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Lipophosphoglycan Blocks Attachment of *Leishmania major* Amastigotes to Macrophages

MICHELLE KELLEHER,[1] SUSAN F. MOODY,[1]† PATRICIA MIRABILE,[1] AMELIA H. OSBORN,[1] ANTONY BACIC,[2] AND EMANUELA HANDMAN[1]*

The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria 3050,[1] and Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Parkville, Victoria 3052,[2] Australia

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Promastigotes of the intracellular protozoan parasite *Leishmania major* invade mononuclear phagocytes by a direct interaction between the cell surface lipophosphoglycan found on all *Leishmania* species and macrophage receptors. This interaction is mediated by phosphoglycan repeats containing oligomers of β(1–3)Gal residues specific to *L. major*. We show here that although amastigotes also use lipophosphoglycan to bind to both primary macrophages and a cell line, this interaction is independent of the β(1–3)Gal residues employed by promastigotes. Binding of amastigotes to macrophages could be blocked by intact lipophosphoglycan from *L. major* amastigotes as well as by lipophosphoglycan from promastigotes of several other *Leishmania* species, suggesting involvement of a conserved domain. Binding of amastigotes to macrophages could be blocked significantly by the monoclonal antibody WIC 108.3, directed to the lipophosphoglycan backbone. The glycan core of lipophosphoglycan could also inhibit attachment of amastigotes, but to a considerably lesser extent. The glycan core structure is also present in the type 2 glycoinositolphospholipids which are expressed on the surface of amastigotes at 100-fold-higher levels than lipophosphoglycan. However, their inhibitory effect could not be increased even when they were used at a 300-fold-higher concentration than lipophosphoglycan, indicating that lipophosphoglycan is the major macrophage-binding molecule on amastigotes of *L. major*. In the presence of complement, the attachment of amastigotes to macrophages was not altered, suggesting that lipophosphoglycan interacts directly with macrophage receptors.

Human leishmaniasis is caused by the obligate intracellular amastigote form of the protozoan parasite species *Leishmania*. Macrophages are the main host cells targeted by *Leishmania* organisms, and infection is initiated by entry of the promastigote into the phagolysosomal compartment of these cells. The promastigote transforms to an amastigote and is not only able to survive but multiplies within this hydrolytic environment (6). Further infection of other macrophages by proliferating amastigotes is essential for disease manifestation, although the precise mode of transmission from macrophage to macrophage has not been defined. The ability of purified amastigotes to bind to and infect macrophages in vitro is consistent with amastigote binding to macrophages following release from overburdened macrophages in vivo. It seems likely that amastigotes bind to macrophages by a receptor-mediated process, as has been found for promastigote attachment (36).

In the case of the promastigote, two cell surface ligands have been implicated in attachment to macrophages: the protease gp63 and lipophosphoglycan (LPG). gp63 contains a Ser-Arg-Tyr-Asp sequence which mimics the cell attachment sequence Arg-Gly-Asp-Ser (RGDS) of fibronectin (40). Thus, gp63 may interact with macrophages via the RGDS binding domains of complement receptor 3 (CR3) and the Fn receptor (40). gp63 has also been shown to interact with CR3 through opsonization with iC3b (37). LPG has been shown to interact indirectly with CR1 and CR3 by opsonization with C3b and iC3b, respectively (7, 35). In addition, LPG can also interact directly in a lectin-like carbohydrate recognition mechanism with the bacterial lipopolysaccharide-binding site of CR3 (42). A component specific for *Leishmania major* LPG, PO_4-6[Gal(β1-3)Gal(β1-3)]Gal(β1-4)Man(α1- (P5b), has been shown to inhibit binding of *L. major* promastigotes to the macrophage cell line J774 (22). However, the macrophage receptor involved in this species-specific interaction has not been defined. Other candidates for lectin-like attachment of promastigotes to macrophages are the mannose-fucose receptor (4, 46) and the advanced glycosylation end product receptor (34). LPG is crucial to parasite survival since isolates deficient in LPG are unable to survive within the phagolysosomal compartment (5, 9, 19, 32).

All LPGs characterized contain four domains: a *lyso*-alkyl phosphatidylinositol anchor linked via a phosphohexasaccharide core to a polymer of phosphorylated oligosaccharide repeats, PO_4-6Gal(β1-4)Man(α1- (P2), capped by a neutral oligosaccharide (for reviews, see references 28 and 44). LPG contains species- and stage-specific structures, with variation occurring in the nonreducing neutral caps and in the side branches extending from the C(O)-3 of the galactose residue of the repeat unit. The repeat units of *Leishmania donovani* promastigote LPG are unsubstituted (43), while 25% of those from *Leishmania mexicana* LPG are substituted by a single glucose residue (20). Approximately 85% of the repeat units of *L. major* promastigote LPG are substituted with side branches containing galactose, arabinose, and glucose (30). *L. major* amastigote LPG is distinct from the promastigote form, as only 30% of its repeats are substituted with side branches (33). These comprise galactose and glucose but not arabinose.

The current study shows that amastigote LPG is present on the surface and in the flagellar pocket of the amastigote of *L. major*. Thus, amastigote LPG is in a position to mediate attachment to macrophages. We show that *L. major* amastigote LPG can block binding of amastigotes to macrophages and discuss the domains of LPG involved in this amastigote-mac-

* Corresponding author. Phone: 61-3-345 2555. Fax: 61-3-347 0852.
† Present address: Weizmann Institute of Science, Rehovot, Israel.

rophage interaction. The ability of complement opsonization to affect attachment mediated by LPG is also examined.

