

# Induction of Th1 Cytokines by Live Logarithmic Promastigotes of *L. major*

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## ABSTRACT

The early cytokines production in response to live logarithmic and stationary phase promastigotes of *Leishmania major* was examined on the peripheral blood cells of the healthy individuals. Whole blood cultures were stimulated by either logarithmic or stationary phase promastigotes. IFN- $\gamma$  and IL-10 productions were assessed by specific sandwich ELISA. The results showed that the logarithmic promastigotes were more potent than the stationary promastigotes in induction of IFN- $\gamma$ . In contrast, IL-10 production was significantly higher in the supernatants of the cells stimulated by the stationary promastigotes compared to the logarithmic phase of the parasites. The effect of BCG on IL-10 and IFN- $\gamma$  productions induced by these two types of promastigotes was also studied. BCG had augmenting effect on the cytokine production. However, the difference between the logarithmic and the stationary promastigotes was still observed, since logarithmic parasites induced higher amount of IFN- $\gamma$  and lower amount of IL-10 compared to the stationary parasites. In parallel, intracellular expression of IL-12 and IL-10 in the CD14<sup>+</sup> cells was studied and the same results were obtained. The logarithmic phase parasites induced significantly higher expression of IL-12 and lower expression of IL-10 compared to the stationary phase parasites which induced lower expression of IL-12 and higher expression of IL-10. These results demonstrated that logarithmic promastigotes of *L. major* are more potent to induce Th1 response than stationary promastigotes which might have implication in vaccine preparation. *Iran. Biomed. J. 5 (1): 15-20, 2001*

**Keywords:** *Leishmania major*, Human cytokines, Logarithmic phase, Stationary phase, Promastigotes

## INTRODUCTION

The control of leishmanial infection requires the induction of immune responses capable of activating macrophages to microbicidal state. The most potent cytokines for the induction of leishmanicidal activity in macrophages are interleukin-12 (IL-12) produced by macrophage and dendritic cells and gamma interferon (IFN- $\gamma$ ) produced by NK and Th1 cells [1]. Clinical studies showed that the onset of parasite killing is typically delayed, since even in the self-limiting cutaneous infections, the parasite is able to amplify its numbers for weeks or months before the lesions begin to resolve. Further evidence shows that the delayed onset of the parasite killing is due to the slow development of the cell mediated immune response (such as delayed production of IFN- $\gamma$ ). In addition, patients are susceptible to re-infection

during the active stage of the lesion development, whereas, they are resistant after healing has begun [2, 3, reviewed in 4]. Furthermore, individuals with healed or healing lesions have significantly higher antigen-specific IFN- $\gamma$  responses than patients with active disease [1, 5]. These data show that IFN- $\gamma$  is indispensable for resolving the infection in human.

Human interleukin-10 (IL-10) is a potent anti-inflammatory cytokine that inhibits the synthesis of the major proinflammatory cytokines and chemokines. IL-10 is the principal Th2 type cytokine that upregulates humoral responses and attenuates cell-mediated immune reactions [reviewed in 6]. It has been shown that IL-10 is involved in early down-regulation of Th1 type of response in human cutaneous leishmaniasis by inhibiting production of IL-12 and IFN- $\gamma$  [7].

Understanding the biology and the life cycle of *Leishmania* is important for vaccine development

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and treatment of leishmaniasis. The differentiation of parasite has been extensively studied and it has been shown that the sequential development of *Leishmania* promastigotes from a non-infective to an infective stage in the sandfly vector so called “metacyclogenesis” can be reproduced *in vitro* [8]. In culture, logarithmic phase promastigotes, like procyclic promastigotes in the midgut of sandfly vector, are non-infective. However, stationary phase promastigotes are infective similar to metacyclic parasites. Since the host immune system encounters the infective form of the parasites, stationary phase promastigotes of *L. major* are used for vaccine preparations against zoonotic cutaneous leishmaniasis (ZCL) [9-10].

The logarithmic and the stationary promastigotes of *L. major* (Friedlin strain) have differential effects on cytokine production [8]. It was shown that logarithmic phase promastigotes induced IL-12 and IFN- $\gamma$  syntheses but stationary phase promastigotes failed to induce production of these cytokines by peripheral blood mononuclear cells of healthy individuals [11].

In this report, we studied the ability of logarithmic and stationary phase promastigotes of *L. major* (a strain isolated from endemic area of Iran) to induce Th1 response. Moreover, the effect of BCG as an adjuvant on cytokine production was studied.

## MATERIALS AND METHODS

**Subjects.** Thirteen healthy individuals (non-smoker, aged 20-40 yr old) without previous history of any serious illness were selected from non-endemic area.

