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Study on immunity of *Leishmania major* new crude antigen as a vaccine against leishmaniasis in out bred (resistant) and Balb/c (sensitive) mice

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ABSTRACT

The aim of this study was to prepare a new formula of *Leishmania major (L. major)* crude antigen and evaluate its effect on immune system. For this purpose *L. major* promastigotes were cultured, harvested, washed, and resuspended in physiologic saline and the suspensions were dispersed in five equal batches. 0.1 ml of various doses of the cocktail antigen were injected intradermaly in three groups of mice [Ninety out bred resistant mice (designated type 1 mice) and 90 Balb/c sensitive mice (designated type 2 mice) of both sexes with age of three months]; group I received antigen and a booster dose of the same antigen, group II received *Leishmania* antigen containing Bacillus Calmette and Guerrin (BCG), group III inoculated with antigen containing BCG and a booster dose of the same antigen, group IV remained intact, groups V and VI received solely BCG, and BCG solvent, respectively without any antigen. Delayed Type Hypersensitivity (DTH) response and spleen white pulp follicles (SWPF) status were evaluated. No significant differences among groups IV, V and VI were seen in two types of mice regarding PPD skin test, in leishmanin skin tests and spleen white pulp statue. However there was a significant difference among three groups of two mice types received the antigens in a dose dependent manner ($P<0.05$). The results showed that, the new formulated crude *L. major* antigen induced reasonable DTH immune responses in both types of mice in a dose dependent manner. It is concluded that the group I that received 100 µg/0.1 ml and 200 µg/0.1 ml antigen had high DTH for SLT and low SWPF increasing ,while low DTH and high SWPF were seen in the groups II and III that received 400 µg/0.1ml and 500 µg/0.1ml antigen ($P<0.005$).

Keywords: *Leishmania major*, antigen, vaccine

INTRODUCTION

Cutaneous leishmaniasis is generally a self-limiting disease followed by lifelong immunity. The leishmanial parasite exists in two forms: promastigote or flagellate form found in the sandfly vector, and amastigote or aflagellate form which is the parasite of the mammalian host macrophage. Human leishmaniasis is a disease caused by several species of the protozoan parasite of the genus *Leishmania*. In human hosts the clinical spectrum induced by different *Leishmania* species can range from a single cutaneous lesion which may undergo

