An evaluation of different vaccine models to protect mice from visceral leishmaniasis

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Abstract:

A soluble cocktail vaccine was prepared from sonicated promastigotes of five Iraqi *Leishmania* isolates with some adjuvants (BCG and Alum), was used experimentally to protect Balb/c mice against visceral leishmaniasis. Groups of mice were immunized with a soluble cocktail of antigens given in 75 µg /0.1 ml of phosphate buffer saline with booster at different intervals. Full protection was observed when mice were challenged one month post vaccination ; the challenged mice were rechallenged after six weeks. At the eighth week post challenge, the results exhibited complete resistance to the rechallenge (no parasites in culture media or impression smears of liver and spleen were noticed). A long run follow-up was performed. Five of the twelve vaccinated mice developed infection, which resolved by 16 weeks. Significant cellular and humoral response (Delayed hypersensitivity, Immunoglobulin levels, Lymphocyte subsets, Cytokine levels, Eosinophil cationic protein and Macrophages migration inhibition) to *Leishmania donovani* were demonstrated in all of the groups that were subjected to 75 µg of antigens. Protein bands of crude antigens of *Leishmania* isolates by SDS - PAGE and Western blotting and the specific protein bands for leishmanial cocktail was determined to be 18 k Da. Results of vaccination with 75 µg antigens and booster doses revealed that immunization against *L.donovani* could be a practical method of protection from visceral leishmaniasis.

Key words: Mice, *L.donovani*, Vaccine, Adjuvants, Cytokines, CD – Marker, SDS –PAGE , and Western blot.

تقييم مختلف انماط تلقيح لحماية الفئران من داء اللشمانية الحشوية

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الخلاصة:

تم تحضير لقاح من الخليط الذائب (المجانس بواسطة الامواج فوق الصوتية) للطور الامامي السوط لطفيلي اللشمانيه الحشوية لخمس عزلات عراقية مع الاستعانة ببعض المساعدات (ب . س . ج. و الشب) حيث أستخدم تجريبيا ً لحماية الفأر الامهق (البالب/ سي) من خمج اللشمانية الحشوية. مُنعّت الفئران عن طريق تجريعها بخليط المستضد الذائب بجرعة مقدارها 75 مايكروغرام0.1/ من

الملح الداريء بجرعات معززة لفترات زمنية مختلفة. أظهرت النتائج وجود حماية تامه للفئران التي تم أخماجها بعد مرور شهر واحد من التمنيع. اعطيت جرعة تحدي ثانية بعد ستة أسابيع من جرعة التحدي الاولي، ومن خلال تشريح الفئران بعد ثمانية أسابيع وجد ان الفئران في حالة مقاومة تامة للخمج وتم التأكد من ذلك من خلال خلو مستنبتات ومسحات الكبد والطحال من الطفيلي . ومن متابعة حيثيات الخمج لفترة أطول نوعما (16 أسبوعاً) فقد لوحظ أن خمسة فئران من مجموع 12فئراً ممنعاً قد تبددت مناعته. من خلال دراسة المناعة الخلوية والخليط كانت هناك فروقات معتد بها لمجاميع الفئران الممنعة وتشمل فرط التحسس الآجل والكوبيوليينات المناعية وجمهرة اللمفاويات والمحركات الخلوية (السايتوكينات) وقيم البروتين الموجب للحمضات وتثبيط هجرة البلاعم ضد أخماج اللشمانية الحشوية في الفئران التي تعرضت الى جرعة 75 مايكرو غرام من المستضد. أن تشخيص الوحده البروتينية 18كيلو دالتن من قبل الاضداد الخام في مصول الفئران المخمجة عند أستخدام تقنية هلام البولي أكريلمايد و الوصمة المناعة يعد بياناً هاماً لخمج اللشمانية الاحشائية وممكن ان يكون اللقاح طريقة فعاله للحماية من خمج الطفيلي.

Introduction:

Leishmania is a protozoan parasite and the causative agent of leishmaniasis. Drug treatment is expensive, and drug resistance is becoming increasingly common. Safe, effective, and cheap vaccine is needed; so many trials with different antigens of *Leishmania* species as a vaccine were introduced for this purpose [1]. The murine model has proven useful in standing the mechanisms of the immunological response to infection in visceral leishmaniasis (VL) [2]. Antileishmanial response is conferred by T-helper type Th1 cells while the susceptibility is conferred by Th2 cells . Th1 cells secrete IL-2 and IFN – γ but Th2 cells secrete IL- 4, IL-5 and IL-10. It has been shown that IFN- γ activates macrophages to express $iNOS_2$, the enzyme catalyzing the formation of nitric oxide. Nitric oxide kills the intracellular amastigotes. In contrast, Th2 immune response limits the action of Th1 function via IL-10 and IL-4 which deactivate macrophages helping intracellular parasite growth and disease progression. Being a parasitic, *Leishm- ania* ensures its own survival by modulating host immune system either by inducing immunosuppression or by promoting pro- parasitic host function. A detailed knowledge of this host parasite interaction would help in designing prophylactic and therapeutic strategies against this infection [3]. [4] reported that, immunity can be elicited against *Leishmania donovani* by subcutaneous immunization of mice with killed promastigotes but adjuvant enhancement is necessary. [5] prepared and used a soluble cocktail vaccine, which showed good capability of inducing protective immunity against visceral leishmaniasis, but higher protection is needed. [6] reported on the use of soluble antigen preparation from *L. donovani* that was used to capture specific IgG and IgM antibodies in VL patients, that can be detect them by immunodiagnostic tests, an

enzyme – linked immunosorbent assay (ELISA). Western blot demonstrating seropositivity and reaction to *Leishmania* species exoantigens and the murine samples gave a consistent pattern where immunodominant bands with estimated mobility appear at certain molecular weight. The clearance of *L. infantum* from the skin of the balb/ c mice was correlated with inflammatory response and the infiltration and activation of CD^+4 and CD^+8 T cell [2]. The inhibition of macrophages migration by lymphokines from activated lymphocytes has studied in detail and shows an approximate correlation within *vivo* immune status on the use of soluble antigen preparation from *L. donovani* [7]. The soluble cocktail antigens (SCA) was prepared from promastigotes of five stocks of *Leishmania* species provided evidence of a development of cellular immunity in Balb/ c against VL which showed significant delayed hypers- ensitivity response (DHR) as compared with control [8] . This report is an evaluation of soluble cocktail antigens (five Iraqi isolates of leishmanial promastigotes) which were initially introduced with known adjuvants giving in higher doses intraperitoneally and with booster at varying time intervals to prevent the subsequent challenge infection and capability of inducing protective immunity against VL. The immune response mechanism of the host to infection was evaluated to detect immunoglobulin M (IgM) and IgG antibodies, lymphocytes subsets (CD^+4 and CD^+8 T-cells), cytokines production (IFN-γ and IL-10) and tumor necrosis factor – alpha (TNF-α) and area percentage of macrophages migration. Evidence clearly indicates that this models system should provide the basis for future vaccine and pathogenesis studies of VL.