**Parasites.** The strain of *L. major* used in this study was the vaccine strain (MRHO/IR/75/ER). The infectivity of the parasites were maintained by regular passage in susceptible BALB/c mice. The parasites were cultured in the Schneider’s medium (Sigma, Chemical Co., St. Louis, USA) supplemented with 10% FBS (Sigma), 292  $\mu\text{g/ml}$  L-glutamine (Sigma) and 4.5 mg/ml glucose [12]. The starting parasite inoculation was  $1 \times 10^6/\text{ml}$ . Under these culture conditions, the stationary phase parasites growth was obtained in 6 days determined by peanut agglutination assay [12]. Logarithmic phase (2 days in culture) and stationary phase (6 days in culture) parasites were harvested and washed three times in PBS.

**Whole blood cultures.** Whole blood was obtained from healthy donors by venipuncture in the sterile blood collecting tubes containing sodium heparin. Then, the blood samples were diluted three times as previously described [13] in RPMI 1640 supplemented with 0.1% FBS, penicillin (100  $\mu\text{g/ml}$ ), streptomycin (100  $\mu\text{g/ml}$ ) and 50 IU/ml sodium heparin. Diluted blood of each donor was cultured in 24-well plates (1 ml/well), and incubated at 37°C for 18-20 h in humidified atmosphere with 5% CO<sub>2</sub>.

**Stimulation of blood cells.** Cultures were either received  $6 \times 10^6$  parasites/ml or parasites plus BCG ( $5 \times 10^6$  CFU/ml, obtained from Pasteur Institute of Iran, Tehran) in total volume of 30  $\mu\text{l}$ . LPS from *Salmonella thiphimurium* (Sigma, 0.01 ng/ml) plus IFN- $\gamma$  (Sigma, 300 pg/ml) were added to the appropriate wells as the positive control. After 18-20-h incubation, the supernatants were harvested and kept frozen at -70°C until use.

**Cytokine determination.** Cytokine levels were determined by the commercially available sandwich based ELISA kits (R&D, Minneapolis, MN, USA). The plates were already coated by specific capture antibodies, and after the addition of serial dilution of standards and samples, the conjugated antibodies were added. The detection limit of each cytokine kits was 5 pg/ml. According to the standard curve, the samples cytokine levels were determined.

**Intracellular cytokine detection.** Since IL-12 production was very low, intracellular cytokine detection was used. Peripheral blood mononuclear cells, isolated by density gradient centrifugation over Histopaque (Sigma) at  $2 \times 10^6$  cells/ml. Cells were cultured in RPMI 1640 supplemented with heat inactivated FBS (10%), penicillin (100 u/ml), streptomycin (100  $\mu\text{g/ml}$ ) and 2mM L-glutamine. The cells were primed with human IFN- $\gamma$  (300 pg/ml) and after 2 h, the cells were stimulated by the freeze-thawed logarithmic and stationary phases *L. major* antigens ( $6 \times 10^6$  parasites/ml) in the presence of monensin, and incubated for additional 12 h. The cells were washed with PBS containing 1% albumin and then stained with anti-human CD14-FITC conjugated antibody for 30 min. After washing, the cells were fixed and permeabilized by cytofix/cytoperm solution (PharMingen, USA). Intracellular IL12/IL-10 were stained by PE-conjugated specific antibodies (PharMingen, USA)

and analyzed by flow cytometer (FACScan, Becton Dickinson, USA).

**Statistics.** Statistical analysis was performed using student's *t-test* for intracellular cytokine production and multiple varieties ANOVA for cytokine determination.  $P < 0.05$  was considered significant.

