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The Analysis of GP63 on *Leishmania* Amastigotes and Promastigotes using Immunofluorescence Method

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Abstract *Leishmania* life cycle consists of two stages; the stage of extracellular promastigotes which multiply in the gut of sandfly insect vector and the stage of intracellular amastigotes which divide in the phagolysosome of mononuclear phagocytic cells of the mammalian host. Promastigotes express a major surface glycoprotein of 63 kDa, refrred to as GP63. The expression of GP63 in both *Leishmania* life stages was studied using rabbit antibodies against native GP63 as well as rabbit antibodies against recombiant GP63 that was synthesized in an *Escherichia coil* expression system. It was demonstrated using immunofluorescence staining that GP63 antigen was not only on the surface of *Leishmania* promastigotes but also on that of amastigotes which were within a macrophage cell line and in freshly isolated lesion. The signal from *Leishmania* detected with anti-recombinant GP63 antibody was less intense than that with anti-GP63. The difference of GP63 antigen distribution on the surface of *Leishmania mexicana* and *major* was also shown in this paper.

Key Words: *Leishmania*, Major surface glycoprotein, Amastigote and promastigote expression, Immunofluorescence method

Introduction

Leishmania is a protozoan parasite with a two-stage life cylcle. The extracellular flagellated promastigotes multiply in the gut of the sandfly insect vector while the intracellular aflagellated amastigotes divide in the phagolysosome of mononuclear phagocytic cells in the mammalian host. Two major surface molecules of *Leishmania* promastigotes, a glycoprotein of 63 kDa, referred to as GP63[1,2], and a lipophosphoglycan[3], have been identified as ligands for specific receptors on host mononuclear phagocytic cells[4,5].

On the other hand, there are conflicting reports as to whether GP63 is expressed in both life stages of *Leishmania*. GP63 has been described as a stage-specific antigen with the use of monoclonal antibodies[6] or as a promastigote-specific protease[7], whereas other studies have described GP63 as an antigen common to both life stages[8]. Analysis of the transcription of GP63 genes, however, has shown that GP63 mRNA is present in both life stages of *Leishmania*[5]. To address whether GP63 protein is present in the amastigote stage of *Leishmania*, antibodies raised against native and also recombiant GP63 (rGP63) purified from an *Escherichia coil* expression system were used to identify GP63 protein in the amastigote life stage of *Leishmania*. We reported GP63 antigen was recognized on the surface of both life stages of *Leishmania mexicana* and *major*[9]. This paper showed more detailed findings such as the localization of GP63

antigen on the two stages of parasites, signal intensity from *Leishmania* using both anti-GP63 and anti-rGP63 and so on.

Materials and Methods

Parasites and a mononuclear cell line

Leishmania major NIH S strain, and *Leishmania mexicana* MNYC/B2/62/M379 were maintained as promastigotes as described previously[5]. In brief, *Leishmania* promastigotes were maintained at 26℃ in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 100u/ml penicillin and 100μg/ml streptomycin (10% 1640). These *Leishmania* promastigotes were passaged every seven days at 10^6 cells/ml. Parasites from stationary phase cultures were injected into footpads for *Leishmania major* or the base of the tail for *Leishmania mexicana* or 10-15 week-old female BALB/c mice bred at the University of British Columbia. Amastigotes were obtained from lesions 8-12 weeks following infection as described[10].

The murine mononuclear cell line P388D_1 was routinely grown at 37℃ in a 5% CO_2/95% air humidified atomosphere incubator. The cells were maintained in·10 ml of 10% 1640 medium in a 260 ml flask (Nunc 147589). Non-adherent cells were discarded every seven days and adherent cells were washed once with 10 ml of warm calcium and magnesium free Hanks balanced salt solution (HBSS/Ca, Mg free). Then the cells were scraped off with cell scraper (Coster 3010) after replenished with 10 ml of HBSS/Ca, Mg free. The P388D_1 cells were washed once in 10% 1640 medium and reseeded at 2.5×10^5 cells/flask in 8 ml of 10% 1640 medium in the flask. For examination, the cells were grown on round cover slips in 24-well plates prior to the addition of amastigotes. Mononuclear cells and parasites at a ratio of 1:10 were maintained at 37℃ for 24 hours prior to fixation.

Antibodies

GP63 was purfied from a lysate of 2.5×10^{10} promastigotes of *Leishmania major*[11]. The GP63 antigen was mixed with complete Freund's adjuvant and used to immunize New Zealand white rabbits (R&R Rabbitries, Stanwood, WA) by three intramuscular injections at two week intervals. Then anti-GP63 rabbit serum was collected. Antibodies to rGP63 were prepared by immunizing a rabbit on three occasions at two week intervals with 100 μg of SDS-PAGE-purified rGP63 in complete Freund's adjuvant[12]. Normal rabbit serum was collected from immunized animals prior to the first injection.

