Monoclonal antibodies to \textit{Leishmania mexicana} promastigote antigens

II. Cellular localization of antigens in promastigotes and infected macrophages

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Summary

In the accompanying paper by Ilg \textit{et al.}, it was shown that \textit{Leishmania mexicana} promastigotes covalently modify a secreted acid phosphatase and other proteins by carbohydrate epitopes characteristic for lipophosphoglycan (LPG). In this study, the reaction of the anti-LPG monoclonal antibodies (mAbs AP3 and L7.25) and of mAb L3.13, an antibody directed against an epitope present on the secreted acid phosphatase but not on LPG, with promastigotes and infected peritoneal macrophages is studied by immunofluorescence and immunoelectron microscopy. AP3 labels the surface, the flagellar pocket and intracellular structures in promastigotes, while L3.13 reacts predominantly with an antigen located in the flagellar pocket. Early after infection with promastigotes, but not amastigotes, AP3 or L7.25 transiently label epitopes at the surface of live macrophages. No L3.13-reactive material is detected at the surface of infected macrophages. In permeabilized, infected macrophages, AP3 heavily labels the surface of amastigotes and the lumen of the parasitophorous vacuole, while L3.13 reveals antigen in the flagellar pocket, intracellular vesicles of amastigotes, and components in the lumen of the parasitophorous vacuole. Possible mechanistic implications for \textit{Leishmania}–macrophage interaction raised by these findings are discussed.

Key words: \textit{Leishmania mexicana}, promastigote, amastigote, macrophage, monoclonal antibodies, immunofluorescence, immunoelectron microscopy.

Introduction

In the accompanying paper (Ilg \textit{et al.} 1991), several monoclonal antibodies (mAbs) against \textit{Leishmania mexicana} promastigote antigens have been characterized. To recapitulate briefly, mAbs AP3 and L7.25 are directed against epitopes shared with lipophosphoglycan (LPG; cf. review by Turco, 1990) and now shown to be present on several promastigote proteins including part of the cell-associated acid phosphatase activity. This cellular enzyme is probably the precursor of the secreted form of the phosphatase, which is also modified by LPG epitopes (Ilg \textit{et al.} 1991; Menz \textit{et al.} 1991). In view of its abundance (0.5 x 10⁶ to 2 x 10⁶ molecules/cell, depending on the species considered; cf. Orlandi and Turco, 1987; T. Ilg, unpublished experiments), LPG is expected to be the predominant molecule reactive with these antibodies in promastigotes. In contrast, very little information is available on components carrying these epitopes in the other stage of the life cycle, the amastigote.

The other monoclonal antibody, L3.13, also reacts with the secreted acid phosphatase but not with LPG. In immunoblots of promastigote or amastigote lysates the antibody binds to several distinct, but so far uncharacterized, high molecular weight components (G. Winter and T. Ilg, unpublished results). In promastigotes, some of these components may be the precursors of the secreted form of the enzyme. Recent evidence suggests that L3.13 is directed against a carbohydrate epitope on the secreted phosphatase (T. Ilg and Y.-D. Stierhof, unpublished experiments).

In this study, we describe the localization of the antigens recognized by these two types of antibodies in promastigotes and infected macrophages. Although antigen complexity imposes obvious limitations on a detailed interpretation, the findings raise several interesting points regarding the cell biology of \textit{Leishmania}–macrophage interaction.

Materials and methods

Monoclonal antibodies

The derivation and characterization of mAbs AP3, L7.25 and L3.13 have been described (Ilg \textit{et al.} 1991; Menz \textit{et al.} 1991).

Cell culture

Promastigotes of \textit{Leishmania mexicana} were grown as described (Ilg \textit{et al.} 1991). Amastigotes of the same strain were isolated from mouse lesions of Balb/c mice. Lesions were excised under sterile
conditions and homogenized in 20 mM sodium phosphate, 100 mM NaCl, 10 mM MgCl₂, 10 mM KCl, 0.5 mM EDTA, 5.5 mM glucose, 0.02% bovine serum albumin (BSA), pH 7.3, in a Potter-type homogenizer. The suspension was freed from larger pieces of tissue by passage through a cotton plug in a 5 ml syringe. The cells were washed three times in buffer (centrifugation 5 min, 2000 g) and then incubated at a density of 5 x 10⁶ cells/ml in modified UM 54 (Pan, 1984; Pan and McMahon-Pratt, 1988) containing per liter: 1 packet of ME 199 (Gibco, Eggenstein, FRG, Cat. no. 071-1200), 2.5 g glucose, 5 g casein digest peptone (BBL, Cockeysville, MD, USA, Cat. no. 89–7023–0), 0.75 g glutamine, 0.2 mg hemin, 25 mM Hepes, 40 mg gentamycin, 150 mM iPCS (inactivated fetal calf serum), pH 6.0, at 34°C in 5% CO₂ in air. After 3 days the cells were diluted to 2 x 10⁷ ml⁻¹. The amastigotes retained their ovoid shape and grew about 20-fold within 2 weeks. They were used after 4–6 days in culture for infection of macrophages.

