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Primary Effects of New *Leishmania major* Antigen on Balb/c Mice Spleen

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Abstract

Background: Cutaneous leishmaniasis is a zoonotic disease transmitted between rodents and canine, mainly induced by phelebotomus sand flies. In Iran, the incidence of this protozoan disease has doubled over the last decade. Regard to its high incidence, in the present study the new antigen preparation was evaluated following challenging with live *Leishmania major* promastigts.

Methods: One hundred and twenty male and female Balb/c mice were randomly divided into four groups as LT, LB, LBT and control groups. Group LT received 100-200 µg /0.1 ml of the crude cocktail antigen preparation plus alcoholic extract of *Teucrium polium* as adjuvant, LB received 100-200 µg /0.1 ml of the crude cocktail antigen preparation plus BCG as adjuvant, LBT received 100-200 µg /0.1 ml of this antigen preparation plus alcoholic extract of *Teucrium polium* and BCG as adjuvants and control group. Groups LT, LB and LBT were injected subcutaneously with the antigen preparation and they were boosted using the same doses with two weeks intervals later. A week after the last booster, all animals was challenged with live *Leishmania major* promastigotes. The animals survived, were euthanized, their spleen was removed, and 4-5 microns paraffin sections were prepared and stained with Harris Hematoxylin and Eosine method. The expansion rates of the spleen white pulp size were evaluated using a light microscope with eye-piece graticule.

Results & Conclusion: Statistically, Higher expansion rate of the spleen white pulp size were recorded in the LBT group. The antigen preparation supplemented with adjuvants had positive effects on spleen white pulp expansion following live *Leishmania major* challenging, nevertheless, based on previous experiment, 100-200 µg of LT group could induces satisfactory DTH response which favors *leishmania* organisms eradication, and higher survival rate after challenging with the live *Leishmania major* organisms in spite of induction of lower spleen white pulp size expansion.

Keywords: *Leishmania major* antigen; Challenge; Spleen

Introduction

Leishmania parasites are vector-borne protozoal pathogens found in the tropical and subtropical regions of both the old and New World. These parasites can cause visceral or cutaneous leishmaniasis which is estimated to be responsible for 500,000 and 1,500,000 new human infections, respectively. Untreated visceral leishmaniasis is often fatal, and there is a significant amount of morbidity associated with the cutaneous disease [1]. *Leishmania* parasites live in the macrophages as round, non-motile 3-5 µm amastigotes. The sand fly ingests the infected macrophages during the blood meal and the amastigotes are released into stomach of insect. Almost immediately the amastigotes transform into the motile, 10-20 µm elongated flagelate promastigote form [2]. Upon intradermal /sub-cutaneous injection with *L. major*, resistance animals develop a small lesion that is limited and spontaneously heals within 6-8 weeks. By contrast Balb/c mice are highly susceptible to the infection with these organisms [3]. These mice fail to control the infection and develop extensive lesions that parasites metastases to the internal visceral organs primarily to the liver, then spleen, and at last bone marrow, is an event that may lead to the animal's death. Many different genes spanning the mouse genome have been implicated in the susceptibility/resistance to *Leishmania*. Most of these genes do not map within the MHC systems of either humans or mice, including the SCL-1 gene implicated in the high susceptibility of Balb/c mice to infection with *L. major* [4]. Lymph node cells of Balb/c mice chronically infected with *L. major*, upon stimulation with the Ldp23 antigen produce high levels of IFN-γ and undetectable amounts of IL-4, a typical Th1 response [5]. Sand fly saliva selectively inhibits parasite killing by macrophages and could suppress nitric oxide production [6]. The resistance to *leishmania* conferred by T-helper type-1 (Th1) cells while the susceptibility is conferred by Th2 cells. Th1 cells secret IL12 and IFN-γ where as Th2 cells secret IL-4, IL-5 and IL-10. It has been shown that IFN-γ activates macrophages to express iNOS2, the enzyme catalyzing the formation of nitric oxide; nitric oxide kills the intracellular amastigotes. In contrast, Th2 immune response limits the action of Th1 functions via IL-10 and IL-4, which deactivate macrophages helping intracellular parasite growth and disease progression [7]. Many vaccine strategies have been pursued, including the use of whole cell lysate, killed the avirulent or irradiated parasites. Additionally, DNA vaccines and purified or recombinant parasite antigens have also

