Comparative immune study on cutaneous leishmaniasis patients with single and multiple sores

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Abstract Ninety-five Iraqi patients with cutaneous leishmaniasis (CL) caused by *Leishmania tropica* at AL-Karama Hospital in Baghdad were included in this study. Sixty patients were with single sore and the remaining with multiple sores. The study also included 10 atopic patients and 30 healthy individuals as a control group. Cellular and humoral immune response at different stages of the disease activity (early and late) were evaluated by estimation of serum IFN- γ , IL-4 and total IgE antibodies using ELISA kits while, the detection of specific anti leishmanial IgE antibodies was done manually. Specific IgE antibodies were only detected in early CL (<2 months) patients 68 (71.57 %) while, were not detected in late CL, atopic and healthy controls 30 (100 %). The results also showed a positive relationship between this antibody and the number of sores. Th-2 predominates during the early stage of the disease then shifts to Th-1 that proceed in the late stage, but both cytokines increased in CL patients in comparison to control group. The immune response of CL infection is possibly regulated by both Th-1 and Th-2. Multiple sores patients showed an increase of anti leishmanial IgE (0.120 ± 0.014), total IgE (120.7 ± 39.58 IU/ml), IFN- γ (87.4 ± 30.52 pg/ml) and IL-4 (63.70 ± 20.32 pg/ml) levels than single sore patients with mean value of 0.108 ± 0.14 , 92.3 ± 35.23 IU/ml, 47.2 ± 27.80 pg/ml and 51.04 ± 15.0 pg/ml respectively. It can be presented also as ratio of INF- γ /IL-4 = 1.37 which is greater than those for single sore 0.9. These results

indicated that the immune response of multiple sores patient's is higher than that with single sores.

Keywords Cutaneous leishmaniasis · Single sore · Multiple sore · IgE · IFN- γ · IL-4

Introduction

Leishmaniasis is one of the most diverse and complex of all vectors borne diseases. It is caused by an obligatory intracellular protozoan parasite belonging to the genus *Leishmania* (Sharma and Singh 2009). *Leishmania* infection is transmitted to susceptible mammalian hosts by the bite of a female sand fly (subfamily Phlebotominae), most sand flies typically bite at dusk, but certain vector species in parts of South America preferentially feed during daylight hours instead (Bailey and Lockwood 2007).

There are over 20 species and subspecies of the genus *Leishmania* that infect humans via the bite of sand flies. Sand flies of the species *Lutzomyia* serve as the vector in the New World, while the *Phlebotomus* species transmit infection in the Old World. They are tiny sand colored the female is blood-feeding that breed in forest areas, caves and burrows in tropical and subtropical regions (Markle and Makhoul 2004).

According to Marovt et al. (2010) the disease is endemic in 88 countries, 21 are in the new world and 67 are in the old world. One of the most common forms of the disease is cutaneous leishmaniasis (CL) that occurs most commonly (over 90 %) in Iran, Afghanistan, Syria, Saudi Arabia, Peru and Brazil. CL is a risk for persons, including military personnel, who travel to or live in areas of the tropics, subtropics, and Southern Europe where the disease is endemic, as in the viscerotropic syndrome caused by

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Leishmania tropica that was identified among a number of American military personnel especially during the aggression on Iraq in 1991 (Herwaldt 1999).

In Iraq, two species are present: *L. tropica*, the agent of anthroponotic cutaneous leishmaniasis (ACL), and *Leishmania major*, the agent of zoonotic cutaneous leishmaniasis (ZCL). Both ACL and ZCL were reported as causative agents of leishmaniasis in Iraq, but ACL is found mainly in suburban areas. The disease is epidemiologically unstable, with large and unpredictable fluctuations in the number of cases. The total incidence rate of cutaneous leishmaniasis in Iraq varies from 2.3/100,000 to 45.5/100,000 (WHO 2003).

The outcome of leishmanial infection in humans depends largely on the immune responsiveness of the host and the virulence of the infecting parasite strain. The protozoan in this genus are capable of producing a broad spectrum of diseases in humans, ranging from asymptomatic infections to horribly disfiguring forms of mucosal leishmaniasis or the potentially fatal visceral form of the disease (Gabriel-Grimaldi and Tesh 1993).