Materials and Methods:

Mice:

Male albino Balb/ c - mice each 8-10 weeks and weighing 20-25 gm(from the Animal House at Al-Nahrain University), were used both for the long – term maintenance of these parasites, through serial passage every 2 months, and as the source of the peritoneal macrophages used for the macrophage migration inhibition (MMI). All the mice had free access to laboratory chow and water. One hundred and twenty mice were divided into six groups of 20 animals each. To provide an easy source of parasites for the experiments, culture on semi- solid medium were set up, using blood samples from infected mice , incubated at 26 ℃, and sub – cultured every 15 days

Parasite strains and in *vitro* cultivation:

Leishmania promastigotes were maint- ained and grown as described by [9]. Five Iraqi isolates were used , obtained from Al- Nahrain College of Medicine , Baghdad/ Iraq. In the present study, *Leishmania* promastigotes of five Iraqi isolates

(visceral and cutaneous) were obtained from different parts of Iraq. In addition to the non – human *Leishmania* sp. (FM 50) which isolated from the fore-gut of the sand fly, *Sergentomya baghdadis*. All these isolates are infection to animals.

1. *L. donovani* (MHOM/IQ/1982/ BRC1 – AA3).
2. FM 50 (IBAG/ IQ/ 1982/ Kal – Irq2).
3. *L. tropica* (MCAN/ IQ/ 1982/ Kal – Irq).
4. *L.major*(MHOM/IQ/1986/ TRC2).
5. *L. donovani* (MHOM/ IQ/ 1990/ RRLL45).

The soluble cocktail antigen was prep- ared from promastigotes of five stocks as described by [10]. Protein concentration was estimated according to [11], and used for immunization of Balb/ c mice . An Iraqi isolate of *L. donovani* (MHOM/ IQ/ 1982/ BRC1 – AA3) was used for infection experiments.

Media Semi – solid medium:

The semi-solid mdium described by [12] was supplemented with antibiotics and used for the routine maintenance of the parasite strains during the study periods.

Biphasic medium:

The biphasic medium employed was a slight modification of Novy – Mac Neal –Nicolle (NNN) medium – one of the first media developed for cultivating *Leishmania* and still widely used [13].

Liquid medium:

The liquid medium used was a modif- ication of RBLM described by [8], itself a modification of the semi – solid medium of [11].

Production of promastigotes:

Promastigotes of *Leishmania* species, from maintenance cultures on semi – solid medium, were inoculated into 100 ml – sterile containers each containing 10 ml liquid medium. These cultures were incubated at 26 °C in an orbital incubator (Sanyo Gallenkamp, Loughborough, U.K.) and checked every 2 days for parasite multiplication and any unwanted contamination. Additional liquid med- ium was added to produce log – phase cultures [8].

Preparation of the cocktail antigens:

The test was done as described by [8] as follows: the promastigote of different *Lieshmania* species were harvested from the liquid media by centrifugation at 1200 xg for 15 min at 4 ℃. The pellets were washed 3 times (1200 xg/ 15 min) in PBS, pH 7.2. The pellet were mixed with an equal volume of triton X – 100 (0.03%) and disrupted by sonication 3 times for 2 min each times, with a break of 30 sec in between the runs. This was done under cooling conditions. The sonicated promastigotes were centri- fuged at 1200 xg for 20 min at 4 ℃. The supernatant (crude antigens) was stored at -20℃ until used.

Aluminum Hydroxide Gel (Alum) Adjuvant:

Aluminum hydroxide gel preparation and the dosage was 50

mg/ ml of vaccine as described by [14].

Adjuvant BCG :

BCG, a freeze – dried preparation of a glutamate *bacille* Calmette – Guérin (BCG – Japan) vaccine, prepared from an attenuated strain of *Mycobacterium bovis* for intradermal use, was reconstituted with the saline provided by the vaccine 's manufacturer (Japan BCG laboratory, Tokyo), to give a suspension containing 10^6 bacteria/ ml. The dosage was 50 mg as described by [15].

Groups of animals:

Group(1): Mice were injected intraperi-toneally three times at 15 days intervals with 25 μg of autoclaved SCA/ 0.1 ml of phosphate buffered saline (PBS) at pH 7.5, receiving a total of 75μg of SCA and challenged subcutaneously one month after the last immunization 1×10^7 proma--stigote of *L. donavani* (BRC1 – AA3).

Group(2): Mice were injected and challenged as group 1, receiving a total of 75 μg of autoclaved SCA plus 50 μg of BCG.

Group(3): Mice were injected and challenged as group 1, receiving a total of 75 μg of autoclaved SCA and combination with 50 μg / ml of Alum.

Group(4): Mice were injected and challenged as group 1, receiving a total of 75 μg of autoclaved SCA and combination with Alum and BCG as in group 2 and group3.

Group(5): Received PBS alone as nor-mal control.

Group(6): Mice were injected subcuta-neously with 1×10^7 promastigotes of *L. donovani* (BRC1- AA3) and served as infected control.

Ten animals of each group were sacrificed six weeks post challenge, at the same time remaining animals for group 1-5 were rechallenged, subcutaneously with 1×10^7 promastigotes of BRC 1 – AA3. Eight weeks post rechallenged, animals were sacrificed by neck dislocation. In both challenged and rechallenged animals, spleen and liver were checked. Small piece of spleen and liver were homogenized and cultured in semi – solid medium for the detection of leishmaniasis promastigotes by culture microtitration method. Impr- ession smear slides were also prepared as fix slide and stained.

Macrophage Migration Inhibition(MMI):

The production of macrophages by [16] requiring culture of peritoneal exudate from normal and immunized mice with 75 μg of SCA /0.1 PBS and challenged with 1×10^7 promastigotes of *L. donovani* (BRC1-AA3). Mice were injected with 2 ml of 2% starch intraperitoneally and killed 3 days later with ether. Under aseptic conditions, 2 ml RBMI – 1940 medium with 10 % fetal bovine serum and heparin (5 IU /ml)was injected intraperitoneally into each mouse.

