

Original Article

Immunological Effects of *Leishmania major* Secretory and Excretory Products on Cutaneous Leishmaniasis in BALB/c Mice

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(Received 24 Feb 2007; accepted 10 Jun 2007)

Abstracts

Background: To evaluate the immunological properties of *Leishmania major* excreted- secreted (E-S) products on the progress of leishmaniasis in susceptible BALB/c mice.

Methods: Promastigotes of the *Leishmania major* were cultured and E-S products were collected during the culture period. Groups of BALB/c mice (n= 12) were immunized with E-S products or whole antigen. Animals were challenged with promastigotes of stationary phase culture, then mortality was followed up to 6 months. In another group of animals drainage lymph nodes cells were removed and cultured for cytokines assay.

Results: Activity of acetylcholinesterase (AChE) and acid phosphatase (ACP) increased time dependently. Using SDS-PAGE two major protein bands of 110 and 75 kDa were seen on the gel. Wound diameter in group receiving 24 h E-S products was significantly lower than the other experimental groups ($P < 0.05$). During the first 4 months of the follow up no mortality was seen in this group, but mortality was started in the second months of the challenge in other groups. The IL-4 and IL-10 level in whole Ag group were significantly higher than the other groups. In cells from animals receiving 24 h E-S products the IL-2 level was significantly higher than the other experimental groups ($P < 0.05$). Also the IFN γ level was significantly higher both in 24 h E-S and whole Ag groups ($P < 0.01$).

Conclusion: The 24 h E-S group corresponded with small wounds, dominant Th1 cytokines response and low level of mortality.

Keywords: *Leishmania major*, Excretory-Secretory proteins, cytokines, Balb/C Acetylcholinesterase

Introduction

Protozoa of the genus *Leishmania* are obligatory intracellular parasites of the mammalian macrophages. They are transmitted to vertebrate hosts by sandfly vector of the genus *Phlebotomus*. Leishmaniasis is one of the most important public health problems world wide that causes a wide spectrum of diseases, including cutaneous, mucosal and visceral leishmaniasis (1). Each year 1.5 million new cases of cutaneous and 500,000 new cases of visceral Leishmaniasis are estimated (2). The disease is prevalent in many areas of Iran in which *Leishmania*

major and *L. tropica* are the primary causative agents of cutaneous leishmaniasis (3). Controls of vectors and reservoirs because of economic, environmental, or ethical considerations are not feasible or are impractical in most situations. Moreover the available drugs for the treatment of leishmaniasis are toxic, need multiple injections, have limited efficacy, and emerge drug resistance in some cases (4). Although vaccination with heat-killed *Leishmania* or recombinant proteins does not induce long-term immunity in humans (5, 6) recovery from natural or deliberate infection with virulent *L. major* on regions of the body, where resultant scarring is hidden,

induce lifelong protection (7, 8). First-generation vaccines, using crude antigens preparations from *Leishmania* promastigotes have been evaluated in human clinical trials, but have failed to confer adequate protection or good efficacy (9-11).

It has been shown that intact parasites are not needed to induce protection in susceptible BALB/c mice, opening the possibility of using defined molecules for vaccine development (12). During the past 15 years, it has become evident that some purified or recombinant proteins can induce effective immunity in animal models (13). New approaches have been investigated in the mouse model of experimental Leishmaniasis. Several *Leishmania* proteins are tentatively identified as candidate vaccines (14, 15). It is obvious that solid protection against a virulent challenge can be achieved with defined proteins; however, not all antigens protect against leishmaniasis in the mouse model, even though some of them induce a strong Th1 response (16).

