Safety and Toxicity of a New Formulated *Leishmania major* Preliminary Vaccine in Animal Model Balb/c and Small White Conventional Laboratory Mice

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SUMMARY: *Leishmania* parasites cause a spectrum of diseases that afflict the populations of 88 countries around the world and all attempts to control leishmaniasis have failed. It seems that preparing a vaccine may be the final solution. The aim of this study was to determine various *Leishmania* (*L.*) *major* antigens vaccine candidates and effects of the vaccine on delayed type hypersensitivity (DTH). Many different methods of vaccine preparation plus or without adjuvant were used. We prepared crude antigen combinations by five different methods using antigens from *L. major* parasites. Phase I was done in animals. The immunogenic effect was evaluated with the delayed type hypersensitivity (DTH) reaction with five different doses, including 100, 200, 300, 400, 500 μg/ml of total protein + BCG in Balb/c and conventional laboratory white mice (out breed). Our results showed that the cocktail antigen was highly specific. No injection of BCG solvent or saline treated controls showed significant results. Taken together, the effect of cellular immune response to the cocktail vaccine induced a significant effect against cutaneous leishmaniasis in the experimental model of vaccine with *L. major*.

Key Words: *Leishmania major*, DTH, Balb/c, vaccine, safety, toxicity.

Yeni Düzenlenen *Leishmania major* Aşısının Güvenlik ve Toksisitesinin Balb/c ve Beyaz Laboratuvar Farelerinde Denenmesi

ÖZET: *Leishmania* parazitleri Dünya'da 88 ülkeyi etkileyen farklı hastalıklara sebep olmakta ve leishmaniasis kontrol yöntemleri yeterince başarılı olamamaktadır. Aşı hazırlanması ise son öneri olarak görülmektedir. Bu çalışmanın amacı, aşı adayı olarak farklı *Leishmania* (*L.*) *major* antijenlerini belirlemek ve geç tip aşırı duyarlılık (DTH) üzerine aşının etkisini belirlemektir. Adjuvanlı veya adjuvansız aşı hazırlanmasında birçok farklı yöntemler kullanılmaktadır. Bu çalışmada, *L. major* antijenlerinden beş farklı yöntemle ham antijen kombinasyonları hazırlandı. İmmunolojik etkisi geç tip aşırı duyarlılık reaksiyonu ile değerlendirildi. Bunun için Balb/c fare ve beyaz laboratuvar faresine (out breed) total protein + BCG uygulaması 5 farklı dozda, 100, 200, 300, 400, 500 μg/ml, uygulandı. Sonuçlarımız, kokteyl antijenin fazlasıyla spesifik olduğunu gösterdi. BCG solvent veya fizyolojik su uygulanan kontroller şimdiye kadar önemli bir etki göstermedi. Hepsi birlikte değerlendirildiğinde, kokteyl aşıya hücresel immun yanıtın etkisi *L. major* ile aşının deneysel modelde kutanöz leishmaniasise karşı önemli derecede spesifik etkisi olduğunu gösterdi.

Anahtar Sözcükler: *Leishmania major*, Geç tip aşırı duyarlılık, Balb/c, aşılama, güvenlik, toksisite

INTRODUCTION

Leishmania major (*L. major*) is the causative agent of cutaneous leishmaniasis in the old world. Infections in humans range from single self healing to disseminating lesions in cutaneous leishmaniasis. To prevent cutaneous leishmaniasis, different antigen preparation has been tried as vaccine. An alternative strategy using attenuated organisms allows the

development of an immune response closest to that of natural infection, with exposure to a much larger range of antigens than achieved by using more refined subunit vaccines.

The live Leishmania vaccine was prepared by in Venezuela to vaccinate subjects against *L. mexicana* and *L. braziliensis* (18). The vaccine was a mixture of equal amount of freeze and thaw intact parasite (10, 26). Irradiated and heated antigen (1, 12) injected as intradermally. Mertiolated killed antigens from five different strains of *Leishmania* has been used for vaccination (6, 7, 9). Despite pursuing such a strategy for human or experimental murine leishmaniasis using naturally a virulent organisms, irradiated organisms or genetically manipulated organisms, there has been little success.

Similarly, killed vaccines have shown limited immunogenicity and efficacy even when combined with adjuvants, either BCG or alum. Interestingly, BCG alone led to a positive leishmanin skin test in some individuals, presumably due to antigenic cross-reactivity between *Mycobacteria* and *Leishmania* (18).

