Stage-specific Binding of *Leishmania donovani* to the Sand Fly Vector Midgut Is Regulated by Conformational Changes in the Abundant Surface Lipophosphoglycan

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Summary

The life cycle of *Leishmania* parasites within the sand fly vector includes the development of extracellular promastigotes from a noninfective, procyclic stage into an infective, metacyclic stage that is uniquely adapted for transmission by the fly and survival in the vertebrate host. These adaptations were explored in the context of the structure and function of the abundant surface lipophosphoglycan (LPG) on *Leishmania donovani* promastigotes. During metacyclogenesis, the salient structural feature of *L. donovani* LPG is conserved, involving expression of a phosphoglycan chain made up of unsubstituted disaccharide-phosphate repeats. Two important developmental modifications were also observed. First, the size of the molecule is substantially increased because of a twofold increase in the number of phosphorylated disaccharide repeat units expressed. Second, there is a concomitant decrease in the presentation of terminally exposed sugars. This later property was indicated by the reduced accessibility of terminal galactose residues to galactose oxidase and the loss of binding by the lectins, peanut agglutinin, and concanavalin A, to metacyclic LPG in vivo and in vitro. The loss of lectin binding was not due to downregulation of the capping oligosaccharides as the same β-linked galactose or α-linked mannose-terminating oligosaccharides were present in both procyclic and metacyclic promastigotes. The capping sugars on procyclic LPG were found to mediate procyclic attachment to the sand fly midgut, whereas these same sugars on metacyclic LPG failed to mediate metacyclic binding. And whereas intact metacyclic LPG did not inhibit procyclic attachment, depolymerized LPG inhibited as well as procyclic LPG, demonstrating that the ligands are normally buried. The masking of the terminal sugars is attributed to folding and clustering of the extended phosphoglycan chains, which form densely distributed particulate structures visible on fracture-flip preparations of the metacyclic surface. The exposure and subsequent masking of the terminal capping sugars explains the stage specificity of promastigote attachment to and release from the vector midgut, which are key events in the development of transmissible infections in the fly.

*L*eishmania donovani is the cause of visceral leishmaniasis or kala-azar, an often fatal form of leishmanial infection that is currently epidemic in parts of Sudan and India. *Leishmania* are digenetic protozoa which exist as flagellated extracellular promastigotes in the alimentary tract of their sand fly vector and as aflagellar obligate intracellular amastigotes within mononuclear phagocytes of their vertebrate hosts. The life cycle of the parasite within the sand fly also includes development of promastigotes into a stage that is uniquely adapted for life in the vertebrate. Sequential development of promastigotes from a dividing noninfective or procyclic stage to a nondividing, infective or metacyclic stage has been observed for

promastigotes growing both within the sand fly midgut and in axenic culture (1, 2). Metacyclic promastigotes display increased resistance to certain microbicidal mechanisms, including complement-mediated lysis, and the oxygen-dependent and -independent leishmanicidal activities of their host macrophages (3). In addition, studies in *L. major*, a widely distributed Old World species that produces self-limiting cutaneous disease, indicate that the two promastigote stages exhibit distinctive patterns of behavior within the sand fly vector (4). Procyclic promastigotes display an inherent capacity to attach to midgut epithelial cells, which allows the parasites to persist in the gut during excretion of the digested blood-

meal. In contrast, metacyclic promastigotes lose this capacity, thereby permitting the detachment and anterior migration of infective forms so that they can be transmitted during a subsequent blood meal. Metacyclic promastigotes are thus well adapted for both arrival at and survival in the vertebrate host (5).

The molecular basis of these adaptions has been investigated largely in the context of the structure and function of the surface lipophosphoglycan (LPG),[1] the dominant surface glycoconjugate on promastigotes of all *Leishmania* species studied to date. LPG is organized as a densely packed glycocalyx that covers the entire surface of the cell, including the flagellum. The structure of LPG has been described in detail, and consists of a polymer of phosphodiester-linked oligosaccharide units linked via a hexasaccharide glycan core to a novel lyso-1-O-alkylphosphatidylinositol lipid anchor (6, 7). Whereas the glycan core and lipid anchor are highly conserved between species, the repeating oligosaccharide-phosphate units display extensive interspecific polymorphisms. The phosphoglycan moieties of all LPGs studied to date share a common backbone consisting of repeating disaccharide units of $Gal\beta1$-4$Man\alpha1$ PO_4-6 where the 3-position of the Gal residue can either be unsubstituted (as in *L. donovani*) or almost completely substituted with a variety of saccharide side chains (as in *L. major*). The nonreducing terminus of the phosphoglycan (PG) chain is capped with a mannose-containing, neutral oligosaccharide, which for *L. major* is primarily the sequence $Man\alpha1$-2$Man\alpha1$ (8). The capping oligosaccharides of *L. donovani* LPG display considerable heterogeneity, with the most abundant structure made up of the branched trisaccharide $Gal\beta1$-4($Man\alpha1$-2)$Man\alpha1$ (9). Molecular dynamics modeling of LPG suggest that the PG chains have an extended helical conformation with side chains oriented perpendicular to the helical backbone; and because several stable conformers can exist about the phosphodiester linkages, the molecule is allowed to expand or contract, somewhat like a slinky spring (10).

Evidence that LPG plays a substantial role in metacyclic virulence is based on a series of studies in *L. major*. During metacyclogenesis of *L. major* promastigotes, the LPG undergoes extensive modifications. These include an elongation of the molecule due to an approximate doubling in the number of oligosaccharide-phosphate units expressed, and a downregulation in the number of side chain substitutions expressing terminal β-linked galactose in favor of side chains terminating in arabinose (11, 12). The latter change appears to account for the greatly reduced binding of the lectins peanut agglutinin (PNA) and ricinus agglutinin (RCA I) to metacyclic promastigotes (13). The elongation of LPG controls complement activation in a nonlethal manner by hindering the access of the membrane attack complex to the cell membrane (14). The reduction of terminally exposed galactose residues controls the stage-specific adhesion of developing promasti-

gotes to the sand fly midgut (4). A similar reduction in lectin binding and increased resistance to complement lysis has been observed during metacyclogenesis of *L. donovani* promastigotes (15, 16), suggesting that the LPG of this species might also be developmentally modified. In these studies we compare the LPG structures of *L. donovani* procyclic and metacyclic promastigotes. We provide evidence that as in *L. major*, *L. donovani* metacyclics display an elongation of the molecule due to an increase in the number of oligosaccharide-phosphate units expressed. The accessibility of terminal sugars is also reduced, but unlike *L. major*, this change is controlled not by a downregulation of their synthesis, but by a conformation change affecting their availability for binding. The functional consequences of these structural changes in terms of promoting parasite transmissibility by the sand fly and survival within the vertebrate are discussed.

Materials and Methods

Parasites. *L. donovani* strain 1S from Sudan (World Health Organization [WHO] designation MHOM/SD/00/1S-2D) was passaged in hamsters before cultivation as promastigotes, and harvested within three serial passages from amastigotes. *L. major* Friedlin strain (WHO designation MHOM/IL/80/Friedlin) was passaged in BALB/c mice. All promastigotes were cultivated at 26°C in medium 199 supplemented with 20% (vol/vol) heat-inactivated FCS, 100 U/ml penicillin, 50 μg/ml streptomycin, and 12.5 mM L-glutamine (all from ABI Adv. Biotechnologies, Inc., Columbia, MD), 40 mM Hepes, pH 7.4, 0.1 mM adenine, and 0.0005% hemin. Parasites were passaged daily in fresh medium and procyclic promastigotes were harvested from 1 to 2-d logarithmic phase cultures. Metacyclic promastigotes were purified from 5 to 6-d stationary cultures by treatment with the lectin PNA (Vector Labs, Inc., Burlingame, CA) at 100 μg/ml as described; 17). Treatment of stationary phase promastigotes of *L. major* and *L. donovani* with PNA yielded metacyclic promastigotes which represented on average 30 and 10%, respectively, of the total stationary populations.

Extraction and Purification of LPG. Promastigotes were washed three times in DMEM (Advanced Biotechnologies) and labeled at 2 × 10^9/ml with 100 μCi/ml D-[6-^{3}H]-galactose (40 Ci/mmol) (Amersham Corp., Arlington Heights, IL). Labelings were done in DMEM for 1–2 h at room temperature and cells washed three times in cold DMEM. The cells were then extracted sequentially as previously described (18) with 1 ml of each solvent for each 10^9 cells as follows: chloroform/methanol/water (3:2:1), 4 mM $MgCl_2$, and chloroform/methanol/water (1:1:0.3). LPG was extracted from the resulting delipidated residue fraction by four extractions with solvent E (water/ethanol/diethyl ether/pyridine/concentrated NH_4OH; 15:15:5:1:0.017 vol/vol). The solvent E extract was dried in a rotary evaporator, resuspended in 0.1 M acetic acid, 0.1 M NaCl, and applied to an octyl-Sepharose column (1 ml) equilibrated in the same buffer. Fractions eluting with solvent E containing [^{3}H]LPG were pooled and lyophilized.