Humans and domesticated animals are accidental hosts for many *Leishmania* spp., which are maintained in cycles between wild animals and sand flies. *L. infantum*, *L. peruviana* and possibly other species can be maintained in dogs, increasing the risk of transmission to people. Other domesticated animals might be involved as secondary maintenance hosts. *L. donovani* and *L. tropica* are adapted to humans, but animals can also be infected occasionally (WHO 2010).

Interest has been shown in the study of immune mechanisms defense against various parasite infections (Roitt et al. 1998). Among these parasites is *Leishmania*, which has become a model system for the study of immune response (Pearson and Sousa 1998).

The immune response to *Leishmania* infection is cell mediated. The organism lies exclusively intracellular, mainly inside macrophages as replicating amastigote. The outcome of infection will depend on whether the host mounts primarily a T-helper Th-1 or Th-2 response (Heinzel et al. 1989). Studies in animals suggest that the same parasite epitope can induce a Th-1 response in animals with resolving infection or a Th-2 response in others with disease progression (Reed and Scott 1993).

The interplay between the host determined delayed type hypersensitivity, antigen-specific T cell reactivity, and cytokine secretion, and the type and virulence of the particular infecting *Leishmania* species determine what type of disease expression develops in the host. Some cases are believed to harbor *Leishmania* organisms for indefinite periods before the disease is expressed, suggesting latent infection (Dereure and Duong-Thanh 2003). Parasites have also been detected in lymph nodes after clinical cure (Sundar et al. 2002).

The serum immunoglobulin E (IgE) and anti leishmanial IgE antibodies have been documented in visceral leishmaniasis (Atta et al. 1998). IgE antibodies are produced in the Th-2 immune response and have been used in serodiagnosis and also as indicators of the disease activity in toxoplasmosis, cerebral malaria, and visceral leishmaniasis. (Wong et al. 1993; Perlmann et al. 1994; Atta et al. 1998).

Cutaneous forms of the disease normally produce skin ulcers on the exposed parts of the body such as the face, arms and legs. The disease can produce a large number of lesions-sometimes up to 200 causing serious disability and invariable leaving the patient permanently scared, a stigma which can cause serious social prejudice (WHO 2001). Due to abundance of different human cases of cutaneous leishmaniasis in Iraq this study was performed.

This study was aimed to compare immunologically between patients with cutaneous leishmaniasis suffer from single and multiple skin lesions To accomplish this aim the following studies were performed:

1. Evaluation of the occurrence of anti leishmanial IgE antibodies in the sera of leishmaniasis patients with single and multiple skin lesions and also to relate the presence of this isotype of antibody and the divers of the disease which is resembled by the number of skin lesions
2. Evaluation of total IgE antibodies in CL patients with single and multiple skin lesions in comparison to atopic patients and control group.
3. Compare the cellular and humoral immune responses in CL patients with single and multiple lesions by the estimation of some cytokines such as IFN- γ & IL-4.
4. Finally we tried to find a relationship if there is any between the number of skin lesions and the rate of regrowth of *Leishmania* parasites in culture media after aspirate isolation.

Materials and methods

Subject selection

The individuals studied were classified into three groups:

Group one leishmaniasis patients

This group included ninety-five patients infected with cutaneous leishmaniasis (Baghdad boil) (35 males and 60 females), age range (6–60) years. They were diagnosed depending on the clinical picture and laboratory diagnosis (smears and cultures).

They are also classified according to the duration of infection newly <2 months and >2 months as mentioned elsewhere:

1. Sixty patients with single sore (one lesion).
2. Thirty-five patients with multiple sores (two-10 lesions).

Group two atopic patients

This group included (10) patients suffering from allergy and rhinitis, (six males and four females), age range (6–30) years.

Group three healthy controls

This group included (30) healthy individuals, age range (6–60) years, (18 males and 12 females)

The following information was taken from each individual listed in questionnaire sheet (part of aspirated): Name, sex, age, address, allergy, number of lesions, duration of lesions, and family history for atopic patients.

