LIVE LOGARITHMIC PHASE PROMASTIGOTES OF LEISHMANIA MAJOR INDUCE HIGH LEVEL OF IFN-γ BUT LOWER LEVEL OF IL-10 IN WHOLE BLOOD CULTURE OF HEALTHY INDIVIDUALS

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Abstract - Stage-specific developmental forms of Leishmania major promastigotes were grown in vitro. Procyclic and metacyclic promastigotes were cultured and separated by peanut agglutinin. The axenic amastigote form were prepared by culturing the promastigotes in acidic medium at 35C. These three forms of parasites were used as antigens to study cytokine production in whole blood culture of healthy individuals (no=13) and early IFN-γ and IL-10 production were determined by specific sandwich ELISA. The results showed that logarithmic promastigotes were more potent to induce IFN-γ production than metacyclic and axenic amastigotes parasites. In contrast, IL-10 production was significantly higher in supernatants of cells stimulated by the two infective forms. In addition, the adjuvant effect of BCG on cytokine production induced by these three types of promastigotes was studied. BCG showed augmenting effect on cytokine production, however there were still differences between logarithmic and the two other forms since logarithmic parasites still induced higher amount of IFN-γ and lower amount of IL-10. These results demonstrated that logarithmic promastigotes of L. major are more potent to induce T helper 1 response which might have implication in vaccine preparation.

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Key Words: Leishmania major, human, cytokines, logarithmic, metacyclic, axenic amastigotes.

INTRODUCTION

Protozoan parasites of Leishmania genus cause a spectrum of diseases which affect more than 12 million people world wide (1). Cutaneous leishmaniasis (CL) is a health threatening disease and major health problem in Iran; therefore extensive efforts have been focused on application of different control measures including immunoprophylaxis. In this respect, since 1990 comprehensive vaccine trials have been programmed by World Health Organization in Iran (2,3).

It is well documented that immunity against CL depends on cellular immunity (4). T helper 1 type of responses, dominated by interleukin-12 and Interferon gamma (IFN-γ) productions, is involved in healing phases of infection (5). However T helper 2 type of responses, dominated by interleukins 4, 5, and 10 productions, is involved in dissemination and exacerbation of disease (6,7). As a result, cytokine pattern is used to assess the immunogenecity of an antigen and the level of cytokines are determined in almost all of the studies pertaining to pathogenesis and immunogenesis of CL (8-10).

Leishmania differentiates from non-infective (procyclic) to an infective (metacyclic) form in sandfly vector and the later form is transmitted to the mammalian host. The same sequential differentiation can be reproduced in vitro. Thus it is possible to propagate different forms of parasites in large scale (11). Biological characteristics of the logarithmic phase promastigotes grown in mono-phasic media are similar to procyclic promastigotes and those of the stationary phase parasites have similar biological characteristics to metacyclic promastigotes. Therefore, stationary phase promastigotes have been successfully used for a long time to infect susceptible hosts and different studies have been performed on the experimental model . Likewise, in leishmanisation practice on human, stationary phase parasites have been applied for control of leishmanisis in hyper-endemic areas. Moreover, the stationary phase promastigotes were selected, as an immunogen, for preparation of killed leishmania vaccine. However, data obtained from efficacy trials in Iran failed to show protectivity of this type of vaccine (2,3).

Recent findings on modulation of immune response by metacyclic promastigotes, resulted from several studies, showed that neither metacyclic nor stationary phase promastigotes enabled to induce IL-12 and subsequently IFN-γ by peripheral blood mononuclear cell culture of healthy individuals. In contrast, procyclic or logarithmic phase promastigotes of L. major is a potent inducer of both immune protective cytokines (11).

In this study, both forms of promastigotes were grown, separated and identified by peanut agglutinin. The ability of different forms of L. major parasites on induction of IFN-γ and IL-10 were assessed in whole blood culture of healthy individuals. In addition, to better understand the adjuvanticity effects of BCG in promoting cytokine responses, the effect of BCG was studied on production of IFN-γ and IL-10 by immune cells of healthy individuals.

