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Heat Shock Proteins Enriched-Promastigotes of *Leishmania major* Inducing Th2 Immune Response in BALB/c Mice

Marzieh Holakuyee[1], Mehdi Mahdavi[2], Zuhair Mohammad Hassan[3] and Mohsen Abolhassani[*1]

[1]*Hybridoma Lab., Dept. of Immunology, Pasteur Institute of Iran, Tehran;* [2]*Dept. of Virology, Pasteur Institute of Iran, Tehran;* [3]*Dept. of Immunology, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Iran*

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ABSTRACT

Background: Heat shock proteins (HSP) are highly conserved molecules with many immunological functions. They are highly immunogenic with important role in cancer immunotherapy and in vaccine development against infectious diseases. As adjuvant, HSP can augment the immunogenicity of weak antigens and can stimulate antigen presenting cells. Although vaccines have been successful for many infectious diseases, progress in leishmaniasis has not been achieved. In this report, the protective effect of HSP-enriched soluble leishmania antigen (SLA) was determined. **Methods**: BALB/c mice were immunized 3× with HSP-enriched SLA and SLA alone and 10 days after final boost. They were infected with 10^6 stationary phase promastigote of *Leishmania major* and immunological responses were followed until nine weeks. **Results**: No significant differences were observed in lymphocyte proliferation, footpad swelling, parasite burden, nitric oxide or IL-12 cytokine between HSP-enriched or SLA groups. Although the levels of IFN-γ, IL-4, TGF-β, IgG1 and IgG2b were increased in both groups, IFN-γ was significantly higher in SLA group and IgG2a in HSP-enriched SLA. **Conclusion**: These results indicate that HSP direct the immune system towards Th2 pattern and does not have protective role in *L. major* infection. *Iran. Biomed. J. 16 (4): 209-217, 2012*

Keywords: Leishmaniasis, Heat shock proteins (HSP), Adjuvant

INTRODUCTION

Leishmaniasis is caused by intracellular protozoan parasites of *Leishmania* species, which has a wide clinical spectrum from cutaneous lesion to fatal visceral form, depending on the strain of parasite and the immune system status of the host [1]. Leishmaniasis is considered as a major public health problem for developing countries after malaria. It has reported in many tropical and semi-tropical regions of the world and 88 countries are involved with Leishmaniasis [2-4].

Control of the disease is a serious problem since current drugs are toxic and expensive with limited efficacy especially in visceral leishmaniasis [5]. There is a need for development of an effective vaccine, which can prevent or control infectious disease with the aim of long lasting immunity, high efficacy, excellent tolerability and with no side effects. Major key for providing a protective vaccine is the shift of immunological responses from IL-4 to IFN-γ [6]. Some strategies for vaccine preparation against Leishmaniasis are based on killed and attenuated parasite, purified or recombinant antigens and DNA vaccine, but all of them showed limited efficacy with low protection [7, 8]. Lacks of suitable adjuvant system that can induce strong and desired immune responses were seen in clinical trials based on the above vaccination strategies. Adjuvant can produce immune stimulatory or danger signals when opposed to pathogen structure or substances released by the host [9].

Heat shock proteins (HSP) are intracellular molecules with different molecular weights, which include cytosolic, mitochondrial, nuclear and endoplasmic reticulum resident proteins [10]. They act as chaperon in peptide folding and under stress conditions such as temperature shock will increase and bind to the cellular proteins to sustain the folding of the proteins [11]. Moreover, HSP have many immune-logical functions such as stimulation of innate immunity. HSP induce dendritic cells to produce pro-inflammatory cytokines such as IL-1, IL-6, TNF-α and IL-12 [12, 13]. Binding of HSP with peptides results in presentation with MHC-I and MHC-II pathways and enhancement of adaptive immunity [14].

*Corresponding author; Telefax: (+98-21) 6649 2596; E-mail: mabolhassani@yahoo.com

The adjuvant effects of HSP are well defined in tumor models and have been shown to induce strong Th1 immune response [15]. Application of HSP as adjuvant in several infection models also revealed a Th1 cytokine pattern and strong cellular immunity [16, 17]. Because of its role in antigen presentation and also potent inducer of cellular immunity, HSP are promising as vaccine adjuvant for a broad spectrum of pathogens [18]. Few conflicting results were obtained in using leishmania HSP in mice and human indicating protecting and non-protecting roles [19, 20]. In this report, we evaluate the immunological roles of HSP-enriched *L. major* promastigote in BALB/c mice.

MATERIALS AND METHODS

Mice. BALB/c mice (6 to 7 weeks old, weight 20 ± 5 g) were obtained from the Pasteur Institute of Iran and were housed in the standard environmental conditions in plastic cages with free access to tap water and standard rodent pellets in an air-conditioned room under a constant 12:12 h light-dark cycle at room temperature and a relative humidity (50-60%). All animals used in this study have received care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). All mice were killed according to Pasteur Institute of Iran Guidelines for Laboratory Animals.