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spontaneous cure, to mucocutaneous lesions which can cause grossly disfiguring lesions. Severe diffuse cutaneous lesions can also occur as hard to treat disorders. Moreover, the disease can evolve to visceralized forms that could be lethal in the majority of the cases if remaining un-treated. Many procedures have been applied to achieve an applicable *Leishmania* immunization until this time (Khamesipour *et al* 2005, Nadim & Javadian 1998, Greenblatt 1980, Greenblatt 1988, Green *et al* 1983, Covit *et al* 2004 and Covit *et al* 1987). Leishmanization is the first approach consisted of vaccination against leishmaniasis with live *Leishmania*. Different experimental human vaccination through controlled infection with virulent parasites was practiced in Russia, Iran and other countries, which were discontinued due to undesirable complications (Greenblatt 1980., Greenblatt 1988 and Nadim & Javadian 1998). In a field trial carried out by Mayrink *et al* in the eastern part of Brazil in 1979, a vaccine containing killed promastigotes of five stocks of *Leishmania* resulted in a relatively satisfactory outcome (Myrink *et al* 1979) and a similar study was done in year 2003 (Ferreira *et al* 2003), *L.major* supplemented with adjuvant *Corynebacterium parvum* was injected intraperitoneally (IP) to mice. Evaluation of immunological protectivity of the antigen against fatal infections showed no protection in the treated animals (Alexander 1982). The LACK *Leishmania* gene (*Leishmania* homolog of receptors for activated C kinase gene) inserted into a eukaryotic expression vector down stream of the CytoMegaloVirus promoter was also used to vaccinate susceptible Balb/c mice (Sacks & Noben-Trath 2002). In three separate studies Scott *et al* (1987), Santos *et al* (2003) and Ahmed *et al* (2004) evaluated immunological properties of sub fractions of *Leishmania infantum* and *L. major* respectively. Ahmed *et al* in 2004 investigated different experimental murine DNA vaccine candidates against leishmanisis due to *L. major*, however no satisfactory results were obtained. Other studies notified altered virulence and vaccination properties of *Leishmania* parasites grown in infected vaccinated mice (Mitchell & Handman 1983, Reiner & Locksley 1995 and Witztum *et al* 1979). In the latest study the greatest protection through challenge with *L. major* was seen in mice immunized with live parasites compared to vaccination with heat killed or soluble antigens. In general, immunized mice produced high level of anti-leishmanial antibodies and T cell stimulation to their respective antigens (Onyalo *et al* 2005). Review of literature indicates no effective vaccine is prepared against *Leishmania* infections till now (Antunes *et al* 1982 , Armijos *et al* 2004, Capron & Dessaint 1988, Convit *et al* 1987, Convit *et al* 2004, Ferreira *et al* 2003.,Goncalves *et al* 1988, Greenblatt *et al* 1980, Hey *et a1* 994, Kato *et al* 1984, Levin *et al* 1970, Monjour *et al* 1988, Nacimento *et al* 1990, Convit *et al* 1989 and Verbon *et al* 1992). Hence in the past few years attentions were focused on developing a non–living prophylactic vaccine (Coler & Reed 2005, Mitchell & Handman 1983), therefore many candidate molecules and adjuvants such as BCG (Alexander 1982, Armijos *et al* 2004 and Verbon *et al* 1992) were studied in the laboratories that have been shown to protect animals against experimental leishmaniasis (Goncalves da Costa *et al* 1988., Sacks & Noben-Trauth 2002 and Satti *et al* 2001). The outcome of infection with the *L. major* is dependent upon whether CD4+ Th_1 or Th_2 cells develop after infection, and experimental infections in mice with *L. major* have been used extensively to define the factors that regulate Th_1 and Th_2 cell development (Ahmed *et al* 2004, Muller *et al* i988, Thomas *et al* 2002, Steven & Locksley 1995 and Mitchell & Handman 1983). In the resistant C57BL/6 (B6) production of Interferon gamma by Th_1 cells leads to parasite control, whereas BALB/c mice developed Th_2 response and a progressive disease. The objective of the present study was to assess the immunogenicity of a new

antigen formula of *L .major* crude antigen in the murine model. To assess the antigen not only with optimum safety without toxicity and endotoxin release, but also it should produces a high level immunity to generate Delayed Type Hypersensitivity response (DTH) for proper skin test reaction. The effect of new *L. major* crude antigen formula in two type mice (genetically resistant and susceptible mice) is also a question. Immune responses were measured and were statistically analyzed. The effects of various doses of antigen on immune response were assessed to find optimal dose or doses. At least three injection groups were used to find out whether any booster dose of antigen is necessary or not.

MATERIALS AND METHODS

Animals. Ninety outbred resistant mice (designated type 1 mice) and 90 Balb/c sensitive mice (designated type 2 mice) of both sexes with age of three moths obtained from Razi Vaccine and Serum Research Institute, Hesarak, Iran were divided into six groups randomly as groups I - VI. Each group of I, II and III were consisted of 25 and each groups of IV, V and VI consisted of 5 animals (control groups).Based on five antigen doses(100,200,300,400 and 500 µ /ml), each group of I, II and III subdivided further into 5 groups at the time of antigen injection. The animals were allocated in the standard polycarbonate boxes located in a well ventilated room. The animals had free access to water and they were fed with commercial mouse chaw.

Antigen preparation, qualifications and quantification. Promastigotes of the *L. major* which were provided kindly by Pasteur Institute of Tehran, Iran, were grown at room temperature for 3-7 days in Novy-MacNeal- Nicolle (NNN) medium supplemented with Hemin and Homa, RPMI 1640 and normal saline. 5–10 percent of heat inactivated fetal calf serum was added to the culture media. By the termination of incubation time, the cultured parasites were harvested, washed and re-suspended in normal saline. The number of the parasites in the suspension was counted using a Neubauer chamber and the suspension was kept within liquid nitrogen until use. At time of experimentation, the suspension was thawed and diluted to a concentration of 3.3×10^8 per ml. The suspension was dispensed in 5 equal batches and each was treated with one of the following methods; freeze and thawing, autoclaving at 121 °C and 15 lbs for 15 minutes, inactivation at 56 °C for 30 minutes, inactivation with dilution of 1/5000 mertiolate in saline and combination of mertiolate inactivation and freeze and thawing methods. The antigen batches treated with different methods were mixed together and the mixture was tested for sterility, toxicity, safety (Armijos *et al* 2004), endotoxin contamination (Levin *et al* 1970), CHN content and total protein (Lowry *et al* 1951). The suspension of five treated batches was tested for complete parasite killing by cultivation on blood agar plate and injection in the footpads of normal mice types 1 and 2. Sterility was checked on by inoculating a sample of suspension in BBL Trypticase soy agar and thioglycolate (Difco) 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 performed for seven days for any clinical manifestations at the Pasteur Institute of Iran, Tehran, Iran. The content of protein in each dose was estimated by Lowry method (Lowry *et al* 1951). Sugar content was determined by the phenol / sulfuric acid method (Greenblatt *et al* 1980). Endotoxin measurement to show possible contamination of antigen preparation was carried out using endotoxin method (Levin *et al* 1970).

