

Survey Protein Vaccine Formulated with Montanide ISA 70 Effects following Immunization and after Challenge with *Leishmania major*

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Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania* which, in the infected host are obliging intracellular parasite . Montanide ISA 70 is an adjuvant composed of a natural metabolizable oil and a highly refined emulsifier from the manide monooleate family. The TSA (thiol-specific antioxidant) is a considerable antigen of *Leishmania major*. The purpose of this work was Protein-vaccine efficacy in the presence Montanide comparing to absence of it. The expression of recombinant protein was confirmed with SDS (sodium dodecyl sulfate) page and Western blotting . 48 BALB/c mice were divided into four groups [TSA/ Freund ,TSA/Alum+BCG , TSA/ Montanide and PBS groups] and immunized with 20 mg of vaccine subcutaneously three times intervals on days 0, 14 and 28. The mice were challenged with parasite 21 days after final immunization . The lymphocyte proliferation was evaluated with Brdu method. Cytokines was evaluated with ELISA method. The vaccine formulated with the recombinant TSA protein with Montanide induced lymphocytes proliferation , cytokines as compared with the control group. Base on results, current candidate vaccine has potency for futher studies.

Keywords: BCG; TSA; Montanide; Leishmaniasis; Vaccine; IFN- γ .

Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania* which, in the infected host are obliging intracellular parasite. *Leishmania* is a genus of trypanosomatid protozoa and reliable for Leishmaniasis. In this group *Leishmania major* is the etiological agent of cutaneous leishmaniasis that is prevalent in many tropical and subtropical areas of the world and transmits via the bite of an infected sand fly. The disease has a high incidence rate in Iran Alvar [Alvar *et al.*, 2010,Farmand *et al.*, 2011] . The manifestation of disease is varying from self-limiting with life-long immunity to developing

shape as chronic disease. When Leishmaniasis accompanied by deficient cellular immunity, the disease can improve to chronic shape[Von Stebut 2007,Douba *et al.*, 2012] .The number of new cases of cutaneous leishmaniasis is estimated a range from 0.7 million to 1.2 million .The human immunodeficiency virus [HIV] and *Leishmania* co infection is reason of rapidly growing of Leishmaniasis in regions where *Leishmania* species are endemic. In the other hand toxicity of chemotherapy and currently available drugs for treatment haven't shown ideal consequence and ever important , resistance to current drugs , above all things underline to need for a safe, effective vaccine[Coler *et al.*, 2005,Masina *et al.*, 2003].In recent years with completion of genome sequencing of *L. major*, researchers find out that some of genes are expressed in the infectious stages of the

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parasite and in particular, in amastigotes, which can help to design effective vaccines [khamespour *et al.*, 2006]. Among *L. major* antigens as vaccine candidate, TSA with molecular weight of 22.1 kDa has been introduced as one of the predominant and one of the important immune system stimulator, homologue to eukaryotic TSA (thiol-specific-antioxidant) protein. Recombinant form of TSA protein composed of 200 amino acids and placed in the chromosome of 15 of *Leishmania major* and is full gene with multiple copy in chromosome. Distribution of TSA protein on the surface of both extracellular and intracellular promastigote and amastigotes is important and in BALB/c mice model infected with *L. major* can persuade Th1 response comparing to the other selected antigens. TSA protein induces strong cellular immune response resulted in protective immunity [mauel 2002, Campos-Neto *et al.*, 2001, 2002, Webb *et al.*, 1998]. Montanide [Incomplete Seppic adjuvants], is an oil adjuvant composed of a natural metabolizable oil and a highly refined emulsifier from the manide monooleate family. Montanide Incomplete Seppic adjuvants, are a group of oil/surfactant based adjuvants in which different surfactants are combined with either a non-metabolizable mineral oil, a metabolizable oil, or a mixture of the two oils. Vaccine formulations with Montanide induce a strong and long term immunity. Compared to traditional oil emulsions, Montanide emulsions are very stable and easy to inject which are having high immunopotential capacity and showing lesser side-effects are available [Aucouturier *et al.*, 2001, 2002, Toledo *et al.*, 2001, Joshua *et al.*, 2010].

In this report we described a novel protein vaccine regimen using recombinant TSA protein in Montanide ISA 70 VG adjuvant, and characterize the immune responses elicited.

