Full Length Research Paper

Evaluation of some cytokines in Iraqi patients with kala-azar

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This study was carried out to investigate some cytokines (INF-γ, IL-10, TNF-α) on blood samples of Iraqi children with *Visceral leishmaniasis* (VL) before and during treatment with stibogluconate and healthy people serving as control, using ELISA kits. Samples were confirmed parasitologically by bone marrow smears. The results of cytokine showed that there were significant increases in the levels of all the investigated cytokines (INF-γ, IL-10, TNF-α) in the sera of patients with VL during active disease in comparison with the control group. On the other hand, patients after treatment showed a dramatic and progressive decline in serum levels of all the tested cytokine during the whole period-21 days- of therapy. These declines were started from day-7 to go back to the normal or comparable to the level of healthy controls after 21-days.

Key words: *Visceral leishmaniasis*, cytokines, ELISA, INF-γ, IL-10, TNF-α, stibogluconate.

INTRODUCTION

Visceral leishmaniasis (VL), also known as kala-azar, black fever and Dumdum fever (James et al., 2006) is the most severe form of leishmaniasis. Leishmaniasis is a disease caused by protozoan parasites of the Leishmania genus. Several species of Leishmania are known to give rise to the *visceral* form of the disease. The "Old World" (Africa, Asia, Europe) species are *L. donovani* and *L. infantum* and the "New World" (South America) species is *L. chagasi*. It is the second-largest parasitic killer in the world (after malaria), responsible for an estimated 500,000 cases each year worldwide (Desjeux, 2001). The parasite migrates to the internal organs such as liver, spleen (hence *visceral*) and bone marrow and if left untreated will almost always result in the death of the host. Signs and symptoms include fever, weight loss, mucosal ulcers, fatigue, anemia and substantial swelling of the liver and spleen. Of particular concern, according to the World Health Organization (WHO), is the emerging problem of HIV/VL co-infection (WHO). Cytokines are soluble low molecular weight proteins produced by variety of cells of innate and adaptive immune system. Their major functional activities are concerned with the regulation of the development and behavior of the immune effecter cells (Benjamini et al., 2000).

The importance of cytokines during leishmania in-fection comes from the demonstration – on experimental murine leishmaniasis- of existence of two distinct CD4^{+} of T-cell subsets named Th1 and Th2 (Mossman and Coffman, 1989).

The Th1 cytokines (INF-γ, IL-2) and Th2 cytokines (IL-4, 5, 10) where associated with resistance and susceptibility to leishmanial infection (Heinzel et al., 1991).

TNF-α mainly secreted by the activated macrophages, antigen stimulated cells, activated NK and mast cells. It has been shown that TNF-α plays a protective role in murine leishmaniasis by inhibiting parasite multiplication *in vitro* and to control infection *in vivo* through the activation of infected macrophage for intracellular killing of amastigotes (Titus et al., 1989).

On the other hand, IL-10 can exerts either immunosuppressing or stimulating effects on variety of cell types by inhibiting the production of some cytokine like INF-γ from Th1 cells, suppress the production of PGE$_2$ by monocytes, suppress the production of ROI and inhibits or facilitates NO production (Liew et al., 1999).

INF-γ is a potent activator of mononuclear phagocytes

during respiratory burst, promotes the differentiation of $CD4^+$ T-cell to Th_1 phenotype and inhibits the proliferation of Th_2 (Benjamini et al., 2000).

The immunology of *Leishmania donovani* has not been studied to quite extent of *L. major* at least in the murine model (Locksley and Louis, 1992).

Patients with symptomatic Kala-azar were found to have high titer of antileishmanial antibodies and their Th2 arm is activated while their Th1 arm is down regulated that is, no Th1 response was developed against *L. donovani* (Pearson et al., 1986).

Therefore, the present investigation was designed to evaluate the level of some cytokines namely IL-10, INF-γ and TNF-α, in a number of Iraqi children positively diagnosed with VL during the active phase of the disease as well as, the evaluation of these cytokines during the treatment with the standard therapy, sodium stibogluconate.

MATERIALS AND METHODS

This study was conducted between May/2003 – December/2005. Follow up was done in Medical Research Centre/Al-Nahrain Medicine College and Central Public Health Laboratory in Baghdad.