Blood samples collection

Five ml of venous blood was collected from each individual. The collected samples were centrifuged and the serum was collected and dispensed in plastic eppendorf tubes then stored at -20°C until used for serological tests.

Parasite isolation

Skin smears were done for CL patients, the skin lesion area was cleaned with 70 % alcohol, and then 0.1–0.2 ml of lock's solution was injected subcutaneously the pus area. The injected solution was re-aspirated without taking the needle out of the skin. This aspirate was directly used to prepare a direct smear and culture.

Skin smear

Part of aspirated material from cutaneous lesion was smeared onto a clean slide, then air dried and fixed with methyl alcohol for 1–2 min after washing with tap water, the slide was stained with Giemsa stain for 30 min washed with tap water and air dried. The slides were microscopically examined under oil immersion lens for amastigotes.

Isolation and regrowth percentage of Leishmania tropica parasites on culture media

The remaining part of the aspirates was inoculated onto semi solid media, and then incubated at 26°C . The culture

was microscopically examined for the presence of the promastigote stage after 7–10 days. It was considered negative if absence of this stage during the first 4 weeks (Rassam and Al-Mudhaffar 1979).

Growth media

Semisolid medium

This medium was used for primary isolation of the parasite from the aspirate and for sub culturing of the parasite. This medium was prepared according to Adler and Theodor (1926) then the pH was adjusted to 7.4 ± 0.1 (Al-Hussayni 1981).

Lock's solution

A liquid phase overlays was prepared according to Dawson et al. (1978).

Liquid media (cell free media/RBM)

This medium was described by AL-Bashir et al. (1992), which is a modification of the semisolid medium of Adler and Theodor (1926). Each liter of this medium contains the following:

NaCl	6.90 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.22 g
NaHCO_3	0.10 g
KCl	0.29 g
D-glucose	0.77 g
Peptone	1.00 g
Beef extract	0.30 g
Brain–heart infusion	10.00 g
Diluted human blood	200 ml
Gentamicin	1.50 ml
Distilled water	800 ml

Immunological assays

Antigen preparation (AL-Bashir et al. 1992; Barbosa-de-Deus et al. 2002)

Antigen for specific antileishmanial IgE antibody was obtained from *L. tropica* promastigotes. The parasites were isolated from patients and were maintained in semisolid culture media and transferred to 100 ml sterile container then 10 ml of liquid medium was added and the container

was incubated at 26 °C. The growth of the parasites was examined after 2 days to ensure a good growth and sterility. Other quantities of liquid medium were added when needed and by this method an active parasites at the log phase can be obtained.

Harvesting the parasites

1. The parasites grown in the liquid media RBM were centrifuged using the cooling centrifuge at (3,000×g) for 10 min to get a pellet.
2. The pellet was then washed three times with normal saline, and stored at −70 °C till used.

Parasites disruption

1. The pellet containing the parasites was taken to which an equal volume of (Triton× 100) and 0.1 % normal saline.
2. A glass homogenizer was used to break down the cells of the parasites at specific time interval inside an ice bath at 4 °C.
3. The homogenate was centrifuged in cooling centrifuge at (4,500×g) for 10 min, the supernatant containing the *Leishmania* antigen was harvested and stored in a aliquots at −70 °C until use.

The amount of protein obtained, assayed by the method of Lowry et al. (1951).

Determination of total IgE and antileishmanial IgE

This method was carried out according to Sousa-Atta et al, (2002) with some modifications using enzyme-linked immunosorbent assay (ELISA) kit as specified in Table 3.

Determination of IFN-γ

Principle of the assay The assay max human IFN-γ ELISA kit is designed for detection of human IFN-γ in plasma (Table 3), serum and cell culture samples. This assay employs a quantitative sandwich enzyme immunoassay technique that measures human IFN-γ in <4 h.

Determination of IL-4

Principle of the assay This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-4 has been pre-coated onto a microplate using the ELISA kit specified in Table 3.

