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# Expression of a Repeating Phosphorylated Disaccharide Lipophosphoglycan Epitope on the Surface of Macrophages Infected with *Leishmania donovani*

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**Murine peritoneal macrophages were infected with living, virulent *Leishmania donovani* promastigotes. At intervals after infection, the macrophage surfaces were probed for the expression of lipophosphoglycan (LPG) epitopes by immunofluorescence with anti-LPG monoclonal antibodies. A repeating phosphorylated disaccharide epitope of LPG was detected as early as 5 to 10 min postinfection and was initially localized to the immediate area of internalization of the promastigote into the macrophage. The epitopes were evenly distributed over the entire macrophage surface by 25 min postinfection. Treatments which inhibited macrophage phagolysosomal degradation processes had no effect on epitope expression, whereas reagents that affected macrophage membrane flow and, thus, phagocytosis drastically reduced or abolished expression. Purified LPG or phosphoglycan, the delipidated form of the LPG molecule, was also shown to bind to a variety of different cell types in a temperature-independent manner. Since LPG has been implicated as having an immunoprotective role in leishmaniasis, these results suggest a further mechanism(s) by which *Leishmania* LPG might be involved in parasite pathogenicity and virulence.**

*Leishmania* parasites are protozoans which have a digenetic life cycle. The parasites alternate between promastigotes that reside within the sandfly vector and obligate intracellular amastigotes that live in the phagolysosomes of host macrophages.

The major leishmania cell surface glycoconjugate is lipophosphoglycan (LPG), which has been reported to exist on the surface of promastigotes (23) and possibly amastigotes (19). It has been estimated that there are approximately $1.25 \times 10^6$ LPG molecules on the surface of a single *Leishmania donovani* promastigote (13). This abundance and the presence of LPG throughout the two life-cycle stages of the parasite suggest that LPG plays an important role in the biology of *Leishmania* organisms and the infections they cause. A variety of activities and functions for LPG have been experimentally demonstrated or at least suggested. These include involvement in attachment of promastigotes to the host macrophage (6), as a protection factor for the parasite within the phagolysosomal system of the host (reviewed in reference 22), and as a recognition molecule for the T-lymphocyte-dependent immune responses characteristic of leishmaniasis (11, 12).

Recently, the structure of *L. donovani* LPG was determined (13, 23, 24). LPG is a tripartite molecule consisting of a polymer of repeating phosphorylated Gal-β1,4-Man disaccharide units linked via a phosphohexasaccharide core (containing three Gal residues, two Man residues, and one unacetylated glucosamine residue) to a novel *lyso*-1-*O*-alkylphosphatidylinositol anchor (see Fig. 1). Similar but immunologically distinct glycoconjugates have been reported for all *Leishmania* species so far examined (reviewed in reference 22).

Several research groups have demonstrated the presence of *Leishmania* glycoconjugates on the surfaces of *Leishmania*-infected macrophages by using monoclonal antibodies (MAbs) in indirect immunofluorescence assays (6, 25). In one study (25), the authors suggested that the *Leishmania braziliensis panamensis* major surface glycoconjugate was not expressed on the surfaces of murine peritoneal macrophages infected in vitro until at least 6 h postinfection (p.i.). These results imply that the molecule had an intracellular origin and that the surface-exposed antigen represented a breakdown product generated via phagolysosomal degradation and recycling events. The other study demonstrated that a macrophage cell line bound an extracellular form of the predominant surface glycoconjugate of *Leishmania major* promastigotes (6).

In previous work, we used LPG-specific MAbs for analysis of the immunochemical structure of *L. donovani* LPG and for the determination of LPG epitope arrangement on the promastigote cell surface (20). In that study we demonstrated that an anti-LPG MAb (CA7AE) bound specifically to the repeating phosphorylated disaccharide epitope of the LPG molecule. Here, we demonstrate that the repeating disaccharide epitope of *L. donovani* LPG appears on the surfaces of infected macrophages as early as 5 to 10 min p.i. and that phagolysosomal degradation processes are not necessary for surface expression to occur.

Antigen presentation by accessory cells to T lymphocytes is central to the induction and maintenance of cell-mediated immune responses. Our findings suggest a mechanism by which *L. donovani* parasites could present a specific parasite antigen to host T lymphocytes early in infection and suggest the possibility of directing a protective immune response to a defined structure which does not require antigen processing.

## MATERIALS AND METHODS

**Murine peritoneal macrophages.** Five milliliters of RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum, $5 \times 10^{-2}$ M 2-mercap-

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toethanol, and 0.1% gentamicin (complete medium) was injected into the peritoneal cavity of 8- to 12-week-old female BALB/c mice. After gentle peritoneal massage, the peritoneal exudate was removed and the body cavity was washed twice with phosphate-buffered saline (PBS) containing 0.34 M sucrose to remove residual membrane-attached macrophages. The cells were pelleted by centrifugation at $400 \times g$ for 10 min, washed once, and suspended at $2 \times 10^5$ cells per ml in complete medium. Macrophage monolayers were prepared by incubating the macrophage suspension on acid-cleaned 12-mm glass cover slips in 24-well tissue culture plates (Becton Dickinson, Lincoln Park, N.J.). Each cover slip received $10^5$ macrophages in 0.5 ml of complete medium. Cells were allowed to adhere for 2 h at 37°C, and the cover slips were washed twice with complete medium to remove nonadherent cells.

