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Production and Characterization of Monoclonal Antibodies Recognizing a Common 57-kDa Antigen of *Leishmania* Species

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ABSTRACT

Background: The therapy of leishmania infection is difficult and each year 1.5 million new cases of cutaneous leishmaniasis and 500,000 new cases of visceral leishmaniasis are estimated, therefore, there is a need for an effective vaccine. Monoclonal antibody (mAb) is one of the suitable methods for isolation and purification of leishmania antigens. In this report, we produced several mAb against *leishmania infantum* antigens for antigen purification to be used as candidate vaccine. **Methods:** BALB/c mice were injected with freeze-thawed promastigote twice together with Freund adjuvant. Three days before fusion, antigen in saline was injected into the tail vain and then mice were killed and the spleen lymphocytes were fused with myeloma SP2/0. **Results:** Five mAb against promastigote form of *Leishmania infantum* parasite were obtained. Western-blot analysis showed that these mAb recognize a band of 57- kDa protein either in parasite lysate or on whole *L. infantum*, *L. tropica*, *L. major* and *L. donovani*. It seems that the 57 kDa-protein is the major surface leishmania antigen (gp63) that is neither stage-specific nor differentially regulated. These mAb do not recognize the recombinant gp63 antigen and seems recognizing only the native form of a gp63 isoform. The IgG1 mAb was purified by affinity column and was used to purify 57 kDa antigens from Leishmania lysate. **Conclusion:** Since these antibodies recognizing one specific protein band in 4 different strains of leishmania, they could be used for leishmania diagnostic kits and also for purification of antigen to be tested for its protective effect against leishmania infection. *Iran. Biomed. J. 13 (4): 245-251, 2009*

Keywords: Monoclonal antibody (mAb), *Leishmania*, 57-kDa protein

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INTRODUCTION

Leishmaniasis is a vector-transmitted disease distributed throughout the world's tropical and subtropical regions. *Leishmania* species give rise to a wide spectrum of clinical manifestations, ranging from self-healing skin ulcer or disfiguring mucosal lesions to fatal visceral infections. *Leishmania* parasites present two forms in their life cycle, promastigotes, which multiply in the midgut of the sandfly insect vectors and amastigotes, the obligate intracellular forms which live within phagolysosomes of vertebrate macrophages. Metacyclic promastigotes are transmitted to the mammalian host by the bite of phlebotomies sand flies. Leishmaniasis is difficult to treat and there is increasing resistance developing against the currently available drugs. New disease foci are identified every year due to the resistance of parasite to the existing chemotherapy and resistance of sandflies towards the insecticides. Therefore, there is an urgent need for vaccine development against leishmaniasis especially against fatal visceral leishmaniasis (VL).

Human VL is one of the most important zoonosis, caused by *Leishmania infantum*. An effective vaccine against kala-azar is not yet available; however, there have been a limited number of vaccine candidates which have been shown to induce protection against VL in animal models [1]. Several vaccination strategies against experimental leishmaniasis have been attempted. Immunization of both susceptible and resistant mice strains with killed parasites, crude antigen fractions, purified membrane proteins and DNA vaccines provided partial protection against parasite challenge. However, few studies on protection by purified

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antigens from *Leishmania infantum* have been performed [2-8].

It has been shown that monoclonal antibodies (mAb) are useful tools for diagnosis of leishmaniasis and for biochemical and immunopathological characterization of the parasites. Species-specific mAb against *L. mexican* and *L. braziliansis* as well as the *L. tropica* and *L. donovani* complexes have been produced for immunodiagnosis and taxonomic classification of *leishmania* species [9-15]. Surface antigenic changes during differentiation *in vitro* of *L. mexicana* were also identified by mAb [16]. The mAb were successfully used to protect BALB/c mice from infection with *L. mexicana* [17] and also, for purification of leishmania antigens. Immunization of BALB/c mice with p30 antigen of *L. chagasi* amastigotes showed a protective role for this protein and induction of Th1 activity [18]. Anti-idiotypic mAb with microbicidal and fungicidal effects also exerted anti-leishmanial activity [19].

