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Inhibition of *Leishmania donovani* Promastigote Internalization into Murine Macrophages by Chemically Defined Parasite Glycoconjugate Ligands

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***Leishmania donovani*, the agent of human visceral leishmaniasis, is an intracellular parasite that must be recognized and internalized by host macrophages to complete its biological cycle. In a search for possible ligands for macrophage surface receptors, glycoconjugates were obtained from *Leishmania* promastigotes by aqueous, phenol-aqueous, and alkaline extraction. A fucose-mannose glycoproteic ligand, a lipopeptidephosphoglycan, and a phosphate mannogalactan ligand were purified from promastigotes and analyzed for their chemical contents, with special attention to their glycidic moieties. Sugars that were identified as components of these glycoconjugates were tested for their capacity to inhibit promastigote internalization by BALB/c peritoneal macrophages in vitro. Neutral hexoses showed little inhibitory activity; fucose, charged monosaccharides, and a mannose polymer showed the highest activity. Two of the glycoconjugates (fucose-mannose glycoproteic ligand and phosphate mannogalactan ligand) purified from promastigotes were potent inhibitors of internalization, 75% inhibition being obtained at concentrations of 6 to 10 μg/ml. The simultaneous presence of both ligands in low concentrations yielded an increase in inhibitory activity above that found for each ligand alone, indicating that promastigotes may use at least two receptor sites for penetration into macrophages. These ligands are specific inhibitors of *L. donovani* promastigote phagocytosis, since 10 μg of each ligand per ml interfered neither with internalization of yeast cells nor with phagocytosis of *Leishmania adleri* promastigotes.**

Leishmania donovani, the etiological agent of human kala-azar (human visceral leishmaniasis), is an obligatory intracellular parasite. *Leishmania* promastigotes are introduced into the host blood circulation by the insect vector. They need to be recognized and internalized by host macrophages to complete their biological cycle. Once they reach the intraphagolysosomal environment, they become amastigotes and multiply until they disrupt the macrophage and propagate the infection to other cells. Recognition and penetration into macrophages is therefore a very critical point in the *Leishmania* cell cycle. It involves several complex phenomena that are still incompletely understood.

In recent research, evidence has been gathered which indicates that both recognition and penetration of leishmanias into macrophages are mediated by carbohydrates. Dwyer et al. (13) suggested that *L. donovani* glycidic coat participates in adhesion of parasites to cells and to other substrates. Chang (9) indicated that mannose and glucosamine are the principal sugars involved in adhesion of promastigotes to hamster macrophages. Wilson and Pearson (41) have shown that mannose-fucose- and mannose-phosphate-containing polymers might be the ligands on the parasite surface that are recognized by receptors responsible for parasite internalization by human macrophages.

These observations are consistent with studies of macrophage receptors that interact with *Leishmania* species and that have been shown to have a lectinlike activity. A mannose-fucosyl receptor, associated with the C3b complement receptor complex, is responsible in mouse macrophages for the general mechanism of internalization of parasites (7, 42) and for the associated respiratory burst (10).

Most of the evidence indicating the participation of sugars in interaction of *Leishmania* species with macrophage receptors and in control of their internalization has been obtained from indirect studies on inhibition of parasite penetration into macrophages in vitro by purified monosaccharides or neoglycoproteins. Knowledge of the composition and structure of the *L. donovani* glycidic ligand is still rudimentary. Recent observations of other *Leishmania* species have demonstrated the relevance of glycidic moieties of surface glycoconjugates in parasite interaction with host cells. An integral membrane glycoprotein containing glucosamine, mannose, and galactosamine was characterized in *Leishmania mexicana* (36), and a lipopolysaccharide containing galactose was identified in *Leishmania major* (19).

In the present work, we report our findings on the chemical and biological characterization of three different carbohydrate-containing fractions obtained from *L. donovani* (LD1S-Sudan): a glycoproteic fraction containing fucose and mannose that was isolated from the aqueous extract, an acidic phosphate-mannogalactan glycoconjugate that was released by alkaline extraction, and a lipopeptidephosphoglycan (LPPD) from the phenolic extract. Monosaccharides identified in these fractions were tested in vitro for their capacity to inhibit parasite penetration into macrophages. Subsequently, the fractions purified from *Leishmania* promastigotes were used in the same in vitro test. Two of them (fucose-mannose glycoproteic ligand [FML] and phosphate mannogalactan ligand [PMGL]) were shown to behave as separate specific ligands for parasite internalization by mouse peritoneal macrophages.

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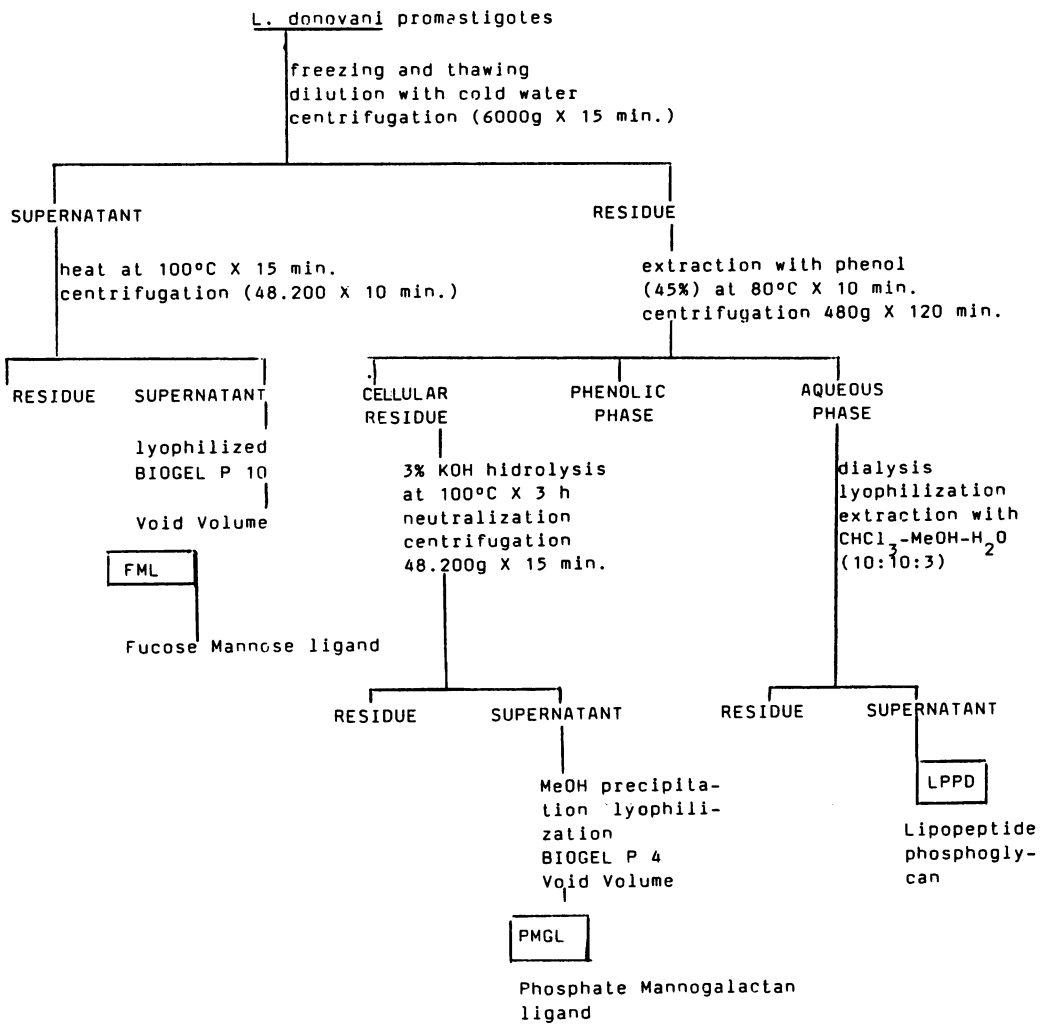

