Immunological, histopathological and immunohistochemical responses to a new B cell epitope-based vaccine against leishmaniasis in experimental mice

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Abstract: Leishmaniasis is a significant worldwide health problem for which no vaccine exists. Recent trend in vaccine design has been shifted to epitope-based vaccines that are more specific, safe and easy to produce. Indeed, as far as we knew, the B cell epitope technique for vaccine development has not been used for preparation of Leishmania vaccine till now. Hence, the aim of the present work was to evaluate a new vaccine using different antigenic B cell epitopes. To achieve this aim, eighty albino mice were used. They were classified into 3 main groups; group I (10 mice) (non- infected, non-vaccinated), group II (10 mice) (infected, non-vaccinated) and group III (60 mice) (infected, vaccinated). Group III was subclassified into 6 subgroups named "a "to" f ", each subgroup was formed of 10 mice. They were vaccinated by epitopes: 239-247 DGMEGSCSG, 6-14 SWGANHYDG, 163-171 SYETGSSTL, 190- 198 NDGDGEEEE, 359- 367 KQKKDEGNQ and477- 485 ASGSADGDE, respectively. Serum IgG OD values were evaluated using ELISA. Histopathological examination of spleen and skin tissues was performed using hematoxylin and eosin staining. Immunohistochemical staining of spleen tissue was accomplished for measuring CD4+ and CD8+T cells counts. The most considerable findings were the detection of higher serum IgG OD values in subgroups IIIa & IIIb than in group II with the presence of high statistical significant difference. No mice related to subgroups IIIa or IIIb showed hyaline changes of spleen (with the presence of statistical significant difference when compared to group II), ulcer in skin or atrophic epidermis (with the presence of high statistical significant difference when compared to group II). CD4+T cells count was significantly higher in all subgroups of group III than group II while the CD8+T cells count was significantly higher in group II than all subgroups of group III. In conclusion, the currently used new B cell epitope-based vaccine proved beneficial in protection against leishmaniasis. Epitopes 239-247 DGMEGSCSG and 6-14 SWGANHYDG gave the best prophylactic yield as evidenced by detection of high serum IgG OD values together with the best resolution of histopathological changes in spleen and skin tissues when compared to the non-vaccinated infected group. It seems that CD4+T cells expressed in spleen tissue in this study were related to Th-1 subset which proved protective against leishmaniasis. It is advised to apply these two promising epitopes in vaccine design against leishmaniasis in clinical trials.

[Nadia S. El-Nahas, Gehan S. Sadek, Amal M. Husein, Amany A. Rady, Salwa F. Oshiba and Reham M. Barakat. Immunological, histopathological and immunohistochemical responses to a new B cell epitope-based vaccine against leishmaniasis in experimental mice. J Am Sci 2015;11(7):1-15]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 1

Key words: *Leishmania*, B cell epitope- based vaccine.

1. Introduction

Leishmaniasis is a vector-borne disease caused by obligate protozoan parasites of the genus *Leishmania* (Kumar and Engwerda, 2014). It is endemic in over 88 countries worldwide, most of which are developing countries located in tropical and subtropical regions (Zand and Narasu, 2013). Approximately 350 million people are at risk of contracting leishmaniasis and 1.5-2 million new cases occur annually (Kumar and Engwerda, 2014). Moreover, a mortality rate of nearly 600,000 per year was recorded (Das and Ali, 2012). This disease ranks second only to malaria in mortality and fourth in morbidity among all tropical parasitic diseases (Schroeder and Aebischer, 2011). Besides, it is considered as one of the world's most neglected diseases, affecting mainly very poor people in developing countries (Kumar and Engwerda, 2014). {Neglected diseases are illnesses of the poor not attracting enough political and financial support, so no adequate research or effective measures to prevent or treat them are available (Schroeder and Aebischer, 2011)}.

Leishmaniasis is characterized by diversity and complexity presenting a wide spectrum of clinical forms in humans ranging from self-healing cutaneous leishmaniasis (CL) to fatal visceral leishmaniasis (VL). The latter emerged as an important public health

concern with major clinical and socioeconomic impacts (Agallou *et al*., 2014). A good understanding of immunity against pathogens is important for developing an effective vaccine (Kumar and Engwerda, 2014). The response of immune system to leishmaniasis could be very complicated and lead to either immediate healing of the lesion or worsen it under certain circumstances. It depends on several factors such as genetic diversity of the mammalian host, genetic diversity of different species of the parasite, location, amount of inoculation and number of infective bites (Zand and Narasu, 2013).It was found to be mediated by both innate and adaptive immune responses and requires activation of macrophages, dendritic cells (DCs) and antigen-specific CD4+ (helper) and CD8+ (cytotoxic) T cells (Evans & Kedzierski, 2012 and Agallou *et al*., 2014). Studies of immune responses associated with experimental murine leishmaniasis has led to the identification of two immunoregulatory subsets of T helper lymphocytes; T-helper-1 (Th1) and T-helper-2 (Th2) (Das and Ali, 2012). In general, a protective role is associated with the cells of the Th1 subset which secrete interleukin-2 (IL-2) and interferon gamma (IFN-γ)whereas the expansion of cells of Th2 subset that produce IL-4 and IL-10 exacerbate the disease (Cardoso *et al*., 2003).Thus, the outcome of infection is determined by the balance between these two types of responses (Pereira *et al*., 2011). IFN-γ produced by Th1 subset of CD4+T cells has been shown to be vital in the process of macrophage activation and parasite destruction. It can perform these actions against both the promastigote and amastigote forms in H_2O_2 dependent manner and nitric oxide production for parasite killing (Cunningham, 2002 and Kumar & Engwerda, 2014). Conversely, cytokines such as IL-4, IL-10 and transforming growth factor-β (TGF-β) produced by Th2 subset of CD4+T cells have been shown to down regulate the Th1 response, hinder macrophage activation and consequently aggravate the disease (Haberer *et al*., 1998; Garg & Dube, 2006 and Agallou *et al*., 2014).

With respect to the humoral immune response, *Leishmania* antibodies are usually present at low levels during the active phase of CL. In contrast, strong anti-*Leishmania* antibodies titres are well documented in VL. The analysis of *Leishmania* antigen- specific immunoglobulin (Ig) revealed elevated level of IgG, IgM and IgE. These highly elevated titres against promastigote or amastigote antigens or recombinant antigens have been extensively exploited for specific serodiagnosis in last two decades (Sharma and Singh, 2009).

Visceral leishmaniasis could be fatal if left untreated (Schroeder and Aebischer, 2011). Current control measures against leishmaniasis are based on chemotherapy to alleviate the disease (Singh *et al*., 2012) and on vector control to reduce transmission (Agallou *et al*., 2014). However, presently employed drugs are associated with severe toxic side effects and increasing parasite drug resistance (Croft *et al*., 2006). As it was found that healing of *Leishmania* lesion was associated with lifelong resistance to reinfection (Schroeder and Aebischer, 2011), researchers were forced to think about development and implementation of an effective vaccine (Garg and Dube, 2006).

Vaccines against leishmaniasis were classified into three main categories namely first generation vaccines, second generation vaccines and third generation ones (Zand and Narasu, 2013). First generation vaccines include a procedure known as leishmanization (Handman, 2001), vaccination with killed parasites (Kobets *et al*., 2012) and vaccination with live attenuated parasites (Evans and Kedzierski, 2012). Second generation vaccines include vaccination with defined proteins (Spitzer *et al*., 1999) and vaccination with parasite subunits (Griffiths and Khader, 2014). Third generation vaccines include recombinant proteins and DNA vaccines (Evans & Kedzierski, 2012 and Zand & Narasu, 2013).

