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Biochemistry

Identification of a macrophage-binding determinant on lipophosphoglycan from *Leishmania major* promastigotes

(glycolipid/host–parasite attachment/carbohydrate epitope)

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ABSTRACT **_Leishmania_ are obligatory intracellular parasites in mammalian macrophages that gain entry by receptor-mediated phagocytosis. Their major cell surface glycoconjugate, lipophosphoglycan (LPG), has been implicated in this process. A monoclonal antibody specific for *Leishmania major* LPG (WIC 79.3), which has been shown to block promastigote attachment to macrophages, was used to identify a macrophage-binding determinant of LPG. WIC 79.3 bound exclusively to the phosphorylated repeats of LPG and not to the saccharide core or lipid anchor. Furthermore, the epitope recognized by WIC 79.3 mapped to the phosphorylated oligosaccharide P5b, PO_4-6[Gal(β1-3)Gal(β1-3)Gal(β1-3)]Gal(β1-4)Man(α1-, which is unique to the LPG of promastigotes of *L. major*. Phosphorylated oligosaccharides P3, PO_4-6[Gal(β1-3)]Gal(β1-4)Man(α1-, and P4b, PO_4-6[Gal(β1-3)Gal(β1-3)]Gal(β1-4)Man(α1-, were also recognized by WIC 79.3 but with considerably lower ($\approx$100-fold) affinities. The phosphorylated oligosaccharide P5b inhibited attachment of promastigotes of *L. major* to the macrophage cell line J774 to the same degree as phosphoglycan (derived from LPG) and Fab fragments of WIC 79.3, suggesting that P5b is a site of *L. major* LPG that is recognized by macrophage receptor(s) and is an important determinant in the attachment of promastigotes to host macrophages and initiation of infection.**

The protozoan *Leishmania* alternates between a free-living flagellate form, the promastigote, in a sandfly vector and an obligate intracellular nonflagellate form, the amastigote, in the macrophage of the vertebrate host. Upon deposition of the promastigote into the host tissue after a sandfly bite, rapid attachment and entry into the macrophage as well as transformation to the amastigote within the phagolysosome are necessary for parasite survival. The initial attachment and internalization of *Leishmania* promastigotes into macrophages is a receptor-mediated process and involves both parasite and host molecules (for review, see ref. 1). Two molecules on the surface of the promastigote have been implicated in this process: the glycoprotein gp63 and the major cell surface glycoconjugate lipophosphoglycan (LPG). LPG was implicated as a macrophage-binding molecule when the phosphoglycan (PG) derived from LPG and the monoclonal antibody WIC 79.3, which is specific for *Leishmania major* LPG, were found to block attachment of *L. major* promastigotes to macrophages (2). LPG from different *Leishmania* species may interact directly with carbohydrate-binding sites of macrophage receptors (1, 3–6) or indirectly with the CR1/CR3 receptors (7, 8) following opsonization of cell surface LPG by complement components (C3b and C3bi).

LPGs form a polymorphic family of similar, but antigenically and structurally distinct, molecules present on all *Leishmania* species. The LPG molecule has a tripartite structure consisting of a series of phosphorylated oligosaccharide repeats (PORs) and a conserved phosphorylated saccharide core attached to a conserved, unusual lyso-alkyl phosphatidylinositol anchor (Fig. 1; for review, see ref. 10). The PORs consist of a backbone of phosphodiester-linked disaccharide repeats [PO_4-6Gal(β1-4)Man(α1-], which is conserved in all species (9, 11). Variability between species involves side chain branches of varying complexity extending out from this common backbone (9, 11). *Leishmania donovani* displays the minimal structure with no branching, whereas *L. major* has the most complex, with the backbone sequence substituted with mono-, di-, tri-, and tetrasaccharide side chains consisting of combinations of galactose, arabinose, and glucose residues (Fig. 1; ref. 9). The nonreducing end of the PORs is terminated with a neutral cap. Structural and antigenic modification have been demonstrated for LPG in promastigotes undergoing maturation (metacylcogenesis; ref. 12) and in amastigotes (13–15), suggesting that LPG is an important molecule in adaptation of the parasite to the macrophage environment. A family of related low-molecular-weight glycolipids, the glycoinositol phospholipids (GIPLs), have similar lipid anchors and saccharide core structures and may function as precursors of LPG (16–18).