Statistical analysis

Experimental data were analyzed by using (ANOVA) test. *P* value <0.01 was considered statistically significant.

Results and discussion

Isolation and regrowth of CL parasites

Only thirty isolates of cutaneous leishmaniasis patients were selected, 16 isolates were grown on culture media, three isolates were contaminated and the remaining eleven isolates did not grow.

The results showed that multiple sores isolates have higher percentage of regrowth 9 (56.25 %) than single sore 7 (43.75 %) and had the lower percentage of no growth 5 (45.46 %). These results give a guide to which type of infection the *Leishmania* parasites can be obtained and maintained in future studies. A decrease in efficacy of local isolates was also observed even those who were grown, they stop growing after a few subcultures, this phenomena was occurred even with our isolates, after months all isolates were died. The explanation of this phenomena is that *Leishmania* parasites become weaker when grown under lab conditions, this may be due to the type of blood used, in the past rabbit blood was used but nowadays human blood is used easily, its constituents may be more harmful to parasites growth as compared to rabbit's blood constituents, another explanation is the un stability of electricity which affects the parasites maintenance this can be another reason for growth failure.

Cutaneous leishmaniasis

Sex distribution

The total numbers of leishmaniasis patients included in this study were 95 individuals. 60 (63.16 %) females and 35 (36.84 %) males.

Age distribution

The ages of patients studied were between 6 and 60 years. The majority of patients were at age of 16–40 years Table 1.

Body site of infection

Different parts of the patients body were observed for infection with Baghdad boil including face, arms, legs and feet, but number of patients infected in arms (upper limbs) had the highest percentage 50 (52.63 %) when compared to other sites of infection, followed by legs 25 (26.32 %), feet 15 (15.79 %) and face 5 (5.26 %) as shown in Table 2, Figs. 1, 2.

Table 1 Distribution of cutaneous leishmaniasis patients according to age and sex

Age (years)	No. of patients according to sex. no. (%)		Total no. (%)
	Females	Males	
6–15	14 (60.86)	9 (39.14)	23 (24.21)
16–40	40 (63.49)	23 (36.51)	63 (66.32)
>40	6 (66.66)	3 (33.34)	9 (9.47)
Total	60 (63.15)	35 (36.85)	95 (100)

Number of lesions

The number of lesions in CL patients ranged between 1 and 10 in different body parts.

One ulcerated lesion was documented in 60 (63.16 %) of 95 CL patients, two and nine ulcerated lesions were observed in 34 (35.79 %), and 10 ulcerated lesions were found in one (1.05 %) patient Table 2 and Fig. 3.

Duration of infection

Period of infection with CL in patients was ranged from <2 months to >2 months Table 2.

The total number of atopic patients was 10; 6 (60 %) females and 4 (40 %) males).

Serological findings by ELISA

ELISA technique was used to measure anti leishmanial IgE antibody, total IgE antibody, gamma interferon and interleukin –4 levels in the sera of the study groups.

Anti leishmanial IgE antibody

The results showed that positive ELISA anti leishmanial IgE antibodies were documented in patients infected with CL, specifically in those recently infected (early CL) <2 months, out off 72 (75.78 %) early infected patients 68 (71.57 %) patients presenting a mean absorbance of (0.114 ± 0.024) while the other four patients (4.21 %) showed absence of IgE anti leishmanial antibody (0.078 ± 0.04) as shown in Table 3. Such antibodies

haven't been documented in late infected patients with CL 23 (24.22 %) which representing a mean absorbance (0.082 ± 0.134) as well as in those atopic patients and control group 10 (100 %) (mean 0.63 ± 0.036), 30 (100 %) (mean 0.519 ± 0.031) respectively Table 3.

The results also showed that multiple sores patients had a higher levels of this isotype of antibody (mean 0.120 ± 0.014) than a single sore patients mean (0.108 ± 0.14) but this elevation is not significant ($P > 0.01$) Table 4.

