PBMCs from healing, non-healing, and control groups stimulated with Ag and mitogen.

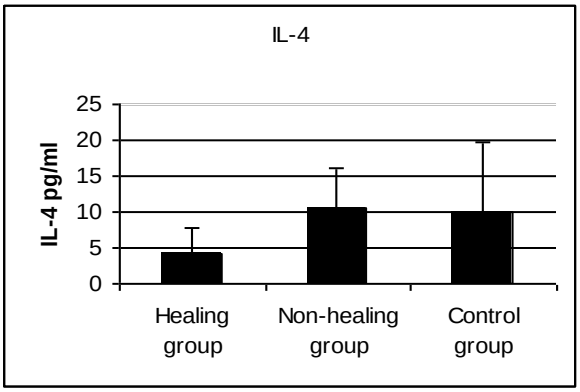

Fig. 2. Concentrations of IL-4 in culture supernatants of PBMCs from healing, non-healing, and control groups stimulated with Ag and mitogen.

Discussion

In this study we measured cytokines produced from PBMCs ex vivo after stimulation with *Leishmania* Ag and PHA in supernatants. Chemotherapeutic cure of leishmaniasis is largely dependent upon the development of an effective immune response that activates macrophages to produce toxic nitrogen and oxygen intermediates to exterminate the amastigotes. CD4+ T cell populations were found to be an essential issue either in disease progression associated with IL-4 or in disease progression associated with IL-4 or in disease prevention related to IFN-γ (6).

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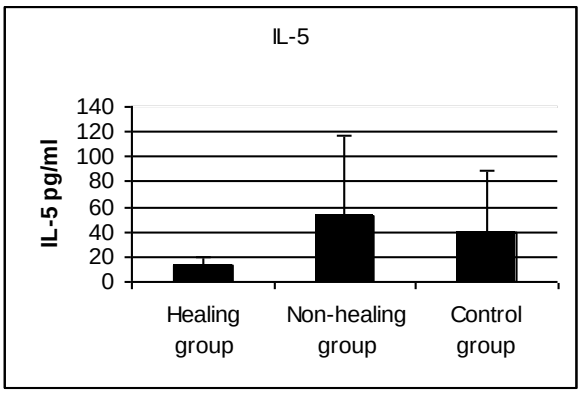

Fig. 3. Concentrations of IL-5 in culture supernatants of PBMCs from healing, non-healing, and control groups stimulated with Ag and mitogen.

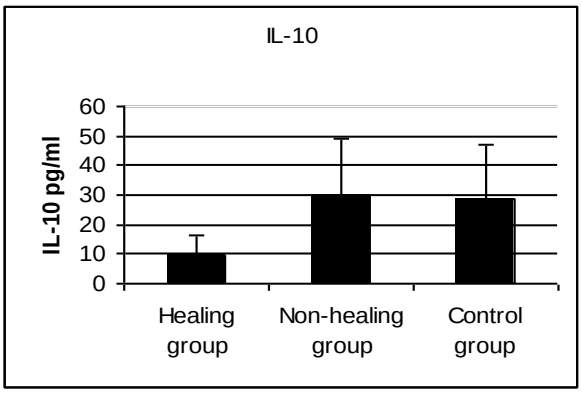

Fig. 4. Concentrations of IL-10 in culture supernatants of PBMCs from healing, non-healing, and control groups stimulated with Ag and mitogen.

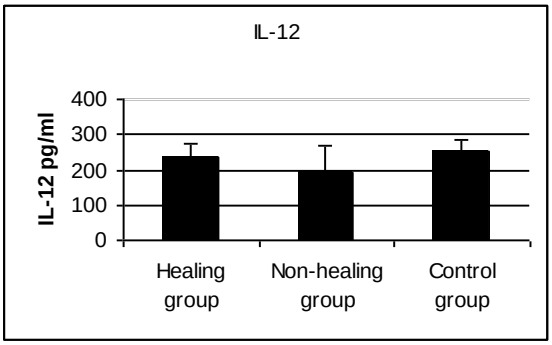

Fig. 5. Concentrations of IL-12 in culture supernatants of PBMCs from healing, non-healing, and control groups stimulated with Ag and mitogen.

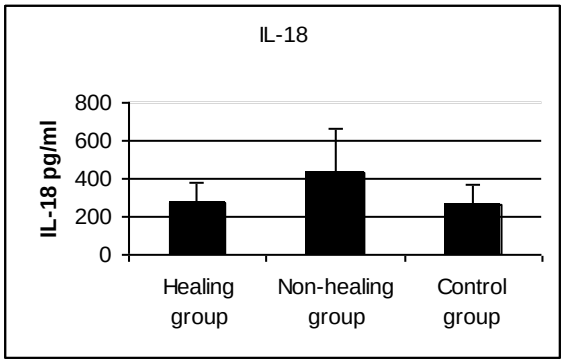

Fig. 6. Concentrations of IL-18 in culture supernatants of PBMCs from healing, non-healing, and control groups stimulated with Ag and mitogen.

Th1-type cellular immune responses within a suitable cytokine (IFN-γ, IL-12) play a significant role in protection against infection with *Leishmania* parasites, whereas activation of Th2-type cells results in progressive disease. CL is often a self-healing disease; however, persistence of lesions lasting for several years is known to occur (non-healing form) (5, 12). There are few reports on the immune status of non-healing patients, particularly those infected with *L. major*. In this study PBMCs from the healing and control groups showed high levels of IL-12. In contrast, a low level of IL-12 was produced in the non-healing group. Similar data were previously reported by Habibi et al. IL-12 is essential for the stimulation of Th1 phenotype-dependent protection (8, 9).