Immunofluorescence

The P388D_1 cells infected with *Leishmania* amastigotes on round cover slips wre washed with 2 ml of 10 mM Na-phosphate, 150 mM NaCl, pH 7.4 (PBS), 3 times for every 10 min, then the cover slips were kept in the bottom of 24-well plates. The cells adhered to cover slips in 1 ml of PBS were fixed with additional 1 ml of 4% paraformaldehyde for 1 hour at room temperature. They were washed with PBS and permeabilized in 1 ml of 0.15% saponin in PBS containing 0.1% bovine serum albumin, for 15 min at room temperature. For examinatin of GP63 expression by lesion amastigotes, tissue from lesions was fixed in 2% paraformaldehyde immediately after killing of the animal. Cells were then cyto-spun and air-dried on slides. While, the pellet of *Leishmania* promastigotes (approx. $1\text{-}2 \times 10^8$ cells) was resuspended in 5 ml of PBS, and then added 5 ml of freshly prepared 4% paraformaldehyde solution. The cells wre mixed well and kept at room temperature for 1 hour. After wahsing, fixed promastigotes were smeared by a cytospin. These specimens were incubated overnight at 4℃ in either 100 μl of antiserum or normal rabbit serum diluted 1/50 with PBS in a humid chamber. Cells were then wahsed in PBS, incubated in 100 μl of fluorescein isothiocyanate-labeled horse $F(ab')_2$ anti-rabbit IgG (25 μg/ml) for 2 hours at 4℃, wahsed in PBS and examined by fluorescence microscopy using a Zeiss III RS microscope.

Results

Expression of GP63 by *Leishmania* amastigotes

To address the question of whether GP63 is expressed by both life stages of *Leishmania*, antibodies against purified *Leishmania major* promastigote GP63 and against rGP63 were used to identify amastigote GP63. P388D_1 macrophage monolayers were infected with amastigotes isolated from lesion tissues, cultured for 24 hours, and stained with rabbit antibodies against GP63. Examination of macrophage cultures maintained at 37℃ for 24 hours with *Leishmania* amastigotes and then co-stained with ethidium bromide and fluorescein diacetate[13] showed that at least

95% of the macrophages were viable and that approximately 30% of the cells were infected with *Leishmania* (data not shwon).

Amastigotes of *Leishmania mexicana* (Fig. 1A) and *Leishmania major* (Fig.1E) contained within infected $P388D_1$ macrophages were clearly labeled with rabbit antibodies to promastigote *Leishmania major* GP63. As a control for non-specific antibody binding, amastigotes of both species were not labeled with normal rabbit serum (Figs. 1B and 1F). *Leishmania mexicana* amastigotes that were fixed directly following isolation from lesions showed punctate and asymmetric labeling with an antibody to GP63, and intense regions of fluorescence were usually recognized at one end of the amastigote (Fig. 1C). In contrast, freshly isolated *Leshmania major* amastigotes had a relatively even distribution of fluorescence staining when labeled with an antibody against GP63 (Fig. 1G). These amastigotes of *Leishmania mexicana* and *major* were not labeled with pre-immune serum (Figs. 1D and 1H). Antibodies against rGP63 gave a similar staining pattern on amastigotes as shwon in Fig. 1, except that the fluorescence signal was less intense (data not shown). In all cases, immunofluorescence labeling with anti-GP63 antibodies was confined to the amastigotes and was not apparent on the surface of the host macrophage.

Expression of GP63 by *Leishmania* promastigotes

Leishmania promastigotes have a body and a flagellum. *Leishmania mexicana* promastigotes were stained evenly with an anti-GP63 antibody on both surface of bodies and flagellums (Fig. 2A). The promastigotes had two shapes of bodies after freshly isolated *Leishmania mexicana* amastigotes were cultured for 24 hours *in vitro*. The one was a football-shaped body and the other was oval-shaped body. The ratio of the former to the latter was about 7-8:2-3. Expression of GP63 antigen on the oval-shaped body was stronger than tnat on the football-shaped one.

The body of *Leishmania major* promastigotes were stained either strongly or moderately with an anti-GP63 antibody. At a half to one third area of the body close to flagellum, GP63 antigen was expressed strongly on the surface and the staining pattern was usually even but sometimes even and spotty. The opposite site of the body and a flagellum were stained evenly with an anti-GP63 antibody and the staining was rather moderate (Fig. 2C).

When *Leishmania mexicana* and *major* promastigotes were stained with normal rabbit serum, no fluorescence signal was seen (Figs. 2B and 2D).