MATERIAL AND METHOD

Expression and purification of the recombinant TSA protein

TSA sequence was optimized and then the sequence was purchased from Biomatrix Company [Canada]. TSA gene was double digested and after purification of gene, it was subcloned into the pET28a expression vector using ligase enzyme. In order to express the recombinant

protein, pET28a-TSA expression vector was transformed into the *E. coli* BL21[DE3] using electroporation technique. Culture of *E. coli* BL21 [DE3] containing pET28a-TSA recombinant plasmid was done in the presence of IPTG 1 mM for 4 h. The expression of recombinant protein was confirmed with SDS-PAGE and also via Western blot technique. Recombinant protein was purified with nickel affinity chromatography [Tabatabaie *et al.*, 2007, Bradford 1976].

Preparation adjuvant

Montanide 70 VG [Seppic france] was used as an adjuvant mixed with antigen at ratio of 30/70 [V/V] according to the manufacturer's instructions.

The MHRO/IR/75/ER [an Iranian strain to be isolated by Nadim *et al.*, in 1964] of *L. major* was provided by Pasteur Institute of Iran. Promastigotes were grown at 26°C in RPMI1640 medium [Sigma®] supplemented with 10% heat inactivated fetal calf serum [Gibco®, BRL], and 100 µg/ml gentamicin [Sigma®]. Stationary phase of the promastigotes was harvested at a density of 2x10⁶/ml.

Immunization and experimental infection of the mice

48 Female Inbred BALB/c [6-8 weeks old] mice were purchased from Iran's Razi Serum and Vaccine Production Research Institute and maintained under standard conventional conditions. Ethical Committee of the University approved this study.

The mice were grouped based on administration content as follows in 4 groups

TSA/Montanide, TSA/BCG-Alum, TSA/Freund and control PBS groups. 12 mice in each group were anesthetized with 25 µLg⁻¹ of mixture of ketamin 10% and xylazin 2% via intraperitoneal [i.p.] injection and were immunized with 100 µL of administration content according to their grouping [20 mg of recombinant protein TSA vaccine and 40 mg of adjuvants] subcutaneously three times intervals on days 0, 14 and 28. Finally, immune responses 3 weeks after the last injection were evaluated. The mice were challenged at the base of tail by the intradermal route with 2x10⁶ promastigotes of *L. major* 21 days after final immunization. The immune responses 3 weeks after challenge were evaluated. The lesions were appeared at the third week in the control groups

and the fifth week in the immunized groups. The measuring of the diameter of lesion [n=12 per each group] at the site of inoculation was monitored weekly by a Vernier caliper thereafter [Rosado-Vallado *et al.*, 2005].

Cell preparation

Responses of specific and proliferation lymphocytes to Brdu technique was done. Spleen was removed from euthanized mice 3 weeks after the last immunization and 3 weeks after the challenge infection. The tissues were macerated and washed in RPMI 1640 and incubated for five minutes in 0.17 M Tris pH 7.2 and 0.16 M NH₄Cl to lyses erythrocytes. The splenocytes were washed and resuspended in RPMI 1640 supplemented with 20 mM sodium pyruvate, 5×10⁵ M mercaptoethanol, 4 mM glutamine, 10% bovine fetal serum, 100U/ml penicillin and 100µg/ml streptomycin. The cell's viability and number were assessed in an improved Neubauer chamber using 0.4% Trypan blue.

Cytokines assay before and after the challenge infection with *L. major*

The splenocyte cultures and cytokine assays were performed. Briefly, single-cell preparations from the spleen tissue were plated in duplicate in 24-well plates [Nunc] at 2 × 10⁶ cells/ml. The cells were incubated in Dulbecco Modified Eagle Medium [DMEM] alone [background control]. Then the cells were stimulated in triplicate with soluble *L. major* antigen separately stimulated with Soluble *Leishmania* antigen [SLA] from *L. major* [20µg/ml], at 37°C in 5% CO₂ for 48 h. The SLA-stimulated cells presented stimulation indexes in the range of 3.5-3.7 for the spleen cells. Stimulated index [SI] greater than 2.5 indicated a positive response. Levels of IFN-α and IL-4 in the supernatants were assessed by sandwich enzyme-linked immunosorbent assay [ELISA] using Inter Test mouse IFN-α and IL-4. [ELISPOT Kit]. All the tests were performed in triplicate for mice [Rafati *et al.*, 2006].

