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Glycosphingolipid Antigens of *Leishmania* (*Leishmania*) *amazonensis* Amastigotes Identified by Use of a Monoclonal Antibody

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Monoclonal antibodies directed against *Leishmania* (*Leishmania*) *amazonensis* amastigotes were produced. One monoclonal antibody (1C3) selected by indirect immunofluorescence reacted with both amastigotes and promastigotes of *L.* (*L.*) *amazonensis*. Glycolipid extraction from *L.* (*L.*) *amazonensis* amastigotes and separation by high-performance thin-layer chromatography followed by immunoblotting demonstrated that 1C3 reacts with two glycosphingolipids which migrate chromatographically similarly to ceramide-*N*-acetylneuraminic acid (GM1) and ceramide-*N*-tetrose-di-acetylneuraminic acid (GD1a). The antibody did not react with glycosphingolipids from *L.* (*L.*) *amazonensis* promastigotes. Immunoprecipitation of ^{125}I- and ^{35}S-methionine-labeled promastigotes demonstrated that 1C3 recognizes gp63 from *L.* (*L.*) *amazonensis* promastigotes. Biosynthetic incorporation of labeled lipids by *L.* (*L.*) *amazonensis* amastigotes indicated that the glycosphingolipids reactive with 1C3 contain oleic acid in their structures. Surface labeling with galactose oxidase and sodium boro[^{3}H]hydride indicated that galactose is present in 1C3-reactive antigens, strongly suggesting that these glycosphingolipids are localized on the surface of *L.* (*L.*) *amazonensis* amastigotes. Inhibition experiments of macrophage infection implicated the 1C3-reactive glycosphingolipids from *L.* (*L.*) *amazonensis* amastigotes in *Leishmania* invasion. The role of gp63 in promastigote-macrophage attachment was also demonstrated by inhibition experiments performed with 1C3, consistent with data from the literature.

Protozoan parasites of the genus *Leishmania* multiply as flagellated promastigotes in the midgut of the sandfly vector. In the vertebrate host, they transform in amastigotes, the obligate intracellular forms which live within macrophage phagolysosomes.

Glycoconjugates have been characterized in *Leishmania* spp., and among them gp63 and lipophosphoglycan (LPG) are the most abundant molecules of *Leishmania* promastigotes (2, 19, 46). These glycoconjugates have been implicated in the attachment of the parasites to macrophages (5, 18, 38) as well as in the induction of protective immune responses in mice (20, 31, 37, 47) and humans (3, 23, 35, 39, 40). Although these molecules seem to be present in *Leishmania* amastigote forms also, there are differences in their expression and/or processing in the amastigote and promastigote forms of the parasite (11, 32, 34, 45).

Another class of glycoconjugate identified in the genus *Leishmania* is a family of low-molecular-weight glycoinositol phospholipids, the major glycolipids present in these parasites. They are expressed in the promastigote and amastigote stages, and structural analysis of glycoinositol phospholipids from *Leishmania donovani* and *Leishmania major* have indicated that there are species-specific as well as stage-specific differences among these molecules (30, 32).

Glycosphingolipids (GSLs) have been identified in *Leishmania* species, especially in amastigote forms of *Leishmania* (*Leishmania*) *amazonensis* (21a, 22), and it was observed that they inhibit lymphoproliferative responses in mice (10).

We report the characterization of a monoclonal antibody (MAb) directed against *L.* (*L.*) *amazonensis* amastigotes which recognizes GSL antigens of this parasite stage. These antigens share common epitopes with gp63 from *Leishmania* (*L.*) *amazonensis* promastigotes and are implicated in the attachment of *L.* (*L.*) *amazonensis* amastigotes to macrophages.

MATERIALS AND METHODS

***Leishmania* cultivation.** *Leishmania* (*L.*) *amazonensis* was kindly provided by J. Shaw, Instituto Evandro Chagas, Belém, Pará, Brasil, and maintained as promastigotes in liver infusion tryptose liquid medium (4).