From these results it can be considered such anti leishmanial IgE antibodies are serum markers of disease activity in CL infections, since they are not documented in individuals with late phase of infection, this result corresponds with previous results of Atta et al. (1998) who reported that this antibody had not been documented in subclinical and late infections with visceral leishmaniasis, but the explanation of the negative results in those four patients with early CL may be due to their infection duration being closer to late infection (50–55 days) than the early infection (3–45 days).

The current result also disagreed with a previous results on *L. amazonensis* which showed that there was an inverse relationship between anti leishmanial IgE antibody and the number of skin ulcers, that is mean patients with single skin ulcer have the highest level of specific anti leishmanial IgE antibody and patients with multiple sores have the lowest levels of this antibody, these differences may be related to different infections resulted from different parasites strains.

These IgE antibodies are produced from the Th-2 immune response and have been used in serodiagnosis and also as indicator of the disease activity in many diseases such as toxoplasmosis, cerebral malaria, and visceral leishmaniasis (Wong et al. 1993; Perlmann et al. 1994; Atta et al. 1998).

Specific IgE antibodies in the current study were only observed in early patients with CL while in atopic or even control groups this antibody was not detectable. This finding indicates that production of specific anti leishmanial IgE antibodies are not merely a consequence of a polyclonal B cell activation, but rather an antigen-driven response.

It is well known that IgE develops its biologic antibody function after fixing the high-affinity receptor $FC_{\epsilon}RI$ on

Table 2 General characteristics of patients the distribution of *Leishmania* sores according to the site of infection, number of lesions and duration of infection

Site of infection				Number of lesions			Duration of infection	
Upper limbs no. (%)	Legs no. (%)	Feet no. (%)	Face no. (%)	One lesion no. (%)	2–9 lesions no. (%)	10 lesions no. (%)	<2 months (3 days–6 weeks) no. (%)	>2 months (3 months–1 year) no. (%)
50 (52.63)	25 (26.32)	15 (15.79)	5 (5.26)	60 (63.16)	34 (35.79)	1 (1.05)	72 (75.78)	23 (24.22)
Total no. (%) = 95 (100)				Total no. (%) = 95 (100)			Total no. (%) = 95 (100)

Fig. 1 Patient with single sore in foot

Fig. 2 Patient with single sore in leg

Fig. 3 Patient with multiple sores in arm

mast cells, basophils, and Langerhans cells, and the presence of IgE antibodies in CL could be interpreted as a protective mechanism to hinder cutaneous dissemination of the infection and to modulate the skin inflammatory response mediated by Th-1 cytokines (Sousa-Atta et al. 2002).

Finding elements of the Th-2 immune response in early CL, demonstrated by serum IgE anti leishmanial antibodies

Table 3 Mean of absorbance $\pm$ SD of anti leishmanial IgE antibody detected by ELISA in the sera of 95 patients infected with CL

No. of patients	Mean of absorbance $\pm$ SD	Patient groups
68 (71.57 %)	0.114 $\pm$ 0.024 (+ve)	Early CL
4 (4.21 %)	0.078 $\pm$ 0.04 (–ve)	Early CL
23 (24.22 %)	0.082 $\pm$ 0.134 (–ve)	Late CL
10 (100 %)	0.063 $\pm$ 0.036 (–ve)	Atopic
30 (100 %)	0.0519 $\pm$ 0.031 (–ve)	Control
0.088		Cut-off value

and high concentrations of this immunoglobulin in the sera of infected patients, is in accordance with previous observations of IL-4 and IL-10 mRNA in cells from biopsies of leishmanial skin ulcers (Pirmez et al. 1993; Gaafar et al. 1999).

Finally, to our knowledge the current study is the first study in Iraq to investigate the role of anti-leishmanial IgE antibodies as a sensitive and specific marker in early CL patients.

Total IgE antibody

The results showed that CL patients had a lower levels of total IgE antibody (mean 106.530 ± 25.52 IU/ml) while atopic patients have a higher levels (mean 813.110 ± 142.95 IU/ml) ($P < 0.01$) in comparison to the control group (mean 114.650 ± 42.41 IU/ml) Fig. 4. The results also showed that total IgE level was higher in multiple sores patients (mean 120.7 ± 39.58 IU/ml) when compared with single sore patients (mean 92.3 ± 35.23 IU/ml), but this difference was not significant ($P > 0.01$) Table 5.