The aim of the present study was to identify and purify antigens from promastigote form of *L. infantum* by hybridoma technique to be used in the future as candidate vaccine against VL.

MATERIALS AND METHODS

Leishmania. Four *Leishmania* species were used in this study: *L. infantum* (MHOM/TN180/IPT1), *L. major* (MRHO/IR/75/ER), *L. donovani* (MHOM/TN /80/IPI1) and *L. tropica* (MHOM/SU/74/R27). Lesion-derived amastigotes of *L. infantum* parasites were isolated from BALB/c mice infected 6-8 weeks before and cultured in Novy-MacNeal-Nicole media and later were transferred to the RPMI-1640 medium enriched with 20% FCS. Axenic amastigotes were grown at 35°C at pH 5 to 5.5 and promastigotes were grown at 22-25°C in the presence of 10% FCS. Soluble leishmania antigens (SLA) was prepared by 6 times freeze-thawed followed by sonication and centrifugation in buffer containing 1 mM anti-protease cocktail phenyl-methanesulfonyl fluoride (Sigma, USA) and stored at -70°C until use.

Production of mAb. Seven-week-old female BALB/c mice were injected i.p. and s.c. with 50 µg of SLA preparation of *L. infantum* promastigote in complete Freund adjuvant and 2 weeks later were boosted with the same amount of antigen in incomplete Freund adjuvant. When 1/1000 dilution of sera had positive reaction with antigen in ELISA, mice were boosted 3 days before fusion with 50 µg antigen in saline in the tail. Spleen cells were fused with SP2/0 myeloma (obtained from Pasteur Institute of Iran) with polyethylene glycol (Sigma, USA). Cells were cultured in hypoxanthine-aminopterin-thymidine medium (HAT) in a 37°C incubator containing 5% CO_2. Two weeks after fusion, the presence of antibody in the supernatant of the colonies was tested by ELISA. The positive colons were subcloned twice with limiting dilutions. Ascitic fluid was prepared by injecting two million hybrid cells i.p. into the BALB/c mice.

Gel electrophoresis and Western-immuno-blotting. SDS-PAGE was performed according to the Laemmli method [20]. Gels were stained with Coomassie Blue and later with silver staining. Proteins separated by SDS-PAGE were transferred to nitrocellulose sheets (Schleicher & Schuell, 0.45 µm pore size) by semi-dry blotting technique [21]. Subsequently, the nitrocellulose sheet was incubated with 1% BSA in PBS buffer for 1 h and then incubated with hybridoma culture supernatant at 4°C overnight. The membrane was washed with PBS containing 0.1% BSA and 0.05% Tween 20, and second antibody (HRP-conjugated goat anti-mouse). After washing, the immune complex was detected by a color reaction using diaminobenzidine as enzyme substrate containing 0.03% $CoCl_2$ for color enhancement.

Purification of mAb by protein G columns. The IgG1 mAb (P3D2) was purified from ascites fluid or cell culture supernatant by precipitation with 45% saturated ammonium sulfate and affinity chromatography on Protein G Sepharose equi-librated in PBS buffer (pH 7.2). After applying the sample, column was washed and antibody was eluted with 0.1 M glycine-HCl buffer, pH 2.5. Eluted antibody was immediately neutralized with 1 M Tris-HCl (pH 9.0), then concentrated and dialyzed against PBS buffer and was used for purification of the 57-kDa protein.

Purification of 57-kDa antigen by affinity column. Purified P3D2 antibody was conjugated to the activated Sepharose 4B and then the crude antigen of *L. infantum* (freeze-thawed) was centrifuged and the clear supernatant was added to the affinity column. The 57-kDa protein was eluted from the column by 0.1 M glycine-HCl (pH 2.5) buffer and the purity of the 57 kDa was determined by gel electrophoresis.

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Protein determination. Protein determination was performed by Bradford's dye binding method [22].

RESULTS AND DISCUSSION

In this report, we obtained five positive clones (P1-A9, P2-G8, P3-D2 P5-E3, and P6-B3) against SLA of *L. infantum*. About 65% of wells showed growth of hybridoma in which 13% were positive. Figure 1 shows the interaction of mAb against different concentrations of SLA of *L. infantum*. These positive clones were subcloned twice by limiting dilution and their isotypes were determined.