Isolation of amastigotes from hamster footpads. *L.* (*L.*) *amazonensis* amastigotes were transferred every 4 to 6 weeks to Golden hamsters by inoculation into footpads. Amastigote suspensions were prepared by homogenization of excised lesions. The remaining tissue was homogenized in RPMI 1640 medium containing 10% fetal calf serum. The suspension was disrupted by four passages through 22-gauge needles and centrifuged at 250 × *g* for 10 min; the resulting supernatant was centrifuged at 1,400 × *g* for 10 min, and the pellet was resuspended in RPMI 1640. The suspension was kept under agitation for 4 h at room temperature and centrifuged at 250 × *g* for 10 min. The final pellet contained purified amastigotes which were essentially free of contamination by other cells.

Production of MAbs. BALB/c mice were immunized with *L.* (*L.*) *amazonensis* amastigotes isolated from footpads as described above. The immunization schedule included, first, a subcutaneous dose of 10^7 dead parasites with complete Freund's adjuvant and then one intraperitoneal injection of 3×10^7 parasites with incomplete Freund's adjuvant and one intravenous injection of 3×10^7 parasites in phosphate-

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buffered saline (PBS; pH 7.2). Hybrid cells secreting MAbs were produced by fusing P3U1 mouse myeloma cells (10^7) with spleen cells (10^8) isolated from immunized mice. Fusion was carried out by the method of Köhler and Milstein (25), as modified by St. Groth and Scheidegger (42). The positive hybrids were cloned by limited dilution and injected by the intraperitoneal route into BALB/c mice to obtain ascitic fluid, tested by indirect immunofluorescence (IFI). The 1C3 MAb described here is an immunoglobulin subclass 1, as determined by radial immunodiffusion.

IFI. Slides containing unfixed or formaldehyde-fixed parasites were soaked for 5 min with PBS and incubated for 30 min with MAb 1C3 ascitic fluid and supernatants. After the slides were rinsed with PBS, they were further incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark). Slides were examined on a Nikon fluorescence microscope.

Immunoprecipitation. *L.* (*L.*) *amazonensis* amastigotes and promastigotes were radiolabeled with ^{125}I by the Iodogen method (9). Alternatively, parasites were radiolabeled with L-^{35}S-methionine, using biosynthetic incorporation. The radiolabeled cells (4×10^8) were solubilized for 10 min at room temperature with 0.5% Nonidet P-40 (400 μl) in PBS containing proteolytic inhibitors and centrifuged at $1,400 \times g$ for 5 min. Radiolabeled supernatant (100 μl) was incubated overnight at 4°C with ascitic fluids (5 μl), and the immune complexes were incubated with a suspension of 10% *Staphylococcus aureus* Cowan I strain (100 μl) for 1 h at room temperature by the method of Kessler (24). Immune complexes bound to *S. aureus* were washed several times with 0.1% Nonidet P-40 and 0.1% Nonidet P-40–0.05% sodium dodecyl sulfate (SDS) in PBS, boiled in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, and subjected to SDS-PAGE on 10% polyacrylamide gels by the method of Laemmli (26). Gels were fixed and stained; after destaining, they were dried and exposed to X-Omat K films (Kodak) at −70°C.

Extraction of glycolipids from *L.* (*L.*) *amazonensis* amastigotes. The glycolipid extraction was based on a modification of the procedure described by Hakomori and Murakami (17) and Svennerholm and Fredman (43). About 10^{11} amastigotes of *L.* (*L.*) *amazonensis* were resuspended in 5 ml of chloroform-methanol (1:2, vol/vol), homogenized, and centrifuged at $12,000 \times g$ for 10 min; the precipitate was resuspended in 5 ml of chloroform-methanol (1:1, vol/vol) and centrifuged at $12,000 \times g$ for 10 min, and then it was resuspended in 5 ml of chloroform-methanol (2:1, vol/vol) and centrifuged at $12,000 \times g$ for 10 min. The three centrifugation supernatants were pooled and dried under N_2. This material, consisting of a glycolipid fraction, was then resuspended in chloroform-methanol (2:1). The glycolipid concentration was estimated by the phenol-sulfuric acid reaction (7). The yield of this procedure ranged from 1 to 1.5 mg of glycolipids per 10^{11} amastigotes.