Recent trend in vaccine design has been shifted to epitope-based vaccines that are more specific, safe, easy to produce and capable of inducing more potent responses than whole protein vaccines (Agallou *et al*., 2014). Epitope can be defined as the minimal structure necessary to invoke an immune response. It was reported that epitopes prediction plays a vital role in the development of immune-diagnostic tests (Youssef *et al*., 2012). Understanding the antibody/epitope interaction provides a basis for the rational design of preventive vaccines. It is assumed that immunization with the precise epitope, corresponding to an effective neutralizing antibody, would elicit the generation of similarly potent antibodies in response to the vaccine. Such a vaccine would be a B-cell epitope-based vaccine (Gershoni *et al*., 2007). The development of peptide based synthetic vaccines is considered one of the most important applications of B-cell epitopes prediction (Agallou *et al*., 2014).

Hence, the aim of the present work was to evaluate a new vaccine using different antigenic B cell epitopes. Evaluation was done by measuring serum IgG OD values, detecting histopathological lesions in spleen and skin and estimating CD4+ and CD8+ Tcells counts in spleen tissue immunohistochemically.

2.Materials and methods

This experimental study was done to prepare a vaccine from *Leishmania* parasite using epitope prediction technique. Experimental mice were immunized by this vaccine then ELISA was used to identify the antibody response. After that, challenge infection was done. Degree of protection by this

vaccine was tested by histopathological examination of spleen, skin , also by immunohistochemical examination of spleen.

I) Vaccine preparation:

This vaccine is a synthetic peptide vaccine. It was prepared by identification of genome of Leishmania parasite from the genbank then the genes of interest were selected. The B cell epitopes of these genes were identified from a certain web site.

A- Sequence analysis

Leishmania major (*L. major*) strain Friedlin genome sequences were compared using genbank database and the applications of National Center for Bio-technology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi.).Nucleotide and amino acid sequences were aligned using the multiple sequence alignment program Clustal W. Two genes were selected for *Leishmania* vaccine, *L. major* elongase (beta ketoacyl) and Pool II LmSTI-1 (Thompson *et al*., 1994).

B- Identification of B cell epitopes

The B cell epitopes of the two genes *L. major* elongase and Pool II LmSTI-1 were predicted in a web based B cell epitope prediction software "antigenic" available at the web site: (http://bioinfo.bgu.ac.il/bsu/immunology/epitope_pred/index.htm). The prediction for the most antigenic epitopes was as follows:

I- For *L. major* elongase (beta ketoacyl): The most antigenic epitopeswere: 239-247 DGMEGSCSG, 6-14 SWGANHYDG and163-171 SYETGSSTL.

II- For pool II LmSTI-1: The most antigenic epitopes were: 190- 198. NDGDGEEEE, 359- 367 KQKKDEGNQ and477- 485 ASGSADGDE.

The code of different amino acids were written according to data of the web site (http://www.ddbj.nig.ac.jp/sub/ref2-e.html).

C-Peptide synthesis:

The above mentioned six epitopes sequences were sent to genscript company, USA (shipping@genscript.com).

Synthetic peptides were synthesized via the above mentioned company using a technique called FLEX peptide technology (Full-length expressed stable-isotope labeled proteins technology).

II) Animals and experimental design:

Eighty male albino mice aged 7-10 weeks were used in this study. Each mouse weighed about 20 g. They were classified into three main groups; group I which included ten non-infected non-vaccinated mice, group II which included ten infected non-vaccinated mice and group III which included sixty infected vaccinated mice. Group III was subclassified into 6 subgroups (a- f). Each subgroup was formed of ten mice which were vaccinated by one epitope (Subgroup IIIa was vaccinated by 239-247 DGMEGSCSG, IIIb by 6-14 SWGANHYDG, IIIc by 163-171 SYETGSSTL, IIId by 190- 198 NDGDGEEEE, IIIe by 359 - 367 KQKKDEGNQ and IIIf by 477- 485 ASGSADGDE).

Concerning group II, mice were infected on the first day of experiment. Concerning group III, each mouse was injected intraperitoneally by the synthetic peptide (fluid phase) combined with equal volume of complete Freund's adjuvant (Star Technology Company, ALX-581-012-L002). Injection was applied for four times with 50, 100, 150 and 200 µg of this peptide with one week interval between every injection and the other.

* One week from last injection, 4 mice from each group & subgroup were sacrificed and blood samples were collected. Serum samples were separated, put in Eppendorf tubes, labeled and kept at -20°C till used for ELISA test.

* The remaining 6 mice from each group& subgroup have completed the experiment and mice related to subgroups of group III were subjected to challenge infection three weeks after the last booster dose.

III- Preparation of soluble *Leishmania* antigen (SLA):

L. major promastigotes were suspended in sterile PBS. Parasites were lysed by five cycles of freezing and thawing. Sonication was done at 4ºC with 20 cycles/second for 10 minutes. Then, centrifugation at 100,000 r.p.m. for 20 minutes was done. The supernatant (SLA) was collected and protein concentration was determined by colorimetric method. The protein concentration was 1.3 gm/dL. Then, it was stored at -70 ºC until use. This antigen was used for coating ELISA plates for antibody assay (Rostami *et al.*, 2010).

IV- Enzyme linked immunosorbent assay (ELISA): Indirect ELISA for determination of antibody titre in serum was done according to the method of Halder *et al*. (1981).

V- Challenge infection:

Three weeks after the last booster dose, the immunized mice (6 subgroups of group III) were challenged subcutaneously with *L. major* (MHOM/IL/81/FEBNI) promastigotes harvested at stationary phase (1.5×10^6/50 µl PBS) into the left footpad and as a control; 50 µl of PBS was injected into the right footpad (Firouzmanda *et al*., 2013).All mice were sacrificed four weeks after infection (6 mice from each group & subgroup), then spleen and skin specimens were used for histopathological and immunohistochemical studies.

N.B. Mice related to group II were infected on the first day of experiment with the same method mentioned above.

VI- Hematoxylin and eosin staining:

Spleen and skin specimens of sacrificed mice of all groups were preserved in 10% formalin and blocks were paraffinized then deparaffinized and stained with haematoxylin and eosin stain (Talumwine *et al*., 2013). Sections were examined by an expert pathologist to detect presence of hyaline changes in spleen, also to exhibit presence of either ulcer or atrophic epidermis in skin. Three slides were evaluated for each organ.

VII- Immunohistochemistry for CD4+ and CD8+ cells in spleen:

It was done according to method of Hald *et al*. (2013) concerning CD4+ cells and according to Lie et al. (2012) regarding CD8+T cells. Positivity was identified when the cell membrane alone or together with the cytoplasm showed brown staining. Cells were counted per ten high power fields (h.p.f.) for each animal, then for each group of animals and the mean was calculated.

Statistical analysis:

Data were collected, tabulated, statistically analyzed by computer using SPSS version 11.

* Chi-squared test was used to test the significance of difference between frequencies of different observations.
* Student t test was used to show the relation between 2 quantitative variables.
* ANOVA was used to compare between more than two groups of quantitative normally distributed data.
* Post hoc LCD was used to differentiate between every two groups.

