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Presence of Immunoglobulins on the Surface of Lesion-Derived Amastigotes of *Leishmania major* As a Tool for Isolation of Amastigote Stage from BALB/c Mice

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ABSTRACT

The onset of infections by *Leishmania* parasites mainly caused by amastigote growth inside the macrophages. One of the important properties of lesion-derived amastigote is thought to be the attachment of various host proteins including immunoglobulins on the surface of amastigote. In this study, the presence of immunoglobulins on the surface of lesion-derived amastigotes was detected by Western blotting using three different peroxidase conjugated anti-heavy chain antibodies and peroxidase conjugated anti-mouse IgG antibody. Then, a new technique was developed for isolation of lesion-derived amastigotes. This technique simply consists of a microbiological plate covered by rabbit anti-mouse immunoglobulins. Overnight incubation of the infected cell suspension isolated from mice lesion on such plate at 4°C, was followed by gentle washing and isolation of the amastigotes. The results showed that the surface of amastigote has covered with different amount of immunoglobulins such as IgG, IgM, and IgA detected by pixel analysis software. With this technique, which was comparable with other techniques, pure amastigote was isolated. This is a simple and reliable method for isolation of real amastigotes from lesion of the infected BALB/c mice. *Iran. Biomed. J. 7 (4): 155-160, 2003*

Keywords: *Leishmania major*, Lesion-derived amastigote, Immunoglobulins , BALB/c mice

INTRODUCTION

Leishmaniasis is a parasitic disease that has been reported from 88 countries and about 350 million people in the world are at risk of infection [1]. Cutaneous, mucocutaneous and visceral leishmaniasis are three distinct types of the disease produced by various species of the *Leishmania* parasite, among them *Leishmania major* usually causes cutaneous form of the disease. Infective form of promastigote caused by differentiation of promastigote in the sandfly midgut, enters into the mammalian host by sandfly inoculation. The promastigote is engulfed by macrophage, and then transforms to a round flagellated form of amastigote in the phagolysosome, that is responsible for all clinical manifestations in the vertebrate host. Some studies on lesion-derived amastigotes have shown that their surface covered by immunoglobulins (Igs) which may mediate internalization of these amastigotes into the other host macrophages [2]. *In vitro* studies have shown that COS cell lines are usually poorly infected by *L. mexicana* amastigotes, because these cells lake immunoglobulins, C3 or other surface receptors. However, if these cells are transfected with Fcγ IIb, they will be susceptible to the infection by lesion-derived amastigote of *L. mexicana* [3]. Other studies indicate that in genetically altered mice lacking circulating antibody, maintenance of the infection is impaired, and there are evidence that the surface Ig, especially IgG, acts as a virulent factor for parasite [2, 4].

Although axenic culture is used for amastigote stage of different *Leishmania* species such as *L. braziliensis* [5], *L. infantum* [6], *L. donovani* [7, 8] and *L. mexicana* [9], there is no suitable axenic culture method for amastigote stage of *L. major*.

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Although, there is a few reports of success in axenic culture of *L. major*, but they have not been confirmed by other investigators [11, 12].

Isolation techniques for amastigote stage of *L. major* are mainly based on the size of amastigote, using various filters [13], or the density and size using Percoll gradient centrifugation [14,15]. In the technique introduced by Glaser *et al.* [13], amastigotes are initially released from macrophages by a homogenizer (tissue grinder) and then isolated by repeated filtration of cell suspension through filters with different pore sizes. In other technique based on Percoll gradient, cell suspension from lesion is transferred into three tubes with different dilutions of Percoll (25, 45 and 70% in PBS), and amastigotes are isolated over a discontinuous Percoll gradient centrifugation [14, 15].

Despite the advantage of isolating a pure form of amastigote by these techniques, they have several disadvantages: I) They are time-consuming and need 2-3 hours continuous laboratory work especially filtering the suspension through different filters or making Percoll gradient tubes. II) They need special filters with different pore sizes (3, 5, 8 μm) for Glaser technique [13], and high-speed centrifugation for Percoll gradient procedure [14]. Therefore, there is a need to develop a new simple inexpensive technique for isolating amastigote stage of *L. major*. In this study, we tested the presence of immunoglobulins on the surface of *L. major* amastigotes by Western blot analysis and detected their relative isotype densities by pixel band analysis software. Based on the presence of host immunoglobulins on the surface of amastigotes**,** a new technique was introduced for isolation of amastigote by rabbit anti-mouse coated plates.