FIG. 1. Fractionation flow diagram for *L. donovani*.

MATERIALS AND METHODS

Parasites and growth conditions. *L. donovani* (LD1S) was kindly supplied by D. M. Dwyer (National Institutes of Health, Bethesda, Md.). Promastigotes were grown at 26 to 28°C in 1-liter flasks containing 37 g of brain heart infusion (Biobras, Montes Claros, Minas Gerais, Brazil) per liter, 10 mg of hemin per liter, 20 mg of folic acid (both from Sigma Chemical Co., St. Louis, Mo.) per liter, and 10% (vol/vol) fetal bovine serum (Microbiologica, Rio de Janeiro, Brazil). After 5 to 7 days of incubation under moderate agitation, cells were harvested by centrifugation (6,000 × *g*, 10 min), washed three times with 0.9% (wt/vol) NaCl, and stored at −20°C until being used for extraction.

Leishmania adleri (LRC L124, World Health Organization collection), a species closely related to *L. donovani* which is infective for mammals, was also grown in the above-described conditions.

Extraction and purification of *L. donovani* glycoconjugates. Promastigotes were subjected to aqueous extraction by the method of Mendonça-Previato et al. (28) (Fig. 1). Briefly, frozen cells were rapidly thawed and extracted twice with water at 4°C. The extract was cleared by centrifugation, and supernatants were pooled and heated to 100°C. The precipitated material was separated by centrifugation and discarded. The resulting soluble material was fractionated by gel filtration on a 100/200-mesh column (120 by 2 cm) containing Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.). The remaining insoluble material was extracted with 45% (wt/vol) phenol-water at 80°C (30) and centrifuged (48,000 × *g*, 120 min at 4°C). The supernatant phenolic extract aqueous layer (PEAL) was dialyzed, lyophilized, and analyzed. A chloroform-methanol-water (10:10:3, vol/vol/vol) extraction (24) of the PEAL of *L. donovani* was subsequently done, releasing a soluble LPPD and insoluble residue. The cellular debris remaining after phenolic extraction was suspended in 3% (wt/vol) aqueous KOH and extracted for 3 h at 100°C. The suspension was neutralized and centrifuged (48,000 × *g*, 20 min), and the supernatant was added to 3 volumes of methanol (32). The precipitate was isolated, analyzed, dissolved in distilled water, and purified by gel filtration chromatography on a column of Bio-Gel P-4 (Bio-Rad) (400 mesh; 99 by 1.25 cm). In all gel filtration column chromatography experiments the carbohydrate content was monitored by the phenol sulfuric acid assay (11).

Composition and size determination of LPPD oligosaccharide. The glycidic moiety of LPPD was analyzed after drastic alkaline hydrolysis in 1 M NaOH and 1 M $NaBH_4$ for 4 h at 100°C (25). The cooled mixture was neutralized with acetic

acid and centrifuged (4,800 × *g*, 10 min). The supernatant containing the carbohydrate moiety was fractionated by gel filtration chromatography on a Bio-Gel P-4 column. The carbohydrate-containing fractions were pooled, lyophilized, and assayed for their total neutral sugar and phosphate composition by chemical assays described below and by gas-liquid chromatography of their alditol acetates. Oligosaccharide size estimation was performed as described by Behrens and Tavora (4), with descending paper chromatography in a butanol-pyridine-water (4:3:4, vol/vol/vol) system. After 24 h, the chromatograms were dried and developed in alkaline silver nitrate. Mannose, cellobiose, inositol, and stachyose (all from Sigma) solutions were used as standards.

PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed under reducing conditions (23) with 10% or 6% polyacrylamide slab gels, 6 to 7 cm long. Periodic acid-Schiff reagent (PAS) (15) and Coomassie brilliant blue R and Sudan black B (both from Sigma) stains (35) were used for identification of carbohydrate, protein, and lipid moieties, respectively. LD1S alkaline extracts were analyzed under nondenaturing conditions (without sodium dodecyl sulfate [SDS]) on 6% polyacrylamide slab gels.

High-voltage paper electrophoresis. High-voltage paper electrophoresis was carried out in 0.3 M acetic acid–pyridine buffer at pH 4.8 and 450 V for 4 h. Chromatograms were developed with standard alkaline silver nitrate reagent (38) for sugars and with molybdate reagent (8) for phosphate.

Amino acid analysis. The PMGL and FML were hydrolyzed in 5.6 N HCl at 110°C, and the amino acids were analyzed in an autoanalyzer (type 119 CL; Beckman Instruments, Fullerton, Calif.) by the method of Fauconnet and Rochemont (16).

Analytical methods. Total carbohydrate content was determined as described by Dubois et al. (11). Proteins were quantified by the method of Lowry et al. (27), phosphates were quantified by the method of Ames (2), and amino sugars were quantified by the method of Belcher et al. (5).

Polarimetric analysis. Optical rotation of optically active glycoconjugates was measured in 10-mg/ml (wt/vol) aqueous solutions by using a model 243 polarimeter (The Perkin-Elmer Corp., Norwalk, Conn.).