Analysis of [^{3}H]LPG by SDS-PAGE and Sephadex G150 Chromatography. Labeled LPG ($\sim$10,000 cpm) was boiled in sample buffer under reducing conditions (1.25% SDS, 60 mM Tris-HCl, 1.3% β-mercaptoethanol, and 8% glycerol) and the samples analyzed by SDS-PAGE using 7.5–15% slab gels. Gels were inpregnated with EN^{3}HANCE (New England Nuclear Research Products, Boston, MA) and dried under vacuum. Fluorography was done at -70°C using X-Omat AR Plus film (Eastman Kodak Co., Rochester, NY).

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[1] *Abbreviations used in this paper:* HPTLC, high performance thin layer chromatography; LPG, lipophosphoglycan; PG, phosphoglycan; PNA, peanut agglutinin.

For analysis of relative size by gel filtration, LPG was delipidated (referred to as PG) with phosphatidylinositol–specific phospholipase C (PI-PLC) (0.1 U) from *Bacillus thuringiensis*, resuspended in 40 mM NH$_4$OH and 1 mM EDTA, and applied to a Sephadex G150 column (1.0 cm × 100 cm) equilibrated in the same buffer. Radioactivity was measured in 0.6-ml fractions.

Determination of LPG Structure. LPG from four separate preparations of procyclic (batch 1 and 2) and metacyclic promastigotes (batch 3 and 4) was purified by octyl-Sepharose chromatography and either analyzed directly or delipidated and further fractionated on a Sephadex G150 column (metacyclic, batch 4). Material eluting near the void volume of the column was pooled for analysis. Before analysis, all LPG/PG preparations were subjected to descending paper chromatography on a 3-mm filters (Whatman Laboratory Products, Clifton, NJ) in 1-butanol/ethanol/water (4:1:1 vol/vol) for 18 h to remove salts and any contaminating monosaccharides, and then quantitatively recovered from the origin with water. The LPG/PG fractions were subjected to mild acid hydrolysis (40 mM TFA, at 100°C for 10 min) which cleaves phosphodiester linkages and depolymerizes the LPG to a mixture of neutral and phosphorylated oligosaccharides, corresponding to the capping and repeating oligosaccharide units of the PG chains, respectively. The acid was removed under reduced pressure and individual components resolved on a CarboPac PA1 column (Dionex Corp., Sunnyvale, CA) via HPLC and a pulsed amperometric detector. All elution buffers contained 0.15 M NaOH and NaOAc. The initial concentration of NaOAc was 12.5 mM which was increased in two linear gradients: from 12.5 to 55 mM (1–30 min), then from 55 to 250 mM (30–50 min), and then maintained at 250 mM for 20 min. Fractions containing individual oligosaccharide species were desalted by passage down a column of AG50 X 12 (H$^+$) over AG3 X 8 (OH$^-$) and then radiolabeled by reduction with NaB^{3}H$_4$. Dried samples were resuspended in 10 μl 1 N NH$_4$OH followed by 3 μl 30 mM NaB^{3}H$_4$ and reduced for 3 h at 25°C. Excess NaB^{3}H$_4$ was destroyed with the addition of 10 μl 1 M acetic acid and the sample desalted by passage down a column of AG 50 X 12 (H$^+$), and four cycles of evaporation with intermediate suspension in methanol (250 μl). Radiochemical impurities were separated from the labeled oligosaccharides by descending paper chromatography in 1-butanol/ethanol/water (4:1:1 vol/vol) as described above, and the oligosaccharides eluted from the paper with water. The identity of the labeled oligosaccharides was determined from their comigration with defined standards on Dionex HPLC and on silica gel 60 high performance thin layer chromatography (HPTLC) before and after treatment with jack bean α-mannosidase. Jack bean α-mannosidase digestions were performed in 0.1 M NaOAc buffer, pH 5.0 (20 μl, 30 U/ml) at 37°C for 18 h. The HPTLC sheets were developed in 1-butanol:acetone:water (5/4/1 vol/vol). The HPTLC mobility of the labeled oligosaccharides was compared with defined standards, obtained from *L. donavani* LPG (9) and a partial acid hydrolysate of dextran oligomers that had been radiolabeled in the same way.

Monosaccharide and lipid analysis of the intact LPG/PG preparations was carried out as previously described (8). After solvolysis in 0.5 M methanolic-HCl, released monosaccharides and alkyglycerols were converted to their corresponding trimethyl derivatives and quantified by gas chromatography-mass spectrometry.

Lectin Binding Profiles of LPG. Agglutination assays using PNA and Con A were performed in 96-well, flat bottomed microtiter plates that contained 2 × 10^7 promastigotes/ml. Equal volumes of the parasite suspension and the lectins in HBSS containing 1% BSA were mixed and incubated at room temperature for 30 min. After gentle mixing, the number of single, unagglutinated pro-

mastigotes was determined in a hemacytometer. Lectin binding was also studied on purified LPG reconstituted onto the surface of hydrophobic silica beads, as described (19). LPG and control beads were generated by application of a monolayer of LPG or phosphatidylcholine to the surface of 5-μm silica beads of the type commonly used in reverse-phase chromatography. LPG was suspended by sonication in chloroform at 1 mg/ml, and 100 μl was added to 100 μl of a 1% suspension of 5-μm-diameter beads in the same solvent. Under such conditions, the LPG was present in excess over the amount needed to coat the beads. The mixture was dried under nitrogen gas, resuspended in PBS containing 1% BSA, and incubated with FITC-PNA, FITC-Con A (both from Vector Lab, Inc.), or mAb CA7AE (a mouse IgM mAb that recognizes the phosphorylated disaccharide repeats) (20), or an irrelevant IgM mAb, followed by FITC-anti–mouse IgG, heavy and light chain specific. Control beads were prepared and labeled in parallel by substituting phosphatidylcholine for LPG. Beads were analyzed for fluorescence using a FACScan ® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Surface Labeling of Promastigotes Using Sodium [³H]Borohydride. LPGs containing terminal galactose residues were surface labeled by treating 10^9 live organisms with 3 U of galactose oxidase (Sigma Chemical Co., St. Louis, MO) in 0.5 ml PBS, pH 7.0, for 1 h at 25°C. Alternatively, 10^9 cells were resuspended in 1 ml PBS, pH 7.0, and treated for 10 min in the dark at 4°C with 10 mM sodium *m*-periodate. The treated parasites were washed twice in PBS, resuspended to 1 ml, and radiolabeled with 2 mCi/ml NaB^{3}H$_4$ (Amersham Corp.; 20 Ci/mmol) for 30 min at room temperature. Labeled cells were washed three times in PBS, and the LPG was extracted and analyzed for relative size by gel filtration through Sephadex G150 as described above.

Ultrastructural Studies. A detailed description of the fracture flip technique is given elsewhere (21). For labeling the surface LPG, parasites were fixed for 30 min at room temperature in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The cells were incubated for 45 min with a 1:100 dilution in Dulbecco's PBS of ascitic fluid containing mAb CA7AE. After several washes in PBS, the cells were incubated with a 1:10 dilution of anti–mouse IgG, heavy and light chain specific, conjugated with colloidal gold (10–15 nm, EY Labs, Inc., San Mateo, CA). Control cells were incubated with an irrelevant IgM mAb. Fixed and labeled parasites were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer solution containing 2 mM CaCl$_2$ and 0.8% potassium ferricyanide, dehydrated in acetone, and embedded in Epon. Routine thin sections of parasites were stained with uranyl acetate and lead citrate. Immunogold-labeled cells were only stained with lead citrate.

In Vitro Assay for Promastigote Binding to Sand Fly Midguts. Phlebotomus argentipes sand flies were reared and maintained in the Department of Entomology at Walter Reed Army Institute of Research (Wash. DC). 3–5-d-old nonfed female sand flies, maintained on 30% sucrose solution, were dissected in PBS, pH 7.4. Heads, crops, hindguts, and Malpighian tubules were removed, and the isolated midguts were opened along the length of the abdominal segment with a fine needle. Midguts (7–10 per group) were placed in the concave wells of a microscope chamber slide. *L. donovani* promastigotes (4.0 × 10^7/ml) in a total volume of 50 μl were added to the guts and incubated for 45 min at room temperature. The guts were then individually washed by placing them in successive drops of PBS. Washed, individual guts were placed into a microcentrifuge tube containing 30 μl of PBS and homogenized using a Teflon-coated microtissue grinder. Released promastigotes were counted in a hemacytometer. The role of LPG in mediating promastigote binding to *P. argentipes* midguts was investigated by

L.major L.donovani
M L M L

200 —
97 —
68 —
43 —
26 —
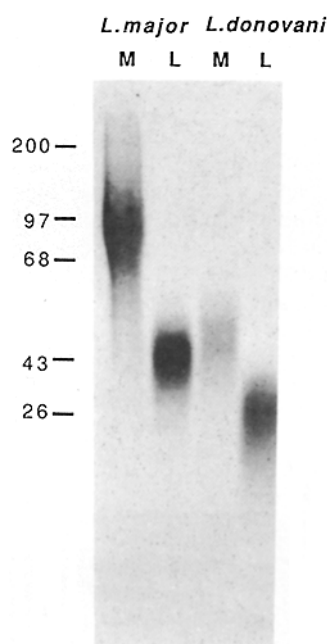
Figure 1. SDS-PAGE analysis of procyclic (L) and metacyclic (M) LPG purified from L. major and L. donovani promastigotes. Cells were metabolically labeled with [3H]galactose, and 15–20,000 cpm of purified LPG was run on each lane.

incubating midguts and parasites in the presence of purified LPG, or mild acid-treated LPG from which domains of either core-PI, or depolymerized dissacharide-phosphate repeats, were purified as previously described.