Macrophages were obtained from Balb/c mice by peritoneal lavage with 5 ml DMEM (10 g Gibco, Cat. no. 074–01600, 3.5 g glucose, 4.76 g Heps, 2.8 g NaHCO₃, per liter, pH 7.3) containing 15% FCS. The suspension was distributed in 0.2 ml samples into 8-well Lab-Tek tissue culture chamber slides (Miles, Naperville, Ill., USA) and kept for 1 h at 37°C in 5% CO₂ in air. Non-adherent cells were removed by washing with medium. After overnight incubation, the macrophages were infected for 2 h at 27°C in 5% CO₂ in air with 2 X 10⁶ promastigotes per ml or 4 X 10⁶ amastigotes per ml per well previously washed twice by centrifugation and resuspension in DMEM/15% FCS. Thereafter, the majority of unphagocytosed parasites were removed by replacing the medium twice. Finally, the wells were supplied with 0.5 ml medium and incubated for up to 10 days at 34°C in 5% CO₂ in air.

Immunofluorescence
Live promastigotes were incubated with antibodies in DMEM, 10% FCS for 60–90 min at 0°C; washed twice in DMEM and fixed with 2% formaldehyde/0.05% glutaraldehyde in PBS for 1–2 h at room temperature. Cells were washed in PBS, blocked with 2% BSA in PBS and then incubated with FITC-labeled goat anti-mouse IgG/IgM (1:250, Dianova, Hamburg, FRG) plus 10 μg/ml T1 bisbenzimide (H 33258) in PBS/BSA for 60 min. After three washings in PBS/BSA, cells were covered with 50% glycerol in PBS and inspected in a Leitz Ortholux microscope using an NPL FLUOTAR objective. Alternatively, the cells were first fixed in formaldehyde/glutaraldehyde and then blocked and incubated with antibodies as described above. For infected macrophages the FITC-labeled goat anti-mouse IgG/IgM was replaced by biotinylated anti-mouse immunoglobulin (1:100, Sigma Cat. no. B-2016) and FITC-labeled streptavidin (1:100, Amersham, Cat. no. RPN1232). Fixed macrophages were permeabilized by adding 0.02% digitonin to all reagents.

Immunoelectron microscopy
Promastigotes and macrophages were pelleted by centrifugation and resuspended in 0.5 ml PBS. After addition of 0.5 ml 4% formaldehyde, 0.1% glutaraldehyde in PBS, cells were fixed for 60 min at 4°C. Thereafter, cells were embedded in agarose, dehydrated at progressively lower temperatures and embedded in Lowicryl K4M or HM20 at 35°C (Carlstenma et al. 1982). Ultrathin sections were treated with 0.5% BSA, 0.2% gelatin in PBS to block non-specific binding sites, and incubated with monoclonal antibodies and protein A–15 nm gold complexes (Slot and Geuze, 1985). Labeled sections were stained with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope at 60 kV.

Lesions from infected mice were cut in small pieces (1 mm x 1 mm x 1 mm) and fixed with 2% formaldehyde, 0.05% glutaraldehyde in PBS for 120 min. Dehydration, embedding and labeling were performed as described above.

Results
The cellular localization of the antigens recognized by the antibodies was studied by immunofluorescence (Figs 1 and 4) and by immunoelectron microscopy (Figs 2 and 3). mAbs AP3 and L7.25 result in a strong fluorescence at the surface of live (not shown) and fixed promastigotes (Fig. 1A). This result was confirmed by on-section immunogold labeling of Lowicryl-embedded promastigotes with mAb AP3 (Fig. 2B); in addition to the surface of the parasite’s body, strong deposition of gold particles is observed in the flagellar pocket and, less intensely, in unidentified intracellular structures between the kinetoplast and the nucleus.

