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Serum Cytokine Production in Patients with Cutaneous Leishmaniasis Before and After Treatment

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Abstract

Background Cutaneous leishmaniasis (CL) is caused by a protozoan from the genus *Leishmania* that infect macrophages of many mammals including humans, their infection induces both humoral and cellular immune responses, but the balance of their expression varies with the type of the disease.

Objective The aim of the present study is to understand the effect of antimonial compounds on some serum cytokines levels that include (IFN-γ, TNF-α, TGF-β, IL-1β, IL-6, IL-8) before, during and after treatment from CL infection.

Methods Eighty people were included in the present study, 60 patients with CL lesions and 20 healthy individuals (control). Patients were diagnosed on the basis of clinical and parasitological criteria. All patients treated with pentostam by intralesional injection. Serum (IFN-γ, TNF-α, TGF-β, IL-1β, IL-6, IL-8) levels were determined by ELISA using a quantitative sandwich enzyme immunoassay technique.

Results Serum levels of (TNF-α, IL-1β, IL-6, and IL-8) were significantly higher in patients group than healthy subjects ($p < 0.05$). INF-γ and TGF-β levels were decreased significantly during infection with CL. During therapy with pentostam, cytokines levels (IFN-γ, TNF-α, TGF-β, IL-1β, IL-6, IL-8) were significantly increased ($p < 0.05$). All cytokines levels returned to the normal values after three months of healing from CL lesions.

Conclusions Cytokines plays an important role in the resolution of CL infection. Pentavalent antimonials compounds may have immuno-stimulating effects which may be responsible for its antimicrobial activities.

Key words Cutaneous leishmaniasis, cytokines, treatment, pentostam.

Introduction

Human leishmaniasis includes a spectrum of diseases with variable severity ranging from cutaneous to visceral diseases, all of them caused by protozoan parasites of the genus *Leishmania* [1,2]. The cutaneous forms are the commonest (1.0 to 1.5 million cases each year), representing 50-75% of all new cases all over the world [2, 3]. In Iraq *L. major* and *L. tropica* are the causes of cutaneous leishmaniasis [4].

Leishmania species are intra-cellular parasites invading monocytes, macrophages, and langerhans cell [5]. Their infection in man induces both humoral and cellular immune responses, but the balance of their expression varies with the type of the disease [6]. A variety of inflammatory mediators are produced by monocytes/macrophages during the course of infection [7], the importance of cytokines during leishmanial infection comes from the demonstration (on experimental murine leishmaniasis) of the existence of two distinct CD4+ Th1 and Th2 subsets [8].

Sodium stibogluconate (pentostam) remain the drugs of choice in the treatment of all forms of *Leishmania* infection, several studies have been demonstrated that there is an important immunological component in response to antimonial therapy [9-11].

The objective of the present study was to determine the serum cytokines levels of IFN-γ, TNF-α, TGF-β, IL-1β, IL-6, IL-8 before, during therapy and after healing in Iraqi patients with CL lesions.

Methods

Patients: This study was conducted at Baghdad Teaching Hospital in Baghdad, during the period from January 2008 to March 2009. Eighty people, 60 patients with CL lesion patients and 20 apparently healthy individuals (control), were included. Their age ranged from 5-40 years.

The clinical diagnosis was confirmed by laboratory demonstration of the parasite in the lesions by direct smears. Lesions were cleaned with ethanol, and punctured at the margins with a sterile lancet. Exudate materials was smeared, dried in air and fixed by methanol. The smears were stained with Giemsa's stain and examined by light microscope. Microscopic diagnosis was made when amastigotes were identified in the smears. In order to confirm the diagnosis, the material was also cultured on Novy Mac – Neal –Nicolle (NNN) medium for up to three weeks to detect the leishmanial promastigotes. Antimonial treatment with (pentostam) was given only through the lesions by intralesional injection, 1-3 ml of this drug was injected, and 1-2 doses were given weekly for two months. The patients were checked weekly for healing or recurrence.