**Other cells.** A doubly cloned hybridoma cell line (TC45/491) which secretes a MAb specific for a procyclinlike molecule on *Trypanosoma congolense* procyclic culture forms (R. P. Beecroft and T. W. Pearson, unpublished data) was used both as a source of MAb and as an experimental cell line in LPG-binding experiments. Thymocytes were obtained from 6-week-old female BALB/c mice. A Gibbon lymphoma line (MLA 144) (14) was obtained from Larry Anthony, Victoria, British Columbia, Canada. Mouse connective tissue fibroblasts (NTC 929, a clone of strain L) were originally obtained from the American Type Culture Collection, Rockville, Md., and were also provided by Larry Anthony. All cell types were maintained in complete medium as described above.

**Parasites.** *L. donovani* LD3 promastigotes represent a recent hamster-passaged, higher infective clone of *L. donovani* 1S2D (4). *Trypanosoma brucei rhodesiense* ViTat 1.1 cloned procyclic culture forms have been described elsewhere (16). All parasites were grown at 26°C in SM medium (3) containing 5% heat-inactivated fetal bovine serum. They were transferred to complete medium at 37°C for the infection experiments (see below).

**MAbs.** The derivation and characterization of the murine anti-LPG MAbs CA7AE, L157, and L98 (20) and the antiprocyclin MAb TBRP1.247 (15, 16) have been described previously.

**LPG and PG.** LPG from *L. donovani* was extracted, purified, and quantitated by phosphate analysis as described previously (13). Phosphoglycan (PG), the delipidated form of LPG, was derived by treating LPG with phosphatidylinositol-specific phospholipase C for 16 h at 37°C as described elsewhere (13), except that no detergent was added.

**Immunofluorescence.** Indirect immunofluorescence was performed to detect LPG epitopes on macrophages and other cell types that had been incubated with *L. donovani* LD3 promastigotes, LPG, or PG. Similarly, *L. donovani*-infected macrophage populations with or without various treatments to affect macrophage functions (see below) were assayed by indirect immunofluorescence. Ascites fluids containing the anti-LPG MAb CA7AE, L157, or L98 or the antiprocyclin MAb TBRP1.247 were used as the first antibody at a dilution of 1/2,000 at 4°C for 30 min. After three washes, the cells were incubated for 30 min at 4°C with a 1/200 dilution of affinity-purified goat anti-mouse immunoglobulin G plus immunoglobulin M conjugated to fluorescein isothiocyanate (Caltag, South San Francisco, Calif.). Finally, the cells were washed three times prior to making permanent slides with polyvinyl alcohol (molecular weight, 10,000; Sigma Chemical Co., St. Louis, Mo.) (5) containing a fluorescence-fading inhibitor, *p*-phenylenediamine (Sigma) (7). Photomicrographs were taken with a Zeiss standard binocular microscope fitted with a Planopro 100/1.3 oil immersion objective and a 35-mm photomicrographic camera.

**Infection of macrophages.** Promastigotes harvested in the late-log to stationary growth phases were used to infect cultures of adherent macrophages on glass cover slips at a ratio of 10 parasites to 1 macrophage. Infection was performed at 37°C, and at several intervals all nonattached or extracellular organisms were removed by washing with ice-cold PBS containing 5% fetal bovine serum and 1% D-glucose. The cells were assayed for surface LPG epitopes by indirect immunofluorescence with anti-LPG MAbs.

**Inhibition of macrophage phagocytic and phagolysosomal functions.** Adherent macrophages were treated with various protocols to inhibit or eliminate their phagocytic and phagolysosomal degradation functions. All methods involved the pretreatment and/or simultaneous treatment of the macrophages as outlined below. Promastigotes were added at a ratio of 10 parasites to 1 macrophage, and the cultures were incubated for 0.5, 2, 7, or 20 h p.i. without removal of the parasite population. At these times, macrophages were analyzed for LPG epitope expression by indirect immunofluorescence. All experiments were repeated at least three times.

The effects of proteases (50 μg of trypsin or 250 μg of proteinase K per ml; Sigma) on the ability of macrophages to bind and then internalize promastigotes were examined. Macrophages were washed extensively with PBS containing 1% D-glucose and incubated in 0.5 ml of the same buffer containing either trypsin or proteinase K for 15 min at room temperature. The macrophages were washed to remove all residual protease and were immediately incubated with promastigotes in complete medium for various times.

For assays with Formalin-fixed macrophages or promastigotes, the cells were washed twice in PBS, suspended in 0.5 ml of a 0.5% Formalin solution, and incubated at room temperature for 30 min. The fixative was removed by extensive washing in PBS and then in complete medium. The infection protocol was performed as outlined above.

To examine the effects of inhibiting the polymerization of cytoskeletal components on membrane transport events and thus parasite attachment and uptake, incubated macrophages in complete medium containing 10 μg of cytochalasin B or D per ml (in a final concentration of 1% dimethyl sulfoxide; Sigma) for 30 min prior to the addition of parasites as well as during the designated period of infection. As a negative control, macrophages were incubated under the same conditions in complete medium containing 1% dimethyl sulfoxide.

Colchicine (10 μg/ml; Sigma) was used to study the effects of an inhibitor of microtubule polymerization on parasite attachment and uptake. The procedure used was identical to that used for the cytochalasin treatment.

The lysosomotropic agents chloroquine (0.1 mM; Sigma) and ammonium chloride (10 mM; Fisher Scientific Co., Fair Lawn, N.J.) were used in a protocol identical to that used for the cytochalasins.

