



Original Article

Evaluation of the Effects of a New Formulation of Leishmania Major Antigen in Balb/C and Conventional White Laboratory Mice

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BACKGROUND/PURPOSE: Leishmaniasis is currently a threat in 80 countries around the world, and cutaneous Leishmaniasis accounts for more than 5% of new cases. It is a problematic disease in Iran, and preparing a protective vaccine has been a major goal of medical investigations. The objective of this study was to compare the protective effects of a cocktail vaccine candidate encoding various Leishmania major antigens in highly susceptible (Balb/C, or type I mice) and resistant (laboratory small white, or type II) mice.

METHODS: A new antigen formulation was evaluated in type I and II mice. Leishmania major promastigotes was cultured and harvested at different growth stages, and a cocktail made from the harvested organisms. The preparations were tested for sterility and contamination by endotoxin. Five different methods were utilized to produce a crude antigen preparation. The protein levels of the antigen preparations were measured using the Lowry method, and the antigens were intradermally injected using different protocols for type I and II mice. After 38 days, all mice were euthanized with diethyl ether, and spleens were removed. Histological sections were prepared, stained with hematoxylin and eosin and changes to the splenic white pulp (SWP) were studied microscopically.

RESULTS: Compared with the control groups, there was a drastic change in white pulp structure. The size increases of the SWP were dependent on the injection group and mouse strain. There was a remarkable expansion of lymphoid follicles in the treated groups in both mice strains.

CONCLUSION: The new antigen formulation was able to stimulate and expand the lymphoid constituents of spleen tissue. The SWP is where immune responses and antibodies are produced. Therefore, the

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effect of the antigen preparations on secondary immune responses, adaptive immunity, and antibody production is important in determining the susceptibility of mice to cutaneous Leishmaniasis and the induction of immunity.

KEYWORDS: antigen, cutaneous Leishmaniasis

Introduction

Leishmaniasis is one of the major illnesses in tropical and subtropical areas, including countries of South and Central America, Africa and Asia. The disease is endemic in Iran and appears as both cutaneous and visceral forms. To prevent cutaneous Leishmaniasis, different antigen preparations have been used as vaccines. Merthiolated killed antigens from five different strains of *Leishmania* had been used as vaccines.¹⁻³ The vaccine comprised a mixture of equal amounts of freeze-thawed intact parasites,^{4,5} and irradiated and heated antigen^{2,5} injected intradermally. An alternative strategy using attenuated organisms allows the development of an immune response similar to that of the natural infection, with exposure to a much larger range of antigens than can be achieved by using more refined subunit vaccines.⁶

A live *Leishmania* vaccine was prepared in Venezuela to vaccinate subjects against *Leishmania mexicana* and *Leishmania braziliensis*.⁶ Evidence for a role for interleukin (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 1982–1986 received such a live vaccine.⁷ Approximately 50% of those who received this vaccine developed skin lesions and of those, 93% demonstrated a positive leishmanin-delayed hypersensitivity skin test, a good field marker of population immunity.⁶ To potentiate the effectiveness of the vaccine, Bacillus Calmette-Guérin (BCG) was also used. There have been attempts to prepare a protective and safe vaccine by several Iranian groups; however, it seems that more research are required to evaluate different preparations of *Leishmania* antigen for vaccination purposes. Clinically, the killed vaccine has not been successful.⁸ However, little success has been made in the treatment of human, or experimental murine, Leishmaniasis using naturally virulent organisms, irradiated organisms, or

genetically manipulated organisms. Similarly, 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*.⁶

Recent evidence has also shown that *Leishmania*-human immunodeficiency virus co-infections are a major health problem in affected areas.⁷ The disease is usually self-limiting, but the time to lesion resolution varies between species and between individuals. The pathology of *Leishmania* infection is determined not only by the parasite species, but also by host genetics and immune factors. Most of the experimental immunological data comes from mouse models, and less is known about the immunology of human Leishmaniasis. Although mouse models have been used for the study of both cutaneous and visceral Leishmaniasis, they more closely reflect the situation in human cutaneous Leishmaniasis than visceral disease. Leishmaniasis in general, particularly cutaneous Leishmaniasis, is probably one of a few parasitic diseases that is most likely to be controlled by vaccines.