After massaging the abdominal area of the mouse, the injected

solution, which contained many macrophages, was collected, via a small abdominal incision, into a sterile Petri dish. The peritoneal washings from three mice were pooled in a sterile silicone – coated tube, which was then centrifuged for 10 min, at 4 ℃ and 800 ×g. The supernatant solution was discarded so that the cell pellets could be resuspended in tissue culture medium. The suspension were then centrifuged down, as before, washed three times with the tissue medium and finally resusp- ended in the tissue medium. After a dye – exclusion test (using 0.4 % trypan blue) [12] to determine the percentage of the cells that were viable, the suspension was diluted with tissue medium to give 2×10^5 viable cells/ ml. Capillaries were filled with cell suspension and one of ends was sealed with softened paraffin wax, then centrifuged at 150 × g for 5 min at 4 ℃, and they were cut just to the cells side of the cell – medium interface. All the capillaries were fixed on the bottom of the chambers with silicone grease (chambers were filled with medium and different SCA dilutions). The chambers were sealed with glass covers slip and incubated at 37 ℃ gassed with 5% CO2 in air for 48 hours. The degree of migration inhibition is calculated as follows

$$\%\text{Migration} = \frac{\textit{Area of the migration of immune cells}}{\textit{Area of the migration of normal cells}} \times 100$$

Culture microtitration

Culture microtitration method was used to determine the parasite burdens in homogenized liver and spleen of mice according to [17].

Delayed Hypersensitivity Response:

DHR was estimated by footpad swelling, with a caliper gauge 24 hours after subcutaneous injection of 10 mg protein (SA) of BRC1-AA3 strain , in 0.1 ml PBS (Right footpad). The left footpad served as control and was injected with 0.1 ml PBS. Experiments were done at six weeks post challenge and eight week post rechallenge.

Enzyme immunoassay for quantitative determination of antibodies.

This assay was done by using ELISA Kit (Vircell, S. L. Spain) as recommended by the manufacturer.

Determination of cytokines Interferon - γ and interleukin–10

Serum levels of IFN - γ and IL – 10 were measured by means of enzyme immunoassay using ELISA kits (Mabtech AB, sweden) as recommended by the manufacturer**.**

Tumor necrosis factor - α (TNF-α):

Serum levels of TNF - α was measured of enzyme immunoassay using ELISA kit (Mabtech AB, Sweden) as recomm- ended by the manufacturer.

Eosinophil Cationic Protein (ECP):

Serum levels of ECP were measured by sandwich ELISA (MBL, Japan) as recommended by manufacturer .

Analysis of lymphocyte subsets:

Blood samples were taken from three mice in each group by cardiac puncture. Lymphocytes separation were according to the isopaque – Ficol technique originally described by [18] . A dye – exclusion test was used (using 0.4 Trypan blue; [12]) to determine the percentage of the cells that were viable. Cells counting were calculated by counting the cells in hemocytometer, and the cells number was adjusted to give 2×10^6 viable cells/ ml. Detection of CD-antigen by means of indirect fluorescent antibody test (IFAT) kits (Immunotech Abeckman Coulter Company, France) as recommended by the manufacturer, 45 µl of lymphocyte suspension was transf-erred in tube and 5 µl of monoclonal antibody (CD 4 and CD 8) was added, well mixed and incubated at 2-8 ℃ for 30 min , then lymphocyte supernatants was centrifuged two times at 400×g for 5 min and the supernatant was aspirated and discard and cell pellets was resuspended in PBS /BSA. Fifty ml of fluorescent conjugate (diluted 1:80 in PBS/ BSA) was added and incubated for 30 min at 2-8 ℃ in the dark. Wash was repeated as in the step 2. The cell pellets were resuspended in 200 ml of PBS/ BSA, a drop was delivered by Pasteur pipette and placed in the center of a clean slide with coverslip were examined under 40 - magnification of a fluorescent micro- scope, their dark green staining identified positively labeled cell; 200 cells were counted to determine percentage of reactivity of the tested monoclonal antibodies.

SDS -PAGE and Western blotting (WB):

Western blotting:

Preparation of the parasite antigen:

The test was done as described by [8] as follows: The promastigotes of different *Leishmania* species were harvested from the liquid media by centrifugation at 1200 xg for 15 minutes at 4 ℃ . The pellets were washed 3 times (1200 xg/ 15 minutes) in PBS, pH 7.2. The pellets were mixed with an equal volume of triton X-100 (0.03%) and disrupted sonication 3 times for 2 minutes each time, with sonicated promastigotes were centrifuged at 1200 xg for 20 minutes at 4 ℃. The supernatant (crude antigens) was stored at - 20 ℃ until used. The crude extract of *Leishmania* antigens were separated on a horizontal SDS-polyacrylamide gel (SDS-PAGE), with a stacking gel of 4% and separating gel of 10% standards of known molecular weight were included, and the separated proteins were electrophoretically transferred to 0.45 µm nitrocellulose paper using semi-dry blotter [19].

SDS-PAGE electrophoresis:

This is one dimensional gel electrophoresis under denaturing conditions (in the presence of 0.1% SDS) which separates proteins based on molecular size.

Preparation of the SDS – gels

a. The separating gel :

The separating gel was prepared as follows:17.5 ml of 30% acrylamide/ 0.8% bisacrylamide + 13.125 ml of 4x tris. HCl/SDS pH 8.8 , and 21.87 ml of deionized D.W. was added .Then 175 µl of 10% ammonium persulfate and 35µl TEMED was added to the solution and gently swirled to mix. The glass plate sandwich was assembled, using two clean glass plates and two 0.75 mm spacers, and then the sandwich was locked to the casting stand. 52.5 ml of the separating gel with final acrylamide 10% was poured. Using a Pasture pipette , the separating gel solution was transferred to the center of the sandwich. Using another Pasture pipette, the top of the gel was slowly covered with a layer (~1 cm thick) of isopropyl alcohol by gently squirting the isopropanol against the edge of one of the spacers, and then the gel was allowed to polymerize 1 hour at ambient temperature.

b. The stacking gel:

The layer of isopropyl alcohol was completely poured off and the gel washed 3 times by D.W. The stacking gel was prepared by mixing 2.6 ml of 30% acrylamide/0.8% bisacrylamide solution, with 5 ml of 4x Tris. HCl/SDS pH 6.8, and 12.2 ml of deionized D.W. was added, then 100 µl of 10% ammonium persulfate and 20 µl TEMED. The mixture was gently swirled to mix and use immediately. Using a Pasture pipette, the stacking gel solution was slowly allowed to trickle into the center of the sandwich along the edge of one of the spacers. The stacking gel was allowed to polymerize 60 – 90 minutes at room temperature.

c. Loading the gel:

The protein sample was dissolved in 50 µl of 2xSDS / sample buffer at a concentration of 300 µg / ml, and boiled for 5 minutes at 100 ^{o}C. Using micropipette, the protein samples were loaded on the stacking gel using sample applicator. Then the wicks (Whatman MM filter paper) were soaked into electrophoresis buffer and attached to the stacking gel from one side and the separating gel from the other side. The electrophoresis apparatus was then covered.