Up to now, successful vaccination strategy against leishmaniasis has been limited to cutaneous leishmaniasis with small doses of living virulent *L.major* promastigotes as a selected site (7).

The effectiveness of immunization against clinical leishmaniasis with the safer killed vaccine is unclear at present.

In our experimental model, protection against *L.major* infection can be induced in genetically susceptibile Balb/c mice with lethally irradiated or heat killed promastigotes (5, 14, 22), frozen thawed *L.major* infected macrophages plus corynebacterium parvum (7) or non-pathogenic clones of *L.major* promastigotes (20). Few researches have been done on radio attenuated *L.major* vaccine (2). A great deal of experience has accumulated which suggests the natural disease can be implanted by a controlled injection of the causative organism (8). The objective of this study was to compare protective effects of cocktail vaccine candidates encoding various *L.major* antigens in highly susceptible Balb/c mice, resistant mice or laboratory small white mice and also, to examine the effects of vaccine on the delayed type hypersensitivity to cocktail vaccine.

MATERIAL AND METHODS

Mice Balb/C and conventional small white laboratory mice were obtained from inbred colonies. Three months old female and male Balb/c and small white mice (n=192) were obtained from the Razi institute. This study was done in compliance with NIH guide for the care and use of laboratory animals, and it was approved by the vice chancellor for research at Tehran University of Medical Sciences.

Leishmania parasites and antigens from promastigotes of *L.major* (WHO strain) were kindly provided by Pasteur Institute of Iran and were grown in NNN medium (14 gr Bactopeptone, 6 gr Nacl, Rabbit blood 300 ml and up to 1200 ml H_2O) supplemented with Hemin, Homa (Triptose 5 gr, phosphate disodic 3 gr, Nacl 4 gr, Liver extract 3 gr, yeast extract 3 gr, Heart extract 1 gr, Glucose 2 gr, Bacto peptone 2.5 gr and up to 1000 ml H2O), RPMI 1640, normal saline and /or 5-10% and heat inactivated FCS. Harvested parasites were washed three times with normal saline solution (0.9%) or phosphate-buffered saline (PBS). The parasites were counted in a Neubar chamber and then kept at $-80\ ^0C$ until use. After parasite accumulation in one flask, it was diluted to a concentration of 3.3×10^8/ml and separated to five equal batches. First batch of parasites was killed by pasteurization in water bath at $56\ ^0C$ for 30 minutes. Second batch was autoclaved in $121\ ^0C$ and 15 pound pressure for 30 minutes.

Third batch was merthiolated with concentration of 0.0001 for 30 minutes and fourth batch was frozen and thawed three times and the fifth batch was left innate. The five batches were mixed together; centrifuged (separated original suspension) and their sediment were dispensed in sterile vials under a laminar flow hood. The *Leishmania* antigen component of five different antigens was tested for complete parasite killing by cultivation on blood agar plate and injection in the footpads of normal Balb/c and small white mice. Sterility was checked on by inoculation in BBL Trypticase soy and Difco thioglycolate liquid media. Routine tests for toxicity were carried out by the intraperitoneal inoculation of one human /animal dose in groups of mice, two guinea pigs and three rabbits. Daily observation was made for seven days for any clinical abnormality at the Institute of Pasteur of Iran, which also verified sterility as indicated above and certified each lot of vaccine dosage according to 100, 200, 300, 400 and 500 μg/ml proteins.

The content of protein in each dose was estimated by the Lowry method (13). Sugar contentment was determined by the phenol / sulfuric acid method (1).

Endotoxin measurements were done to show possible contamination of antigen preparation with endotoxin (12).

The vaccine was stored at 4 ^0C until use. Just before injection BCG vaccine "SSI" (Mycobacterium bovis, Bacillus Calmette Guerin, BCG Strain, Pasteur Institute of Iran Frozen – dried BCG Vaccine Pasteur France. 1173 P2 secondary seed lot C. batch No. 179. Feb. 1995) was suspended in diluted solution SSI solvent (125 mg Mg SO_4, 125 Mg K_2HPO_4, 1 mg L – aspargine, H_2O, 12.5 mg iron ammonium citrate, 18.4 mg 85% glycerol, 0.5 mg citric acid, H_2O 1 ml for injection / manufacturer's insert) and 0.1 mg BCG (first dose) or 0.01 mg (successive dose) was added to each vial containing promastigotes, All doses were injected intradermally in tails (or footpads) in both genetically resistant laboratory conventional white mice and susceptible Balb/C mice.