During the past several decades, extensive efforts have been made to search for an effective *Leishmania* vaccine. Vaccine formulations incorporating killed, live attenuated parasites, recombinant *Leishmania* proteins, or DNA encoding *Leishmania* proteins, as well as immunomodulators from sandfly saliva have been examined. A typical inoculum contains around 100–1,000 metacyclic promastigotes, which quickly become engulfed by leucocytes, particularly macrophages, neutrophils, and dendritic cells.⁶

Although there is no vaccine against *Leishmania* to date, several of the vaccine preparations are at advanced stages of clinical testing. In the present report, the kinetics of the splenic white pulp (SWP) expansion in two types of mice were studied to compare the effects of the different *Leishmania* cocktail vaccines, and injection doses

(100–500 µg/mL of protein) with placebo groups. The aim of this study was to compare the protective effects of a candidate cocktail vaccine encoding various *Leishmania* major antigens in highly susceptible (Balb/C or type I) and resistant (laboratory small white, or type II) mice.

Methods

This 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.

Animal model

For detail procedure please refer to Latifynia and Mohaghegh Hazrati, 2008.⁹ In brief, Balb/C and traditional white laboratory mice ($n = 192$) were obtained at three months old from the Razi institute.

Culture and isolation of Leishmania parasites

Leishmania parasites and promastigote antigens from the *L. major* WHO strain were kindly provided by the Pasteur Institute, and grown in NNN (Novy-MacNeal-Nicol) medium supplemented with Hemin, Homa, RPMI 1640, normal saline, and/or 5–10% heat inactivated fetal calf serum.

Harvested parasites were washed three times with either 0.9% normal saline or phosphate buffered saline. The parasites were counted using a Neubauer chamber and then kept at -80°C until use. Accumulative parasites were then diluted to a concentration of $330 \times 10^6/\text{mL}$ and separated to five equal batches. The first batch was killed by pasteurization in water at 56°C for 30 minutes. The second batch was autoclaved at 121°C for 30 minutes. The third batch was merthiolated for 30 minutes at a dilution of 1:10,000. The fourth batch was freeze-thawed three times and the fifth batch was left innate.

Vaccine preparation

The five batches were mixed and centrifuged, and the sediments were dispensed into sterile vials. The *Leishmania* component of the vaccines was tested for complete parasite killing by cultivation on blood agar plates, and by injection into the footpads of type I and II mice. Sterility and routine tests for toxicity were described previously.⁹ These tests were carried out at the Institute of Pasteur, which

also verified the sterility of the preparations and certified each lot of vaccine as containing dosages of 1,000–5,000 µg/mL protein. The sugar contentment was determined by the phenol/sulfuric acid method.¹⁰ Endotoxin measurements were performed to determine any possible contamination of the antigen preparations with endotoxin method.¹¹ The vaccine was stored at 4°C until use.

Vaccine injection

Detailed procedures were described previously.⁹ In brief, just before injection, the BCG vaccine “SSI” was suspended in Sauton SSI diluent and 0.1 mg of BCG (first dose) or 0.01 mg of BCG (successive dose) was added to each vial containing promastigotes. All doses were injected intradermally into the tails (or foot pads) of type II mice.

Three injection groups [(1) *Leishmania* antigen plus booster dose of *Leishmania* antigen; (2) *Leishmania* antigen plus BCG; and (3) *Leishmania* antigen plus BCG plus booster dose of *Leishmania* antigen], and five injection dosages (100, 200, 300, 400, or 500 µg protein) were used. Each dosage group contained five mice. Five mice received no injection, and five mice were injected with sterile saline alone. Among the mice were genetically susceptible and resistant mice.

The protective response was evaluated by analysis of secondary immune system responses against *Leishmania* antigens 38 days after antigen injection. The mice were euthanized, and their spleens removed. The spleens were preserved in 10% formalin solution, and pathologic sections were prepared.

Hematoxylin and eosin staining

Fixed spleens in paraffin blocks were processed and sectioned in a tissue processor. Tissue sections (5–6 µm thick) were prepared and stained with Hematoxylin and Eosin. SWP section diameters were measured and compared with each other, and with the controls.

Statistical analysis

Data were analyzed using SPSS (SPSS Inc., Chicago, IL, USA). Means were compared by standard analysis of variance/simple factorial tests, and by one-, two- and three-way Student–Newman–Keuls methods. Correlation coefficient analysis was determined using a Pearson bivariate, two tailed test.