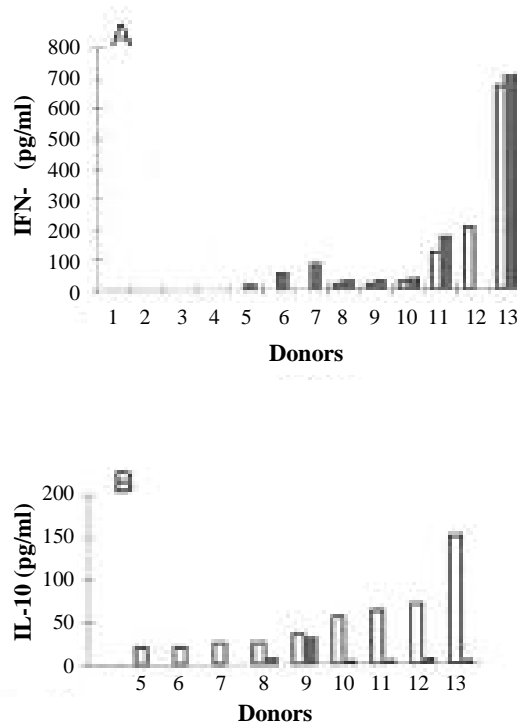
## RESULTS

The first part of the study was measuring IFN- $\gamma$  and IL-10 productions in the supernatant of whole blood cultures stimulated by different forms of the live parasites. Great variability in IFN- $\gamma$  production was observed with live stationary phase promastigotes (mean:  $65.55 \pm 150$  ranging from 0 to 670 pg/ml) and to the logarithmic promastigotes (mean:  $97.05 \pm 162.7$  ranging from 0 to 700 pg/ml). A slight increase of IFN- $\gamma$  production was obtained in response to logarithmic phase parasites compared to the stationary phase parasites (Fig.1A). Four out of thirteen individuals (30.8%) did not produce IFN- $\gamma$  in response to logarithmic phase parasites; however IFN- $\gamma$  production in response to stationary phase parasites was negative for seven out of thirteen individuals (53.9%).

IL-10 production was significantly induced by stationary promastigotes (mean:  $22.5 \pm 14.8$  pg/ml) in contrast to IFN- $\gamma$  which was induced less by these promastigotes. The ability of logarithmic promastigotes to induce IL-10 was remarkably lower than stationary phase parasites (Fig.1B). However, 3 out of 7 individuals who did not produce IFN- $\gamma$  in response to stationary phase parasites were positive for IL-10.

To verify the effect of BCG on IFN- $\gamma$  and IL-10 productions, the cultures were simultaneously stimulated with parasites and BCG.

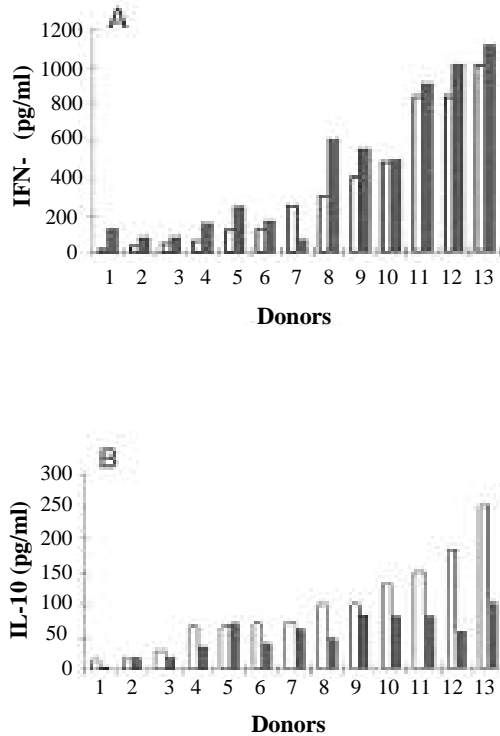
Similarly, variable results but significantly different from experiments with parasites alone were obtained (Fig. 2A). In response to stationary phase promastigotes plus BCG, IFN- $\gamma$  production was from 15 to 1,000 pg/ml, (mean:  $345.2 \pm 342.5$ ) and to logarithmic phase parasites plus BCG from 62 to 1,100 pg/ml, (mean:  $419 \pm 376$ ). BCG was able to induce IFN- $\gamma$  production in the cultures that were negative when parasite alone was used as stimulant.



**Fig. 1.** Cytokine productions in whole blood cultures of healthy individuals induced by live Metacyclic (□) and Procyclic (■) promastigotes of *L. major*. Fig.1A shows IFN- $\gamma$  results and Fig.1B shows IL-10 results.

In parallel, IL-10 production was measured in wells stimulated by these two forms of parasites plus BCG (Fig. 2B). Logarithmic promastigotes produced less IL-10 (mean:  $65.5 \pm 21.3$  pg/ml) than stationary promastigotes (mean:  $118 \pm 60$  pg/ml). Based on statistical analysis, this difference was shown to be significant.

Intracellular cytokine assay was used to look at the expressions of IL-12 and IL-10 in CD14<sup>+</sup> cells in response to the different logarithmic and the stationary phase promastigotes antigens. The cells were primed with IFN- $\gamma$  and the percentage of CD14<sup>+</sup> IL-12<sup>+</sup> or CD14<sup>+</sup> IL-10<sup>+</sup> cells in response to various stimulants were determined. As shown in Table 1, the percentage of IL-12 producing cells in response to logarithmic phase antigen (mean:  $34 \pm 7.6\%$ ) was significantly higher than the percentage of the cells stimulated by stationary phase antigen (mean:  $11 \pm 5.5\%$ ,  $p < 0.01$ ). In contrast, the percentage of cells producing IL-10 augmented in cultures stimulated by stationary phase parasites (mean:  $32.4 \pm 16.5\%$ ) when the results were compared with the cells stimulated by logarithmic phase parasites (mean  $11 \pm 5.5\%$ ) (Table 1).