Subject selection

Blood samples were collected from children below six years of age in a number of children hospitals in Iraq. Al-Zahraa Children and Maternity Hospital/Najaf and Babil. AL-Khadhmiya Teaching Hospital, Central Children Hospital, Al-`Ilwia Children Hospital, Al-Mansoor Children Hospital and Central Public Health Laboratory In Baghdad. All samples were taken from the patients under the permission of Iraqi Ministry of Health/Centre of Control of Communicable Diseases according to the current local applied legislations; permissions of parents have also been taken. This study was divided into (3) groups:

(a) Confirmed VL group: Eighty six patients with splenomegaly, positive - microscopic examination or culture- bone marrow aspirate and positive dipstick assay for *Visceral leishmaniasis*. These samples were used as confirmed samples in this study.
(b) Healthy control: Thirty six blood samples were collected from children below six years of age from different primary health centers with no history of living in endemic areas with VL and were apparently healthy by physical examination.
(c) Treated group: Forty five of the confirmed VL patients were treated by the conventional therapy sodium stibogluconate (Pentostam® 100 mg/ml) and were followed up until the end of therapy. The standard course of therapy for VL is 20 mg/kg/day for 28 days (Croft and Coombs, 2003).

Sample collection

A sufficient amount of blood was collected in an anticoagulant container and plain tube from each patient in each group: normal healthy children, children with positively confirmed active VL -before administration of sodium stibogluconate injection- and children who were followed up until the end of therapy - 28 days of sodium stibogluconate therapy-(see above). Each blood sample was centrifuged as soon as possible and serum separated, liquated into portions to avoid repeated freezing - thawing and stored at -20°C till use.

Parasitological diagnosis

Bone marrow samples (biopsies) were aspirated from children suspected of being infected with VL. About 1 ml of bone marrow was aspirated; portion of the aspirate were introduced aseptically into different culture media (Rassam and AL-Mudhaffar, 1979).

Bone-marrow aspirate was also smeared on a clean slide, air dried, fixed with methyl alcohol for 1 - 2 min and washed under tap water. Then stained with 10 - 15% Giemsa stain for 30 min, washed under tap water and air dried.

The slides were examined microscopically under oil-immersion lens for the detection of *L. donovani* (LD) bodies, the smears were considered negative if these bodies were not seen after examination.

Determination of cytokines: Interferon-γ (IFN-γ), interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α)

Serum levels of IFN-γ, IL-10 and TNF-α were measured by means of enzyme immunoassay using ELISA kits (Mabtech AB, Sweden) as recommended by the manufacturer. The assays have detection limits of (2, 0.5 and 8) pg / ml for IFN-γ, IL-10 and TNF-α respectively.

Assay procedure

A cytokine-specific monoclonal antibody (MoAb) was coated onto the microtitre plate by adding 100 μl/well and was incubated overnight at 4 - 8°C then washed twice with phosphate buffer saline (PBS) (200 μl /well). Blocking solution was added to block the plate by adding (200 μl /well) of example, PBS with 1% bovine serum albumin (BSA) for IFN-γ, IL-10 and 0.1% (BSA) for TNF-α. Plate was then incubated for 1 h at room temperature, washed five times with (PBS) containing 0.05 % Tween .

Human IFN-γ, IL-10 and TNF-α standards were prepared by reconstituting the contents of each recombinant human cytokine standard vial with suitable buffer, then diluted with (PBS) containing 0.1% (BSA) to make up a stock solution of 10 μg/ml. Using the standard range as a guideline in the kit leaflet, several dilutions of the stock were prepared. 100 μl/well of samples or standards diluted in (PBS)-Tween containing 0.1% (BSA) -incubation buffer- were added into the plate and incubated for 2 h at room temperature, washed as before.

Then, 100 μl/well of biotinylated antibodies (MoAb) for each cytokine containing 1 μg/ml in the incubation buffer were added into the plate, incubated for 1 h at room temperature, washed as before. Then 100 μl/well of streptavidin -ALP (diluted 1: 1000) in the incubation buffer were added into the plate and incubated for 1 h at room temperature, washed as before.