Results

To evaluate of the kinetics of the SWP expansion in the two types of mice, and to compare the efficiency of the cocktail vaccine candidates, we first identified the optimal size of the SWP after inoculation of the cocktail vaccine, and compared the results with those obtained after injections of saline, the BCG placebo, and no injection at all.

Analysis of variance results were based on the amount of SWP increase in the different injection groups/mouse types. The effects of the dosage of the cocktail vaccine (100–500 µg) were compared within, and between all groups (1, 2, and 3).

The peak increase in SWP size was seen after injection of 300, 400 and 500 µg vaccine (Figure 1). In contrast, lower injection doses (100–200 µg) resulted in little or no increase above that seen in normal controls ($p < 0.005$). A confidence interval (CI) of more than 0.95 was considered significant. The SWP of the mice in all the test groups (1, 2, and 3) showed an increase in size, and was notably different from the controls. This difference was independent of the injection group and dose. The smallest expansion was seen in group 1 (antigen+booster), and the largest in group 3 (antigen+BCG+booster), with a 100% difference between these two groups.

The minimum size increases were seen in type II mice, the maximum in type I ($p < 0.001$; $CI > 0.99\%$). A vaccine dose of 400 µg was associated with the largest increases, and 200 µg with the smallest ($p < 0.001$; $CI > 0.99\%$).

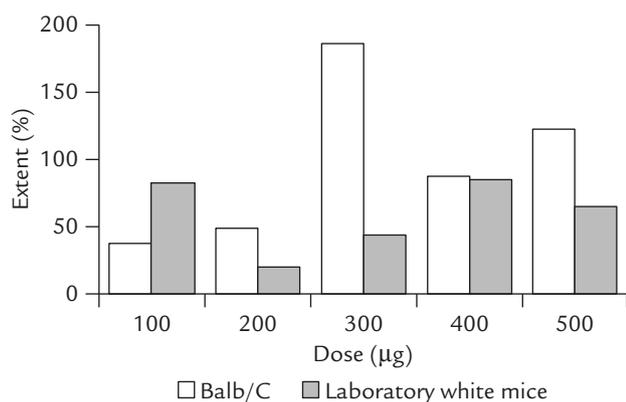


Figure 1. Sectional spleen pathology showed white pulp size expansion 38 days after *Leishmania* antigen intradermal injection plus a booster dose after 14 days.

Measurements showed that the lowest white pulp size was seen in type II mice in group 1 (100 µg antigen). The same result was found type I mice in group 1 (200 µg antigen; Table 1). However, when the mouse type was not considered, the lowest overall increase in white pulp size was seen in group 1 (200 µg antigen). When both the group and mouse type were not considered, the largest white pulp sizes were seen in animals injected with either 400 or 500 µg of antigen. The analysis of variance results show a significant difference in white pulp size between two mouse types ($p < 0.005$; Table 2).

The relationship between the mouse type and injection group, and vice versa, was significant ($p < 0.001$; $CI > 99\%$) and all groups showed significant differences between type I and type II mice (Table 2). When considering the mouse type against the different injection groups, the injected dose of antigen against mouse types, and the different injection groups against the injected dose of antigen, significant differences were seen. The increase in SWP size was notably different within each group, irrespective of injection group and dose.

Discussion

Leishmaniasis is a parasitic infection caused by an obligate, intracellular protozoan of the genus *Leishmania* (family Trypanosomatidae).⁶ Localized, cutaneous Leishmaniasis is characterized by one or more ulcers with limited self-healing. Patients develop antigen-specific cell mediated immunity to the microorganism. The parasite has a digenetic life cycle, with an extracellular developmental stage in the insect vector (a female phlebotomine sandfly), and a developmental stage in mammals, which is mostly intracellular.⁶

An ideal anti-leishmanial vaccine would need to possess several attributes; however, not all of them are easily achievable. These include (1) safety; (2) affordability to the populations in need; (3) induction of CD4⁺ and CD8⁺ T cell responses, and long-term immunological memory that can be boosted by natural infections thus minimizing the number of immunizations required; (4) effectiveness against species causing cutaneous leishmaniasis and visceral leishmaniasis; (5) stability at room temperature, eliminating the need for a cold chain to preserve potency; and (6) effectiveness as a prophylactic as well as a therapeutic