This report describes the characterization of the epitope of LPG from promastigotes of *L. major* that is recognized by the monoclonal antibody WIC 79.3, which blocks attachment of the parasite to macrophages.

MATERIALS AND METHODS

Monoclonal Antibodies. The anti-LPG monoclonal antibodies WIC 79.3 and WIC 108.3 were prepared as described (2). WIC 79.3 specifically recognizes LPG from *L. major*, whereas WIC 108.3 is cross-reactive with LPGs from all species of *Leishmania* (19–21). Fab fragments of the purified monoclonal WIC 79.3 were prepared by papain digestion (2). The IgG1 myeloma MOPC-21 (2) was used as a nonspecific control in the macrophage-binding experiments.

Isolation of Parasites. Parasites were obtained from the World Health Organization Reference Center for Leishmaniasis (Israel). The cloned, virulent *L. major* parasite clone V121 was produced from the human isolate LRC L137 (22). Promastigotes of V121, of the avirulent *L. major* strain LRC L119 (23), and of *L. donovani* LRC L52 were grown in Schneider's *Drosophila* medium supplemented with 10% fetal calf serum (GIBCO).

Preparation of LPG and GIPLs. LPG and GIPLs 1–6 were isolated from V121 and L119 promastigotes in stationary growth phase as described (16, 24).

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Abbreviations: LPG, lipophosphoglycan; PG, phosphoglycan; POR, phosphorylated oligosaccharide repeat; NOR, neutral oligosaccharide repeat; GIPL, glycoinositol phospholipid.

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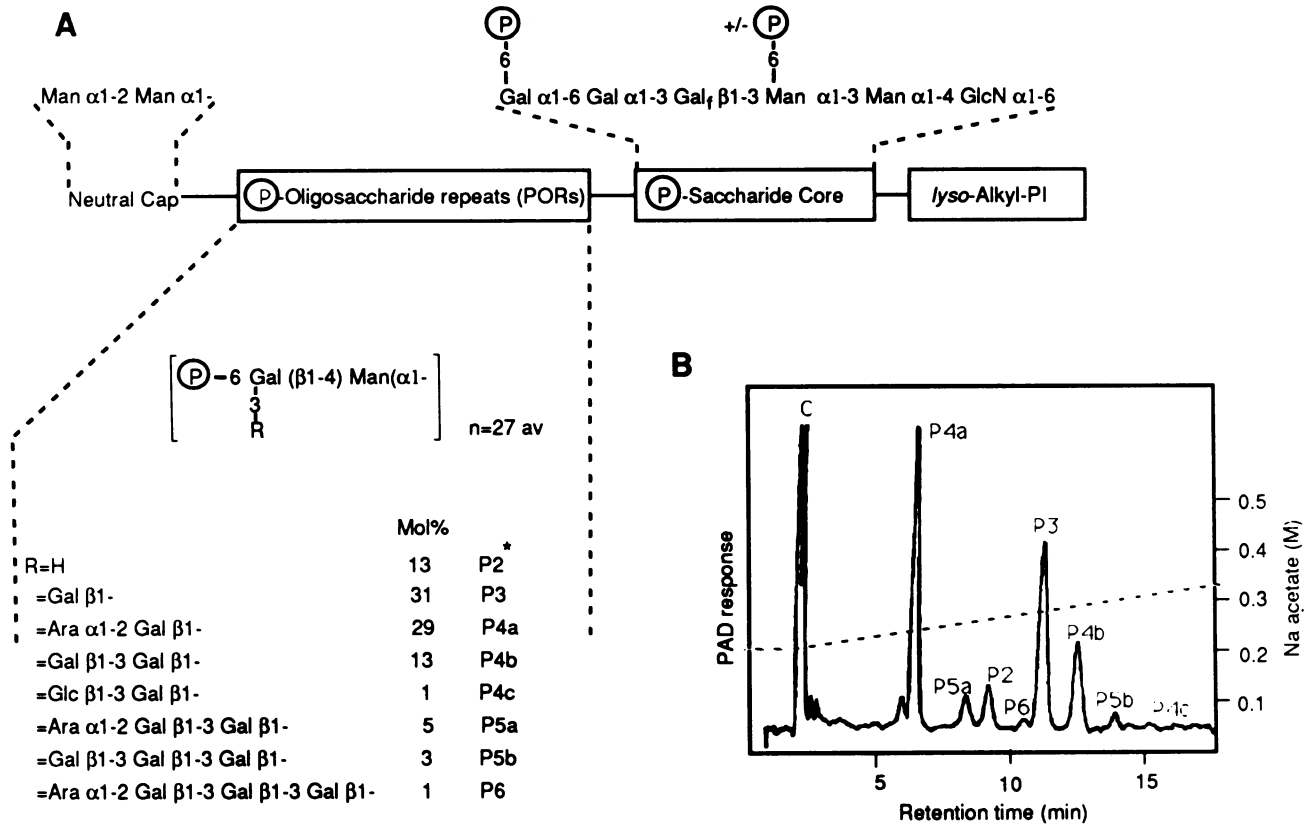