Alternatively, the glycolipid dry residue was kept overnight under vacuum in the presence of P_2O_5. Total lipid extract was acetylated as described by Saito and Hakomori (41), and GSLs were separated from other lipids by using a Florisil column. The GSL fraction was eluted with 1,2-dichloroethane-acetone (1:1, vol/vol), dried under reduced pression, and deacetylated with sodium metoxide (0.5%, wt/vol). Neutral GSLs were isolated by DEAE-Sephadex ion-exchange chromatography as described by Yu and Ledeen (48), passed three times through a C_{18} Bondelut reversed-phase column (Variant/Analytichen), and washed with 3 volumes of 0.1 M NaCl and then with 3 volumes of distilled water; the GSLs were recovered by elution with 5 volumes of chloroform-methanol (2:1, vol/vol).

GSL separation by TLC. The GSLs were subjected to high-performance thin-layer chromatography (HPTLC) on silica gels plates (type 60 F254; Merck) developed in chloroform-methanol-water (60:35:8, vol/vol/vol). The GSLs were visualized after the plates were sprayed with 0.5% (wt/vol) orcinol in 3 M H_2SO_4 at 120°C for 10 min.

Immunoblotting. Immunoblotting was performed by the procedure of Towbin et al. (44). Briefly, after chromatographic GSL separation, the silica plates were dried and sprayed with isopropanol-water (2:1, vol/vol) until visibly wet. The plate was then pressed against a nitrocellulose sheet (type HAW; Millipore Corp.) supported by a glass plate for 15 min. The nitrocellulose sheet was cut into 5-mm-wide strips which were blocked in 0.5% powdered skim milk in PBS (SMB) for 1 h at 25°C. The blocked strips were incubated with antisera or ascitic fluid of MAb 1C3 diluted 1:40 in SMB. After incubation for 2 h, the strips were washed for three 30-min periods with PBS and incubated for a further 1 h with anti-mouse peroxidase-conjugated secondary antibody (Sigma Chemical Co., St. Louis, Mo.) diluted in SMB and washed three times for 30 min each with PBS. Bound immunoglobulin G was visualized after reaction with 0.016% (wt/vol) 3,3′-diaminobenzidine (tetrahydrochloride; Merck)–0.005% (wt/vol) H_2O_2 in PBS, and the reaction was stopped by rinsing with distilled water.

Biosynthetic labeling of amastigotes. *L.* (*L.*) *amazonensis* amastigotes isolated from hamster foot lesions were washed three times in PBS. Incorporation of fatty acids was performed by adding 300 μCi of either [9,10(*n*)-^3H]oleic acid (10 Ci mmol^{-1}) or [9,10(*n*)-^3H]palmitic acid (53.6 Ci mmol^{-1}) (Amersham International, Amersham, England) to 10^9 parasites in 5 ml of RPMI containing 1.5% delipidated bovine serum albumin for 4 h at 28°C.

Surface labeling of live amastigotes. Galactose oxidase and sodium boro[^3H]hydride were used to label surface galactose residues. Amastigotes, 10^9, were resuspended in 200 μl of RPMI containing 20 μl of galactose oxidase (100 U/ml; Sigma) for 45 min at 28°C with agitation. The cells were washed three times in RPMI and maintained in 200 μl of RPMI containing 1.5 mCi of boro[^3H]hydride (specific activity, 13.47 Ci mmol^{-1}; Amersham International) for 45 min at 28°C with agitation.

After biosynthetic and surface labeling, the cells were added to 10^{10} unlabeled amastigotes and then subjected to GSL extraction, HPTLC, and immunoblotting. Autoradiography was performed by exposing the HPTLC plates and nitrocellulose strips sprayed with Amplify (Amersham International) to X-Omat K films (Kodak) for 3 to 7 days at −70°C.