been tested. Most of these strategies have shown some degree of effectiveness in animal models but little or no protection in humans. There is now a general consensus among *Leishmania* vaccine researchers that parasite persistence may be important for effective protective response and could be achieved by the live attenuated parasite immunization [8]. Although there is no vaccine against cutaneous leishmaiasis to date, several of the vaccine preparations are at advanced stages of clinical testing. In the previous reports of author upon the present preliminarily studied candidate vaccine on two types of mice (susceptible and resistance), results of the DTH responses and the spleen white pulp size expansion rates were satisfactory. An increase in Th1 cytokines (IL-12, IFN-γ) and a reduction in Th2 cytokines (IL4, IL10) observed in the recent experiments of the author (unpublished) could indicate beneficial effects of the new formulated *Leishmania* antigen. The author for the first time used the alcoholic extract of *Teucrium polium* as an adjuvant to potentiate immune responses to the new preparation of *L. major* antigen on Balb/c mice with the satisfactory results. *T. polium* plant is an herb native to the Mediterranian region and the Middle East. Traditionally the herb is used as a remedy in various disease conditions including bacterial and inflammatory diseases. To continue our assessment, we formulated a new *Leishmania major* cocktail crude antigen as a provisional vaccine and decided to evaluate the effects of this new antigen preparation of *Leishmania* antigen on Balb/c mice following challenging with live *Leishmania* promastigotes using the same methodology as we had done in our previous studies including antigen preparation an standardization [9-12].

Materials and Methods

One hundred and twenty young adult female and male Balb/c mice were obtained from Razi Vaccine and Serum Research Institute and randomly assigned to four standard polycarbonate boxes and they were kept and fed under standard condition and four treatment groups. All groups were fed ad lib with the commercial mice chaw and kept in a well ventilated animal room located in the University of Medical Sciences of Tehran, School of Medicine, Tehran, and Islamic Republic of Iran. The experimental design consisted of three antigen injected groups (LT, LB, LBT) and a control group which received no antigen injection. For detail of the antigen and adjuvant preparations, standardization of the *Leishmania* antigen preparation including formulation, safety and sterility of the preparations, determination of optimum dosages of antigen for Balb/c mice, please visit the authors previous publications [9-12]. The new formulated antigen combined with adjuvant BCG at levels of 2×10^5 CFU live BCG /0.1 ml or 400 µg/0.1 ml alcoholic extract of *Teucrium polium* [13] or an admixture of BCG and *T. polium*. Group LT received 100-200 µg/0.1 ml of the crude cocktail antigen preparation plus alcoholic extract of *Teucrium polium* as adjuvant, GroupLB received 100-200 µg /0.1 ml [14] of the crude cocktail antigen preparation plus BCG as adjuvant, group LBT received 100-200 µg/0.1 ml of the crude cocktail antigen preparation plus alcoholic extract of *Teucrium polium* and BCG as adjuvants. Groups LT, LB and LBT were injected subcutaneously with the antigen preparations at the base of the tail and they were boosted two times using the same doses with a week interval. A week after the last booster, all animals including the control (or normal) group were challenged with 3×10^5/0.1 ml live *L. major* promastigotes. The protective response to the antigen was observed based on cutaneous lesion induction and survival rate during 70 days from the beginning of the challenge. The protective response to the antigen was observed based on cutaneous lesion induction and rate of the mortality. The

animals survived post challenging were euthanized using diethyl-ether, necropsies and spleen was removed and fixed in 10% buffered formaldehyde solution. The fixed spleen tissues were processed in a tissue processor, paraffin blocks were made and 4-5 microns tissue sections were prepared and stained with Harris Hematoxyline and Eosine method. The expansion rates of the spleen white pulp size was evaluated using a light microscope with eye-piece graticule.

Data obtained from the experiment were analyzed using SPSS (SPSS Inc., Chicago, IL, USA). Means were compared by a standard analysis of variance/ simple factorial tests, and by one and two way, student Newman-Keuls methods. Correlation coefficient analysis was determined on a Pearson average two tailed test of significance.

The study was done in compliance with the Helsinki Declaration, and the protocol was approved by research deputy of Tehran University of Medical Sciences, Tehran, Iran.