MATERIALS AND METHODS

Subjects: 13 healthy individuals (females, non-smoker and aged 20-40 yr) 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 and Leishmanin strain (MRHO/IR/75/ER). The infectivity of parasites are maintained by regular passage in susceptible Balb/c mice. The parasites were cultured at 25 C in Schneider's medium (Sigma Chemical Co., St. Louis, USA) supplemented with 10% FBS (Sigma), 292 pg/ml L-glutamine (Sigma) and 4.5 mg/ml glucose (Sigma) (12).

Peanut agglutination test: 100 μl of peanut agglutinin (Sigma) suspension (50 pg/ml in PBS) was mixed with 100 μl of promastigotes (10^7 promastigotes / ml in PBS) in a 96 well micro titer plate and incubated at room temperature for one hour. At the end of the incubation, 20 μl of the supernatants were harvested and the free promastigotes were counted by haemocytometer. The percentage of agglutination was determined as follows:

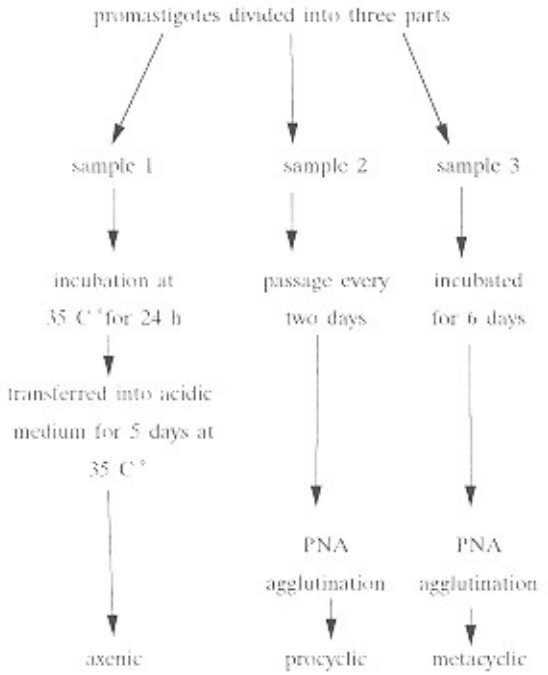
$$\frac{\text{total number of promastigotes-total number of free promastigotes}}{\text{total number of promastigotes}} \times 100$$

Discrimination of logarithmic and stationary phase promastigote: In order to determine when the metacyclogenesis process completes, the parasites were cultured (10^6 parasites/ml of the medium). Every day a sample was prepared from the cultures and the percentage of PNA agglutinated parasites was determined. Promastigotes with more than 85% agglutination were considered as logarithmic and with less than 30% agglutination were considered as stationary.

Preparation of procyclic and metacyclic promastigotes: Stationary phase parasites were harvested and washed three times in PBS. In order to isolate procyclic promastigotes contamination from the metacyclic promastigotes, a suspension of 4×10^8 promastigotes in PNA (50 μg/ml in PBS) was prepared and incubated for one hour. Then, the suspension was layered over a cushion of FCS (50% v/v in PBS) and incubated for another half an hour at room temperature. After completion of the incubation, the supernatant was collected and washed with PBS. The obtained promastigotes were pure metacyclic form which was confirmed by PNA Agglutination Assay.

Axenic amastigotes were cultured under heat and low pH stress. The procyclic promastigotes were cultured for 24 h at 35° C. Then the parasites were transferred into the same medium with pH 5.5 at 35C for 5 days.

In order to have these three types of promastigotes in the same day the following procedure is designed:



Whole blood cultures: Whole blood was obtained by venipuncture from normal healthy donors in sterile blood collecting tubes containing sodium heparin and was diluted three times as previously described (13) in RPMI 1640 supplemented with 0.1% FBS, penicillin (100u/ml), streptomycin (100 μg/ml) and 50 IU/ml sodiumheparin. Diluted whole blood of each donor was cultured in 24-well culture plates (Nunc, Roskilde, Denmark) , 1 ml/well and incubated for 18-20 h at 37C in humidified atmosphere with 5% CO2.