Significance of results:

- P>0.05 means non significant results.
- P<0.05 means significant results.
- P<0.01 means moderately significant results.
-P<0.001 means highly significant results.

3.Results

I-ELISA results:

Comparison between main groups:

Difference between groups I and II and between groups I and III was highly significant (p<0.001) while it was significant between II and III (p<0.05) (data not shown).

N.B. Results of group III were before challenge infection with Leishmania promastigotes.

Comparison between group II and subgroups of group III:

There were high significant differences between the mean±SD of the serum IgG OD values of vaccinated non infected mice subgroups IIIa, IIIb, IIIe and IIIf when compared to infected non vaccinated group (group II) (P1, P2, P5, P6<0.001). Also, there was a moderately significant difference between subgroup IIId and group II (P4<0.01). On the other hand, no statistical significant difference was found between subgroup IIIc and group II (P3>0.05) (Table 1, T test).

Comparison among subgroups of group III:

Among all subgroups of group III, there were high significant differences between the serum IgG OD values of subgroups IIIa and IIIc, IIIa and IIId, IIIa and IIIe, IIIa and IIIf, IIIb and IIIe, also IIIb and IIIf (P2, P3, P4, P5, P8, P9 <0.001). Also, there were significant differences between subgroups IIIb and IIIc, IIIb and IIId, IIIc and IIIe, also IIIc and IIIf (P6, P7, P11, P12<0.05). On the other hand, there were no significant differences between the serum IgG OD values of subgroups IIIa and IIIb, IIIc and IIId, IIId and IIIe, IIId and IIIf, also IIIe and IIIf (P1, P10, P13, P14, P15 >0.05) (Table 1, Post hoc LCD).

II- Histopathological examination results:

A) Spleen:

Comparison between main groups:

Difference between the three main groups was highly statistically significant regarding hyaline changes of the spleen (p<0.001) (data not shown) (Figure 1).

Comparison between group II and subgroups of group III:

Hyaline changes of spleen tissue were detected in the majority (83.3%) of mice related to group II. Lower percentage rates (16.7%, 16.7%, 33.3% and 33.3%) were detected in subgroups IIIc, IIId, IIIe & IIIf, respectively. No changes were detected in subgroups IIIa & IIIb. There was a significant statistical difference (p<0.05) between all subgroups of group III versus group II (Table 2, chi-squared test) (Figures 1 & 2).

Comparison among subgroups of group III:

There was no statistical significant difference ($p>0.05^{*}$) among all subgroups of group III regarding hyaline changes of spleen (Table 2, chi-squared test*) (Figures 4, 5 & 6).

B) Skin:

Comparison of main groups:

Difference between the three main groups was highly significant regarding skin lesions (p<0.001) (data not shown) (Figure 7).

Comparison between group II and subgroups of group III:

The majority of mice (83.3%) related to group II showed ulcer in skin while only 16.7% showed atrophic epidermis. All mice (100%) related to subgroups IIIa and IIIb had normal skin. More than half of mice related to either group IIIc or IIId (66.7%) showed atrophic epidermis while 33.3% of the same subgroups had normal skin. Only one mouse out of 6 (16.7%) related to subgroup IIIe showed ulcer in skin, 2 (33.3%) showed atrophic epidermis and 3 (50%) had normal skin. Also, only one mouse out of 6 (16.7%) related to subgroup IIIf showed ulcer in skin, 3 (50%) showed atrophic epidermis and 2 (33.3%) had normal skin. There was a high significant statistical difference

between group II and subgroups of group III regarding grade of skin lesion (p<0.001) (Table 3, chi-squared test) (Figures 8, 9 & 10).

Comparison among subgroups of group III:

Difference among all subgroups of group III regarding grade of skin lesion was not significant (p>0.05*) (Table 3, chi-squared test*) (Figures 9 & 10).

Immunohistochemical examination results:

A) CD4+T cells:

Comparison of main groups:

Concerning CD4+T cells number, difference between group I and II was significant (p<0.05) while it was highly statistically significant between groups I and III and between groups II and III (p<0.001) (data not shown) (Figure 11).

Comparison between group II and subgroups of group III:

The mean ± SD of the number of CD4+T cells was significantly higher in all infected vaccinated subgroups of group III than the infected non vaccinated group II (P1, P2, P3, P4, P5, P6< 0.001) (Table 4, T test) (Figures 12, 13, 14 & 15).

Comparison among subgroups of group III:

There was a high significant statistical difference between the mean ± SD of the number of CD4+T cells of infected vaccinated subgroups (IIIa and IIIc, IIIa and IIId, IIIa and IIIe, IIIa and IIIf, IIIb and IIIc, IIIb and IIId, IIIb and IIIe, IIIb and IIIf, IIIc and IIId, IIIc and IIIe, IIIc and IIIf, IIId and IIIe and also IIId and IIIf) (P2, P3, P4, P5 , P6 , P7, P8, P9, P10, P11, P12, P13, P14<0.001). Also, there was a significant statistical difference between subgroup IIIb and IIIa (P1<0.05). On the other hand, no significant difference was detected between subgroup IIIe and IIIf (P15>0.05) (Table 4, ANOVA).

B) CD8+T cells:

Comparison of main groups:

Difference between the three main groups was highly significant (p<0.001) regarding CD8+T cells number (data not shown) (Figure 16).

Comparison between group II and subgroups of group III:

The mean number of CD8+ T cells was significantly higher in the infected non vaccinated group (group II) than all the infected vaccinated subgroups of group III (P1, P2, P3, P4, P5, P6<0.001) (Table 5, T test) (Figures 17, 18, 19 & 20).

Comparison among subgroups of group III:

There was a high significant statistical difference between subgroups IIIa and IIIc, IIIa and IIId, IIIa and IIIe, IIIa and IIIf, IIIb and IIIf, IIIc and IIIe, also IIId and IIIe (P2, P3, P4, P5, P9, P11, P13<0.001). Moderate significant difference was detected between subgroups IIIb and IIIc, IIIb and IIId, IIIb and IIIe, IIIc and IIIf, also IIId and IIIf (P6, P7, P8, P12, P14<0.01). A significant difference was detected between subgroups IIIa and IIIb(P1<0.05). On the other hand, no significant difference was present between subgroups IIIc and IIId or IIIe and IIIf (P10, P15>0.05) (Table5,ANOVA).

Table(1):The mean ± standard deviation ($\overline{X}$ ±SD) of the serum IgG OD values of group II and all subgroups of group III (resultsofgroupIIIwerebeforechallengeinfectionwith *Leishmania* promastigotes).

ELISA IgG	$\overline{X}$ ± SD	T test	P value	Post hoc LCD
roup II (n=4)	0.79±0.037	T1 9.03	P1 < 0.001	P1>0.05
ubgroup IIIa(n=4)	1.06±0.087	T2 13.59	P2 < 0.001	P2<0.001
Subgroup IIIb(n=4)	0.965±0.017	T3 0.50	P3 >0.05	P3<0.001
Subgroup IIIc(n=4)	0.797±0.025	T42.90	P4 < 0.01	P4<0.001
Subgroup IIId(n=4)	0.715±0.073	T5 10.1	P5 < 0.001	P5<0.001
Subgroup IIIe(n=4)	0.660±0.017	T6 12.79	P6 < 0.001	P6< 0.05
Subgroup IIIf(n=4)	0.635±0.010			P7<0.05
				P8<0.001
				P9<0.001
				P10>0.05
				P11<0.05
				P12<0.05
				P13>0.05
				P14>0.05
				P15>0.05

T test: P1: between group II and subgroup IIIa, P2: between group II and subgroup IIIb, P3: between group II and subgroup IIIc, P4: between group II and subgroup IIId, P5: between group II and subgroup IIIe, and P6: between group II and subgroup IIIf.