Excretory and secretory molecules are released from parasite in all stages of the parasite life cycle, and may have immunomodulatory and protective effects. Promastigotes of various *Leishmania* species produce and release a variety of proteins during their growth *in vitro* (17). One way narrowing down the choice of candidate vaccines might be to select antigens that are highly expressed and secreted in the infective metacyclic promastigote stage of the parasite that are inoculated into the vertebrate host by the sand fly bite. The molecular basis of the parasites survival, within the phagolysosomal system of the human macrophages is poorly understood. This presumably mediated by properties of the surface membrane itself or factors released by the parasite into its immediate surroundings. In the latter regard, promastigotes from a variety of species of *Leishmania* release a soluble acid phosphatase activity during *in vitro* cultivation (18). Another important secretory protein in nematodes seems to be enzyme acetylcholinesterase. Different isoforms of acetylcholinesterase are encoded by multiple genes in nematodes, and

analysis of the pattern of expression of these genes in *Caenorhabditis elegans* suggests that they perform non-redundant functions. In addition, many parasitic species which colonise host mucosal surfaces secrete hydrophilic variants of acetylcholinesterase, although the function of these enzymes is still unclear (19).

The aim of present study was to evaluate the immunological properties of *Leishmania major* E-S products on the progress of leishmaniasis in susceptible BALB/c mice.

Materials and Methods

Acetylthiocholine iodide (ATC), 5, 5'- dithio bis (2-nitrobenzoic acid) (DTNB), 1, 5-Bis (4 allyldimethylammoniumphenyl) pentan-3-one dibromide (BW), fast garnet GBC and α naphthyl phosphate were obtained from Sigma-Aldrich. Cytokines assay kits was from Bender Med Systems (Vienna, Austria). All other chemicals were obtained from Merck (Darmstadt, Germany).

Parasite culture

L. major strain (MRHO/IR/75/ER) was maintained in BALB/c mice. Amastigotes were isolated from mice spleens, and then transformed to promastigotes in Novy-Nicolle-Mac Neal (NNN) medium supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml) and 20% heat-inactivated fetal calf serum (FCS) at 22 \pm 1 °C. Subsequently the third passage promastigotes from NNN medium were progressively adopted to RPMI 1640 media (GIBCO) supplemented with antibiotics, glutamine and FCS (complete medium).

Excretory and secretory products

E-S products were prepared from stationary growth phase of *L. major* (MRHO/IR/75/ER) promastigotes. In order to determine the growth phase of the populations, promastigote numbers were assessed daily using a Helber bacteriological counting chamber. Promastigotes were centrifuged at 3000 \times g, then cell pellet washed four times in serum free RPMI medium. Washed promastigotes inoculated into RPMI media (supplemented with antibiotics and glutamine but without FCS) to give a final density of 10⁸ cells/ml and then in-

cubated at 22 °C. Thereafter, at 0, 6, 12, 24, 48 and 72 h of incubation, the medium was removed, separated by centrifugation procedure at different centrifugal force (from 3000 to 25000 g) at 4 °C for 15 min. Then supernatant was examined through microscopy. Because of the motility of the parasite, supernatant was not free of parasite in any centrifugal force examined. Therefore 3000g was selected in all experiments and then supernatant was filtered through 0.22 micron Amicon filters to remove any remaining parasites. This filtrate was called E-S product and used for enzymes assay and gel electrophoresis. For animals immunization, the supernatant collected, filtered (0.22 µm-pore-size filter, Millipore) to eliminate promastigotes and concentrated approximately 100-fold by ultra filtration with a 3-kDa-cutoff filter unit (Pall). The concentrated filtrate was stored at -80 °C until use.

Enzymes and protein assay

Acetylcholinesterase (AChE) activity in E-S products was measured at 30 °C with a modified version of the spectrophotometric method of Ellman (20), using spectrophotometric kinetic assay. Activity was measured after a 10-min pre incubation of enzyme with DTNB in buffer to allow free sulfhydryls to react. Each cuvette contained; 1000 µl potassium phosphate, pH 7.4, containing 1.2 mM DTNB, 500 µl of culture filtrate and 100 µl acetylthiocholine chloride (ATC). The final concentration of phosphate buffer, DTNB and ATC in optimized assay was 50 mM, 0.6 mM and 1.25 mM, respectively. Increase in absorbance was recorded at 412 nm, in 30 second intervals up to 10 min. Two different blanks were included; one containing 10 µM BW for non-acetylcholinesterase hydrolysis of ATC, and one containing all components except cell culture filtrate in order to account for non-enzymatic reaction. Blanks reading were subtracted from all test samples reading. Enzyme activities are reported as the number of nanomoles of substrate hydrolyzed per minute at 30 °C.