Parasites and sample preparation. *L. major* (MHRO/IR/75/ER) was maintained by serial passages in BALB/c mice and promastigotes were isolated from infected spleens and maintained in NNN/RPMI-1640 medium. Promastigote were cultured at 26ºC in RPMI 1640 complete medium containing 10% heat inactivated FBS, 4 mM L-glutamine, 25 mM HEPES, 0.1 mM non-essential amino acid, 1 mM sodium pyruvate, 50 µm 2-ME, streptomycin (100 µg/ml), Penicillin (100 u/ml). The parasites were collected from the logarithmic phase and heat treated in a water bath at 37°C for 30, 60, 120 and 240 min. The promastigotes were cultured at 26°C for 18 h and then isolated by centrifugation at 3,000×g at 4°C for 15 min. and washed 3 times with PBS buffer. The promastigotes (1×10^9 cells/ml) were subjected to 5 times freeze-thaw cycles using liquid nitrogen and 37°C water bath in the presence of protease inhibitor cocktail (Sigma 037K4012, Germany). Cell lysate was sonicated three times at 60 HZ and then dialyzed against PBS buffer at 4°C overnight. Protein concentration was determined by Bradford method [21].

SDS-PAGE analysis of HSP expression. Expression of HSP at different time periods was investigated by 10% SDS-PAGE. The gel was stained by Coomassie blue and the intensity of HSP 70 band (as an indication of increased HSP) in each time periods was analyzed with Quantity One software (Bio-Rad, CA, USA).

Mice immunization and infection. Three groups of mice (20 mice per group) were immunized intraperitoneally three times with three week intervals. Group I was immunized with 100 µg of 60 min heat shocked promastigote lysate (60 min heat induced higher HSP). Group II was immunized with 100 µg of normal promastigote lysate (no heat shock) and group III was immunized with 100 µl of PBS as control group. Ten days after the final boost, all mice were infected subcutaneously with 1×10^6 stationary phase promastigote. Two months after challenge, mice were sacrificed for immunological assays.

Assessment of footpad swelling. Ten days after last immunization, all groups (5 mice per group) were challenged subcutaneously in the left footpad with 1×10^6 stationary phase promastigote (in 50 µl PBS). As a control, right footpads were injected with 50 µl PBS alone. The footpad thickness was monitored weekly for eight weeks by a metric caliper. The lesion size was characterized as the mean of width and thickness of the footpad.

Lymphocyte proliferation assay. Five mice of each group were scarified before and eight weeks after infection. Spleen cells were removed and 2×10^6 cells/ml was cultured in complete RPMI-1640 medium in the presence or absence of 20 µg/ml of non-heated soluble leishmania antigens (SLA). For positive lymphocyte proliferation, 5 µg/ml phytohemagglutinin PHA was added to the cultures in the presence or absent of SLA. Cells were incubated in 96-well flat-bottom plates in 5% CO_2 at 37°C for 72 h. Twelve hours before harvest, cells were pulsed with bromodeoxy uridine (BrdU) (BD Biosciences, USA) and DNA synthesis was measured by cell proliferation based ELISA kit (Roche Applied Science, Germany) based on manufacturer's instruction. Stimulation index was calculated according to the following formula: OD of the wells stimulated with antigen/OD of the wells having control cells.

Cytokines analysis. Spleen cells were removed as above and after 72 h of culture the levels of cytokines IFN-γ, IL-4, IL-12 and TGF-β in the culture supernatant were measured by commercial ELISA kit (R&D, Minneapolis, MN, USA).

Parasite burden. Three mice from each group were sacrificed and parasite burden was determined using quantitative limiting dilution [22] at weeks 3, 6 and 9 after infection. Briefly, popliteal lymph nodes of the infected foot were removed, weighed and homogenized in 4 ml complete RPMI medium supplemented with 30% FBS and antibiotics. In a 96-well flat-bottom microtiter plates, homogenates were serially diluted (10^{-1} to 10^{-15}) and 200 µl of each dilution was cultured. After two weeks at 26°C, the wells were examined for motile promastigotes at 3-day intervals by an inverted microscope, and the reciprocal of the highest dilution which was positive for parasites was considered to be the number of parasites per milligram of tissue. Data reported are the calculated mean and standard error of mean of the last positive well multiplied by dilution factor.

Determination of nitrite oxide concentration. Nitrite level was determined in the macrophage supernatant using nitrate/nitrite colorimetric assay kit (Alexis, USA). Ten days after final immunization and two months after infection, macrophages from each group were derived from the mice peritoneal cavity and 5×10^6 cells/ml were cultured in the complete RPMI 1640 medium in 24-well plates and pulsed with 20 µg/ml of *L. major* SLA. As positive control, 500 ng/ml LPS (Sigma, Aldrich) and 2 µg/ml of recombinant mouse IFN-γ (Allexis, USA) were added to the cell. After 48 h of incubation, culture supernatant was collected and nitrite level was measured according to the kit instruction.