Post hoc LCD: P1: between subgroups IIIa and IIIb, P2: between subgroups IIIa and IIIc, P3: between subgroups IIIa and IIId, P4: between subgroups IIIa and IIIe, P5: between subgroups IIIa and IIIf, P6: between subgroups IIIb and IIIc, P7: between subgroups IIIb and IIId, P8: between subgroups IIIb and IIIe, P9: between subgroups IIIb and IIIf, P10: between subgroups IIIc and IIId, P11: between subgroups IIIc and IIIe, P12: between subgroups IIIc and IIIf, P13: between subgroups IIId and IIIe, P14: between subgroups IIId and IIIf, and P15: between subgroups IIIe and IIIf.

***P < 0.001means highly significant. P < 0.01 means moderately significant. P<0.05 means significant. P >0.05 means not significant.

Table (2): Histopathological examination of spleen tissue of group II and all subgroups of group III regarding hyaline changesafterchallengeinfection with*Leishmania* promastigotes.

Hyaline change		Yes	%	No	%	X^2	P –value	X^{2*}	P –value*
Group II	(n=6)	5	83.3	1	16.7	11.65	< 0.05	4.80*	0.05*>
Subgroup IIIa	(n=6)	0	0	6	100				
Subgroup IIIb	(n=6)	0	0	6	100				
Subgroup IIIc	(n=6)	1	16.7	5	83.3				
Subgroup IIId	(n=6)	1	16.7	5	83.3				
Subgroup IIIe	(n=6)	2	33.3	4	66.7				
Subgroup IIIf	(n=6)	2	33.3	4	66.7				

Table (3): Histopathological examination of skin tissue of group II and all subgroups of group III after challenge infection with*Leishmania* promastigotes.

	Normal	%	Atrophic epidermis	%	Ulcer	%	X^2	P- value
Group II (Inf.+non vac.) (n=6)	0	0	1	16.7	5	83.3	38.29 16.57*	<0.001 > 0.05*
Subgroup IIIa (n=6)	6	100	0	0	0	0		
Subgroup IIIb (n=6)	6	100	0	0	0	0		
Subgroup IIIc (n=6)	2	33.3	4	66.7	0	0		
Subgroup IIId (n=6)	2	33.3	4	66.7	0	0		
Subgroup IIIe (n=6)	3	50	2	33.3	1	16.7		
Subgroup IIIf (n=6)	2	33.3	3	50	1	16.7		

Table (4):The mean ± standard deviation ($\overline{X}$ ±SD) of the CD4+T cell numbers of group II and all subgroups of group III after challengeinfectionwith*Leishmania* promastigotes.

CD4	$\overline{X}$ ±SD	T test	P- value	ANOVA	P value
Group II (Inf.+nonvac.)(n=6)	18.0±1.67				P1<0.05 P2<0.001 P3<0.001 P4<0.001 P5<0.001 P6<0.001 P7<0.001 P8<0.001 P9<0.001 P10<0.001 P11<0.001 P12<0.001 P13<0.001 P14<0.001 P15>0.05
Subgroup IIIa (n=6)	21.16±1.83	T1 4.03	P1 <0.001	264.83	
Subgroup IIIb (n=6)	26.16± 3.54	T2 8.16	P2 <0.001		
Subgroup IIIc (n=6)	38.00± 2.68	T3 30.04	P3 <0.001		
Subgroup IIId (n=6)	48.33±1.63	T4 27.55	P4 <0.001		
Subgroup IIIe (n=6)	73.00±2.09	T5 65.01	P5 <0.001		
Subgroup IIIf (n=6)	70.50±5.92	T6 29.56	P6 <0.001		

T test: P1 between group II and subgroup IIIa, P2 between group II and subgroup IIIb, P3 between group II and subgroup IIIc, P4 between group II and subgroup IIId, P5 between group II and subgroup IIIe, and P6 between group II and subgroup IIIf.
ANOVA: P1 between subgroup IIIa and subgroup IIIb, P2 between subgroup IIIa and subgroup IIIc, P3 between subgroup IIIa and subgroup IIId, P4 between subgroup IIIa and subgroup IIIe, P5 between subgroup IIIa and subgroup IIIf, P6 between subgroup IIIb and subgroup IIIc, P7 between subgroup IIIb and subgroup IIId, P8 between subgroup IIIb and subgroup IIIe, P9 between subgroup IIIb and subgroup IIIf, P10 between subgroup IIIc and subgroup IIId, P11 between subgroup IIIc and subgroup IIIe, P12 between subgroup IIIc and subgroup IIIf, P13 between subgroup IIId and subgroup IIIe, P14 between subgroup IIId and subgroup IIIf, and P15 between subgroup IIIe and subgroup IIIf.

Table (5):The mean ± standard deviation($\bar{X}$ ±SD) of the CD8+T cells number of group II and all subgroups of groupIIIafterchallengeinfectionwith*Leishmania* promastigotes.

CD8	$\bar{X}$ ±SD	T test	P- value	ANOVA	P value
Group II (Inf. + non vac.) (n=6)	37.66±3.20				P1<0.05
Subgroup IIIa (n=6)	8.00±1.26	T1 27.26	P1 <0.001		P2<0.001 P3<0.001 P4<0.001
Subgroup IIIb (n=6)	12.83±4.35	T2 14.54	P2 <0.001		P5<0.001
Subgroup IIIc (n=6)	19.00±0.89	T3 17.77	P3 <0.001	27.68	P6<0.01 P7<0.01 P8<0.01
Subgroup IIId (n=6)	19.66±1.36	T4 16.37	P4 <0.001		P9<0.001 P10>0.05
Subgroup IIIe (n=6)	29.00±6.78	T5 3.65	P5 <0.001		P11<0.001 P12<0.01
Subgroup IIIf (n=6)	25.33±2.94	T6 8.97	P6 <0.001		P13<0.001 P14<0.01 P15>0.05

T test: P1 between group II and subgroup IIIa, P2 between group II and subgroup IIIb, P3 between group II and subgroup IIIc, P4 between group II and subgroup IIId, P5 between group II and subgroup IIIe, and P6 between group II and subgroup IIIf.

ANOVA: P1 between subgroup IIIa and subgroup IIIb, P2 between subgroup IIIa and subgroup IIIc, P3 between subgroup IIIa and subgroup IIId, P4 between subgroup IIIa and subgroup IIIe, P5 between subgroup IIIa and subgroup IIIf, P6 between subgroup IIIb and subgroup IIIc, P7 between subgroup IIIb and subgroup IIId, P8 between subgroup IIIb and subgroup IIIe, P9 between subgroup IIIb and subgroup IIIf, P10 between subgroup IIIc and subgroup IIId, P11 between subgroup IIIc and subgroup IIIe, P12 between subgroup IIIc and subgroup IIIf, P13 between subgroup IIId and subgroup IIIe, P14 between subgroup IIId and subgroup IIIf, and P15 between subgroup IIIe and subgroup IIIf.