d. Running the gel:

The power supply was connected to the cell and run at 50 mA of constant current. After the bromophenol blue tracking dye had reached the bottom of the separating gel, the power supply was disconnected. The total run time for gel was 6 hr. after disassembling the gel, the part of gel for detection of protein bands by coomassie blue staining was cut and separated from the other part of gel Western blotting, and placed in plastic box.

e. Coomassie blue staining:

The polyacrylamide gel that was placed in a plastic box, was covered by fixing solution for 2 hr. and agitated slowly on a shaker, then the fixing solution was poured out, and the gel was covered with coomassie blue solution for 1 hr. and slowly agitated. The staining solution was then poured out and the gel rinsed briefly with fixing solution, and covered with destaining solution over night, slowly agitated, and the destaining staining solution was then poured out (Fig .1)

Immunoblotting

The separated components in the gel were immediately transferred electrop- horetically onto nitrocellulose membrane (0.45 µm) with the use of transport apparatus and blotting buffer (pH 8). The procedure went as follows: A piece of Whatman 3 MM filter paper, cut to the same size as the gel and prewetted with electro-blotting buffer was placed on the blotter being near the anode, then a piece of cut, marked and wetted nitrocellulose membrane was directly placed on the filter paper facing on the anode, all air bubbles between the filter paper and the membrane were removed by gently pushing with gloved fingers. The gel was then placed on the membrane and the surface of the gel was moistened with electro-blotting buffer. Any air bubbles were also removed as above. Another piece of wetted Whatman filter paper was placed on the cathode side of the gel, also all air bubbles were removed, and then the cover of the blotter was applied. The current was adjusted so as to be 0.8 x surface area of the gel; transfer time is 3 hr. Following the blotting, the membrane was stained with 0.5% ponceau-s solution for five minutes and destained with distilled water for 2 minutes to reversibly stain the transferred proteins. It was completely destained by soaking in water for 10 minutes, then strips of nitrocellulose membrane were cut out longitudinally and incubated overnight with blocking buffer using constant agitation with rocking platform , to block non specific binding sites. Patient´s serum samples were diluted 1:50 in blocking buffer . The strips were incubated with the diluted serum for 1 hr., at room temperature using constant agitation with rocking platform , then non specifically bound primary antibody was washed away by washing for three times using PBS for minutes each wash.The bound antibody was detected by HRP-anti-IgG conjugate diluted 1:250 in blocking buffer. It was also incubated with the strips for 1 hr. at room temperature using constant agitation with rocking platform, and then the strips were also washed 3 times by agitation with PBS , 15 minutes each time. Finally, the strips were stained with freshly prepared DAB (diamino benzidine) substrate solution (10 – 15 min.) and the reaction was

stopped by rinsing briefly with water (fig. 2).

Results:

In the present experiment , the kinetic of *L. donovani* infection was examined by culture microtitration: a sensitive method for quantifying parasite burdens (ama-stigotes) in tissue (liver and spleen) of infected mice, in addition to imprints method. Parasite burdens were determ- ined in 80 mice sacrificed in groups 1, 2, 3 and 4 after six weeks post challenge and eight weeks post rechallenge. Upon infection, at six weeks post challenge, only animals of group 6, (infected control) had positive cultures and smear and the same results for group 5 and 6 at eight weeks post rechallenge (tab.1:a). A long run follow-up was performed. An efficacy of 58.3% was observed in mice vaccinated against visceral lishmaniasis that five of the twelve vaccinated mice developed infection, which resolved by 16 weeks (tab. 1:b).

Table 1:a. Parasitic burdens in vaccinated Balb/ c mice, challenge and rechallenge with *L. donovani*.

Group no .of mice	**Six weeks post challenge**				**Eight weeks post rechallenge**			
	Liver		Spleen		Liver		Spleen	
	Mean no .of parasites by microtitration	No.of mice (+ve) by imprint method	Mean no . of parasites by microtitration	No.of mice (+ve) by imprint method	Mean no . of parasites by microtitration	No.of mice (+ve) by imprint method	Mean no . of parasites by microtitration	No.of mice (+ve) by imprint method
1	All negative				All negative			
2	All negative				All negative			
3	All negative				All negative			
4	All negative				All negative			
5	All negative				**$5\times10^{5}\pm2.5\times10^{3}$**	**3/3**	**$6\times10^{7}\pm4.5\times10^{2}$**	**3/3**
6	**$4\times10^{4}\pm5\times10^{2}$**	**3/3**	**$3.5\times10^{6}\pm4\times10^{2}$**	**3/3**	**$6\times10^{8}\pm1.2\times10^{4}$**	**3/3**	**$8\times10^{6}\pm1\times10^{3}$**	**3/3**

Table 1:b. Parasitic burdens in vaccinated Balb/ c mice, rechallenge with *L.donovani*.

Group no .of	Sixteen weeks post rechallenge			
	Liver		Spleen	
	Mean no . of parasites by microtitration	No.of mice (+ve) by imprint	Mean no . of parasites by	No.of mice (+ve) by imprint
1	negative	2/3	negative	2/3
2	negative	1/3	negative	1/3
3	negative	1/3	negative	1/3
4	negative	1/3	negative	1/3
5	$8\times10^{6} \pm 3\times10^{3}$	3/3	$8\times10^{8} \pm 3\times10^{2}$	3/3
6	$6\times10^{7} \pm 2\times10^{3}$	3/3	$5\times10^{5} \pm 4\times10^{2}$	3/3

The results of cellular immunity response in immunized animals were shown in Tab.2. At week six post challenge all groups revealed significant DHR in comparison with normal control and the highest value was seen in group 4 ($P< 0.05$). At week 8 post rechallenge, the groups 1 - 4 and 6 revealed signi- ficant DHR in comparison with control. Group 2 and 4 showed significant DHR in comparison with group 6 ($P< 0.05$).

Table 2: Footpad swelling test in Balb/ c with soluble cocktail antigens, challenged and rechallenged mice with *L. donovani*. Values are mean swelling thickness ±SE(mm).

Group	One month post immunization	Six weeks post	Eight weeks post
	Diameter in mm		
1	1.05 ± 0.9	1.28 ± 0.10	1.90 ± 0.10
2	1.13 ± 0.3	1.36 ± 0.80	1.95 ± 0.05
3	1.17 ± 0.2	1.40 ± 0.02	1.96 ± 0.15
4	1.27 ± 0.4	1.50 ± 0.02	2.04 ± 0.03
5	0.06 ± 0.6	0.06 ± 0.01	0.14 ± 0.15
6	0.17 ± 0.2	0.40 ± 0.30	1.20 ± 0.05

Humoral immunity was detected by ELISA (tab. 3). The level of IgG + IgM in immunized animals showed significant variation among different treated groups. At six weeks post challenge, the IgG + IgM titer was increased in immunized groups. In comparison, the control group showed significant differences with the other groups (1, 2, 3 and 4) ($P > 0.05$).