The protective response was evaluated by the analysis of delayed type hypersensitivity reaction against *Leishmania* antigen and PPD. Delayed type hypersensitivity (intradermal reaction to leishmanial antigen) was determined 29 days after leishmanin test (Pasteur institute Iran). In the test 0.1 ml of antigen was injected intradermally, in the right front footpad. The footpad thickness was measured using a Mitutoyo apparatus, at 24, 48 and 72 hrs after injection. Each animal was also injected with 0.1 ml sterile saline in the left front footpad as control. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. PPD intradermal test was determined thirty-three days after injection 0.1 ml of PPD in the right front footpad. The footpad thickness was measured as mentioned for PPD test at 24, 48 and 72 hrs after injection. Each animal was also

injected with 0.1 ml sterile saline in the left front footpad for control. At each time, the values of the saline control were subtracted from the reaction due to PPD. PPD and various dosages of leishmania antigen results were compared together. *Leishmania* antigen contains three injection groups: (I) Antigen plus booster dose of *Leishmania* antigen after14 days, (II) *Leishmania* antigen plus BCG, (III) *Leishmania* antigen plus BCG plus booster dose of *Leishmania* antigen after 14 days. And five injection dosages: 100, 200, 300, 400, 500 μg/ml protein. There were five mice in each dosage group. Five mice had no injection, five mice had sterile saline injection, and five mice had BCG injection. Mice included genetically susceptible mice (Balb/c) and resistant mice (W.LS).

Statistical analysis: Means were compared by a standard analysis variance and by one/two and three way, student-Newman-Keuls method (SPSS for windows). Correlation coefficient analysis was determined on a Pearson bivariate, two tailed test of significance.

RESULTS

In the present study, laboratory white and Balb/c mice differed significantly in their susceptibility to type of cocktail dose vaccine with regard to their skin leishmanin test (SLT) results ($p < 0.0001$). The SLT results were significantly and specifically enhanced in animals treated in 48 h, over their saline and PPD controls, in all types of mice and all dosages (100 – 500 μg/ml) in *Leishmania* injection plus booster (group I) (Figure 1). The intradermal response to the cocktail antigen of *L. major* was higher in cocktail vaccine plus BCG (group II) in all dosages (100 – 500 μg/ml) than in saline or BCG or PPD controls ($p < 0.005$) at all tested times (24, 48 h) (Figure 2). No Swelling was achieved 72 h after antigen injection characterizing a delayed type of hypersensitivity. The intradermal response to the cocktail antigen was also high in cocktail vaccine plus BCG plus booster (group III) in all dosages (100 – 500 μg/ml) (Figure 3). BCG was highly correlated to the increase of intradermal reaction, (24 h and 48 h) and revealed the high effect of the vaccine. Control animals treated with saline or no injection or BCG solvent only showed a very low increase- almost zero-over antigen or PPD. Our results showed that cocktail antigen was highly specific, since no BCG solvent or injecting saline treated controls showed any significance. Difference in SLT and PPD results: The SLT increased positively with the resistant mouse: small white laboratory (SWL) ($p < 0.005$), injection group I, II ($p < 0.001$) and injection doses 100, 200, 300 ($p < 0.001$) and negatively correlated to enhancement of genetically susceptible mouse (Balb/c) and type of injection group (III) and injection doses 400, 500 (Figure 1, 2, 3). Taken together, the effect on cellular immune responses, the coctail vaccine induced a significant effect against cutaneous leishmaniasis experimental model of vaccine with *L.major*.
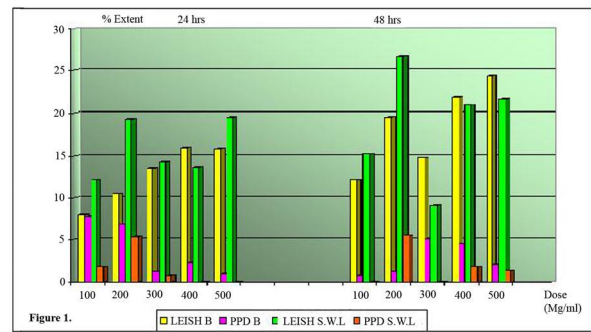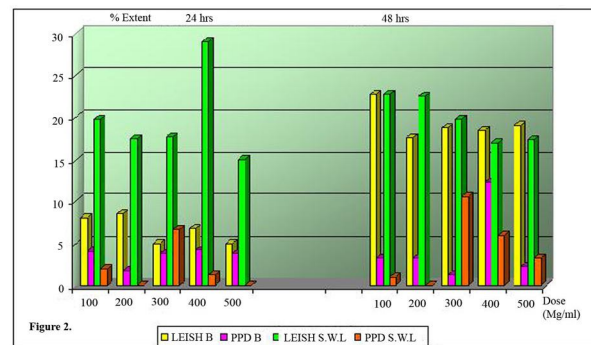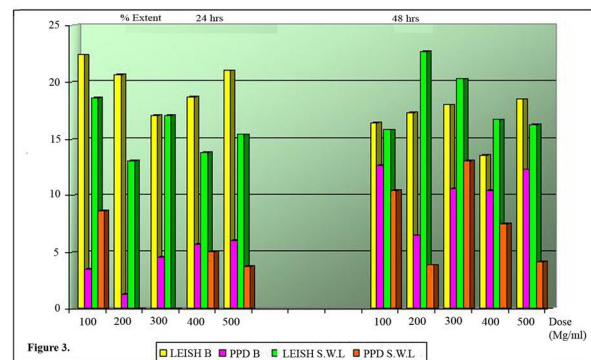

