Study of *Leishmania major*-Infected Macrophages by Use of Lipophosphoglycan-Specific Monoclonal Antibodies

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*Leishmania major* infection of macrophages is followed by a time-dependent appearance of lipophosphoglycan (LPG) that can be detected on the surface of infected cells by monoclonal antibodies. The origin of these LPG epitopes is probably the intracellular amastigote. LPG epitopes could be detected on the amastigote and the infected macrophage by a number of monoclonal antibodies directed to several distinct determinants on the phosphoglycan moiety. The macrophage-expressed LPG may be modified because, unlike the parasite LPG as expressed on promastigotes or amastigotes, it could not be radiolabeled by galactose oxidase or periodate treatment of infected cells followed by reduction with 2H-labeled sodium borohydride. Some LPG epitopes displayed on the macrophage may be anchored with glycosylphosphatidylinositol, and some may be in the water-soluble phosphoglycan form bound to macrophage integrins involved in its specific recognition. The water-soluble population could be released from the infected macrophage by gentle protease treatment.

**MATERIALS AND METHODS**

**Parasites.** Promastigotes of the *L. major* cloned cell line V121 (13) were grown in Schneider *Drosophila* medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum. In all experiments promastigotes were used in the stationary phase of growth, usually on day 5 in culture.

**In vitro infection of macrophages.** The macrophage cell line J774 (4) or peritoneal macrophages were seeded in RPMI medium with 10% fetal calf serum on glass cover slips in 24-well tissue culture trays (Flow Laboratories, Inc., McLean, Va.). Cells at a concentration of 5 x 10⁶ per ml were allowed to adhere (500 μl per well) for 30 min at 37°C and were infected with promastigotes at a ratio of 5:1. Cells were cultured in RPMI for 2 h at 37°C, free organisms were removed by vigorous washing, and the cells were incubated at 37°C for various times. At each time point, the cover slips were washed three times in warm phosphate-buffered saline (pH 7.3) (PBS) and used for immunofluorescence. At this infection ratio, about 50 to 60% of cells harbored multiple amastigotes after overnight incubation.

**Immunofluorescence and flow cytometry.** For the flow cytometry analysis, the macrophages were infected in suspension by using the protocol described previously (13). Free promastigotes were removed by centrifugation and subsequently gated out of the analysis on the basis of size. Infected macrophages, or normal macrophages which had been incubated with LPG or the water-soluble phosphoglycan (PG) derived from it by *Staphylococcus aureus* phosphatidylinositol-specific phospholipase C (PIPLC), were incubated for 60 min on ice with the monoclonal antibodies described below and then with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin G F(ab) (Silenus Laboratories, Melbourne, Australia). For some experiments, PG was purified from promastigote culture supernatant by monoclonal antibody WIC-79.3 affinity chromatography (6, 12). They were then washed again and fixed in 1% formaldehyde–2% glucose in PBS and were analyzed by microscopy and by flow cytometry using a fluorescence-activated cell sorter (FACS II; Becton Dickinson FACS System, Mountain View, Calif.).

Extraction of LPG was by sequential solvent extraction as
described by McConville et al. (21), and LPG was purified to homogeneity by hydrophobic interaction chromatography on octyl-Sepharose. LPG was quantitated by using the sulfuric acid method of Dubois et al. (7).

Surface labeling of macrophages. LPG molecules on the surface of infected macrophages were labeled by treatment with galactose oxidase or sodium periodate followed by reduction with \(^3\)H-labeled sodium borohydride (11, 16). Macrophages were seeded (10\(^5\) cells) on large petri dishes in 10 ml of RPMI medium with 10% fetal calf serum and infected as described above. Monolayers were washed in PBS, covered with 2 ml of PBS containing 10 U of galactose oxidase, and incubated at 37°C for 30 min. The PBS was then removed and replaced with 2 ml of fresh PBS containing 1 mCi of \(^3\)H-labeled sodium borohydride (Dupont, NEN Research Products, Boston, Mass.). The cells were incubated for an additional 20 min at room temperature, washed three times in PBS, and solubilized in situ in 1% Triton X-114 in PBS. The core of LPG has been shown to contain a galactofuranose residue (18; McConville and Bacic, in press) which can be radiolabeled by very mild periodate oxidation followed by reduction with \(^3\)H-labeled sodium borohydride. Macrophage monolayers prepared as described above were treated with 2 mM sodium periodate in 2 ml of PBS for 10 min in the dark at room temperature. They were washed in PBS and incubated with 2 ml of PBS containing 1 mCi of \(^3\)H-labeled sodium borohydride for 20 min at room temperature before solubilization in 1% Triton X-114 as described above.