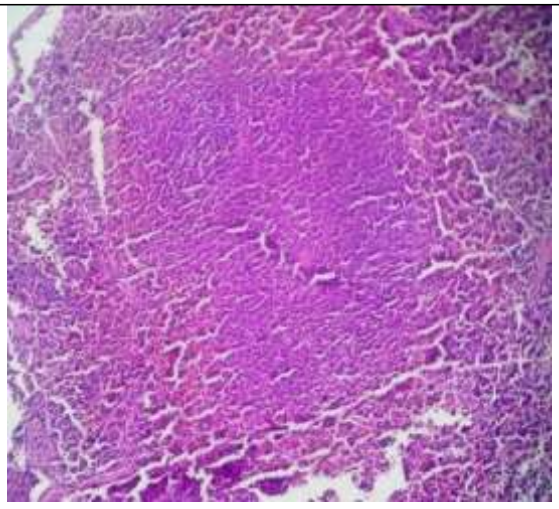

Figure (1): Spleen tissue of non infected non vaccinated group (group I) showing normal spleen with preserved red and white pulp and thin capsule (H&E, x200).

Figure (2): Spleen tissue of infected non vaccinated group (group II) showing extensive hyaline changes of the spleen, the capsule is thick with hyaline deposition in the capsule (white arrows) and trabeculae (red arrows) (H&E, x200).

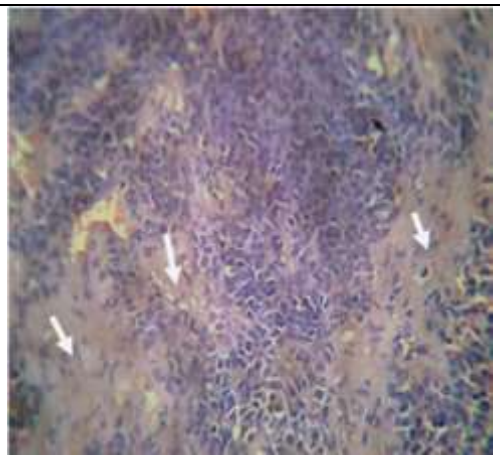

Figure (3): Spleen tissue of infected non vaccinated group (group II) showing extensive deposition of the hyaline material in the stroma (white arrows) surrounding the white pulp (H&E, x400).

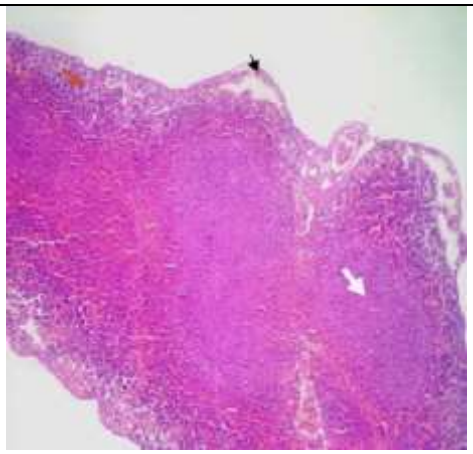

Figure (4): Spleen tissue of infected vaccinated subgroup IIIa showing normal spleen with preserved red (white arrow) and white pulp (red arrow) and thin capsule (black arrow) (H&E, x200).

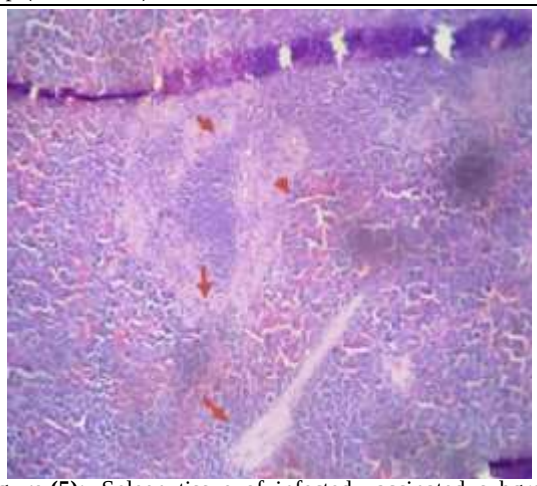

Figure (5): Spleen tissue of infected vaccinated subgroup IIId showing deposition of the hyaline material in the stroma (red arrows) (H&E, x200).

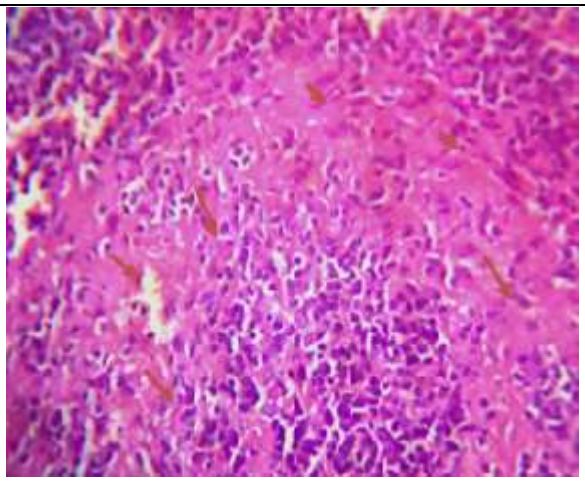

Figure (6): Spleen tissue of infected vaccinated subgroup IIIf showing extensive hyaline deposition in the matrix (red arrows) (H&E, x400).

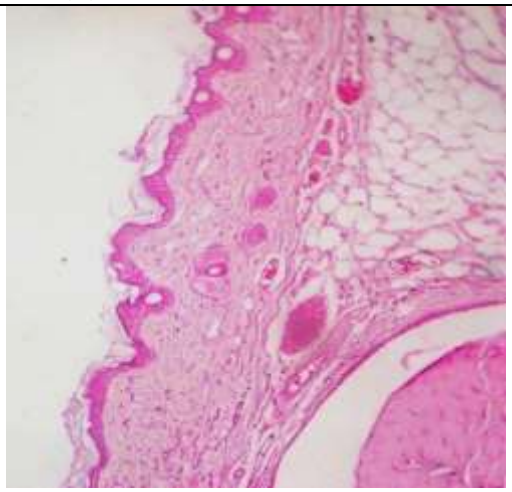

Figure (7): Skin tissue of non infected non vaccinated group (group I) showing normal skin with normal epidermal covering (H&E, x200).

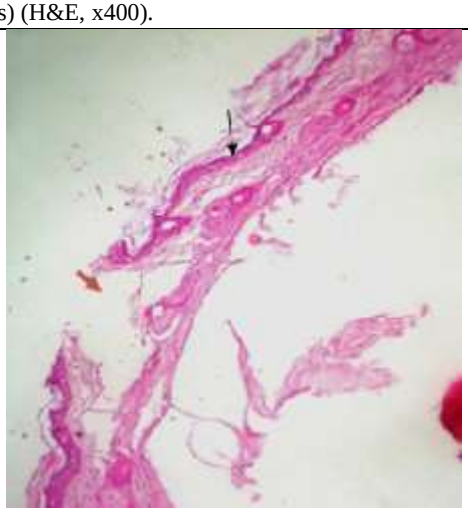

Figure (8): Skin tissue of infected non vaccinated group (group II) showing atrophic skin (black arrow) with focal ulceration (red arrow) (H&E, x100).