100 μl/well of appropriate substrate solution example, 1 tablet of phosphatase substrate p-nitrophenyl phosphate (PNPP) (sigma) = 2mM $MgCl_2$ in 5 ml were added into the plate and the optical density was measured at 405 nm in an ELISA reader after a developing time of 15 min.

The concentrations of IFN-γ, IL-10 and TNF-α were calculated by referring to standard curves constructed with known amounts of recombinant human IFN-γ, IL-10 and TNF-α as discussed before. All assay results were expressed as the mean of duplicate determinations in pg/ml.

Statistical analysis of the results

The data were represented as a mean $\pm$ SD (standard deviation). Student t-test was used for comparison between two groups. In all cases a P-value < 0.05 was considered to be significant.

Table 1. Mean cytokines concentration ± SD (pg/ml) in sera of 86 patients with VL and 36 healthy controls measured by ELISA.

Type of cytokine	Patients	Healthy controls	P-value	Average fold increase infection/control
IFN-γ (pg/ml)	112.1 ± 4.6	3.22 ± 1.1	< 0.05	34.81
IL-10 (pg/ml)	202.3 ± 9.8	5.45 ± 3.4	< 0.05	37.11
TNF-α (pg/ml)	59.3 ± 6.7	16.23 ± 1.2	< 0.05	3.65

Table 2. Mean cytokines concentration ± SD (pg/ml) follows up during treatment with sodium stibogluconate in a total of 45 patients with VL, measured by ELISA.

Type of cytokine	Days of therapy			
	0 (un treated control)	7	14	21
IFN-γ	112.1±4.6	18.49 ± 4.3	7.19 ± 3.6	5.02 ± 2
IL-10	203.3±9.8	46.05 ± 8.3	17.36 ± 7.8	9.04 ± 4.6
TNF-α	59.3±6.7	21.4 ± 9.7	19.18 ± 9.6	18.2 ± 3.4

RESULTS

During active disease

There were significant increases P < 0.05 in all tested cytokines (INF-γ, IL-10 and TNF-α) levels in the sera of VL patients compared to that of the control group during active disease. These levels were ranged for IFN-γ from 11.53 - 201.92 pg/ml and 0.96 - 5.78 pg/ml with a mean of 112.1 ± 4.6 pg/ml and 3.22 ± 1.1 pg/ml for the VL and the control group respectively. The average fold increase was 34.81.

Considering IL-10, these levels were ranged from 10.71 - 276.92 pg/ml and 2.97 - 6.17 pg/ml with a mean of 202.3 ± 9.8 pg/ml and 5.45 ± 3.4 pg/ml for the VL and the control group respectively. The average fold increase was 37.11.

While for TNF-α, the levels were ranged from 5.35 - 160.41 pg/ml and 4.86 - 18.45 pg/ml with a mean of 59.3 ± 6.7 pg/ml and 16.23 ± 1.2 pg/ml for the VL and the control group respectively. The average fold increase was 3.65 (Table 1).

During treatment

Considering the treated group of VL patients with stibogluconate, all serum levels of the tested cytokines (INF-γ, IL-10 and TNF-α) were continued to be decreased during the whole period of treatment -21 days-H starting from day 7.

These reductions in the levels of all the tested cytokines (INF-γ, IL-10 and TNF-α) during treatment were significantly lower than in the pretreated VL group P < 0.05 (Table 2). On the other hand, no significant difference was observed in the mean level of (INF-γ, IL-10 and TNF-α) after 21 days of treatment compared with that of the control (Tables 1 and 2).

In case of INF-γ, serum levels were decreased starting from a range of 1.92 - 150 pg/ml with a mean of 18.49 ± 4.3 pg/ml at day 7 and continued to be decreased at day 14 with a value ranging from 0.96 - 7.69 pg/ml with a mean of 7.19 ± 3.6 pg/ml (Figure 1).

To be after 21 days of treatment at a range of 0.64 - 6.72 pg/ml with a mean of 5.02 ± 2 pg/ml.