Macrophage cultures and inhibition of *L.* (*L.*) *amazonensis* macrophage infection. Macrophages were collected in PBS from peritoneal cavities of BALB/c mice. About 4×10^5 cells were allowed to attach to round 13-mm glass coverslips. The non-adherent cells were removed by rinsing the coverslips with PBS and were placed in 16-mm-diameter wells of Costar plates containing 0.5 ml of RPMI 1640 plus 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml and kept in a 5% CO_2 humid atmosphere at 37°C. After 24 h, the macrophage cultures were infected with control amastigotes or parasites treated with 1C3 Fab fragments prepared with papain (36) and dialyzed against PBS. *L.* (*L.*) *amazonensis* amastigotes were treated with 1C3 Fab fragments for 2 h at 37°C, washed with PBS, and added to the macrophage monolayers at a cell ratio

of 2:1 for 4 h at 37°C. For experiments performed with *L.* (*L.*) *amazonensis* promastigotes, parasites from stationary growth phase in liquid medium were treated for 2 h with Fab fragments at 24°C, washed with PBS, and used to infect the macrophage cultures in a 5:1 ratio for 4 h. After 18 and 48 h of incubation at 37°C in 5% CO_2 for cultures infected with amastigotes and 48 h of incubation for macrophages infected with promastigotes, the coverslips were washed with PBS, fixed in methanol, stained with Giemsa (Merck), dried, mounted on glass slides, and examined microscopically.

The phagocytic index was determined by counting the percentage of infected macrophages and multiplying by the average number of amastigotes per macrophage.

RESULTS

Reactivity of MAb 1C3 with GSLs from *L.* (*L.*) *amazonensis* amastigotes. The screening of MAbs directed against amastigote forms of *L.* (*L.*) *amazonensis* was performed by IFI, and one MAb (1C3) which reacts with both developmental stages of *L.* (*L.*) *amazonensis* was selected. Figure 1 shows the IFI pattern following the reaction of MAb 1C3 with amastigotes (Fig. 1A) and promastigotes (Fig. 1B). Similar results were obtained when unfixed parasites were tested by IFI.

The initial steps in the characterization of 1C3-reactive antigens involved immunoprecipitation of ^{125}I- and L-^{35}S-methionine-labeled amastigotes and immunoblotting tests, and under no circumstance were amastigote proteins reactive with MAb 1C3 (data not shown).

In contrast, the antigens which react with MAb 1C3 were identified when GSLs from *L.* (*L.*) *amazonensis* amastigotes were used as antigens. Thus, the parasites were subjected to a chloroform-methanol extraction, and the resulting GSLs were used in immunoblotting experiments after HPTLC separation on silica gel plates, as described in Materials and Methods.

Figure 2 shows the results from these experiments. The GSL pattern of *L.* (*L.*) *amazonensis* amastigotes in HPTLC after staining with orcinol is shown in Fig. 2D. Immunoblotting revealed that GSLs recognized by MAb 1C3 migrate chromatographically in HPTLC similarly to ceramide-*N*-tetrose-*N*-acetylneuraminic acid (GM1) and ceramide-*N*-tetrose-di-acetylneuraminic acid (GD1a) (Fig. 2C). Figure 2A shows the control immunoblotting reaction of GSLs from *L.* (*L.*) *amazonensis* amastigotes after HPTLC and incubation with P3U1 ascitic fluid.

The *L.* (*L.*) *amazonensis* promastigote antigens recognized by MAb 1C3 were also examined. Figure 2E shows the GSL patterns from *L.* (*L.*) *amazonensis* promastigotes after HPTLC and staining with orcinol, and Fig. 2B shows the GSL profiles from *L.* (*L.*) *amazonensis* promastigotes after HPTLC and immunoblotting with MAb 1C3. As observed in Fig. 2D and E, amastigote forms contain higher concentrations of GSLs compared with promastigote forms. In addition, GSLs from *L.* (*L.*) *amazonensis* promastigotes are not recognized by MAb 1C3 (Fig. 2B).

The possibility that the antigens recognized by MAb 1C3 represent other classes of glycolipids or proteins soluble in chloroform-methanol was discarded after experiments in which the chloroform-methanol parasite extracts were subjected to fractionation in Florisil, DEAE-Sephadex, and C_{18} columns to separate the neutral GSLs from other lipids and proteins. Similar antigenic profiles were obtained when a neutral GSL pool was used in immunoblotting experiments performed with 1C3 (data not shown).