3-Antigen evaluation on animal model: At the time of trial, the antigen was thawed and homogenized gently and the doses of antigen were adjusted to 100, 200,300,400 and 500 µg /0.1ml.Just before injection, 2×10^5 (200,000) CFU BCG

(*Mycobacterium bovis*, Bacillus Calmette Guerin, (BCG Strain / Pasteur Institute of Iran Freeze–dried BCG vaccine from Institute Pasteur France. 1173 P_2 secondary seed lot c. Batch NO. 179) suspended in diluted Sauton SSI solvent was added to each vial of groups II and III containing different doses of antigen. For each doses of antigen, 5 animals were selected randomly from each groups of I, II and III from both mice types (type1 and type2). All groups of animals were treated as follows:

Group I received 0.1 ml of five doses *Leishmania* antigen followed by a single same dose injection as booster, group II was injected with 0.1 ml of the antigen containing BCG, group III received 0.1 ml of antigen containing BCG and a booster of the same dose of antigen, group IV remained untreated, group V was injected with 2×10^5 CFU BCG and group VI received only 0.1 ml BCG solvent. All animals were injected subcutaneously at the back of the tail base. At day 35 the foot pad of front right leg of the animals was injected intradermally with 0.1 ml of PPD skin test and foot pad of posterior right foot was injected intrademally with 0.1 ml of leishmanin as well. The foot pads of left were injected intradermally with physiologic saline as control. The results of skin testing were read and recorded after 24, and 48 hours using Mitutoyo apparatus .To evaluate the statue of spleen white pulp follicles (SWPF), 25 animals from each injection group (including all five injection doses 100-500µg/ml), and 5 mice from each control groups IV, V and VI and from both mice types were euthanized, necropsies promptly and the spleen of each animal was removed, sliced, and fixed in 10% buffered formaldehyde solution. The fixed spleen tissues were processed and stained with Haematoxylin and Eosine (H&E) staining method for histological examination. The number of the spleen lymphoid follicles was counted and mean of the diameters of all of the follicles were measured using an eyepiece graticule and were compred with the control. At each time, for measuring the percentages of SWPF diameter excess of normal control values (without injection) were subtracted from the values of the treated animals. Values of the saline or control for DTH (foot pads of left feet) were subtracted from the levels of reactions due to Leishmanin skin test (posterior right foot) .The same procedure was applied for PPD skin test.This study was performed in compliance with the Helsinki Declaration, and it was approved by the vice chancellor for research at Tehran University of Medical Sciences, Tehran, Iran.

Statistical analysis. The means were compared by standard analysis of variance, simple factorial test and by one, two or three ways student Newman–Keuls method (SPSS for windows), and also using POST-HOC test, Correlation coefficient analysis was determined on a Pearson bivariate two tailed test of significance (SPSS for windows).

RESULTS

Twenty four and 48 hours post-inoculation, the highest value of skin leishmanin test was seen in group I and mice type 1. The same result was found 48 hours post-inoculation in group I , mice type 2 which had received 200µg of the antigen .The highest skin indurations of 24 hours post-inoculation(P I) of PPD skin test was noticed in mice type 1, group III and mice type 2, group III that had received 400µg and 100µg of the antigen respectively. However without considering the types of mice, the highest DTH response at 24 hours post-inoculation was found in group I that received 100 µg of the antigen. The same result was obtained 48 hours post-inoculation of skin leishmanin test (SLT) in group I that had received 100 µg of the antigen. However without considering the types of mice, the highest DTH response was found at 24 hours post-

Table 1. Distribution of mean and standard Deviation for SLT and PPD in the 24 and 48h and W.P.S on the basis of mouse type and ground object of studies and basis of received dose.