Triton X-114 phase separation of integral membrane glycoconjugates. Cell lysates were prepared as described above and left on ice for 30 min, and then insoluble material was removed by centrifugation at 37,000 \(\times\) g for 30 min at 4°C. Integral membrane glycoconjugates were separated from the cytoplasmic aqueous components by using the protocol devised by Bordier (3) as modified by Murray et al. (26).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described previously (15) using the Laemmli system (19).

Monoclonal antibodies. Monoclonal antibody WIC-79.3 has been characterized in detail (6, 11, 12) and shown to recognize a polymeric epitope specific for \(L.\) major LPG. Monoclonal antibodies CA7AE, recognizing the repeating unit of LPG, and L157, recognizing the core of LPG, were a generous gift from D. Tolson and T. W. Pearson, and they recognized determinants on \(L.\) donovani LPG which are also present on \(L.\) major (35).

Monoclonal antibodies 4A2-A2 and 5E6-B4 were raised to \(L.\) major LPG (unpublished results), and they recognized distinct epitopes which are also different from the WIC-79.3, CA7AE, and L157 epitopes. Antibody 4A2-A2 may be directed to a single epitope, since on a two-site enzyme-linked immunosorbent assay, once the trapping antibody 4A2-A2 bound LPG, the peroxidase-conjugated detecting antibody 4A2-A2 did not bind any longer. Monoclonal antibody 5E6-B4 recognized a repeating epitope, but judging from the weak signals obtained by two-site enzyme-linked immunosorbent assay, it may not be a very abundant epitope.

RESULTS

Detection of LPG epitopes on infected macrophages. Macrophages were infected with promastigotes for 2 h at 37°C, and after 2, 4, 6, and 24 h they were examined for the presence of LPG epitopes on their surface by indirect immunochemistry. At 2 h postinfection, parasites were observed both intracellularly and attached to the surface of the macrophages. The distribution of LPG examined by immunochemistry with monoclonal antibody WIC-79.3 was spread around the parasite surface and the point of attachment to the macrophage membrane. Most infected and noninfected macrophages displayed a speckled pattern of weak immunochemistry. This pattern changed after 4 h of infection, when all macrophages in the culture were negative for WIC-79.3 binding. At 6 h of infection some, but not all, infected macrophages showed surface fluorescence with WIC-79.3, and by 24 h all infected cells displayed LPG on their surface. Thus, the appearance of LPG detectable with monoclonal antibodies on the surface of the infected macrophages was a time-dependent phenomenon, suggesting that its origin was the intracellular amastigotes and not the original inoculum of promastigotes.

Mode of attachment of LPG to the macrophage surface. As a parasite ligand for the macrophage integrins (28; Talamas-Rohana et al., in press), LPG detected on the infected macrophage may be attached to these receptors via its carbohydrate recognition domain. Alternatively, LPG may be anchored to the macrophage membrane via its GPI anchor in a manner similar to that of its attachment to the parasite itself. Another possibility is that small LPG-derived fragments are present, these epitopes being associated with integrins or major histocompatibility complex molecules, for example. In order to examine the possible mode of LPG attachment, macrophages were infected in suspension, washed, and treated with 10 or 100 \(\mu\)g of pronase. The presence of remaining LPG epitopes was detected by immunofluorescence with WIC-79.3. Pronase treatment of infected macrophages reduced the amount of LPG detectable on these cells, but it did not eliminate it (Fig. 1). In contrast,
FIG. 2. Detection by immunofluorescence and flow cytometry of *L. major* LPG bound by normal macrophages. Cells were incubated on ice with V121 PG and then treated with pronase as described for Fig. 1. Remaining PG was detected by monoclonal antibody WIC-79.3 and FITC-conjugated sheep anti-mouse F(ab)2.

[Graph showing control, 10µg Pronase, and 100µg Pronase treatments with fluorescence counts.]
Numbers represent gel weights of the inserted LPG material before being loaded on the gel. Numbers represent the following molecular weight standards: 94,000 (phosphorylase B), 67,000 (bovine serum albumin), 43,000 (ovalbumin), and 33,000 (carbonic anhydrase).