Acid phosphatase (ACP) assay were performed in acetic acid- sodium acetate buffer pH 4.8, containing *p*-nitro phenyl phosphate as substrate

as described by Doyle and Dwyer (21). Assay contained 1000 µl buffer, 500µl culture filtrate and 100µl substrate. The final concentration of acetate buffer, and *p*-nitro phenyl phosphate in optimized assay was 50 mM, and 5 mM respectively. Absorbance was recorded at 405 nm, in 30 second intervals up to 10 min. Enzyme activities are reported as the number of nanomoles of substrate hydrolyzed to *p*-nitrophenol per minute at 30 °C.

Protein concentration was determined by the method of Bradford (22) using bovine serum albumin as a standard.

SDS and Native Poly acryl amide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure of Laemmli (23) in a 1 mm thick slab gel with 12% acryl amide in separating and 6% in stacking gels. Boiling with 5%, mercaptoethanol for 5 min reduced the disulphide bonds. Each lane was loaded with equal volume of various time-course culture filtrates (100 µl). Protein bands on the gel, was stained with silver staining protocols. For ACP native gel electrophoresis, samples were subjected to non-denaturing gel electrophoresis (6% polyacrylamide) at constant 30 mA at 10 °C. The ACP activity on the gel was detected using fast garnet GBC (1 mg/ml) and α naphthyl phosphate (1 mg/ml) in 0.1M sodium acetate buffer, pH 5.0.

Animals Immunization and challenges

Female BALB/c mice 6-8 wk old were obtained from Institute of Razi Tehran-Iran. Animals were housed in a colony room 12/12 h light/dark cycle at 21 °C and had free access to water and food. Baqiyatallah University of Medical Sciences, Ethical Committee Acts, approved the study.

Promastigotes whole antigen (Whole Ag) was prepared by washing promastigotes three times in cold PBS (pH 7.5). The suspension was frozen and thawed six times in PBS containing 0.1% Triton X-100, and then sonicated at 4 °C with 20 cycles at 2-s blasts. Four groups of BALB/c mice (n=12) were chosen in a double-blind randomized fashion and assigned to one of follow-

ing experimental groups: group receiving 0 h cell culture filtrate (0 h E-S), group receiving 24 hour cell culture filtrate (24 h E-S), group receiving whole promastigote cells homogenate (whole Ag) and control group receiving RPMI without any cell culture filtrate (Control). Final protein concentration in E-S products and whole promastigotes homogenate was adjusted to 50 µg/100 µl for all group except control group, which was without any protein. All samples were mixed 1:1 (v/v) with complete and incomplete Freund's adjuvant to make a homogeneous emulsified solution. Seven- to eight week-old female BALB/c mice were received two subcutaneous injections at a 2-wk interval. After 28 d of the first immunization all animals were challenged with 3×10^5 metacyclic promastigotes of stationary phase culture at the back near the tail. Animals weight, wound diameter and mortality were then weekly recorded.

Cell culture and cytokine assay

Another groups of BALB/c mice (n= 8) were assigned to the different experimental groups as mentioned above. Two months after the challenge with *L. major* promastigotes, these animals were sacrificed and draining lymph node cells were removed aseptically. Mononuclear cells were adjusted to 2×10^6 cells/ml in Dulbecco modified Eagle's medium containing antibiotics without any serum added. Cells were stimulated *in vitro* (37 °C in 5% CO₂) with 50 µg of 24 h E-S products. Supernatant were collected after 48 h of culture and assayed for IL-2, IL-4, IL-10 and IFN- γ using commercial mouse cytokine assay kits (Bender Med systems GmbH, Vienna, Austria). Absorbance values were recorded at 450 nm in an automatic microplate reader. Standard curves for cytokines, were performed by the use of supplied recombinant mouse proteins.