Table3: **Measurement of antibodies (IgM + IgG) means ± SE in Balb/ c mice immunized with soluble cocktail antigens of *L. donovani* .**

Group no. of mice	Antibody values		
	One month post immunization	Six weeks post challenge	Eight weeks post rechallenge
1	1.634 ± 0.24	1.965 ± 0.04	2.274 ± 0.09
2	1.987 ± 0.46	2.293 ± 0.14	2.373 ± 0.20
3	2.332 ± 0.25	2.560 ± 0.5	2.830 ± 0.13
4	2.497 ± 0.25	2.672 ± 0.4	2.990 ± 0.05
5	0.158 ± 0.85	0.136 ± 0.02	1.946 ± 0.4
6	1.639 ± 0.26	1.832 ± 0.6	986 ± 0.4 1.

Interferon–gamma (IFN - γ).

A significant increase ($p< 0.05$) of sera IFN - γ was recorded in challenged and rechallenged mice (group 1, 2, 3 and 4) in comparison with that control (group 5). The highest value for IFN - γ appeared in group 4 (1.44.2 ± 2.0) in comparison with other groups (tab .4).

Interleukin – 10 (IL – 10).

A significant increase ($P < 0.05$) of sera 1L – 10 was recorded in challenged and rechallenged mice (group 1,2,3 and 4) in comparison with that control (group 5). The highest value for IL – 10 appeared in group 4 (243 ± 5.7) in comparison with other groups (Tab.4).

Tumor necrosis factor (TNF - α)

A significant increase ($P < 0.05$) of sera TNF - α was recorded in challenged and rechallenged mice (group 1, 2, 3 and 4) in comparison with that control (group 5). The highest value for TNF - α appeared in group 4 (71.6 ± 5.7) in comparison with other groups (tab .4).

Table 4: **Mean cytokines concentration ± SE (pg/ml) in Balb/ c mice with soluble cocktail antigens, challenged and rechallenged mice with *L. donovani* .**

Group no. of mice	One month post immunization			Six weeks post challenge			Eight weeks post rechallenge		
	IFN- γ	**IL – 10**	**TNF - α**	**IFN- γ**	**IL – 10**	**TNF - α**	**IFN- γ**	**IL – 10**	**TNF - α**
1	99.2 ± 6.7	168 ± 5.6	44 ± 1.8	110.3 ± 3.4	198 ± 7.6	52.6 ± 1.3	201 ± 1.8	210 ± 3.7	66 ± 3.7
2	108 ± 2.0	186 ± 4.5	48 ± 4.3	123.1 ± 2.6	4.8 ±208	58.9 ± 2.9	220 ± 4.5	236 ± 5.6	71 ± 2.3
3	122 ± 1.2	193 ± 2.7	53 ± 2.7	137.4 ±7.8	217 ± 3.4	63.7 ± 4.8	225 ± 3.2	245 ± 7.9	78 ± 1.8
4	133.2 ± 9.6	206 ± 1.5	68 ± 2.5	144.2 ± 2.0	234 ± 5.7	71.6 ± 5.7	242 ± 6.1	280 ± 8.1	85 ± 3.7
5	3.22 ± 3.4	4.9 ± 1.2	8.4 ± 2.8	3.55 ± 1.2	5.0 ± 6.3	13.2 ± 2.3	112 ± 4.6	186 ± 9.8	62 ± 3.8
6	102 ± 6.2	179 ± 2.8	52.3 ± 4.6	115.2 ± 4.6	202 ± 1.5	56.8 ± 2.4	135 ± 1.1	290 ± 3.2	79 ± 3.1

Table 5: Mean percentage ± SE of lymphocyte subsets of Balb/ c mice injected with soluble cocktail antigens, challenged and rechallengedmice . with *L. donovani*promastigotes.

Group no. of mice	One month post immuniztion			Six weeks post challenge			Eight weeks post rechallenge		
	CD 4	**CD 8**	**CD 4 / CD 8 ratio**	**CD 4**	**CD 8**	**CD 4 / CD 8 ratio**	**CD 4**	**CD 8**	**CD 4 / CD 8 ratio**
1	38.7 ± 4.3	56.4 ± 2.1	0.68	43.0 ± 2.3	58.6 ± 3.6	0.73	40.8 ± 3.2	63.7 ± 3.2	0.64
2	37.6 ± 2.7	59.7 ± 1.3	0.62	42.2 ± 4.2	62.4 ± 2.8	0.67	38.4 ± 2.3	68.2 ± 1.8	0.56
3	32.1 ± 3.5	61.2 ± 2.5	0.52	38.3 ± 3.4	68.2 ± 2.3	0.56	36.7 ± 2.6	73.3 ± 2.4	0.50
4	30.4 ± 2.9	72.3 ± 1.4	0.42	36.1 ± 2.3	75.0 ± 1.6	0.48	32.0 ± 5.7	83.1 ± 5.7	0.38
5	49.8 ± 5.3	38.4 ± 1.6	1.29	52.4 ± 1.0	42.2 ± 0.8	1.24	30.2 ± 7.0	66.0 ± 6.5	0.45
6	36.3 ± 4.5	60.0 ± 3.8	0.60	40.3 ± 0.9	63.0 ± 3.3	0.63	38.3 ± 8.2	73.9 ± 4.4	0.51

T– helper/inducer lymphocytes:

A significant decrease ($P < 0.05$) of sera, CD4 was recorded in challenged and rechallenged mice (group 1, 2, 3 and 4) in comparison with that control (group 5). The lowest value for CD 4 appeared in group 4 (36.1 ± 2.3) in comparison with other groups (tab .5).

T–cytotoxic /suppressor lymphocytes:

A significant increase ($P < 0.05$) of sera CD8 was recorded in a challenged and rechallenged mice (group 1, 2, 3, and 4) in comparison with that control (group 5). The highest value for CD 8 appeared in group 4 (75.0 ± 1.6) in comparison with other groups(tab.5).

CD 4 / CD 8 ratio:

The CD4/ CD8 ratio was lower in visceral leishmaniasis mice (group 1, 2, 3 and 4) in challenged and rechallenged mice than that of control (group 5) (tab.5)

Eosinophil Cationic Protein:

There was an increase of ECP levels in the sera of mice , immunized and challenged with leishmanial proma- stigotes (group 1, 2, 3, and 5), when compared with that normal control (group 5). The highest value for ECP appeared in group 4 (36.8 ± 3.5) (tab. 6).