MATERIALS AND METHODS

Parasites. The isolates *L. major* LRC-L137, *L. donovani* LRC-L52, and *L. mexicana* LRC-L94 were obtained from the World Health Organization Reference Centre for Leishmaniasis, Jerusalem, Israel. The virulent cloned line V121 of *L. major* was derived from the LRC-L137 isolate by limiting dilution cloning (18) and maintained by passage in BALB/c mice. Amastigotes of *L. major* were harvested from 4-week-old lesions at the base of the tail of hypothymic CBA/H *nu*/*nu* mice by the method of Glaser et al. (12). The avirulent *L. major* isolate LRC-L119 was originally isolated in Kenya from a *Tatera nigricaunda*, has been shown to be *L. major* by genomic characterization and isoenzyme analysis, and is LPG deficient (19). A cloned line of this parasite has been used in these studies. Promastigotes of *L. major*, *L. donovani*, and *L. mexicana* were grown in Schneider's Drosophila medium supplemented with 10% fetal calf serum.

Purification of LPG and GIPLs. LPG was purified from promastigotes of *L. major*, *L. donovani*, and *L. mexicana* in stationary growth phase (26) and from lesion amastigotes of *L. major* (33). The low-molecular-weight glycoinositolphospholipids (GIPLs) 1 to 6 were purified from LRC-L119 promastigotes (25). Individual GIPLs were separated by thin-layer chromatography and extracted from the silica with isopropanol-hexane-water (50:20:25, vol/vol) as described before (25).

Preparation of LPG and GIPL fragments. The phosphoglycan (LPG without the lipid anchor) and phosphorylated repeats were prepared and purified from LPG (25, 26, 30). The glycan portion of GIPLs 2 and 3 were prepared from purified GIPLs 2 and 3, respectively (25, 45).

Enzyme treatments of LPG. LPG from either *L. donovani* or *L. mexicana* promastigotes was digested with jack bean α-mannosidase (30 U/ml; Boehringer Mannheim) in 50 mM sodium acetate buffer (pH 5.0), at 37°C for 16 h. The enzyme was inactivated by boiling for 2 min. The terminal phosphate was subsequently removed with calf intestine alkaline phosphatase (20 U/ml; Boehringer Mannheim) in 0.1 M ammonium bicarbonate buffer (pH 8.0) at 37°C for 16 h. The phosphatase was inactivated by boiling for 2 min, and the enzymes were separated from the LPG by phenol-chloroform extraction (38). Salts were removed from the enzyme digests by mixed-bed ion-exchange chromatography (30).

Potential inhibitors of amastigote attachment to macrophages. Mannose 6-phosphate, galactose 6-phosphate, lactose (4-*O*-β-D-galactopyranosyl-D-glucose), 4-*O*-β-D-galactopyranosyl-D-mannose, and yeast mannan, containing α(1-3), α(1-6), and α(1-2) mannopyranose, were obtained from Sigma Chemical Co., St. Louis, Mo. Chondroitin 4-sulfate, chondroitin 6-sulfate, and dextran sulfate were a generous gift from C. R. Parish, Australian National University, Canberra, Australia. These carbohydrates were used at the following concentrations: mannose 6-phosphate and galactose 6-phosphate, 66 μM; lactose and 4-*O*-β-D-galactopyranosyl-D-mannose, 58 μM; dextran sulfate (M_r 7,300), 2.74 μM; chondroitin 4-sulfate (M_r 50,000), 0.4 μM; and chondroitin 6-sulfate (M_r 50,000), 0.4 μM.

MAbs. The monoclonal antibody (MAb) WIC 108.3, an immunoglobulin M (IgM), recognizes LPG from all *Leishmania* species (8, 14). The epitope recognized by WIC 108.3 has not been precisely defined but includes at least a single and probably several P2 backbone repeat units. The IgM monoclonal antibody 4C12 directed to gp63 (15a) was used as a specificity control. The MAbs L-5-27, L-5-28, and L-5-34 recognize GIPLs 2 and 3 from promastigotes and amastigotes of *L. major* (10, 17). Fab fragments of the MAbs L-5-27, L-5-28, and L-5-34 were generated by using papain and purified by ion-exchange chromatography (13, 16). The purity of the Fab preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The rat MAb M1/70, which reacts with the α chain (CD11b) of Mac-1 (CR3) (34), was obtained from the American Type Culture Collection. The antibody was precipitated with 50% ammonium sulfate from ascitic fluid, dialyzed against phosphate-buffered saline (PBS), and used at 72.5 μg/ml, thus saturating Mac-1 present on the macrophages used. The IgG1 myeloma MOPC-21 (3) was used as a nonspecific control in the macrophage-binding experiments. Fab fragments of the IgG1 MAb 4F12, directed to a surface protein antigen of amastigotes, was also used as an additional specificity control.

Macrophage-binding assay. Peritoneal macrophages were obtained by flooding the peritoneal cavity of normal BALB/c mice with RPMI 1640 medium (6, 17). Bone marrow-derived macrophages were obtained by culturing the cell population from normal mouse femurs in the presence of L cell-conditioned medium, rich in the macrophage growth and differentiation factor macrophage colony-stimulating factor (15). Monolayers of peritoneal macrophages, bone marrow-derived macrophages, or the murine macrophage cell line J774 were prepared on glass coverslips in four-well Multidish tissue culture trays (Nunclon). Each coverslip was coated with 2×10^5 macrophages in 400 μl of RPMI 1640 with 10% heat-inactivated fetal calf serum and allowed to grow overnight at 37°C in 5% CO_2. Lesion amastigotes were labelled by incubation with 2′,7′-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes, Inc., Eugene, Oreg.) at a concentration of 10 μg/ml at 37°C for 15 min. Potential inhibitors of parasite binding were added 10 min before the addition of parasites to the macrophages. Fab fragments of MAbs (100 μg per coverslip) and pooled mouse complement (10 μl per coverslip; Cappel Research Products, Durham, N.C.) were added to parasites, while LPGs and P2 (12 μg per coverslip) and various sugars and M1/70 (25 μg per coverslip) were added to macrophages. Amastigotes were added to macrophage monolayers at a ratio of 10:1 unless specified and incubated for 35 min at 37°C. The binding assay was terminated by three gentle washes in PBS and fixation in 1% paraformaldehyde–2% glucose in PBS for 10 min at room temperature. The coverslips were mounted onto microscope slides, and the number of macrophages with bound amastigotes was counted using light and fluorescence microscopy. A minimum of 500 macrophages were counted from each coverslip. Each treatment was repeated at least three times in duplicate. The results shown are the averages for these experiments with the standard deviation. In some experiments, the infected cells were stained with Giemsa stain before counting.

