Expression of an Unusual Acidic Glycoconjugate in *Leishmania donovani*

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An acidic glycoconjugate containing mannose, galactose and phosphate in approximately equimolar amounts was extracted from *Leishmania donovani* promastigotes and partially characterized. The glycoconjugate could be metabolically labeled with either \([3H]\)mannose or \([3H]\)galactose and was extractable from a delipidated residue fraction with water/ethanol/diethyl ether/pyridine/concentrated NH\(_4\)OH (15:15:5:1:0.017) at 25 °C. The radioactively labeled glycoconjugate was found to possess the following characteristics: 1) comprised 45–60% of the total \([3H]\)mannose label incorporated into macromolecules; 2) was soluble in alkaline solvents and 0.5% Triton X-100; 3) migrated as a broad band upon electrophoresis on sodium dodecyl sulfate-polyacrylamide gels with an approximate molecular weight of 15,000–30,000; 4) bound to DE52 cellulose and was eluted with a salt gradient of 0–0.1 M NaCl; 5) was insensitive to Pro-nase, hyaluronidase, chondroitinase, endo-\(\beta\)-N-acetylglucosaminidase H, and endo-\(\beta\)-galactosidase; and 6) possessed hydrophobic properties. An unusual feature of the glycoconjugate was its lability to mild acid hydrolysis (0.02 N HCl, 15 min, 60 °C). As determined by alkaline phosphatase and glycosidase digestion and paper chromatographic analysis, the major fragment generated by mild acid hydrolysis was found to be a phosphorylated galactosyl-\(\beta\)-mannose disaccharide. All of these characteristics suggest that the glycoconjugate may be a polysaccharide and, possibly, may be important in parasite-host cell interactions.

The trypanosomatid *Leishmania donovani* is the etiologic agent of human visceral leishmaniasis, an often fatal disease. These protozoan parasites are transmitted by the sand fly and upon infection of humans, they become parasitic mainly in the cells of the reticuloendothelial system. It is unknown how the parasite is recognized by the macrophages of the host and more importantly, how it avoids destruction. Possibly, a component of the cell surface of the parasite, such as a glycoconjugate, is responsible for these host-parasite interactions. However, relatively little is known about the chemistry, biosynthesis, and function of glycoconjugates of *L. donovani*. Cell surface studies of the parasite by Dwyer (1, 2) have revealed the existence of molecules rich in carbohydrate, which bind specifically to lectins, as well as entities that confer a negative charge to these structures. Using antisera against whole organisms, a number of investigators working with several distinct human leishmanial species have reported that the promastigote forms release antigenically active glycoproteins into culture media (3–6). In related work, Kaneshiro et al. (7) have shown by immunoprecipitation techniques with antisera against purified *L. donovani* promastigote cell surface preparations that the parasites produce extracellular carbohydrate-containing antigens.

We have begun a biochemical analysis of the glycoconjugates of the promastigote form of *Leishmania donovani*. In this paper, we report the isolation and partial characterization of a major glycoconjugate containing an approximate equimolar ratio of mannose, galactose, and phosphate. An unusual feature of the glycoconjugate is its lability to mild acid hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Materials** — All materials were obtained as follows: Dulbecco's modified Eagle's medium (DME 430-2100) and fetal calf serum from Gibco; [2-\(3H\)]mannose (18.4 Ci/mmol) and [6-\(3H\)]galactose (9.1 Ci/mm) from New England Nuclear; [\(35S\)]methionine (1300 Ci/mmol) from Amersham; Sephadex G-100 (40-120 µ), jack bean \(\alpha\)-mannosidase, and green coffee bean \(\alpha\)-galactosidase from Sigma; bacterial alkaline phosphatase from Worthington; and DE52 cellulose and 3-mm chromatographic paper from Whatman. \(\beta\)-Galactosidase from jack bean was provided by Dr. Roger Laine (Louisiana State University) who obtained it from Dr. Y.-T. Lee (Tulane University).

**Cells and Cell Culture** — Promastigotes of *Leishmania donovani* were obtained from Dr. Buddy Ullman (University of Kentucky) and were passaged according to his procedure (8). The cells were grown at 25 °C in Dulbecco's modified Eagle's medium supplemented with 2.5–5% fetal calf serum, adenosine (0.05 mM), xanthine (0.05 mM), biotin (1 mg/liter), Tween 80 (40 mg/liter), hemin (5 mg/liter), and triethanolamine (0.5 ml/liter).