Determination of monosaccharide components. Neutral monosaccharides were identified and quantified by hydrolysis of samples in 3 M trifluoroacetic acid for 3 h at 100°C, followed by reduction with alkaline $NaBH_4$ and acetylation and gas-liquid chromatography of the remaining alditol acetates (1, 37).

Mouse peritoneal macrophages. Three-month-old BALB/c mice, bred at the Federal University of Rio de Janeiro, were used to obtain peritoneal macrophages. They were sacrificed by ether overdose. Three milliliters of McCoy 5A medium (Sigma), supplemented with 10% (vol/vol) fetal bovine serum (Cultilab, Campinas, São Paulo, Brazil), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (pH 7.4), was injected into the peritoneal cavity. After the abdomen was massaged gently, the peritoneal fluid was aspirated under sterile conditions, and cells were counted and stored on ice. Circular glass cover slips were cleaned with sulfochromic acid; extensively washed in tap water, distilled water, and double-distilled water; sterilized; and placed into 4-well tissue culture plates (Nunclon; Nunc, Roskilde, Denmark). Cell suspensions were plated onto the cover slips and incubated at 37°C for 2 h, under a 5% CO_2 atmosphere. Nonadherent cells were removed by rinsing plates with the supplemented McCoy 5A medium described above. Approximately 10^6 macrophages were obtained in each well. Adherent peritoneal cells were incubated for an additional 16 h under the same conditions and used for phagocytosis tests.

Phagocytosis assays. Plates with peritoneal macrophages were washed with fresh McCoy 5A medium. Living LD1S promastigotes were obtained from 7-day cultures in the above-described medium and placed in 5-ml screw-cap tubes. They were added to macrophages in appropriate concentrations (2, 10, 25, and 50 parasites per macrophage), incubated for periods ranging from 5 to 120 min, and washed three times in McCoy 5A medium. Cover slips were fixed in methanol for 5 min and stained by standard Giemsa stains (E. Merck AG, Darmstadt, Federal Republic of Germany). Cover slips were dried, mounted, and analyzed microscopically. The phagocytic index was determined by counting the percentage of all macrophages in 10 microscopic fields under ×1,000 magnification (a total of approximately 200 cells) that had phagocytosed at least one *Leishmania* parasite and multiplying this count by the average number of *Leishmania* parasites per macrophage observed in 100 macrophages (6). The adhesion index (6) was determined in a similar way, except that experiments were carried out at 4°C.

Inhibition of *Leishmania* internalization by monosaccharides, polysaccharides, and *Leishmania* promastigote glycoconjugates. The following carbohydrates were tested in final concentrations of 10 and 100 mM: D-(+)-xylose, D-(+)-mannose, D-(+)-galactose, D-(+)-glucose, *N*-acetylglucosamine, D-glucose-6-phosphate, D-mannose-6-phosphate, *N*-acetylneuraminic acid, and D-(+)-glucosamine hydrochloride (all from Sigma) and L-fucose (Nutritional Biochemical Corporation, Cleveland, Ohio). In addition, an α-D-mannopyranan obtained from *Saccharomyces cerevisiae* as previously described (17) was used in final concentrations of 2.5 and 0.25 mg/ml. The carbohydrate-containing *Leishmania* extracts were used in the same test, in final concentrations ranging from 2 to 1,000 μg/ml.

Macrophages, prepared on glass cover slips as described previously, were preincubated with carbohydrate or with carbohydrate-containing *Leishmania* extracts for 60 min. They were washed and incubated in 1 ml of the above-described medium containing the test molecules and 10^7 LD1S promastigotes for 45 min. Cover slips were then extensively rinsed with fresh medium, fixed, dried, and stained, and the phagocytic index was determined as described previously. Controls were preincubated, washed, and incubated under the same conditions but without the test molecules.

S. cerevisiae cells (strain S288 C), used as controls for phagocytosis assays, were obtained from A. Panek. They had been grown in YED medium containing yeast extract (Sigma) and D-glucose (pH 5.2) at 28 to 29°C under agitation. Yeast cells were harvested in the stationary phase of growth, washed three times with McCoy 5A medium, and used for incubation with macrophages.

RESULTS

***L. donovani* promastigote glycoconjugates.** Three major carbohydrate-containing fractions were obtained from *Leishmania* promastigotes: (i) aqueous extract and FML; (ii) phenol-aqueous extract and LPPD; and (iii) alkaline extract and PMGL.

(i) Aqueous extract and FML. The chemical analysis of the purified aqueous extract of *L. donovani* is shown in Table 1. The principal component of the aqueous extract was a complex glycoproteic fraction that was excluded in the void

TABLE 1. Chemical analysis of *L. donovani* glycoconjugates

Extract	% (dry wt)[a]				Distribution (%) of neutral sugar fraction[b]							$[\alpha]_D^{20}$ of extract[c]
	Neutral sugar	Protein	Phosphate	Hexosamine	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose	Inositol	
FML	29	44	11	tr	10		1	47	12	30		−27°
PMGL	19	10	11	2				45	55			+41°
Phenol-aqueous extract	21	40	6	ND[d]	tr	6	tr	52	21	20	1	ND
LPPD	10	19	2	ND	4			37	34	22	3	ND
LPPD glycidic fraction	ND	ND	ND	ND				30	38	27	5	ND

[a] Percentages were determined as described in Materials and Methods.
[b] Distributions were determined by integration of gas-liquid chromatography curves.
[c] Optical rotations were determined for 10 mg (dry weight) of extract per ml in aqueous solution.
[d] ND, Not done.

volume of the Bio-Gel P-10 column (Fig. 2A). Since it contained 10% fucose and 47% mannose (Table 1) and behaved as a potent ligand, we have designated it the FML. The SDS-PAGE analysis of this fraction disclosed major proteic components with approximate molecular sizes corresponding to 9, 28, 39, 43 to 45, 58 to 64, 68, and 92 to 95 kilodaltons (Fig. 3, lane A). The 39- and 58- to 68-kDa fractions showed a positive staining reaction for carbohydrates (Fig. 3, lane B). The polarimetric analysis of FML showed negative values ($[\alpha]_D^{20} = -27°$). The FML amino acid analysis disclosed the presence of asparagine (10%), glutamic acid (16%), alanine (12%), and glycine (10%) as major components.

