SHORT PAPER

Antioxidants and Proinflamatory Cytokines in the Sera of Patients with Cutaneous Leishmaniasis

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ABSTRACT

Background: *Leishmania* is a significant health problem in many parts of the world. Tumor necrosis factor (TNF) plays an essential role in *Leishmania major* infections. **Objective:** To study the pro-inflammatory cytokines and antioxidants in four groups of cutaneous leishmaniasis patients. **Methods:** 39 patients were divided into four groups of: 1) active (acute phase of treatment); 2) non-healing (received treatment for almost two years without recovery); 3) healing (recovered upon treatment); and 4) healed (previously received treatment and achieved complete remission) patients. Serum levels of pro-inflammatory cytokines (IL-1B, TNF-α, IL-6) and serum antioxidant levels were measured by ELISA and FRAP assays, respectively. **Results:** While serum antioxidant levels were elevated in the non-healing group, there was no difference among other groups of patients and healthy controls in this regard. Interleukin-1β showed the highest level in the non-healing group followed by the other groups of patients. The mean serum IL-6 level was highest in the non-healing group, but showed no significant change in the other groups. TNF-α and IL-1β levels were non-significantly elevated in the sera of active and non-healing patients. **Conclusion:** Pro-inflammatory cytokines IL-1β, TNF-α, IL-6 maybe related to the progression of leishmaniasis. Serum antioxidant levels maybe correlated with patient response to drug treatment.

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Keywords: Antioxidants, Cutaneous Leishmaniasis, Proinflamatory Cytokines

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INTRODUCTION

Leishmania infection is estimated to affect more than 15 million people worldwide, and presents with 400,000 new cases annually (1). Humans infected with the Leshmania pathogen develop species-specific pathologies (e.g., cutaneous, mucocutaneous, and visceral). In its mammalian host, *Leishmania* can promote numerous phagocyte dysfunctions leading to the inability of these cells to elicit an effective innate and cell-mediated immune response, thus favoring the persistence and progression of the infection (2). Tumor necrosis factor-α (TNF-α) secretion is one of the major effector mechanisms of memory CD8+ T cells believed to be required for immunological protection in vivo. Inflammatory cytokines play an important role in controlling infections such as *Leishmania* and other intracellular parasites. Antioxidants play a coordinated role along with cytokines while regulating the microenvironment in which they function. TNF-α is an essential cytokine in the control of inflammatory lesions and, to a lesser degree, parasite killing in *Leishmania major* infections (3). TNF-α is also considered as an important inflammatory mediator for cell recruitment (6) and healing of pathogen-induced lesions (7). It is shown that viable motheaten SHP-1 deficient mice have a stronger inflammatory response against *Leishmania* infection than wild–type mice. The response in these mice was accompanied by higher pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokine expression and secretion, in addition to increased chemokine and chemokine receptor expression. These inflammatory molecules may be responsible for enhanced cellular recruitment, mainly of neutrophils, seen at the site of infection in viable motheaten mice 6 h post-inoculation (4). It is important to evaluate the differences in the inflammatory response among different types of responses to the parasite. Targeting different elements of the inflammatory responses is a logical approach, yet it needs to deal with a delicate balance in the immune response. Inhibition of the inflammatory responses of synovial cells through induction of apoptosis is known as the main target of therapeutic intervention in other diseases (5).

This study intends to evaluate the level of pro-inflammatory cytokines and antioxidants in four groups of cutaneous leishmaniasis patients compared together and with healthy controls.

MATERIALS AND METHODS

This study consisted of four groups of unequal numbers of *Leishmania*-infected patients in addition to a control group. Comparisons were made among the experimental groups, as well as between the control group and every experimental group.

Samples. Included were patients who fulfilled the Leprosy and Dermal Disease Center, Tehran University of Medical Sciences, Tehran, Iran criteria for leishmaniasis. Serum samples were obtained from clotted blood of 39 patients diagnosed with Zoonotic cutaneous *leishmania* **(**ZCL) and Antroponotic ccutaneous *leishmania* (ACL) leishmaniasis.