**Metabolic Labeling and Extraction** — Exponentially growing cells (2 × 10\(^5\)-2 × 10\(^7\) cells/ml) were centrifuged at 3000 × g for 5–7 min, washed with 5 ml of phosphate-buffered saline (9), and centrifuged again. The cells were resuspended and labeled with 100–200 µCi of \([6-\(3H\)]\)mannose or \([3H]\)galactose in 1.5 ml of Dulbecco's modified Eagle's medium containing 0.1 mg/ml of glucose and supplemented with 5% dialyzed fetal calf serum, adenosine (0.05 mM), xanthine (0.05 mM), biotin (1 mg/liter), Tween 80 (40 mg/liter), hemin (5 mg/liter), and triethanolamine (0.5 ml/liter). The period of incubation with the isotope was 1 h at 25 °C in a 5% CO\(_2\) atmosphere unless otherwise stated. Subsequently, the cells were separated from the medium by centrifugation and washed with 5 ml of phosphate-buffered saline. The cells were extracted sequentially with chloroform/methanol/water (3:2:1), water, and chloroform/methanol/water (1:1:0.3) to remove radiolabeled metabolites and lipids (10). A delipidated residue fraction remained and was extracted as described in the text.

**SDS-Polyacrylamide Gel Electrophoresis** — Radiolabeled samples were resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions according to Laemmli (11). The separating gel was 10% acrylamide and the stacking gel was 3.6% acrylamide. Electo-
phoresis was carried out for 5-6 h at 25 mA. Following electrophoresis, the gel was subjected to fluorography according to Bonner and Laskey (12) using Kodak XAR 5 film.

Enzyme Digestions—All enzyme digestions were performed at 37°C for 16 h unless otherwise stated. Alkaline phosphatase (0.1-0.3 unit) digestion was done in 1 mM Tris-HCl, pH 8. α-Galactosidase (0.004 unit) digestion was conducted in 0.05 M sodium citrate, pH 5. β-Galactosidase (0.01 unit) and α-mannosidase (0.6 unit) digestions were performed as described elsewhere (13, 14).

Phosphate Determination—Phosphate was quantitated by the procedure of Bartlett (15).

Gas-Liquid Chromatography—Prior to gas-liquid chromatography, samples were subjected to mild acid hydrolysis (0.02 M HCl, 5 min, 100°C), dried, and treated with alkaline phosphatase. The samples were then desalted by passage through a column of AG 50W-X4 (H+ form) and AG 1-X4 (acetate form) and lyophilized. Following methanalysis of these samples and preparation of the trimethylsilyl derivatives of the methyl glycosides (16, 17), gas-liquid chromatography was performed on a Shimadzu gas chromatograph model GC mini 1 equipped with a flame ionization detector.

RESULTS

Characterization of the Radioactive Glycoconjugate

Extraction of the Glycoconjugate—In higher eukaryotic cells, a delipidation of [3H]mannose-labeled cells by the procedure described under "Experimental Procedures" or by similar procedures results in an insoluble residue fraction. This fraction contains radiolabeled glycoproteins and usually is solubilized with detergents or is digested with Pronase to generate soluble glycopeptides (10, 14, 18, 19). As shown in the flow diagram in Fig. 1, a similar fraction generated from Leishmania donovani labeled with [3H]mannose or [3H]galactose can be further extracted with several solvents. Treatment of the delipidated residue fraction with 0.04 M NH4OH at 100°C solubilized 97% of the radioactivity (Table I). Treatment with solvent E which contains water, ethanol, diethyl ether, pyridine, and NH4OH in a proportion of 15:15:5:1:0.017 at 25°C resulted in the extraction of 86% of the radioactivity from an identical residue fraction. Other buffers or solvents were not nearly as efficient in extracting the tritium label from this fraction.

The material extracted by solvent E accounted for 30–35% of the total [3H]mannose label incorporated into macromolecules (glycoconjugate, lipid-linked oligosaccharides, and glycoproteins) during 1 h of radiolabeling. After 3 h or longer of metabolic labeling, the percentage increased to 45–60%.

Chromatographic Analysis—To characterize the material extracted by solvent E, the [3H]mannose-labeled glycoconjugate was subjected to gel filtration chromatography. As shown in Fig. 2, the glycoconjugate was applied to a column of Sephadex G-100 (1 × 80 cm) equilibrated in 0.04 M NH4OH and 0.001 M EDTA; 0.6 ml was collected and measured for radioactivity. Recovery of radioactivity was 75%.