FIG. 1. (*A*) Tripartite structure of LPG from promastigotes of *L. major* (adapted from ref. 9). The variable regions of LPGs of different species of *Leishmania* lie within the PORs and the neutral cap. The structure of the conserved disaccharide backbone (P2, where R = H) is shown in the square brackets, while the unique substitutions (R) of *L. major* LPG are listed below. Nomenclature assigned for the PORs P2–P6* is as described (9). (*B*) Fractionation of PORs of *L. major* LPG by HPLC. The PORs generated by acid hydrolysis were chromatographed on a CarboPac PA-1 column by using gradient 1. The acetate gradient is indicated by the dashed line. The neutral cap was purified after rechromatography of material corresponding to peaks in region C by using gradient 2. PAD, pulsed amperometric detector.

Preparation of the Phosphorylated Saccharide Core and PG. PG was isolated by phosphatidylinositol-specific phospholipase C hydrolysis of LPG (16). The phosphorylated saccharide core from *L. major* LPG was prepared as described for the preparation of the core from *L. donovani* LPG (25) except that chromatography was performed using octyl-Sepharose (9).

Preparation of PORs of LPG. PORs were prepared from purified LPG as described (9), except that, after hydrolysis of LPG with trifluoroacetic acid (40 mM, 100°C, 8 min) and removal of the acid, lipid was extracted (three times) by adding an equal volume of *n*-hexane. The aqueous phase was freeze-dried and resuspended in H_2O or 100 mM NaOH for analysis by HPLC.

Preparation of Neutral Oligosaccharide Repeats (NORs) from LPG. NORs were prepared from purified LPG by hydrofluoric acid (HF) hydrolysis of intact LPG (17). NORs were also generated from PORs by removal of the phosphate group with alkaline phosphatase (9).

HPLC. PORs and NORs were separated on a Dionex model BioLC carbohydrate analyzer (Dionex) equipped with a pulsed amperometric detector (PAD) essentially as described elsewhere (9). Chromatography was performed in 100 mM NaOH, and separation was achieved by using a gradient of NaOAc as described below. The PORs were separated on a CarboPac PA-1 analytical column (4 × 250 mm) using gradient 1 at a flow rate of 1.0 ml/min (Fig. 1). Gradient 1 started at 200 mM NaOAc, was held for 2 min, and was increased linearly to 400 mM NaOAc at 30 min. For large-scale purifications, a CarboPac PA-1 preparative column (9 × 250 mm) was used at a flow rate of 4.0 ml/min, using 200 mM NaOAc in 100 mM NaOH. Fractions were collected and immediately neutralized by the addition of the cation-exchange resin AG 50W-X12 (H^+) (Bio-Rad). Each fraction was analyzed for carbohydrate by the phenol/sulfuric acid procedure (26), and appropriate fractions were pooled. The structures of the PORs (P2, P4a, P4b, P5a, and P5b; Fig. 1) were confirmed by chemical analysis (9). NORs and the disaccharide cap were purified by chromatography using gradient 2, which started at 10 mM NaOAc, was held for 2 min, and was raised linearly to 70 mM NaOAc at 80 min. All separations gave profiles (Fig. 1) essentially as described elsewhere (9).