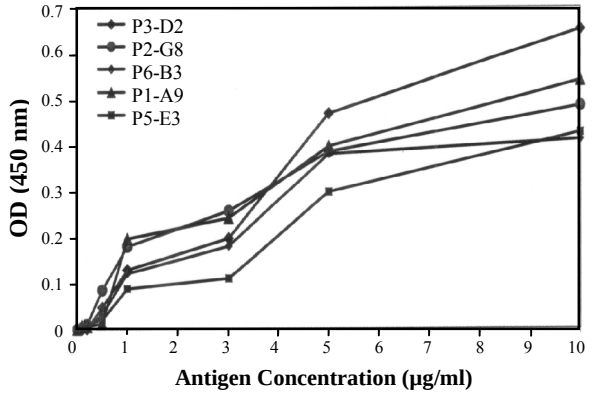


Fig. 1. ELISA titration curve of five hybridomas supernatant (100 µl) against different concentrations of freez-thawed *L. infantum* antigens.

Clones P1-A9, P2-G8, P5-E3 and P6-B3 produced IgM and P3-D2 produced IgG1 subclass.

To determine the specificities of these mAb, Western-blot analysis was performed. SLA either in reduced or non-reduced forms was electro-phoresed and then Western-blotted on nitrocellulose and developed with mAb. All mAb recognized only a band with apparent molecular weight of 57 kDa in both reduced and non-reduced forms (data not shown) indicating that the antigenic epitope is not conformational. Soteriadou *et al.* [23], also raised mAb against a 58-kDa protein of *L. infantum* that did not recognize the reduced form of protein, indicating that this antibody is against conformational epitope. In ELISA, these antibodies could recognize both freeze-thawed antigens as well as whole parasites of logarithmic and stationary phases of promastigote and amastigote forms, indicating that the 57-kDa protein is located on the surface of parasites and is neither stage-specific nor differentially regulated. Also, these

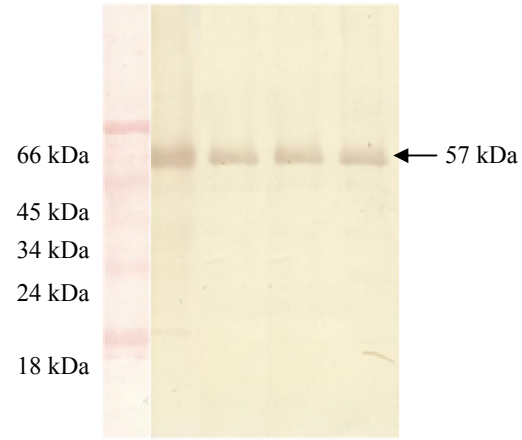


Fig. 2. Western-blot analysis of 57-kDa antigens (10 µg) from four different species of leishmania by P3D2 monoclonal antibody. Lane 1, *L. infantum;* lane 2, *L. major*; lane 3, *L. tropica* and lane 4, *L. donovani.*

mAb recognized not only *L. infantum* antigen, but also *L. major*, *L. donovani* and *L. tropica* antigens in freez-thawed forms or as a whole parasite. Figure 2 shows that P3D2 mAb recognizes the same 57-kDa membrane antigen on all four *Leishmania* species in Western-blot. These data and other studies indicate that 57-kDa protein is an abundant surface protein of all leishmania [24]. Different concentrations of *L. infantum* cell lysate were used in Western-blot analysis. Figure 3 shows that P3D2 mAb could detect approximately less than 1 ng of 57-kDa antigen in cell lysate.