To determine whether an anti-LPG MAb could inhibit parasite attachment and/or the expression of LPG epitopes on the macrophage cell surface, we incubated macrophages with a 1/40,000 dilution of the anti-LPG MAb CA7AE (to prevent the extensive agglutination of promastigotes that is characteristic of this MAb at lower antibody dilutions) under conditions identical to those used for the cytochalasins. The

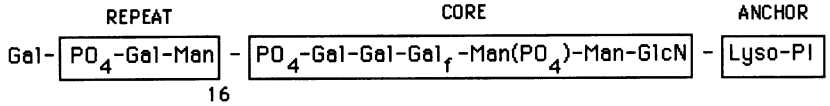


FIG. 1. Schematic representation of the structure of *L. donovani* LPG (4). The molecule is a polymer of repeating phosphorylated Gal-β1,4-Man disaccharide units linked via a phosphosaccharide core (containing three Gal residues, two Man residues, and one unacetylated glucosamine residue) to a novel *lyso*-alkylphosphatidylinositol (Lyso-PI) anchor.

antiprocyclin MAb TBRP1.247 (15, 16) was used as a negative control.

**LPG epitope expression on the surfaces of different cell types.** A selection of different living cells was incubated with 50 μg of LPG or PG at 4 or 37°C, either as adherent populations (macrophages and fibroblasts) or as suspension cultures (hybridomas, lymphomas, and thymocytes), for 2 h. The cells were washed extensively with PBS containing 5% fetal bovine serum and subsequently assayed for the presence of LPG or PG epitopes on the cell surface by indirect immunofluorescence. Alternatively, cells were incubated with leishmania promastigotes or procyclic culture forms of African trypanosomes at a 10:1 parasite/cell ratio as described above.

## RESULTS

**Expression of a specific LPG epitope on the surfaces of murine macrophages.** Murine macrophages were incubated in vitro with *L. donovani* promastigotes or with purified *L. donovani* LPG or PG. At 20 h p.i., the macrophage cell surface was probed for the presence of LPG epitopes with three anti-LPG MAbs. The epitopes within the LPG molecule recognized by these MAbs have been previously described (20). Briefly, two of the MAbs, L157 and L98, recognized the core region of the LPG molecule, while MAb CA7AE bound to the phosphorylated disaccharide repeat region (Fig. 1). The epitope recognized by MAb CA7AE and its surface orientation are shown schematically in Fig. 2. Neither of the anti-core MAbs nor the antiprocyclin MAb TBRP1.247 bound to the *L. donovani*-infected or LPG- or PG-pulsed macrophages. In contrast, MAb CA7AE bound strongly to the entire surface of macrophages incubated with *L. donovani* promastigotes (Fig. 3D). Similar binding was seen when the macrophages were incubated with purified LPG or PG (Fig. 3B and C). None of the MAbs bound to the control (uninfected) macrophages (Fig. 3A).

**Kinetics of LPG epitope expression.** Our initial experiments involved incubation of *L. donovani* promastigotes with macrophages for 2 h and examination of the macrophage surface for LPG epitope expression at 20 h p.i. In addition, macrophages were incubated with *L. donovani* promastigotes for 2 h and immediately tested for LPG epitope expression. The fluorescence intensity exhibited at 2 h p.i. was as strong as that exhibited at 20 h p.i. We therefore assayed the macrophage surface at 5-min intervals over a 2-h period after infection with *L. donovani* promastigotes (Fig. 4). The CA7AE epitope was detected on the surfaces of infected macrophages as early as 5 to 10 min p.i. (Fig. 4B and C). Initially, fluorescence was specifically localized to the area of promastigote attachment to the macrophage (Fig. 4B to D); subsequently, fluorescence was seen to be evenly distributed over the entire surface of the macrophage within 20 to 25 min p.i. (Fig. 4E and F).

Only those macrophages with attached parasites exhibited expression of the LPG epitope, in contrast to neighboring uninfected macrophages, which did not (Fig. 4B, E, and F). Similar results were observed at all times, even at 20 h p.i. These results also clearly demonstrated that MAb CA7AE, of the immunoglobulin M isotype, did not react with the surface membrane of uninfected macrophages, even when they were cocultured with leishmania-infected cells.

Expression of LPG epitopes on the macrophage cell surface was also apparent within 2 h of incubation with purified LPG or PG but was never localized, as was observed when macrophages were infected with promastigotes. Rather, LPG epitopes appeared to accumulate in an evenly distributed pattern over the entire macrophage surface. The immunofluorescence intensity increased as the length of macrophage and LPG or PG incubation increased.

**Effect of inhibition of macrophage functions on LPG expres-**

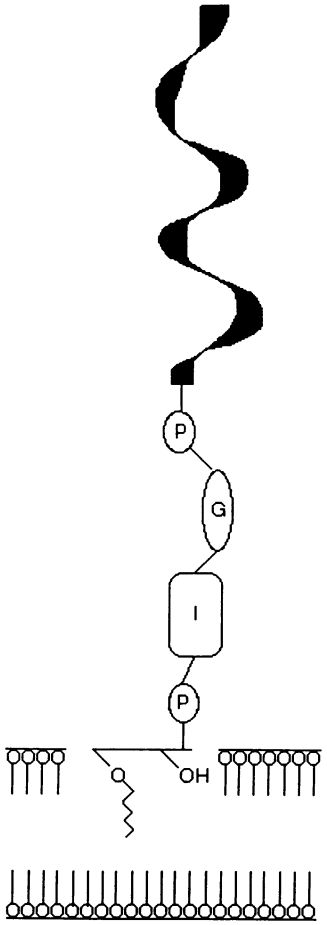


FIG. 2. Schematic representation of *L. donovani* LPG as it would appear on the promastigote cell surface. The epitope recognized by MAb CA7AE is indicated by the shaded ribbonlike structure, which represents the phosphorylated disaccharide repeat units. Abbreviations: I, inositol; G, carbohydrate core; P, phosphate.