**Fig. 2.** Cytokine productions in whole blood cultures of healthy individuals induced by live Metacyclic (□) and Procyclic (■) promastigotes of *L. major* plus BCG. Fig. 2A shows IFN- $\gamma$  results and Fig. 2B shows IL-10 results.

## DISCUSSION

Our results showed that the ability of the stationary phase promastigotes to induce Th1 response is lower than the logarithmic phase promastigotes which, to some extent, is in agreement with the previous study on Friedlin strain of *L. major* [11]. They showed that the infective form of promastigotes inhibit IL-12 and IFN- $\gamma$  productions, but induce IL-10 by peripheral blood mononuclear cells (PMNC) of healthy individuals. On the contrary, the logarithmic phase promastigotes were able to induce increased amount of IL-12 and IFN- $\gamma$ . In the present study, the stationary phase promastigotes alone induced IFN- $\gamma$  production but

**Table 1.** Induction of intracellular IL-12 and IL-10 expressions by different *L. major* antigens in CD14<sup>+</sup> cells of the healthy individuals

	CD14 <sup>+</sup> /IL-12 <sup>+</sup>	CD14 <sup>+</sup> /IL-10 <sup>+</sup>
Control	2.0 ± 1.0	0.5 ± 0.2
Metacyclic	32 ± 16.5	11 ± 5.5
Procyclic	12.8 ± 3.7	34 ± 7.6

lower than the logarithmic phase parasites. The difference between our results and the study on Friedlin strain of *L. major* [11] might be due to the different strains and/or different culture conditions used in two studies. We measured the cytokines in whole blood cultures to avoid intentional exclusion of several immune components (such as complement system and the cells involved in the innate immunity like neutrophils) which are excluded in PBMC cultures. Nonetheless, based on IFN- $\gamma$  and IL-10 productions, the results obtained from the logarithmic phase promastigotes were similar to that of previous study showing that this form of parasites were more potent to induce Th1 type of response.

Since BCG is believed to be a Th1 inducer agent, its effect as an adjuvant on cytokines production was investigated in the presence of both forms of parasites. BCG augmented IFN- $\gamma$  production in response to all stimulants, compared to the cultures without addition of BCG. However, IL-10 results were different to IFN- $\gamma$  results. BCG augmented the level of IL-10 stimulated by stationary phase promastigotes, similar to the IFN- $\gamma$  results however such increment was not obtained by the logarithmic phase promastigotes. Therefore, the augmenting effect of BCG on IL-10 production was seen only in the presence of the stationary phase promastigotes. It should be indicated that the stationary form of *L. major* in conjunction with BCG has been used for vaccine studies in different trials [9, 10, 14]; however the efficacy of killed stationary phase parasites of *L. major* (MRHO/IR/75/ER) vaccine was demonstrated to be very low and it was indicated that another adjuvant(s) should be studied [14].

Once promastigotes were transferred into the host skin, CD14<sup>+</sup> cells were the first permissive cells that uptake the invasive parasite. Therefore, intracellular cytokine assay was performed on these cells. Intracellular studies showed that human IFN- $\gamma$  primed monocytes responded to *L. major* antigens and induced intracellular expression of IL-12 in CD14<sup>+</sup> cells. However, there was significant difference between the logarithmic and the stationary phase promastigotes. IL-12 production in IFN- $\gamma$  primed CD14<sup>+</sup> cells was remarkably induced in response to the logarithmic phase promastigotes. In contrast, the percentage of CD14<sup>+</sup> IL-12<sup>+</sup> was very low when the stationary phase promastigotes was used as the antigen.

The expression of IL-10, an anti-inflammatory cytokine produced by monocytes, was also

determined. Our results showed that the stationary promastigotes are potent inducer of IL-10 in CD14<sup>+</sup> cells but logarithmic parasites induced low level of IL-10 (Table 1). These findings were in agreement with the data of cytokine production in whole blood cultures. Since IL-12 is a potent inducer of IFN- $\gamma$  and the logarithmic parasites induced expression of IL-12 in addition to IFN- $\gamma$ . Moreover, the results of IL-10 production by the stationary promastigotes was in agreement with the intracellular study. To our knowledge, this is the first study on intracellular IL-12 expression in human monocytes (CD14<sup>+</sup>).

In conclusion, the results of this study might have implications in vaccine design since the antigen profile of stationary and logarithmic phase promastigotes of *L. major* is very similar [15]; therefore logarithmic phase promastigotes might be used as an protective vaccine against cutaneous leishmaniasis. In addition, it would be worth characterizing the specific antigen(s) involved in IL-12/IL-10 induction.

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