RESULTS

LPG from *L. major* amastigotes was previously shown to be present on the surface of the parasite by immunofluorescence with a rabbit anti-amastigote antiserum, which recognized primarily LPG spanning the region of 55,000 to 100,000 M_r on Western blots (immunoblots) of amastigote integral membrane fractions. This antiserum immunoprecipitated LPG labelled on live organisms with sodium [^{3}H]borohydride after treatment with galactose oxidase (11). This label was present in the integral membrane fraction, as expected for a cell surface molecule. We have further confirmed the surface location of amastigote LPG by using immunofluorescence and immunoelectron microscopy with the anti-LPG MAb WIC 108.3 (data not shown).

LPG can block attachment of amastigotes to the macrophage cell line J774. Previous studies have shown that promastigote LPG from *L. major* inhibited *L. major* promastigote binding to the macrophage cell line J774 (22). We were prompted to investigate the effect of LPG on the binding of amastigotes to macrophages since we detected LPG on the cell surface of *L. major* amastigotes. LPG from *L. major* amastigotes (1.42 μM) and promastigotes (1.26 μM) inhibited the attachment of amastigotes to the macrophage cell line J774 by 82 and 63%, respectively (Fig. 1, Table 1). At the ratio of 10 amastigotes per macrophage, with the short incubation time used, only one or two parasites attached to each cell. Therefore, the reduction in the number of macrophages with parasites attached or internalized was not accompanied by a reduction in the number of parasites per cell.

Attachment of *L. major* promastigotes to the macrophage cell line can be inhibited by both the *L. major*-specific LPG repeat unit P5b and the anti-P5b MAb WIC 79.3 (8). The repeat unit P5b constitutes 2 and 3 mol% of the total number of repeats in promastigote and amastigote LPG, respectively (30, 33). Despite the presence of P5b in *L. major* amastigote LPG, Fab fragments of WIC 79.3 were unable to block amastigote attachment to the macrophages (Fig. 1). These results indicated that although LPG can mediate attachment between amastigotes and macrophages, the domain involved in the interaction is distinct from that utilized by the promastigote.

To rule out the possibility that nonspecific charge interactions on the highly phosphorylated LPG molecule were responsible for mediating attachment, we examined the ability of a number of charged carbohydrate molecules to block attachment. There was no significant inhibition of amastigote binding when mannose 6-phosphate, galactose 6-phosphate, chondroitin 6-sulfate, or dextran sulfate was present (Table 1). The best inhibitor among the haptens tested was the neutral disaccharide lactose (4-*O*-β-D-galactopyranosyl-D-glucose), which was able to reduce binding of amastigotes to macrophages by about 20%. Yeast mannan also had no significant effect on binding (Table 1). Hence, the interactions between amasti-

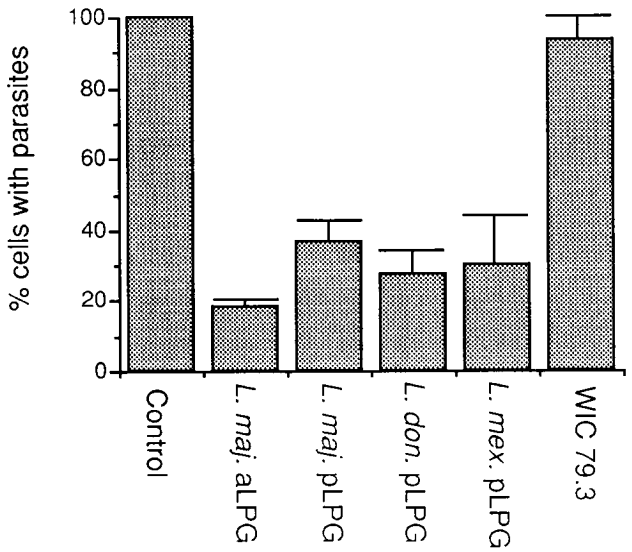

FIG. 1. Inhibition of attachment of *L. major* amastigotes to the macrophage cell line J774 in the presence of LPG from different *Leishmania* developmental stages and species and the MAb WIC 79.3. The number of macrophages with parasites attached was counted and expressed as percent adherence compared with the control. In this experiment, at a ratio of 10 parasites per macrophage, about 50% of control cells had parasites attached. The control represents the binding of amastigotes alone and in the presence of Fab fragments of the nonrelated MAb MOPC21. *L. maj.* aLPG, LPG from *L. major* amastigotes (1.42 μM); *L. maj.* pLPG, LPG from promastigotes of *L. major* (1.26 μM); *L. don.* pLPG and *L. mex.* pLPG, LPG from promastigotes of *L. donovani* (0.34 μM) and *L. mexicana* (1.89 μM), respectively. Fab fragments of WIC 79.3 (12 μM) were used. Values are means ± SD for three experiments testing each condition in duplicate.

gotes and macrophages could not be attributed to nonspecific charge interactions.