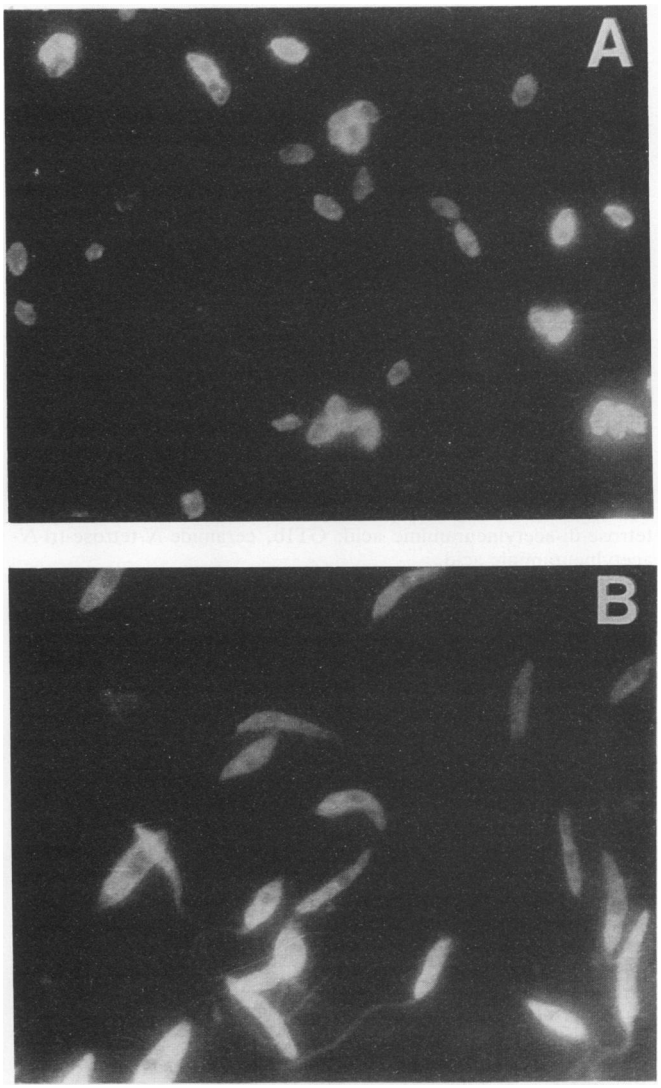

FIG. 1. Binding of MAb 1C3 to *L.* (*L.*) *amazonensis* amastigotes (A) and promastigotes (B) visualized by IFI. Magnification, ×1,000.

Although the *L.* (*L.*) *amazonensis* amastigote population obtained from hamster foot lesions is of a relatively high degree of purity, some controls were carried out to check for the presence of hamster macrophage glycolipids contaminating the *Leishmania* GSL preparations. Thus, GSLs were extracted from hamster starch-activated macrophages, separated by HPTLC, and probed by immunoblotting with MAb 1C3. The results of these experiments (Fig. 3) show that not only is the GSL pattern of hamster macrophages (Fig. 3D) different from that of *L.* (*L.*) *amazonensis* amastigotes (Fig. 3C), but also 1C3 did not recognize any GSL component from hamster macrophages (Fig. 3B), confirming its restricted specificity for *L.* (*L.*) *amazonensis* amastigote GSLs (Fig. 3A).

Characterization of *L.* (*L.*) *amazonensis* promastigote antigens recognized by MAb 1C3. IFI experiments showed that MAb 1C3 also reacts with *L.* (*L.*) *amazonensis* promastigotes (Fig. 1B). The presence of common epitopes between amastigotes and promastigotes reactive with MAb 1C3 and the lack of reactivity of this MAb with GSLs from promasti-