Figure 1. Skin test to Balb/c and small white laboratory (S.W.L) mice **LL group**: *Leishmania* plus same booster dose of Comparition results of leishmanin skin (Lst) and PPD test percentage. For two animal models: susceptible (Balb/c) and resistance (S.W.l) mice for 24 and 48 hrs. Five doses (100-500) Mg/0.1) injected ,the same booster doses injected 14 days after first injection, both skin and PPD tests performed 29 and 33 days after antigen first injection.

Figure 2. Skin test to Balb/c and small white laboratory (S.W.L) mice. **LB group**: *Leishmania* plus BCG. Comparison results of both leishmanin skin test (LST) and PPD percentage in two animal Models: susceptible (Balb/c) and resistance (S.W.L) in 24 and 48 hrs. Five dose (100-500) Mg/0.1ml plus BCG injected Both skin and PPD test performed 29 and 33days after first injection.

Figure 3. Skin test to Balb/c and small white laboratory (S.W.L) mice. **LBL group**: *Leishmania* plus BCG plus booster dose of *Leishmania*. Comparison results of leishmanin skin (Lst) and PPD test percentage For two animal models: susceptible (Balb/c) and resistance (S.W.l) mice for 24 and 48 hrs. Five doses (100-500) Mg/0.1) plus BCG injected ,the same booster doses injected 14 days after first injection, both skin and PPD tests performed 29 and 33 days after antigen first injection.

DISCUSSION

The parasites exist in the digestive tract of sand flies as flagellated promastigotes that (upon entry into humans) transform into amastigotes inside phagolysosomes in macrophages (15). According to the world health organization, (25) leishmaniasis is a serious public health problem in several countries. Hypersensitivity skin tests are also used for diagnosis of leishmaniasis and indicate the presence or absence of a cellular immune response (17, 25). During the past several decades, extensive efforts have been made to search for an effective *Leishmania* vaccine. Vaccine formulations including killed, live attenuated parasites, recombinant *Leishmania* proteins or DNA encoding leishmanial proteins, as well as immunomodulators from sandfly saliva have been examined. A typical inoculum contains around 100–1000 metacyclic promastigotes which quickly become engulfed by leucocytes, particularly macrophages, neutrophils and dendritic cells (18). In addition, to reinforce the effectiveness of the vaccine, BCG was used. There have been attempts to prepare a protective and safe vaccine by different Iranian groups; however, it seems that more research needs to evaluate different preparations of *Leishmania* antigen for vaccination purposes. The clinical effectiveness of leishmaniasis immunization with the killed vaccine was not successful (21).

Over the past few years, several reports of various experimental vaccines against cutaneous leishmaniasis came out with promising but sometimes discordant results. In experimental models protection against *L.major* infection can be induced in genetically susceptible Balb/c mice with lethally irradiated or heat killed promastigotes plus *Corynebacterium parvum* and others. Further evidence of a role for IL-10 comes from studies demonstrating increased IL-10 mRNA expression in bone marrow, lymph nodes and spleen. Nearly 1.2 million people in Iran between 1982 and 1986 received such a live vaccine (16). Approximately 50% of those who received this vaccine developed skin lesions and of those, 93% demonstrated a positive leishman-delayed hypersensitivity skin test, a good field marker of population immunity (18). Recent evidence has also shown that *Leishmania*-HIV co-infections are major health problem in affected areas (16).