LPGs from other *Leishmania* species can block amastigote attachment. Candidates for the LPG domain(s) involved in the blocking of amastigote binding to macrophages include one or more of the four domains that constitute LPG: the neutral cap structures, the phosphorylated repeat backbone units, the glycan core, and the lipid anchor. The lipid anchor and the hexasaccharide core are largely conserved between species, with minor variation in the lipids constituting the anchor and the degree of glucosylation of the core hexasaccharide. In contrast, the compositions of the side branches on the repeat units and of the major cap structures vary significantly between species. The majority (70%) of the backbone repeat units of *L. major* amastigote LPG are not branched (33). The presence of a significant proportion of unsubstituted repeats is shared with LPG from other species. The repeat units of *L. donovani* promastigote LPG are totally unsubstituted with side branches (43), and only 30% of the repeat units from *L. mexicana* promastigote LPG are substituted (20). In contrast, *L. major* promastigote LPG contains only 15% unbranched repeats (30). All *L. major* amastigote LPG molecules are capped by a single structure, Gal(β1-4)Man(α1-, while LPGs from *L. major* promastigotes and promastigotes of other species comprise a number of structures which contain the sequence Man(α1-2)Man(α1- or Gal(β1-4)Man(α1- (20, 31, 43).

TABLE 1. Effects of haptens on the binding of *L. major* amastigotes to macrophage cell line J774

Hapten	Binding[a] (% of control ± SD)
None (control)	100
L. major amastigote LPG	18.2 ± 1.2
Mannose 6-phosphate	112 ± 12
Galactose 6-phosphate	97.4 ± 3.6
Chondroitin 6-sulfate	97.0 ± 4.3
Dextran sulfate	98.6 ± 1.9
Lactose	76.1 ± 9.6
Mannan	96.6 ± 4.9

[a] Values shown are the percentage of macrophages with parasites compared with that in the control pretreated with buffer alone and are representative of duplicate experiments.

In order to determine whether the LPG domain involved in the attachment of amastigotes to macrophages is species specific or not, LPGs isolated from other species were used. LPG from both *L. donovani* (0.34 μM) and *L. mexicana* (1.89 μM) promastigotes inhibited attachment of amastigotes by 72 and 70%, respectively (Fig. 1). These results indicate that the LPG domain involved in the interaction between amastigotes and macrophages is likely to be a conserved and not a species-specific domain.

Amastigote binding to peritoneal macrophages and bone marrow-derived macrophages displays a pattern similar to that observed in J774 cells. We compared the behavior of primary macrophages with respect to amastigote attachment and its inhibition by promastigote LPG and by the MAb WIC 108.3 with that of the macrophage line J774. With increasing ratios of parasites to cells, there was a concomitant increase in the percentage of cells with attached amastigotes (Fig. 2) and in the number of amastigotes attached to each cell (data not shown). The only difference was that a higher percentage of primary macrophages showed parasites attached compared with J774, possibly because of the presence of a mixed population of cells in the primary isolates. We examined the ability of LPG at a constant concentration of 1.26 μM to block amastigote attachment at the different parasite-to-cell ratios. We observed, as expected, a dose-dependent decline in LPG's ability to inhibit binding of amastigotes irrespective of the macrophage source (Fig. 2). However, the inhibition was still significant even at a ratio of 20:1 in the case of J774 (Fig. 2C) and 10:1 in the case of primary peritoneal macrophages (Fig. 2A) and less so in the case of bone marrow-derived macrophages (Fig. 2B). Similar results were obtained with MAb WIC 108.3 (Fig. 2), but not with an isotype-matched MAb directed to gp63 (4C12) or Fab fragments of a control IgG1 antibody, 4F12, directed to an as yet unidentified amastigote surface protein antigen (21a). There were some differences between the different cells in the levels of inhibition, but the overall pattern was similar. These results validate the data obtained with the cell line and indicate that promastigote LPG specifically inhibits amastigote attachment to macrophages derived from different sources.

Which domain of LPG is involved in amastigote attachment? The regions of LPG conserved among different promastigote species and in *L. major* amastigotes include the lipid anchor, the glycan core, the phosphorylated disaccharide repeats (P2), and some of the neutral cap structures which contain the sequence Man(α1-2)Man(α1- and Gal(β1-4)Man(α1-. To determine which of these was involved in amastigote attachment to the macrophage cell line J774, fragments of LPG were generated and used as potential inhibitors of binding. Since amastigote LPG is difficult to isolate in large amounts, some experiments were performed with purified LPG from promastigotes of *L. major* and other species. *L. major* promastigote phosphoglycan (1.14 μM), which comprises the intact molecule without the lipid anchor, inhibited amastigote attachment by 58% (Fig. 3), comparable to intact *L. major* promas-