**FIG. 4.** Analysis of the *L. major* LPG in normal macrophages with passively inserted LPS (lanes a, b, and c) or in macrophages infected with V121 for 24 h (lanes d, e, and f) by SDS-PAGE. Cells were lysed in 1% Triton X-114, and integral membrane components were separated from aqueous material before being loaded on the gel. Numbers represent the following molecular weight standards: 94,000 (phosphorylase B), 67,000 (bovine serum albumin), 43,000 (ovalbumin), and 33,000 (carbonic anhydrase).

**FIG. 5.** Analysis of *L. major* LPG in infected macrophages by SDS-PAGE and immunoblotting. A duplicate gel of that shown in Fig. 4d through f was analyzed by immunoblotting with radioiodinated monoclonal antibody WIC-79.3 (specific activity, 10 μCl/μg). Molecular weight standards are as in Fig. 4.

**DISCUSSION**

Early studies by Farah et al. (8), Handman et al. (10), Berman and Dwyer (2), and Delbarra et al. (6) have demonstrated the presence of leishmanial antigens on the surfaces of infected macrophages. One of the antigens containing these epitopes has been shown to be LPG (6, 11). This is one of the few examples of a well-characterized parasite antigen displayed on the surface of the infected macrophage, which should be available for immune recognition by sensitized T cells.

The mechanism by which structurally intact LPG and/or fragments of it reach the external surface membrane is not known. It has been shown that amastigotes of at least some
**Leishmania** species attach to the phagolysosome membrane in a way resembling gap junctions (1). This close apposition may enable the transfer of parasite glycolipids which do not span the lipid bilayer onto the host membrane. Alternatively, the hydrophilic PG, which may be released by the amastigote into the phagolysosome, may bind to the same macrophage receptors that allowed parasite attachment initially and recycle to the cell surface. Our data are consistent with the presence of both types of LPG molecules, one population which is bound to macrophage proteins and may be removed from the macrophage surface by pronase treatment, and the other population which is resistant to pronase. Caution should be exercised in interpreting pronase digestion results, since the enzyme preparation is far from pure and contains glycosidases shown to degrade LPG (L. Morris and S. Turco, personal communication). We could not positively identify the GPI anchor of this LPG population by PIPLC hydrolysis of live infected macrophages. The *S. aureus* PIPLC was not effective in cleaving LPG from live promastigotes or live infected macrophages. This enzyme could not hydrolyze other well-characterized GPI-anchored targets, the *T. brucei* variable surface glycoprotein on live trypanosomes (9) and the *Leishmania* gp63 on live promastigotes (unpublished observation). Early studies suggested that parasite antigens may be present on noninfected macrophages in in vitro-infected cultures (10), implying uptake and possible processing of soluble parasite antigens by normal cells. However, in the present studies using monoclonal anti-LPG antibodies, the appearance of LPG was limited to infected cells, with very low background on uninfected cells. In the present study, our observations were limited to 24 h to prevent new rounds of infection and to avoid the presence of extracellular amastigotes which may release hydrophilic PG. This restriction to a 24-h culture period was not the case in the longer-term cultures described previously (10).

Despite the detection of LPG on infected macrophages by using a battery of monoclonal antibodies directed to distinct epitopes of LPG, LPG could not be efficiently radio-labeled on the surface of these cells. The protocol used relies on the presence and availability of terminal galactose and could successfully radiolabel purified promastigote LPG passively inserted into the uninfected macrophage membrane. This would suggest that there were no inhibitory factors on the macrophage interfering with the reaction. Moreover, amastigotes purified from lesions and surface labeled by using an identical protocol expressed significant amounts of LPG. The amastigote LPG was similar in many respects to the high-molecular-weight metacyclic form of LPG, and amastigotes were recognized by all the monoclonal antibodies used in this study (T. Glaser et al., submitted for publication). The possibility exists that the form of LPG transported to the macrophage surface may be modified in the phagolysosome or in transit and thus differ from the parasite form. This modification may be chemical and effected by lysosomal enzymes, or it may be a masking effect due to the binding of LPG to macrophage surface molecules such as the integrins or class II major histocompatibility complex molecules. If indeed the macrophage LPG is distinct from the parasite LPG, and this is the form seen by gamma interferon-producing Th1 cells of the immune system, vaccination with this form of LPG may improve the often variable success rate observed with parasite LPG as a vaccine.

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**LITERATURE CITED**