Detection of mouse-anti-Leishmania antibody. Ten days after final immunization and two months after infection, mice sera from each group were collected and anti-leishmania antibodies (IgG1, IgG2a, IgG2b and total IgG) were detected by ELISA assay. Briefly, 96-well plates (Nunc, Denmark) were coated with 20 µg/ml of SLA at 4°C overnight. Plates were blocked with 2% BSA in PBS at 37ºC for 1 h. Sera were diluted 1/200 and 100 µl was added to each well and incubated at 37°C for 30 min. After washing five times, 100 µl of HRP-conjugated rabbit anti-mouse (Sigma, USA) was added to each well and after 2 h, the plates were washed five times and 100 µl of TMB (3,3', 5,5"-tetra-methylbenzidine) substrate (KEM-EN-TEC, Denmark) was added to each well and incubated in the dark at 37°C for 30 min. The reaction was stopped by adding 100 µl of 2N SO_4H_2, and the absorbance was measured at 450/620 nm. For isotype determination, goat anti-mouse IgG1, IgG2a and IgG2b (Sigma, USA) and HRP-conjugated mouse anti-goat antibody were used according to the manufacture's instruction.

Statistical analysis. All experiments were done in triplicates and the data expressed as means ± S.E.M. The data were analyzed with SPSS V.13 software by one-way ANOVA followed by Tukey's test. *P* values <0.05 were considered to be statistically significant.

RESULTS

Analysis of HSP production at different time periods by SDS-PAGE. To assess the level of HSP expression, the logarithmic phase promastigotes were cultured at 37°C in different time periods. Figure 1A shows the production of HSP heat shock-treated promastigote for 30, 60, 120 and 240 min. The data show that 60 min heat shock at 37°C has the best effect on HSP production. The density of HSP 70 (as an example) at different time periods was analyzed with Quanty One software and then the relative HSP 70 expression was calculated (Fig. 1B).

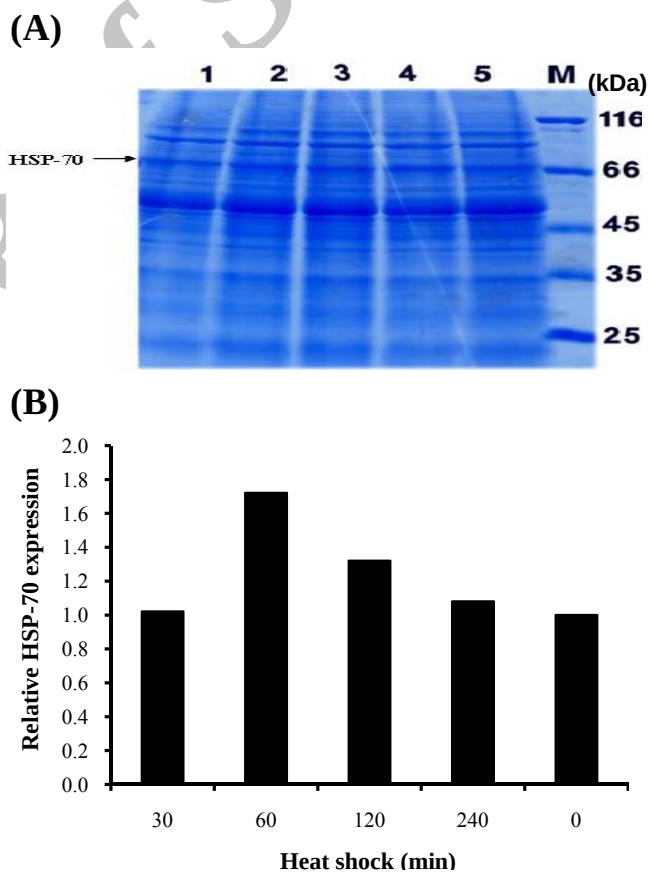

Fig. 1. Kinetic of HSP-70 synthesis based on molecular weight under heat stress at different times by 10% SDS-PAGE. **(A)** Lane 1, time zero (control); lane 2, 240 min; lane3, 120 min; lane 4, 60 min; lane 5, 30 min heat stress and M, standard molecular weights. Samples (20 µg) were boiled in SDS loading buffer and applied to the gel. **(B)** Relative HSP-70 expression under heat shock treatment at different times. Density value of each sample was calculated with Quanty One software and relative HSP-70 expression was also calculated.