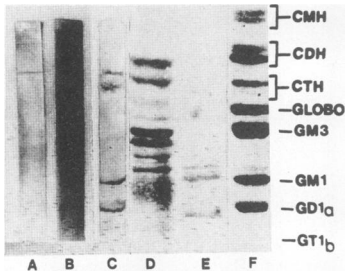

FIG. 2. Immunoblotting of *L.* (*L.*) *amazonensis* GSLs after HPTLC separation: GSLs from *L.* (*L.*) *amazonensis* amastigotes incubated with ascitic fluids from (A) P3U1 and (C) MAb 1C3; GSLs from *L.* (*L.*) *amazonensis* promastigotes incubated with MAb 1C3 ascitic fluid (B); GSLs from *L.* (*L.*) *amazonensis* amastigotes (D) and promastigotes (E) stained with orcinol; standard GSLs stained with orcinol (F). CMH, ceramide monohexosyl; CDH, ceramide dihexosyl; CTH, ceramide trihexosyl; GLOBO, ceramide tetrahexosyl; GM3, ceramide-lactose-*N*-acetylneuraminic acid; GM1, ceramide-*N*-tetrose-*N*-acetylneuraminic acid; GD1a, ceramide-*N*-tetrose-di-acetylneuraminic acid; GT1b, ceramide-*N*-tetrose-tri-*N*-acetylneuraminic acid.

gotes indicated that the 1C3-reactive promastigote antigens might be of a proteic nature.

Thus, promastigotes were radiolabeled with ^{125}I, incubated with 1C3, immunoprecipitated, and subjected to SDS-PAGE and autoradiography, as described in Materials and Methods. Figure 4 shows that one band of 63,000 molecular weight from *L.* (*L.*) *amazonensis* promastigotes is recognized by 1C3 (Fig. 4E). The efficient iodine incorporation and the apparent molecular weight of this component led us to conclude that 1C3 recognizes gp63 from *L.* (*L.*) *amazonensis* promastigotes. This result is strengthened by immunoprecipitation experiments in which 1C3 was incubated with *L.* (*L.*) *amazonensis* promastigotes radiolabeled with L-^{35}S-methionine. Again, it could be seen that MAb 1C3 recognized a single band of 63,000 apparent molecular weight (data not shown).

Biosynthetic and surface labeling of *L.* (*L.*) *amazonensis* amastigotes. To characterize the lipid components from the GSLs reactive with 1C3, *L.* (*L.*) *amazonensis* amastigotes were metabolically labeled with radioactive fatty acids. Galactose oxidase and sodium boro[^3H]hydride were used to label surface galactose residues. GSLs extracted from labeled parasites were subjected to HPTLC, immunoblotting, and autoradiography. The results obtained from these experiments are shown in Fig. 5. Figure 5A shows the results from sodium boro[^3H]hydride labeling after treatment with galactose oxidase. It can be seen that the two 1C3-reactive antigens comigrate with the labeled species. This result is consistent with the presence of galactose residues in GSL antigens recognized by 1C3 and strongly suggests that these antigens are expressed on the surface of *L.* (*L.*) *amazonensis* amastigotes.

In relation to the lipid composition of the GSLs, it was possible to see radiolabeled bands which comigrate in HPTLC with MAb 1C3-reactive GSL when the parasites were labeled with [9,10(*n*)-^3H]oleic acid (Fig. 5C). In contrast, no radiolabeled bands comigrating with MAb 1C3-reactive GSLs were detected when the parasites were labeled with [9,10(*n*)-^3H]palmitic acid (Fig. 5B).

Effect on MAb 1C3 on macrophage infection by *L.* (*L.*) *amazonensis*. The effect of MAb 1C3 on the infection of peritoneal macrophages by *L.* (*L.*) *amazonensis* amastigotes is shown in Fig. 6. MAb 1C3 Fab fragments at 250 μg/ml inhibited about 60% of the penetration of amastigotes into macrophages. Preincubation of parasites with similar concentrations of a different MAb directed to *L.* (*L.*) *amazonensis* amastigotes, 15D10, which does not recognize GSLs, did not significantly inhibit macrophage infection. These results are consistent with the notion that the GSLs recognized by 1C3 represent surface molecules implicated in

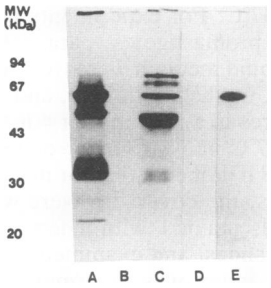

FIG. 4. Immunoprecipitation and SDS-PAGE of *L.* (*L.*) *amazonensis* promastigotes radiolabeled with ^{125}I: total cellular extract (A) and cellular extract immunoprecipitated with: normal mouse serum (B), serum from mice immunized against *L.* (*L.*) *amazonensis* promastigotes (C), P3U1 ascitic fluid (D), and MAb 1C3 ascitic fluid (E). MW, molecular weight.