From this study it was observed that early CL patients had a non significant increase ($P < 0.01$); in the levels of total IgE antibody (115.58 ± 25.99 IU/ml) when compared to late CL patients (97.49 ± 10.59 IU/ml) Tables 2, 5; Fig. 5.

IL-4 has paradoxical effects on Th cell development depending on the nature of the cells (dendritic cells or primed T cells) targeted for IL-4 signaling. In the initial period of dendritic cells (DCs) activation by *Leishmania* preceding T cell priming, IL-4 directs DCs to produce IL-12 and promotes Th-1 cell maturation and resistance to *Leishmania* parasites but later, during the period of T cell priming, IL-4 induces Th-2 differentiation and progressive leishmaniasis (Biedermann et al. 2001; Louis et al. 2003). These antagonistic effect of IL-4 could describe this unexpected finding of low serum IgE level in leishmaniasis patients, but the explanation of the reversed result in early CL in comparison to late CL patients is not clear.

Atopic disease is a localized immediate hypersensitivity reaction to an allergen. Response to atopic disease is mediated by T-lymphocytes. Antigen exposure to T- lymphocytes leads to their differentiation into active T-cells,

Table 4 Distribution of CL patients according to number of lesions and period of infection with means of absorbance $\pm$ SD of anti leishmanial IgE antibody

No. of lesions	<2 months	Mean of absorbance $\pm$ SD	>2 months	Mean of absorbance $\pm$ SD	Total
Single	41 (45.05 %)	0.108 $\pm$ 0.14	15 (16.48 %)	0.077 $\pm$ 0.05	56 (61.54 %)
Multiple	27 (29.67 %)	0.120 $\pm$ 0.014	8 (8.79 %)	0.087 $\pm$ 0.1	35 (38.46 %)
Total	68 (74.73 %)	0.114 $\pm$ 0.024	23 (25.27 %)	0.082 $\pm$ 0.134	91 (100 %)
Cut-off		0.088

Fig. 4 Total IgE, INF- γ and IL-4 concentrations in atopic and control CL patients groups

which secrete a series of biologically active proteins called cytokines, these cytokines includes IL-4 and IL-5 which activate B-lymphocyte to produce IgE antibody (Vannichpuntu et al. 1991).

Kjellman et al. (1976) found that there is a wide distribution of expected serum IgE values in healthy individuals of the same group because IgE is a mediator of allergic response, and this was found in the current study.

Jarvis et al. (2005) found in their study that normal individuals have IgE concentrations that vary widely and increase steadily during childhood, reaching their highest levels at age 15–20, and remaining constant then they slowly decline. This could reflect either a decrease in sensitization with aging or a higher prevalence of sensitization in more recent birth cohort and this correspond with our findings.

Immune dysfunction and Th-2 overactivity is engaged in pathogenesis of many diseases like atopic dermatitis where it is associated with IL-4 production by Th-2 cells and IgE production by B cells (Kristal and Clark 1996). This effect is evident by high IgE level in the sera of atopic patients more than the study subjects.

Gamma-interferon (IFN- γ)

This study showed that IFN- γ levels were significantly higher ($P < 0.01$) in leishmaniasis patients (mean

67.30 $\pm$ 59.41 pg/ml) in comparison to control group (mean 3.81 $\pm$ 1.79 pg/ml) and atopic patients (mean 1.98 $\pm$ 1.33 pg/ml); Fig. 5.

The results in general showed that IFN- γ level was significantly ($P < 0.01$) higher in multiple sores patients (mean 87.4 $\pm$ 30.52 pg/ml) than its level of single sore patients (mean 47.2 $\pm$ 27.80 pg/ml) Table 5.

Outcome of the infection is critically dependent on the activation of one of the two subsets of CD4 T cells, Th-1 and Th-2.