Results

The LPGs of Procyclic and Metacyclic L. donovani Promastigotes Differ in Average Molecular Size but Express the Same Terminal Sugars. Solvent E extraction of [3H]galactose-labeled cells resulted in extraction of 72–90% of the radioactivity contained within the delipidated residue fractions of L. donovani and L. major procyclic and metacyclic promastigotes. The LPG was further purified by octyl-Sepharose chromatography. Analysis of this labeled material by SDS-PAGE is shown in Fig. 1. All of the LPGs migrated as broad bands consistent with their polydisperse nature. The extracts from procyclic and metacyclic promastigotes of L major migrated as previously observed (17), with the metacyclic LPG displaying a relative twofold increase in size compared with the procyclic form. A similar shift in relative molecular size was seen when L. donovani LPGs were compared, with the procyclic LPG migrating between protein standards of 20 and 35 kD, and the metacyclic LPG between 35 and 50 kD.

The entire [3H]carbohydrate domains released by PI-PLC hydrolysis of the L. donovani procyclic and metacyclic LPGs, termed PG, were subjected to gel filtration in order to examine their relative sizes in the absence of lipid. The PGs eluted in two broad but clearly resolvable peaks (data not shown). The metacyclic PG eluted near the void volume, substantially ahead of the procylic PG. Taken together, these analyses indicate that whereas both procyclic and metacyclic LPGs have polydisperse molecular weights, there is a significant increase in the overall average size of the PG chain during metacyclogenesis.
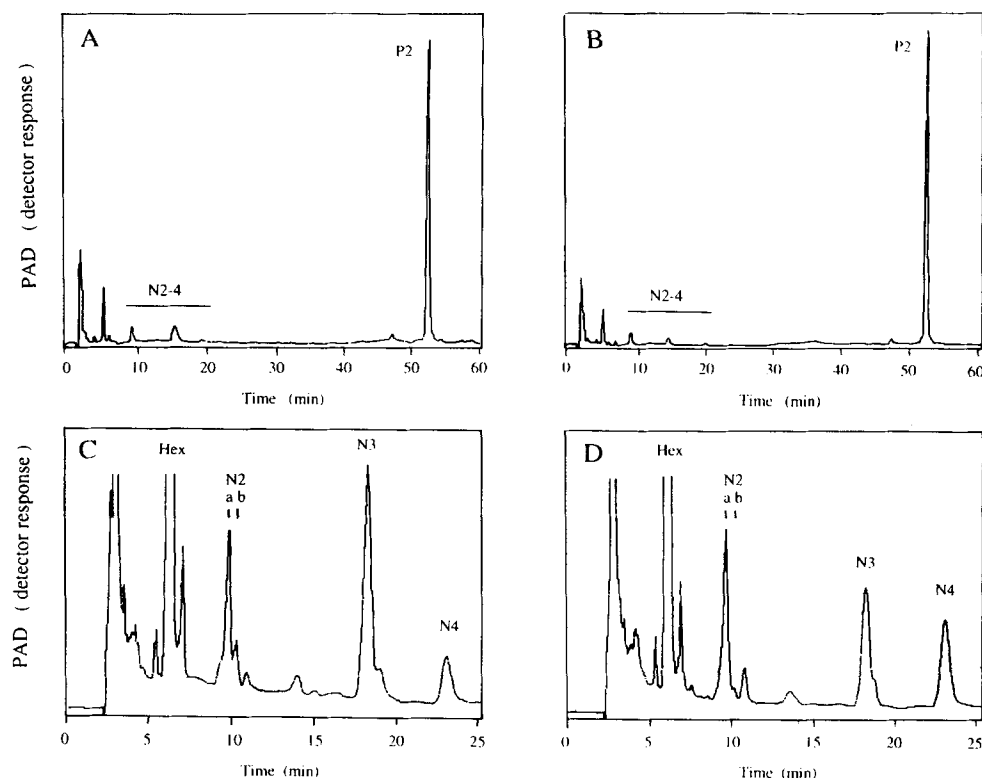

Figure 2. Dionex HPLC analysis of depolymerized procyclic and metacyclic LPG. LPG from procyclic (A and C) and metacyclic (B and D) promastigotes (batch 1 and 3, respectively) was depolymerized by TFA hydrolysis and the released neutral (N2-4) and phosphorylated (P2) oligosaccharides resolved by Dionex HPLC using gradient program as described in Materials and Methods. A and B show the entire profile where the major peak comigrates with the phosphorylated disaccharide, PO₄-6Galβ1-4Man. C and D show the region of the chromatogram containing the neutral oligosaccharides (0–25 min) with approximately five times more material loaded. The structures of the neutral oligosaccharides, given in Table 1, were determined from their elution position and migration on Dionex HPLC and HPTLC, respectively, monosaccharide analysis, and enzyme digestions (see text). Monosaccharide analysis of the hexose peak (Hex) revealed predominantly glucose (95%), consistent with this residue being derived from the glucose residue which is linked through phosphodiester linkage to the LPG core (9).

To probe for differences in the fine structure of the LPGs, unlabeled LPG was purified from the respective stages and depolymerized by mild acid hydrolysis. This treatment releases the phosphorylated oligosaccharide repeat units and the neutral capping oligosaccharides that are linked together by the acid-labile linkages. These carbohydrate fragments were then resolved by Dionex HPLC before further characterization (the diphosphorylated glycolipid which is also generated by the acid hydrolysis is not detected with the gradient conditions used). The LPG from procyclic promastigotes contained the expected structures which have previously been characterized: a single major phosphorylated oligosaccharide, which comigrated with authentic PO_4-6Galβ1-4Man (Fig. 2 A), and a series of minor, neutral oligosaccharides. The structure of the phosphorylated disaccharide was confirmed by monosaccharide analysis and its comigration with Galβ1-4Man on Dionex HPLC and HPTLC after alkaline phosphatase digestion (data not shown). The neutral oligosaccharides were indistinguishable from previously defined oligosaccharide caps (Galβ1-4Man, Manα1-2Man) and branched tri- and tetrasaccharide caps (Manα1-2[Galβ1-4]Man, Manα1-2Manα1-2 [Galβ1-4]Man, respectively) in their behavior on Dionex HPLC and HPTLC (Fig. 2 C, Fig. 3). These structures were consistent with the monosaccharide analysis (data not shown) and their susceptibility to jack bean α-mannosidase digestion (Fig. 3). The ratio of neutral cap oligosaccharides to phosphorylated repeat units was 1:9.4, suggesting that the procyclic LPG contained, on average, 9.4 repeat units per chain (Table 1).

The LPG from metacyclic promastigotes was structurally similar to the procyclic LPG. Importantly, it only contained the single phosphorylated disaccharide and the same series of neutral cap oligosaccharides (Fig. 2, B and D and Fig. 3). The main difference occurred in the ratio of cap to repeat unit oligosaccharides which indicated that metacyclic LPG contained, on average, 17.6 repeat units per chain (Table 1). Minor differences were seen in the relative abundance of the different cap structures. In particular, there was a decrease in the number of mannose-terminating oligosaccharide caps (N3, N4) and a corresponding increase in the amount of the Galβ1-4Man cap (Fig. 2 D, Table 1). This difference was a feature of the high molecular weight fraction obtained by gel filtration as it was not as apparent when the unfractionated metacyclic LPG preparation was analyzed (Table 1).