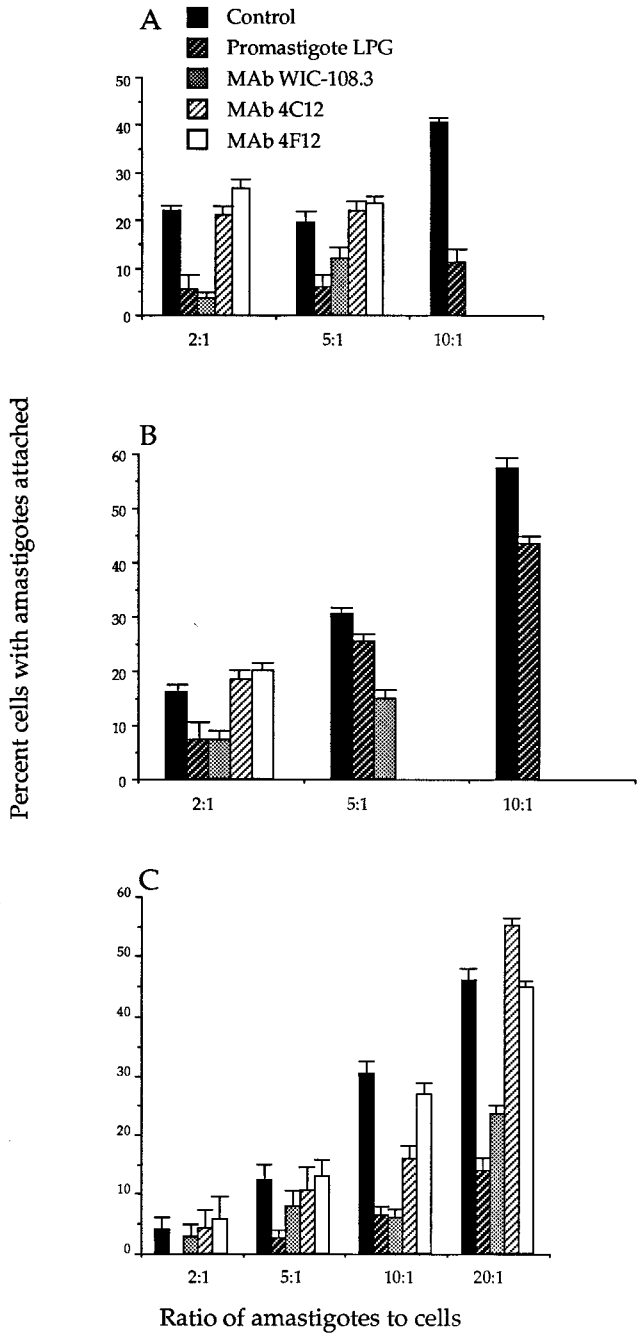

FIG. 2. Binding of *L. major* amastigotes to (A) peritoneal macrophages, (B) bone marrow-derived macrophages, and (C) J774 cells, at different ratios of parasites to cells, and inhibition of binding by promastigote LPG (1.26 μM), MAb WIC 108.3 (0.2 μM), MAb 4C12 (0.2 μM), and Fab fragments of MAb 4F12 (12 μM). Values are means ± standard deviations (SD).

tigote LPG (63%; Fig. 1), indicating that the lipid anchor is not involved in the interaction. To determine whether the conserved glycan core was involved, a mixture of the glycan portions of GIPLs 2 and 3, Gal(α1-3)Gal$_f$(β1-3)Man(α1-3)Man(α1-4)GlcN(α1-6)Inos and Gal(α1-6)Gal(α1-3)Gal$_f$(β1-3)Man(α1-3)Man(α1-4)GlcN(α1-6)Inos, respectively (29), were used, as they are similar to the glycan core of LPG, Gal(α1-6)Gal(α1-3)Gal$_f$(β1-3)[Glc(α1-)PO$_4$-6]Man(α1-3)Man(α1-4)GlcN(α1-6)Inos, except for the Glc-(α1-)PO$_4$ residue. This glycan mixture (24 μM) decreased the binding of amastigotes to macrophages by only 26% (Fig. 3). However, the anti-GIPL 2 and 3 MAbs L-5-34, L-5-27, and L-5-28, the epitope for which is expressed on the surface of promastigotes and amastigotes, had no effect (data not shown). The presence of the entire LPG core inhibited parasite attachment to the same degree as the glycan portions of GIPLs 2 and 3. A mixture of GIPLs 3 through 6 (16 to 24 μM) from the *L. major* isolate L119, which included GIPL 6, whose glycan portion, Manα1-PO$_4$-6Gal(α1-6)Gal(α1-3)Gal$_f$(β1-3)[Glc(α1-)PO$_4$-6]Man(α1-3)Man(α1-4)GlcN(α1-6)Inos, spans the entire core region of LPG and part of the first phosphorylated repeat unit, inhibited binding of amastigotes to macrophages by 28% (Fig. 3). At a much higher concentration (~400 μM), a mixture of GIPLs 1 to 6 still inhibited attachment by only 26% (data not shown). These results imply that the core of LPG, and hence also the GIPLs, has a small but consistent potential to block the attachment of amastigotes to macrophages. However, the glycan core is much less effective than LPG, since intact LPG used at 1.26 μM can inhibit attachment by 63 to 82%, whereas the type 2 GIPLs used at 400 μM can only reduce attachment by 28%.

The inhibitory effects of the conserved backbone repeat, P2, and the neutral cap(s) were then examined. Removal of the major neutral cap structure, Man(α1-2)Man(α1-, and a minor cap structure, Man(α1-2)Man(α1-2)Man(α1-, from *L. donovani* LPG by mannosidase digestion, to expose the phosphorylated repeat unit P2, resulted in essentially the same inhibition of binding of amastigotes to macrophages (77% compared with 74%) as unmodified *L. donovani* LPG. When the terminal phosphate group was removed from the demannosylated LPG with alkaline phosphatase, the capacity to inhibit increased slightly, to 87% (Fig. 4). When LPG from *L. mexicana* was similarly modified, the capacity to inhibit also increased slightly, from 70 to 80% (Fig. 4). After removal of these cap residues, LPG from *L. donovani* and *L. mexicana* promastigotes was able to inhibit to the same degree as LPG from *L. major* amastigotes (Fig. 1 and 4). Removal of Man(α1-2)Man(α1-)PO$_4$ and Man(α1-2)Man(α1-2)Man(α1-)PO$_4$ leaves Gal(β1-4)Man(α1- at the reducing end of the LPG molecule, which is the neutral backbone repeat of LPG from all *Leishmania* species.