Table 1. Distribution of splenic white pulp size on the basis of mouse type, injection group, and received dose^a

Group	Dose (µg)	Balb/C		White laboratory		Total	
		n	SWP (µm)	n	SWP (µm)	n	SWP (µm)
Leishmania antigen only	0	15	11.00±0.00	15	11.90±0.00	30	11.45±0.46
Leishmania antigen + booster	100	3	10.83±0.29	2	41.50±0.00	5	23.10±16.80
	200	4	26.63±4.39	3	20.8±4.62	7	24.14±5.13
	300	2	59.75±3.18	4	28.75±4.27	6	39.08±16.41
	400	5	31.80±9.52	2	42.00±3.54	7	34.71±9.34
	500	8	41.63±11.96	9	34.72±3.41	17	37.97±9.00
	Subtotal	22	34.11±15.54	20	32.85±7.44	42	33.51±12.24
Leishmania antigen + BCG	100	4	34.50±8.29	3	36.00±12.97	7	35.14±9.54
	200	5	23.70±3.96	7	27.71±13.88	12	26.04±10.73
	300	4	18.00±3.11	3	41.50±0.87	7	28.07±12.76
	400	4	49.75±9.49	4	45.63±7.33	8	47.69±8.15
	500	3	34.17±12.66	5	21.60±11.41	8	26.31±12.75
	Subtotal	20	31.50±13.20	22	32.59±13.63	42	32.07±13.27
Leishmania antigen + booster + BCG	100	5	15.50±0.00	3	52.83±20.82	8	29.50±22.30
	200	5	42.00±15.57	4	45.50±4.08	9	43.56±11.44
	300	2	19.00±3.54	3	39.00±2.60	5	31.00±11.25
	400	5	28.80±4.38	4	37.50±5.77	9	32.67±6.57
	500	4	41.13±7.39	5	39.70±18.92	9	40.33±14.14
	Subtotal	21	30.19±13.67	19	42.42±12.90	40	36.00±14.52
Total	0	15	11.00±0.00	15	11.90±0.00	30	11.45±0.46
	100	12	20.67±11.26	8	43.69±15.31	20	29.88±17.14
	200	14	31.07±12.52	14	31.32±13.79	28	31.20±12.93
	300	8	28.69±19.37	10	35.65±6.64	18	32.56±13.80
	400	14	35.86±11.81	10	41.65±6.72	24	38.27±10.25
	500	15	40.00±10.74	19	32.58±12.79	34	35.85±12.33
	Subtotal	78	27.94±15.13	76	31.03±14.61	154	29.47±14.91

^aData presented as mean ± standard deviation. SWP = spleen white pulp.

Table 2. Test of between-subjects effects dependent variable white pulp size distribution

Source	Type III Sum of Squares	df	Mean square	F	p
M. TYPE	674.38	1	674.38	9.81	0.002
IN. G	167.33	2	83.66	1.22	0.300
DOSE	957.87	4	239.47	3.49	0.010
M.TYPE*IN.G	993.45	2	496.72	7.23	0.001
M.TYPE*DOSE	2,661.41	4	665.35	9.68	0.000
IN.G *DOSE	4,578.82	8	572.35	8.33	0.000
M.TYPE*IN.G*DOSE	3,523.77	8	440.47	6.41	0.000
Error	8,384.11	122	68.72		
Total	167,737.40	154			

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 is achieved by using more refined subunit vaccines. However, despite pursuing such a strategy for human, or experimental murine Leishmaniasis using naturally a virulent organisms, or irradiated or genetically manipulated organisms, there has been little success. Similarly, 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*. *Leishmania* parasites possess a variety of virulence mechanisms that enable the amastigote stage to survive in the hostile environment of the phagolysosome.⁶

A different type of problem has been the loss of virulence after repeated *in vitro* passage of the parasites, which makes the standardization of the vaccine difficult. Efforts are being made to improve safety of Leishmanization by the inclusion of drug-sensitive *Leishmania* mutants containing suicide genes for controlled infection,^{12,13} the inclusion of killed parasites to reduce the size and duration of lesions, or by using adjuvant that promote more rapid onset of anti-Leishmanial immunity and swift healing of lesions.^{14,15} Killed parasite vaccines have been proposed as both prophylactic and therapeutic vaccines. The need for vaccine(s) against cutaneous Leishmaniasis, and the population at risk for whom such vaccines should be developed, are briefly discussed.