The Terminal Capping Oligosaccharides of LPG Are No Longer Exposed on the Surface of Metacyclic Promastigotes. The structures of the neutral capping sugars on procyclic and metacyclic LPGs suggest that whereas the proportion of the various oligosaccharides may differ, each nonetheless expresses β-galactose-, as well as α-mannose–terminating oligosaccharide caps. This was surprising given that the metacyclics were purified, as previously described by Howard et al. (16) and confirmed in these studies, based on their loss of agglutination by PNA, which bind to *L. donovani* promastigotes via recognition of β-linked terminal galactose residues on LPG (22). Lectin binding profiles of *L. donovani* promastogotes are shown in Fig. 4. At concentrations of 50–200 μg/ml PNA, 100% of logarithmic phase promastigotes were agglutinated, whereas 10–20% of stationary phase parasites remained free. A similar change in lectin binding was found when agglutination by
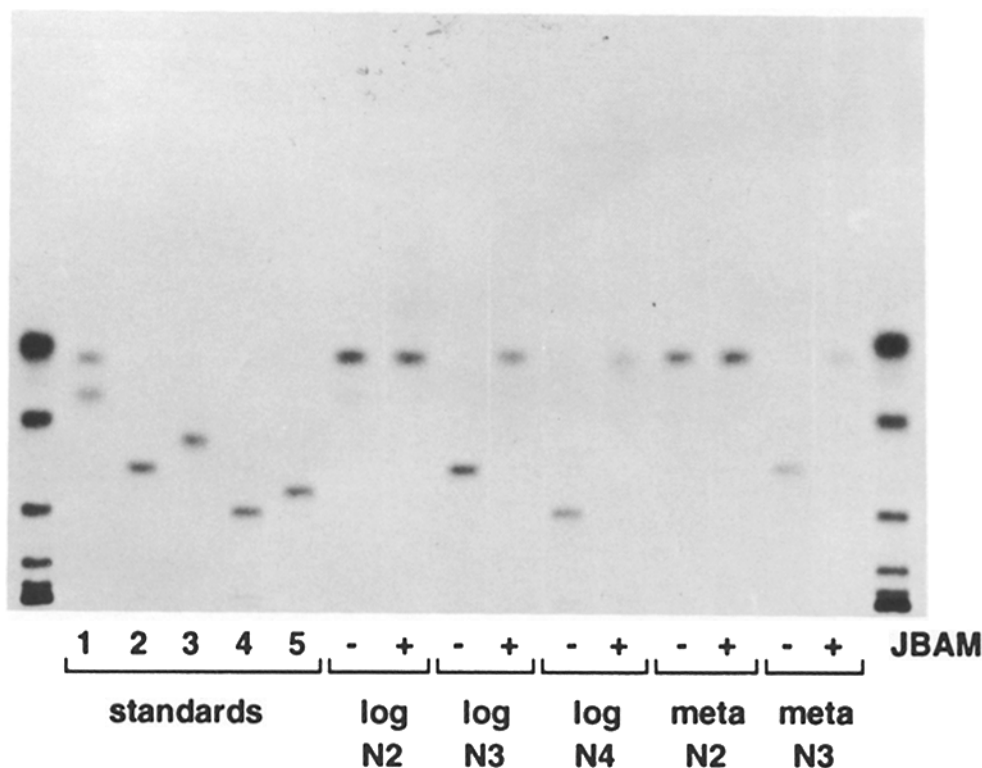
Figure 3. Characterization of the cap oligosaccharides of procyclic and metacyclic LPG. Neutral cap oligosaccharides were released from procyclic and metacyclic (batch 2 and 4) LPG by mild acid hydrolysis and purified by Dionex HPLC. Each cap species (N2-4) was labeled by reduction with NaB³H₄ and radiochemical contaminants removed as described in Materials and Methods. Half of the labeled fractions were treated with jack bean α-mannosidase and the untreated and treated oligosaccharides analyzed by HPTLC together with defined LPG cap oligosaccharides. The HPTLC was developed in 1-butanol/acetone/water (5:4:1 vol/vol) and the glycans detected by fluorography. Lanes 1–4 contain the following standards: 1, Galβ1-4Man (*upper band*), Manα1-2Man (*lower band*); 2, Manα1-2-[Galβ1-4]Man; 3, Manα1-2Manα1-2 Man; 4, Manα1-2Manα1-2[Galβ1-4] Man; and 5, Manα1-2Manα1-2 Manα1-2Man. The lanes at either end of the HPTLC contain ³H-labeled reduced dextran oligomers.

Table 1. *Ratio of Different Cap Structures and Average Chain Length of the LPGs from L. donovani Procyclic and Metacylic Promastigotes. Results of Analyses of LPG from Different Preparations (Batch 1–4) of Parasite Are Given*

	Procyclic		Metacyclic	
Structure	1	2	3	4*
N2a Galβ1-4Man	26	10	36	64
N2b Manα1-2Man	7	5	1	ND
N3 Manα1-2[Galβ1-4]Man	51	75	27	36
N4 Manα1-2Manα1-2[Galβ1-4]Man	11	10	32	ND
Average number of repeat units per chain‡	ND	9.4	ND	17.6

* LPG from metacyclic promastigotes (batch 4) was further size fractionated by Sephadex G150 and corresponds to the [³H]galactose-labeled high molecular weight fraction.
‡ Average chain length of the PG chains was determined from the ratio of repeat units to cap structures, based on the pulsed amperometric detection of these species in the same Dionex HPLC chromatographic run.

Con A was compared. The parasites from stationary cultures that were obtained based on their loss of agglutinability with either 100 µg/ml PNA or Con A were in each case metacyclic forms by morphologic and biologic criteria (i.e., complement resistance and intramacrophage survival). Their identity was further established by the fact that PNA-negative

forms were uniformly Con A negative, and vice versa. The lectin binding profiles of *L. major* promastigotes are also presented for comparison (Fig. 4). As previously reported (13), PNA-negative cells arise within stationary cultures and represent homogeneous populations of metacyclic promastigotes. In contrast, no reduction in Con A binding was observed within the population of stationary phase *L. major* promastigotes, including the metacyclics that were purified based on their loss of PNA binding. The results are consistent with the reported structural modifications of *L. major* LPG during metacyclogenesis, which are confined to the side chain substitutions terminating in β-linked galactose, whereas the predominant Manα1-2Man capping structure remains unchanged and available for binding by Con A. By contrast, whereas *L. donovani* metacyclics also continue to synthesize LPG capping oligosaccharides which are common to procyclic forms, including those containing terminal β-linked galactose, α-linked mannose, or both, these residues nonetheless appear to become cryptic on the surface of metacyclic cells.