(ii) Phenol-aqueous extract and LPPD. A hot phenol-water extract of *Leishmania* promastigotes released in the aqueous phase a fraction containing 40% protein and 21% carbohydrate (Table 1). This fraction gave PAS-positive bands on SDS-PAGE at 10 to 25 and 58 to 64 kilodaltons (Fig. 3, lane C). The chloroform-methanol-water-soluble complex of the phenol-water extract migrated near the front in SDS-PAGE. It contained 2% phosphate and 19% protein and showed a positive staining reaction for carbohydrates (Fig. 3, lane D) and lipids (Fig. 3, lane E). It was consequently designated LPPD. It contained 10% neutral carbohydrates, among which mannose and galactose were predominant. The glycidic moiety of LPPD was isolated through a hot alkaline hydrolysis (Table 1). Gel filtration column chromatography on Bio-Gel P-4 (Fig. 2B), descending paper chromatography, gas-liquid chromatography, and chemical analysis demonstrated that the glycidic fraction of LPPD contained oligosaccharides ranging from 3 to 10 sugar units (results not shown). A ribose-free oligosaccharidic fraction consisting mainly of 10 sugar units, containing mannose, galactose (1:1), and phosphate (7%), was recovered in the void volume of the Bio-Gel column (Fig. 2B). Three fractions were composed of shorter oligosaccharidic chains (Fig. 2B). Fraction 1 on Bio-Gel P-4 (F_1-P_4) (mostly 7 sugar units) was composed of mannose, galactose, and glucose (1:1:2) and contained 3% phosphate. F_2-P_4 (tri- to pentasaccharide units) and F_3-P_4 (mostly trisaccharides) contained mannose, galactose, and glucose in ratios of 1:1:2 and 1:1:1, respectively. In addition, a fourth fraction, F_4-P_4, composed of galactose was eluted in the monosaccharide region.

(iii) Alkaline extract and PMGL. The hot alkaline hydrolysis of LD1S cellular residue released a compound containing sugar and phosphate with an $[\alpha]_D^{20}$ of +16°. This phosphate-containing polysaccharidic fraction migrated at the front in 6 and 10% gels (Fig. 3, lane F). It revealed one acidic spot on paper electrophoresis (not shown). Upon purification of this fraction by gel filtration chromatography on Bio-Gel P-4, a peak was released in the void volume that contained carbohydrate (19%) and phosphate (11%) and was free of nucleic acids (Table 1; Fig. 2C). This purified fraction contained 45% mannose and 55% galactose (Table 1). It also migrated in 10 and 6% PAGE, with and without SDS (Fig. 3, lane G). In paper electrophoresis, it migrated as an acidic spot that, in our conditions, stained for phosphate but not for sugar. It did not migrate in descending paper chromatography as expected from its highly hydrophilic phosphate moiety, stained at the origin of migration (results not shown). The presence of the phosphate group associated with sugar interfered in the colorimetric assays. Taken together, these results identify it as an acidic phosphate glycoconjugate, to which we have assigned the term PMGL. Despite the extensive alkaline extraction, this fraction contained 10% protein. Amino acid analysis disclosed the presence of glutamic acid (28% of all amino acid residues), glycine (18%), aspartic acid (10%), alanine (8%), serine (6%), and the others in minor proportions. Ethanolamine was also detected in trace amounts.

***Leishmania* promastigote-macrophage interactions.** The interaction between *Leishmania* promastigotes and BALB/c mouse macrophages was studied in an in vitro phagocytosis assay. The effect of the promastigote/macrophage ratio on phagocytosis is shown in Fig. 4A. The ratio of 10 promastigotes per macrophage was used in all further experiments. Assays carried out at 4°C showed that the adhesion contributed only to 10% or less of the phagocytosis index (results not shown), indicating that the observed internalization of promastigotes was a physiological phenomenon representing a normal macrophage activity.

The effect of time on promastigote internalization is shown in Fig. 4B. The phagocytosis index was proportional to the incubation period up to 60 min, when the maximal number of promastigotes per macrophage had been internalized. After 60 min, 98% of macrophages had internalized promastigotes, and afterwards their phagocytic capacity seemed to be saturated. We have not observed any morphological evidence of rapid digestion of promastigotes during this period, and we have concluded that the observed plateau was not an equilibrium between the ingestion and digestion of parasites in macrophages. On the contrary, the morphology of internalized parasites corresponded to that of amastigotes, indicating that they were completing their intracellular life cycle normally (Fig. 5A).

Control experiments performed with promastigotes of *L. adleri*, a closely related *Leishmania* species, and *S. cerevisiae* showed a lower phagocytic index (see Fig. 9).

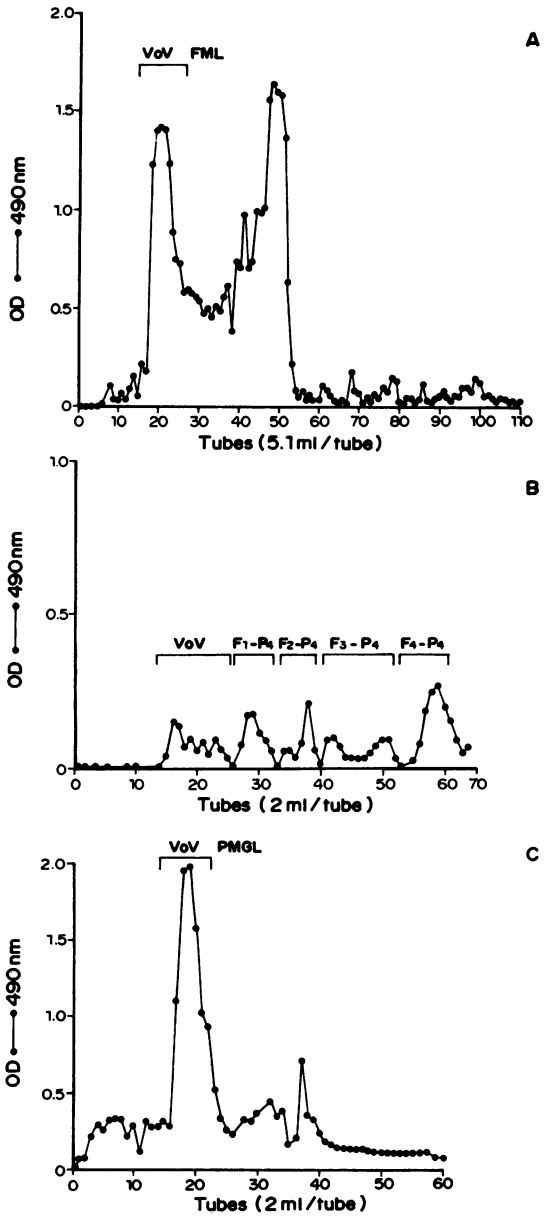

FIG. 2. Gel filtration column chromatography of *L. donovani* glycoconjugates. Carbohydrate content (12) is given for aqueous extract on Bio-Gel P-10 column (A), glycidic moiety of LPPD (B), and total alkaline extract on Bio-Gel P-4 column (C). VoV, Void volume; F_1-P_4 to F_4-P_4, LPPD glycidic fractions (see text and Table 1).