The monomeric backbone repeat unit P2 (7.4 μM) inhibited amastigote binding to macrophages by 46% (Fig. 5C), while in the same experiments, intact *L. donovani* LPG (0.34 μM), which contains a mean of 16 copies of the repeat unit, reduced binding by 77% (Fig. 5B). When the LPG was preincubated with MAb WIC 108.3, it did not block attachment (Fig. 5D). This result implicated the backbone in attachment of amastigotes to macrophages but suggested that a single repeat unit is not sufficient. The MAb WIC 108.3 was used in the binding studies because it recognizes P2, probably in a tandem array. In the same experiment, WIC 108.3 was able to reduce binding by 48% when used at 87.5 μg/ml and by 50% when used at 150 μg/ml. At this concentration, the antibody should be saturating all potential binding sites on the amastigotes. Because WIC 108.3 was an IgM, it was difficult to obtain Fab fragments to examine their effect on binding (13).

Effects of complement and anti-Mac-1 antibody on amastigote attachment to macrophages. Amastigotes have been shown to be resistant to complement-mediated lysis (47) yet bind complement components, making it possible for them to interact with the complement receptors CR1 and CR3. The amastigotes used in the binding assays described here had

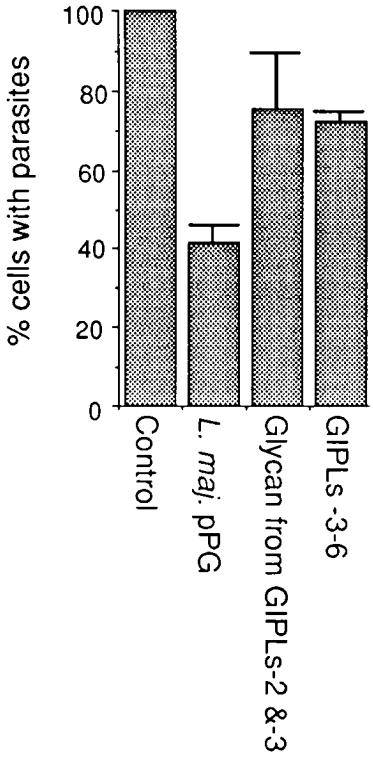

FIG. 3. Effect of components of LPG and type 2 GIPLs on the binding of *L. major* amastigotes to the macrophage cell line J774. *L. major* promastigote phosphoglycan (*L. maj.* pPG; 1.14 μM) and the glycan from GIPLs 2 and 3 (24 μM), which is structurally similar to the glycan core of LPG, were released from their lipid anchor by phosphatidylinositol-specific phospholipase C hydrolysis. GIPLs 3 to 6 were used at 16 to 24 μM. The control is the same as described in the legend to Fig. 1. Values represent the mean number of macrophages with attached parasites ± SD, expressed as a percentage of the control value, for three experiments testing each treatment in duplicate.

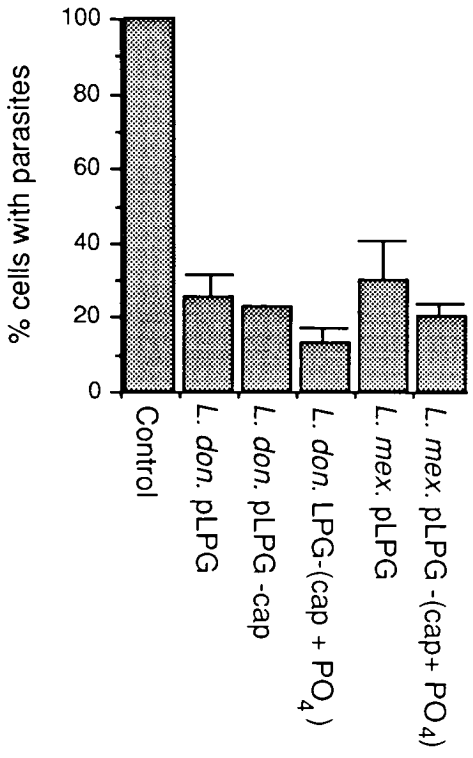

FIG. 4. Effect of LPG from different *Leishmania* species on the attachment of *L. major* amastigotes to the macrophage cell line J774. *L. don.* pLPG and *L. mex.* pLPG, LPG from promastigotes of *L. donovani* (0.34 μM) and *L. mexicana* (1.89 μM), respectively; *L. don*. pLPG-cap, LPG from *L. donovani* promastigotes which was demannosylated; *L. don.* pLPG-(cap+PO_4) and *L. mex.* pLPG-(cap+PO_4), LPG from *L. donovani* and *L. mexicana* promastigotes, respectively, which has been demannosylated and dephosphorylated. Modified LPGs were used at the same concentrations as intact LPG from a particular species. The control is as described in the legend to Fig. 1. Values represent the mean number of macrophages with attached parasites ± SD, expressed as a percentage of the control value, for three experiments testing each treatment in duplicate.

complement on their surface, which was deposited either while the amastigotes were present in the mammalian host or during their purification, prior to the addition of exogenous complement. This was detected by an anti-C3 MAb by immunofluorescence (data not shown). The anti-Mac-1 (CR3) MAb M1/70 (3) was only able to reduce binding of amastigotes to macrophages by about 10% (Table 2). This suggests that amastigotes were unlikely to interact with the iC3b-binding site of CR3 but could possibly interact with CR1. In the presence of exogenous complement (6% pooled mouse serum), further deposition of complement was detected by immunofluorescence (data not shown). However, this had no significant effect on either the percentage of macrophages infected or the number of parasites per infected macrophage (Table 2). These results indicate that neither C3b nor iC3b mediates an interaction between the amastigotes and CR1 and CR3. In triplicate experiments, *L. donovani* promastigote LPG was able to reduce binding of amastigotes by 70% in the presence of complement, compared with 80% in the absence of complement (Table 2). This suggests that the inhibitory effect of LPG was essentially independent of the presence of exogenous complement.

