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Characterization of a Monoclonal Antibody Specific for the Parasite Surface Antigen-2 of *Leishmania major*

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Abstract

The *Leishmania major* Parasite surface Antigen-2 (PSA-2) is a family of glycoinositol phospholipids anchored glycoprotoins expressed in both promastigotes and amastigotes. Promastigote PSA-2 comprises three polypeptides with approximate molecular weight of 96, 80 and 50 kDa. Amastigote express a distinct but closely PSA-2 polypeptide with molecular weight of 50 kDa. In this study fusion of SP2/0 myeloma cells with immunized mice spleenocytes infected with promastigotes of *L. major* intraperitoneally resulted to a clone of hybridoma producing a specific antibody that only reacts with *L. major* parasite surface antigen (PSA-2). This mAb showed no crossreactivity with either other *Leishmania* species including *L. tropica*, *L. donovani* and *L. infantum* or recombinant gp63. Western blot analysis of culture supernatant revealed multiple bands with molecular weight of 50, 58, 80 and 96 kDa only in *L. major*.

Keywords: Leishmania major, *Monoclonal antibody, PSA-2*

Introduction

Members of genus *Leishmania* are responsible for a wide variety of diseases, affecting man and other mammals in different parts of the world. *Leishmania* has a relatively simple digenetic life cycle, existing in the sand fly vector as flagellated promastigotes and in mammalian host macrophages as intracellular non-motile amastigotes (1).

Promastigotes cell surface molecules are critical for recognition and infection of the mammalian host and possibly for subsequent survival in the vector. Two major classes of membrane antigens have been identified on the surface of the *Leishmania* parasite; a family of glycolipids consisting of lipophosphglycan (LPG) which has been shown to be involved in parasite attachment to host macrophages and initiation of infection (2) and a family of glycoprotein, the two best characterized ones in *L. major* are the surface protease gp63 (the promastigote surface antigen complex 1) and the parasite surface antigen-2 (PSA-2) (3, 4). Gp63 has been implicated to be a receptor for the host macrophages and is encoded by multiple genes (5). PSA-2 belongs to a family of genes, which are highly polymorphic and produce multiple mRNA transcripts (4). These complex gene loci and its protein products have been detected in all *Leishmania* species examined except *L. braziliensis* (6).

The function of PSA-2 in the biology of the parasite is poorly known. However, areas of PSA-2 show similarity to *Trypanosome cruzi* and human mucins and to *Drosophila* glu protein (7), suggesting a possible role in protein-protein interaction. *L. major* PSA-2 has been shown to protect vaccinated mice subsequent to challenge with parasite by a Th1 type immune response (8). In this study, we report

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the production of a mAb that can specifically recognize an epitope of *L. major* PSA-2.

Materials and Methods

Parasites *L. major* (MRHO/IR/75/ER) was generously provided by Dr E Javadian, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. *L. tropica* (MHOM/SU/74/K27) and *L. infantum* (MHOM/TN/80/IPT1) were obtained from Department of Medical Parasitology, School of Hygiene and Tropical Medicine, London, England. The parasites were grown at 25°C in RPMI culture medium supplemented with 15% FCS, 100U/ml penicillin and 100 µg/ml streptomycin. Recombinant gp63 was obtained from Dept. of Biotechnology, Pasteur Institute of Iran, Tehran, Iran.

Axenic amastigotes culture To obtain axenic amastigotes, *L. major* Promastigotes were cultured in complete medium (Ph 5.5) in CO_2 incubator at 35°C. After 5 days, the axenic amastigotes were harvested and kept at -70°C.

***Preparation of murine monoclonal antibody to* L. major** Six–week old female BALB/c mice were given 500µl of inoculums comprising equal amounts of 10^7 promastigotes/ml and complete Freund adjuvant intraperitonealy. The same doses of antigen were given but in incomplete Freund adjuvant four weeks later and then the sera from the mice were tested for IgG by enzyme linked immunosorbent assay (ELISA) when four weeks elapsed. The final 200µl inoculum was given in phosphate-buffered saline intravenously into the base of the tale of the animals and then they were sacrificed 3 days later. The Spleen cells of immunized mice were fused with SP2/0 mouse myeloma cells by the method of Goding using polyethylene glycol (PEG) 1500 as a fusing agent (9). Hybridomas were screened initially for anti-*L. major* by ELISA and those producing specific antibody were subcloned at least twice by limiting dilution method. The class and subclass of mAb were determined by ELISA using Isotype reagent Kit (ISO-2 Sigma).

Enzyme Linked Immunosorbent Assay Flat bottom 96-well polyvinyl chloride plates were coated with either 10^6/well of intact axenic amastigotes of different species of *Leishmania* or 5 µg/ml of recombinant gp63 diluted in PBS (pH7.5) and incubated overnight at 37°C. The plates were washed with PBS con- taining 0.05% Tween 20 (PBS-T) and blocked with 1% bovine serum albumin at 37°C for 1h. After three washes with PBS-T, hybridomas supernatants were added and incubated at 37 °C for 90 min. The plates were further washed and commercial HRP conjugated goat anti-mouse IgG (Sigma) was added for 1h at 37°C. The plates were washed and tetramethylbezidine (TMB) was added as substrate, color was allowed to develop for 20 min at 25°C before the reaction was stopped with HCl and A450 values were measured.