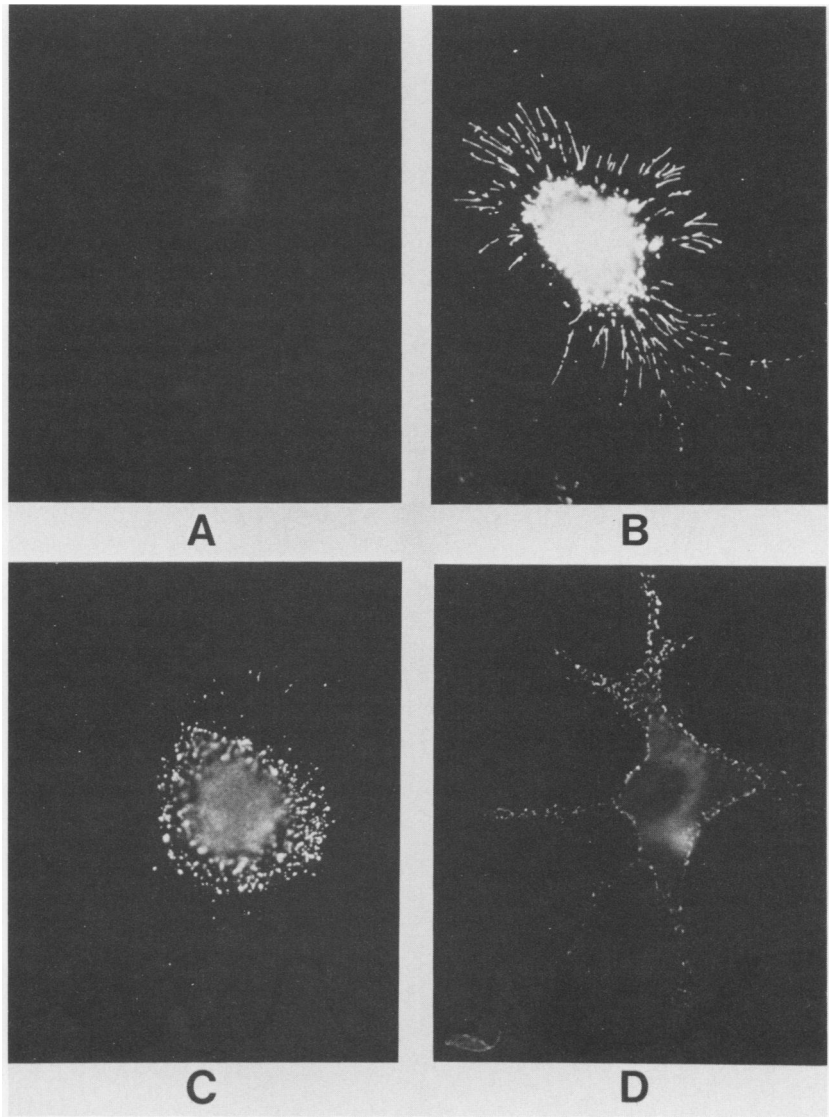


FIG. 3. Indirect immunofluorescence of murine macrophages with anti-LPG MAb CA7AE. Macrophages were incubated wtih promastigotes for 2 h and assayed at 20 h p.i. or, alternatively, incubated with purified LPG or PG for 20 h and assayed. (A) Uninfected macrophages. (B) Macrophages incubated with purified LPG. (C) Macrophages incubated with purified PG. (D) Macrophages incubated with *L. donovani* promastigotes. Magnification, ×660.

**sion.** Macrophages were treated under conditions which affect phagocytic and phagolysosomal processing events (Table 1). The values in Table 1 represent the number of macrophages exhibiting immunofluorescence per 100 total macrophages. As demonstrated by the population of untreated macrophages, the number of parasites bound (and the number of macrophages subsequently expressing LPG epitopes) was seen to increase over time.

Reagents such as ammonium chloride or chloroquine had no effect on LPG epitope expression on *L. donovani*-infected macrophages. These chemicals interfere with phagolysosomal antigen degradation processes by increasing the lysosomal pH, resulting in the depression of acid hydrolase activity. Similar results were seen with colchicine treatment, which inhibits microtubule polymerization.

Formalin-fixed promastigotes, when incubated with normal macrophages, were still able to transfer LPG epitopes to the macrophage surface. Formalin-fixed promastigotes are able to bind the anti-LPG MAb CA7AE as strongly as are living organisms (D. L. Tolson, unpublished observations). The incubation of Formalin-fixed promastigotes with macrophages resulted in immunofluorescence patterns similar to those observed with untreated promastigotes. In contrast, treatments (decreased temperature, the addition of cytochalasin B or D, or mild fixation of macrophages with Formalin) which inhibited membrane flow and, thus, phagocytosis resulted in the abolition or drastic reduction of LPG epitope expression.