Statistical Analysis

Statistical analysis was performed with Sigma Stat (SPSS). Data are mean \pm S.E.M.; n= 12 animals per group. Groups comparison were assessed via analysis of variances (ANOVA) followed by the Dunnett's test, and t tests was used for other analysis. A $P < 0.05$ was considered significant.

Results

Excretory and secretory Products

The protein content of the culture filtrate was gradually increased up to 24 h and then reached a plateau (Table 1).

Activity of AChE gradually increased in culture supernatant and reached plateau at 24 h and then was almost constant during next 48 and 72 h (Table 1). However when activity was divided to protein concentration (specific activity), the specific activity was higher at 6 h period and then was significantly decreased in following hours (Fig. 1). There are two explanations for this phenomenon. First, it may be other proteins are secreted at later hours from cultivated promastigotes, so protein content is increased and enzyme activity remains constant but specific activity decreases. Second, enzyme are inactivated during longer time in the culture media.

ACP activity in culture filtrate showed similar pattern to the AChE activity, but both activity and specific activity were higher at 6 h period (Table 1 and Fig.1). Using optimized assay as described above, sodium tartarate (5 mM) as an inhibitor of ACP, inhibited more than 80% of enzyme activity (data not shown).

SDS and Native PAGE

Using denatured SDS-PAGE, the results showed no detectable protein band in 0 h E-S filtrate, but two strong protein bands corresponding to 75 and 110 kDa were seen in 24 h E-S products lane stained with silver nitrate. Three separate repeated experiments showed the same reproducible protein band (Fig. 2 A). In E-S filtrates from 48 and 72 h one extra weak band corresponding to 130 kDa was also appeared on the gel (Fig. 2 A). Because of lack of specific antibodies to *L. major* ACP protein band shown on the SDS PAGE (Fig. 2A), the native gel was done at low temperature (10 °C) without SDS. Results showed a single dominant activity band in 24 h E-S filtrate corresponding to ACP. The 0 h lane did not show any active protein band (Fig. 2B). This results confirms that 110 kDa protein band most probably corresponds to *L. major* ACP.

Animal chaleng with E-S antigenes

During 6 mo of the study there were no significant diffences in animal weight among different experimental groups. After 1 mo of promastigote inoculation (challenge) wound was induced and gradually increased in diameter in all experimental groups. Wounds diameter in group receiving 24 h E-S concentrate were significantly ($P < 0.05$) smaller than other experimental groups, and also stayed smaller during next 5 mo of study (Table 2). None of the animals showed complete cure. Animal mortalities were followed up to 5 mo after the challenge. Mortality was started in the second months in all study groups, except groups receiving the 24 h E-S and whole antigene. During first 4 mo of the follow up, no mortality was seen in 24 h E-S group, but after then mortality was occured in this group too. Total mortality in group B (24 h E-S) was two-fold lower than all experimental groups, although whole Ag group also had lower mortality rate (Table 3).

cytokine production

The cytokines levels in supernatants of cultured cells from different vaccinated groups of mouse receiving E-S products, whole antigen or RPMI alone were evaluated. The IL-2 content of cells supernatant of group receiving 24 h E-S concentrate and *in vitro* restimulated with 24 h E-S product was significantly higher than those of other experimental groups ($P < 0.01$). There were no significant differences between other experimental groups (Fig. 3A).

The concentration of IL-4 in cells supernatant from group which previously was challenged with whole Ag and incubated with 24 h E-S concentrate was significantly increased ($P < 0.01$) compared to the other groups (Fig. 4B). The IL-4 concentration did not show any significant differences in other experimental groups.

The IL-10 levels followed the same pattern as IL-4, significantly increased in whole Ag group comparing to the other experimental groups ($P < 0.01$). The IL-10 level was also increased in 24 h E-S group (Fig. 3C), but this increase was only significantly differed from control group ($P < 0.05$).