Table 6: Mean concentration (Pg/ ml) ± SE of Eosinophil Cationic Protein of Balb/ c mice injected with soluble cocktail antigens challenged with *L. donovani* promastigotes.

Group no.	one month post immunization	Six weeks post challenge
1	24.2 ± 5.3	28.3 ± 2.6
2	26.3 ± 2.5	30.6 ± 1.7
3	29.2 ± 3.4	32.0 ± 2.4
4	34.5 ± 4.6	36.8 ± 3.5
5	7.6 ± 4.7	8.9 ± 2.8
6	23.9 ± 3.2	27.0 ± 1.2

Macrophage Migration Inhibition:

The macrophage migration inhibition test was used in assessing the cellular immune response in all groups. The results of this parameter was found significantly positive in immunized mice (group 2, 3, 4 and 5) in comparison with negative control (group 1). The highest positive value of low macrophages migration appe- ared in group 5 (4.0 ± 2.7) (tab . 7).

Table 7 : Area percentage of macrophages migration obtaind from Balb/ c mice immunized with different soluble cocktail antigens (75 µg/ 0.1 ml) by intraperitoneal injection and challenged dose (1×10^7 promastigote) of *L. donovani* (BRC1-AA3) subcutaneously.

Group no.	Macrophage migration before		Macrophage migration after	
	Area ($mm)^2$	%	Area ($mm)^2$	%
1	12.56	100	12.56	100
2	9.6 ± 1.8	76.43	8.0 ± 3.2	63.69
3	8.4 ± 1.9	66.87	7.7 ± 2.5	61.30
4	6.3 ± 2.7	50.15	4.8 ± 1.9	35.82
5	5.4 ± 2.4	42.99	4.0 ± 2.7	30.84

Group (1): PBS only.

Group (2): Autoclaved SCA* only.

Group (3): Autoclaved SCA* plus BCG.

Group (4): Autoclaved SCA* plus Alum .

Group (5): Autoclaved SCA* with BCG + Alum .

***SCA: Soluble cocktail Antigens (leishmanial promastigotes).**

Table 8: The efficacy of each isolate administered separately.

No.	Isolates	Protective efficacy (%)
1	*L. donovani* (MHOM/ IQ/ 1982/ BRC1 – AA3)	10
2	FM 50 (IBAG/ IQ /1982/ Kal – Irq 2)	66
3	*L. tropica* (MCAN/ IQ /1982/ Kal – Irq	8
4	*L. major* (MOHM/ IQ /1986/ TRC2)	7
5	*L. donovani* (MHOM/ IQ/ 1990/ RRLL45)	9

Table 9:Parasitic burdens reduction produce from each of the adjuvant used either alone or in combination (without antigen) administrated separately into mice.

Adjuvants	Parasitic burdens reduction (%)
BCG	60
Alum	69
BCG + Alum	75
PBS	0.0

SDS – PAGE and Western blot:

The whole protein profile and mole- cular weight of different autoclaved cocktail of leishmanial promastigote were obtained in SDS – PAGE (Fig. 1). To facilitate the calculation of mole- cular weight these subunits, zymograph for different bands were done as shown in Fig.2. Molecular weight of each band was arranged according to their antigenic groups: 13, 18, 28, 30, 45, 53, 66 and 74 k Da. The serum IgG of the different antigens in WB was based on the banding patterns that appeared on nitrocellulose paper strips (Fig. 2). The results showed that sera from mice with active visceral leishmaniasis recognize numerous antigens with molecular weight: 18, 20, 28, 36, 45 and 53 k Da. Control strips did not show any banding pattern appeared on nitroce- llulose paper strips, that the sera which were applied in WB obtained from healthy mice were confirmed by the leishmanial promastigote was the most reactive one and 100 % of mice recog- nized this band.

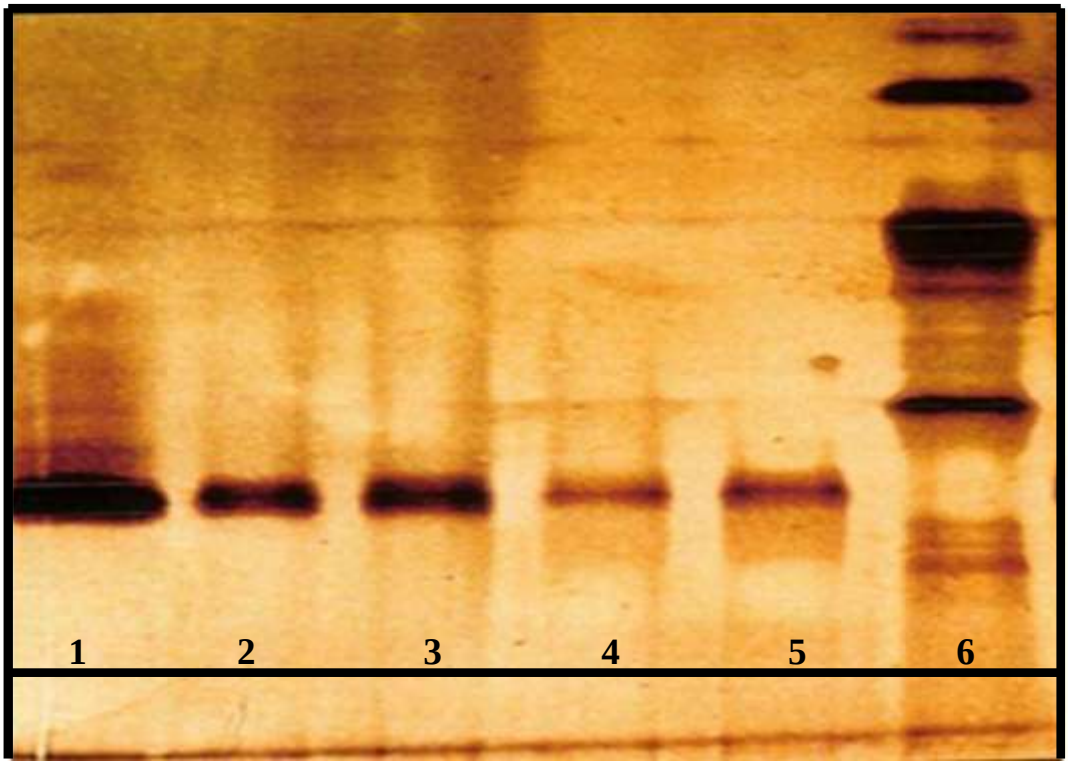

Fig. (1): Electrophoretic patterns photograph (Conventional Technique) of leishmanial promastigote.