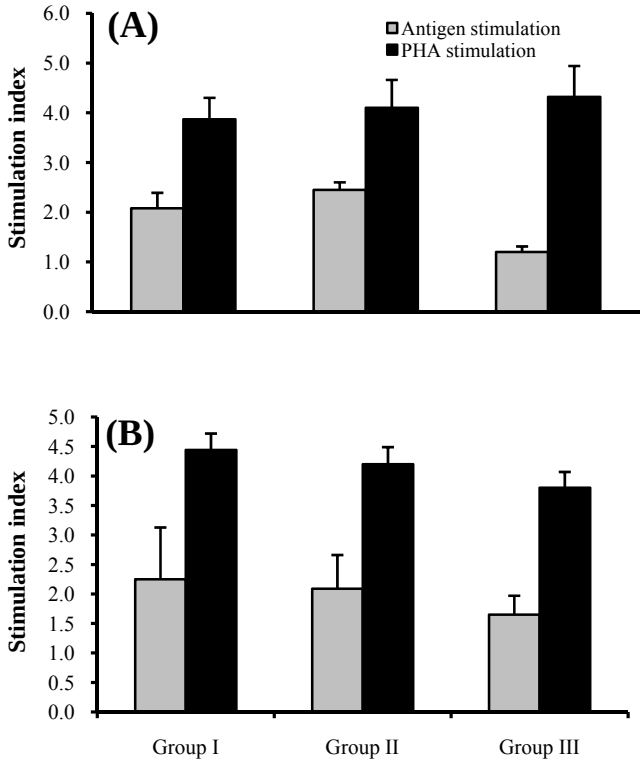

Fig. 2. Spleen cell proliferation assay of BALB/c mice (n = 5) before and after challenge with parasite. **(A)** Proliferation indexes of group I (HSP-SLA), group II (SLA) and group III (control group) before challenge. **(B)** Stimulation indexes of lymphocytes eight weeks after challenge with parasite. Spleen cells were stimulated with SLA in the presence or absence of PHA. Values are the mean ± SE of the mean of 5 experiments.

Cell proliferation assay. In order to evaluate lymphoproliferative response of immunized mice with either HSP-enriched SLA or SLA alone before or after challenge with parasite, proliferation assays were performed. The results showed that before infection, the HSP-enriched SLA (group I, P = 0.042) and SLA alone (group II, P = 0.005) had significantly higher stimulation index than control group (group III). However, there was no significant differences between HSP-enriched SLA and SLA alone (P = 0.461). When PHA was used, HSP-SLA and SLA had lower stimulation indexes than control group (Fig. 2A). Lymphocyte proliferation was also performed after mice were challenged with *L. major* (Fig. 2B). Spleen cell proliferation with HSP-enriched SLA and SLA alone) was significantly higher than control (P = 0.016, but the differences were not statistically significant (P = 0.109). When PHA was used, lymphocyte proliferation was increased as compared to the control group; however, the lymphoproliferation of HSP-enriched or non-enriched was not significant (P = 0.547).

Footpad swelling of mice challenged with parasite. Ten days after last immunization, mice were challenged with *L. major* into the footpad and the lesion size was measured weekly (Fig. 3). The results showed that there were no significant differences between footpad swelling in immunized mice (group I and II) as compared with the control (group III).

Parasite burden in lymph node. To determine the protective effect of enriched heat shock SLA, the parasite burden was measured in immunized mice after challenge with *L. major*. The popliteal lymph node was isolated and the parasite burden was determined. No significant differences were observed among groups after weeks 3, 6 and 9 post infection (Fig. 4).

Cytokines assays. To determine the cellular immune responses, we analyzed IFN-γ, IL-12, IL-4 and TGF-β cytokines in the supernatants of spleen cells of vaccinated mice before or after infection with *L. major*. IFN-γ analysis showed that immunization with enriched HSP-SLA (group I) and non-enriched SLA (group II) have significantly increased before challenge (P = 0.001) and after challenge (P = 0.021) as compared to the control group (group III). The level of IFN-γ in group II was significantly higher (P = 0.011) than group I, Fig. 5A). Increased IL-4 was observed in groups I and II before challenge (P = 0.012, P = 0.004 respectively) and after challenge (P = 0.012, P = 0.004, respectively) as compared to the control group. However, no significant differences were observed between group I and II before challenge, P = 0.134 or after challenge, P = 0.795 (Fig. 5B). Figure 5C shows

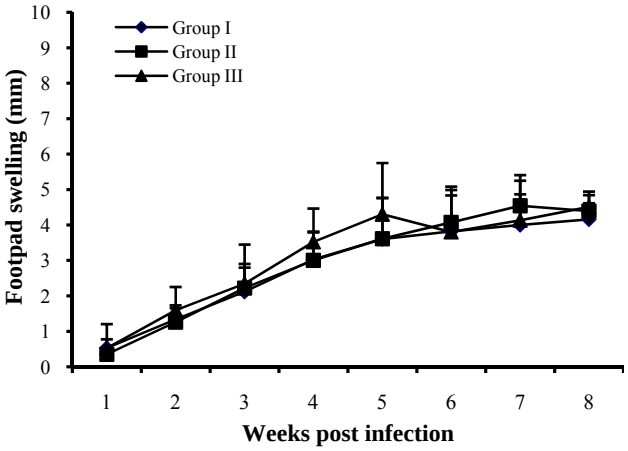

Fig. 3. Footpad swelling of BALB/c mice immunized with HSP-enriched SLA and SLA alone as compared with the control group after challenge with parasite. Ten days after last immunization, mice (n = 5) were challenged in the left footpad with 1×10^6 *L. major* promastigotes. Lesion size was measured for 8 weeks. Data are expressed as mean ± SE of 5 experiments.