Discussion

Many kinds of antigens are expressed on the surface of *Leishmania*, and molecular weight of these antigens were reported as 17 kDa, 34 kDa, 43 kDa, 53 kDa, 63 kDa, 65 kDa, 83 kDa, > 200 kDa and so on[1,2,3,14,15,16,17,18], analyzed by western blots of the lysate of *Leishmania* using antibodies to *Leishmania*. Among these antigens, GP63 (glycoprotein, molecular weight 63 kDa) is one of the major surface antigen of *Leishmania*.

We showed that GP63 antigen was expressed on the surface of both life stages of the *Leishmania mexicana* and *major* by a previous[9] and in this paper using immunofluorescence technique. *Leishmania* promastigotes express large amounts of surface GP63 antigen, and GP63 has been described as a stage-specific antigen with the use of monoclonal antibodies[6,7]. Whereas, other studies have described GP63 as an antigen common to both life stages of *Leishmania*[8]. When *Leishmania mexicana* and *major* amastigotes were stained with an anti-GP63 polyclonal antibody following paraformaldehyde fixation and pretreatment with saponin, these amastigotes infected to macrophages or freshly isolated amastigotes from lesions were clearly labeled (Figs. 1A, 1C, 1E and 1 G). The reason why I succeeded to show GP63 antigen on the surface of *Leishmania* amastigotes infected to macrophages might be due to the use of polyclonal antibody which recognizes many epitopes of GP63 antigen. Chang *et al.*[6] and Bordier[7] used monoclonal antibodies which recognized only one epitope of GP63 antigen, so, signal from fluorescence labeled antibody might be weak.