Preparation of the Phosphorylated Disaccharide Backbone Repeat [PO_4-6Gal(β1-4)Man(α1-] from *L. donovani* LPG. LPG was purified from promastigotes of *L. donovani* by solvent extraction and hydrophobic chromatography on octyl-Sepharose (24). LPG-containing fractions were detected by carbohydrate analysis (26) and immunoblotting with monoclonal antibody WIC 108.3. The phosphorylated disaccharide backbone repeat was released from the LPG by mild acid hydrolysis as described above. A single peak appeared on the HPLC profile using gradient 1 (see above), which coincided with the phosphorylated disaccharide backbone repeat (P2) (Fig. 1) of *L. major* LPG.

Competitive RIA. Aliquots (50 μl) of purified LPG (2 μg/ml) were coated onto the wells of 96-well round-bottom flexible polyvinyl chloride microtiter plates (27). Modified LPGs and fragments of LPG to be tested for their ability to inhibit binding of WIC 79.3 to LPG were added at various concentrations in the range of 10 nM to 1 mM, together with ^{125}I-labeled WIC 79.3 monoclonal antibody (specific activity = 10 uCi/μg; 1 Ci = 37 GBq) in 5% bovine serum albumin in phosphate-buffered saline. After 1–2 h at room temperature,

plates were washed and cut, and wells were assayed for radioactivity in a Packard autogamma counter. Quantities of some PORs (e.g., P3 and P4b) were limited, and therefore saturating concentrations were not possible.

Promastigote–Macrophage Binding Experiments. Monolayers of the murine macrophage cell line J774 were prepared on glass coverslips in 24-well Linbro tissue culture trays (Flow Laboratories; ref. 2). Each coverslip received 10^4 cells in 1 ml of medium, and cells were allowed to grow in RPMI 1640 with 10% fetal calf serum for 48 h prior to assay. Promastigotes in the stationary phase of growth, day 5–6 in culture, were added to the macrophage monolayers at a cell number ratio of 20:1. Potential inhibitors of parasite binding were added 10 min before the parasites. Parasites were allowed to attach for 1 h at 37°C. The binding assay was terminated by two gentle washes in mouse tonicity (0.02 M) phosphate-buffered saline at pH 7.3. Cells were fixed in methanol and stained in Hemacolor rapid blood smear staining (Merck). The coverslips were mounted on microscope slides, and the number of promastigotes bound to cells was determined by counting a minimum of 500 macrophages in each of the duplicate preparations.

RESULTS

Previous studies showed that *L. major* LPG bound specifically to macrophages and that Fab fragments of the monoclonal antibody WIC 79.3, directed against *L. major* LPG, could block attachment of promastigotes to the macrophage cell line J774 (2). To determine which domain of the LPG molecule is recognized by WIC 79.3, LPG from *L. major* promastigotes was fragmented into its three major structural domains, the lipid anchor, the phosphorylated saccharide core, and the PORs including the neutral cap (see Fig. 1). In competitive RIAs, inhibition of WIC 79.3 binding to LPG could only be achieved by domains of LPG that included the PORs (Fig. 2). Binding of WIC 79.3 to LPG was reduced 50% by intact LPG at a concentration of 22.3 ng of carbohydrate per ml (1.40 nM LPG), by PG at a concentration of 28.6 ng of carbohydrate per ml (1.76 nM PG), and by a mixture of the PORs at a concentration of 1.88 mg of carbohydrate per ml (125 μM) (Fig. 2). Similar levels of inhibition were obtained if Fab fragments of WIC 79.3 were used in these assays. The phosphorylated saccharide core (Fig. 2) could not inhibit WIC 79.3 binding to LPG when used at 1 mM. GIPL 3, which has an identical structure to the saccharide core and similar lipid anchor of LPG (10), was also unable to compete for the binding to LPG (data not shown), confirming that these two domains were not part of the WIC 79.3 epitope. This was further demonstrated when GIPL 6, which may represent a truncated form of LPG (16), a mixture of GIPLs 1–6, and the glycolipids obtained from trifluoroacetic acid hydrolysis of LPG also failed to affect the binding of WIC 79.3 to LPG in competitive RIAs (data not shown). Therefore, we concluded that WIC 79.3 recognizes site(s) within the region of the PORs.