***TX-114 preparation of membrane proteins of* L. major** 10^{11} stationary phase promastigotes of *L. major* were solubilized in 100ml PBS containing 0.5% Triton X-114, vortexed vigorously and kept on ice for 90 min. Insoluble materials were removed from the lysate by centrifugation at 400g for 7 min at 4°C. The procedure was continued as described by the detergent phase separation method of Murray et al (10).

Immunoblotting of* Leishmania *species
Different antigens from promastigote of *Leishma nia* species as well as axenic amastigote, rgp63 and TX-114 preparations were separated by 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)using the discontinuous buffer systems of Laemmli(11). The antigens were solubilised by incubation for 2h at 37°C in 1% SDS and reduced by boiling in 5%2-Mercaptoethanol and 1% SDS. After running the gel, the antigens were transferred to nitrocellulose membrane (0.2A, 16h, 25mM sodium phosphate buffer, pH 6.5). The membrane was treated for 2h in 0.25% gelatin, 25mM sodium phosphate pH 6.5 and then incubated with 1:10 dilution of supernatants of positive clone for 3h at 25°C. The strips were washed for 1h with several changes of buffer

and incubated with HRP conjugated goat anti-mouse IgG for 2h. After washing, the bands were detected with Diaminobanzedine (DAB) substrate.

Results

***Characterization of mAb Specific for* L. major**

Fusion of spleen cells from BALB/c mice immunized with whole parasite of *L. major* promastigote, yielded a hybridoma (4B10) secreting an IgG1 antibody which in ELISA bound specifically to stationary and logarithmic phase of *L. major*. The antibody reacted neither with recombinant gp63 of *L. major* nor with other species of *Leishmania*.

Immunoblotting To examine the antigen(s) recognized by 4B10, promastigote of *L. major*, *L. tropica*, *L. infantom*, axenic amastigote of *L. major*, rgP63 and lysates of L. *major* promastigotes phase separated in TX-114 were fractionated by SDS-PAGE and blotted onto nitrocellulose filters. As shown in Fig.1 the mAb only revealed four bands of 50, 58, 80 and 96 kDa MW in promastigote and two bands of 80 and 90 kDa in axenic amastigote form of *L. major*. This mAb also recognized same bands in TX-114 phase separated *L. major* (Fig.2).

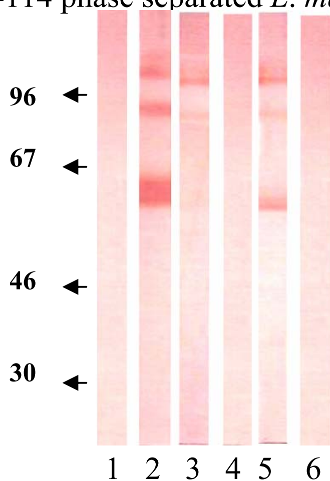

Fig. 1: Western blot analysis of different species of *Leishmania* and gp63 using antibody produced by hybridoma 4B10. line1, gp63; line 2, *L. major* stationary phase; line3, *L. major* axenic amastigote; line 4, *L. infantum* promastigotes; Line 5, *L. major* log. phase; line 6, *L. tropica* promastigots.

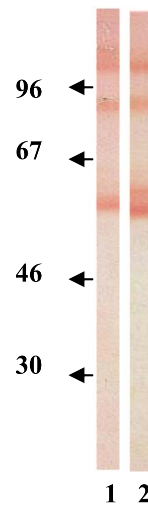

Fig. 2: Western blot analysis of intact and Tx-114 treated parasites. Line 1, Intact *L. major*: line2, Tx-114 treated *L. majors*.

Discussion

The identification of parasite component is very important for preparation of a vaccine against human leishmaniasis. To be effective, this kind of vaccine is expected to induce and maintain a Th1 type response without expansion of cells with a Th2 cytokine secretion pattern.

Immunization of BALB/c mice with PSA-2 antigen from *L. major* promastigotes has been shown to provide a high degree of protection against challenge infection with *L. major* which is associated with Th1 cytokine pattern (8). It has also been shown that immune individuals to cutaneous leishmaniasis display a Th1 response upon re-stimulations with PSA-2 antigens (12). The presence of Th1–type memory to PSA-2 in human and the ability of the antigen to protect the mice against *L. major* infection make it an attractive vaccine candidate against human cutanous leishmaniasis. It has recently been shown that PSA-2 has a leucin-rich motif which binds to macrophages through complement receptor 3 (13).

In this report we have prepared a clone (4B10) which, secret a specific antibody against *L. ma-*

jor PSA-2 antigens. This mAb could detect four polypeptides of approximate molecular weight of 50, 58, 80 and 96 kDa with no reactivity with rgp63 or other *Leishmania* species such as *L. tropica* and *L. infantum*. Although others have raised antibodies against *L. major* rPSA-2 fragment that could recognize three polypeptides of 96, 80 and 50 kDa in promastigotes of Israeli *L. major* isolates, but the produced antibodies were not specific to *L. major* and reacted with polypeptides in *L. tropica* and *L. donovani* as well (4). The epitope reacted with this mAb would be important for further research on antigens responsible for infectivity or immunity in *L. major*. In view of the high degree of homology between different members of the PSA-2 family, these relationships will be different to establish, and require analysis using a monoclonal antibody to show peptides that are unique to each PSA-2 isoform. By blocking the PSA-2 in parasites grown in medium culture supplemented with this mAb it would be possible to study its role during the course of infection. This mAb can also be exploited as a valuable tool for purification of *L. major* PSA-2 to be used as a subunit vaccine in animal models.

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