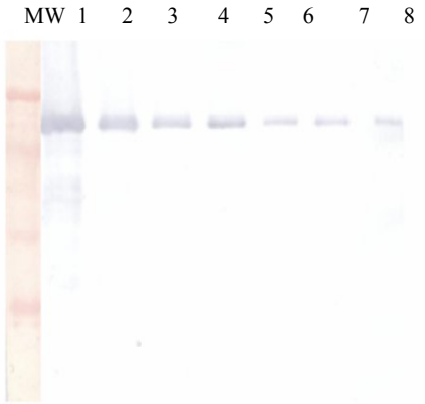


Fig. 3. Western-Blot Analysis of P3-D2 Monoclonal Antibody against different concentration of *L. infantum* cell lysate antigens. After 10% SDS-PAGE, proteins were transferred to nitrocellulose sheet and incubated with P3-D2 culture supernatant. Lanes 1-8 different concentrations of parasite lysate (1, 0.5, 0.3, 0.08, 0.04, 0.01, 0.001, and 0.0001 µg, respectively).

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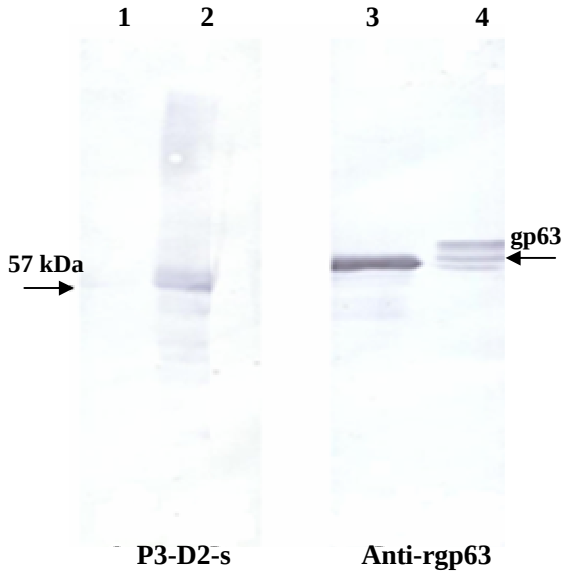


Fig. 4. Western-blot analysis of 57 kDa-antigen from *L. infantum* cell lysate and recombinant gp63 molecule by subcloned P3D2 (P3D2-s) and anti-gp63 monoclonal antibodies. Lanes 1 and 3, rgp63; lanes 2 and 4, *L. infantum* cell lysate. P3D2 does not recognize the rgp63 molecule.

Is 57-KDa protein gp63 isoform? Several investigators identified gp63 in different species of leishmania and in both promastigote and amastigote life stages of *L. major* and *L. Mexican* [24-26]. In order to determine whether the 57-kDa protein is actually gp63, Western-blot analysis was performed using anti-gp63 mAb. As Figure 4 shows, anti-gp63 could detect recombinant gpP63 (rgp63) as well as natural gp63 with apparent molecular weight of 57 kDa from *L. infantum* cell lysate. However, our subcloned P3D2 antibody did not recognize rgp63. The molecular weight differences between gp63 and 57-kDa proteins may be due to the lack of glycolization of rgp63. The antibody may recognize one of the gp63 isoforms [27]. GP63 that is called leishmanolysin is an abundant leishmania cell surface metalloproteinase. The genes of GP63 are highly conserved across the genus and all major surface proteases share high sequence identity; however, there are species-specific differences in gene organization and number [27]. The identification of 57-kDa protein on the surface of all four *Leishmania* species, its abundance and immunogenicity may indicate that this protein is gp63 or one of its isoforms. It has been shown that there are seven GP63 genes in *L. major*, genes 1-5 are expressed in promastigotes only, gene 6 is expressed in promastigotes and amastigotes, while gene 7 is expressed predominantly in the stationary phases of promastigotes and amastigotes [27]. *L. Mexican* has ten GP63 genes, *L. donovani* has seven, *L. guyanensis* has 22 and *L. chagasi* has three distinct gene clusters with more than 10 isoforms in both promastigotes and amastigotes [28]. Several groups have suggested that gp63 have several important roles in leishmaniasis. It acts as an acceptor for C3 components of complement (C3b and iC3b) mediating attachment of promastigotes to macrophage [29]. Purified gp63 from *L. Mexican amazonensis* by mAb inhibits leishmania-macrophage binding and that of *L. mexican mexicana* involves in the attachment of *Leishmania* promastigotes to macrophages [30]. The gp63 enhances parasite survival in macrophage phagosomes by cleaning at least one macrophage cytoplasmic molecule. Its proteolytic activity may protect intracellular *Leishmania* species from lysosomal hydrolases and/or provide nutrition for amastigotes in the host cell [31, 32].