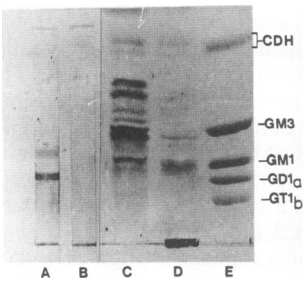

FIG. 3. Comparison of *L.* (*L.*) *amazonensis* amastigotes and hamster macrophage GSLs: GSLs extracted from *L.* (*L.*) *amazonensis* amastigotes (A) and hamster macrophages (B) after HPTLC separation and immunoblotting with MAb 1C3 ascitic fluid; GSLs from *L.* (*L.*) *amazonensis* amastigotes (C) and hamster macrophages (D) stained with orcinol after HPTLC separation; standard GSLs after HPTLC separation and orcinol staining (E). See the legend to Fig. 2 for explanation of abbreviations on the right.

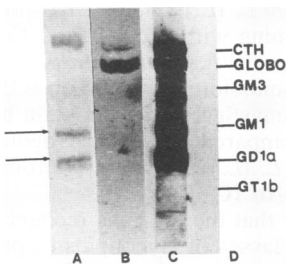

FIG. 5. Profiles of *L.* (*L.*) *amazonensis* amastigote GSLs after biosynthetic labeling with (B) [9,10(*n*)-^3H]palmitic acid or (C) [9,10(*n*)-^3H]oleic acid. Parasites radiolabeled with sodium boro[^3H] hydride after treatment with galactose oxidase are in lane A. Arrows indicate the GSL antigens recognized by MAb 1C3. Standard GSL chromatographic migration is shown in lane D. See the legend to Fig. 2 for explanation of abbreviations on the right.

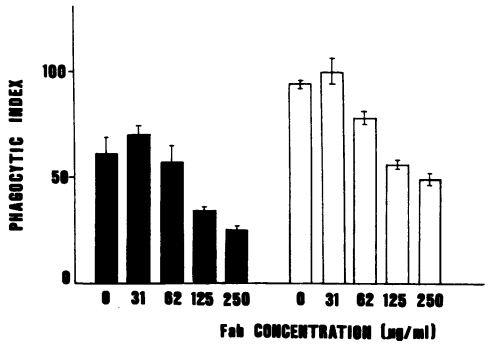

FIG. 6. Effect of MAb 1C3 Fab fragments on infection of mice peritoneal macrophages by *L.* (*L.*) *amazonensis* amastigotes. Parasites were treated with Fab fragments of 1C3 at the indicated concentrations and used to infect macrophage cultures. The results were obtained by counting at least 200 macrophages per triplicate coverslip and are expressed by the phagocytic index after 18 (closed bars) and 48 (open bars) h of incubation. Error bars show standard deviations of five experiments.

Leishmania invasion. The effect of GSLs from *L.* (*L.*) *amazonensis* amastigotes on parasite binding to macrophages was also investigated. *L.* (*L.*) *amazonensis* amastigotes (one parasite per macrophage) were incubated for 4 h in the presence of 4 μg of GSLs per ml, after which they infected four times less macrophages compared with the control (amastigotes in the absence of GSLs). These data corroborate a role for GSLs as ligands of *L.* (*L.*) *amazonensis* amastigotes to macrophages.

Similar results were obtained when *L.* (*L.*) *amazonensis* promastigotes were treated with MAb and used to infect peritoneal macrophages in vitro. Figure 7 shows that promastigotes incubated previously with 1C3 Fab fragments at 66 μg/ml infect approximately 60% less than control, untreated parasites.