To further compare the availability of LPG-terminal sugars for protein interactions, promastigotes were surface labeled using galactose oxidase/NaB[³H]₄, whereby the 6-carbon of terminal galactose is oxidized by the enzyme to an aldehyde and then radiolabeled by the reductant. Surface LPG was also labeled by treating promastigotes with 10 mM periodate/NaB[³H]₄, which labels sugars containing adjacent hydroxyl groups (e.g., carbons 2 and 3 of each mannose residue on LPG). After labeling, the LPG was extracted, purified, and PI-PLC-treated material was applied to a column of G-150 Sephadex. The elution profiles of periodate- and galactose
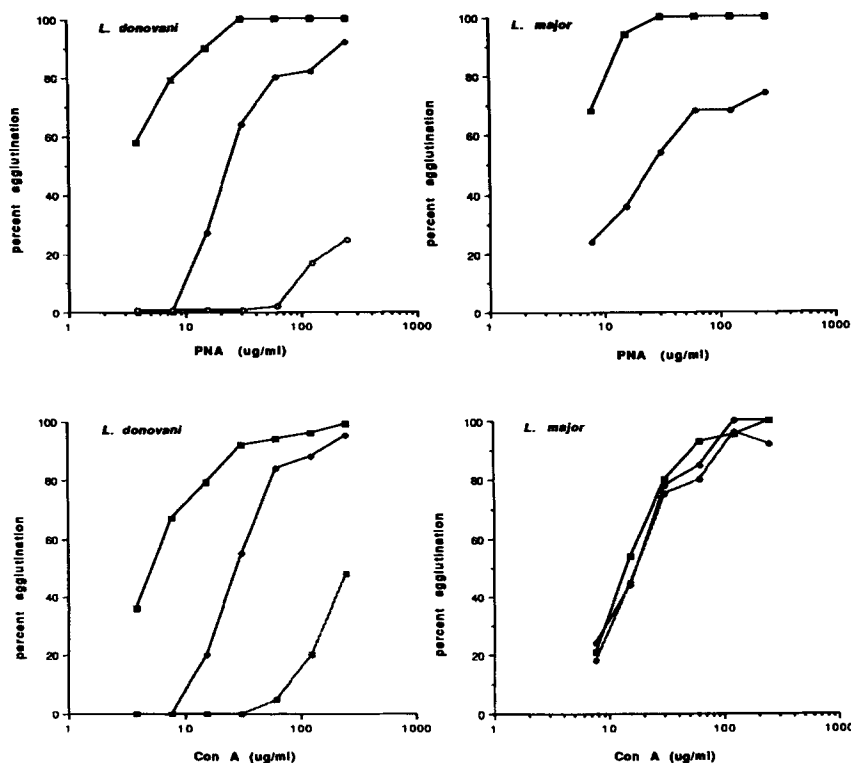
Figure 4. Lectin-mediated agglutination profiles of *L. donovani* and *L. major* promastigotes using PNA (*top*) or Con A (*bottom*). Logarithmic phase, procyclic promastigotes (*open squares*); stationary phase promastigotes (*diamonds*); PNA-negative metacyclic promastigotes (*closed circles*); and Con A-negative metacyclic promastigotes (*open circles*).
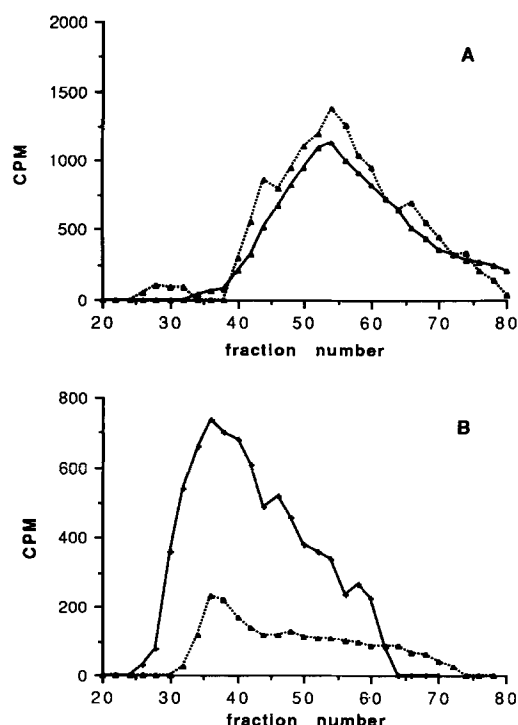
Figure 5. G-150 Sephadex chromatography of NaB³H₄-labeled *L. donovani* LPG after PI-PLC hydrolysis. (*A*) Procyclic LPG; (*B*) metacyclic LPG; (*solid line*) periodate treated; and (*broken line*) galactose oxidase treated.
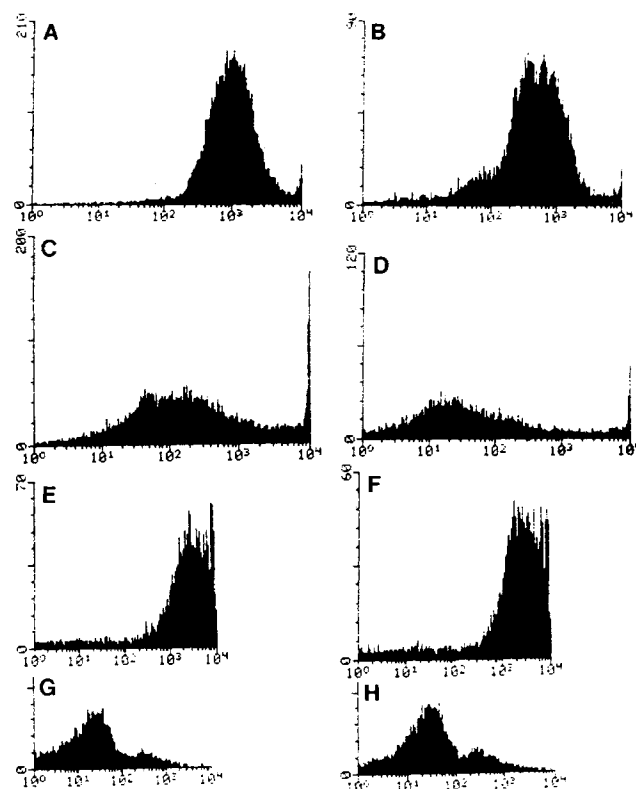

Figure 6. FACScan® flow cytometric analysis of FITC-labeled, LPG-coated hydrophobic beads. (*A*) FITC-PNA/procyclic LPG; (*B*) FITC-

oxidase–labeled procyclic PGs were identical to each other and to [³H]galactose biosynthetically labeled procyclic PG, each eluting as a single broad peak of radioactive material between fractions 38 and 80 (Fig. 5 *A*). Periodate-treated metacyclic PG eluted as a single broad high molecular weight peak (Fig. 5 *A*). Periodate-treated metacyclic PG eluted as a single broad high molecular weight peak (Fig. 5 *B*), consistent again with the biosynthetically labeled metacyclic PG. The periodate labeling confirmed that the LPG expressed on the metacyclic surface is comprised almost exclusively of the elongated form. Surface labeling of galactose oxidase–treated metacyclics resulted in extremely poor labeling of high molecular weight LPG, suggesting that the terminal galactose residues on the neutral, capping sugars were inaccessible to the enzyme.

Loss of Terminally Exposed Sugars on the Surface of Metacyclic Promastigotes Appears to be Due to a Conformational Change in Metacyclic LPG. The inability of LPG on the metacyclic surface to provide reactive substrate or lectin binding sites might be explained by the expression of other stage-specific molecules that mask exposure of their terminal sugars. To determine if the loss in lectin binding to the parasite surface was in fact related to structural changes in the LPG itself, binding studies were performed on purified LPG reconstituted onto C18 derivitized hydrophobic beads. The FACScan® analyses of LPG and control beads stained with CA7AE, PNA, or Con A, are shown in Fig. 6. The CA7AE antibody, which recognizes the PO₄-6Gal(β1-4)Man repeats, bound comparably to the procyclic and metacyclic LPG beads, indicating that the LPGs in each case were efficiently reconstituted onto the surface of the beads (Fig. 6, *C* and *D*). LPG beads were not stained with an irrelevant antibody (Fig. 6, *E* and *F*). Binding of FITC-PNA or FITC-Con A also resulted in a sharp increase in fluorescence intensity of virtually all of the procyclic LPG-coated beads (Fig. 6, *A* and *B*), but in only a small proportion (<10%) of the metacyclic LPG-coated beads (Fig. 6, *C* and *D*). Control beads coated with phosphatidylcholine did not stain with CA7AE or either lectin (data not shown). The lectin binding profiles are thus similar to those observed with the parasites themselves, and suggest that the terminal capping sugars are poorly exposed on the metacyclic surface because of a conformational change in the LPG.

Surface Ultrastructure of Procyclic and Metacyclic Promastigotes. We have previously shown that the technique of fracture flip preserves the micro and macroanatomic details of the parasite surface (21). Fracture flip preparations of *L. donovani* procyclic promastigotes show a random and sparse distribution of single rounded particles over the cell surface (Fig. 7, *A* and *C*). In contrast, the metacyclic surface is covered with an approximately sixfold higher density of particles distributed over the entire cell body (Fig. 7, *B* and *D*). Fracture flip images of the surface of *L. major* promastigotes have been published