Animal models of cutaneous leishmaniasis indicate that Th1 responses are essential for protection by vaccination. This has been usually predicated on the induction of high levels of IFN-γ and low levels of IL-4. However, recent studies indicate that even vaccines triggering high levels of IFN-γ do not protect in the presence of high levels of the regulatory cytokine IL-10 (23). Moreover, IL-4 and IL-10 act together in the presence of exacerbatory antigens (19). Hence, it would appear that IL-10 may be the most appropriate cytokine to serve as an indicator of failure or success of vaccination. Another, essential requirement seems to be IL-12 that is a critical cytokine in the

initiation and maintenance of immunity, as well as a very effective adjuvant (20). To complicate things, the use of the mouse model for disease mimics only some aspects of the human disease. For example, the outcome of infection and the immune response induced is affected by the strain of mouse used, by the site of infection (4) and type of a challenge, i.e. by sand fly bite or injection by syringe.

The present studies were designed to compare under similar conditions. Protective effects were observed in the highly susceptible Balb/c mice, and resistant mice or white Laboratory Small (WLS) mice of cocktail vaccine candidates encoding various *L.major* antigens. The candidate vaccine encode to the following forms: autoclaved, mertiolated, freezed and thawed, pasteurized and innate Leishmanial promastigotes (3). Thirty five days post vaccination, measurement of DTH response against leishmanin and PPD in Balb/c and (WLS) mice injected intradermally. The effects of vaccine on the delayed type hypersensitivity to cocktail vaccine DTH were examined and induced various DTH responses. The DTH increased significantly in the vaccinated animals over the saline and controls in doses 100-500 µg/ml, three group injections and two type mice. The DTH response against cocktail antigen were also significantly higher than controls (P<0.005). It was highly specific, in doses 100-500 µg /ml , three injection dose I ,II ,II and two type mice.

Regard to this results, we guess that differences of DTH results belong to various cytokines amounts. We guess that, our research cytokine patterns is, increase of Th1 cytokines amounts such as IL-12 and IFN-γ, for high positive DTH response, and oppositely,Th2 cytokines likely IL-4, IL10, levels enhancement in low DTH response. In view of the fact that, we have high DTH response for injection doses 100, 200, 300 and injection groups LL and LBL, we do not know actually optimum dose or optimum injection group with high DTH positive only produce TH1 cytokines pattern or both TH2 cytokines pattern that will lead to border line disease. The immune responses needed for protection in humans are not surprisingly less clear than in the mouse models. Bacille Clamette-Guerin (BCG) is the only vaccine in use that relies on the generation of T cell responses, thus can be used as a prototypic vaccine for other diseases. Interestingly, BCG alone led to a positive leishmanin skin test in some individuals, presumably due to antigenic cross-reactivity between *Mycobacteria* and *Leishmania* (18) and leishmaniasis in particular (11). Based on BCG, a *Leishmania* vaccine needs to trigger strong IFN-γ production through Th1 responses as well as activation of CD8+T cells in order to develop and maintain the protective response. Results of our study show that group I or LL, that didn't receive BCG, not only have response to BCG, but also had similar response to LB group, whenever LBL group that received BCG plus booster, have highest response to BCG in all doses (Figures 1, 2, 3). The same results show that skin leishmanin test (SLT) increased highly

for injection group I, II (P<0.001), meaning that booster have similar BCG effect and must have regard to BCG has various side effects. The essential requirement for any vaccine is long-lasting immunity. Recent insights into the generation of immunological memory (3, 27) indicate that central memory T cells are generated during the early stages of infection and persist in the absence of antigen (28). Thus, defining the requirements for the generation and maintenance of central memory T cells is crucial for Leishmania vaccine development if long-lived central memory T cells do not require parasite persistence. In this manner, interesting point for our study is high response for injection doses: 100, 200, 300 (P<0.001) over doses 400, 500 µg/ml. This results lead to, high dose and low dose concept's (24). To Notice this concept, three doses: 100, 200, 300, measure in future that will measure cytokines and challenge in animal model. Preventive vaccines are recognized as the best and most cost-effective protection measure against pathogens, and *Leishmania* is no exception. *Leishmania* vaccine development has proven to be a difficult and challenging task, which is mostly hampered by inadequate knowledge of parasite pathogenesis and the complexity of immune responses needed for protection. Nevertheless, one of the candidate, this antigen has reached Phase I clinical testing following promising results in animal model studies. Then, the issues of delivery systems, antigen formulation and adjuvant would have to be resolved. Currently, there seem to be as many problems and questions as there are solutions, but given the rapid progress in the vaccinology field, a successful anti-*Leishmania* vaccine should be achievable.

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