Determination of cytokines: The blood samples were collected after the diagnostic procedure from healthy subjects, CL patients, during therapy and after three months when they healed from their infection. Ten ml of venous blood was withdrawn from each individual, and allowed it to clot for 30 minutes at 37 °C. The tubes were then centrifuged for 10 minutes at 4 °C and 2500 rpm. The serum was collected and stored at -20 °C until the time of the serological test. Serum IL-1β, IL-6, IL-8 and TNF- α levels were determined by ELISA using a quantitative sandwich enzyme immunoassay technique (EASIA kits for IL-β, IL-6, IL-8 by Bio source, Europe) and ELISA kits (Mabtech AB, Sweden) for IFN-γ, TNF-α, TGF-β. All tests were carried out by vigorously following manufacturer instructions. Serum cytokine levels were calculated by interpolating the standard absorbance readings of the test samples calculated from samples of known concentrations supplied with the kits and assayed in parallel. The data were processed using the SPSS PC statistical program (Statistical Package for Social Sciences, PC version 10.0). Initially, mean cytokine levels were compared between groups using Student t-test.

Results

The serum levels of IFN-γ, TNF-α, TGF-β, IL-1β, IL-6 and IL-8 in patients responsive and refractory to antimonial therapy (pentostam) as well as normal controls were determined by ELISA before and after treatment were shown in table 1. The results of the pretreatment determinations of the cytokines levels (TNF-α, IL-1β, IL-6 IL-8) in CL patients were higher significantly ($p < 0.05$) than those in normal controls (Table 2). Serum cytokine levels in patients during treatment are listed in table 1 also. CL patients who were responsive to therapy presented with elevated levels of these cytokines ($p < 0.05$) and they were significantly higher than in control group (Table 2). Low levels of IFN-γ and TGF-β were determined in serum of patients group when compared to control group (healthy), but during therapy these concentrations were increased significantly

($p < 0.05$) when compared to patient group (before treatment). After three months all patients were healing from the infection due to successful therapy, serum IFN-γ, TGF-β, TNF- α, IL-1β, IL-6 and IL-8 levels were returned to the normal values (Table 1) and did not show significant differences ($p > 0.05$) between control group (healthy) and healing group (Table 2).

Table 3 shows the comparative significance (*p*-value) for the repeated measurements at different periods of contrasts (before, during and post healing).

Table 1: Concentrations of cytokines in serum of different study groups

Cytokine	*Concentrations of cytokines (pg/ml)			
	Control group	Patients groups		
		Before treatment	During treatment	Post healing
IFN-γ	102.7 ± 11.46	77.5 ± 15.95	183.4 ± 26.55	103.3 ± 12
TNF-α	14.12 ± 2.359	28.25 ± 3.61	63.5 ± 7.5	14.75 ± 2.25
TGF-β	44 ± 7.17	30.4 ± 4.15	37.8 ± 1.48	43.2 ± 7.29
IL-1β	4.01 ± 0.89	9.31 ± 1.38	14.12 ± 2.23	3.87 ± 2.16
IL-6	5.47± 1.66	12.85 ± 1.28	48.36 ± 6.82	5.08 ± 0.70
IL-8	16.38 ± 1.61	32.22 ± 2.16	333.6 ± 69.97	15.51 ± 1.10

* All cytokine concentrations were expressed as means ± standard deviation

Table 2: Comparisons Significant between Control and treated groups in its' different periods of treatments (Before, during and post healing)

Parameters	Control X Before		Control X During		Control X Healing	
	t-value	P-value	t-value	P-value	t-value	P-value
IFN-γ	4.057	0.001	-8.825	0.000	-0.114	0.910
TNF-α	-9.257	0.000	-19.57	0.000	-0.542	0.596
TGF-β	3.666	0.006	1.892	0.126	0.175	0.866
IL-1β	-8.051	0.000	-12.71	0.000	0.004	0.997
IL-6	-9.936	0.000	-19.18	0.000	0.683	0.504
IL-8	-15.87	0.000	-14.33	0.000	1.204	0.000

$p > 0.05$ Non significant

Table 3: Comparison between Significant (p-value) for the repeated measurements by different periods of contrasts (Before, during and post healing)

(i)	(j)	IFN-γ	TNF-α	TGF-β	IL-1β	IL-6	IL-8
Before	During	0.000	0.000	0.025	0.001	0.000	0.000
	Healing	0.006	0.000	0.003	0.002	0.000	0.000
During	Healing	0.000	0.001	0.175	0.000	0.000	0.000

$P > 0.05$ Non significant

Discussion

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 kill the amastigotes. This process is suppressed

by the infection itself which down regulates the requisite signaling between macrophage and T cells [8].