The ability of the host to control infection and resolve disease requires the generation of cell-mediated immune responses capable of activating host macrophages to eliminate intracellular parasites. In both human and experimental models of CL, control of infection is mediated by T-lymphocytes and is critically dependent upon the early induction of an IL-12-driven Th-1 type immune response and the production of IFN- γ by CD4 T cells (Rosas et al. 2005; Tripathi et al. 2007).

From the results obtained it can be considered that the immune system is more prone towards cell mediated immunity due to increased levels of IFN- γ in CL patients in general in comparison to the control group. This elevation capable of activation of host macrophages to eliminate *Leishmania* parasites, because macrophages are the terminal effector cells for the survival and multiplication of *Leishmania* parasites, any attack of the immune system to kill the parasite has to involve this cell (Overath and Aebischer 1999; Singal and Singh 2003). Although macrophages are the major host cells for leishmaniasis, reports using animal models have shown that neutrophils and DCs are also able to uptake leishmania parasite early after infection (carvalho et al. 2012). The decisive question therefore is how to activate the macrophage to use its lethal machinery to kill *Leishmania*. It is now clear that NO produced by the inducible NO synthase (iNOS) is the most important mechanism of the macrophage to kill *Leishmania* in vitro and in vivo. IFN- γ is by far the most important cytokine leading to induction of iNOS (MacMicking and Xie 1997).

Alexander and Russell (1992) also showed that IFN- γ plays a critical role in the activation of macrophages to kill intracellular parasites by inducing production of NO which is critical for elimination of parasites.

Table 5 Mean concentration $\pm$ SD of IgE, IFN- γ & IL-4 in the sera of 95 cutaneous leishmaniasis patients in comparison to atopic and control groups

Patients	No. of patients	IgE mean (IU/ml)	Mean conc. $\pm$ SD of IFN- γ (pg/ml)	Mean conc. $\pm$ SD of IL-4 (pg/ml)	Ratio of IFN- γ /IL-4
CL	95 (100 %)	106.535 $\pm$ 25.520	67.30 $\pm$ 59.41	57.37 $\pm$ 68.62	1.17
Single sore	60 (63.61 %)	92.3 $\pm$ 35.23	47.2 $\pm$ 27.80	51.04 $\pm$ 15.0	0.92
Multiple sore	35 (36.84 %)	120.7 $\pm$ 39.58	87.4 $\pm$ 30.52	63.70 $\pm$ 20.32	1.37

Fig. 5 Total IgE, INF- γ and IL-4 concentrations in late and early CL patients

IFN- γ production is to a large extent induced by IL-12 and down-regulated by IL-4 (Leal et al. 1993; Scharton-Kesten et al. 1995) the resistant hosts mount a Th-1 response (high IFN- γ low IL-4) whereas in susceptible hosts a Th-2 response (low IFN- γ high IL-4) predominates (Locksley and Scott 1991).

Scott et al. (1991) and Wang et al. (1994) revealed in their studies that depletion of IFN- γ leads to a Th-2 response with disseminating disease in healer mice, while Sadick et al. (1990) and Chatelain et al. (1992) showed that depletion of IL-4 leads to a Th-1 response and allows no healer mice to cure the infection.

Many studies had shown that during the early infection with *Leishmania major* hosts exhibited mixed Th-1/Th-2 responses of CD4 cell population, then induces a transient Th-2 type response and then shifts to a Th-1 response associated with healing (Uzonna et al. 2004) induction of this Th-1 type of response partly depends on the activation of IFN- γ producing CD4 T cells and in the absence of CD8 T cells, the Th-2 response is sustained.

In the current study the results showed this type of cytokine shift, characterized by lower levels of IFN- γ in the early stage of the disease (mean 28.00 ± 11.31 pg/ml) and then the levels increase later (106.68 ± 32.33 pg/ml); Fig. 4 these findings agreed with Bourreau et al. (2003) who suggested that in patients with CL a Th-2 response

transiently predominated during the early phase of infection and was followed by the development of a Th-1 response during the late course of lesion development, this suggestion is completely corresponded with our results of high levels of Th-1 cytokine (IFN- γ) and low levels of Th-2 cytokine (IL-4).