DISCUSSION

The interaction of the intracellular form of *Leishmania* species, the amastigote, with the mammalian macrophage is of critical importance in determining immunological host responses and parasite survival and propagation within the phagolysosomal compartment. Although the process of propagation of amastigotes from macrophage to macrophage is not understood, it is essential for disease manifestation. By analogy with the attachment of promastigotes to macrophages, amastigotes released from infected macrophages may be involved in a receptor-mediated uptake into uninfected macrophages. LPG is an attachment ligand for promastigotes which is involved in a direct saccharide interaction with a macrophage receptor(s) (42) or can be opsonized with complement

TABLE 2. Effects of complement and MAb M1/70, directed against the iC3b-binding site of CR3, on the attachment of *L. major* amastigotes to macrophage cell line J774

Treatment[a]	Binding[b] (% of control ± SD)	No. of parasites/ macrophage ± SD
None (control)	100 ± 0.6	1.4 ± 0.1
L. donovani LPG	20.0 ± 5.2	1.0 ± 0.1
Complement	93.1 ± 8.3	1.6 ± 0.7
Complement + *L. donovani* LPG	30.8 ± 15.6	1.7 ± 0.3
MAb M1/70	88.8 ± 8.2	1.3 ± 0.1

[a] Complement, 6% mouse serum.
[b] Values shown are the percentage of macrophages with parasites compared with that in the control pretreated with buffer alone and are representative of duplicate experiments.

components C3b and iC3b to interact with CR1 and CR3, respectively (7, 35). The promastigote LPG fragment P5b has been shown to be involved in the attachment of promastigotes of *L. major* to the macrophage cell line J774 (22).

Having shown that LPG from *L. major* amastigotes was localized at the cell surface, we set out to explore its role in attachment between amastigotes and macrophages. Binding of *L. major* amastigotes to primary macrophages and the macrophage cell line J774 could be inhibited by *L. major* amastigote LPG. The binding of the amastigotes to macrophages could also be reduced by LPGs from promastigotes of *L. major*, *L. donovani*, and *L. mexicana*, indicating that a conserved nonspecies-specific LPG domain was involved. This inhibition was dose dependent and decreased with increasing ratios of amastigotes to cells. From the known structure of LPG, we can infer that the conserved domains include the lipid anchor, the glycan core, the unsubstituted phosphorylated repeat unit, and some cap structures. The lipid anchor and most of the promastigote cap structures were unable to affect attachment, but both the backbone disaccharide repeat unit and, to a much lesser extent, the glycan core could inhibit the binding of amastigotes to macrophages. The backbone repeat units are the primary domain of LPG involved in amastigote attachment. Evidence supporting the idea that the amastigote binding domain is larger than a single backbone repeat unit (P2) is that the MAb WIC 108.3, the epitope for which probably extends beyond a single repeat unit, was a more effective inhibitor than the single repeat unit P2. Among several haptens used to inhibit amastigote attachment, only lactose (4-*O*-β-D-galactopyranosyl-D-glucose) was able to reduce attachment by 24%. The inhibitory effect of lactose may be due to its similarity in structure to the Gal(β1-4)Man of the backbone repeat unit of LPG. Lactose was shown to inhibit binding of *Leishmania amazonensis* amastigotes to activated mouse macrophages (41).

The effect of the conserved glycan core on amastigote attachment was determined by using GIPLs, whose glycan portion is structurally similar to the glycan core of LPG, and anti-GIPL antibodies. Amastigote binding to macrophages was modestly but consistently reduced by these reagents. There was little difference in inhibition of binding whether the entire core (GIPL 6) or part of the core (GIPL 2) was used in the assay, indicating that the effect was contained in the sequence Gal(α1-3)Gal$_f$(β1-3)Man(α1-3)Man(α1-4)GlcN(α1-6)Inos. Furthermore, the inhibition was saturated at this level even when the concentration was raised by 300-fold. These results indicate that if the glycan core of LPG plays a role in attachment of *L. major* amastigotes to macrophages, it is unlikely to be a major role.

In many other cell-cell interactions studied, the process of attachment has been demonstrated to be a multistep process. For example, attachment of leukocytes to endothelial cells may be mediated initially by a low-affinity binding interaction to the endothelial surface via selectins and the sialyl Lewis^x saccharide, followed by a high-affinity interaction, with CD18 integrins of the endothelial cells providing stable association before transmigration (23). Evidence also suggests that the attachment of *Plasmodium falciparum* merozoites to human erythrocytes is mediated by a two-stage binding process utilizing two distinct binding domains of the 175-kDa erythrocyte-binding antigen (21). Similar interactions may also occur for the attachment of *Leishmania* amastigotes to macrophages via two binding domains of LPG or two different cell surface molecules. The involvement of two cell surface molecules in a multistep infection process has already been demonstrated for attachment of *Leishmania* promastigotes to macrophages. Beads coated with promastigote gp63 were not internalized by

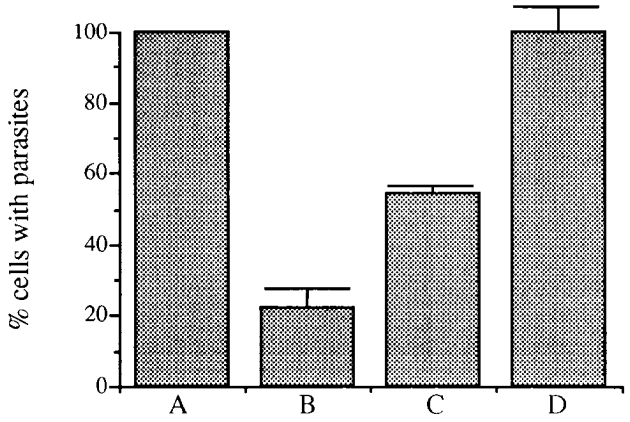

FIG. 5. Effect of *L. donovani* LPG on binding of *L. major* amastigotes to the macrophage cell line J774. (A) The number of macrophages with parasites was counted and expressed as a percentage of the control value. Potential inhibitors used were (B) *L. donovani* LPG (0.34 μM), (C) P2 (7.4 μM), and (D) *L. donovani* LPG (0.34 μM), all preincubated with intact MAb WIC 108.3 (0.17 μM) for 30 min before addition to the macrophages. The data represent the average ± SD for a single experiment performed in duplicate.