The glycoconjugate was judged to be anionic by experiments employing ion exchange chromatography. The 3H-glycoconjugate was applied to a column of DE52 cellulose equilibrated with 0.04 M NH4OH and 0.5% Triton X-100 and was quantitatively eluted as a broad peak with a gradient of 0–0.1 M NaCl (Fig. 3). Pretreatment of the intact glycoconjugate with

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**FIG. 1. Scheme of radiolabeling and extraction.**

**FIG. 2. Sephadex G-100 chromatography of the [3H]mannose-labeled glycoconjugate.** The radioactive glycoconjugate was applied to a column of Sephadex G-100 (1 × 80 cm) equilibrated in 0.04 M NH4OH and 0.001 M EDTA; 0.6 ml was collected and measured for radioactivity. Recovery of radioactivity was 75%.

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**TABLE I**

| Addition | Temperature | Per cent extractable |
|----------|-------------|----------------------|
| 0.04 M NH4OH | 100 | 97 |
| 0.04 M NH4OH | 25 | 30 |
| Solvent E | 25 | 86 |
| 0.1 M Tris-HCl, pH 8 | 100 | 64 |
| 0.1 M Tris-HCl, pH 8 | 25 | 25 |
| 0.7 M NH4OH | 25 | 7 |
| 0.1 M NaCl | 25 | 8 |
alkaline phosphatase did not alter its behavior on DE52 cellulose.

In a number of thin layer and paper chromatographic systems, the glycoconjugate did not migrate unless the solvent contained a high proportion of H2O and usually a small amount of Triton X-100. A SG-81 paper chromatographic system, for example, permitted migration of the glycoconjugate in the presence of a solvent containing n-propyl alcohol/0.04 M NH4OH and 0.001 M EDTA (1:1.5) in 0.5% Triton X-100. The glycoconjugate migrated as a single but diffuse spot in this system. It should be noted that all of the above chromatographic results obtained with the [3H]mannose-labeled glycoconjugate were also observed with material metabolically labeled with [3H]galactose.

Electrophoretic Analysis on Polyacrylamide Gels—SDS-polyacrylamide gel electrophoresis of the [3H]mannose-labeled glycoconjugate was consistent with the results of gel filtration on Sephadex G-100 in that the material migrated as a broad band with an approximate molecular weight range between 15,000–30,000 (Fig. 4, lane 1). No other [3H]mannose-labeled macromolecules were seen in this extract. A comparable labeling of the parasites with [35S]methionine, followed by extraction with solvent E and electrophoresis on SDS-polyacrylamide gels, revealed that the extract was contaminated with a number of proteins (Fig. 4, lane 2).

Identification of the Radiolabel—In order to determine the identity of the radiolabel, the [3H]mannose- and the [3H]galactose-labeled glycoconjugates were subjected to strong acid hydrolysis (2 N trifluoroacetic acid, 2.5 h, 110 °C) and analyzed by descending paper chromatography in butanol/pyridine/water (6:4:3). Acid hydrolysis of the [3H]mannose-labeled glycoconjugate yielded a single radioactive peak that co-migrated with standard mannose (Fig. 5A), indicating that the [3H]mannose was not metabolized to any other radioactive sugar during the labeling period. A similar analysis of the [3H]galactose-labeled glycoconjugate revealed that approximately 14 and 20% of the radioactivity co-migrated with authentic mannose and galactose, respectively, while the bulk did not migrate (Fig. 5B). However, treatment of the [3H]galactose-labeled hydrolysate with alkaline phosphatase prior to chromatography on paper showed 86% of the label as galactose and 14% as mannose (Fig. 5C). Since the amount of liberated mannose remained constant (Fig. 5, B and C), these results suggest that a phosphate ester of galactose is generated by strong acid hydrolysis of the glycoconjugate which is then susceptible to alkaline phosphatase. An important observation was that radioactive glucose was not detected in the chromatograms and that the ratio of galactose and mannose was not changed following treatment of an identical sample (Fig. 5C) with glucose oxidase. Untreated glycoconjugate remained at the origin in this solvent system.

Analysis of the Mild Acid-hydrolyzed Fragments of the [3H]-Glycoconjugate

Chromatography of the Hydrolysate—Of particular interest was the finding that the [3H]mannose- or [3H]galactose-labeled glycoconjugate was labile to mild acid hydrolysis. Fig. 6 shows the gel filtration profiles of the mild acid hydrolysates (0.02 N HCl, 60 °C) of the [3H]mannose-labeled glycoconjugate as a function of time. Treatment of the radioactive glycoconjugate under these conditions converted the substance into lower molecular weight products that eluted close to the retention volume of the column. It took about 15 min of hydrolysis at 60 °C (Fig. 6, B–E) or several minutes at 100 °C to complete this conversion. As shown in Fig. 6A, preincubating the glycoconjugate for 15 min at 60 °C with no acid did not result in any appreciable breakdown.