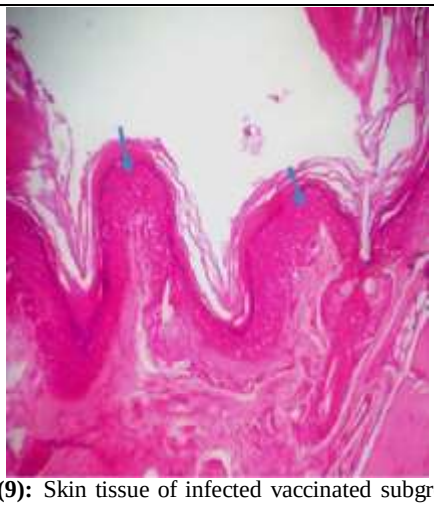

Figure (9): Skin tissue of infected vaccinated subgroup IIIb showing normal skin with keratinizing stratified squameous epidermal covering (blue arrows) (H&E, X200).

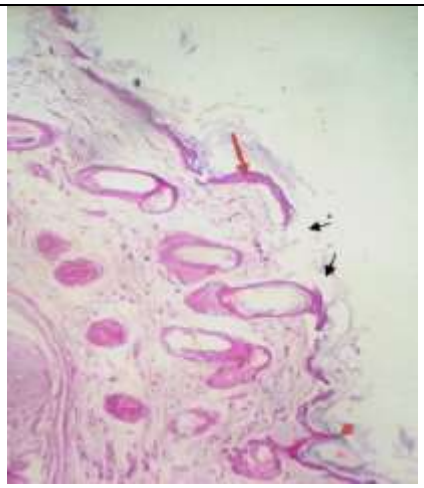

Figure (10): Skin tissue of infected vaccinated subgroup IIIe revealed atrophic skin (red arrows) with focal ulceration (black arrows) (H&E, x100).

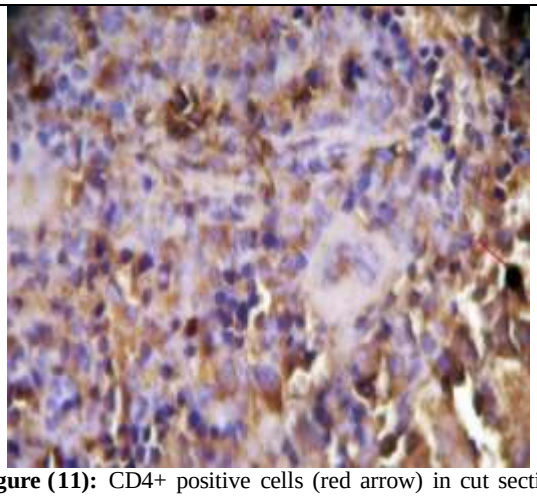

Figure (11): CD4+ positive cells (red arrow) in cut section of spleen tissue in non infected non vaccinated group (group I) (immune stain reaction of CD4, x400).

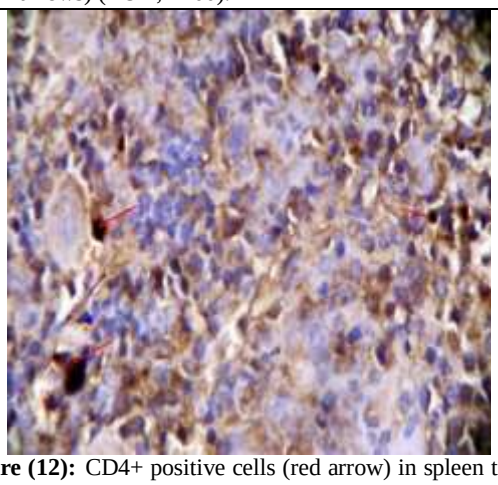

Figure (12): CD4+ positive cells (red arrow) in spleen tissue showing membrano-cytoplasmic expression of CD4 in infected non vaccinated group (group II) (immune stain reaction of CD4, x400).

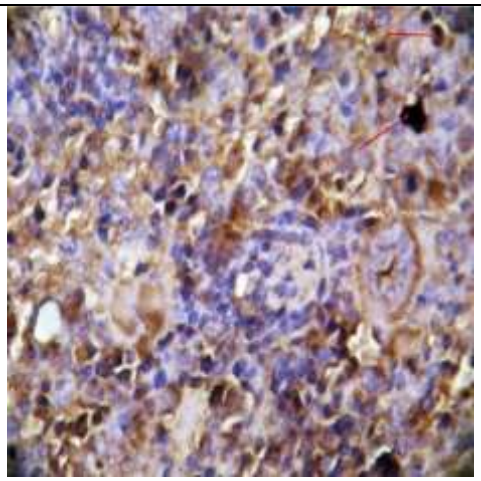

Figure (13): CD4+ positive cells showing membrano-cytoplasmic expression of CD4 (red arrows) in cut section of spleen in infected vaccinated subgroup IIIa (immune stain reaction of CD4, x400).

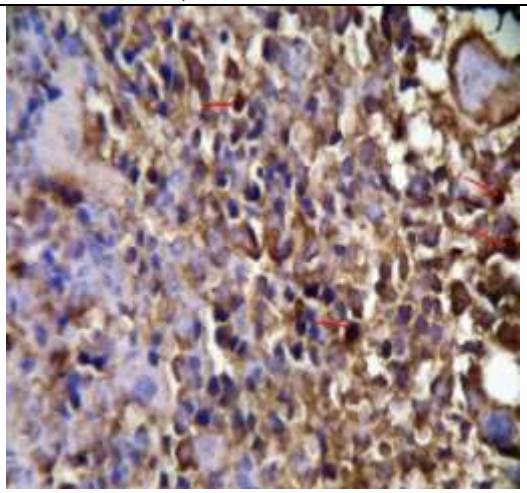

Figure (14): Spleen tissue of infected vaccinated subgroup IIIc showing CD4 expression in many cells (red arrow) (immune stain reaction of CD4x, 400).

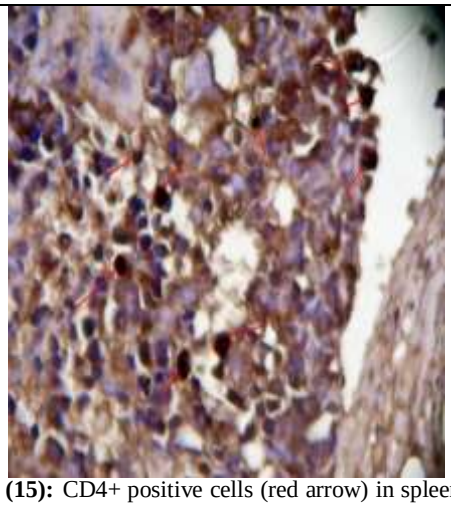

Figure (15): CD4+ positive cells (red arrow) in spleen tissue in infected vaccinated subgroup IIIf (immune stain reaction of CD4, x400).

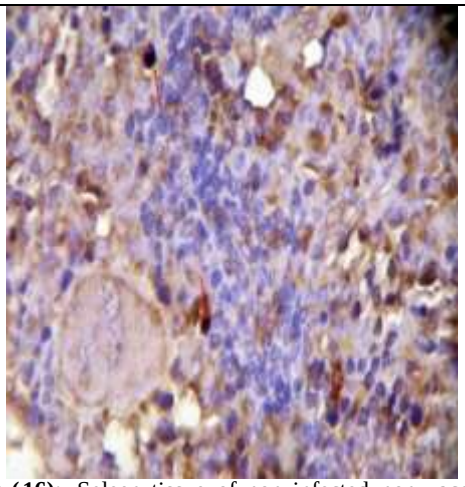

Figure (16): Spleen tissue of non infected non vaccinated group (group I) showing CD8 expression in many cells (red arrow) (immune stain reaction of CD8, x400).