IFN- γ levels in supernatants of cells derived from vaccinated 24 h E-S and whole Ag groups (Fig. 3D) were significantly higher than the other experimental groups ($P < 0.01$). This increase in whole Ag group was comparatively higher than 24 h E-S group ($P < 0.01$).

Table 1: AChE and ACP activities and Protein content in *L. major* promastigotes culture filtrate

Time (h)	AChE activity (nmol/L)	ACP activity (nmol/L)	protein (μ g/ml)
0	403 \pm 202*	309 \pm 22*	1.3 \pm 0.05
6	1310 \pm 193	1060 \pm 18	5.2 \pm 2.19
12	1300 \pm 57	1040 \pm 70	6.3 \pm 1.70
24	1500 \pm 54	1020 \pm 54	9.3 \pm 1.30
48	1490 \pm 106	970 \pm 14	10.5 \pm 1.10

* Significantly lower from other hours cell culture filtrates ($P < 0.01$)

Table 2: Wound diameter (mm) in experimental groups chalenged with *L. major* promastigotes

Monthts/ Groups	0hr E-S	24 h E-S	WA	Control
First	1.75 \pm 0.7	1.3 \pm 0.7	3.1 \pm 0.9	1.2 \pm 0.4
Second	6.10 \pm 0.8	4.0 \pm 0.5*	7.2 \pm 1.0	7.4 \pm 0.8
Third	9.30 \pm 0.9	7.2 \pm 0.2*	10.8 \pm 1.3	10.8 \pm 1.0
Fourth	9.40 \pm 0.9	8.8 \pm 1.3*	14.3 \pm 2.4	13.6 \pm 1.7
Fifth	10.80 \pm 1.2	8.1 \pm 2.4*	10.9 \pm 1.1	13.2 \pm 1.6

N= 12 animals per experimental group, *. Significantly different from other experimental groups ($P < 0.05$)

Table 3: Mortality in the experimental groups chalenged with *L. major* promastigotes *

Monthts/ Groups	0hr E-S	24 h E-S	WA	Control
First	0	0	0	0
Second	1	0	0	2
Third	1	0	1	1
Fourth	2	0	1	1
Fifth	2	3	3	2
Total death	6	3	5	6

*.After challenge with *L. major* Promastigotes, mortalities were recorded for animals up to five months. N= 12 animals per experimental group

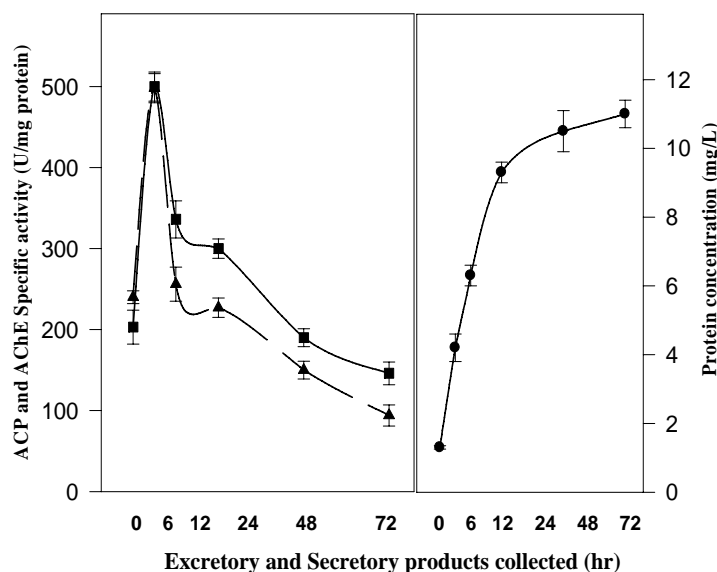


Fig. 1: Left panel shows time- course of AChE and ACP specific activities in cell culture filtrate. Squares (■) are AChE and triangles (▲) ACP specific activity. Right panel shows protein content (●) versus time in cell culture filtrate. Promastigotes culture supernatant was removed at different time and filtered. Enzymes and protein content were measured, as described in the materials and the method section.