Considering IL-10, serum levels were continued to be decreased starting from a range of 7.14 - 95.83 pg/ml with a mean of 46.05 ± 8.3 pg/ml at day 7 day. These levels were continued to be decreased after 14 days with a range of 2.67 - 23.21 pg/ml and a mean of 17.36 ± 7.8 pg/ml. After 21 days, these levels were still continued to be decreased ranging from 1.78 - 28 pg/ml with a mean of 9.04 ± 4.6 pg/ml (Figure 2).

In case of TNF-α serum levels, these levels were lowered ranging from 5.31 - 81.24 pg/ml with a mean of 21.4 ± 9.7 pg/ml at day 7 and continued to be lowered with a range of 3.87 - 80.21 and a mean of 19.18 ± 9.6. After 21 days, serum levels became at a range of 1.78 - 81.11 pg/ml and a mean of 18.2 ± 3.4 pg/ml (Figure 3).

DISCUSSION

Cytokine levels

The role of cytokines in human infectious diseases is becoming more and more recognized. Most of the knowledge gained on their importance in leishmania infections has been obtained with animal models. In the murine model of infection with *Leishmania major*, a clear dichotomy is observed between cytokine productions by draining lymph node cells of susceptible versus resistant mouse strains. Infection with *L. major* results in the development of a protective T helper-1 (Th1) immune response with high levels of IFN-γ and resistance to re-infection. In contrast, infection of susceptible mouse strains leads to the development of a Th2 immune response
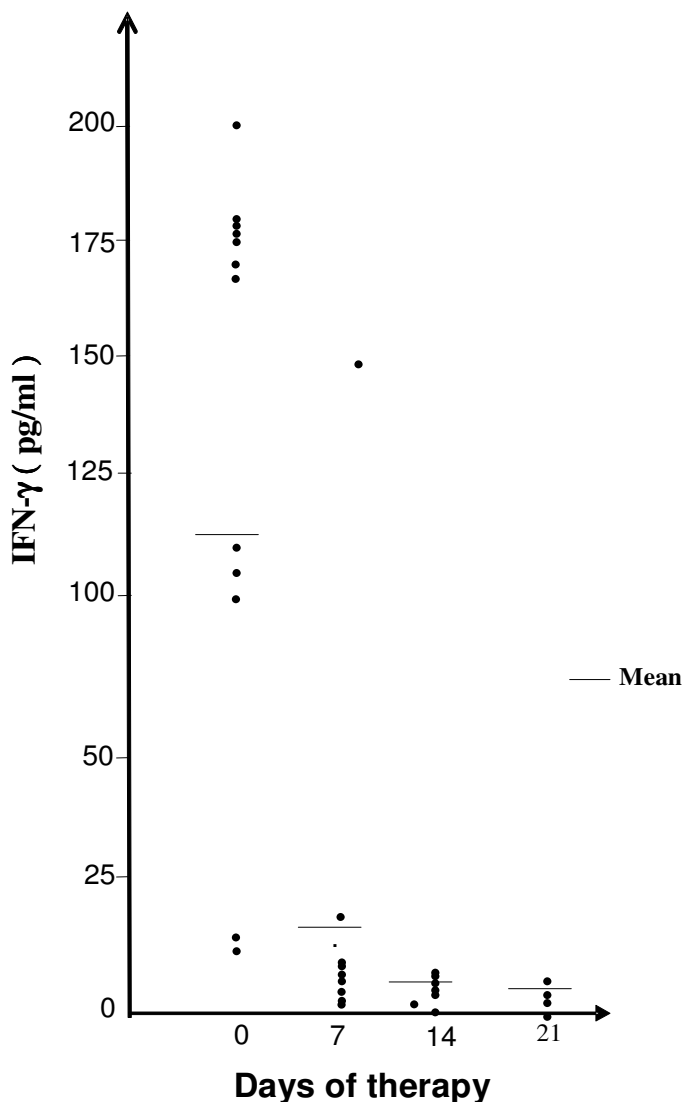
Figure 1. Serum levels of INF-g (pg/ml), measured by ELISA in 15 patients with Visceral leishmaniasis before and during therapy.