Purification of antibody and antigen. To purify the mAb from hybridoma culture supernatant and from ascites fluid, protein G Sepharose was used. Approximately 4.4 mg antibody was purified from 450 ml of culture supernatant (9.7 µg/ml) and 1.3 mg from 11 ml of ascites fluid (120 µg/ml). Figure 5 shows the purified P3D2 antibody from protein G column. Purified P3-D2 antibody was coupled to the activated Sepharose 4B and 57-kDa antigen was purified from *L. infantum* cell lysate (Fig. 6).

The purified mAb will be used for diagnosis of 57-kDa antigen in urine, blood and the identification of parasite in the patient's tissues. Since major surface proteases differ biochemically and localize differently in two parasite stages [28], we can use the purified mAb in immune-electron microscopy to find out the distribution of 57-kDa protein in promastigote and amastigote [28]. The anti-leishmania antibody could also be used for inhibition of leishmania development in the sand flies.

Antibody against *L. donovani* could inhibit *L. major* development by 82% in sand flies fed on 10^6 amastigotes [33]. The mAb raised against *L. infantum* promastigotes could inhibit the life cycle of several *Leishmania* species from the Old and New World and these antibodies recognize three constant antigens of 40, 70 and 113 kDa among the *Leishmania* species [34]. Antibodies may also be useful for leishmania typing and were used for species and stage specificity against leishmania

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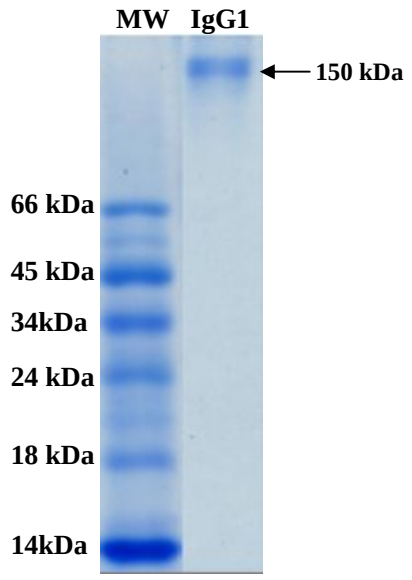


Fig. 5. Purification of P3D2 monoclonal antibody from protein G column. Monoclonal antibody was eluted from the column and after concentration was electrophoresed on 10% SDS-PAGE under non-reduced condition and stained with Coomassie blue.

amazonensis [35]. It also could be used for affinity purification, immunoprecipitation [36], epitope mapping of parasite antigens and characterization of private and public epitopes.

Recombinant gp63 have been used as subunit vaccine with appropriate adjuvant and induced protection against *L. major* infection in BALB/c mice. Since we can have plenty of purified 57 kDa, it would be possible to use this natural purified protein to investigate the protective effect against *L. infantum* infection as experimental subunit vaccine and also as a reagent for serodiagnosis tool against leishmaniasis.

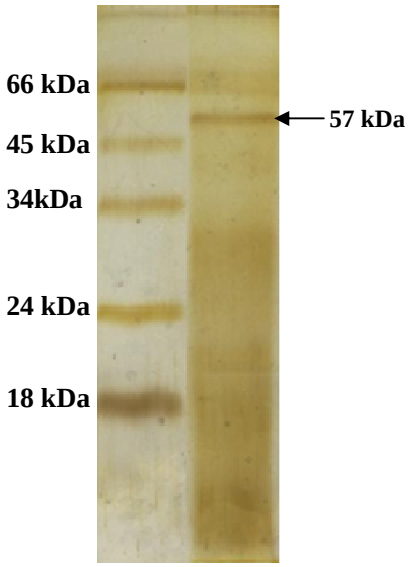


Fig. 6. Purification of 57-kDa antigen from *L. infantum* cell lysate by P3D2 affinity column. 57-kDa protein was eluted from the column and electrophoresed on 10% SDS-PAGE and silver stained.

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