Stimulation of cultures: Cultures either received 6×10^6 parasites/ml or parasites plus BCG (5×10^6 CFU/ml, provided from Pasteur Institute of Iran, Tehran) in total volume of 30 μl. Lipopolysaccharide from Salmonella thiphymurium (Sigma, 0.01 ng/ml) plus IFN-γ (Sigma, 300pg/ml) were added to the appropriate wells as positive control. After 18-20 h incubation, the supernatants were harvested and kept frozen at -70° C until used. Cytokine assay: cytokine levels were determined by means of commercially

available ELISA kits (R&D, Minneapolis, MN, USA). The detection limit of each cytokines was 7.5 pg/ml. Based on standard curve, cytokine level of each supernatant was determined.

Statistical analysis: Multiple variates ANOVA (SPSS Ver. 10) was used for comparison between individuals and antigens.

RESULTS

Determination of PNA concentration:

The optimum concentration of PNA used for isolation of stationary phase parasites were determined by testing serial dilutions of PNA. The best concentration for agglutination which discriminate between procyclic and metacyclic promastigotes was 50 μg/ml (data not shown).

Discrimination of logarithmic and stationary phase promastigotes:

Figure 1 showed the changes of PNA agglutination rate of promastigotes in culture medium. It was clearly demonstrated that under the indicated conditions, the promastigotes in the second day of culture are the log phase promastigotes since the majority of parasites were agglutinated by PNA (80-85%). However, the promastigotes in the sixth day of culture showed the least ability to be agglutinated by PNA (25%) which were considered as stationary phase promastigotes. These promastigotes were used for further experiments.

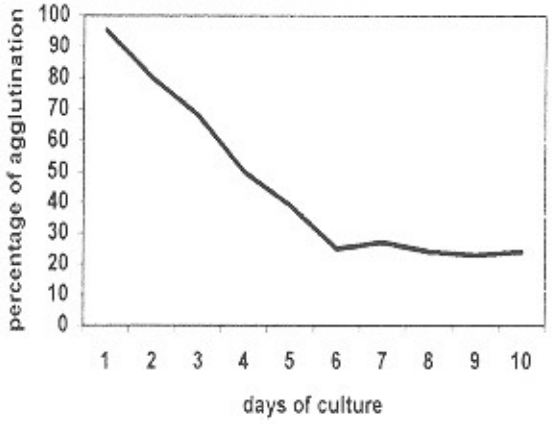


Fig. 1. The percentage of L. major promastigotes agglutination by PNA : changes of promastigotes binding to PNA by day of culture

Cytokine production in supernatant of whole blood cultures: There was great variability in IFN-γ production of healthy individuals to live axenic amastigote (mean: 47.9 ± 134.6 ranging from 0 to 490 pg/ml), stationary (mean: 65.55 ± 150 ranging from 0 to 670 pg/ml), and logarithmic promastigotes (mean: 97.05 ±162.7 ranging from 0 to 700 pg/ml). However when the results of IFN-γ production induced by different forms of parasites were compared for each individual (Fig.2).

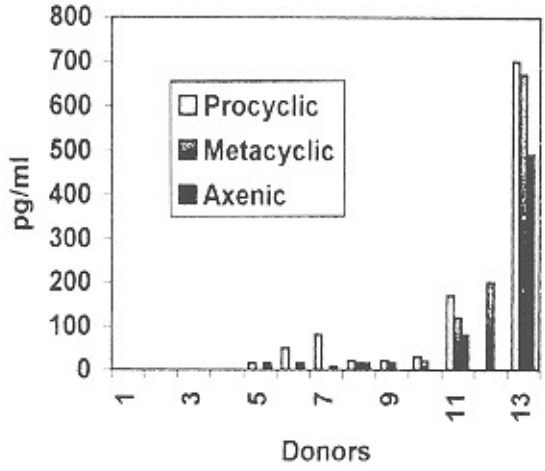


Fig. 2. Comparison of IFN-γ production by peripheral blood cells of healthy individuals in response to different forms of L. major parasite

A difference was observed since all individuals, except one case, produced higher amount of IFN-γ in response to logarithmic than the other forms of parasite (Fig2). It is worthinoting that only 4 out of 13 individuals (30.8%) did not produce IFN-γ in response to logarithmic phase parasites; however IFN-γ production in response to stationary phase and axenic amastigote parasites were negative for 7 out of 13 individuals (53.9%).