The fragments generated by mild acid hydrolysis of the [3H]mannose-labeled glycoconjugate could be resolved by application to a column of DE52 cellulose, resulting in quantitative recovery of radioactivity in at least three peaks (Fig. 7A). One product did not bind the ion exchange support, whereas the bulk of the material was bound and eluted with a NaCl gradient. A similar profile was obtained with the
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Fig. 5. Paper chromatography of the strong acid hydrolysis products of the [3H]mannose- and [3H]galactose-labeled glycoconjugate. Aliquots of the labeled glycoconjugates were treated with 2 N trifluoroacetic acid for 2.5 h at 110 °C. Following hydrolysis, the samples were dried by evaporation under N₂ and chromatographed on paper in n-butyl alcohol/pyridine/water (6:4:3) for 16 h. Quantitation of radioactivity was accomplished by cutting the paper strips into 1-cm segments and counting in a vial containing scintillation fluid. Standard sugars were detected by staining with alkaline silver nitrate (24). A, [3H]mannose-labeled hydrolysate; B, [3H]galactose-labeled hydrolysate; C, [3H]galactose-labeled hydrolysate, dried under N₂ to remove the acid, and then treated with alkaline phosphatase, corresponding [3H]galactose-labeled material (data not shown). As shown in Fig. 7B, alkaline phosphatase digestion of the mild acid-hydrolyzed products resulted in none of the radioactivity binding to the ion exchange support. On the other hand, pretreatment of the intact [3H]glycoconjugate with alkaline phosphatase prior to mild acid hydrolysis, resulted in a profile similar to the one shown in Fig. 7A.

Identification of the Major Fragment—The identity of the major [3H]mannose-labeled fragment (Fig. 7A, fractions 20–29) was determined by sequential hydrolase degradation followed by paper chromatographic analysis of the products. The untreated fragment remained at the origin (Fig. 8A) as did samples treated with β-galactosidase, α-galactosidase, or α-mannosidase (Fig. 8C). However, the fragment treated with alkaline phosphatase did migrate with a mobility slightly faster than the disaccharide lactose (Fig. 8B). Furthermore, digestion of the alkaline phosphatase-treated fragment with β-galactosidase yielded a single radioactive peak that co-migrated with standard mannose (Fig. 8D). The alkaline phosphatase-treated fragment was resistant to both α-galactosidase and α-mannosidase. Additional information was obtained by reduction of the alkaline phosphatase-treated [3H]mannose-labeled fragment with NaBH₄ followed by strong acid hydrolysis and chromatographic analysis of the products on paper. Using another solvent system, the radioactivity

Fig. 6. Sephadex G-100 chromatography of the mild acid hydrolysis products of the [3H]mannose-labeled glycoconjugate. Aliquots of the labeled glycoconjugate were treated with 0.02 N HCl at 60 °C for the time periods indicated. The samples were neutralized with NaOH and chromatographed on a column of Sephadex G-100 (1 × 80 cm) equilibrated in 0.04 N NH₄OH and 0.001 M
prior to methanolysis, galactose was not detected in appreciable amounts. Glucose was the only other sugar detected and quantitatively co-migrated as mannitol (Fig. 8E), indicating that mannose is located at the reducing end of the fragment. Taken together, the major mild acid hydrolysis fragment of the glycoconjugate is believed to be a phosphorylated galactosyl-β-mannose disaccharide.

**Analysis of Unlabeled Glycoconjugate**

The glycoconjugate was extracted by solvent E from a relatively large number of exponentially growing cells in order to isolate enough material to perform direct sugar analysis by gas-liquid chromatography. The yield of hexose in the solvent E extract was estimated to be approximately 0.2 to 0.4 μmol per 10^9 cells (equivalent to about 100 ml of culture). The carbohydrate composition was determined on 11 separate preparations and revealed the presence of mannose and galactose in a ratio ranging from 1.3:1 to 0.9:1 (Fig. 9). Also, it was observed that when the glycoconjugate was not subjected to mild acid hydrolysis and alkaline phosphatase treatment prior to methanolysis, galactose was not detected in appreciable amounts. Glucose was the only other sugar detected and was believed to be a contaminant since it was present in the blank samples in similar amounts as appeared in the glycoconjugate-containing samples.

EDTA; 0.6 ml was collected and measured for radioactivity. A, control sample heated at 60 °C for 15 min with no acid; B-E, aliquots of the glycoconjugate treated with acid for 1, 3, 5, and 15 min, respectively.