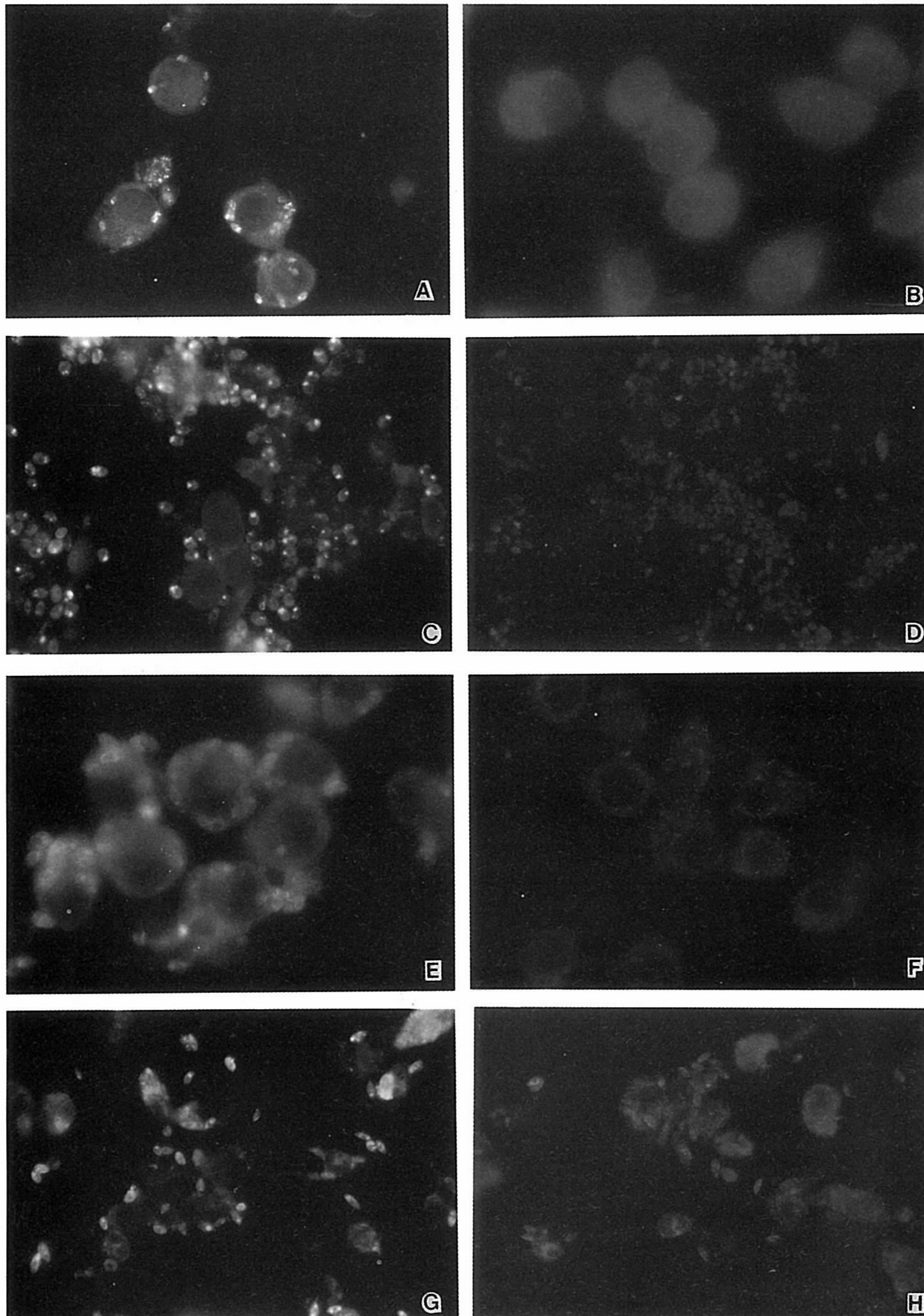

Fig. 1 Identification of GP63 antigen in *Leishmania* amastigotes. P388D$_1$ macrophages infected with amastigotes for 24 hours (A, B, E and F) or freshly isolated lesion amastigotes (C, D, G and H) were fixed with 2% paraformaldehyde and then stained with rabbit antibodies against GP63 (A, C, E and G) or preimmune rabbit serum (B, D, F and H) followed by fluorescein isothiocyanate conjugated horse F(ab')$_2$ anti-rabbit IgG. A and B show the macrophage cell line P388D$_1$ infected with *Leishmania mexicana* amastigotes and E and F with *Leishmania major* amastigotes. Freshly isolated *Leishmania mexicana* amastigotes are C and D, *Leishmania major* are G and H. (× 450)

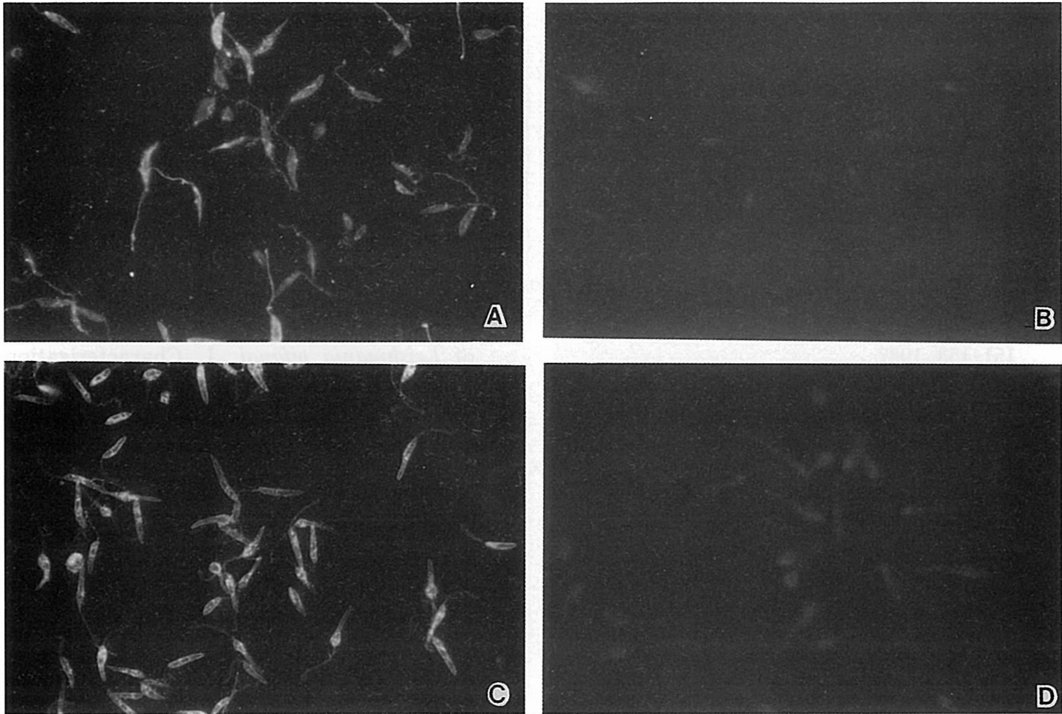

Fig. 2 *Leishmania mexicana* promastigotes fixed wth 2% paraformaldehyde were stained with anti-GP63 (A) or normal rabbit serum (B). *Leishmania major* promastigotes were fixed and stained with anti-GP63 (C) or normal rabbit serum (D). (× 450)

On the other hand, when *Leishmania* amastigotes and promastigotes were stained with anti-recombinant GP63 polyclonal antibody, the fluorescence intensity was weak, though the staining condition was similar to that of antibody to native GP63 antigen. This might be due to the antigenic difference of recombinant GP63 from the native structure of GP63 on the *Leishmania* cell surface, because, antigen to make anti-rGP63 was produced by pBS10Rb.1 in *Escherichia coil* system[4] and lacked carbohydrate composition.

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