macrophages, although attachment of these beads to the surface of the cells was observed (37). In contrast, beads coated with LPG attached and were phagocytosed. In the experiments described here, the anti-gp63 antibody 4C12 had no effect on the attachment of amastigotes to either the J774 line or primary macrophages. Love et al. (24) have also suggested that the interaction of *L. mexicana amazonensis* amastigotes with mammalian cells via interactions with heparan sulfate proteoglycans may be a general mechanism to bind to host cells. However, binding of amastigotes to macrophages was inhibited by only 60% after treatment with heparin, suggesting additional mechanisms for macrophages recognizing amastigotes. Similarly, in our studies, LPG was unable to completely block attachment, suggesting the involvement of other ligands.

The role of complement in the attachment of *L. major* amastigotes to the macrophage cell line J774 was examined because complement has been shown to affect binding of promastigotes to macrophages (7, 35). Preincubation of macrophages with the MAb M1/70, directed against the iC3b-binding site of CR3, only slightly reduced binding of amastigotes to macrophages. This antibody was also ineffective in inhibiting the binding of *L. major* and *L. donovani* amastigotes to murine peritoneal macrophages (4, 15). These results suggest that only a minor proportion of binding of amastigotes may be mediated by opsonization by iC3b and subsequent interactions with CR3. Furthermore, the addition of exogenous complement, which results in further deposition of complement on the amastigote surface, did not affect the percentage of macrophages infected or the number of parasites per macrophage, indicating that neither CR1 nor CR3 is a major receptor mediating attachment or that attachment is mediated by a different part of the receptor. These findings are consistent with data showing that attachment of *L. donovani* amastigotes to murine and hamster peritoneal macrophages is unaffected by complement (4, 6). In the presence or absence of exogenous complement, promastigote LPG was able to inhibit attachment of amastigotes to macrophages to the same extent. Surprisingly, despite the presence of complement on the amastigote surface prior to the addition of exogenous complement, it did not seem to opsonize LPG to any significant degree. In the in vitro system used here, LPG was able to interact with the macrophage independent of complement opsonization.

A number of studies focusing on the macrophage side of the interaction with *Leishmania* species showed that several host receptors may be involved. Amastigotes of *L. mexicana amazonensis* could be inhibited from binding to human monocytes by an antiserum to fibronectin (48). Another study suggested that *L. major* amastigotes gain entry into bone marrow-derived murine macrophages via Fc receptors following opsonization with antiparasite IgG (15). Binding of amastigotes obtained from mice with severe combined immunodeficiency and from macrophages infected in vitro (which lack antibodies on their surface) to bone marrow-derived murine macrophages was enhanced when parasites were incubated with IgG prior to infection (15). The evidence also indicates some interaction with CR3 independent of complement, as binding of amastigotes of *L. donovani* and *L. major* was reduced only slightly with anti-CR3 MAbs (4, 15, 41). The role of the mannose-fucose receptor is uncertain, as *L. donovani* amastigotes were inhibited by mannan from binding to mouse peritoneal macrophages by 30%, but mannan did not alter the binding of *L. major* amastigotes to macrophages (4, 15). This is consistent with our result that yeast mannan had no effect on the binding of *L. major* amastigotes to the macrophage cell line J774 (Table 1). Amastigotes of *L. mexicana amazonensis* and *L. major* displayed binding to heparan sulfate proteoglycans of mammalian cells (24). These results indicate that a number of macrophage receptors may be utilized by amastigotes but this may depend on the species of parasite and the subpopulation of macrophages involved. However, in our study, bone marrow-derived and peritoneal macrophages displayed patterns of interaction with the amastigotes similar to those of the cell line. The only difference was that the primary macrophages bound more parasites than the cell line did.

The interaction of *L. major* amastigotes with the macrophage is clearly distinct from that of *L. major* promastigotes. A macrophage-binding domain of promastigotes is the epitope of MAb WIC 79.3, P5b, which had no significant effect on the binding of amastigotes to macrophages. The glycan core and repeat unit P2, which could inhibit binding of amastigotes to macrophages, had no effect on the attachment of promastigotes (22). This phenomenon of distinct attachment interactions of the two stages of the parasite has also been observed for *L. donovani* (4).

The involvement of LPG in the interactions between the amastigote and the macrophage clearly distinguishes *L. major* from other *Leishmania* species. LPG is not detectable in amastigotes of *L. donovani* (27) or *L. mexicana* (1), but the GIPLs are abundantly expressed in both. These GIPLs may be important for the attachment of some amastigote species to macrophages. They may also play a role in the interaction of amastigotes with inflammatory macrophages encountered during the chronic phase of the disease. Here, we examined the interaction of the amastigotes with normal macrophages which would be encountered early in the development of the lesion. The host and parasite molecules involved in the attachment and uptake of amastigotes into activated macrophages remain to be determined.

In two recent studies, antibodies directed against glycosphingolipids of *L. amazonensis* were able to inhibit attachment of *L. amazonensis* amastigotes to murine peritoneal macrophages (2, 41). The use of different amastigote cell surface molecules may reflect a preference for homing to different macrophage subpopulations, as displayed by the different *Leishmania* species.

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