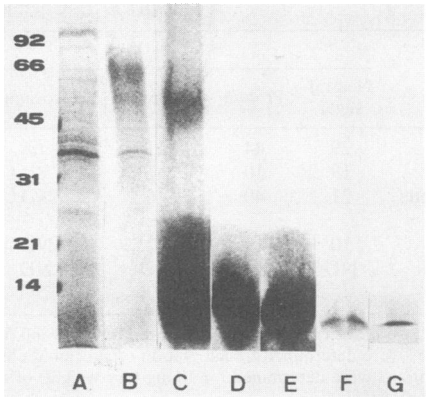

FIG. 3. PAGE of *L. donovani* glycoconjugates (150 to 200 μg). Lanes: A and B, fucose-mannose ligand; C, phenolic aqueous extract; D and E, LPPD; F, total alkaline extract on 10% PAGE with SDS; G, PMGL, run without SDS. Lanes B, C, D, F, and G were stained with PAS reagent, lane A was stained with Coomassie brilliant blue R, and lane E was stained with Sudan black B. Positions of low-molecular-size standards (Bio-Rad) in the SDS gels (lanes A to F) are shown in kilodaltons on the left.

To determine whether the penetration of promastigotes into BALB/c peritoneal macrophages was mediated by carbohydrates, we tested the inhibition of phagocytosis by using several monosaccharides, as well as an α-D-mannopyranan extracted from the yeast cell wall. Cells were preincubated with carbohydrates to saturate free receptors and coincubated with promastigotes and carbohydrates to maintain saturation of receptors with high turnover or with possible up-regulation during the promastigote-macrophage interaction.

At 100 mM, all monosaccharides except xylose inhibited internalization of promastigotes, with effects ranging from 45 to 95% (data not shown). The neutral hexoses were less effective than the charged ones (amino sugars and phosphate sugars) and fucose. At 10 mM (Fig. 6), glucosamine, neuraminic acid, and fucose—typical components of glycidic moieties of complex glycoproteins—as well as phosphorylated acidic sugars (mannose-6-phosphate and glucose-6-phosphate) were much more effective inhibitors than the neutral hexoses. The α-mannan was an especially potent inhibitor; at 0.25 mg/ml, it induced 64% inhibition, compared with the 15% inhibition obtained with 1.8 mg of D-(+)-mannose per ml (Fig. 6).

In the next experiments, we tested the hypothesis that the surface glycoconjugates of *Leishmania* promastigotes may include ligands for macrophage receptor recognition and subsequent internalization. The phagocytosis inhibition mediated by FML, PMGL, and LPPD is shown in Fig. 7. The LPPD showed only low inhibitory activity, the 75% decrease of phagocytosis index being observed only with the addition of 1,000 μg of LPPD per ml. Both FML and PMGL behaved as potent ligands and completely inhibited promastigote phagocytosis at a concentration of 1,000 μg/ml and decreased it considerably at 100 μg/ml. For both compounds, saturation of inhibition curves began near 6 μg/ml, reaching more than 60% inhibition of phagocytosis at this concentration. In all concentrations tested, macrophages showed no overt signs of abnormality in the presence of ligands (Fig. 5C and E). The additive effect of inhibition of phagocytosis by low concentrations of both ligands is shown in Fig. 8. While the addition of 3 μg of either ligand per ml induced approximately 40% inhibition, the simultaneous addition of both ligands increased the inhibition to 60%, attaining the saturation level expected for 6 μg of either ligand per ml.

In contrast to results obtained in phagocytosis of *L. donovani*, no inhibition was observed in phagocytosis of *L. adleri* promastigotes and *S. cerevisiae* cells in the presence of the glycoconjugates tested (Fig. 9).

DISCUSSION

In recent research, different complex glycoconjugates have been characterized in some *Leishmania* species: *L. adleri* (32), *L. major* (19), *L. mexicana* (36), *Leishmania tarentolae* (43), and *L. donovani* (39, 40). The relevance of