MATERIALS AND METHODS

Mice and Parasites. Female BALB/c mice (6-8 weeks old) were obtained from Animal Breeding Division of the Pasteur Institute of Iran (Tehran). *L. major* strain (MRHO/IR/75/ER) was initially provided by Dr. E. Javadian, School of Public Health, Tehran University of Medical Sciences, Iran. Pathogenicity of parasite was maintained by regular passage through BALB/c mice.

Isolation of amastigote. Mice were inoculated with 10^6 *L. major* promastigotes in the stationary phase (a 7-days-old culture after changing the color) i.m. on the hind leg. Two months later, the mice were sacrificed and the lesions were removed and amastigotes were separated according to the technique introduced by Glaser *et al*. [13]. Briefly, the isolated lesions were transferred into the isolation buffer (10 mM KH_2PO_4/K_2HPO_4, 150 mM NaCl,
2 mM EDTA, 5.5 mM glucose, pH 7.2) in the sterile plates on ice, and forced through a wire mesh. Amastigote suspensions were prepared using homogenization of the infected macrophages, disruption by seven passages through a tissue grinder, and centrifugation at 1,300 × g, at 4ºC for 20 min. The pellet was resuspended in 2.5 ml of 0.14 M ammonium chloride in 17 mM Tris-HCl for lysing the red blood cells. Then**,** amastigotes were washed three times in isolation buffer, filtered through polycarbonate filters of 8, 5 and 3 μm, respectively, counted by haemocytometer chamber and stored at -20ºC until use (6 months).

Isolation of amastigotes by Percoll gradient was carried out according to the procedure described elsewhere [15]. In brief, the amastigote suspension was prepared from infected tissues and large particles were left to settle out. The supernatant was centrifuged at 1300 ×g for 15 min and the pellet was suspended in 6 ml of 45% Percoll in isolation buffer. Then 1.5 ml of 70% Percoll in isolation buffer was overlaid with 4 ml of 45% Percoll containing the amastigote and finally overlaid with 4.5 ml of 25% Percoll in isolation buffer and centrifuged at 1300 ×g at 4ºC for 60 min. Amastigotes were collected from the band at the interface of 45/70% Percoll, resuspended in isolation buffer, counted and then stored at -20ºC.

Western blot and pixel analyses of bands. A sample from isolated amastigotes was electrophoresed under reducing condition in 10% SDS-PAGE using Goding protocol [16]. Proteins from the gel were electroblotted onto the nitrocellulose membrane and blocked with 2% BSA for 30 min. Individual strip was incubated with appropriate peroxidase conjugated anti-mouse heavy chain antibodies including anti-mouse-μ, γ and α chains, as well as anti-mouse-IgG (all from Sigma, Germany). The bands were visualized with diaminobenzidine (DAB) substrate [17], and then scanned and analysed by pixel analysis software (TotaLab Company, USA) to compare their intensity and relative concentration.

Isolation of amastigotes by the new method.

a) Preparation of coated plate. Antibody against mouse serum immunoglobulin was prepared in a rabbit by a standard protocol [18]. The anti-serum

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was adjusted to 10 μg/ml (total protein) by PBS and 5 ml of it was poured into the sterile microbiological plates. The plates were incubated overnight at 4°C or for 2 hour at room temperature and then washed three times using cold PBS + 0.05% Tween 20. Each step took 5 min with gentle shaking until all free antibodies were removed. The plates were blocked with 2% BSA at room temperature for 1 hour and then washed as before and kept frozen in -20°C until use.

b) Isolation of amastigotes by coated plates: Lesions from *L. major* infected mice were removed carefully and suspended in a cold PBS buffer, containing 5 mM glucose, 100 μg/ml gentamicin and a protease inhibitors cocktail including 1 mM Phenylmethylsufonylfluride (PMSF) and 2 mM EDTA (complete PBS) under the microbiological hood. Large particles of lesion were ground and the resulting suspension was transferred quickly into a sterile tube on ice to remove the remaining large particles. Supernatant was transferred into a new sterile tube and amastigotes were released by grinding the infected lesion-derived macrophages through a homogenizer for several times until all macrophages were disrupted. The suspension was centrifuged at low speed (3 min, at 300 ×g at 4°C) to remove large cells aggregates and then the supernatant was transferred into a new tube. For lysing the red blood cells, the suspension was centrifuged at high speed (at 1300 ×g**,** at 4°C for 20 min) and the pellet was resuspended in 2.5 ml of 0.14 M ammonium chloride in 17 mM Tris-HCl at room temperature for 2 min. Then, the cells were mixed with 7 ml of cold complete PBS and centrifuged at 1200 ×g at 4°C for 20 minutes. The supernatant was discarded and the pellet was re-suspended in 5 ml cold complete PBS and then transferred into the coated plates where rabbit anti-mouse immunoglobulins have been coated. The plates were covered firmly with a cellotape to prevent contamination and kept at 4°C overnight, and then were washed gently 2 times with complete PBS and checked under inverted microscope to ensure complete attachment of amastigotes onto the plate bottom. Finally, 10-20 ml of cold complete PBS was added into the plates and the attached amastigotes were isolated from the plate surface using a sterile plastic loop or a sterile syringe with narrow needle.