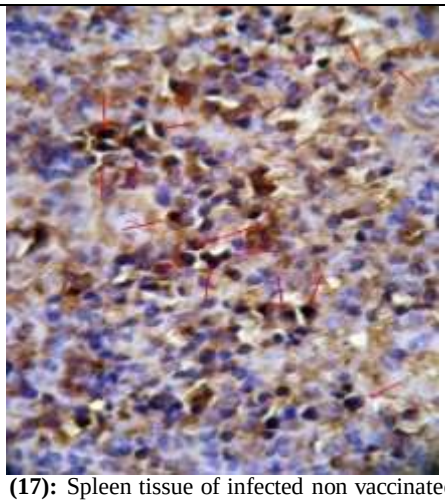

Figure (17): Spleen tissue of infected non vaccinated group (group II) showing CD8 expression in many cells (red arrow) (immune stain reaction of CD8, x400).

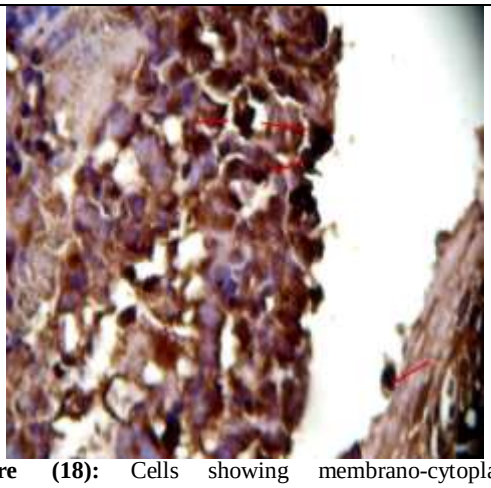

Figure (18): Cells showing membrano-cytoplasmic expression of CD8 (red arrows) in cut section of spleen tissue in infected vaccinated subgroup IIIa (immune stain reaction of CD8, x400).

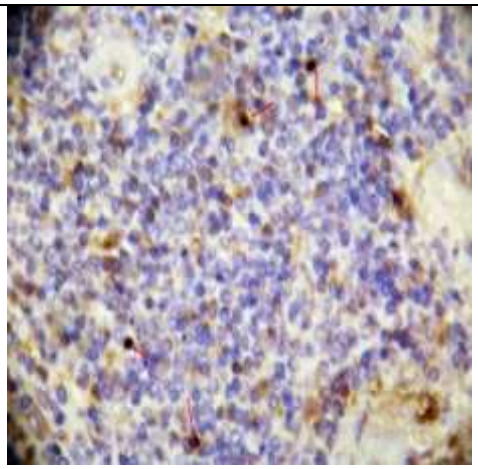

Figure (19): Cells showing expression of CD8 (red arrows) in cut section of spleen tissue in infected vaccinated subgroup IIIe (Immune stain reaction of CD8, x400).

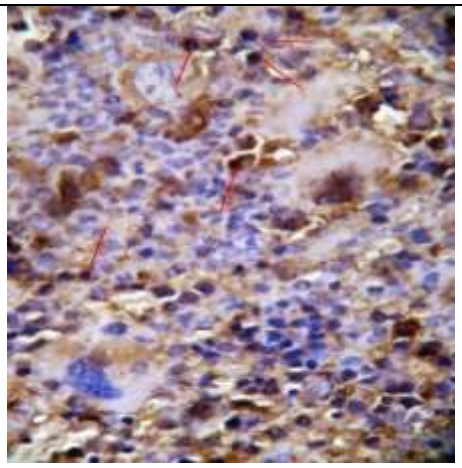

Figure (20): CD8 positive cells (red arrows) in spleen tissue showing expression of CD8 in infected vaccinated subgroup IIIf (immune stain reaction of CD8 , x400).

4. Discussion

Leishmaniasis is a major killer around the world. An effective vaccine can suppress the disease, but no licensed vaccine is currently available for the disease in spite of hard efforts made in this field (de Sousa Junior *et al*., 2015). Further work will not only improve current vaccination strategies but also will raise hope about developing new methods of vaccine production (Zand and Narasu, 2013). An ideal vaccine against leishmaniasis should fulfill some criteria such as being safe, healthy, cost-effective and able to create long-term immunity through minimum immunization. To develop a standard vaccine with such characteristics, it is necessary to identify proper antigens to be used in the process of production (Coler and Reed, 2005). It is striking that only few types of *Leishmania* proteins have been tested as vaccines, suggesting that many antigens remain to be identified for research. For instance, *Leishmania major* has a genome size which expresses about 8300 proteins, all of which are antigenic but not all of them were tested as vaccines (Herrera-Najera *et al*., 2009).

Unfortunately, there are many obstacles for developing a successful vaccine. One of these obstacles is antigenic identification which is considered as a significant barrier in vaccine design, as this is usually achieved through time consuming and labor intensive in vitro and in vivo experiments. Efforts have thus focused on developing novel strategies for more rational and faster antigen identification among large number of pathogen proteins (De Groot *et al*., 2005). An equally important consideration for the design and implementation of anti-parasite vaccines in general is the contribution of the genetics of the target host population and their susceptibility to infection and disease (Garg and Dube, 2006).

One of the most recent methods of recombinant vaccine is the application of epitope prediction for antibody production against antigen (Youssef *et al*., 2012). Vaccine prepared using this technique represents one of the most promising approaches to vaccine development as it allows reduction of time and ensures the availability of a vaccine with good efficacy (Zhou *et al*., 2011). T cell epitope vaccines have been tried against leishmaniasis and proved protective in a rodent model of infection (Das *et al*., 2014). Indeed, as far as we knew, the B cell epitope technique for vaccine development has not been used for preparation of *Leishmania* vaccine till now. Hence, the aim of the present work was to evaluate a new vaccine using different antigenic B cell epitopes. Evaluation was done by measuring serum IgG OD values, detecting histopathological lesions in spleen and skin and estimating CD4+ and CD8+ T cells counts in spleen tissue immunohistochemically.

Concerning serum IgG OD values in the present work, the most considerable findings are the presence of high statistical significant differences between groups I and II and between groups I and III ($p<0.001$). Also, the serum values were higher in subgroups IIIa and IIIb than group II with the presence of a high statistical significant difference ($p<0.001$). Comparing of subgroups IIIa and IIIb showed that difference between them was not significant ($p>0.05$). So, it could be said that they gave nearly similar results. Our results are in agreement with those of Zhou *et al*.(2011) who tested multiple linear B-cell epitopes of classical swine fever virus glycoproteins E2 expressed in *E. coli* as multiple epitope vaccine. Those authors reported that this vaccine provided adequate protection against the disease in pigs by producing high level of neutralizing antibodies. Also, Youssef *et al*. (2012) used successfully B cell epitope prediction technique for antibodies production against Potato leafroll virus. Those antibodies were rapidly produced, highly specific and cheap. The same authors also stated that the use of this method had eliminated the need to obtain large amounts of viral expressed protein or purified virus.