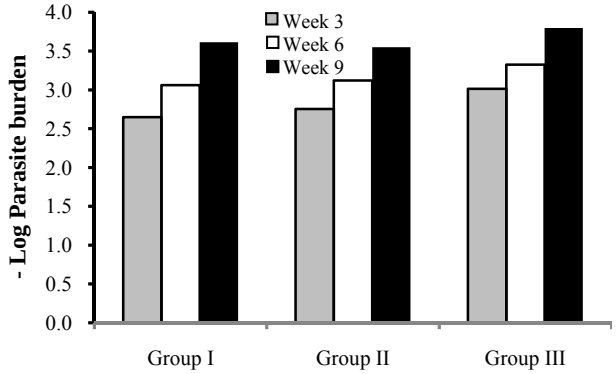

Fig. 4. Lymph node parasite burden of infected BALB/c mice immunized with HSP-enriched SLA and SLA alone. Three, 6 and 9 weeks after infection, a limiting dilution analysis was performed. Lymph node cells were isolated and cultured in triplicate in RPMI containing 30% FCS for 12 days at 25°C in serial 12-fold serial dilution. Data represent the calculated mean of the last positive well multiplied by dilution factor.

that there are no significant differences between IL-12 secretions of groups I and II before or after infection when compared with the controls. There were also no significant differences between groups I and II (P = 0.323). TGF-β was also determined. Figure 5D shows that HSP-enriched promastigotes and SLA antigen alone before and after infection could significantly increase the TGF-β secretion as compared to the control group (P = 0.011). Group I had significantly higher TGF-β than control group (P = 0.038). However, there were no significant differences between groups I and II (P = 0.119).

Total IgG responses. Serum samples of immunized mice were examined for detection of specific IgG anti-*L. major* Ag. Figure 6A shows that HSP-enriched lysate and SLA alone increased the specific IgG antibody as compared to the control group (P = 0.002). This increased was significantly higher than group I (P = 0.021). Total specific IgG after challenge was significantly increased in groups I and II as compared to the control group (P = 0.001), but there was no significant differences between groups I and II (P = 0.127).