Pretreatment of the macrophage population with trypsin or proteinase K significantly reduced or abolished promastigote binding and, thus, LPG epitope expression up to and including 30 min p.i. From 2 h p.i. on, however, protease-treated macrophages expressed LPG epitopes at levels similar to those seen in untreated infected macrophages. No fluorescence was seen when uninfected protease-treated macrophages were assayed with MAb CA7AE or when

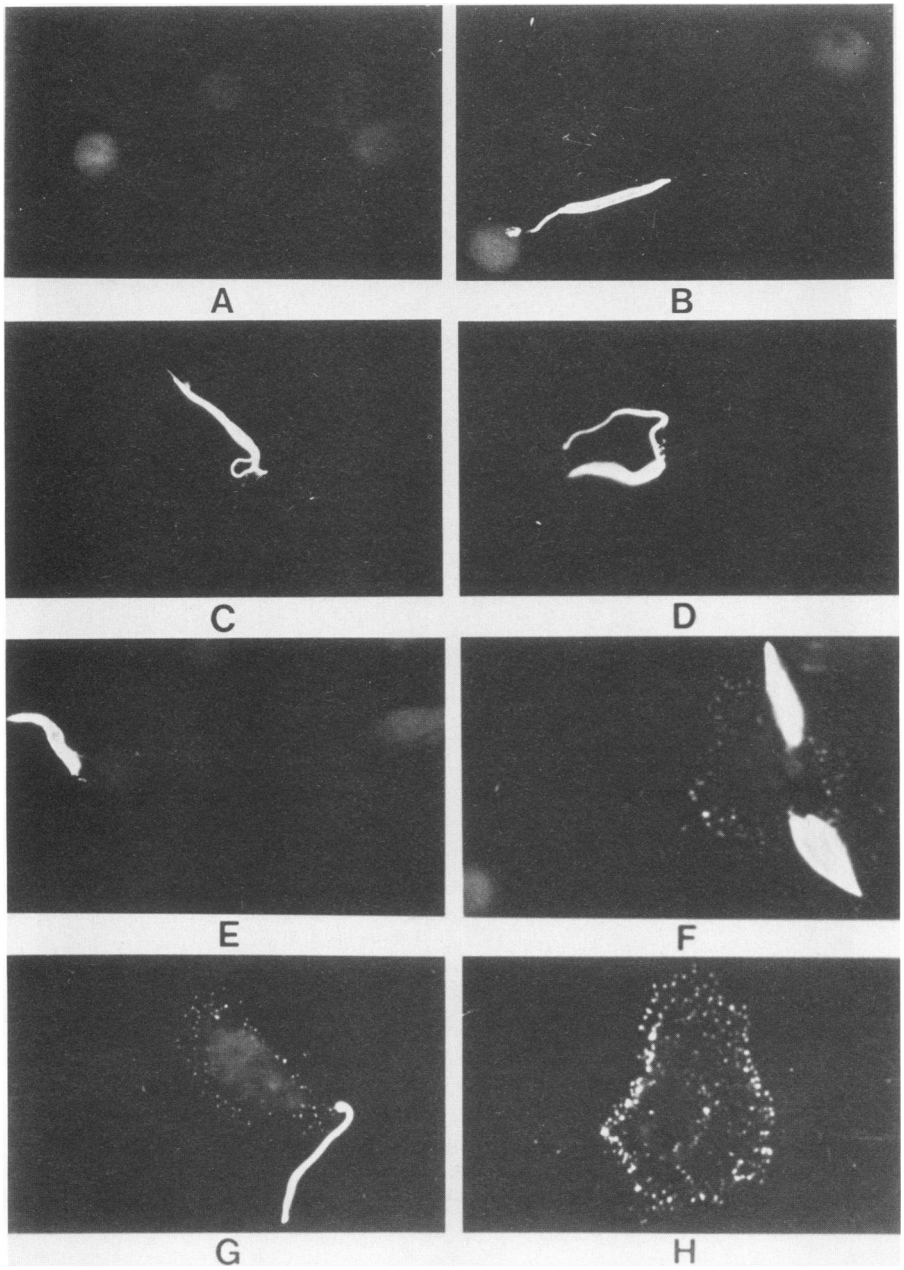


FIG. 4. Temporal expression of LPG epitopes on the surfaces of *L. donovani*-infected murine macrophages as detected by immunofluorescence. (A) 0 min p.i. (B) 5 min p.i. (C) 10 min p.i. (D) 15 min p.i. (E) 20 min p.i. (F) 25 min p.i. (G) 30 min p.i. (H) 35 min p.i. Magnification, ×700.

infected protease-treated macrophages were assayed with the antiprocyclin MAb TBRP1.247. That the proteases were active was confirmed by treating procyclic trypanosomes with trypsin or proteinase K. The treated trypanosomes did not show fluorescence after incubation with MAbs that detect surface epitopes on the trypanosome glycoprotein procyclin (15). *L. donovani* promastigotes treated with either trypsin or proteinase K bound the anti-LPG MAb CA7AE as well as untreated parasites did when assayed by indirect immunofluorescence (D. L. Tolson, unpublished observations). The treated macrophages were judged by light microscopy to be viable at all times.

**Binding of LPG epitopes to different cell types.** Several cell types were tested at 37 or 4°C (to inhibit membrane flow) for their ability to express LPG epitopes when incubated with living promastigotes, LPG, or PG (Table 2). Promastigote-derived LPG epitopes were expressed only on parasite-infected macrophages and only at 37°C. None of the other cell types examined expressed LPG epitopes after incubation with living parasites. However, all of the cell types tested strongly bound LPG or PG and did so at either 4 or 37°C. Strong antibody-induced agglutination was exhibited by cells that were tested as suspension cultures (i.e., hybridomas, thymocytes, and lymphomas). None of the cells assayed with the antiprocyclin MAb TBRP1.247 showed any fluorescence.