In a number of studies, recombinant IFN- γ was capable of activating infected macrophages from both resistant and susceptible mice to clear *L. major* in vivo these findings that were achieved by Scott et al. (1989) and Green et al. (1991) correspond with our results of high levels of this cytokine in late CL patients and this fact reveal the importance of IFN- γ in the elimination of infection.

Interleukin-4

The results showed that there was a significant increase of IL-4 levels in the sera of CL patients (mean 57.37 ± 68.62 pg/ml) in comparison to the control group (mean 33.42 ± 9.36 pg/ml) while, atopic patients showed the highest levels ($P < 0.01$) of this cytokine (mean 769.910 ± 178.85 pg/ml); Fig. 4.

The current results showed that this cytokine level in multiple sores patients (mean 63.70 ± 20.32 pg/ml) is higher than the levels in single sore patients (mean 51.04 ± 15 pg/ml) but this difference was not significant ($P > 0.01$) Table 5 and early CL patients have a significantly ($P < 0.01$) higher level of IL-4 (mean 78.70 ± 62.40 pg/ml) in comparison to late CL patients (mean 36.05 ± 5.94 pg/ml); Fig. 5.

IL-4 is the signature cytokine associated with Th2-type immune responses and is associated with non healing forms of cutaneous disease in mice (Tripathi et al. 2007).

The higher levels of IL-4 that we noticed in these results especially with those of multiple sores agreed with the fact which suggests that IL-4 has been shown to be associated with disease progression and susceptibility was also reported by Locksley et al. (1987).

IL-4 stimulates the differentiation of naive CD4 T cells into Th-2 cells capable of producing Th-2 associated cytokines IL-4, IL-5, IL-10 and IL-13, and promotes antibody production and IgE class switching by B cells. IL-4 also functions as a powerful inhibitor of IFN- γ -producing CD4 T cells and suppressor of protective Th-1 immune responses (Nelms et al. 1998), this fact corresponded with

our results of low levels of IFN- γ and the high levels of IL-4 in early infection with CL. IL-4, along with related cytokine IL-13, trigger macrophages to undergo alternative activation and is associated with parasite survival and persistence of infection (Rosas et al. 2003; Tripathi et al. 2007).

CL is characterized by a mixed Th-1/Th-2 immune response during the early phase then Th-2 is detected and converts to an evident Th-1 type of immune response documented by lymphocyte proliferation and IFN- γ secretion. (O'Neil et al. 1993).

The results also showed that both cell population Th-1 and Th-2 are highly expressed in comparison to that control group. The co-expression of IFN- γ and IL-4 at high levels suggest that the immune response to *L. tropica* infection is possibly regulated by both Th-1 and Th-2 cytokines.

Studies that had been achieved by Sadick et al. (1990) and Chatelain et al. (1992) revealed that the apparent resolution of infection with *L. major* in BALB/c mice treated at the time of infection with an anti-IL-4 monoclonal antibody (or in IL-4-deficient BALB/c mice) helped to establish the view that early production of IL-4 drives the polarized Th-2 response that is responsible for suppressing Th-1 cell development and inhibiting the high-level secretion of IFN- γ that is required to activate infected macrophages for parasite killing, and this agreed with high levels of IL-4 and low levels of IFN- γ that we noticed in early CL patients included in this study.

Number of mechanisms by which IL-4 may interfere with the production or activity of IFN- γ has been identified, including the direct inhibition of macrophage activation to kill intracellular amastigotes (Lehn et al. 1989). The ability of IL-4 to block the activation of macrophages has been most consistently demonstrated following prior treatment with IL-4.

Th-1 cells, through the production of IFN- γ , evoke cell-dependent immunity and inhibit both the production of IL-4 by Th-2 cells and Th-2 cell proliferation in vivo (Fernández-Botrán et al. 1988; Gajewski et al. 1989). In contrast, Th-2 cells, through the production of IgE can promote immunoglobulin class switching to IgE (Randolph et al. 1999) and also stimulate IgE gene recombination and transcription to IgE mRNA, thereby enhancing IgE production by B cells (Lagier et al. 1995; Ryan 1997).

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