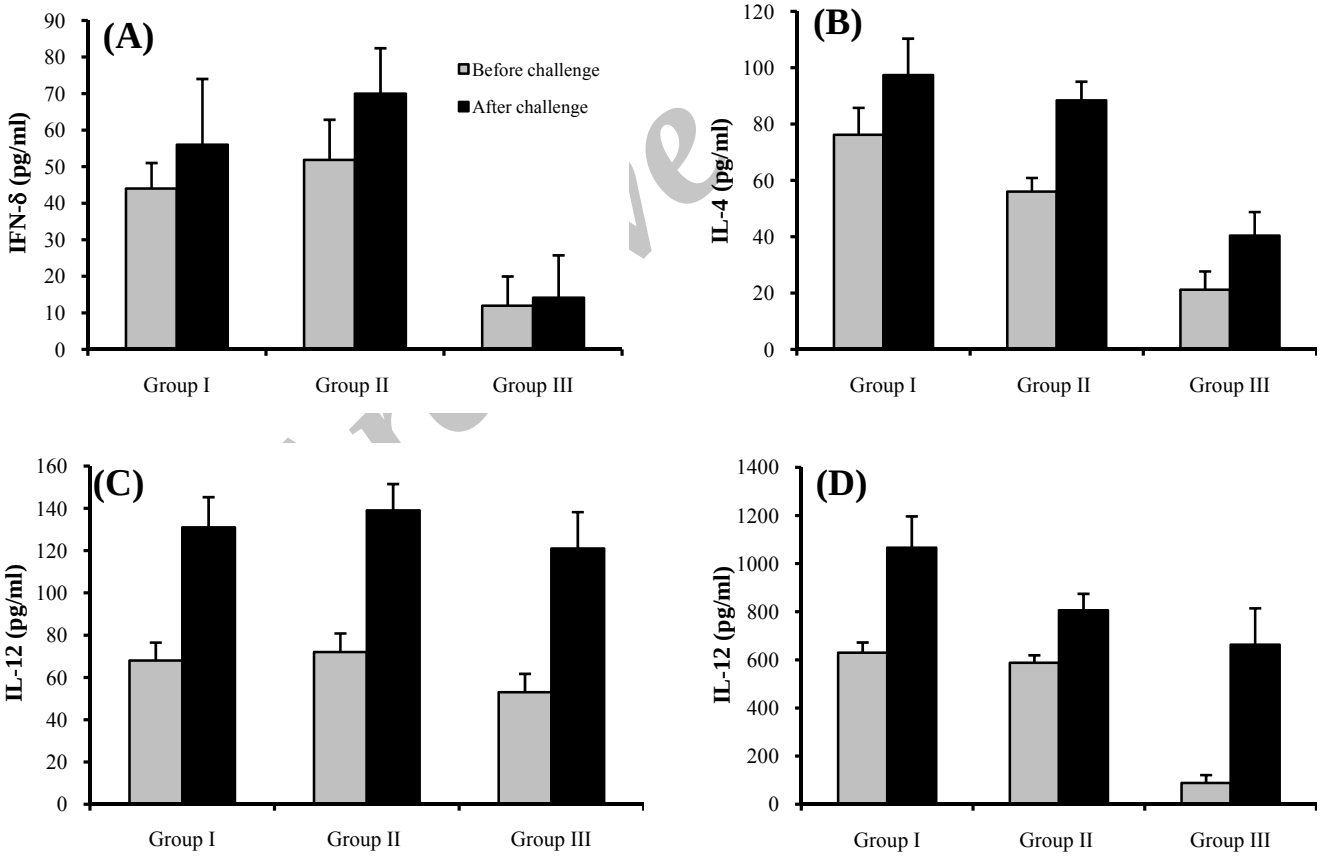

Fig. 5. Effect of HSP-enriched SLA immunization on Th1 and Th2 cytokines. The levels of IFN-γ, IL-4, IL-12 and TGF-β cytokines were determined in the supernatant of spleen cells of vaccinated BALB/c mice before and after infection with *L. major*. **(A)** IFN-γ responses of BALB/c mice immunized three times with HSP-SLA (group I), SLA alone (group II) and control group (group III) before and after challenge with *L. major*. **(B)**, IL-4 responses; **(C)**, IL-12, and **(D)** TGF-β responses. The data express mean ± S.E. of 5 mice in each group in triplicates.