It has been reported that IFN-γ improved the efficacy of antimonials in the treatment of visceral leishmaniasis (VL) and CL (6). IFN-γ is crucial, but not sufficient, to control leishmaniasis. It is known that IFN-γ is one of the major macrophage-activating cytokines, and activated macrophages are a major source of IL-12, which induces autocrine macrophage activation (10). Our data reveals that higher levels of IFN-γ were secreted by PBMCs from the healing and control groups than the non-healing group. *Leishmania*-specific central memory $CD4^+$ T cells require IL-12 to produce IFN-γ, demonstrating that this population needs additional signals to develop into Th1 cells (11).

Ajdary et al. showed strong IFN-γ production from PBMCs from healing cases in response to soluble *Leishmania* antigen (SLA) (5).

Our results show that IL-4 was secreted at a high level in the non-healing and control groups compared to the healing group. It has been shown that IL-4 is associated with the down-modulation of IFN-γ-mediated macrophage activation and the development of vulnerability in leishmaniasis (8, 12). Habibi et al. reported the expression of IFN-γ and IL-12 gene in PBMCs from non-healing cases infected with *L. major* in response to in vitro stimulation

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with recombinant gp63 (rgp63) was decreased, but the expression of IL-4 was increased, in these cases (8).

The findings of the present investigation demonstrated high levels of IL-10 in non-healing and control groups and a low level of IL-10 in the healing group. Non-healing forms of leishmaniasis in humans are commonly associated with elevated levels of the deactivating cytokine IL-10, and in the mouse, normally chronic infections can be cleared in the absence of IL-10. These findings are similar to those reported by Anderson et al., which demonstrated IL-10-producing T cells, activated early in a strong inflammatory setting as a mechanism of feedback control, are the major mediators of T cell-derived IL-10-dependent immune suppression in a chronic intracellular infection (13). In the present study the level of IL-5 was low in the healing group relative to the non-healing and control groups. A Th2 response, with production of interleukin IL-5, can aggravate the disease (14).

Interleukin-18 (IL-18), originally recognized as the IFN-γ-inducing factor, plays an important role in both innate and adaptive immune responses against intracellular pathogens (15). Unexpectedly, the present investigation showed strong IL-18 production from PBMCs from non-healing cases in response to *Leishmania* Ag relative to the healing and control groups. No reports have compared secretion of IL-18 from PBMCs in healing and non-healing CL cases in response to in vitro stimulation with *Leishmania* Ag in human. Despite its importance in inducing and regulating immune responses, relatively little is known about its production in *Leishmania* infections. Wei et al. have shown that BALB/c IL-18-/- mice were more resistant to *L. major* infection than WT BALB/c mice, whereas DBA/1 IL-18-/- mice were markedly more susceptible than their WT littermates. BALB/c IL-18-/- mice produced less IFN-γ and IL-4, whereas DBA/1 IL-18ko mice produced more IFN-γ and IL-4 than their respective WT controls. These results clearly demonstrate that the role of IL-18 in resistance or susceptibility to *L. major* is determined by host genetic background (16). Nakanishi et al. demonstrated IL-18 alone or without help of IL-12 could stimulate Th2 cytokine production as well as allergic inflammation. Therefore, the functions of IL-18 in vivo are very heterogeneous and complicated. In principle, IL-18 enhances IL-12-driven Th1 immune responses, but it can also stimulate Th2 immune responses in the absence of IL-12 (17). However IL-12 synergizes with IL-18 in exciting IFN-γ production by NK cells (18). IL-18 regulates either Th1 or Th2 responses depending on the cytokine microenvironment. One study in BALB/c mice has shown administration of recombinant IL-18 (rIL-18) alone does not advance Th1 response, but rather induces Th2 response and exacerbates *L major* infection in susceptible BALB/c mice (19). The findings of the present investigation demonstrate low levels of IL-12 secreted in the non-healing group. In addition, the level of IL-18 that was previously thought to enhance secretion of IL-12 and activate Th1 responses, in this study showed higher secretion in non-healing patients than healing patients and induced Th2 responses with disease progression. In addition to the above mentioned factors, this unexpected finding maybe depend on decreasing IL-12 secretion from PBMC in non-healing patients.

In the present investigation the level of secreted cytokine was determined after incubating PBMC with Ag and mitogen for two days. The most highly-secreted cytokine was IFN-γ, and Th2 cytokines were secreted at lower levels than Th1 cytokines.
IFN-γ was the most highly-secreted cytokine in PBMCs from the control group following exposure to *Leishmania* Ag. In this study all the evaluated cytokines in the three groups were secreted in measurable amounts, whereas Ajdary et al. reported PBMCs from control and non-healing donors produced low or negligible levels of IFN- γ (5).

In conclusion, the response of PBMCs from subjects with healing cutaneous leishmaniasis to the stimulus of *Leishmania* Ag and mitogen contrasts with that of non-healing subjects. PBMCs from the healing group produced significantly more IFN-γ and IL-12 than the non-healing group. However, non-healing group PBMCs secreted higher levels of IL-4, IL-5, and IL-10, which activate the Th2 response along with IL-18 in non-healing CL subjects with lack of IL-12, activates the Th2 response and causes progression of the disease.

It is tempting to evaluate these cytokines and other immune cells in these study groups with other techniques such as PCR, especially IL-18 to speculate an association between the increased secretion of IL-18 from PBMCs from the non-healing group and their lack of IFN-γ and IL-12 in company with progression of their disease. Finally, we propose it is possible to find an effective treatment policy for these patients.

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