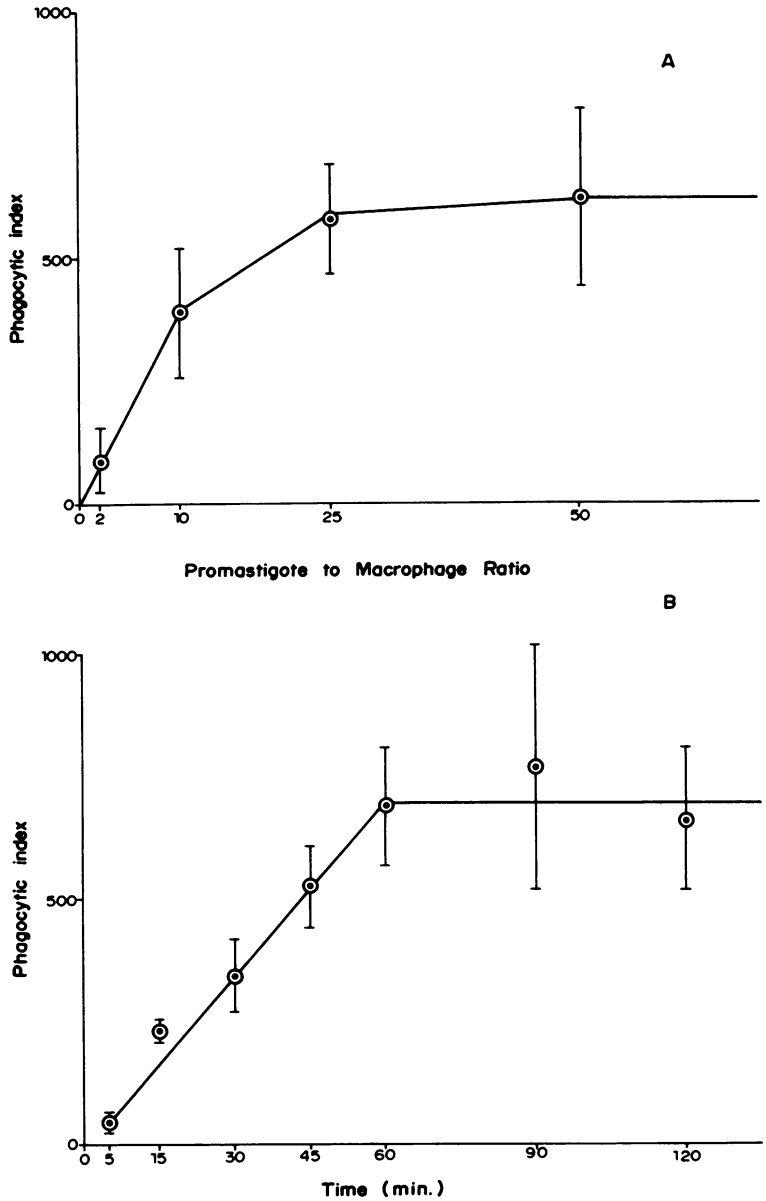

FIG. 4. Effect of promastigote/macrophage ratio (45-min incubation) (A) and effect of time (B) on phagocytosis at a ratio of 10:1. Phagocytic index was determined as described previously. Error bars show standard deviations for four experiments.

these molecules for internalization of parasites into macrophages has been demonstrated for the 63 glycoprotein purified from *L. mexicana* (36) and for a glycolipid of *L. major* (18). Indirect evidence suggests that mannose-phosphate (41) and mannose-fucose (7, 41) might be involved in macrophage internalization of *L. donovani* promastigotes.

In the present work, we have isolated three *L. donovani* glycoconjugates, two of which are ligands for the internalization receptors on BALB/c macrophages. We have analyzed their glycidic moiety and further shown the importance of charged carbohydrates, mannan, and fucose, in this phenomenon. These glycoconjugates appear to mediate specifically the internalization of *L. donovani* promastigotes, as they inhibit neither internalization of unrelated *S. cerevisiae* cells nor phagocytosis of the closely related *Leishmania* species *L. adleri*.

One of the ligands, FML, is a complex glycoproteic fraction composed of several bands with distinct molecular weights. This complexity is a characteristic of *L. donovani* cells and has been reported in other studies of glycoproteic extracts obtained either from pellicular membrane preparations (12) or from a glycoproteic residue obtained after organic extraction of *L. donovani* cells (22). However, chemical analysis in which nearly 100% of glycoprotein is recovered among sugar, proteic, phosphate, and hexosamine molecules indicated that although complex, FML is an homogeneous glycoproteic fraction.

The gp63 glycoprotein, considered to be one of the major ligands for parasite internalization (36), is not present in FML and PMGL. The aqueous extraction of FML includes heating to 100°C and removing the precipitated proteins by centrifugation. On the other hand, the presence of a membrane integral protein would be expected in phenolic extract, corresponding possibly to the upper band in PEAL (Fig. 3C).

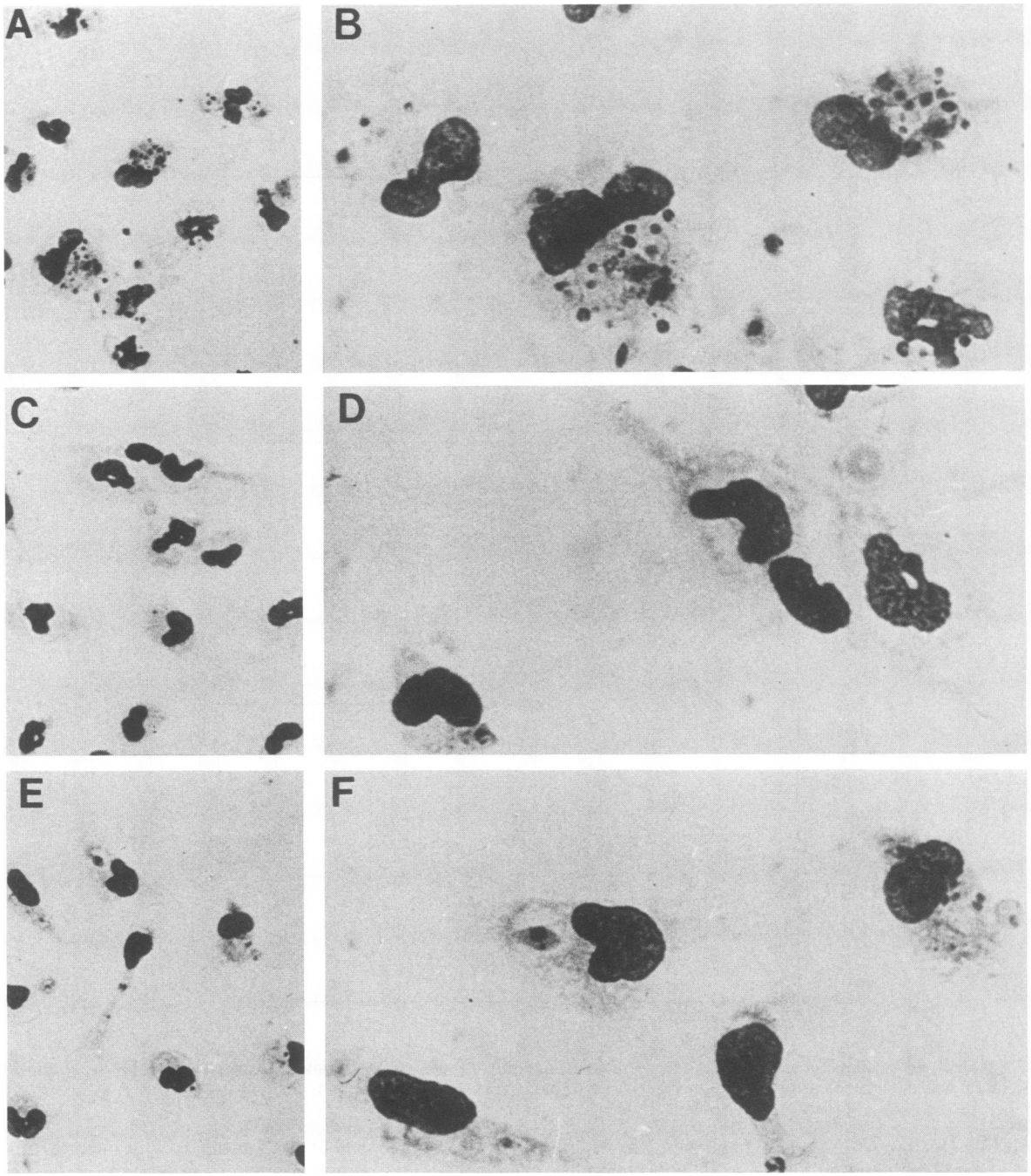

FIG. 5. Phagocytosis of promastigotes by macrophages (ratio, 10:1) is shown alone (A and B), in the presence of 1,000 μg of FML per ml (C and D), and in the presence of 10 μg of PMGL per ml (E and F). Magnifications, ×1,450 (A, C, and E) and ×3,200 (B, D, and F).