Gamma interferon (IFN-γ) secreted by Th1 cells is the most potent macrophage-activating cytokine, leading to the host resistance to infection with *Leishmania* parasite [12]. A marked decrease in the production of IFN-γ was observed in patients group (Table 1). The deficient production of IFN-γ after exposure to *Leishmania* antigens is one of the commonly reported factors which associated with increase expression of CD4+ T cells. The disease susceptibility is associated with the inability to produce macrophage-stimulating profile including IFN-γ, IL-2, and IL-12 [13]. Coutinho et al. [14] detected a decrease in the level of *Leishmania* in supernatants from *Leishmania* stimulated cell cultures 2255±653 pg/ml before antimonial therapy and 3005±900 pg/ml at the end of the treatment. A significant increase in the level of IFN-γ was detected in the serum of patients during treatment with pentostam when compared to its level before treatment, this explain that a successful drug therapy were restored T-cell proliferation and IL-2, IFN-γ production in response to *Leishmania* antigen [15]. Furthermore, these findings confirmed the role of IFN-γ in the healing of the lesion due to involvment of CD4+ T cells in the healing process and elevated IFN-γ production at the end of the treatment in human [16]. Also, IFN-γ could be used as immunopotentiator for augmenting the capacity of macrophages to eliminate *Leishmania* infection [17].

Transforming growth factor (TGF-β) is a multipotential cytokine with diverse effects on immune cells, including the down-regulation of certain macrophages and the blockade of IFN-γ induced macrophages activation [18,19]. In this study the concentrations of TGF-β in patients and treated groups decreased significantly as compared to controls (30.4±4.15 pg/ml and 37.8±1.48 respectively). Li et al [20] showed that using anti-TGF-β treatment promotes rapid healing of murine leishmaniasis through enhancing *in vivo* nitric oxide (NO) production by activated macrophages.

In this study, TNF-α and IL-1 levels were found to be significantly higher for CL patients than for control group, and during therapy their concentrations were significantly elevated also.

T cells mediate activation of macrophages to produce NO, resulting in killing or control *L. major* parasites and the secretion of TNF-α by macrophages is sufficient to mediate production of NO and killing of *L. major* parasites [21]. Melby et al. [22] found significant increase in the expression of IL-1β, TNF-α, IL-10 and TGF-β in late lesions compared with that in early lesions, these finding were in agreement with our study.

IL-1 is primarily produced by cells of the mononuclear phagocytic lineage but is also produced by endothelial cells, keratinocytes, synovial cells, astrocytes, osteoblasts, neutrophils, glial cells, and numerous other cells. IL-1 production may be stimulated by a variety of agents, including endotoxins and other cytokines, microorganisms, and antigens. IL-1 is also cytotoxic to cancerous and virus – infected cells [8]. Sodhi et al. [23] demonstrated that IL-1 levels were significantly increased when *L. donovani* infected animals were treated with antimonium salts 14 days post infection; their findings appear to support our study. It is thought that TNF-α and IL-1 levels increase as a part of host defense strategies, and induction of the cytokines by antimonial therapy might be dependent on macrophage activation.

T cells, monocytes and fibroblast produces IL-6, which is a major cytokine involved in T and B cell regulation and also in some aspects of the inflammatory response [24]. Serum IL-6 levels were significantly

elevated in CL patient group compared to values seen for the controls. This cytokine was increased during antimonial therapy in patient group. Several *in vitro* studies demonstrated that some herb powders, such as Echinacea, activate macrophage to produce TNF-α, IL-β, and IL-6 as well as oxidative burst and killing of *Leishmania* parasite [25].

IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells and endothelial cells. It is a proinflammatory cytokines that chemoattract and activates blood cells, beside its central role in inflammation; other biological functions of IL-8 include T cell chemotaxis, angiogensis, and hematopoiesis [26,27]. IL-8 concentrations were significantly higher ($p < 0.05$) in CL patients before treatment (32.22±2.16) than in the control subjects (16.38±1.61) and this increment was still significantly higher during treatment (333.6±69.97). Lejon et al. [28] detected a significant elevation of IL-6 and IL-8 levels in patients of the late stage *Trypanosoma gambiense*. TNF-α stimulate release of IL-8 which may in turn play an important role in the inflammation reaction. The chemokines IL-8 essential to bring the more neutrophils at the site of infection, also other proinflammatory cytokines might induce production of IL-8 to a reactive oxygen species, which caused a direct intracellular killing to *Leishmania* parasite during treatment with antimonial salts [8,29].

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