Con A/procyclic LPG; (*C*) FITC-PNA/metacyclic LPG; (*D*) FITC-Con A/ metacyclic LPG; (*E*) CA7AE/procyclic LPG; (*F*) CA7AE/metacyclic LPG; (*G*) control antibody/procyclic LPG; and (*H*) control antibody/metacyclic LPG.
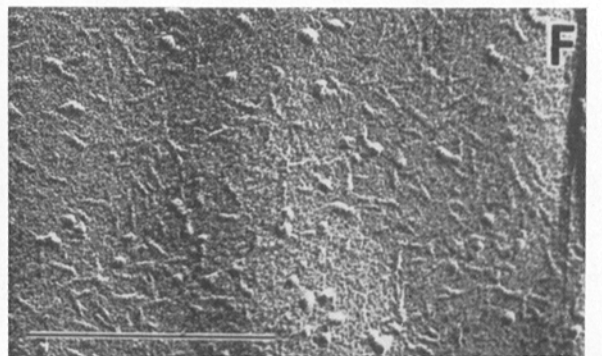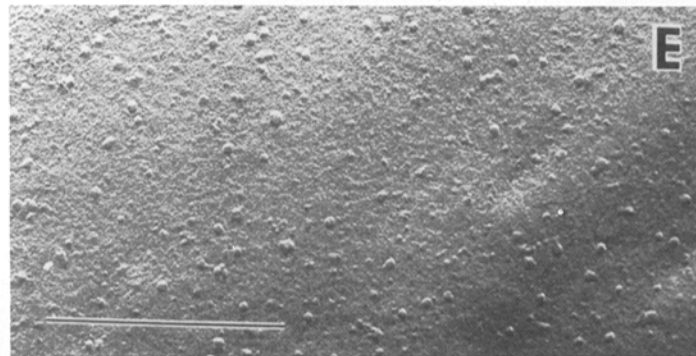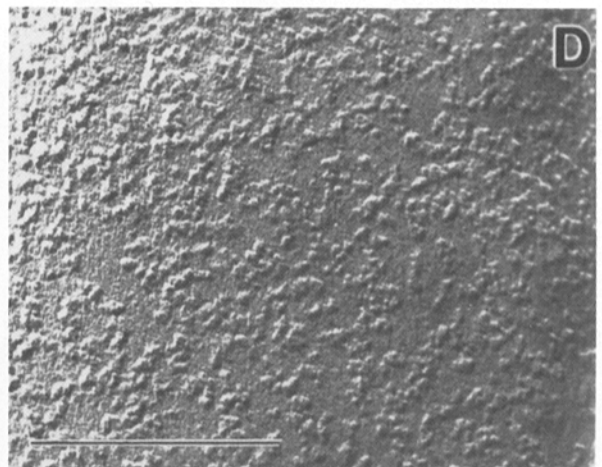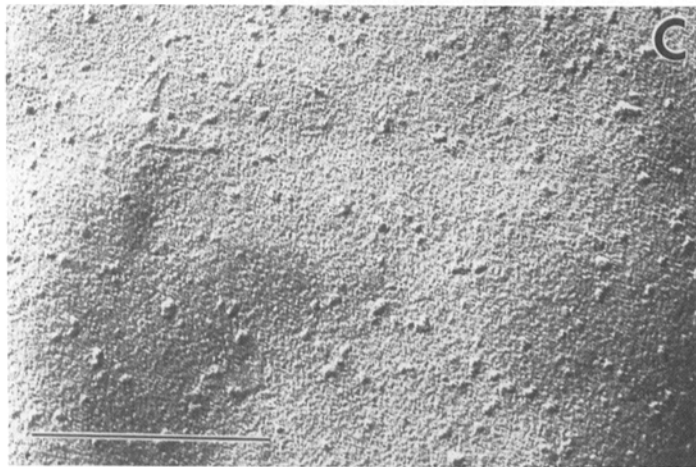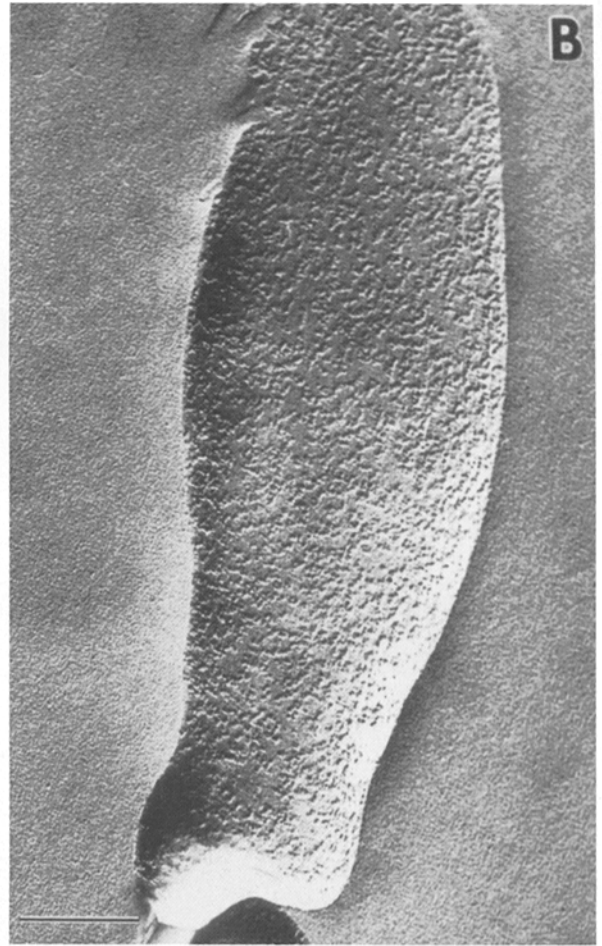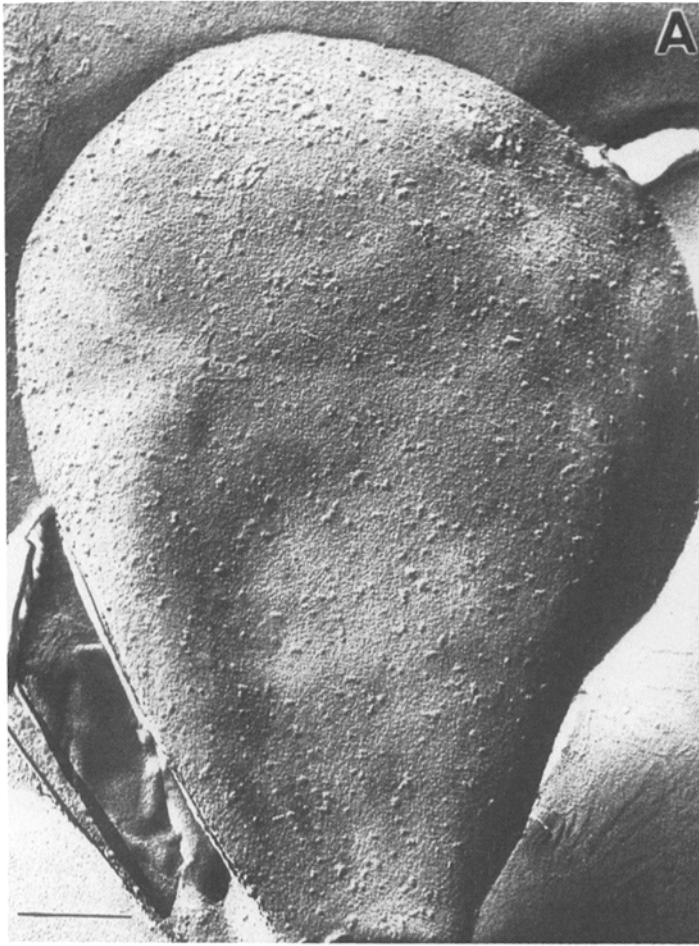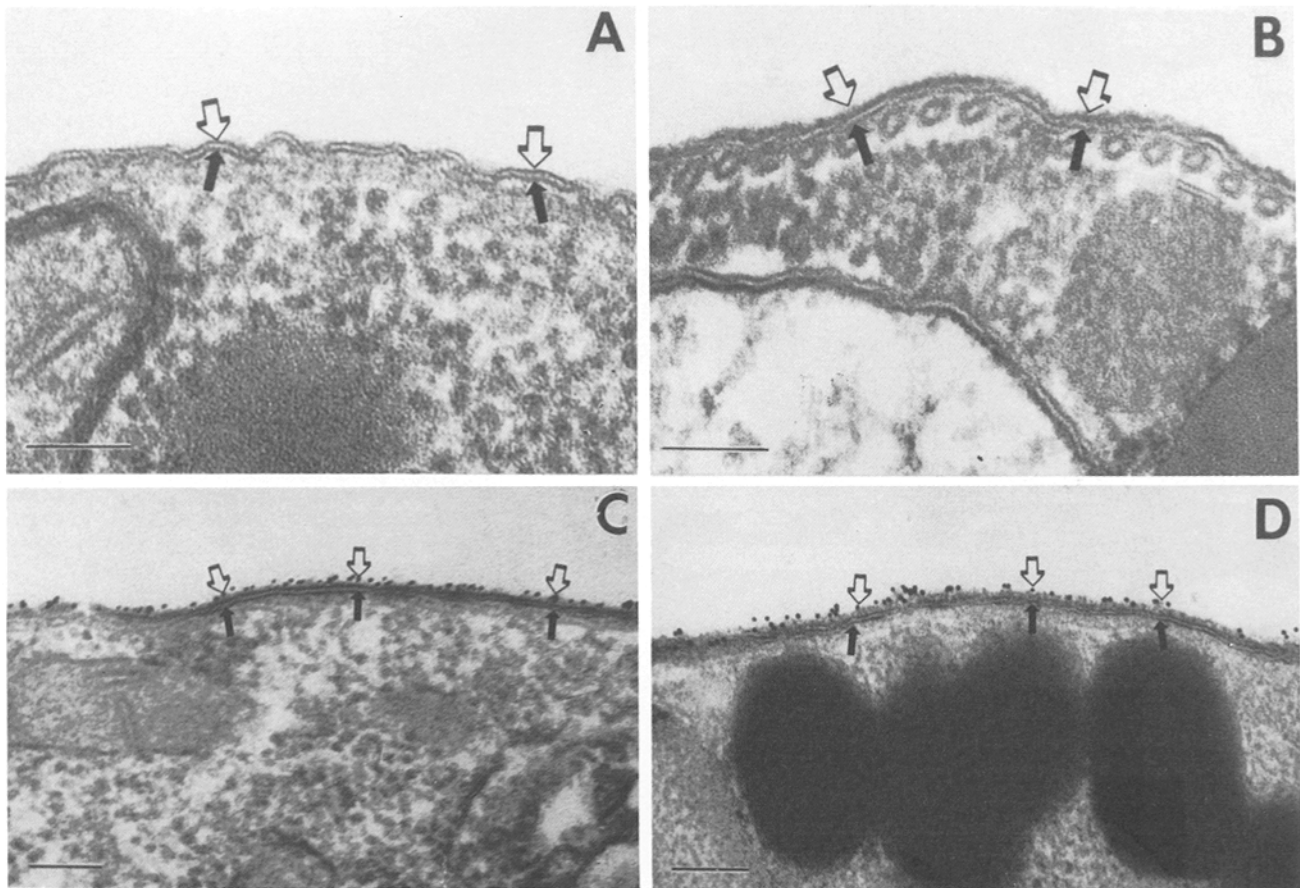
Figure 8. Thin section views of the cell coat (*arrows*) of *L. donovani* procyclic (*A* and *C*) and metacyclic (*B* and *D*) promastigotes, including immunogold-labeled promastigotes after incubation in mAb CA7AE. Bar, 0.1 μm.

previously (21) and are reproduced here for the purpose of comparison with the structures on *L. donovani*. Whereas the procyclic surface features of *L. donovani* and *L. major* procyclics (Fig. 7, *C* and *E*) appear quite similar, the changes in surface ultrastructure accompanying metacyclogenesis of the two species are strikingly different. The cell surface of *L. major* metacyclics is covered by a meshwork of fusiform filaments (Fig. 7 *F*), quite distinct from the particulate structures which appear on *L. donovani*. In each case the novel structures could be labeled with mAbs against the LPG, although their images were considerably distorted by the colloidal gold particles (data not shown).