In contrast to IFN-γ, production of IL-10 was remarkably higher in cultures receiving stationary promastigotes (mean: 51.3 ± 41.6 pg/ml) or axenic amastigote (mean: 50.7 ±41.4pg/ml) compared to the logarithmic promastigotes (Fig.3).

The IL-10 production was only determined for individuals who responded to at least one antigen and produced IFN-γ.

The effect of BCG on cytokine production: To study the adjuvant effect of BCG on production of IFN-γ and IL-10, cells were stimulated with parasites plus BCG. Similarly heterogeneous responses but significantly higher than responses to parasites alone were obtained. IFN-γ production in response to stationary promastigotes plus BCG was ranging from 15 to 1000 pg/ml, (mean: 345.2 ± 342.5). Axenic amastigote plus BCG induced IFN-γ with the range from 0 to 1000 pg/ml (mean: 252.5± 276.7).

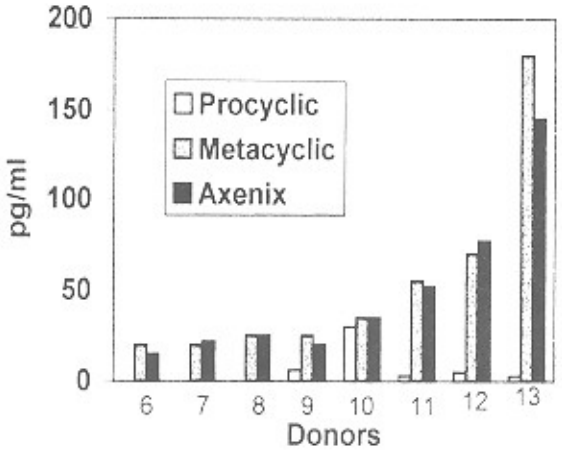


Fig. 3. Comparison of IL-10 production in whole blood cultures stimulated by different forms of L. major parasite

Logarithmic parasites plus BCG induced IFN-γ with the range from 62 to 1100 pg/ml, (mean: 425.9 ± 376.6). A remarkable difference was still observed when the results of each individual response to different forms of parasites were compared (Fig.4).

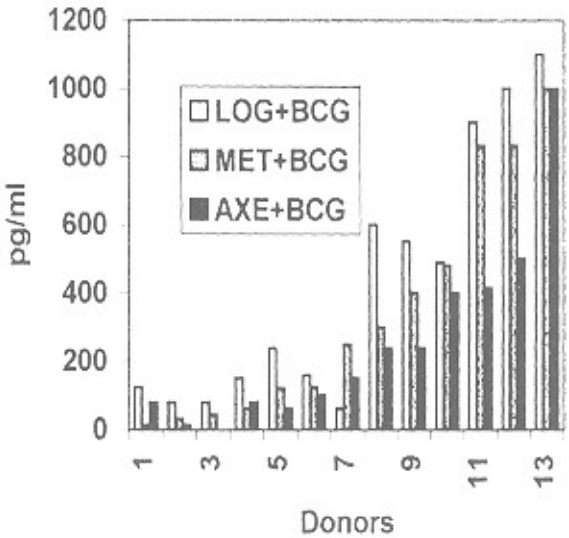


Fig. 4. Comparative demonstration of IFN-γ production in response to logarithmic, stationary phase promastigotes, and axenic amastigote of L. major plus BCG

These results indicate that even in the presence of BCG logarithmic promastigotes still induce higher amount of IFN-γ than stationary and axenic amastigote parasites. It should be noted that all of individuals who did not produce IFN-γ in response to parasites alone, showed IFN-γ production in response to L. major promastigotes plus BCG.