TABLE 1. LPG epitope expression on the surfaces of *L. donovani*-infected macrophages (MOs) after various treatments[a]

| Treatment | No. of MOs/100 MOs exhibiting fluorescence at: | | | |
|---|---|---|---|---|
| | 30 min p.i. | 2 h p.i. | 7 h p.i. | 20 h p.i. |
| None (untreated cells) | 14 | 45 | 64 | 86 |
| Uninfected MOs | 0 | 0 | 0 | 0 |
| MOs at 4°C | 0 | 0 | 0 | 0 |
| Trypsin + MOs | 6 | 45 | 65 | 88 |
| Proteinase K + MOs | 0 | 36 | 68 | 86 |
| Formalin-fixed MOs | 0 | 0 | 0 | 0 |
| Formalin-fixed promastigotes | 20 | 48 | 70 | 90 |
| Colchicine + MOs | 13 | 39 | 53 | 60 |
| $NH_4Cl$ + MOs | 11 | 32 | 59 | 87 |
| Chloroquine + MOs | 16 | 49 | 56 | 73 |
| Cytochalasin D + MOs | 0 | 4 | 8 | 6 |
| Cytochalasin B + MOs | 0 | 5 | 11 | 20 |
| Anti-LPG MAb CA7AE + MOs | 16 | 52 | 73 | 96 |

[a] The results from one of two experiments are shown. Both experiments gave similar results.

## DISCUSSION

In this study, murine peritoneal macrophages expressed on their surfaces an LPG epitope after infection with *L. donovani* promastigotes or after incubation with purified *L. donovani* LPG or PG. The epitope recognized by the identifying MAb, CA7AE, has previously been shown to be a phosphorylated Gal-β1,4-Man disaccharide unit (20)—the base unit of the carbohydrate repeat moiety of LPG from *L. donovani* promastigotes. Since neither of the anti-LPG core MAbs bound to the surfaces of infected or antigen-pulsed macrophages, a result similar to that observed with these two MAbs and promastigote cell surfaces (20), the LPG and PG molecules are probably expressed on the macrophage membrane in an orientation similar to that found on the promastigote cell surface.

Purified LPG or PG, the delipidated form of the LPG molecule, also bound to a variety of other cell types in a temperature-independent manner. This result suggests that passive incorporation into the host cell membrane does not require the lipid anchor of LPG. Recently, the incorporation of *Chlamydia trachomatis* lipopolysaccharide into host cell membranes was found to be independent of host cell type (8). In contrast, purified membrane-form *T. brucei* variant surface glycoprotein (which has a glycolipid anchor) bound sheep erythrocytes, whereas purified soluble variant surface glycoprotein (which has lost the glycolipid anchor) did not (17).

TABLE 2. Expression of LPG epitopes on the surfaces of different cell types incubated with *L. donovani* promastigotes or purified LPG or PG for 2 h as determined by indirect immunofluorescence

| Temp (°C) | Antigen | Immunofluorescence[a] of the following cell type: | | | | |
|---|---|---|---|---|---|---|
| | | Macrophages | Fibroblasts | Hybridomas | Thymocytes | Lymphomas |
| 37 | *L. donovani* LD3 promastigotes | + | − | − | − | − |
| | LPG | + | + | + | + | + |
| | PG | + | + | + | + | + |
| | None | − | − | − | − | − |
| 4 | *L. donovani* LD3 promastigotes | − | − | − | − | − |
| | LPG | + | + | + | + | + |
| | PG | + | + | + | + | + |
| | None | − | − | − | − | − |

[a] +, Strong immunofluorescence observed over the entire cell surface; −, no immunofluorescence. For the suspension cultures (hybridomas, thymocytes, and lymphomas), extensive agglutination was also exhibited.

LPG epitope expression occurred at the initial area of parasite-macrophage attachment within 5 to 10 min p.i. By 35 min p.i., LPG epitopes were displayed over the entire macrophage surface. These observations contrast markedly with those reported previously (25) which suggested that the *L. braziliensis panamensis* major surface glycoconjugate did not appear on the macrophage membrane until several hours after parasitization. However, it should be noted that the exact identity of this glycoconjugate was not determined. A similar observation was made with *Chlamydia trachomatis*-infected cells: the lipopolysaccharide antigen from these microorganisms was not detectable on the macrophage surface by an anti-chlamydia lipopolysaccharide MAb until about 24 h p.i. (8). These previous results support the idea that the *L. braziliensis panamensis* and *C. trachomatis* glycoconjugates detected on the cell surfaces were of intracellular origin. A similar hypothesis was proposed for *L. major* glycoconjugate expression on macrophages (6).

A recent review outlined four possible pathways for handling and presentation by accessory cells (including macrophages) of antigens which were membrane-bound, intracellular, or excretory-secretory molecules (9). These pathways were as follows. (i) Soluble excretory-secretory antigens are taken up after specific binding or fluid-phase pinocytosis and then processed. (ii) Host cell proteases cleave cell surface molecules to liberate soluble antigens, which then follow pathway i. (iii) Degradation within host cell phagolysosomal compartments disrupts parasite membrane integrity, allowing parasite antigens to be recycled to the macrophage membrane in a processed form. (iv) Cell membrane antigens may be sloughed off or directly transferred onto the host cell surface during parasite attachment and internalization. Since we observed LPG epitopes on the macrophage cell surface within 5 to 10 min of infection and since they were initially localized to the area of promastigote attachment, we suggest that the *L. donovani* LPG made its way to the surfaces of infected macrophages via pathway iv. Presumably, LPG epitope accumulation on the macrophage surface occurs as soon as parasite internalization begins.