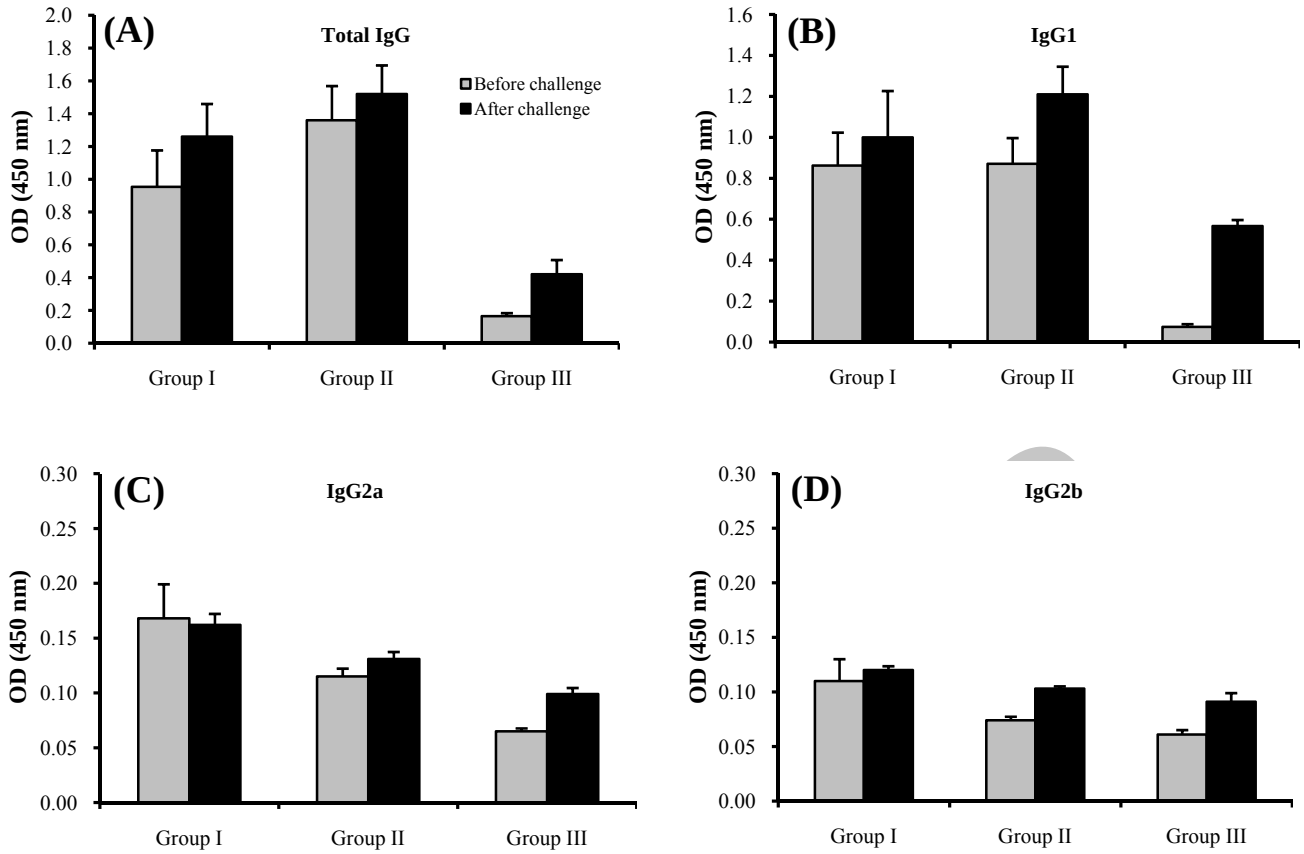

Fig. 6. Specific antibody responses of immunized BALB/c mice with HSP-SLA and SLA alone before and after infection. **(A)** Specific total IgG level in the sera of experimental groups (n = 5) after immunization with HSP-SLA (group I), SLA alone (group II) and control group (group III) before and after challenge with *L. major.* **(B)**, IgG1 level; **(C)**, IgG2a response and **(D)** IgG2b response. The data express as mean ± S.E. of triplicate experiments.

IgG subtyping. The IgG subclasses (IgG1, IgG2a and IgG2b) were also determined. IgG1 isotype before and after infection were increased in groups I and II as compared to the control group (P = 0.0001 and P = 0.001, respectively) and there was no significant difference between groups I and II (P = 0.986). As seen in Figure 6B, after infection, IgG1 in group II was increased significantly when compared to the group I (P = 0.048)). Figure 6C indicates that IgG2a was increased significantly in group I before infection as compared to the control group III (P = 0.032), but no differences were seen between group II with group I (P = 0.144) and group III (P = 0.187). However, after challenge with *L. major,* the level of IgG2a was increased significantly in group I (P = 0.001) and group II (P = 0.038) as compared to the control group. The level of IgG2a in group I was significantly higher than group II (P = 0.045). Analysis of IgG2b subtype in the sera of experimental groups shows that after three times immunization with candidate vaccine, IgG2b level in group I that immunized with HSP-enriched promastigotes lysate increased significantly compared to control group (P = 0.035) and the difference was not significant with group II (P = 0.131) (Fig. 6D). The differences between groups I and II was not statistically significant (P = 0.729). Evaluation of sera IgG2b level after challenge with *L. major* shows that IgG2b level in group I (P = 0.006) was significantly increased compared to the control group (Fig. 6D). There was no significant difference among other groups.

Nitric oxide (NO) production by peritoneal macrophages. NO production in the culture supernatant of peritoneal macrophages was determined in mice immunization with promastigote HSP-enriched SLA or SLA alone (Fig. 7). NO production in HSP-enriched and SLA alone immunized mice were increased significantly when compared to the control group (P = 0.037 and P = 0.008, respectively). However, there were no significant differences between groups I and II. Analysis of NO production after challenge with *L. major* showed that NO only was increased significantly in SLA immunized mice when compared with HSP-enriched mice (P = 0.011) and control group (P = 0.001).

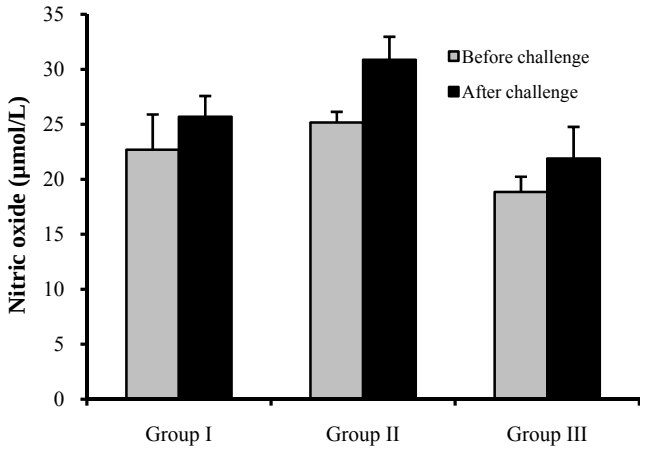

Fig. 7. Nitric oxide determination of peritoneal macrophage culture supernatant. BALB/c mice (n = 4) were immunized with HSP-SLA (group I), and SLA alone (group II) and after 3, 6, and 9 weeks after infection with *L. major*, the levels of NO were compared with the control (group III). The data express as mean ± S.E. of triplicate experiments.

DISCUSSION

In this study, we have shown that heat stress in *L. major* procyclic promastigote caused a marked induction of HSP, especially HSP-70. Immunization of BALB/c mice with HSP-enriched SLA or SLA alone and challenging with stationary phase promastigote of *Leishmania major* did not show any significant differences in immunological responses until nine weeks after infection. No significant differences were observed in lymphocyte proliferation, footpad swelling, parasite burden, NO or the level of IL-12, and INF-γ cytokines between HSP-enriched or SLA groups.