Lane (1): FM 50 (IBAG/ IQ/ 1982/ Kal – Iraq2) antigens.
Lane (2): *L. tropica* (MCAN/ IQ/ 1982/ Kal – Irq) antigens.
Lane (3): *L. major* (MHOM/ IQ/ 1986/ TRC2) antigens.
Lane(4): *L. donovani* (MHOH/ IQ/ 1982/ BRC1 – AA3) antigens.
Lane (5): *L. donovani* (MHOM/ IQ/ 1990/ RRLL45) antigens.
Lane(6): The soluble cocktail antigens for Iraqi isolates.

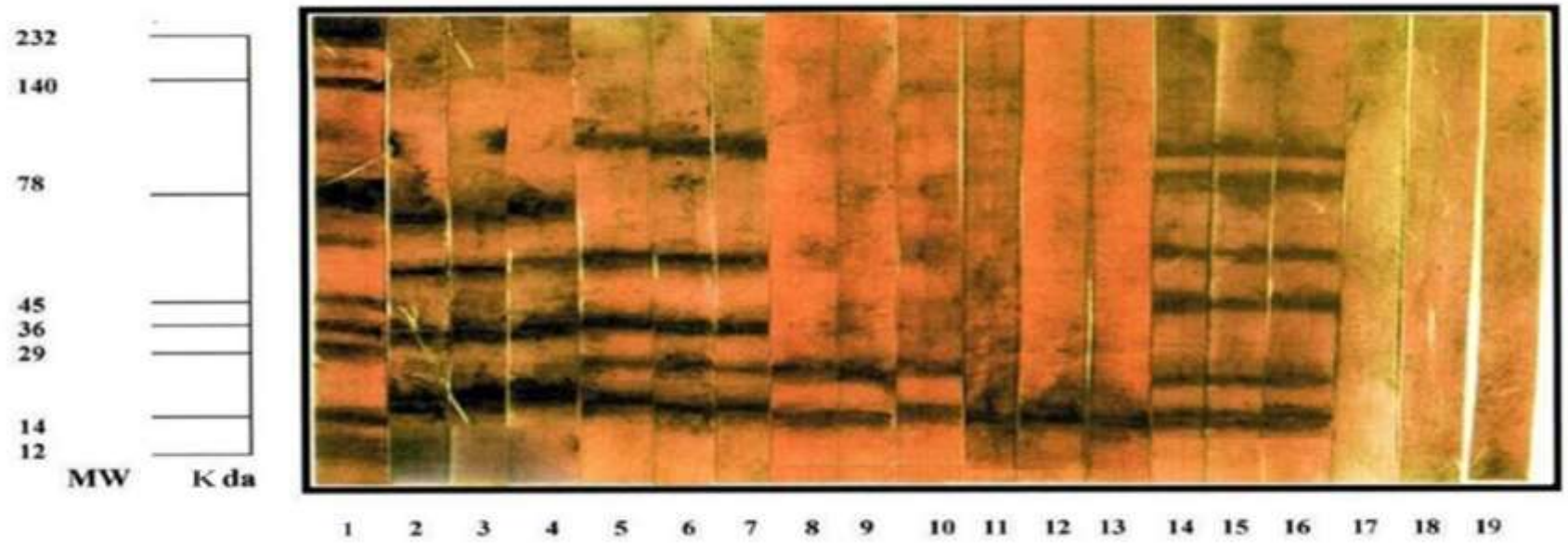

Figure (2): Description of different bands for standard molecular weights (SMS), Western blot of serum IgG.antbodies to . *Leishmania* antigens and control.

Lane (1) strip: standard molecular weights.
Lane (2,3 & 4) strips: *L. major* (MHOM/ IQ/ 1986/ TRC2)
Lane (5,6 & 7) strips: *L. tropica* (MCAN/ IQ/ 1982/ Kal – Irq).
Lane (8, 9 & 10) strips: *L. donovani*(MHOM/ IQ/ 1982/ BRC1 – AA3).
Lane (11, 12 & 13) strips: *L. donovani* MHOM/ IQ / 1990/ RRLL 45).
Lane (14, 15 & 16) strips: FM 50 (IBAG/ IQ/ 1982/ Kal – Irq2).
Lane (17, 18 & 19) strips: control (serum healthy mice).

Discussion:

The use of soluble cocktail antigens with some adjuvants (BCG and Alum) as a vaccine and the feasibility of using subcutaneously challenge to establish visceral infection, Balb/c mice and subcutaneously rechallenge infection with *L. donovani*, parasite burdens. Ana- lyse were performed at various times post infection in spleen and liver tissues. The parasite burdens decreased with time post infection and the elimination of parasites had occurred in challenged mice (Six weeks post challenge) and rechallenged mice (eight weeks post challenge). In the terms of prospective of a vaccine for VL, it is encouraging that, in general, protection persists and that the reduction (n-fold) in parasite burdens in vaccinated mice increase with time post infection. However, it should be noted that this protection does reach those found in vaccine studies of murine comparable times post infection. These results are constituent with those of previous studies [20] that demonstrated those mice vaccinated with the D-13 *L. donovani* antigens were better protected against *L. major* than against *L. dono- vani* infection. Taken together, these results suggest that the immunological mechanisms important in the control of visceral infection are adequate induced and/or activated by using the vaccination approaches that are effective inducing protection against cutaneous disease. Hence, further development vaccination methods directed toward the control of VL need to be considered. Complete protective effect of the vaccinated mice was reported of soluble antigens from Iraqi *Leishmania* isolates against *L. donovani* in highly susceptible Balb/ c mice, the efficacy and was evaluated for a very limited duration (8 weeks) post challenge a long run follow – up was performed, and an efficacy of 58.3% was observed in mice vaccinated against visceral leishmaniasis that the five of the twelve vaccinated mice developed infection, which resolved by 16 week, this resolve may be due to less develop Th1 responses against *L. donovani* and their macrophages do not secrete more IFN - γ or IL-2 in the presence of diminished antigens. However, these mice regularly have high titers of antileishmanial antibodies [21] that is their Th2 arm is activated and Th1 arm is downregulated, Th2 immune response limits the action of Th1 function via IL-10 and IL- 4, which deactivate macro- phages of appropriate immunity depends on the properties of antigen. Living vaccines have the great advantage of providing increasing antigenic chall- enge that lasts days or weeks and likely to contain the greatest number of antigens. The killed vaccine required more than one dosage to long lasting immunity, while live vaccines need one dose to do that [22]. In conclusion the possible long

lasting immunity required higher dose of vaccine was given at varying time.