The chloroform-methanol-water extract isolated from PEAL contained only the LPPD without any proteic contaminants (Fig. 3, lanes D and E).

Both FML and PMGL are much more potent inhibitors than gp63. The inhibition of internalization attained only 40% in the presence of 1 to 2 mg of specific anti-gp63 antibody per ml, while our molecules induced similar levels of inhibition in concentrations 3 orders of magnitude lower. The protein described as gp63 or gp65 is one of the widespread proteins in *Leishmania* species (14, 26) being considered as a marker of this genus. On the other hand, FML and PMGL are species specific. They have a very low inhibitory activity on *L. adleri* internalization (Fig. 9). In recent research, a similar result was obtained when their inhibitory activity for nine other Old and New World *Leishmania* species (31) was compared.

Our results recognizing FML as one of the major mediators of promastigote internalization into macrophages are complementary to recent reports (42) indicating that the mannose-fucose receptor is one of the relevant mediators of *L. donovani* internalization.

In our tests for inhibition of promastigote internalization, mannose was less effective than mannan, fucose, and charged monosaccharides. It may be that, similar to other

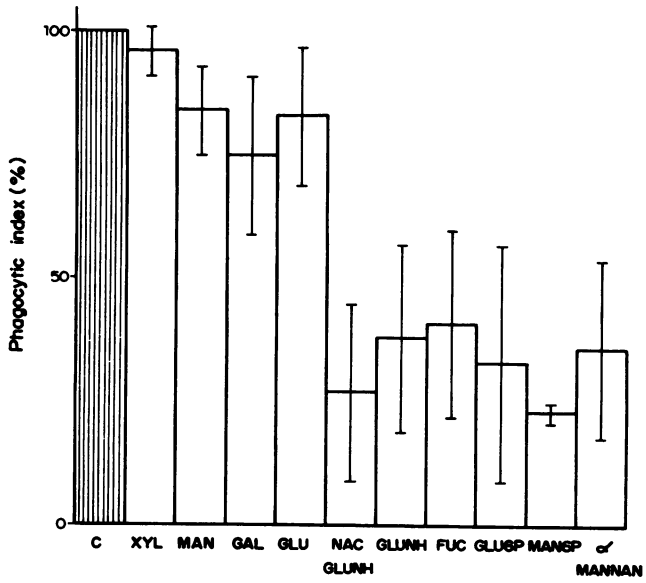

FIG. 6. Effect of incubation with 10 mM monosaccharides or 0.25 mg of yeast mannan per ml on the phagocytic index, compared with result of control assay (C) without addition of sugar. Error bars show standard deviations of four experiments.

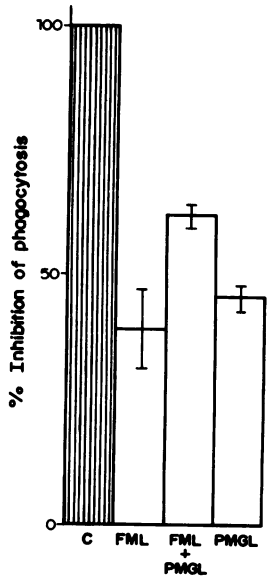

FIG. 8. Additive inhibitory effect of coincubation with 3 μg of PMGL and 3 μg of FML on promastigote phagocytosis by macrophages, compared with the effects of incubation of each compound alone and control assay (C) with no additions. Mean values and standard deviations of four experiments are shown.

complex glycoproteins, the α-mannan represents a core of the glycidic moiety of FML, while fucose and glucosamine, present in lower quantities, have exposed positions and consequently act as ligands during the promastigote interaction with macrophages, as confirmed by their high inhibitory activity in our in vitro tests. Size may be another factor in determining inhibitory effectiveness, since mannose was less potent on a size basis than the mannose polymer.

PMGL is a pure compound, as confirmed by paper electrophoresis, SDS-PAGE and chemical assays, since A_{260} and ribose are absent, thus eliminating the contamination with nucleic acids. It is a very electronegative glycoconjugate (11% phosphate), as seen in PAGE and paper electrophoresis.

The presence of 10% protein in PMGL, after extensive alkaline treatment that hydrolyzes all of the possible proteic contaminants, is surprising. The amino acid composition of the proteic moiety is constant among different extractions, indicating its close association with the glycidic moiety. It may be speculated that the glycidic moiety protects this proteic component. Whether this may occur also in vivo, where the protection of *Leishmania* structures in the very aggressive environment of secondary phagosomes is of vital importance for parasite survival, is an interesting objective for future studies.

Turco et al. (39, 40) have recently characterized an acidic lipid-containing glycoconjugate obtained from *L. donovani*, the glycidic moiety of which is a phospho-6-galactosyl-β-1→4-mannose in a repetitive sequence. This type of molecule has been isolated and characterized from several

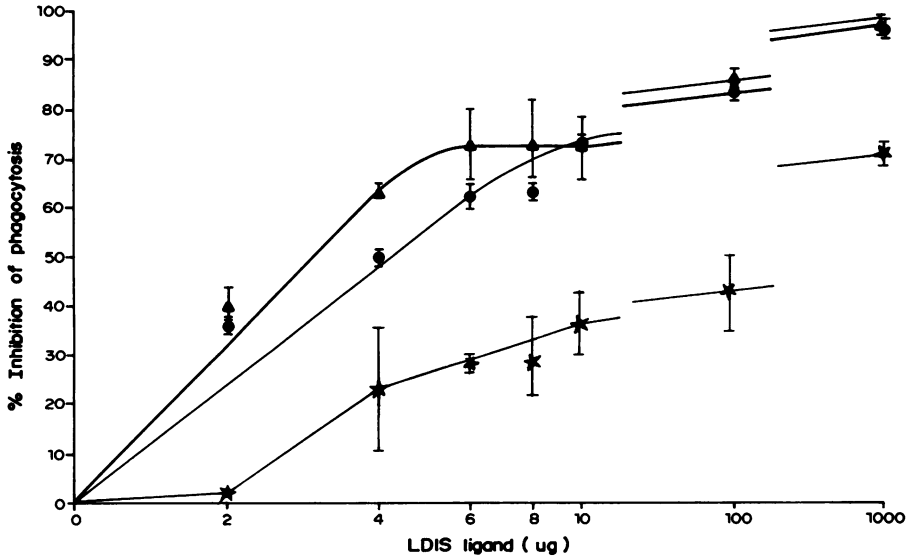

FIG. 7. Inhibition of phagocytosis by PMGL (▲), FML (●), and LPPD (★). Error bars show standard deviations of four experiments.