In order to access an effective vaccine against infectious diseases such as Leishmaniasis, scientists intended to design a vaccine that has ability to induce protection and elicit Th1 immune responses. Several vaccine candidates have been developed and evaluated against Leishmaniasis but all of them have limited efficacy. Vaccination strategies based on HSP have induced considerable protection in various infectious diseases [23]. Application of HSP in combination with many vaccines or vaccines that enriched with HSP has shown an increase in the immunogenicity of vaccine with a pro-inflammatory cytokine pattern [24]. Among the main HSP families, HSP60, HSP70 and HSP90 have been mentioned as major antigens produced by protozoa, fungi and bacteria in infectious diseases [23].

Few conflicting results were obtained using leishmania HSP in mice and human, indicating protecting and non-protecting roles of HSP [19, 20]. Induction of protective immune responses to Leishmania HSP seems to depend on differential patient immune responses. When two different *L. braziliensis* HSP homologous to the eukaryotic 83- and 70-kDa HSP was studied on patient immune response, different results were obtained [19]. rLbHSP-83 could stimulate PBMC from all *L. braziliensis*-infected individuals to proliferate and to produce IL-2, IFN-γ and TNF-α. However, rLbHSP-70 only stimulated PBMC proliferation from patients with mucosal disease but not from self-healing individuals. In contrast, rHuHSP-70 did not stimulate proliferation or production of cytokines of PBMC from mucosal patients [19]. Recent study shows that immunization of both susceptible BALB/c and resistant C57BL/6 mice with rHSP-70 from *Leishmania* had no efficient protective responses when challenge with *Leishmania major* [20]. Also, no significant proliferation against HSP70 in the cellular immune responses of 16 cured cutaneous leishmaniasis patients and 5 normal individuals was obtained. However, humoral immune responses against HSP70 suggested a mixed TH1/TH2 response *in vivo* [20]. Our results also show that mixture of induced HSP does not have protective immune responses in BALB/c mice. Evaluation of lymphocyte proliferation response revealed that enriched HSP lysate of *L. major* did not stimulate the proliferation of spleen cells significantly. However, rHSP70 was shown to induce proliferation in patients with cutaneous or mucosal Leishmaniasis [19]. Footpad swelling after infectious with *L. major* was measured and the results demonstrated that the HSP-enriched promastigotes did not induce protection against *L. major* infection. Parasite burden was also shown that there were no significant differences between groups I and II. Therefore, our results suggest that the immune responses elicited by enriched HSP of *L. major* in vaccinated mice were not effective in controlling the parasite in the macrophages. Because, the parasite loads in mice immunized with lysate from enriched HSP of *L. major* (group I) did not change as compared to the other groups. These results may be correlated with the dose of HSP or the route of injection that needs some delivery systems to maintain and enhance the immunostimulatory activity of these proteins. It is also possible that the administration of HSP alone without any suitable adjuvant could not provide effective immunity against leishmaniasis. However, in tumor models [15] and in several infection models [16, 17], HSP have adjuvant effects that induce strong Th1 immune responses and cytokine.

It is known that secretion of IgG2a is associated with IFN-γ production and the development of TH1 immune response, but IgG1 response is associated with IL-4 production that is a marker for Th2 response [25]. In this report we showed that HSP-enriched SLA induces TH2 response. In both groups (I and II) that received HSP-enriched SLA or SLA alone, the level of IgG1 before infection were increased significantly.

However, SLA alone had higher IgG1 than group I with HSP. The levels of IgG2a and IgG2b were increased in group I before challenge; however, eight weeks after challenge, the isotype level increased slightly. These results demonstrate that HSP-enriched *L. major* induces multi-isotypic humoral immune response toward Th2 response. Cytokine assays also showed that in HSP-enriched group, IFN-γ level decreased and IL-4 level increased when compared to the SLA group. These results contradict the data obtained in pulmonary tuberculosis model [26] in which, HSP-enriched SLA stimulate strong Th1 cytokine pattern. Although IL-12 is associated with the production of Th1 cytokines in infectious diseases [27], we could not detect any significant changes in both groups. TGF-β, that is importance for parasite to survival in macrophages [28], showed significant increases in both groups before infection.

In vaccine preparation strategies, antigen and adjuvant alone can have a distinct influence on the immune response and the effect of combination and fusions between antigen and adjuvant have different results for effective immune response. It seems that HSP-enriched promastigote lysate inhibits NO production and may affect the parasite burden. Our data show that HSP did not induce strong NO production by peritoneal macrophages in contrast to SLA alone before and after challenge. We have prepared HSP-enriched promastigotes in the logarithmic phase of leishmania growth where the HSP change occurs. Since the infective phase of parasites is in the stationary phase, there are no detectable changes of HSP synthesis occurred after heat shock induction [29]. Therefore, antigens of non-infective promastigote and induced HSP by heat shock may be incapable to induce protective immune responses. More studies are needed to elucidate the effect of leishmania HSP on the mice immune system.

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