The current human vaccines are reviewed, as are some experimental mouse studies with emphasis on *Leishmania major* infection relevant to vaccine development. In this regard in Venezuela, a clinical healing rate of more than 95% was achieved, and cure was associated with a Th1-like immune response in the patients.^{16,17} A 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.¹⁸

Based on the information from mouse models and human studies, the benign nature of the cutaneous disease, the ease with which *L. major* can be manipulated to yield the required material, and the ongoing practice of Leishmanization which allows rapid evaluation of

candidate vaccine(s), little can be done to control the reservoirs or vectors. The observation that vaccinated individuals develop a Th1-mediated immune response, without being protected against infection, is in agreement with several similar observations in mice and vervet monkeys,^{19,20} and suggests that the induction of a Th1 immune response may be necessary, but not sufficient, for protection against cutaneous Leishmaniasis. However, a vaccine consisting of autoclaved *L. major* and BCG was used, shown to be safe and, significantly, it induced Interferon-gamma (IFN- γ) production in healthy volunteers.²¹ Care should be taken to avoid establishing settlements at the edge of, or within, involved forests. Insect repellents can provide limited protection for travelers.^{3,4} WHO have designated Leishmaniasis as a category 1 (emerging and uncontrolled) disease, with prevention focused on vector control, the control of animal reservoirs, and research into potential vaccines.⁶

In Brazilian studies, a killed promastigote vaccine derived from five *Leishmania* strains reduced the annual incidence of cutaneous Leishmaniasis in military recruits by 86%.^{3,4} When used as a recombinant protein expressed in *Escherichia coli* gp63 either failed to protect mice against *L. major* infection, or offered only partial protection in monkeys.^{22,23} In contrast, immunization with the native protein purified from *L. major* led to the protection of mice against challenge with either *L. mexicana* or *L. major*.^{24,25} These promising findings were overshadowed by variable (mostly negative) T cell responses in humans²⁶⁻²⁹ and the inability of T cells from mice immunized with gp63 to elicit protective responses in macrophages.²⁵ Nonetheless, researchers have focused their efforts on gp63 DNA-based vaccines (described below), and gp63-derived synthetic peptides. The latter were successfully tested in an animal model of cutaneous Leishmaniasis, and triggered long-lasting T cells responses.^{30,31}

To date, successful vaccination strategies against Leishmaniasis have been limited to cutaneous Leishmaniasis, with small doses of living virulent *L. major* promastigote at a selected site.¹⁷ The clinical effectiveness of Leishmaniasis immunization with the killed vaccine is not high at present.³² Phase III clinical trials in Ecuador and Colombia show that the *Leishmania amazonensis* vaccine is safe, but not effective.^{33,34}

Spleen is the most important organ of the secondary lymphatic system and comprises white pulp (lymphocytes)

and red pulp (sinuses and blood vessels). The white pulp is where immune responses are induced, and antibodies are produced; important for secondary immune responses and adaptive immunity. The main concerns are reliable correlates of immunity that need to be developed to evaluate vaccines, as well as a need for a uniform testing system for new vaccine candidates. Only later can the issues of delivery, antigen formulation and adjuvant be resolved. There is a pressing need to develop better animal models of visceral Leishmaniasis that can help the design of vaccines to control both canine and human disease in endemic areas. Currently, there seem to be as many problems and questions as there are solutions, but given the rapid progress in the field of vaccinology, a successful anti-Leishmania vaccine should be achievable.

In this study, the results from each treatment group (1, 2, and 3) were evaluated statistically. The differences between the various injection doses used, the two types of mice used, and all three parameters (mouse type, injection dose, and injection group) were evaluated (Figures 1–3). Further studies are planned to investigate whether the mice in groups 1 and 3 produce IFN- γ or IL-4. We will also determine whether long term immunological memory is induced in the mice in groups 1 and 2. A limitation of this study is that we did not measure IFN- γ as a response indicator.