Measurement of Cytokine Levels in Sera. We compared the levels of inflammatory cytokines (IL-1B, TNF-α, IL-6) among all groups by sandwich ELISA, according to the manufacturer's recommendations. The following ELISA cytokine kits were used: TNF-α (BMS2034), Il-6 (BMS213/2), and IL-1β (BMS224/2). Human serum levels of

cytokines were measured using an automated micro-plate reader set at 405 nm with a sensitivity limit of 20 pg/ml for TNF-α, IL-β, and IL-6. Serum antioxidant levels were measured by FRAP method (8).

Statistical Analysis. Arithmetic mean, standard error of mean, and the minimum and maximum values were measured for every dependent variable. One-way ANOVA was used for statistical analysis of the data.

RESULTS AND DISCUSSION

Innate pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) and antioxidants in the four groups of patients were measured and the results are summarized in Table 1. In comparison with the other four groups, the highest amount of TNF-α belonged to the active and healing group. The non-healing group had the lowest level of TNF-α followed by the healed group (Figure 1a).

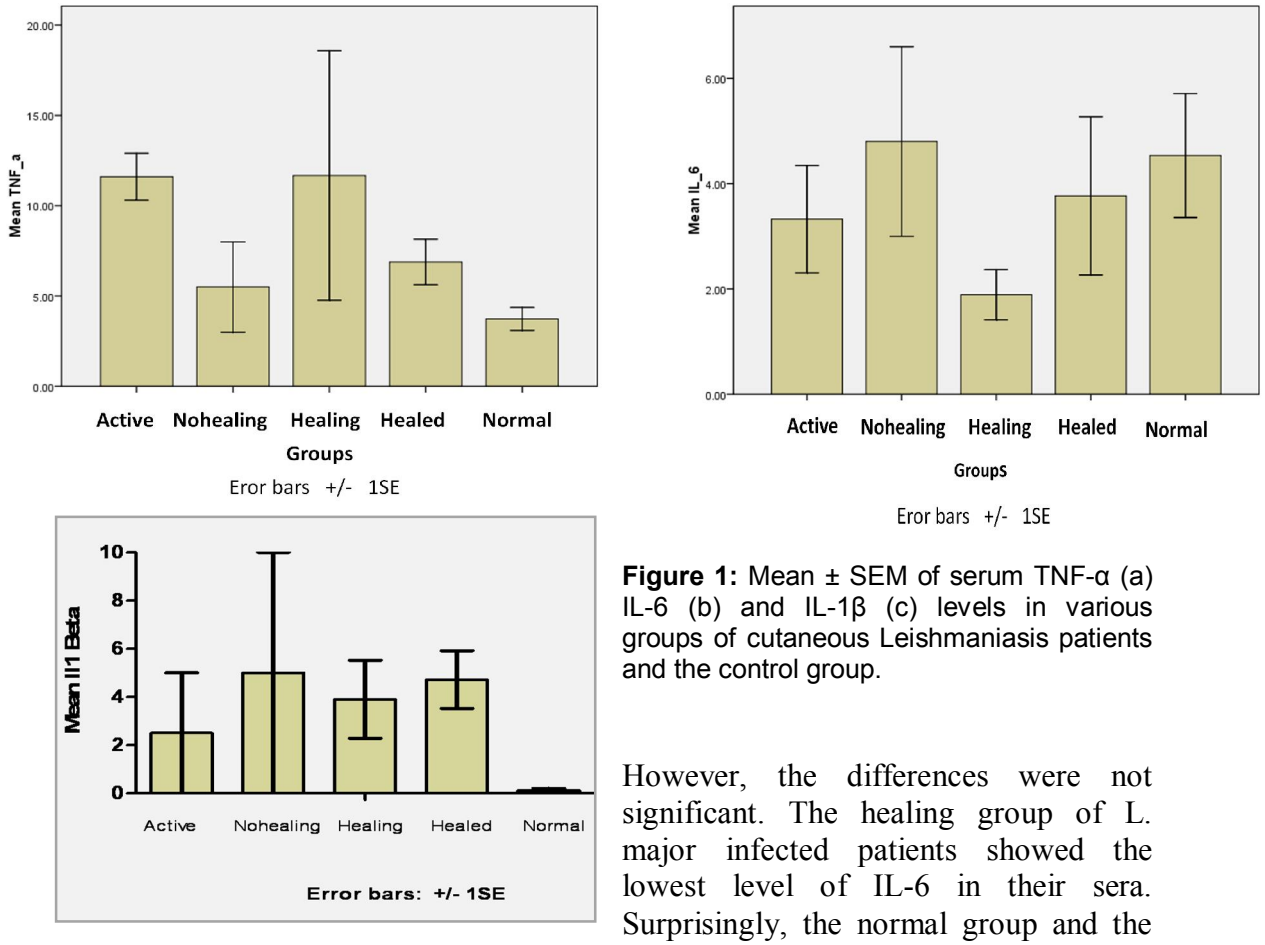

Figure 1: Mean ± SEM of serum TNF-α (a) IL-6 (b) and IL-1β (c) levels in various groups of cutaneous Leishmaniasis patients and the control group.

However, the differences were not significant. The healing group of L. major infected patients showed the lowest level of IL-6 in their sera. Surprisingly, the normal group and the non-healing patients had the highest levels of serum IL-6 (Figure 1b). None of these differences were found to be significant. Although there was no IL-1β in the sera of control individuals, the level of this cytokine was increased in all groups of patients with no significant differences among them (Figure 1c). The highest levels of antioxidants were in the non-healing group compared with the control group. The mean values of the remaining groups were lower than the control group (Figure 2).

Pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are recognized for their role in the inflammatory response (4). The innate inflammatory response is detrimental for survival of *Leishmania* parasite and disease progression. In this regard, our results have shown that TNF-α, and IL-1β levels increased in active and healing patients. The same results were also observed when *Leishmania* lipid treatment in normal rats resulted in elevated serum TNF-α and IL-1β levels (5). The highest amounts of antioxidants were seen in the non-healing group compared with the control group. The mean values of other groups were lower than the control group. Both TNF-α, and the oxidative burst have been described to contribute to the control of *Leishmania major* in vitro and in vivo (9). TNF-α has also been reported to be essential in the healing process of *Leishmania*-induced lesions (4).

We also observed that in patients of the non-healing and active groups, the antioxidant level was higher than the other three patient groups and the control. *Leishmania* lipophosphoglycan suppresses TNF-α, IL-1β, and NO production by lipopolysaccharide-stimulated or PMA-stimulated macrophages. TNF-α induces mononuclear phagocytes and neutrophils to produce Reactive Oxygen Intermediates (ROIs)**.** TNF-α is an in vitro inducer of ROIs that directly allows infected myeloid cells to kill bacteria (10). Accordingly, macrophages deficient for either gene could clear an infection with both *Leishmania major* strains in a manner similar to that of wild-type macrophages and release comparable amounts of nitrite after stimulation with IFN-c alone or in combination with TNF or Lipolysaccharide (LPS) (11). It is possible that an increase in antioxidants causes a decrease in ROIs which are necessary for the removal of intracellular pathogens and containing the disease course in patients. When antioxidants augment, they cause a decline in ROIs which subsequently allows the infection to progress, leading to drug resistance.

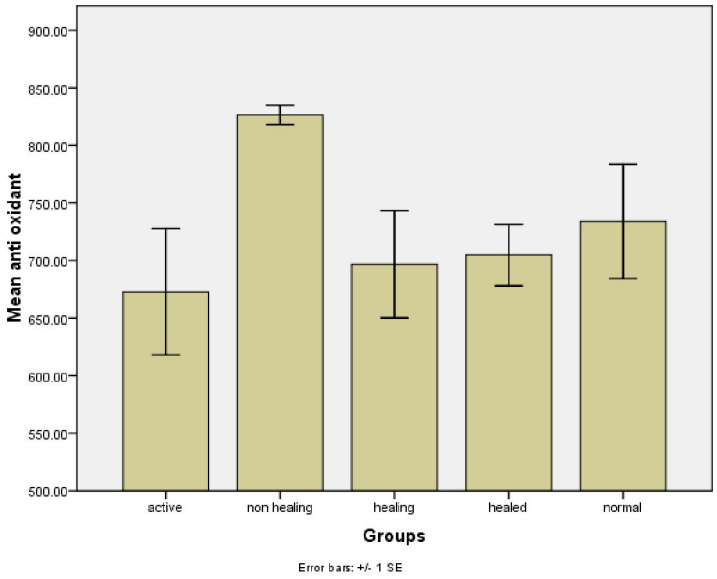

Figure 2. Mean +/-SEM of serum antioxidant levels in four groups of cutaneous *Leishmania* and the normal group.