The adjuvants were used in vaccines tested in Balb/ c mice model of visceral leishmaniasis through subcutaneous routes. The parasitic burdens reduction (%) produce from using each of the adjuvant used either alone or in combination (without antigen) admin- istered separately into mice are shown in table 9. The Alum + BCG treatment accounted for 75% of this protection. In our conditions, vaccination with Alum + BCG was superior to other treatments and had no toxic effect was found. It appears that the effected of adjuvants is due to mainly to two activities: the concentration of antigen in a site where lymphocytes are exposed to it (the 'depot' effect) and the induction of cytokines which regulate lymphocyte function. BCG acts mainly by stimulating the formation of the appropriate cytokines. This theory is supported by the fact that cytokines themselves have been shown to be effective adjuvants, particularly when coupled directly to antigen [23]. A vaccine prepared from sonicated pormas- tigotes of non-human *Leishmania* Spe- cies, isolated from *Sergentomya baghdadis*, IBAQ/ IQ/ 1982/ Kal – Irq 2, was used expermentally to protect Balb/ c against visceral leishmaniasis. Exami- nation of culture media and impression of liver and spleen indicated that the vaccine immunized 66% of the mice (free from infection), this observation suggests that there was enhanced killing or suppression of amastigote prolife- ration. It may be speculation that agents such as sensitized lymphocytes process soluble factor (s), fostering the development of an immune response. In conclusions, the present study demon- strated that no human *leishmanial* antigens enhance Balb/c mice resistance (humoral and cellular immunity) to visceral leishmaniasis giving possibly, to some extent, considerable length of time. The efficacy of other isolates was only partial protection rate was achieve and indeed, the efficacy was evaluated for a limited role (fig. 2).

In delayed type hypersensitivity (DTH), Th1 cells, activated by soluble cocktail antigens of leishmanial promastigotes secrete several cytokines that lead to inflammation. In DTH the principal effector cells are macrophage, but many cells types participate [14].In the present work, SCA provided evidence of develo- pment of cellular immunity against visceral leishmaniasis, which showed significant DTH as compared with normal control. The highest DTH was in group 4 and remained so far a long period. [24] mentioned that lymphocytes activity affects the ability to generate macrophage activation lymphokines, as a key determination of acquired cellular resistance of visceral leishmaniasis. Concerning humoral immune response, antibodies showed variable

elevation levels after challenge and rechallenge. This elevation may be due to polyclonal activation of B-cells by leishmanial antigens that induces proliferation and differentiation of B-cells into plasma cells that secreted antibodies [25]. At eight weeks post challenge, antibody titer raised as shown in Tab.3. This obser- vation came in accordance with [26] who stated that, specific antibodies play an important role in the *vivo* expression of resistance against infection, in the present work, it is possible that the so called (lytic antibodies) involved in com plement – mediated lysis of parasites are the best target for protection from VL.

Leishmania donovani infection is known to induce endogenous secretion of IL-10 as a mechanism of parasitism because IL-10 seems to be responsible for inhibition synthesis of IFN $-\gamma$, the main macrophage – stimulating cytokine involved in the defense against *Leishmania* which facilitated the intracellular survival of parasite by down – regulating the oxidative and inflammatory response [27]. In fact in human severity of VL has been closely associated with increased levels of IL-10 and the use of anti IL-10 antibody to block the IL-10 activity or IL-10 receptor blockade can be effective approach for treatment of leishmaniasis [23]. A different observation of CD4 in VL, it was significantly lower than control. These results are in agreement with [28] who confirmed that acute and chronic VL cases are depressed in peripheral blood CD4 count. This lowering of CD4 may result from apoptosis as reported by [28] who suggested that CD4 derived from susceptible mice undergo rapid apoptosis, produce less IL-2 and IFN- γ and fail to mediate DTH. The role of CD8 T-cells against *Leishmania* infection was suggested by [30] who reported that CD8 T-cells were shown to be responsible for the conversion of susceptible Balb/c mice into resistant phenotype after depletion of CD4 T-cells against *L. major* infection. Despite the fact that CD8 T-cells also produce IFN- γ on activation and can directly destroy the infected macrophage [31]. Results showed that CD4/ CD8 ratio was significantly lower than control group; this may be considered as an index of immune suppression in VL patients. These results agree with [32] who reported that at diagnosis CD4 cells showed a significant decrease while CD8 cells had significantly increased when compared with control and CD4/ CD8 ratio was inverted.

The level of ECP was significantly raised in serum compared to the control group. This elevation was due to the attachment of parasites to the eosinophils induced their degranulation with the release of granules contents onto parasite surface causing its destruction. Ingestion of parasite by eosinophils, induced drastic morphological changes, and finally leading to

leucocytes lysis with liberation of intact granules. This fact may increase the extracellular parasite killing [33].

The activation of macrophages migration system through the secretion of migration inhibition factor (MIF). A factor released from lymphocyte exposed to antigen will prevent this migration by causing the macrophage to stick to – gether. This factor is likely to release also in *vivo* and may be responsible for the accumulation of macrophages in cell-mediated immune reaction and they serve three main functions (1) recruitment of uncommitted lymphocytes, (2) retention of such cells and phagocytes at the inflammatory sites and (3) activation of the retained cells so that they can take part in the inflammatory response [15]. These products can be through of as chemical messengers, IFN- γ , IL-2 and TNF- α , the sensitized macrophages play an important role in stimulation Th1 cells through the subsequent exposure to leishmanial antigens or secretion of IL-1 and IL-12 results in an inflammatory reaction at the infection sites [34].

In WB for different types of *Leishmania* antigens were used , IgG recognized especially low to medium molecular bands between 13 -17 k Da. Surveys of different parasites infections by SDS-PAGE and Western blotting [35] [36] have shown that some specific protein bands obtained from both human and animals´ sera have closer molecular weights. The differences in banding pattern may be due to different parasites antigens expressed in different host, or related to intraspecific variations of *Leishmania* parasite genome, host relationship to parasite and due to differences in laboratory procedures [37]. The results showed that 18 k Da subunit of *Leishmania* promastigote was the most reactive one and 100% of mice recognized this band. These polypeptide fractions had been identified as nuclear proteins of parasite [37]. The subunit 18 k Da was a powerful and good antigen for serology , because it contained a large spectrum of epitopes, which covered variations in the response among mice. However, it represents a complex structure, and there was always a balance between diagnostically relevant epitopes versus crossreactive ones, which sometimes compromises the specification of the system. In conclusion, this work was demonstrated for the first time that these antigen (SCA) facilitated the in *vivo* study and enhanced Balb/c mice resistance (cellular and humoral immunity) to visceral leishmaniasis giving possible long lasting immunity. The results of this work could be a practical method protection from visceral leishmaniasis.

Statistical Analysis:

The data were analyzed using the available soft – ware package. The results were presented as number,

percentage and mean ±SD whenever possible. The data was analyzed by using analysis of variance (ANOVA) test taking $P < 0.05$ as lowest limit significance. These manipulations are carried out according to statistical analysis system [38].

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