Detection of antibody responses before and after the challenge infection with *L. major*

The blood samples were collected by retro-orbital puncture from the immunized mice 3 weeks after immunization and 3 weeks after challenge infection. The samples were centrifuged in 3000rpm for 15 min for serum preparation. The sera were tested for the presence of total anti-*L. major* IgG antibodies by ELISA. Briefly, the

microtitre plates were coated overnight at 4 °C with soluble *L. major* antigens [10 µg/ml] in 100 mM carbonate-bicarbonate buffer pH9.6 [100µLper well] and sealed. The plates were washed in PBS [pH7.4]. Blocking was carried out with 1% dried skim med milk in PBS [pH7.2] for 1 h at 37°C. After washing with PBS containing 0.05% Tween 20 [PBST20], the sera were diluted 1/200 in 1% dried skimmed milk-PBST20 [100µLper well] and incubated for 1 hour and 30 minutes at 37° C. After washing, the bound antibodies were detected by incubation at 37°C for 1 hour and 30 minutes with horseradish peroxidase [HRP]-labeled goat anti-mouse IgG [DAKO, Denmark] at 1/2000 dilution in 1% dried skimmed milk PBST20 [100µLper well]. Peroxidase activity was revealed by adding 100 µL per well of Tetra methyl benzidine [TMB] substrate. The reaction was stopped after 15 minutes with adding 100µ L of 2 MH₂SO₄ and the optical density [OD] was read at 450 nm in an ELISA micro plate reader [Bio-Rad, USA] [Sasaki *et al.*, 2003].

Determination of parasite burden

Three mice from each group were sacrificed seven weeks after challenge and parasite burden was determined as follows: A piece of spleen was excised, weighed and then homogenized with a tissue grinder in 2 ml of Schneider's *Drosophila* medium supplemented with 20% heat-inactivated fetal calf serum and Gentamicin [0.1%]. Under sterile conditions, the serial dilutions ranging from 1 to 1/4 × 10⁻⁴ were prepared in wells of 96 well micro titration plates. After 7 and 15 days of incubation at 26° C, the plates were examined with an inverted microscope at a magnification of 40x. The presence or absence of mobile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one parasite. The number of parasite per gram was calculated in the following way : Parasite burden = -log₁₀ [parasite dilution/ tissue weight] [Rafati *et al.*, 2006, Buffer *et al.*, 1995].

Statistical analysis

Statistical comparisons between the experimental groups were carried out with an analysis of variance [ANOVA] and post hoc Turkey test. Differences were statistically considered significant when p values was less than 0.05 [p<0.05].

RESULTS

Our results showed that recombinant protein was produced in the *E. coli* BL21 [DE3] containing pET 28a-TSA plasmid after induction with 1mM IPTG that expression of recombinant protein was confirmed with SDS page and Western blot analysis[a ~22 kDa band]. In the next, we intended that use this protein as a vaccine candidate against Leishmania infection in BALB/c mouse model.

To evaluate the proliferative responses of lymphocytes was used to Brdu test. The 3 weeks

after three protein vaccination the proliferative responses of lymphocytes markedly were increased in the vaccinated groups, which were significantly higher than in control group. [$P < 0.05$]. Before and after challenge infection in the vaccinated groups were no statistical significant difference but was statistical significant difference with control group [Fig. 1].

We analyzed the levels of IFN- γ and IL-4 production in the supernatants of the spleen cells of all the 4 groups 3 weeks after immunization and 3 weeks after challenge infection with *L. major*. The 3 weeks after three protein vaccination IFN- γ

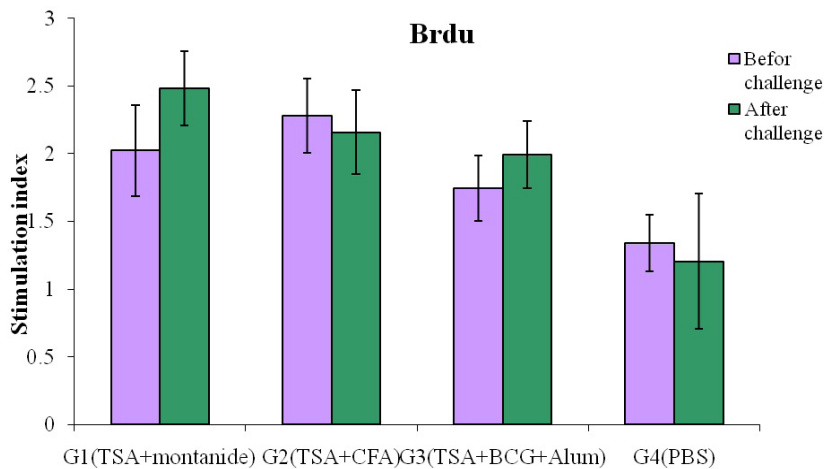


Fig. 1. The proliferative responses of lymphocytes (Brdu test) in vaccinated and control groups