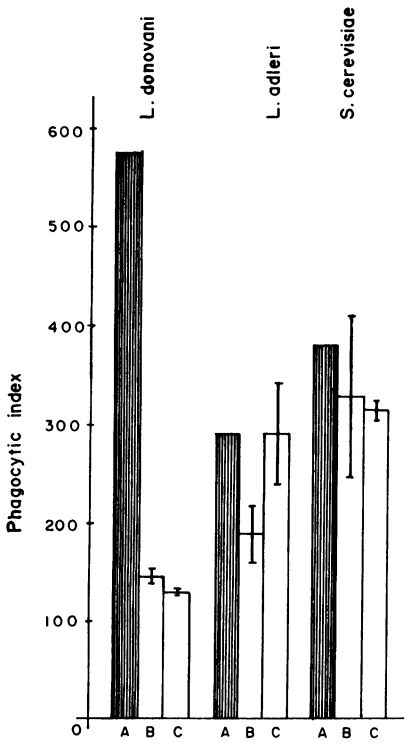

FIG. 9. Specificity of FML and PMGL inhibition of *L. donovani* promastigote phagocytosis, compared with that of *L. adleri* and *S. cerevisiae* cells. Bars: A, standard medium; B, 10 μg of FML per ml; C, 10 μg of PMGL per ml. Mean values and standard deviations of four experiments are shown.

trypanosomatids. We have characterized the presence of galactofuranose and 2-O,3-O- and 2,3-di-O-substituted mannopyranosil units in LPPD, in *Trypanosoma cruzi*. Galactofuranose was directly linked (1→3) to mannopyranose (28). In *L. adleri* lipopeptidephosphomannan (LPPM), we have found mainly α-(1→2/1→6) mannopyranose short oligosaccharidic chains, with terminal units of mannopyranose and, in minor proportions, galactopyranose (32). We recognized the presence of lipopeptidephosphoxylan (LPPX) in *Leptomonas samueli* and *Herpetomonas samuelpessoai* (33). This xylose-enriched uronic acid glycoconjugate was mainly composed of (1→4)- or (1→2)-linked xylopyranose units, or both, forming a xylan backbone with nonreducing end units of hexoses. All of these compounds are composed of glycidic, proteic, phosphate, and lipidic moieties. They show similar patterns of migration in SDS-PAGE, staining strongly for carbohydrates and lipids and faintly for proteins. The variation in LPPD composition is detected mainly in their glycidic moiety, which is responsible for their specificity either for a parasite species or for the evolutionary pathway of adaptation to a parasitic environment (33). LPPD of *L. donovani* contains phosphate-sugars associated with lipid, among which mannose and galactose are predominant. Phosphate-sugars are common among yeasts (3), but in trypanosomatids they were described only in *Trypanosoma conorhini* (34). LPPD contains smaller oligosaccharides than the compound described by Turco et al. (39, 40). In recent studies of LPPD chemical structure (L. Mendonça-Previato, C. B. Palatnik, and J. O. Previato, First International Workshop on Glycophosphatidyl Inositide Anchored Membrane Proteins, in press), we have shown through methylation-mass spectrometry analysis the presence of nonreducing ends and 3-O- and 3,6-di-O-substituted units of mannopyranose in a ratio of 3:3:1 and of 2-O- and 4-O-substituted mannopyranose units in minor proportions, indicating that the glycidic moiety of LPPD is mainly composed of (1→3)-linked mannopyranosidic chains. Signals characteristic of tri-O- and di-O-substituted galactofuranosidic units were also detected. Similar to results reported by Kaneshiro et al. (21) for total lipid analysis of *L. donovani*, the results of the gas-liquid chromatography-mass spectrometry analysis of LPPD fatty acids indicated the presence of stearic, palmitic, and myristic acids in a 14:2:1 proportion (Mendonça-Previato et al., in press). All of this evidence shows that the LPPD and lipophosphoglycan described by Turco et al. (39) are different compounds. However, we believe that they are both derived from a major surface component of *Leishmania* promastigotes.

Although much attention has been paid to lipophosphoglycan complexes in *Leishmania* species as molecules possibly involved in the infectivity of parasites (18, 20, 29, 39), under our conditions the LPPD did not seem to be the major mediator of promastigote internalization. Indeed, 70% inhibition of phagocytosis was reached only in concentrations of 1,000 μg of LPPD per ml, while similar levels of inhibition were obtained with 6 to 10 μg of FML and PMGL per ml. In agreement with our results, King and Turco (22) recently reported that a *L. donovani* mutant lacking lipophosphoglycan is taken up by macrophages. In an in vitro test, its internalization into human macrophages was not inhibited but, on the contrary, increased by 50%. The same authors suggest that a glycoproteic mannose-containing fraction may be responsible for parasite internalization, a hypothesis which is confirmed by our results for FML.

In our system, FML and PMGL purified from *Leishmania* promastigotes are particularly potent inhibitors of their internalization into macrophages. It should be emphasized that over 75% inhibition is obtained in concentrations (compared in terms of weight/volume ratio) at least 3 orders of magnitude lower than those observed for monosaccharides or pure polysaccharides. Both PMGL and FML have similar levels of inhibitory activity, and each one can inhibit completely the macrophage phagocytosis of promastigotes. Simultaneously, they are specific for *L. donovani* interaction with macrophages. They do not interfere with a nonspecific phagocytosis of yeast cells or with internalization of a closely related *Leishmania* species, such as *L. adleri*.

The observed increase of internalization inhibition obtained by simultaneous addition of both compounds indicates that promastigotes may use the two ligand-receptor sites independently and simultaneously. Our data do not allow us to distinguish whether both ligands act through the same, bivalent receptor or through two independent receptor systems.

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