This theory is further substantiated by the experiments which showed that inhibition of macrophage phagolysosomal degradation processes had no effect on epitope expression, while reagents that affected macrophage membrane flow drastically reduced or abolished expression. Earlier publications (2, 26) clearly indicated that attachment and internalization of *Leishmania* parasites were inhibited by low temperatures, cytochalasins, and mild fixation of macrophages, demonstrating that parasite uptake was dependent on the integrity of the macrophage phagocytic mechanisms.

It has been shown previously that LPG can mediate the attachment of promastigotes to macrophages (reviewed in reference 18). Our results showed that treatment of macrophages with trypsin or proteinase K prior to infection significantly reduced or abolished LPG epitope expression in a transient fashion. This result was almost certainly due to

the inhibition of parasite attachment (D. L. Tolson, unpublished observations). Attachment and, therefore, LPG epitope expression attained normal levels from 2 h p.i. on, suggesting that the reexpression and redistribution of the appropriate *L. donovani* receptors on macrophages occurred during this period.

Leishmania organisms are known to release a form of LPG, termed excreted factor (EF), both in vitro and in vivo (19); however, the chemical structure of EF and its mechanism of release from promastigotes are currently unknown. MAb CA7AE binds to *L. donovani* EF (D. L. Tolson, L. F. Schnur, and T. W. Pearson, unpublished data). The possibility that *L. donovani* EF is secreted into the extracellular fluid and then readsorbed onto the macrophage plasma membrane can be discounted, at least for the experiments presented here. Only parasitized macrophages exhibited the LPG epitope; nonparasitized neighboring macrophages did not. This exclusivity of epitope expression occurred as late as 20 h p.i., when the amount of EF in the culture medium had accumulated to levels easily detectable in a double-antibody sandwich enzyme-linked immunosorbent assay with MAb CA7AE (D. L. Tolson, unpublished observations). Previous work (1), in which *L. donovani* culture supernatants (presumably containing EF) were incubated with uninfected macrophages, produced equivocal results in that in some experiments no antigen was detectable on the macrophage surface while in others the macrophage surface was clearly labeled by anti-*L. donovani* serum. *L. major* EF has been shown to accumulate on the surfaces of macrophages (6). However, in that study the authors incubated the macrophages with a glycoconjugate purified from a culture medium that was 10-fold more concentrated than the late-log-phase growth medium from which the glycoconjugate was obtained—a situation unlikely to reflect the environment surrounding the macrophage during infection. Also, since LPG is thought to be released from promastigotes in two structurally distinct forms (23), one of which binds very tightly to serum albumin and appears to be similar in structure to cellular LPG, it may be that the authors merely concentrated this form to levels sufficient to accumulate on the macrophage membrane. Alternatively, it is possible that since *L. donovani* and *L. major* have different LPG carbohydrate structures (reviewed in reference 22), they may utilize different mechanisms for the expression of epitopes on the macrophage surfaces.

Our results indicate that *L. donovani* LPG disaccharide repeat epitopes accumulate on the surfaces of infected macrophages during parasite penetration. This process does not require active antigen processing. Epitope incorporation is not limited to lipid-tail interactions with the bilayer of the macrophage plasma membrane and may also occur via interactions between the carbohydrate portion of the LPG molecule and host cell receptors, such as the integrins, that are present on many cells, including macrophages. These results do not, however, discount the possibility that processed LPG epitopes could also contribute to the expression of LPG epitopes exhibited on the macrophage surface as infection proceeds. We have also observed that while intracellular amastigotes do not appear to express the epitope recognized by MAb CA7AE (determined by immunofluorescence on acetone-permeabilized leishmania-infected macrophages), there does seem to be an accumulation of residual promastigote LPG surrounding the amastigotes within the phagolysosomal vacuole as late as 5 to 6 days p.i. (D. L. Tolson and T. W. Pearson, unpublished observations).

The mechanism by which leishmanial parasites are killed is thought to be by T-lymphocyte recognition of the infected macrophage and subsequent lymphokine-induced oxidative leishmanicidal activity of the macrophage (reviewed in reference 10). Whether carbohydrate epitopes, such as those of LPG, are recognized by T cells has been a contentious issue with immunologists. However, LPG has been implicated in T-cell immunity (11, 12). Indeed, recent evidence suggests that phosphorylated carbohydrate epitopes of the LPG molecule are capable of stimulating T lymphocytes of the $CD4^+$ $CD8^-$ phenotype (A. Jardim, D. L. Tolson, S. J. Turco, T. W. Pearson, and R. W. Olafson, submitted for publication). Also, we have recently demonstrated that murine macrophages either infected with *L. donovani* promastigotes or pulsed with purified LPG are able to stimulate LPG-primed T cells in vitro (D. L. Tolson, A. Jardim, S. J. Turco, H. Teh, R. W. Olafson, and T. W. Pearson, submitted for publication). Therefore, understanding the mechanism by which promastigote LPG is transferred to macrophage membranes during parasitization could provide insight into the mechanisms involved in the pathogenesis and immunology of leishmaniasis. LPG molecules incorporated into the macrophage plasma membrane could alter the antigenic properties of the host cell, resulting in the preferential triggering of specific T-cell phenotypes and leading to resolution or exacerbation of the disease. Alternatively, membrane-incorporated LPG molecules could disrupt macrophage metabolic and/or functional properties, resulting in parasite evasion of the macrophage leishmanicidal mechanisms. Interestingly, *L. donovani* LPG has recently been proposed to play an important protective role for the parasite by effectively disarming the oxidative burst of the macrophage (reviewed in reference 22). Studies to determine the role of LPG epitopes in parasite intracellular survival and in macrophage–T-cell interactions are currently under way.