Groups	Sex	Mean	N	Std.Error of Mean	Minimum	Maximum
LT	Male	5.1185E2	6	49.49877	308.00	657.50
	Female	5.7302E2	3	122.55497	392.70	806.96
	Total	5.3224E2	9	48.74903	308.00	806.96
LB	Male	5.8800E2	1	-	588.00	588.00
	Female	6.2052E2	4	28.44757	565.72	699.20
	Total	6.1401E2	5	22.97494	565.72	699.20
LBT	Female	9.1794E2	6	114.28406	623.40	1386.10
	Total	9.1794E2	6	114.28406	623.40	1386.10
Control	Male	4.6177E2	3	6.36562	451.20	473.20
	Female	6.1445E2	2	58.4500	556.00	672.90
	Total	5.2284E2	5	41.86321	451.20	672.90
Total	Male	5.0444E2	10	31.00569	308.00	657.50
	Female	7.2918E2	15	63.95588	392.70	1386.10
	Total	6.3928E2	25	45.61674	308.00	1386.10

Table 1: The results of analysis variance show a significant differences in white pulp size between male and female Balb/c mice (P<0.001).

Results

The results of the experiment are appeared in the Table 1 and Figure 1. As can be noticed from the Table 1, the differences between the LT, LB, LBT and control group were different statistically (P=0. 001). Compared to control group, the spleen white pulp size increased in the groups LBT and LB but not in LT group. The higher expansion rate of the spleen white pulp size was found in the LBT group that received the crude cocktail antigen preparation plus BCG and alcoholic extract of *T. polium* as adjuvants; statistically the differences were significant. In this regard, the p values for LT, LB, and control groups were 0.02, 0.036 and 0.005 respectively. Among the groups LT, LB and LBT, the lower spleen white pulp size was seen in the LT group which received the crude cocktail antigen preparation supplemented with alcoholic extract of *T. polium* as adjuvant. In this regard, there was a significant difference between LBT and LT groups (P<0. 002)

and the differences were significant as well when LBT was compared to the groups of LB (0. 03) and control (0. 05). It seems that, when BCG and alcoholic extract of *T. polium* are used together, they show a remarkable synergistic effects (Table 1). Comparing to control group, the crude cocktail antigen supplemented with alcoholic extract of *T. polium* had a negligible effect on spleen white pulp size expansion rate. Spleen is the most important organ of the secondary lymphoid tissue which is structurally composed of white pulp (periarterioral lymphoid tissue with primary or secondary lymphoid follicles) and red pulp (sinuses and blood vessels). The white pulp is where the immune response is induced. The white pulp could react to antigen administration via the expansion of lymphoid constituents including B cell and T cell dependant areas, results in antibody production. The SWPS expansion rates differed in the female and male LT, LB and LBT and control groups. The largest white pulp size were seen in the female Balb/c LBT group, and smallest white pulp size were seen in the male Balb/c LT group (Table 1). The data obtain from the experiment indicate that comparing to control group, the higher survival rate was seen in LT group which received the antigen preparation plus the alcoholic extract of *T. polium* followed by live *L. major* challenge. The protection rates 70 days post challenging in the LT, LB, LBT and control groups were 50.0%, 25.0%, 30.0% and 20.0% respectively. Comparing, four groups of LT, LB, LBT and control, the lower survival rate was seen in control group challenged by live *L. major* promastigotes.

Figure 1: The effects of the crude cocktail *L. major* antigen preparation on spleen white pulp size in the groups LT, LB and LBT challenged with the live *L. major* ptomastigotes, compared to control group. Differences between LBT groups and three groups of LT, LB and control groups is significant statistically (P<0.05), but differences between three groups of LT, LB and control group is not significant (p>0.05).