In addition, vaccines against *Streptococcus pyogens* infections (Pandey *et al*., 2013) and influenza virus (Sun *et al*., 2014) using B cell epitopes prediction gave better results than other types of vaccines. Ponomarenko and Regenmortel (2009) summarized that the B cell epitopes were considered as candidates for safe and inexpensive vaccines which can elicit antibodies that can efficiently neutralize the pathogen than do other vaccines.

However, serum IgG OD values of the current study were lower than those of Agallou *et al*. (2014) who analyzed four known antigenic *Leishmania infantum* proteins, cysteine peptidase A (CPA), histone H1, KMP-11, and *Leishmania* eukaryotic initiation factor (LeIF) for the prediction of T cell epitopes. They designed synthetic multi-epitope peptide in complete Freund's adjuvant as a vaccine for leishmaniasis in BALB/c mice. That higher serological immune response may be attributed to use of multi-epitope vaccine unlike the present work in which each individual epitope was used as a vaccine.

As regards histopathological examination results of spleen, it was found that the difference between the three main groups regarding hyaline changes was highly significant ($p<0.001$). The majority (83.3%) of mice related to group II showed hyaline changes while lower percentage rates (16.7%, 16.7%, 33.3% and 33.3%) were detected in subgroups IIIc, IIId, IIIe and IIIf, respectively. Noteworthy information is the absence of hyaline changes in all mice related to subgroups IIIa and IIIb, so it could be considered that epitopes "a" and "b" were the best regarding

prophylactic yield on spleen tissue. Difference between group II and all subgroups of group III was significant ($p<0.05$) while it was not significant among all subgroups of group III ($p>0.05$). The above mentioned findings concerning the currently used vaccine support the findings of Grimaldi *et al*. (2014) who tested clinical and parasitological protection in a *Leishmania infantum*-Macaque model vaccinated with adenovirus and recombinant A2 antigen. Those authors cited that histological changes of spleen tissue in infected non vaccinated group were more severe than those in the infected vaccinated one.

Concerning histopathological examination results of skin, it was found that the difference between the three main groups was highly significant ($p<0.001$) regarding skin lesions. The majority (83.3%) of mice related to group II showed ulcer in skin while only 16.7% showed atrophic epidermis. Subgroups of group III showed different degrees of improvement except mice related to subgroups "a" and "b" which were totally free. There was a high significant statistical difference between group II and all subgroups of group III ($p<0.001$) while there was no significant difference among subgroups of group III ($p>0.05$).

The above mentioned results were consistent with Iborra *et al*. (2003) who tested the acidic ribosomal protein of *Leishmania major* as DNA vaccine in BALB/c mice. The authors confirmed that pathological changes of skin tissue were predominantly detected in non vaccinated infected group. On the other hand, our results are not in accordance with those obtained by Carneiro *et al*. (2012) who evaluated the immune response of recombinant DNA cocktail *Leishmania* vaccine in BALB/c mice plus unmethylated synthetic oligonucleotides as immune adjuvants. They reported that this vaccine did not give protection against pathological changes with accumulation of dense and widespread inflammatory infiltrate containing infected macrophages. However, the same authors explained that this vaccine did not elicit a strong immune response due to its limited ability to stimulate macrophages and dendritic cells to synthesize cytokines as shown by lack of a significant increase in cytokine production in immunized mice.

Concerning immunohistochemical examination of spleen tissue, it was observed that CD4+T cells count was significantly higher in all subgroups of group III than group II ($p<0.001$) while the CD8+T cells was significantly higher in group II than all subgroups of group III ($p<0.001$). These results are in agreement with those of other investigators such as Zhou *et al*. (2011) who mentioned that the cells of the immune response in tissues of vaccinated infected group were mainly Th1 and Th2 cells. Peters *et al*. (2012) also tested recombinant *Leishmania* poly-proteins delivered in a stable emulsion with TLR 4 agonists as monophosphoryl lipid A or glucopyranosyl lipid A in C57BL/6 mice. They reported that the number of CD4+T lymphocytes was higher in vaccinated infected group than non vaccinated infected one. However, Iborra *et al*. (2003) tested the acidic ribosomal protein of *Leishmania major* as a DNA vaccine in BALB/c mice and Dey *et al*. (2013) tested the live attenuated *Leishmania* p27 gene knockout parasites in BALB/c mice. Those authors reported that CD4+ and CD8+T cells were equally represented in the immune response. This could be explained by the presence of extensive antigenic diversity in both vaccines. On the contrary, Giunchetti *et al*. (2008) evaluated the immunogenicity of a whole parasite vaccine as a promising candidate against leishmaniasis in dogs and reported that the immunization elicited a strong cellular reactivity and increase in T lymphocytes counts particularly the subpopulation CD8+T cells which were important for the control of tissue parasitism. This response may be due to non-specificity of this type of vaccine. Besides, other studies have shown that CD8+T cells share in the protective immune responses against *Leishmania* parasites. Those studies cited that these cells either contribute in the destruction of *Leishmania* infected cells by activating macrophages to oxidative burst via cytokines produced upon antigen stimulation (Tsagozis *et al*.,2003 and Diez *et al*., 2006) or regulating CD4+T cell mediated immune responses (Uzonna *et al*., 2004). Also, Stager *et al*. (2000) and Zand & Narasu (2013) reported that these cells were found to play a role in generation of memory response. Anyhow, it must be mentioned that controversy still remains concerning the route of activation of CD8+T cells in leishmaniasis, since *Leishmania* resides within the parasitophorous vacuoles of the macrophage and it is not clear how these cells present *Leishmania* antigens to CD8+T cells through major histocompatability complex (MHC) class I (Seyed *et al*., 2011).

Focusing on the previously mentioned results of the currently used B cell epitope vaccine as regards association of elevated ELISA IgG OD values with higher counts of CD4+T cells in most subgroups of vaccinated group than the non vaccinated infected one was explained by Dantas *et al*. (2014). They reported that B cell depletion leads to disease exacerbation, suggesting that B cells are necessary for the activity of T cells that mediate lesion healing; thus humoral immune responses may in fact play a role in mediating protective immunity against leishmaniasis.

Analysis of whole results clearly shows that subgroups IIIa and IIIb gave the best prophylactic yield on spleen and skin tissues as evidenced by

histopathological examination. Also, they gave the highest values of the serum IgG OD values by ELISA examination. This was supported by Gershoni *et al*. (2007) who elucidated that B-cell epitope-based vaccines allow the production of highly potent neutralizing antibodies which can intercept the microbe before it attaches to its target cell and thus inactivates and minimizes the tissue affection. This ability is based on the antibodies specific recognition of epitopes and the sites of the antigen to which antibodies bind.

In conclusion, the currently used new B cell epitopes vaccine proved beneficial in protection against leishmaniasis. Epitopes 239-247 DGMEGSCSG and 6-14 SWGANHYDG gave the best prophylactic yield as evidenced by detection of high serum IgG OD values together with the best resolution of histopathological changes in spleen and skin tissues when compared to the non vaccinated infected group. It seems that CD4+T cells expressed in spleen tissue in this study were related to Th-1 subset which proved protective against leishmaniasis. It is advised to apply these two promising epitopes in vaccine design against leishmaniasis in clinical trials.

Acknowledgment

The authors would like to express their gratitude to Dr. Noha El Kady (Lecturer of Pathology, Faculty of Medicine, Menoufia University) for her assistance in reading the pathological data in H& E sections and in reading results of immunohistochemical staining.

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5/16/2015