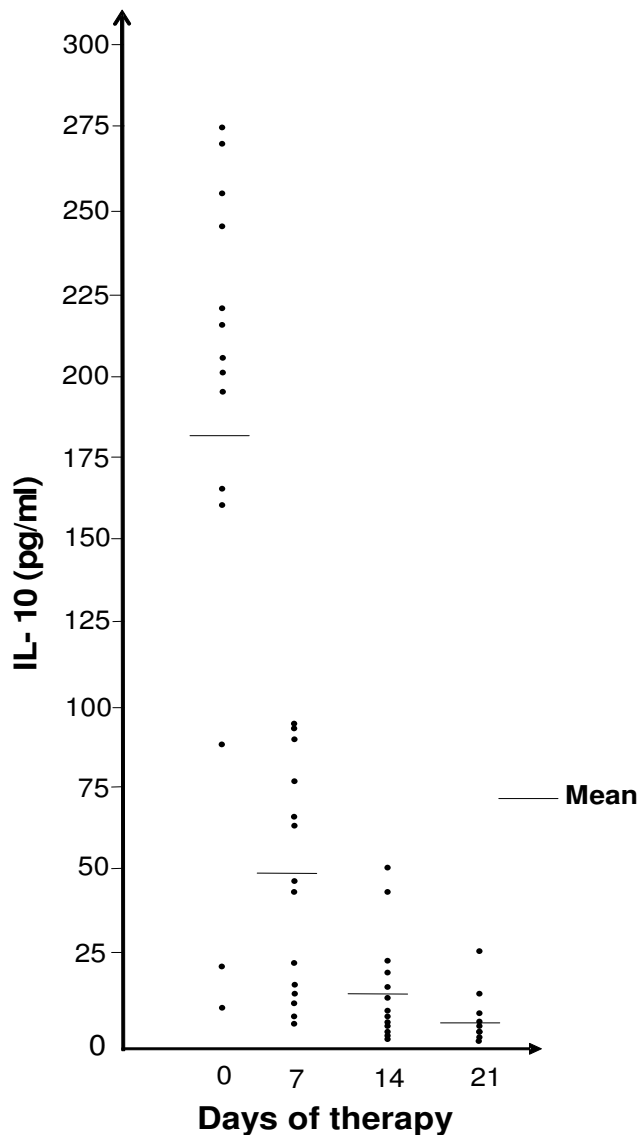**Figure 2.** Serum level of IL-10 (pg/ml), measured by ELISA in 15 patients with Visceral leishmaiasis before and during therapy.

characterized by the production of IL-4 by draining lymph node cells (Tacchini-Cottier and Launois, 2008).

In most parasitic diseases, a predominantly cellular Th1 or humoral Th2 immune response offers the best control over pathogens, the induction of an appropriate T-helper cell response is essential in determining a successful immune reaction. Th1 cells are effective mediators for delayed type hypersensitivity reaction (DTH) and secrete IL-2 and IFN-γ, the prime effectors of cell - mediated immunity (Perez et al., 1995). In contrast, Th 2 cells do not transfer (DTH), but they produce IL-4, IL-5, IL-6 and IL-10 and cooperate with B - cells to generate IgM, IgG, IgA and IgE responses (Finkelman et al., 1990).

The results showed a significant increase in the serum levels of all the tested cytokines (INF-γ, IL-10, TNF-α) during active VL disease state which then started to

decline after treatment with the conventional therapy sodium stibogluconate. These declines were also shown to be so dramatic and progressive starting from day 7 of therapy to go back to the normal or comparable to the level of health control after 21 days of treatment.