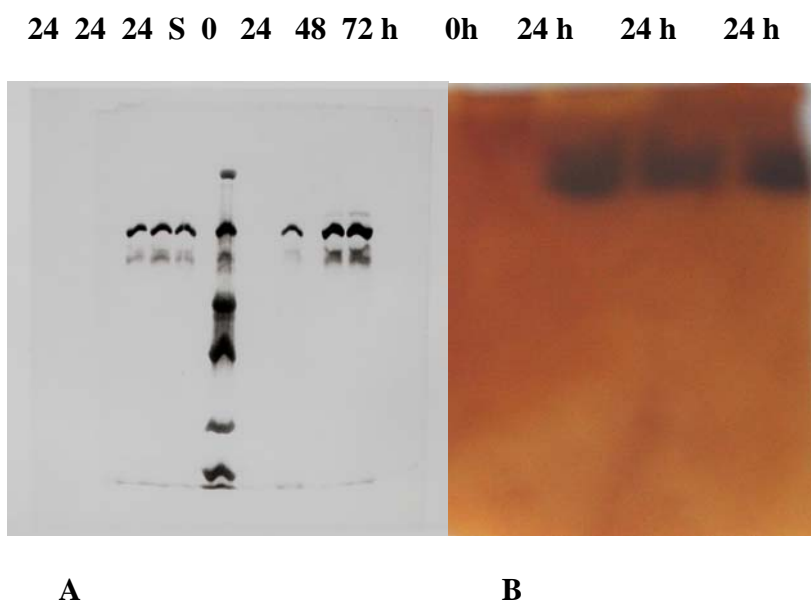
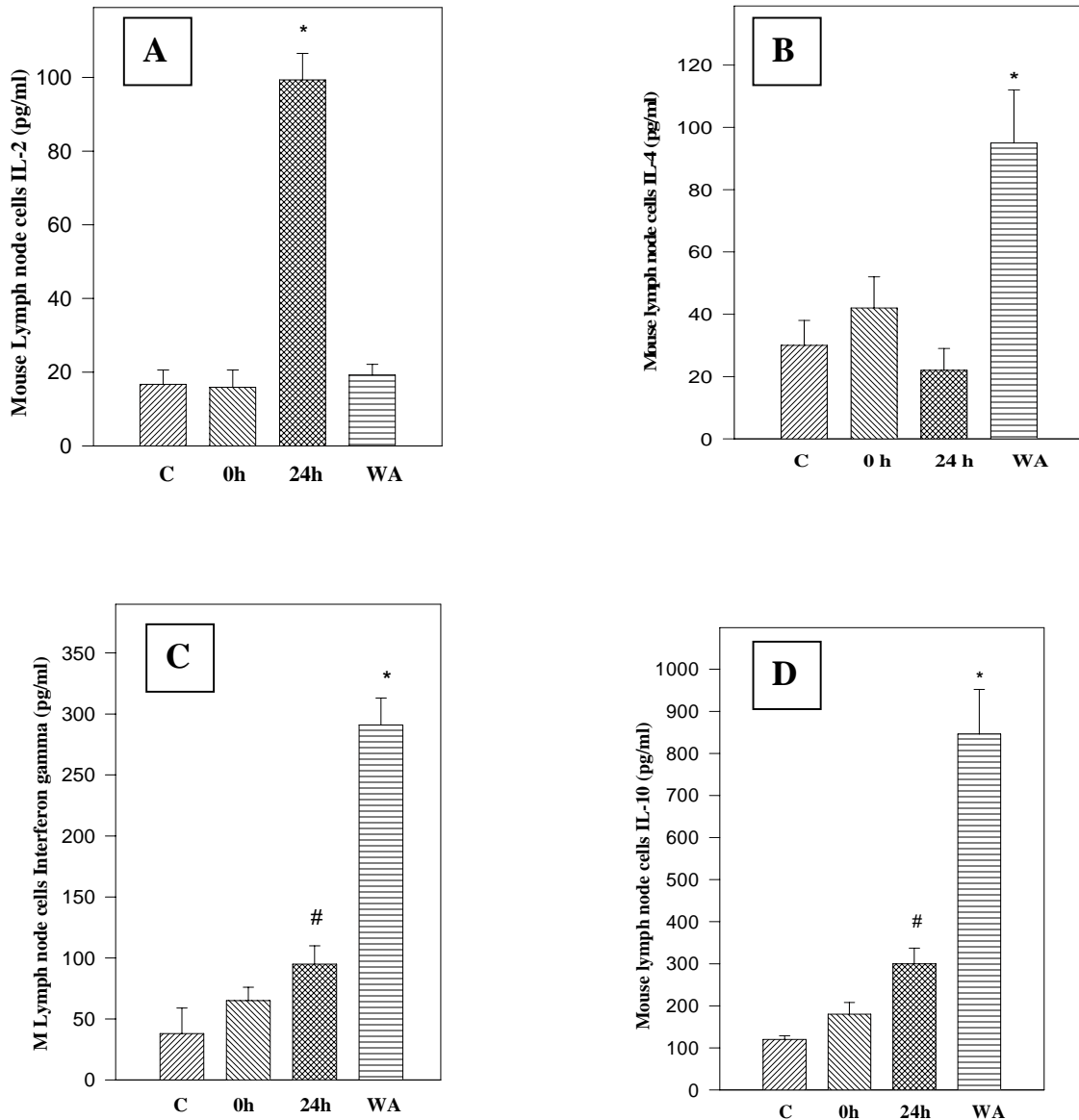