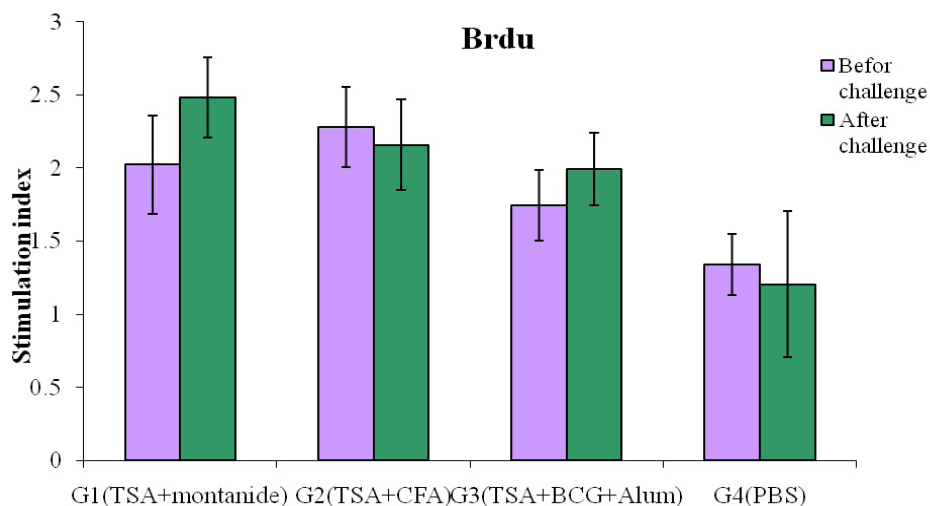


Fig. 2. Cytokine production (IFN- γ) by the splenocytes in vaccinated and control groups

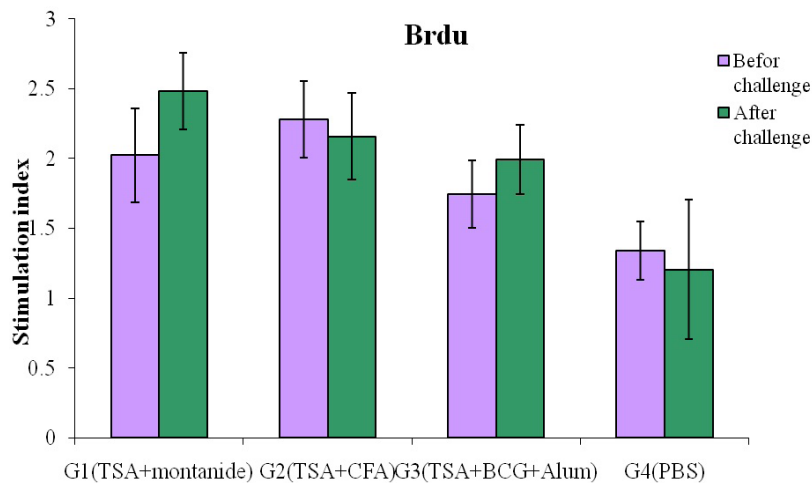


Fig. 3. Cytokine production (IL-4) by the splenocytes in vaccinated and control groups

values markedly increased in the vaccinated groups, which were significantly higher than in the control group [$p < 0.05$]. Between vaccinated groups were no statistical significant difference [$p > 0.05$]. After challenge infection IFN- γ values markedly increased in the vaccinated groups, which were significantly higher than in the control group [$p < 0.05$]. Between the vaccinated groups were no statistical significant difference [$p > 0.05$] [Fig2]. Before booster injection and after challenge with *Leishmania major* IL-4 values Significant differences werenot observed between vaccinated and control groups [$p > 0.05$]. Though Before booster injection and after challenge IL-4 values increased in the vaccinated groups but there were no statistically significant differences between vaccinated and control groups [$p > 0.05$] [Fig 3].