DISCUSSION

MAbs directed against promastigotes have been used for the identification of species-specific determinants of *Leishmania* antigens (1, 12, 33) as well as for the purification of species-specific components such as gp63 and LPG (5, 20). In contrast, fewer studies have been performed with the amastigote stage of the parasite (8, 21). However, the importance of the intracellular forms of the parasite in mediating the interaction with the host cell, leading to immunological host responses and parasite survival inside the macrophage, is unquestionable. The present work describes a MAb, 1C3, which recognizes GSLs from *L.* (*L.*) *amazonensis* amastigotes. The 1C3-reactive GSLs comigrate, in HPTLC, with GM1 and GD1a (Fig. 2C).

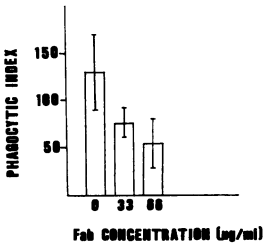

FIG. 7. Effect of MAb 1C3 Fab fragments on infection of mice peritoneal macrophages by *L.* (*L.*) *amazonensis* promastigotes. Promastigotes from stationary growth phase in liquid medium were treated with Fab fragments of 1C3 at the indicated concentrations and used to infect macrophage cultures. The results represent counts of at least 200 macrophages per triplicate coverslip and are expressed by the phagocytic index 48 h after incubation. Error bars show standard deviations of four experiments.

GSLs are important differentiation and malignant transformation markers (13, 15, 16), and their role as immunomodulators has also been recognized (29). Characterization of *Leishmania* GSLs has been carried out, and we have shown that the amastigote form of the parasite contains high concentrations of this class of glycoconjugates (21a, 22).

Here we have characterized a MAb obtained against *L.* (*L.*) *amazonensis* amastigotes which recognizes GSLs. IFI experiments carried out with 1C3 showed a prevalent distribution of fluorescence on the surface of parasites (Fig. 1A), suggesting that these GSLs are expressed on the amastigote surface. Data from surface labeling of *L.* (*L.*) *amazonensis* amastigotes with galactose oxidase and sodium boro[^{3}H] hydride strengthen these results (Fig. 5A).

The existence of 1C3 epitopes in both promastigotes and amastigotes of *L.* (*L.*) *amazonensis* was demonstrated by IFI (Fig. 1A and B). However, the antigens recognized by 1C3 do not belong to the same glycoconjugate category. In fact, it was possible to show that in *L.* (*L.*) *amazonensis* promastigotes the MAb does not recognize GSLs (Fig. 2B) but reacts with the major surface glycoprotein, gp63 (Fig. 4E). These results strongly suggest that the shared 1C3 epitope might be of a carbohydrate nature. It has already been described by others that antibodies directed against carbohydrate determinants may react with the appropriate sugar sequences linked to either a lipid or a peptide backbone. Some examples of such antibodies are represented by MAbs directed against the ABH blood group and Lewis and Ii antigens (6, 14). Analysis of cell membranes or crude tissue extracts with these antibodies showed that they may react with GSLs or glycoproteins or both. An example of such reactivity is a MAb elicited by immunization with a colon cancer cell line which predominantly reacts with GSL extracts from these tumors (27) and mucin from sera of cancer patients (28).

Results from Fig. 6 demonstrated that GSLs recognized by 1C3 are important in the attachment of amastigotes to macrophages. Although several surface molecules present in *Leishmania* promastigotes have been implicated as determinants in cellular interactions, few studies have focused on amastigote surface ligands. Results from Fig. 7 corroborate previous data from the literature which have demonstrated the participation of gp63 in *Leishmania*-macrophage binding (5, 38). In vivo experiments with *L.* (*L.*) *amazonensis* amastigotes treated with 1C3 are being performed.

Inhibitory effects of GSLs from *L.* (*L.*) *amazonensis* amastigotes on lymphoproliferative responses in mice have been demonstrated in vitro (10). The existence of a MAb which recognizes GSLs from *L.* (*L.*) *amazonensis* amastigotes enables us to examine the relevance of the GSL inhibitory effect on the lymphocyte proliferation in vivo. Preliminary data from our laboratory show that treatment of BALB/c mice with MAb 1C3 partially protects them from *L.* (*L.*) *amazonensis* amastigote infection.

The availability of MAb 1C3 opens new perspectives in the evaluation of the role of amastigote GSLs in cellular immune responses in leishmaniasis.

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