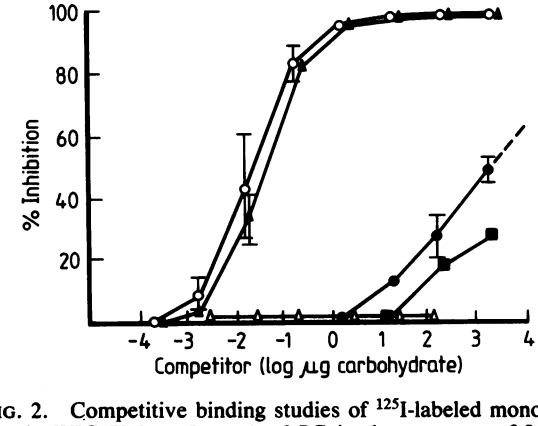

FIG. 2. Competitive binding studies of ^{125}I-labeled monoclonal antibody WIC 79.3 to *L. major* LPG in the presence of LPG or domains generated from LPG. Competitors used were LPG (○), PG (▲), a mixture of the PORs and the neutral cap (●), the NORs (■), and the phosphosaccharide core (△).

Before proceeding with the purification of the PORs, it was necessary to determine whether the phosphate group of the oligosaccharide repeats contributed to the binding domain of WIC 79.3. A mixture of NORs was not as effective an inhibitor of WIC 79.3 binding to LPG as the PORs (Fig. 2). At a concentration of 1.88 mg of carbohydrate per ml, the inhibition of WIC 79.3 binding to LPG was only 23% by the NORs compared to 50% by the PORs (Fig. 2). This indicated that the phosphate group was part of the epitope recognized by WIC 79.3. Hence, subsequent binding experiments were performed with PORs.

The PORs containing only galactose residues in their side chains, P5b, P4b, and P3, were able to inhibit the binding of WIC 79.3 to LPG (Fig. 3). P5b was ≈100 times more effective as an inhibitor than P3 and P4b. Binding of WIC 79.3 to LPG was reduced 50% by P5b at 4.74 μM, by P3 at 416 μM, and by P4b at 520 μM (Fig. 3). The repeats P4a, P5a, P6, and P4c, the backbone disaccharide P2 isolated from LPGs of promastigotes of both *L. major* and *L. donovani*, and the neutral cap [Man(α1-2)Man(α1-] did not affect the binding of WIC 79.3 to intact LPG, when used at 1 mM [i.e., a 10-fold higher concentration than P5b (data not shown)].

In an earlier experiment (Fig. 2), we had shown that the phosphate group of the oligosaccharide repeat was important for recognition by WIC 79.3. NORs were generated by alkaline phosphatase treatment of pure PORs P5b, P4b, and P3. The ability of P5b to inhibit binding of WIC 79.3 to LPG was severely diminished upon removal of the phosphate group (Fig. 3). NORs corresponding to P3 and P4b, used at