Table 1. Statistical analysis of the means of serum innate proinflammatory cytokines (TNF α, IL-6, IL-1β) and antioxidant level in four groups of cutaneous Leishmaniasis patients.

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
TNF-α	Active	4	11.6000	2.59743	1.29872	7.4669	15.7331	9.60	15.20
	non healing	2	5.5000	3.53553	2.50000	-26.2655	37.2655	3.00	8.00
	Healing	9	11.6667	20.72318	6.90773	-4.2626	27.5959	3.00	66.00
	Healed	18	6.8778	5.35389	1.26192	4.2153	9.5402	1.00	18.00
	Normal	3	3.7333	1.10151	0.63596	.9970	6.4696	3.00	5.00
	Total	36	8.2611	10.97819	1.82970	4.5466	11.9756	1.00	66.00
IL-6	Active	4	3.3250	2.03859	1.01929	.0812	6.5688	2.00	6.30
	non healing	2	4.8000	2.54558	1.80000	-18.071	27.6712	3.00	6.60
	Healing	9	1.8889	1.42517	0.47506	.7934	2.9844	0.00	4.40
	Healed	18	3.7667	6.37172	1.50183	.5981	6.9352	0.00	28.00
	Normal	3	4.5333	2.04287	1.17945	-.5414	9.6081	2.20	6.00
	Total	36	3.3694	4.67350	0.77892	1.7882	4.9507	0.00	28.00
IL-1B	Active	4	2.5000	5.00000	2.50000	-5.4561	10.4561	0.00	10.00
	non healing	2	5.0000	7.07107	5.00000	-58.5310	68.5310	0.00	10.00
	Healing	9	3.8889	4.85913	1.61971	.1538	7.6239	0.00	10.00
	Healed	18	4.7222	4.99182	1.17658	2.2398	7.2046	0.00	15.00
	Normal	3	0.0000	0.00000	0.00000	0.0000	0.0000	0.00	0.00
	Total	36	3.8889	4.79749	0.79958	2.2657	5.5121	0.00	15.00
Anti- oxidant	Active	4	6.7275E2	109.61866	54.80933	498.3223	847.1777	567.00	823.00
	non healing	2	8.2650E2	12.02082	8.50000	718.4973	934.5027	818.00	835.00
	Healing	9	6.9667E2	139.51613	46.50538	589.4251	803.9083	531.00	883.00
	Healed	18	7.0483E2	112.98790	26.63150	648.6458	761.0209	548.00	897.00
	Normal	3	7.3400E2	86.12201	49.72256	520.0611	947.9389	641.00	811.00
	Total	36	7.0842E2	114.66532	19.11089	669.6195	747.2138	531.00	897.00

In a previous study we found that differences in delayed type hypersensitivity (DTH) responses are due to varying amounts of cytokines provoked by *Leishmania* parasites. We have proposed that increased amounts of Th1 cytokines such as IL-12 and IFN-γ cause a highly positive DTH response. In contrast, Th2 cytokines (e.g., IL-4 and IL-10) levels enhance low DTH response in cutaneous Leishmaniasis (12). IL-4 and IL-10 act together in the presence of exacerbatory antigens (13). When *Leishmania major* causes a single cutaneous lesion, or undergoes a spontaneous cure, the subject is resistant. In this case, probably, the infection is inhibited in the macrophage via innate immunity and production of IFN-γ and IL-12 by a Th1 response that eliminates the parasite. Most likely, in a future challenge, the subject would be immune (14). However, Scott et al. have suggested that low antigen doses may preferably promote a CD4+ Th2 response in vivo, whereas high doses may favor the development of Th1 cells (15). The link between the antigen dose, cytokine level, DTH response and healing remains a matter of debate.

In conclusion, the most visible disparity in our results, though not statistically significant, was an increase in the Il-6 and decrease in TNF-α in non-healing group versus an increase in IL-6 and TNF-α in the healing group of patients. This may be suggestive of the unwanted versus protective kind of inflammation against the *Leishmania* parasites.

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