Fig. 1. Immunofluorescence of promastigotes and infected macrophages. (A) Fixed promastigotes probed with mAb L7.25. (B and C) Fixed promastigotes probed with or without mAb L3.13, respectively; comparison of the photographs shows the specific fluorescence in the flagellar pocket (cf. arrows). (D and E) Live peritoneal macrophages treated with mAb L7.25 24 h after infection with promastigotes; (D) the surface fluorescence specific for the reaction with antibody; (E) the DNA staining of the same cell with bisbenzimide revealing the macrophage nucleus, and the nuclei and kinetoplast of the intracellular amastigotes derived from multiple infections by promastigotes. (F–G) Fixed and digitonin-permeabilized macrophages 92 h after infection with promastigotes treated with mAb L7.25 and bisbenzimide. (F) The intense L7.25-specific fluorescence at the inner lining of the extended parasitophorous vacuole and at the surface of the ovoid amastigotes derived from one infecting parasite by division. (G) (antibody staining) and (H) (DNA staining) show the same cell. Note the absence of fluorescence at the surface and the cytoplasm of the macrophage extensions. The staining of the macrophage nuclei (G) is non-specific because it was also observed on nuclei of uninfected cells.

(F) Fixed and permeabilized macrophages 220 h after infection with promastigotes treated with mAb L3.13. The amastigotes are arranged side by side at the periphery of the extended parasitophorous vacuole with the fluorescent flagellar pocket (cf. lower arrow) always facing the lumen of the vacuole. The non-uniform (vesicular?) staining opposite the flagellar pocket at the posterior half of the cells suggests the presence of antigenic material in megasomes. The diffuse staining of the macrophage is non-specific because it is likewise observed in uninfected cells (cf. upper arrow).
With all three techniques, a specific reaction of L3.13 is observed in the flagellar pocket of amastigotes (Fig. 11, cf. arrow, Figs 3 A and C, 4 A and B). Judged by immunofluorescence, the antibody also reacts in a punctate manner with intracellular structures, at the posterior end of the amastigotes (Fig. 11, reaction opposite to the flagellar pocket, compare also with the surface fluorescence of L7.25 in F and G). This pattern corresponds to a weak immunogold labeling on megasomes (not shown). By immunofluorescence, the host macrophage is apparently devoid of antigen (Fig. 11), and neither does the antibody react with the surface of live, infected macrophages. On the other hand, immunoelectron microscopy gives a weak reaction in the lumen of the parasitophorous vacuole, suggesting that the antigen might be secreted by the parasites (Fig. 3A).

**Discussion**

As may be expected for an antibody recognizing a secreted glycoprotein, L3.13 reacts specifically with the lumen of the flagellar reservoir of promastigotes (Figs 1 B and 2 A). This observation agrees with experiments of Bates et al. (1989), although, due to fixation and detergent permeabilization, the staining of the flagellar pocket with antibodies against the secreted acid phosphatase of *L. donovani* was highly diffuse. Whether some antigen is also located on the cell surface is debatable. In fixed cells we were able to detect surface fluorescence using L3.13, while Bates et al. (1989) using different antibodies against the secreted phosphatase of *L. donovani*, did not; from which he concluded that the surface phosphatase activity, demonstrated by cytochemical techniques (Gottlieb and Dwyer, 1981) is unrelated to the secreted form of the enzyme.

The most straightforward interpretation of the staining pattern observed with L3.13 in amastigotes (Fig. 11) is that the antigen is located in the flagellar pocket (see also Fig. 4 A) and in the lysosome-like organelles called megasomes, which are located at the posterior cell pole, i.e. opposite to the flagellar pocket (Antoine et al. 1988). By immunoelectron microscopy, some labeling was observed in the lumen of the parasitophorous vacuole in addition to the strong reaction in the flagellar reservoir (Fig. 3 A and C), suggesting that the molecules carrying the reactive epitope may be secreted by amastigotes and resist degradation by the lysosomal hydrolases of the macrophage. The nature of labeled molecules in the parasitophorous vacuole remains unknown; neither we (unpublished results) nor others (Antoine et al. 1987) have been able to detect acid phosphatase activity in the supernatant of cultured amastigotes. Several groups (Lewis and Peters, 1977; Barbieri et al. 1985; Pimenta and De Souza, 1986; Antoine et al. 1987, 1988; Hassan and Coombs, 1987) have investigated the cellular distribution of acid phosphatase activity in amastigotes by cytochemical techniques; enzyme was consistently detected in megasomes but only rarely in the flagellar pocket or on the cell surface.