RESULTS

Detection of surface immunoglobulins. The analysis of lesion-derived amastigotes by Western blotting revealed the presence of three distinct types of heavy chain immunoglobulins as well as IgG as prototype of immunoglobulins on the surface of amastigotes (Fig. 1). The results showed that the concentration of IgG has greater intensity in pixel analysis than IgM followed by IgA based on their corresponding to heavy chain concentration. (Fig. 2)

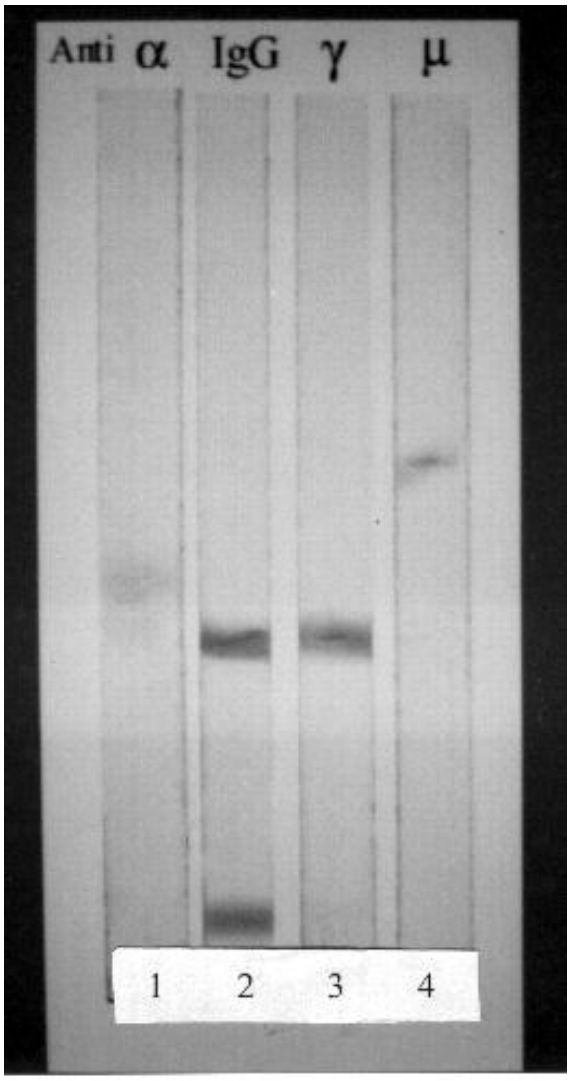

Fig. 1. Western blot analysis of amastigotes isolated from BALB/c mice lesion. Strips incubated with peroxidase-conjugated anti-mouse-α (lane 1); anti-mouse-IgG (lane 2); anti-mouse-γ (lane 3) and anti-mouse-μ (lane4) chains**,** respectively.

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Isolation of amastigote. Based on the presence of surface Igs the amastigotes were isolated successfully by adhering the amastigoate suspension to the plates coated with anti-Igs. A pure suspension of amastigotes was observed under light microscope. No more cell residuals and debris were associated with amastigote suspension in this method in comparison to other (Glaser and Percoll gradient based) technique. Using this new technique, a homogeneous preparation of amastigotes was obtained (1-10 × 10^6 cell/ml) that depends on the size and number of plates used.