Fig. 2: SDS –PAGE of *L.major* Promastigotes cell culture filtrate. Part A: Secreted –excreted proteins of *Leishmania major* promastigotes were analyzed using SDS-PAGE and silver staining. Lanes from left to right are: three separate experiments of 24 h cell culture filtrate, S molecular weight standards, 0, 24, 48 and 72 lanes are cell culture filtrate collected at this time after promastigotes cultured in serum free media. Standards from top are: Myosin (205 kDa), β -galactosidase (116 kDa), glycogen phosphorylase (98 kDa), bovine serum albumin (66 kDa), ovalbumine (45 kDa), lactate, dehydrogenase (35 kDa) and lysozyme (14.3 kDa). Part B: Native gel activity staining of ACP. Lanes from left to right are, 0 h and three separate 24 hour cell culture filtrates.



*. Significantly different from other experimental groups $P < 0.01$

#. Significantly different from other experimental groups $P < 0.05$

Fig. 3: Cytokines produced by BALB/c mice vaccinated and challenged by *L.major* promastigotes. After two months of animals challenge, mononuclear cells from the immunized animals lymph nodes were restimulated *in vitro* with 24 h E-S products. After 48 h, the supernatant of the cultures were harvested and tested by ELISA. IL-2 (A), IL-4 (B), IL-10 (C) and IFN- γ (D). Data are expressed as means \pm SEM. N= 8 animals in each group (WA stands for whole Ag group). All measurements were in triplicate.

Discussion

Previous reports stressed that excretory and secretory molecules from intracellular pathogens such as *Mycobacterium bovis* and *Toxoplasma gondii* have highly immunogenic and protective effects in vaccine models (24, 25). *Leishmania* parasites are able to survive in host macrophages despite the harsh phagolysosomal vacuoles conditions. This could reflect, in part, their capacity to secrete proteins that may play an essential role in the establishment of infection and serve as targets for cellular immune responses. In this study, SDS-PAGE was used to analyse *L. major* promastigotes proteins excreted/secreted *in vitro*. Our results showed that after 24 h incubation of promastigotes, two proteins band with molecular weight of 75 and 110 kDa appeared in SDS PAGE in repeated experiments. More recently, it has been shown that the biologically active phosphoglycan structures of lipophosphoglycan are also present on several proteophosphoglycans. This group of molecules includes the secreted acid phosphatase produced by all *Leishmania* species except *L. major* (26). Previously it was reported that promastigotes of virtually all pathogenic *Leishmania* species, except *L. major*, release a structurally conserved soluble acid phosphatase (ACP) activity during their growth *in vitro* (21). Later Shakarian and Dwyer (27) showed that also all *L. major* strains produced and released an enzymatically active ACP into their culture supernatants during growth *in vitro*. Similar result to this study has also shown that *L. donovani* secretory ACPs consisted of two heterodispersed, 110 and, 130 kDa glycosylated isoforms. This heterodispersity was directly attributed to a wide array of both N-linked and O-linked glycosylations (28). Furthermore, Soares *et al.* (29) showed that *Leishmania chagasi* secreted acid phosphates on native gel appears to be 215 kDa. Taken together 110 kDa protein band on the SDS PAGE and activity staining in native gel in current experiment most probably corresponds to secreted acid phosphatase (Fig. 2A and B). Our results also show that there was