Discussion

Leishmaniasis is a parasitic infection by an obligate, intracellular protozoan of the genus *Leishmania*. Localized, cutaneous leishmaniasisis characterized by one or more ulcers with limited self-healing. The parasite has a developmental stage in mammals, which is mostly intracellular [15]. Using defined vaccination protocols is an interesting unorthodox alternative approach to the discovery of a *Leishmania* vaccine. Paradoxically with respect to the conventional Th1/Th2 paradigm, many investigators have observed that several leishmanial antigens against which a Th1 response is developed during the infection are not necessarily protective antigens [4,5]. Recent experiments using the virulent mutant phosphoglycan-deficient *L. major* lpg-2 demonstrated that infection of mice with this parasite results in minor disease and long survival of the mutant in the animals' tissue. The protection induced by lpg2 parasites is basically not associated with enhanced INF-γ production in response to leishmanial antigens but clearly with a dramatic suppression of IL-4 and IL-10 response to the same antigens [3,4]. Killed vaccines have shown limited immunogenicity and efficacy, even when combined with adjuvant 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* organism. *Leishmania* parasites posses virulence mechanisms that enable the amastigote stage to survive in the hostile environment of the phagolyzosome [16]. Efforts are being made to improve safety of leishmanization by inclusion of drug-sensitive *Leishmania* mutants containing suicide genes for controlled infection [17,18]. The current human vaccines which reviewed related to some experimental studies based on murine models with emphasis on *Leishmania major* infection relevant to vaccine development. In a human study in Venezuela, a clinical healing rate of more than 95% was achieved following vaccine administration, and cure was associated with a Th1–like immune response invaccinated patients [19,20]. Another modified form of vaccine using pasteurized *L. braziliensis* promastigotes and live BCG was effective in the treatment of both refractory mucocutaneous Leishmaniasis and early cases of diffuse cutaneous Leishmaniasis [21]. To date, successful vaccination strategies against Leishmaniasis have been limited to cutaneous Leishmaniasis, with small doses of living virulent *L. major* promastigotes at a selected site, however the results of the vaccination has not been satisfactory [20]. The clinical effectiveness of immunization with the killed vaccine is not acceptable at the present [22]. Phase III clinical trials in Ecuador and Colombia showed that the *Leishmania* amazonensis vaccine is safe, but not effective [23,24]. One study's results show that NKT cells should be considered both when treating active *Leishmania* infection as well as in the development of vaccines. The reported effects of *L.major*-activated NKT cells observed in various models of *Leishmania* infection have been variable and often conflicting which, most of this is probably due to both different infection models and *Leishmania* strains applied [24]. Recent progress in understanding how the innate immune system recognizes microbial stimuli and regulates adaptive immunity is being applied to vaccine discovery in what is termed "systems vaccinology" [25]. Systems vaccinology is an offshoot of systems biology for which tools of a number of high- throughput technologies including DNA microarrays, RNA-seq., protein arrays, deep sequencing, mass spectrometry along with sophisticated computational tools have been originally developed [26]. The role of IL-10 in promoting pathology of VL has long been demonstrated in human studies. It must be noted that in several of the heat killed *Leishmania* vaccines, BCG was a common adjuvant and the immune reaction caused by BCG compounded the LST-based interpretation significantly. A meta- analysis further confirmed that LST conversion maybe associated with an immune response that can provide some protection by its ability to distinguish as an population of responders to leishmanial antigen or BCG after vaccination even though such response had a huge variability (16-68% conversion rate) in these studies [27]. Regarding to above researches and based on the previous
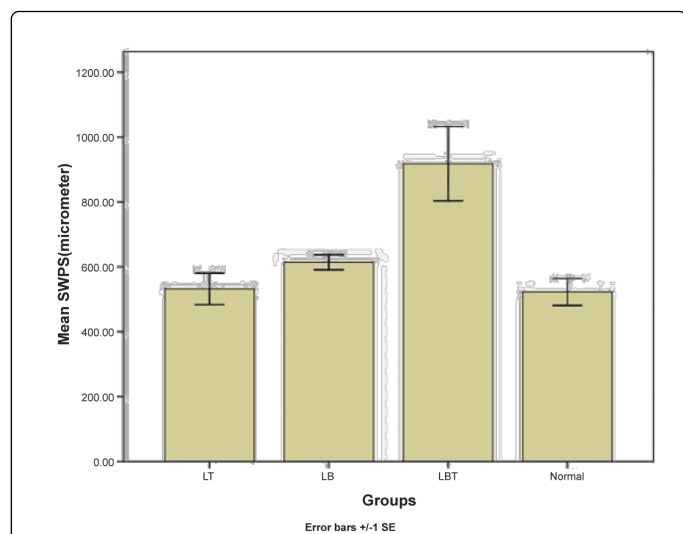
studies of the author, the new formulated crude cocktail preparation of *L. major* antigen supplemented by BCG alone or BCG plus new recommended alcoholic extract of plant *Teucrium polium* as an adjuvant showed a satisfactory immune response which could help animal to contain *leishmania* infection. In the aforementioned experiments conducted by the author, safety, toxicities and immunity (DTH) of the new preparation and protective results accompanied by cytokine assessment after challenging were evaluated in the murine models [10-12].