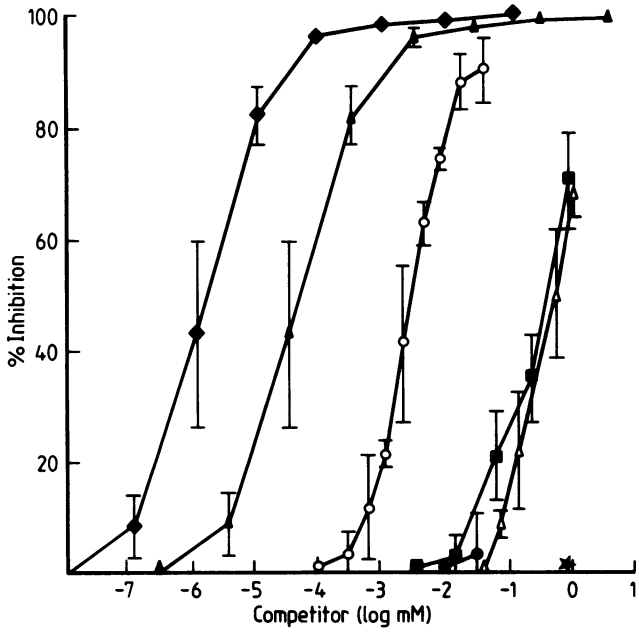

FIG. 3. Competitive binding studies of ^{125}I-labeled monoclonal antibody WIC 79.3 to *L. major* LPG in the presence of *L. major* LPG and purified PORs. Competitors were the PORs P5b (○), P4b (■), and P3 (△) and also the three corresponding NORs of P5b (●) and P3 and P4b (★) generated by alkaline phosphatase treatment. To calculate the concentration of LPG we have used a M_r assuming that each LPG molecule has 27 PORs (9). The data for intact LPG as a competitor (◆) has been reexpressed (▲), using the concentration of PORs in this LPG preparation.

concentrations of 1 mM, also failed to inhibit WIC 79.3 binding to LPG (Fig. 3).

LPG, PG, and WIC 79.3 had previously been shown to inhibit attachment of promastigotes of *L. major* to the macrophage cell line J774, indicating that LPG bound to the macrophage (2). We thus addressed the question of whether the macrophage-binding domain of LPG was the epitope recognized by WIC 79.3 or whether it was an epitope in the near vicinity of this WIC 79.3 epitope such that steric hindrance was caused by the bound antibody, rendering the macrophage-binding domain inaccessible. A number of PORs (P5b, P4a, and P4b) were used as potential inhibitors of parasite binding to macrophages (Fig. 4). The arabinose-containing fragment P4a gave a similar level of parasite attachment to macrophages as the control Fab fragments of the antibody MOPC-21 (Fig. 4). Fragment P4b gave a slightly lower level than the control, while P5b inhibited promastigote attachment to the same degree as PG and Fab fragments of WIC 79.3 (Fig. 4). There was no difference in the degree of inhibition of attachment if serum was absent from the assay.

To demonstrate the species and strain specificity of the binding of *L. major* promastigotes to the macrophage cell line J774, macrophage-binding assays were performed with promastigotes of *L. donovani* and the LPG-deficient strain L119 in the presence of *L. major* promastigote LPG and PG as well as Fab fragments of WIC 79.3. Both of these parasites do not bind to the monoclonal antibody WIC 79.3. For *L. donovani*, 84% of macrophages had parasites attached in the control (no added inhibitors), and this did not change when LPG (91%), PG (81%), and WIC 79.3 (88%) were present. Similarly for L119, 92% of macrophages had parasites attached in the control, and this did not change when LPG (90%) and WIC 79.3 (90%) were present.