Mice type	Injection group	Ag. doses	Mean 24hrs SLT-	Mean 48hrs SLT	Mean 24hPPD	Mean 48hPPD	Mean (SWPF)	N
	I	0	2.0000	2.0000	2.0000	2.0000	11.0000	15
		100	2.8000	3.0333	2.2000	2.1333	10.8333	3
		200	2.6000	2.9000	2.1500	2.1750	26.6250	4
		300	2.8000	2.7000	2.1500	2.2500	59.7500	2
		400	2.6000	2.7800	2.0800	2.1000	31.8000	5
		500	2.5875	3.0875	2.1000	2.0750	41.6250	8
Balb/C (type2)	II	100	2.4750	2.9500	2.2750	2.2000	34.5000	4
		200	2.5000	2.7200	2.0800	2.1800	23.7000	5
		300	2.4750	2.7500	2.2750	2.3750	18.0000	4
		400	2.4000	2.7250	2.3000	2.3000	49.7500	4
		500	2.5000	2.7333	2.1333	2.0333	34.1667	3
	III	100	2.5000	2.7000	2.2600	2.3400	15.5000	5
		200	2.5400	2.8600	2.2200	2.3800	42.0000	5
		300	2.6000	2.8500	2.1000	2.5500	19.0000	2
		400	2.5800	2.7200	2.3600	2.5000	28.8000	5
		500	2.7250	2.8750	2.3000	2.3250	41.1250	4
	I	100	2.5000	2.6500	2.0500	2.0500	41.5000	2
		200	2.5000	2.7000	2.0667	2.1667	20.8333	3
		300	2.3000	2.5750	2.0750	2.0750	28.7500	4
		400	2.3500	2.6000	2.0500	2.0500	42.0000	2
		500	2.3889	2.6222	2.0556	2.1333	34.7222	9
Conventional	II	100	2.5667	2.7333	2.1333	2.2000	36.0000	3
(type 1)		200	2.5000	2.7857	2.1143	2.1571	27.7143	7
		300	2.5333	2.7667	2.2000	2.3667	41.5000	3
		400	2.6750	2.8250	2.1750	2.2500	45.6250	4
		500	2.5000	2.8200	2.1600	2.1000	21.6000	5
	III	100	2.4333	2.7333	2.2667	2.4667	52.8333	3
		200	2.5750	2.8500	2.0250	2.1000	45.5000	4
		300	2.5333	2.7667	2.1000	2.3000	39.0000	3
		400	2.6750	2.8250	2.2250	2.2750	37.5000	4

I:Leishmania antigen plus booster dose of antigen,
II: Leishmania antigen accompanied by BCG plus booster dose of leishmania antigen
III: Leishmania antigen plus booster dose of antigen.
SWPF : diameter of Spleen White Pulp Follicles (increasing against injection antigen) (micron)
leishmania Doses: microgram protein of leishmania antigen (µg)/ 0.1 ml **N:** number
SLT: Skin Leishmanin test (millimeter)

inoculation of SLT in group I receiving 100 µg of antigen. The highest DTH response to PPD skin test at 48 hours post-inoculation (PI) was shown in mice type 1, group III, and mice type 2, group III received 200 µg and 100 µg antigens respectively.

However without considering the types of mice, the highest DTH response at 24 hours post-inoculation was found in group III received 100 µg of antigen. The same result was obtained at 48 hours post-inoculation of PPD skin test in group III mice injected

Table 2. DTH response and Multivariate tests for SLT 24h, 48h and PPD 24h &48h

Effect	Value	F	sig	Value	F	Sig
FACTOR1 Wilk's Lambda	.381	198.310	.000	.928	9.455[a]	.003
FACTOR1* M. Type Wilk's Lambda	.998	.185	.668	1.000	.009[a]	.923
FACTOR1* IN.G Wilk's Lambda	.986	.870	.422	.944	3.609[a]	.030
FACTOR1* DOSE Wilk's Lambda	.958	1.326	.264	.928	2.360[a]	.057
FACTOR1* M. Type* IN.G Wilk's Lambda	.983	1.036	.358	.986	.882[a]	.417
FACTOR1* M. TyPE* DOSE Wilk's Lambda	.972	.864	.488	.979	.648[a]	.630
FACTOR1*.IN.G*DOSE Wilk's Lambda	.913	1.461	.178	.959	.651[a]	.733
FACTOR1* M. Type* IN.G* DOSE Wilk's Lambda	0.848	2.743	0.008	.982	.272[a]	.974