High levels of IFN-γ have been detected in serum of patients with VL in comparison with control. High levels of IFN-γ are necessary in the maintenance of the balance between Th1 and Th2 responses. These results matched the results of previous researchers Gomes and Dos (1998) who found a mixed Th1/Th2 response of parasite - specific T - cells from both acute and chronic murine *Visceral leishmaniasis*. This was supported by the later study of Kemp and Theander (1999) who found an elevation of both IL-10 and IFN-γ mRNA in patients with VL.
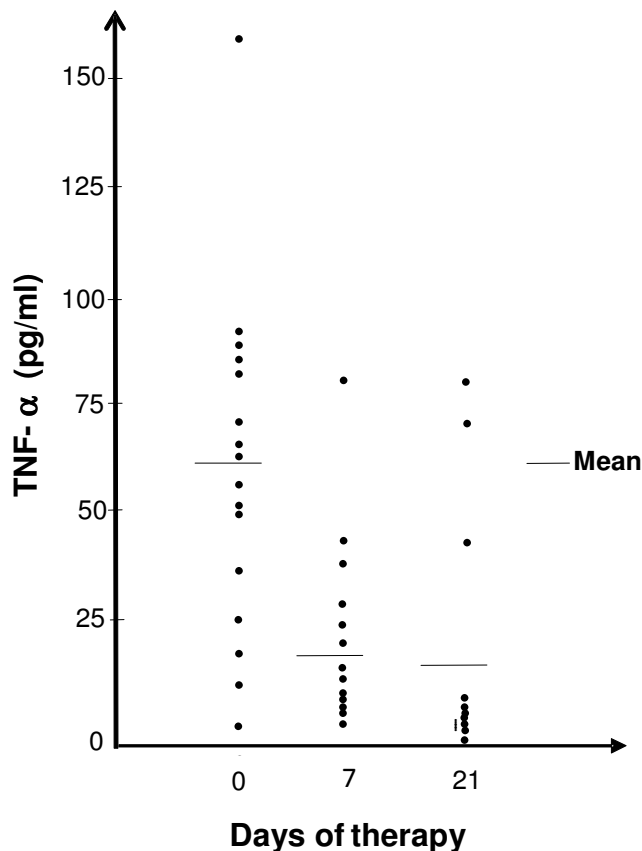
Figure 3. Serum level of TNF- (pg/ml), measured by ELISA in 15 patients with Visceral leishmaniasis before and during therapy.

Results of IL-10 confirmed the results showed by Bogdan and Rollinghoff (1998) who noted that IL-10 blocks Th1 activation and consequently a cytotoxic response bydown regulating IL-12 and IFN-γ production, IL-10 also inhibits macrophage activation and decrease the ability of these cells to kill leishmania.

Moreover, later study has found that VL patients have increased expression of IL-10 mRNA as important strategy for down regulating T-cell response (Kane and Mosser, 2001).

L. donovani infection is known to induce endogouns secretion of IL-10 as a mechanism of parasitism because IL-10 seems to be responsible for the inhibition of the synthesis of IFN-γ -the main macrophage stimulating cytokine involved in the defense mechanism against leishmania - which facilitated the intracellular survival of parasite by down regulating the oxidative and inflammatory response (Bhattacharyya et al., 2001). In fact in human, the severity of VL has been closely associated with increased levels of IL-10 and the use of anti IL-10 antibody to block the IL-10 activity or IL-10 receptor blockade can be effective approach for the treatment of leishmaniasis (Murrary et al., 2002).

Decrease of IL-10 mRNA following therapy and detection of IFN-γ mRNA in acute and treated patients was previously

reported. Thus, it was suggested that, the presence of IL-10, rather than the absence of IFN-γ , is characteristic of acute leishmaniasis (Ghalib et al., 1993).

Circulating levels of TNF-α (which is mainly secreted by the activated macrophages) were detected with active kala - azar and during disease. TNF-α showed raised levels. It is interesting to consider that the higher levels of TNF-α are detected simultaneously with the highest levels of IL-10, a known inhibitor of TNF-α secretion. We found that circulating levels of TNF-α declined as patients improved, as but less than that of IL-10 and IFN-γ. In experimental Visceral leishmaniasis, TNF-α has been shown to be a critical factor in disease control. In human VL circulating levels of TNF-α have been related to the activity of disease (SalomAo et al., 1996).

Finally, the Th1/Th2 concept cannot entirely account for the true complexity of their in vivo activities. Survival of the parasite depends on various mechanisms rather than a generic ability to ignore the Th1 or Th2 type responses. Failure to control or resolve infectious disease often results from an inappropriate rather than an insufficient immune response (Powire and Coffman, 1993).

Furthermore, understanding differences in the role of cytokines in infections with different strains of leishmania should contribute to the design of better and more efficient therapies (Tacchini-Cottier and Launois, 2008).

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