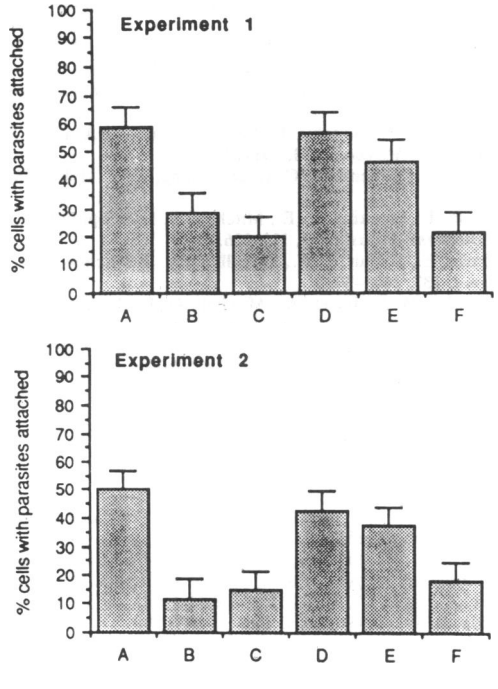

FIG. 4. Binding of *L. major* promastigotes to macrophages. Histograms represent the fraction of macrophages with promastigotes of *L. major* attached or internalized after a 1-h incubation at 37°C in the presence of the potential attachment inhibitors: bar A, Fab fragments of control myeloma MOPC-21 (12 μM); bar B, Fab fragments of monoclonal antibody WIC 79.3 (12 μM); bar C, PG from *L. major* LPG (1.5 μM); bar D, POR P4a (7.0 μM); bar E, POR P4b (6.7 μM); bar F, POR P5b (5.5 μM). The two histograms show replicate experiments.

DISCUSSION

LPG has been shown to be a multifunctional molecule crucial to the survival of *Leishmania* parasites (for review, see ref. 10). However, to date the functional domains of LPG have not been defined. In the present study, we define the functional unit of LPG isolated from promastigotes of *L. major*.

We chose to work with the anti-*L. major* LPG monoclonal antibody WIC 79.3, as it had previously been shown to inhibit the attachment of promastigotes of *L. major* to the macrophage cell line J774 (2). The epitope of this species-specific monoclonal antibody was mapped to the POR P5b, PO_4-6[Gal(β1-3)Gal(β1-3)Gal(β1-3)]Gal(β1-4)Man(α1-, which is unique to the LPG from promastigotes of *L. major*. Two other PORs, P3 and P4b, were also recognized by WIC 79.3 but with considerably lower affinities. The PORs P3, P4, and P5b may be regarded as a series of repeats consisting of the phosphorylated disaccharide backbone with the addition of one, two, or three (β1-3)-linked galactopyranosyl residues, respectively (Fig. 1). The addition of the third galactose residue has a marked effect on the ability of this repeat to competitively inhibit binding of WIC 79.3 to LPG and is an essential part of the WIC 79.3 recognition site. This is further supported by the inability of P4a, P5a, P6, and P4c to affect binding of WIC 79.3 to LPG because P4a, P5a, and P6 have the same structures as P3, P4b, and P5b, respectively, except that each has the addition of a terminal arabinose residue, whereas the fragment P4c has the same structure as P3 but with the addition of a terminal glucose residue (Fig. 1). The epitope that is recognized by WIC 79.3 includes more than saccharide residues alone, because removal of the phosphate group from fragments P5b, P4b, and P3 by alkaline phosphatase reduced their inhibitory capacity significantly. Whether the phosphate group is part of the antigenic determinant or whether it is important for maintaining the correct presentation of the epitope remains to be determined.

The POR P5b is the minimum determinant of LPG recognized by WIC 79.3. The intact structure of LPG also appears to be important for maintenance of the WIC 79.3 epitope because LPG and PG are much more effective inhibitors than a mixture of the PORs or the purified POR P5b (Figs. 2 and 3). This effect is not due to an increased avidity of divalent antibody molecule for polyvalent antigens as monovalent Fab fragments of WIC 79.3 gave a similar inhibition curve when used in competitive RIAs (data not shown). The reduction of the inhibitory capacity of LPG upon hydrolysis must be a result of the cleavage of the phosphodiester bonds, which disturbs the epitope. A number of factors may contribute to this effect. First, the tertiary structure of LPG may be fundamental in the presentation of the epitope. Steric interactions with neighboring branches of other PORs and the intact backbone may enhance the presentation of the epitope. Second, the sequence of the various PORs may be critical for binding as a particular combination of repeats (e.g., P4b–P5b or P3–P5b) may increase the affinity of WIC 79.3 for its epitope. Last, the size of the binding domain of WIC 79.3 may encompass a larger portion of LPG than P5b. Carbohydrate determinants of antibodies that have been analyzed generally range in size from three to seven residues (28, 29). For example, the IgA monoclonal antibody J539 recognizes a linear tetrasaccharide fragment of a β(1-6)-D-galactan (30) and anti-i Den recognizes a linear hexasaccharide, Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc from mouse embryonic cells (31, 29), whereas C14 recognizes a branched pentasaccharide, [Fuc(α1-2)]Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-3/4)GlcNAc, from the Le^b-related blood group antigen (29, 32). The POR P5b fits well into this size range. However, the active site of an antibody may involve a number of subsites (33), be more of a groove-like depression than a cleft (30), and/or be driven by hydrophobic