Examination of routine thin sections of procyclic *L. donovani* promastigotes reveals a sinuous cell surface covered by a thin cell coat about 4–6 nm in thickness (Fig. 8 *A*). The metacyclic surface reveals a prominent electron-dense cell coat about 10–12 nm thick which covers the entire cell, including the flagellum and the internal surface of the flagellar pocket (Fig. 8 *B*). Immunogold surface labeling of LPG using the mAb CA7AE, which recognizes the disaccharide-phosphate repeats,

shows comparable levels of expression of LPG on the two developmental stages (Fig. 8, *C* and *D*), and confirms that the unique structures visible on the metacyclic surface reflect an altered form or presentation of LPG structure rather than an increase in the number of molecules expressed.

Involvement of LPG in the Stage-specific Binding of L. donovani Promastigotes to the Sand Fly Midgut. In a previous report (4), LPG differing in expression of terminally exposed sugars was shown to control the stage-specific attachment of *L. major* promastigotes to midgut epithelial cells of their natural vector, *Phlebotomus papatasi*. We wanted to investigate first of all whether the attachment of *L. donovani* promastigotes to the midgut of an appropriate vector also displays stage specificity, and second whether such differences in binding can be similarly explained by the developmental modifications of LPG just described. *P. argentipes* is the natural vector of *L. donovani* in India, and it is also a permissive sand fly for the complete development of the *L. donovani* 1S strain from Sudan, as demonstrated by studies in laboratory-reared flies (23). In in vitro binding assays using dissected *P. argentipes* midguts, procy-

Figure 7. Surface views of promastigotes after fracture-flip. *L. donovani* procyclic (*A* and *C*); *L. donovani* metacyclic (*B* and *C*)l; *L. major* procyclic (*E*); *L. major* metacyclic (*F*). Bars, 0.5 μm.
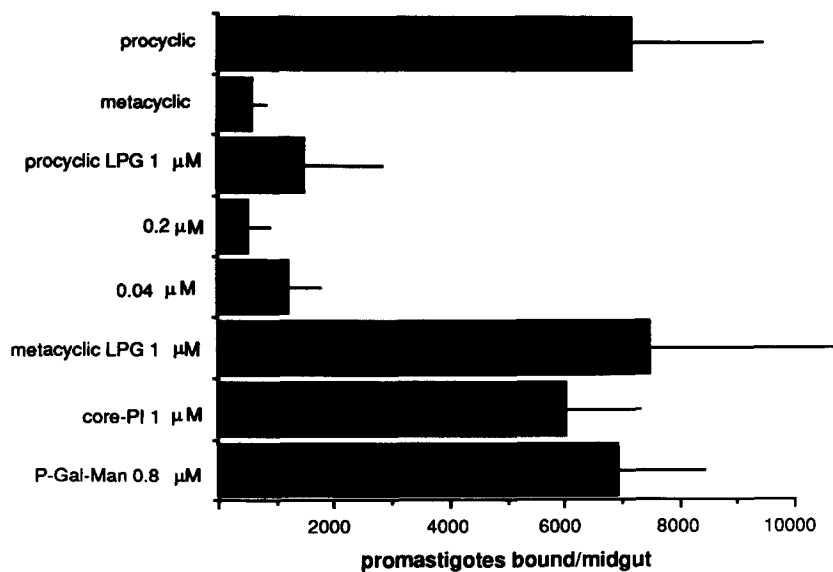
Figure 9. Attachment of *L. donovani* promastigotes to *P. argentipes* midguts in vitro. Procyclic promastigotes were incubated alone or in the presence of various LPG-derived oligosaccharides as inhibitors. Metacyclic promastigotes were only incubated alone. The data represent the mean number of promastigotes bound per midgut, ± 1 SD, 7–10 guts per group.
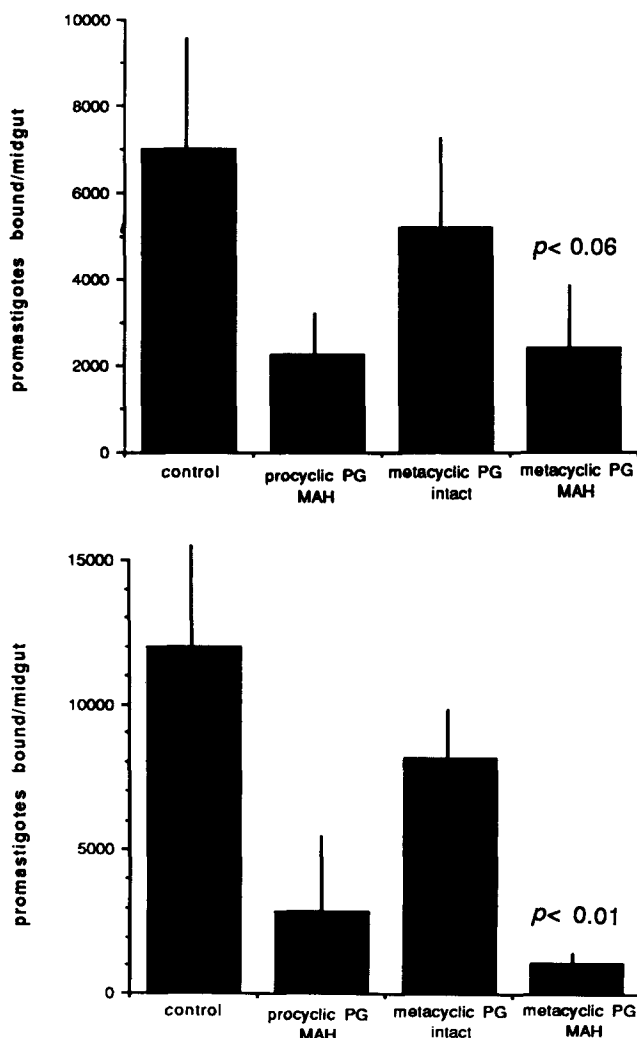
clic promastigotes of the 1S strain bound well (7,400/gut, Fig. 9). In contrast, the binding of metacyclic promastigotes was negligible (<600/gut). Procyclic attachment was efficiently inhibited (85%) by as little as 0.04 μM of procyclic LPG, but was not inhibited at all by up to 1 μM metacyclic LPG. Whereas it was not possible to obtain sufficient amounts of the various neutral capping sugars from procyclic LPG to use them directly to inhibit procyclic binding, no inhibition was observed with either core-PI (1 μM), or the PO_4-6Galβ1-4Man repeats (0.8 μM), implying that the capping domains were involved. The failure of the capping sugars on metacyclic LPG to either mediate the binding of metacyclic cells or to inhibit the binding of procyclic cells, was again attributed to a conformational change which in this case made the terminal sugars inaccessible to the LPG recognition sites lining the vector midgut. Intact metacyclic LPG was therefore compared with equimolar concentrations of metacyclic LPG which had been depolymerized by mild acid, which releases the phosphorylated disaccharide repeat units as well as the neutral capping oligosaccharides. In this experiment, depolymerization of procyclic LPG did not significantly affect its ability to inhibit procyclic binding, whereas treatment of metacyclic LPG with mild acid converted it from an inactive molecule into one capable of inhibition which was at least as strong as the procyclic material (92%) at the higher concentration tested (2 μM) (Fig. 10).

Discussion

Despite the fact that *L. donovani* produces the most severe form of leishmanial disease in humans, virtually nothing is known about the molecules controlling the biology of this

Figure 10. Inhibition of procyclic attachment of *P. argentipes* midguts using 0.5 μM (*top*) or 2.0 μM (*bottom*) of intact or mild acid hydrolysis

treated (*MAH*) LPG fragments. The data represent the mean number of promastigotes bound per midgut, ± 1 SD, seven to eight guts per group. *p* values were calculated from the comparison of intact and depolymerized metacyclic LPG.

parasite in its sand fly vector. In these studies, the abundant surface LPG of *L. donovani* promastigotes was shown to undergo major structural modifications during metacyclogenesis which function to promote the transmissibility of the parasite by the fly and the infectivity of the parasite in the vertebrate host. The size of the molecule is dramatically increased because of a twofold increase in the number of phosphorylated disaccharide repeat units expressed. In addition, the display of terminally exposed neutral capping sugars is affected, resulting in the loss of lectin binding domains, including those involved in the binding of the parasite to the vector midgut.