no activity in 0h filtrates, but three separate repeated experiments showed reproducible active ACP band in 24 h cell culture filtrate (Fig. 2B).

Larazi *et al.* (30) have reported two different AChE isoform from the bovine lungworm *Dictyocaulus viviparus* with molecular weight ranging from 70-75 kDa. So far, acetylcholinesterase activity is not reported in *Leishmania* species. We believe that this is the first report of this enzyme in *L. major* excreted or secreted into the culture media.

Here, we report an experimental vaccination trial on BALB/c mice using *L. major* excreted secreted antigens of promastigotes as vaccine candidate. Access to a serum-free system for culturing promastigotes of *Leishmania* improves the feasibility of large-scale production of well-defined parasite material. Using this methodology we immunized groups of animals with the same amounts of protein (50 µg/animal) in complete Freund's adjuvant but, results show that 24 h E-S product offers better protection (small wound diameters, low level mortality, dominant Th1 pathway) against the challenge with *L. major* promastigotes. Tonui *et al.* (31) found similar results using *L. major* exogenous antigens in BALB/c mice. It seems that during the *in vitro* culture, parasites secrete some proteins, which could stimulate immune system in vaccinated animals. Whole Ag has many different proteins, which may have complex interactions in cytokine patterns.

It is generally thought that a *Leishmania*-specific Th1-type response is required for effective protection against *Leishmania* infection in humans and mice. Ideally, following infection, a Th1 response is generated that will be responsible for activating leishmanicidal activities by host macrophages and the subsequent control of the infection. Moreover, it has recently been shown that controlling the Th1 response also enables parasite persistence (32), which aids the maintenance of a robust and protective population of antigen-specific CD4⁺ memory T cells (33). Beyond inducing an effective Th1-type response against *Leishmania*, the establishment of long-lasting protection is crucial for both the well-

being of individuals living in endemic areas and the design of vaccines against *Leishmania*. IL-10 is a potent inhibitor of IFN- γ production and has been shown to be a key cytokine that favors the persistence of the parasites in skin lesions (34). In this experiment, low IL-10 and IL-4 levels and high IL-2 level in *in vitro* stimulated lymph node cells from vaccinated animals with 24 h E-S Ag, seems to play dominant Th1 response. In the absence of IL-4 and IL-10, the low levels IFN γ produced may be sufficient for protection. Development of a vaccine from a well-defined antigen has advantage of reproducibility over crude *Leishmania* vaccine. Most of the *Leishmania* antigens, which were tested in animal models showed to be protective when, used with an appropriate adjuvant (35). This study showed that using 24 h E-S products of cell culture filtrate with complete Freund's adjuvant improved wound healing and reduce mortality in mice model of leishmaniasis. Most probably there are other proteins secreted and excreted during promastigotes culture media. Obviously, there are other proteins secreted and excreted during promastigotes culture media. The applied procedures showed only 2 predominant 110 kDa and 75 kDa protein band in SDS-PAGE stained with silver nitrate.

In conclusion, this study may provide a useful model for the characterization of a protective immune response, against secretory and excretory product of *L. major*.

Acknowledgements

Authors are thankful to Baqiyatallah University Research Branch for financial supports.

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