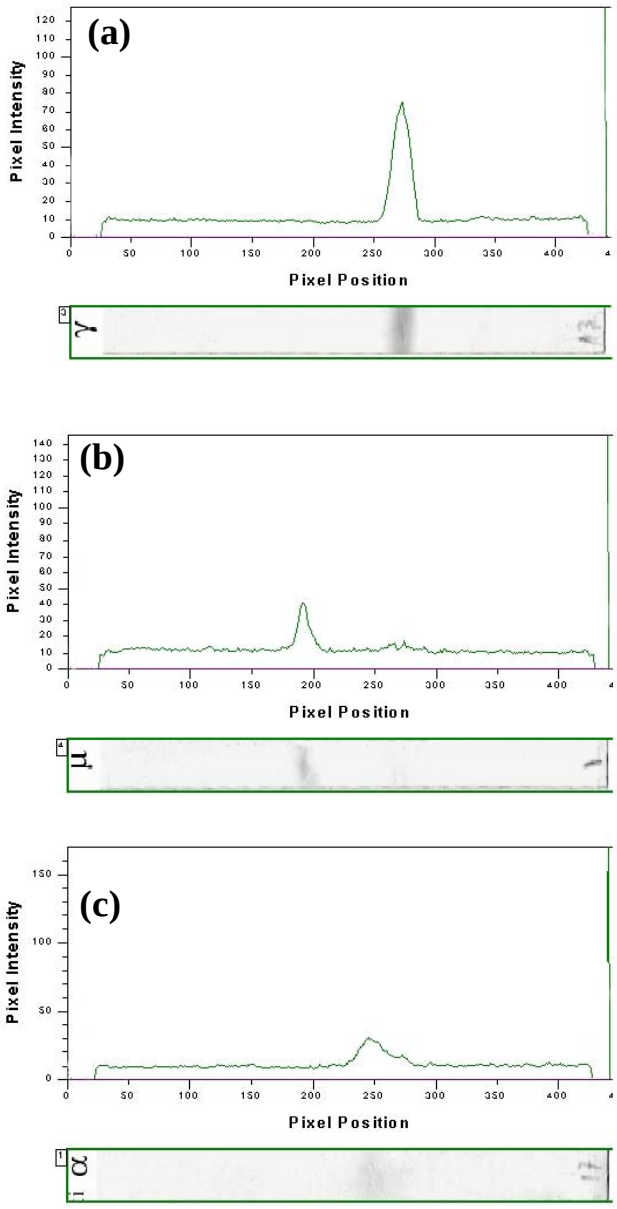

Fig. 2. Band intensity of heavy chain concentration of **(a)**, γ; **(b)**, μ and **(c)**, α heavy chain on the surface of lesion-amastigotes detected by pixel band analysis (densitometry).

DISCUSSION

Amastigotes are intracellular form of *Leishmania* parasite that are able to survive and replicate inside the phagolysosomes by binary fission, until the infected macrophages can no longer sustain them. In this stage, the infected cells are disrupted and amastigotes are internalized by uninfected macrophages [2]. In this study, the amastigotes isolated from lesions of BALB/c mice infected with *L. major* were tested for possible surface immunoglobulins. The results showed that different classes of host immunoglobulins present on the surface of *L. major* amastigote, among them the amount of IgG was higher than the other immunoglobulins. These data are consistent with another study suggesting the presence of host proteins and immunoglobulins on the surface of lesion-derived amastigote [19]. The reasons for the presence of these Igs and their possible role are not clear; however, some reports have shown that the parasite exploits receptors from host proteins such as Igs to enter into the macrophages [19, 20]. Other studies have shown that the parasite uses host IgG as a virulence factor and it allows amastigote to ligate Fcγ receptors on inflammatory macrophages to preferentially induce the production of high amounts of IL-10. [4]

During screening antibody against tissue-derived amastigote by ELISA or indirect immunofluorescent antibody test, a false positive reaction was observed which seems to be produced by the amastigote surface Igs. Hence, the surface Igs of amastigotes should be removed before the detection of antibody against real amastigotes in immunized mice.

Based on amastigote surface Igs, a technique was developed for isolation of lesion-derived amastigotes. This technique could be used for isolation of antibody-coated amastigotes of other species of *Leishmania* including *L. donovani*, *L. mexicana*, etc. In comparison to other conventional techniques, this new technique showed several advantages: 1) In contrast to other techniques, that need 2-3 h, this technique needs only 1 hour for isolation of amastigotes. The prepared coated plates with Igs can be used easily for isolation of amastigotes; 2) this technique is a simple procedure that can be used in many research laboratories without need to expensive and special equipment and supplies. Although, in contrast to other methods, a limited number of amastigotes (1-10 × 10^6) will be obtained from one plate. By mass production of anti-serum and also preparation and

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storage of a large number of plates, this problem can be overcome. Moreover, after a complete removal of the amastigotes, the plates, could be used again for isolation of the amastigotes; 3) Because of specific binding of amastigotes to coated antibodies in plates, a pure preparation of amastigote without cell debris will be obtained.

It should be noted that this technique is just developed for lesion-derived amastigote. Due to the lack of Igs on the surface of *in vitro* grown amastigotes including those which are grown in macrophage cell-lines (U937, J774, etc.), this technique is not applicable for isolation of axenically grown amastigotes. Moreover, the possible presence of mice tissue Igs mixed with lesion-derived amastigotes may be interfered with initial stage of this protocol, but they will be removed during washing steps. Therefore, it seems by this method a pure suspension of lesion-derived amastigote will be isolated in a short time and with lower cost compared to the other techniques used for isolation of amastigotes.

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