Comparison of the dose and injection groups shows that there were significant differences in SWP size (the minimum difference was seen at a dose 200 μ g in group 1, the maximum at a dose 400 μ g in group 2). Therefore, the optimal dose(s) to stimulate an immune response, IFN- γ

production and long term immunological memory requires further investigation.

The question to ponder here is whether BCG is of benefit for the production of a delayed-type hypersensitivity response or not. Our results show that type I mice that are susceptible to cutaneous Leishmaniasis exhibited the greatest SWP expansion, but the effects of BCG on this expansion requires further study.

The vaccine dose, mouse type, and injection groups all affected each another. Each of the three factors had a notable affect on SWP size (minimum increase with a dose of 200 μ g in group 1, and maximum increase with a dose of 400 μ g in group 2, and 500 μ g in group 3).

Further evidence of a role for IL-10 comes from studies demonstrating increased IL-10 mRNA expression in bone marrow, lymph nodes and spleen.⁶ We hypothesize that the greatest increase in SWP expansion may be related to IL-4 and IL-10 production, resulting in humoral immune responses.⁶ Both IL-4 and IL-10 are associated with systemic disease without long term immunity to cutaneous Leishmaniasis, as suggested by our results in type I mice (susceptible) in injection group 3 at doses of 400–500 μ g. A cure was associated with a fall in IL-10 mRNA levels.⁶ SWP expansion is related to the production of cell mediated immune responses, IFN- γ and (most likely) IL-4 suppression, that may lead to long term immunity to cutaneous Leishmaniasis, similar to our results in type II mice (resistant), in injection group 1 at doses of 100–200 μ g.

The results of this study suggest that the optimal injection dose(s) and injection protocol must be selected to

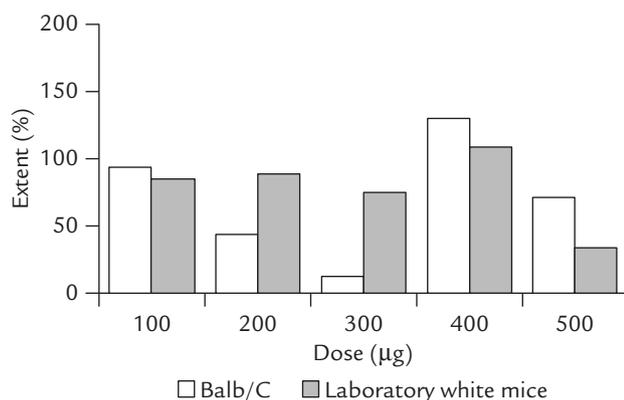


Figure 2. Sectional spleen pathology showed white pulp size expansion 38 days after Leishmania antigen intradermal injection plus a Bacillus Calmette-Guérin vaccine.

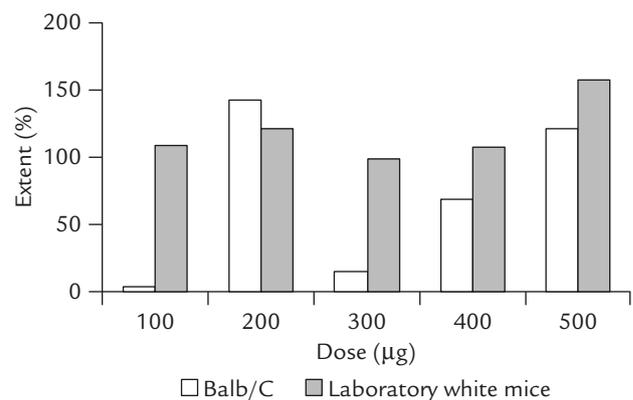


Figure 3. Sectional spleen pathology showed white pulp size expansion 38 days after Leishmania antigen intradermal injection plus a Bacillus Calmette-Guérin vaccine plus booster dose after 14 days.

stimulate cell-mediated immune responses, while inhibiting the humeral immune system and preventing antibody production. These points must be addressed for progress to be made in the search for an effective vaccine against cutaneous Leishmaniasis.

In conclusion, the greatest increases in SWP size were seen at doses of 400–500 µg, with the greatest effect observed in injection group 3. Injection group 1 showed a high response to the primary booster dose, which induced variable increases in SWP size, but BCG was not very effective. Therefore, the optimal injection dose, the type of experimental model, and the use of BCG as an adjuvant need to be determined in future studies.

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