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## LITERATURE CITED

1. **Berman, J. D., and D. M. Dwyer.** 1981. Expression of *Leishmania* antigen on the surface membrane of infected human macrophages *in vitro*. Clin. Exp. Immunol. **44**:342–348.
2. **Chang, K. P.** 1979. *Leishmania donovani*: promastigote-macrophage surface interactions *in vitro*. Exp. Parasitol. **48**:175–189.
3. **Cunningham, I.** 1973. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J. Protozool. **24**:325–329.
4. **Dwyer, D. M.** 1977. *Leishmania donovani*; surface membrane carbohydrates of promastigotes. Exp. Parasitol. **41**:341–358.
5. **Freer, S. M.** 1984. A permanent wet-mount for fluorescent microscopy of surface stained lymphoid cells. J. Immunol. Methods **66**:187–188.
6. **Handman, E., and J. W. Goding.** 1985. The *Leishmania* receptor for macrophages is a lipid containing glycoconjugate. EMBO J. **4**:329–336.
7. **Johnson, G. D., and G. M. C. de Nogueira Araujo.** 1981. A simple method of reducing the fading of immunofluorescence during microscopy. J. Immunol. Methods **43**:349–350.
8. **Karimi, S. T., R. H. Schloemer, and C. E. Wilde III.** 1989. Accumulation of chlamydial lipopolysaccharide antigen in the plasma membranes of infected cells. Infect. Immun. **57**:1780–1785.

9. **Kaye, P. M.** 1987. Antigen presentation and the response to parasitic infection. Parasitol. Today **3**:293–299.
10. **Liew, F. Y.** 1989. Functional heterogeneity of CD4+ T cells in leishmaniasis. Immunol. Today **10**:40–45.
11. **Mitchell, G. F., and E. Handman.** 1985. T-lymphocytes recognise *Leishmania* glycoconjugates. Parasitol. Today **1**:61–63.
12. **Moll, H., G. F. Mitchell, M. J. McConville, and E. Handman.** 1989. Evidence for T-cell recognition in mice of a purified lipophosphoglycan from *Leishmania major*. Infect. Immun. **57**:3349–3356.
13. **Orlandi, P. A., and S. J. Turco.** 1987. Structure of the lipid moiety of the *Leishmania donovani* lipophosphoglycan. J. Biol. Chem. **262**:10384–10391.
14. **Rabin, H., R. F. Hopkins III, F. W. Ruscetti, R. H. Neubauer, R. L. Brown, and T. G. Kawakami.** 1981. Spontaneous release of a factor with properties of T cell growth factor from a continuous line of primate tumor cells. J. Immunol. **127**:1852–1856.
15. **Richardson, J. P., R. P. Beecroft, D. L. Tolson, M. K. Liu, and T. W. Pearson.** 1988. Procyclin: an unusual immunodominant glycoprotein surface antigen from the procyclic stage of African trypanosomes. Mol. Biochem. Parasitol. **31**:203–216.
16. **Richardson, J. P., L. Jenni, R. P. Beecroft, and T. W. Pearson.** 1986. Procyclic tsetse fly midgut forms and culture forms of African trypanosomes share stage- and species-specific surface antigens recognized by monoclonal antibodies. J. Immunol. **136**:2259–2264.
17. **Rifkin, M. R., and F. R. Landsberger.** 1990. Trypanosome variant surface glycoprotein transfer to target membranes: a model for the pathogenesis of trypanosomiasis. Proc. Natl. Acad. Sci. USA **87**:801–805.
18. **Russell, D. G., and P. Talamas-Rohana.** 1989. *Leishmania* and the macrophage: a marriage of inconvenience. Immunol. Today **10**:328–333.
19. **Schnur, L. F., A. Zuckerman, and C. L. Greenblatt.** 1972. *Leishmania* serotypes as distinguished by the gel diffusion of factors excreted *in vitro* and *in vivo*. Isr. J. Med. Sci. **8**:932–942.
20. **Tolson, D. L., S. J. Turco, R. P. Beecroft, and T. W. Pearson.** 1989. The immunochemical structure and surface arrangement of *Leishmania donovani* lipophosphoglycan determined using monoclonal antibodies. Mol. Biochem. Parasitol. **35**:109–118.
21. **Turco, S. J.** 1988. The lipophosphoglycan of *Leishmania*. Parasitol. Today **4**:255–257.
22. **Turco, S. J.** 1990. The leishmanial lipophosphoglycan: a multifunctional molecule. Exp. Parasitol. **70**:241–245.
23. **Turco, S. J., S. R. Hull, P. A. Orlandi, S. D. Shepherd, S. W. Homans, R. A. Dwek, and T. W. Rademacher.** 1987. Structure of the major carbohydrate fragment of the *Leishmania donovani* lipophosphoglycan. Biochemistry **26**:6233–6238.
24. **Turco, S. J., P. A. Orlandi, S. W. Homans, M. A. J. Ferguson, R. A. Dwek, and T. W. Rademacher.** 1989. Structure of the phosphosaccharide-inositol core of the *Leishmania donovani* lipophosphoglycan. J. Biol. Chem. **264**:6711–6715.
25. **Williams, K. M., J. B. Sacci, and R. L. Anthony.** 1986. Identification and recovery of *Leishmania* antigen displayed on the surface membrane of mouse peritoneal macrophages infected *in vitro*. J. Immunol. **136**:1853–1858.
26. **Zenian, A., P. Rowles, and D. Gingell.** 1979. Scanning electron-microscopic study of the uptake of *Leishmania* parasites by macrophages. J. Cell Sci. **39**:187–199.