Finally, IL-10 production was measured in wells stimulated by these three forms of parasites plus BCG (Fig.5) . Logarithmic promastigotes produced less IL-10 (mean:53.9.5 ± 29.0 pg/ml) than stationary promastigotes (mean: 118± 60 pg/ml) and axenic amastigote (mean: 94.4 ± 66.4 pg/ml)

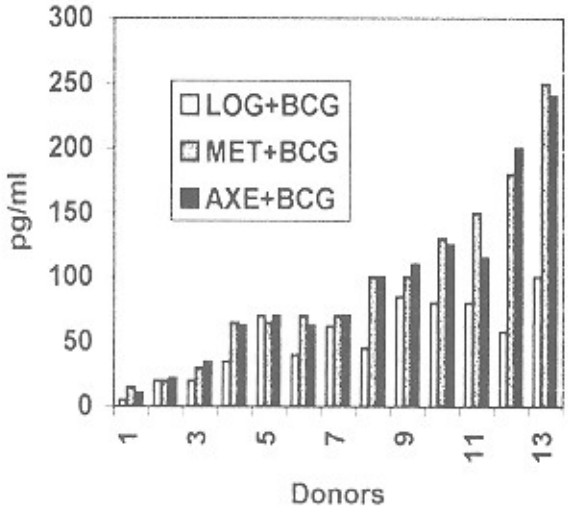


Fig. 5. IL-1a production in response to different forms of L. major parasite plus BCG

DICUSSION

Leishmania parasites sequentially develop into logarithmic (non- infective) and stationary phase promastigotes (infective) in the culture with similar biological characteristics to procyclic and metacyclic promastigotes. Using PNA agglutination test, we could discriminate and isolate logarithmic and stationary phase promastigotes and used them as inducer in the blood cell cultures. Moreover, pure metacyclic promastigotes were separated from stationary parasites for induction of immune cells in vitro. The cytokine induction potency of parasites with different developmental stages in culture was studied. Two major cytokines of Th1 (IFN-γ) and Th2 (IL-10) were selected.

Similar to the previous study on Friedlin strain (11), our results showed that the ability of infective forms of promastigotes of L. major, including stationary phase promastigotes and axenic amastigote, on stimulation of T-helper 1 responses was lower than logarithmic parasites. This effect was proved by higher production of IFN-γ and lower production of IL-10. In contrast, the infective forms induced higher level of IL-10 production than non-infective form.

To avoid intentional exclusion of several immune components (i.e. complement system and the cells involved in innate immunity such as neutrophils) which are normally excluded in PBMC culture, induction of immune cells for cytokine production was carried out in whole blood cultures. This culture condition is very similar to the in vivo events, since it has been shown that parasites are transferred to the skin of mammalian host in conjunction with a vasodilator peptide of the sandfly saliva so called maxdilan, which facilitate the blood meal for the insect (14), and therefore, the parasites encounter blood soon after entering the skin (15). It had been proposed that logarithmic promastigotes would not be able to induce acquired immunity since they were very susceptible to the effect of complement system(16). The present study showed that procyclic promastigotes were able to induce IFN-γ production and not IL-10 secretion, so it would be able to promote T helper 1 responses.

Since BCG is used as adjuvant in several vaccine trials against CL in Iran (2-3), we investigated its effect on cytokine production stimulated by the three forms of parasites. As expected, BCG as an adjuvant increased cytokine production, however the difference between the effect of these developmental stages parasites was not changed and the non-infective form still induced higher level of IFN-γ but lower level of IL-10 compared to the infective forms.

So far, studies on efficacy of killed L. major (KLM) vaccine have not shown very promising results. One of the possible explanation for low efficacy of this vaccine may be attributed to the usage of stationary phase promastigotes in preparation of vaccine. Therefore, the results of this study might have implications for vaccine design. In addition, characterization of factor(s) involved in IFN-γ and IL-10 production would be interesting.

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