As expected (Tolson et al. 1989), the antibodies AP3 or L7.25 detect LPG on the surface of promastigotes (Fig. 1 A). In addition, molecules carrying these epitopes, i.e. the secreted acid phosphatase, are located in the flagellar pocket and in intracellular structures between kinetoplast and nucleus (Fig. 2 B). The display of these epitopes, most likely LPG itself, on the plasma membrane of macrophages after infection with promastigotes (Fig. 1 D); and De Ibarra et al. 1982; Handman and Hocking, 1982; Handman et al. 1984; Williams et al. 1986) led Handman et al. (1987) to suggest that this antigen should signal the presence of the intracellular parasite to the immune system. However, in agreement with Williams et al. (1986), we find that this phenomenon is only transient and, in addition, promastigote-specific. The antigenic components appear to be transferred to the macrophage surface during attachment and invasion by

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Fig. 2. Ultrathin sections of Lowicryl HM-20 (A) or K4M (B)-embedded *L. mexicana* promastigotes labeled with mAbs L3.13 (A) or AP3 (B) and protein A—15 nm gold. L3.13 labels the flagellar pocket (fp); AP3 the flagellar pocket, the surface and intracellular structures between kinetoplast (k) and nucleus (n). Bar, 1 μm.
Fig. 3. Ultrathin sections of Lowicryl K4M-embedded amastigotes in infected macrophages (A and B) and mouse lesions (C and D) labeled with mAbs L3.13 (A,C) or AP3 (B,D) and protein A-15nm gold. L3.13 labels the flagellar pocket (fp) (A and C); and, in some cells, the parasitophorous vacuole (pv) (A). AP3 labels the FP and intracellular vesicles (v) (B and D). No significant labeling was obtained in controls using mouse serum or omitting antibody. n, nucleus. Bar, 1 μm.
promastigotes; they may subsequently be lost by a mechanism similar to the release of LPG from the promastigote surface (King et al. 1987). In support of this interpretation, we have not been able to detect antigen on the surface of live macrophages either several days after infection with promastigotes or at any time after infection with amastigotes, in spite of the heavy labeling of the surface of live macrophages either several days after infection with promastigotes; they may subsequently be lost by a mechanism similar to the release of LPG from the promastigote surface (Barbieri et al. 1985; Prina et al. 1990). Possibly, the amastigotes inhibit the fusion of vesicles from the vacular membrane, thereby prohibiting the recycling of Leishmania antigens to the macrophage surface.

It should be noted that there are several qualitative and quantitative discrepancies between the light and the electron microscopic results, which may arise either from differences in the accessibility of the antigens to the respective antibodies or from loss or relocation of antigens during specimen preparation. For example, labeling of the flagellar pocket of promastigotes with mAbs AP3 or L7.25, which both belong to the IgM subclass, is readily observed in sectioned (Fig. 2B) but not in whole cells (Fig. 1A). In this latter case, the IgM antibody probably cannot enter the pocket, in contrast to the smaller IgG1 (L3.13, cf. Fig. 1B). A second example is the lack of L7.25/AP3-reactive material in the parasitophorous vacuole of most infected macrophages as judged by immunoelectron microscopy, in contrast to the strong reaction observed on the light microscopic level (Fig. 1F and G). This discrepancy can be explained by the extraction of molecules that cannot be sufficiently fixed by aldehydes, from the parasitophorous vacuole during dehydration and infiltration with Lowicryl. It is evident that for the antigens considered in this study, the combination of several techniques leads to more reliable conclusions regarding their cellular localization.

Our findings stimulate further investigations in several directions. First, since mAb L3.13 specifically labels antigen(s) in the flagellar pocket of live promastigotes, it should be possible to characterize this material using the antibody as a probe. Second, it will be rewarding to isolate the component(s) recognized by mAb AP3 or L7.25 which are released by amastigotes into the parasitophorous vacuole of macrophages. Finally, further studies are warranted to clarify the transfer mechanism of AP3-reactive molecules from promastigotes to the macrophage plasma membrane during attachment and invasion.

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