bonding, thus creating a mesh of interactions between the antibody and antigen (33–35). Additional subsites or hydrophobic bonds may arise from interactions of WIC 79.3 with the neighboring repeats or the backbone when fragment P5b is present in an intact LPG molecule, thus increasing the affinity of WIC 79.3 for this epitope.

After mapping the epitope of WIC 79.3, we were able to determine whether WIC 79.3 inhibited promastigote attachment to macrophages by binding directly to the macrophage-binding determinant of LPG. The epitope for WIC 79.3, the POR P5b, was able to inhibit the attachment of promastigotes to macrophages, implying that this fragment is in the macrophage-binding domain of LPG from *L. major* promastigotes. Fragment P4b gave only a slight inhibition in attachment, indicating that this epitope recognized by WIC 79.3 is not important for promastigote attachment by LPG. Initially we did these experiments in the presence of serum. There was no difference in the degree of inhibition if serum was absent, suggesting that the interaction of P5b with the macrophage was independent of added complement components and rather that P5b was directly associating with a macrophage receptor(s). However, we cannot rule out the secretion of complement components by the macrophage in sufficient quantities for opsonization (36), allowing the possibility of an indirect association with a macrophage receptor(s). Evidence suggests that LPG from promastigotes of *L. major* acts as an acceptor of complement (C3b and, to a lesser extent, C3bi), thus interacting indirectly with CR1, allowing the promastigote to enter the macrophage without triggering the oxidative burst (7, 8). Other target receptors (for review, see ref. 1) may be the lectin-like binding site of CR3, similar sites of p150 and p95, and possibly LFA-1 (3, 4, 6), the mannose-fucose receptor (3), or the advanced glycosylation end products receptor (5). It is also possible that promastigotes of various species and strains of *Leishmania* may enter the macrophage via other cell surface molecules such as glycoprotein gp63. For example, the LPG-deficient strain L119 may enter the macrophage via gp63 but is rapidly killed after gaining entry into the phagolysosome (23). Under certain conditions, a particular mode of entry may be favored by the parasite, thus increasing chances of its survival.

As fragment P5b is found only on *L. major* promastigote LPG, a unique attachment mode may exist for this species. It is unknown whether structural differences between LPGs reflect different attachment and internalization modes, leading to the tissue tropism found among the different *Leishmania* species. The WIC 79.3 epitope is expressed on the surface of both noninfective and infective promastigotes (37). However, noninfective promastigotes are so-called due to their inability to survive in the host macrophage, rather than their inability to attach and enter (38). The WIC 79.3 epitope cannot be detected on amastigote LPG (13, 15), suggesting that amastigotes may enter macrophages at a different site than promastigotes of the same species (36). The inability of *L. major* LPG and the Fab fragments of WIC 79.3 to inhibit attachment of *L. donovani* and the LPG-deficient strain L119 to the macrophage cell line J774 provides further evidence for the specificity of this mode of attachment, via the POR P5b for *L. major* promastigotes. Although the interaction between the POR P5b from *L. major* LPG and receptor(s) of the macrophage cell line J774 appears to be species, strain, and stage specific, studies of this interaction are fundamental to understanding attachment of *Leishmania* parasites to the host macrophage.

We can conclude that the binding domain of the monoclonal antibody WIC 79.3 includes the POR P5b, but the factor(s) contributing to the greater affinity of the antibody for intact LPG remains to be determined. In spite of this limitation, P5b is an effective inhibitor of promastigote attachment to macrophages, indicating that P5b is a site of *L. major* promastigote LPG that is recognized by macrophage receptor(s).

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