**DISCUSSION**

The data presented in this paper provide evidence that *Leishmania donovani* parasites express an unusual acidic glycoconjugate. While the glycoconjugate was removable from a delipidated residue fraction with several solvents at 100 °C, extraction with solvent E at 25 °C was preferable. This solvent was desirable due to the lower temperature and pH during the extraction process, which reduced the possibility of degradation of the glycoconjugate. Solubility of the glycoconjugate in solvent E which contains ethanol, ether, and pyridine suggested that the substance may possess hydrophobic regions. This possibility was further strengthened by observations that the migration of the material was enhanced in thin...
layer and paper chromatographic systems by the inclusion of Triton X-100 in the mobile phase.

As judged by gel filtration on Sephadex G-100, ion exchange chromatography on DE52 cellulose, and SDS-polyacrylamide gel electrophoresis, the glycoconjugate appeared to be heterogeneous in size with a tentatively molecular weight range of 15,000–30,000. The glycoconjugate was not degradable by treatment with several endoglycosidases: hyaluronidase, chondroitinase, endo-β-galactosidase, and endo-α-N-acetylglucosaminidase. Although Pronase also did not seem to degrade the material, the presence of small amounts of amino acids cannot be ruled out.

An unusual feature of the glycoconjugate was its lability to mild acid. Breakdown of the glycoconjugate began to be apparent within several minutes using conditions of 0.02 N HCl at 60 °C. Following 15 min of incubation under these hydrolytic conditions, complete conversion to low molecular weight products occurred. The degree of sensitivity of the glycoconjugate to mild acid would categorize its labile bonds as "extra labile" according to the classification scheme of Leloir and Cardini (20). Moreover, since there was a series of intermediate sized products that were generated during the acid-catalyzed breakdown, our results suggested the existence of more than one labile bond.

The products of mild acid hydrolysis were fractionated into several species by chromatography on DE52 cellulose. The data obtained by digestion with alkaline phosphatase indicated that the source of the binding of the acid-generated fragments to DE52 cellulose is due to the presence of phosphate. These phosphate groups were susceptible to the enzyme only when the glycoconjugate was mild acid hydrolyzed, suggesting that the phosphate groups of the glycoconjugate are labile and that the glycosidic linkage between the carbohydrate components as well as the identities of the other acid-generated fragments remain to be established. Since this disaccharide unit had a much greater proportion of the radioactivity (75–80% of the total [3H]mannose-labeled or [3H]galactose-labeled material, Fig. 7A) relative to the other observed fragments separated by DE52 cellulose, there may be a number of these disaccharide units in the overall glycoconjugate structure.

Consistent with the observations made with the radioactively labeled glycoconjugate were the results obtained with corresponding unlabeled material. Sugar compositional analysis was determined by gas-liquid chromatography and phosphate analysis was conducted by the procedure of Bartlett (15). Mannose, galactose, and phosphate were present in an approximate equimolar proportion of 1:1:1, the ratio of the major radioactive fragment. Galactose was detected only upon prior treatment of the glycoconjugate with mild acid and subsequent alkaline phosphate digestion. A likely interpretation is that methanolysis of the glycoconjugate released a stable galactosyl phosphate unit which was not detectable with the usual procedure for gas-liquid chromatography. In an analogous observation, strong acid hydrolysis of [3H]galactose-labeled glycoconjugate yielded a product that did not migrate upon chromatography on paper and which was converted to galactose upon alkaline phosphatase digestion.

Glucose was also seen following gas-liquid chromatographic analysis of the glycoconjugate. However, we have concluded that glucose is not a component of the glycoconjugate on the basis of the following observations. 1) Glucose was present in similar amounts in blanks prepared for gas-liquid chromatography; 2) radioactive glucose was not found upon strong acid hydrolysis of [3H]galactose-labeled glycoconjugate. Labeling of cells with radioactive galactose has been used to incorporate label into the glucose moiety of complex carbohydrates (9, 21, 22).

All of the observed characteristics suggest that the glycoconjugate may be a polysaccharide. It is tempting to speculate on the structure of the glycoconjugate. In some respects, it may be similar to the teichoic acid components of some bacterial cell walls. The glycoconjugate may be a polymer of short galactosyl-β-mannose units linked together via acid-labile phosphate bridges. Experiments are in progress to examine these and other possibilities as well as the cellular location and function of the glycoconjugate. If the glycoconjugate is a component of the cell surface, it may have an important role in parasite-host cell interactions.

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