Sig: significantly effects (p value)

with 300µg or 400µg of the antigen. Tukey HSD test evaluation of skin leishmanin test showed that the values in the groups I, II and III were significantly different when compared with groups IV, V and VI (P<0.001). The same results were found for PPD skin test. PPD skin test only correlates with BCG and booster dose plus BCG. Measurement of white pulp statue showed that, the lowest white pulp size was seen in the resistant mice type, group I that had received 100 µg of the antigen. The same result was found in group I, mice type 1 injected with 200 µg of antigen (Table 1). Multivariate test of repeated measures, has shown that in relation to PPD and leishmanin skin test at 24 and 48 hours post-inoculation, the results were statistically significant when the mice types, the injection groups, doses of antigen were considered (P<0.005). The same results were noticed for PPD test induced DTH response (P<0.005) (Table2). In this regard, comparing two groups of mice using POST-HOC test, indicated that there is a significant difference between mice types in relation to SLT, and between the mice type and among injection groups (P<0.005).The same results were found for PPD test when compared various dose groups and two mice types, in which a significant difference were found (P<0.005) (Table 3). However when the types of mice were not considered, the lowest increase in SWPF size was shown in group I that had received 200 µg of the antigen. When the groups and types of mice were not considered, the highest SWPF size was seen in animals who that received 400 or 500 µg of the antigen. The results of analysis of variances show that, there is a significant difference between the size of SWPF of two types of mice (P<0.005). Comparing the effects of antigen doses on SWPF size of both types of mice, show that there is a significant difference (P<0.005). Effect of mice type upon injection groups and vice versa and reliability more than 99 percent between mice type and injection groups was obvious, and one of the groups I, II or III have significant differences in the type 1 mice and type 2 mice (P<0.001) (Table 4).

Table 3. Test of Between-Subjects effects Dependent variable DTH response and significantly POST-HOC test of Between-Subjects effects for SLT (24h 48h), PPD (24h 48h).

Source	Type III Sum of Suqares	F	SLT Sig	Type III Sum of Suqares	F	PPD Sig
M.TYPE	.333	6.768	.010	.270	7.137	.009
IN.G	.046	.469	.627	.854	11.279	.000
DOSE	.048	.243	.913	.365	2.408	.053
M.TYPE*IN.G	.948	9.635	.000	.106	1.399	.251
M.TYPE*DOSE	.267	1.359	.252	.040	.262	.902
IN.G*DOSE	.345	.877	.538	.379	1.250	.276
M.TYPE*IN.G*DOSE	.122	.309	.961	.262	.365	.548
Error	6.000			4.618		

Sig: significantly effects (p value)

Table 4. Test of Between-Subjects Effects Dependent variable W.P.S. significantly effects (p value), 1-three Factors against each other, 2- type ,3- injection group and ,4- injection dose.

Source	Type III Sum of Squares	F	Sig.
M. TYPE	674.377	9.813	.002
IN. G	167.328	1.217	.300
DOSE	957.869	3.485	.010
M.TYPE*IN.G	993.448	7.228	.001
M.TYPE*DOSE	2661.411	9.682	.000
IN.G *DOSE	4578.823	8.328	.000
M.TYPE*IN.G*DOSE	3523.766	6.409	.000

Sig: significantly effects (p value)

In general, significant differences are present no matter considering types of mice and injected groups or injected doses of antigen, types of mice or injected groups and injected doses of antigen and also types of mice, injected groups and injected doses of antigen. ($P<0.001$) (Table 4). Correlation of DTH 24h and 48h showed when SLT (24 hrs) increasing, SLT (48hrs) increased too. Correlation of PPD skin test at 24hrs post inoculation (PI) with 48hrs PI there is a complete direct linear correlation between two parameters (0.866) that show PPD skin test at 24 hrs PI and 48hrs PI increased together. Correlation of DTH at 24hrs PI and 48 hrs PI with percentage of SWPF size expansion is reversed and linear (-0.0797), that is near to (-1.0). There is a correlation between groups injected with antigen plus BCG and PPD skin test response. The skin thickness at 24hrs PI and 48hrs post leishmanin skin test was increased in all groups injected with the antigen.