These developmental modifications of *L. donovani* LPG are similar to those recently described for *L. major*, particularly with respect to the increase in the number of repeat units expressed. The elongation of the phosphoglycan chain results in an approximate twofold increase in the thickness of the glycocalyx on *L. donovani* metacyclics which could be labeled using mAbs specific for the LPG. Similar surface changes in *L. major* were shown to protect metacyclics from complement-mediated damage by hindering access of the membrane attack complex to the cell membrane, thereby preventing channel formation and lysis (14, 24). We believe that LPG chain elongation serves a similar function on *L. donovani* metacyclics, which like *L. major*, activate complement efficiently, yet are far more resistant to complement-mediated lysis than procyclic forms (15, 16). There is substantial data to suggest that complement activation and C3 deposition on the promastigote surface is important in targeting the parasite to macrophages and in promoting their subsequent intracellular survival (25–27). The present data support a common molecular mechanism for accomplishing compliment activation on the metacyclic surface in a nonlethal manner.

In these studies we have directly addressed an additional aspect of LPG developmental modification which appears to function not in the vertebrate host but in the sand fly vector. We have previously reported that for *L. major–P. papatasi* interactions, the stage-specific adhesion of procyclic promastigotes to midgut epithelial cells is mediated by terminal β-linked galactose residues, the downregulation of which during metacyclogenesis was shown to control the selective release of infective-stage parasites for anterior migration and subsequent transmission by bite (4). The loss of exposed terminal sugars on *L. donovani* metacyclic LPG was clearly demonstrated in the current studies by the reduction of both PNA and Con A binding to metacyclic cells and to metacyclic LPG-coated hydrophobic beads. The availability of terminal sugars as enzyme substrates was also affected insofar as galactose oxidase followed by reduction with $NaB[^3H_4]$ failed to introduce a label into the galactose containing capping sugars. The relevance of these changes to the vector biology was confirmed in binding studies involving midguts of *P. argentipes*, which is a permissive vector for the *L. donovani* 1S strain, and the proven vector of *L. donovani* transmission in India. Proyclic promastigotes bound efficiently, whereas metacyclic promastigotes did not. Procyclic binding was strongly inhibited by nanomolar concentrations of procyclic LPG, but not by metacyclic LPG, and required the presence of the capping sugars,

insofar as the disaccharide-phosphate repeats did not inhibit, nor did the core-lipid anchor domain.

Because different species of *Leishmania* express structural polymorphisms in procyclic LPGs, different types of developmental changes might be required in order to downregulate the midgut binding properties of the parasite. Since *L. donovani* LPG does not express side chain substitutions off the disaccharide-phosphate backbone repeats, the only possibility for modification of terminal sugars which might affect the binding

□ mannose
○ galactose
● arabinose

terminal sugars   phosphoglycan chain   glycolipid anchor
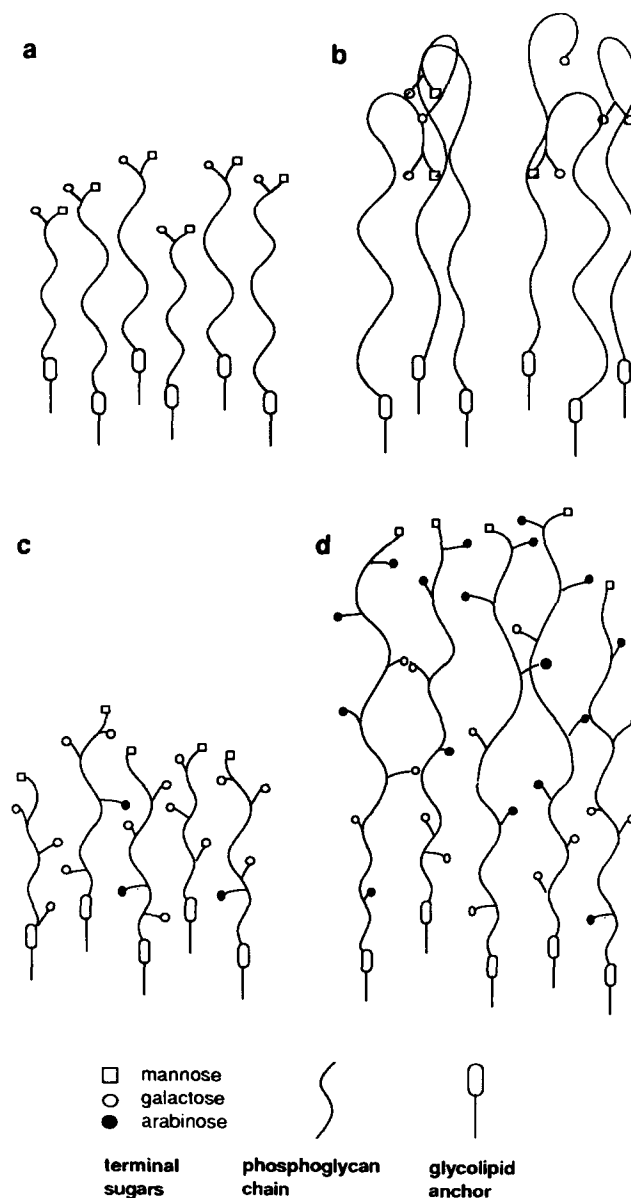
Figure 11. Schematic representation of possible changes in surface LPG during metacytogenesis of *L. donovani* (*a* and *b*) and *L. major* (*c* and *d*) promastigotes. For *L. donovani*, there is a twofold increase in the size of the LPG chains and a decrease in the accessibility of the terminal capping sugars to lectins, as well as the parasite recognition sites on the vector midgut. Evidence is presented that the loss of these ligands is due to clustering and folding of the longer metacyclic LPG. For *L. major*, the elongated PG chain remains unfolded and highly branched, and the side chains with terminally exposed β-linked galactose residues which mediate the binding of procyclics to the vector midgut are capped with arabinose.

properties of the molecule lies within the neutral capping domain. Complete analysis of the capping oligosaccharides from the respective LPGs revealed only minor differences in the relative abundance of these caps and showed that all LPGs contained both β-Gal– and α-Man–terminating structures. Therefore, the loss of the lectin- and midgut-binding properties of the molecule suggests that the cap oligosaccharides become cryptic as a consequence of the elongation of the phosphoglycan chain. Since depolymerization of metacyclic LPG released oligosaccharides that were fully capable of inhibiting procyclic attachment, this confirms that metacyclics continue to synthesize midgut binding ligands which require masking in order to alter the binding properties of the cell.

The fracture flip images suggest that the elongated LPG molecules of L. donovani assume a rounded, particulate appearance, quite distinct from the filamentous structures found associated with LPG elongation on L. major metacyclics. It is tempting to speculate that these particulate structures are formed by folding and clustering of the extended glycan chains. Despite the fact that the LPG on L. major metacyclics is also increased in size, it might be prevented from folding in a similar manner because it remains so highly branched. L. major LPG might therefore assume a filamentous, rather than particulate appearance. The absence of folding is supported by the observation that the Manα1-2Man capping oligosaccharides remained exposed on metacyclic forms, to the extent that the Con A binding profile did not change.

We have recently reported that the relatively complex structure of L. major LPG is driven at least in part by differences in the parasite recognition sites expressed by its vector in na-

ture (23). P. papatasi midguts possess a relatively low affinity receptor for terminal galactose residues. Its vectorial competence for only L. major sp. is attributed to the unique, highly substituted nature of L. major procyclic LPG that provides for multiple side chains with terminally exposed β-linked galactose residues, thereby increasing its affinity. P. argentipes, in contrast, is a competent vector for many leishmanial species, including those with unsubstituted LPGs (e.g., L. donovani), by virtue of their midguts having receptors for both terminal β-galactose– and α-mannose–containing oligosaccharides (Pimenta, P.F.P., unpublished observations) which in variable proportion, comprise the neutral capping domains of all LPGs studied to date (6). The development of L. donovani infections in P. argentipes might therefore require the loss of all terminally exposed sugars expressed in each of the different capping oligosaccharides, and this might be more easily achieved by masking these structures rather than by the addition or deletion of specific terminal sugars. The proposed mechanisms by which L. donovani and L. major LPGs downregulate terminal sugars involved in binding to their respective vector midguts is depicted in Fig. 11.

In summary, despite their considerable differences in structure, the LPGs of L. major and L. donovani promastigotes each undergoes modifications in size and expression of terminally exposed sugars that reflect common molecular strategies for survival of infective-stage parasites in the vertebrate and invertebrate hosts. It will be of interest to determine the nature of the developmental modifications expressed by other leishmanial species for which species-specific polymorphisms in LPG structure have also been described.

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