Higher white pulp size in the LBT group indicates induction of humoral immunity which it could not protect the animals against *Leishmania* infection or progression. In addition this antigen preparation also could induce cell mediated immunity which it seems to be a protective response as shown in previous studies. Therefore, supplementation of crude cocktail antigen at levels of $2x10^5$ CFU live BCG /0.1 ml and 400 µg/01 ml of alcoholic extract of *Teucrium polium* simultaneously could induce cell mediated and humoral immune responses at 100-200 µg of the antigen in the Balb/c mice model. However as far as LT and LBT groups were concerned, in spite of lower expansion rates of spleen white pulp size which indicates lower non protective humoral immunity, they were shown a satisfactory DTH response.

In the authors previous experiments [9-12] the new formulation of crude cocktail of *Leishmania major* antigen fortified by alcoholic extract of plant *Teucrium polium* could be recommended as a candidate vaccine, since compared to the LBT an LB groups, the antigen preparation caused lower mortality and a satisfactory DTH response which could be considered as a protective responses to *Leismania major* infection. With regard to antigen preparation, a mixture of antigens prepared by using different methods including freezing and thawing, autoclaving, heat inactivation, chemical inactivation and combination of chemical inactivation and freezing and thawing method, offer very many *leishmania* epitopes. It could be expected that some of these epitopes could induce such an immune response which capable to protect the vaccinated subjects. In the other hand, as shown previously, it seems that the pure alcoholic extract of *Teucrium polium* could be a safe adjuvant as shown in the Balb/c mice. However, since the extract is used parentally, further investigation is required to elucidate the safety of the adjuvant. In contrast, the crude cocktail antigen preparation plus alcoholic extract of *Teucrium polium* and BCG or plus BCG alone is not recommended for the provisional vaccine in spite of their potency in both cell mediated or humoral immunity induction since the live BCG could produce mycobacterial infection particularly in the immune compromised or immunodefficient subjects. As seen in the Table 1, the gender of the animals affects the spleen white pulp size expansion rates against the antigen preparation, in this regard, the differences between two groups of female and male Balb/c mice were statistically significant. In fact, it could be attributed partly to different types of hormones produced by the female and male animals including estrogenic/progestronic versus androgenic hormones. At the present time, there are unsolved problems and many questions must be answered, nevertheless the rapid progress in the field of vaccine, vaccination and development of safe and effective vaccines against intracellular infectious agents including lieshmania organisms is promising. We expect that the new antigen formula would show the following characteristics; firstly, could induce a reasonable immune response which activate macrophages therefore it enable macrophages to inhibit propagation or destroy intracellular amastigotes at the early stages of infection, secondly, could inhibit transformation of promastigotes organisms to intracellular forms or amstigotes at the site of the parasite entrance, thirdly, to induce a safe and potent immune response which could help clinically infected individuals to eradicate intracellular form of *leishmania* organisms, fourthly, from our point of view the side effect of the antigen preparation must be negligible or tolerable when it is administrate, since induction of high levels of cytokines including inflammatory cytokines or activation of a large proportion of lymphocytes will have a grave consequences and finally mass production of antigen preparation as a vaccine must be feasible commercially. On the other hand, this preparation is safer than those supplemented with the live BCG as adjuvant since they could produce mycobacterial infection particularly in the immune deficient individuals.

Conclusion

The results of the present experiment and previous findings were obtained by the author indicate that the crude cocktail antigen preparation at doses of 100-200 µg/0.1ml which had alcoholic extract of *Teucrium polium* as adjuant could induce an acceptable and satisfactory immune response in the Balb/c mice. To evaluate the preparation as a provisional vaccine against *Leishmania major* infection, the author recommends further investigations conducted in other susceptible species including canine species.

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