DISCUSSION

Killed *Leishmania* or soluble promastigote extract have been used for immunizing animals (Scott *et al* 1987) and human (Greenblatt 1988 and Mayrink *et*

al 1979) without convincing results. The injection of purified *Leishmania* subunit proteins, conferred protection in the mouse model of leishmanasis against subsequent challenges, but such vaccines seems to require continuous boosters and presence of immune adjuvant. Understanding how antigen dose influences the development of Th_1 and Th_2 cells is important for designing vaccines and until the time of being, experiments that have addressed this issue have had conflicting results. The last approach is what we call cocktail based vaccination which has been already used in various pathologic conditions and in animal models. Our studies include two types of mice: susceptible (type 1) and resistant (type 2), and five injection doses of antigen (100,200,300,400,500 μg /0.1ml) and three injection groups: group I (*Leismania* plus the same dose booster) group, group II (*Leismania* plus BCG), and group III (*Leismania* plus BCG plus the same dose booster). The results obtained in the present study indicated that the DTH responses and spleen white pulp statue differed significantly when comparing mice type 1 and 2. When comparing groups I, II and III in each types of mice, results indicated a statistically significant difference among the groups in an antigen dependent manner. The higher DTH responses and lower spleen white pulp size were noticed in animals that had received 100 or 200 μg of the antigen with a single booster either in mice type 1 or 2. Previous studies (Reiner & Locksley 1995 and Sacks & Noben-Trauth 2002) show that CD4+ Th_1 and Th_2 regulate infection development. When *L. major* causes a single cutaneous lesion, or undergo spontaneous cure, subject is resistant and probably infection is inhibited in macrophage via innate immunity and production of interferon gamma and IL_{12} by Th_1 response that lead to parasite killing, and probably in future challenge subject is immune. Scott *et al* suggested that low antigen doses may preferably promote a CD4+ Th_2 response *in vivo*, where as high doses may favor Th_1 cells develop (Boonstra *et al* 2003, Constant *et al* 1995, Constant & Bottomly 1997, Hosken *et al* 1995). Antigen doses could effect T helper cell development and our results provide additional insight that the doses of antigen might influence the efficacy of vaccines and immunotherapy. Spleen is a lymphoid organ of the secondary lymphatic system that contains two types of tissues, red pulp and white pulp. The white pulp is the place where induced immune response is induced that subsequently results in antibody production. Upon antigenic challenge, these primary follicles develop into characteristic secondary follicles containing germinal centers, where rapidly dividing B cells (centroblasts) and plasma cells are surrounded by dense clusters of concentrically arranged lymphocytes (Nairn & Helbert 2007, Abul Abbas *et al* 2005). Our findings show that DTH response and spleen white pulp statue were significantly different when comparing two types of mice, three injection groups and five injection doses. It is very noticeable that highest DTH of SLT related to group I that received 100 μg antigen and lowest increasing of SWPF size also related to group I that received 100 μg and 200 μg antigen. An important finding in these studies is that, lower expansion of WPSF and highest increasing in DTH was seen in groups received 100 and 200 μg antigen. These findings may indicate that in the resistant animal and human subjects the infection will be probably resolved in the macrophage via innate immunity and production of interferon gamma and IL_{12} by Th_1 response that lead to parasite inhibition which in turn it confer immunity to future challenge (Reiner & Locksley 1995, Sacks & Noben-Trauth 2002). Our results show that group I that received 100 μg/0.1 ml and 200

μg/0.1 ml antigen had high DTH for SLT and low WPSF increasing, while low DTH and high WPSF was seen in group II, III that received 400 μg/0.1ml and 500 μg/0.1ml antigen This results lead to high dose and low dose concepts that proposed by Uzonna *et al* in 2004. Preventive vaccines are recognized as the best and most cost effective protection against pathogens. Leishmania vaccine development has proven to be a difficult and challenging task, which is mostly hampered by inadequate knowledge of parasite pathogenesis and the complex of immune responses needed for protection.

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