DISCUSSION

Leishmania spp are responsible for a large variety of parasitic diseases in almost 90 countries. The disease is prevalent in many parts of the world, with about 12 million total cases worldwide. As many as 1.5–2 million new cases of cutaneous leishmaniasis and 500 000 cases of visceral leishmaniasis are reported every year [Handman 2001]. Immunity against re infection is acquired following cutaneous infection with *Leishmania* spp., suggesting that prophylactic immunization is feasible. A number of vaccine strategies have been

tested, ranging from killed parasites to recombinant antigens or DNA or protein vaccines. No protective and effective anti-*Leishmania* vaccine is available at the moment in spite of several tested vaccine protocols Despite enormous effort of researchers to preparation of effective vaccine formula there is no protective vaccine and this failure turned to cell mediated immune system response in primarily impact to parasite [Handman 2001, Campos-Neto *et al* 2001]. In this level vaccine progresses strategy against *L. major* depends on proper and good defined antigen[s] and application of strong adjuvant in vaccine formulation to reach optimal immunity results. The availability of hundreds of adjuvants has prompted a need for identifying rational standards for the selection of adjuvant formulation based on immunological principles for human vaccines [Benhni *et al.*, 2009]. TSA family from humans to *Saccharomyces cerevisiae* has conserved domains and distinctive similarity in amino acid sequence has been seen in this group. *S. cerevisiae* thiol specific antioxidants confer safety against oxidative stress and damage. Mechanism of TSA action embedded in a thiol oxidation-based enzyme inhibition mode. Function of TSA protein depend on thioredoxin, thioredoxin reductase, and NADPH as reducing equivalents, then been define as thioredoxin peroxidase. thioredoxin as a reducing equivalent In *S. cerevisiae* help to reduction of H_2O_2 molecules and participate in protection of mechanism against peroxide-mediated oxidative damage Similar situation

occurs inside macrophages. Respiratory burst in macrophages mediated by production of H₂O₂ and TSA molecules as leishmania product, guarantee *Leishmania* survival inside the macrophages. *Leishmania* TSA protein is known as antigenic in both murine and human systems and is constitutive expressed in both promastigote and amastigote life stages [Mendez *et al.*, 2002, Chae *et al.*, 1993, 1994, Netto *et al.*, 1996, James *et al.*, 1993, Mehlotra 1996, Ahmed *et al.*, 2004]. The recombinant *leishmanial* antigens LmSTII and TSA have been shown that they can induce excellent protection in both murine and non human primate models of human cutaneous leishmaniasis. The recombinant TSA protein with IL-12 induces excellent protection in the BALB/c mice. recombinant proteins, LACK and TSA have produced at least partial protection against *L. major* in BALB/c mice [Ovendale *et al.*, 1998, Mougneau *et al.*, 1995]. Successful immunization that induces protection against leishmaniasis is highly dependent on adjuvant that preferentially stimulates the Th1 phenotype of immune response. In this research was used Montanide ISA 70 as a adjuvant . Montanide ISA 70 has been approved for experimental use in humans as an alternative adjuvant to aluminium hydroxide. It has been shown to be immunogenic, inducing both Th 1-type cellular and humoral immune responses in humans. Montanide ISA 70 has also shown good results in non-human primate vaccination studies. A safety and immunogenicity study of a malaria vaccine containing single, intramuscular doses of ICC-1132 formulated in Montanide ISA 720 showed that the vaccine was safe and well tolerated. All vaccines that received either 20 µg or 50 µg of ICC-1132/ISA 720 developed anti immunogen antibodies, predominantly of opsonizing IgG subtypes. Peripheral blood mononuclear cells of ICC-1132/ISA 720 vaccinees proliferated and released cytokines when stimulated with recombinant *Plasmodium falciparum* CS protein, and CS-specific CD4+ T-cell lines were established from volunteers with high levels of antibodies to the repeat region. [Joshue *et al.*, 2010] Kusakabek, *et al.*, showed IFN -γ induction vaccinated group [with Montanide] was significantly higher than controls. Furthermore, the parasite load of vaccinated group was significantly lower than control groups [khoshgoo *et al.*, 2008]. Qiu et al

showed that protein immunization using CpG ODN and Montanide ISA 720 as adjuvants greatly enhanced cellular as well as humoral immune responses against HCV in Balb/c mice and the use of adjuvants appears critical to the induction of Th1 immune responses during HCV vaccination with recombinant proteins [Qiu et al 2008]. In this study our results showed that proliferative responses of lymphocytes and IFN-γ values before and after challenge infection in the vaccinated groups were no statistical significant difference but was statistical significant difference with control group but in IL-4 values Significant differences were not observed between vaccinated and control groups . In this study, we demonstrated that Montanide can effect on efficacy of a protein vaccine-encoding TSA against *L. major* infection and elicits cellular immune responses to the antigen delivered . The vaccine formulation described here may be an protection and excellent candidate for further vaccine development against *Leishmania*.

Conflicts of interest

We declare no conflicts of interest.

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