THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 271, No. 34, Issue of August 23, pp. 20573–20579, 1996
Printed in U.S.A.

Deficiency in β1,3-Galactosyltransferase of a *Leishmania major* Lipophosphoglycan Mutant Adversely Influences the *Leishmania*-Sand Fly Interaction*

(Received for publication, May 29, 1996)

Barbara A. Butcher‡, Salvatore J. Turco§¶, Beth Ann Hilty§, Paulo F. Pimenta‖, Marialaura Panunzio, and David L. Sacks‡**

*From the ‡Laboratory of Parasitic Diseases, Intracellular Parasite Biology Section, National Institutes of Health, Bethesda, Maryland 20892, the §Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky 40536, the ‖Laboratorio de Parasitiologia, Centro de Biocencias e Biotecnologia, Universidade Estadual do Norte Fluminense, Rio de Janiero, Brazil, and the **Institute of Medical Microbiology, University of Bari, Bari, Italy*

To study the function of side chain oligosaccharides of the cell-surface lipophosphoglycan (LPG), mutagenized *Leishmania major* defective in side chain biosynthesis were negatively selected by agglutination with the monoclonal antibody WIC79.3, which recognizes the galactose-containing side chains of *L. major* LPG. One such mutant, called Spock, lacked the ability to bind significantly to midguts of the natural *L. major* vector, *Phlebotomus papatasi*, and to maintain infection in the sand fly after excretion of the digested bloodmeal. Biochemical characterization of Spock LPG revealed its structural similarity to the LPG of *Leishmania donovani*, a species whose inability to bind to and maintain infections in *P. papatasi* midguts has been strongly correlated with the expression of a surface LPG lacking galactose-terminated oligosaccharide side chains. An *in vitro* galactosyltransferase assay using wild-type or Spock membranes was used to determine that the defect in Spock LPG biosynthesis is a result of defective β1,3-galactosyltransferase activity as opposed to a modification of LPG, which would prevent it from serving as a competent substrate for galactose addition. The results of these experiments show that Spock lacks the β1,3-galactosyltransferase for side chain addition and that the LPG side chains are required for *L. major* to bind to and to produce transmissible infection in *P. papatasi*.

Protozoan parasites of the genus *Leishmania* spend the extracellular phase of their life cycle as flagellated promastigotes within the gut of their sand fly vectors. The lipophosphoglycan (LPG)[1] is the most abundant molecule on the surface of *Leishmania* promastigotes. The molecule consists of a neutral oligosaccharide cap followed by a backbone structure made up of repeating PO_4-6-Gal(β1, 4)Manα1 units, which are linked by a phosphosaccharide core to a phosphoinositide lipid anchor. Species-specific polymorphisms in LPG structure may occur in the structure of the cap and in the composition or number of oligosaccharide side chains that branch from the repeat units (1).

Three types of LPG have been described, based upon the nature of side chain substitutions (2). Type-1 LPG bears no side chains and is represented by the LPGs of the East African *Leishmania donovani* strains (Fig. 1) (3). Type-2 LPGs have glycan chains linked to the C-3 of galactose in the repeat units. Included in this group is *Leishmania mexicana* LPG, which bears glucose-containing side chains on approximately 30% of its repeats; *Leishmania tropica* LPG, which is highly substituted with over 19 different oligosaccharides (3, 4); and *Leishmania major* LPG, in which virtually every repeat is substituted with β1,3-galactose-terminated side chains (Fig. 1). Type-3 LPG is exemplified by *Leishmania aethiopica*. In this case, the C-2 position of mannose is substituted with a single α-mannose residue on approximately 35% of the repeat units.

LPG has been implicated as an adhesion molecule that mediates the interaction of procyclic promastigotes with the midgut epithelium of the sand fly vector (5). In order for *Leishmania* promastigotes to maintain infection in their sand fly vector, they must be anchored to the midgut epithelium during the passage of the digested bloodmeal. We previously demonstrated the significance of LPG polymorphisms for the establishment and maintenance of infection in *Leishmania*:vector pairs (6). Specifically, we showed that the β1,3-galactose-terminated side chains of *L. major* are critical for both *in vitro* binding to and *in vivo* infection of *Phlebotomus papatasi* midguts. The finding that only *L. major*, and not species which lack terminal β-galactose-containing side chains, is capable of this interaction implies the species-specific co-evolution of receptor:ligand pairs.

One approach to understanding how the structure of LPG dictates its function is to generate parasites that are deficient in distinct steps of LPG synthesis (6–10). The structural mutations of LPG can then be correlated with phenotypic characteristics such as infectivity for the vector (5, 6) and host (7). Using this strategy, the present report describes the biochemical and biological characterization of the *L. major* mutant, Spock. This represents the first description of a defect in LPG side chain biosynthesis resulting from a deficiency in β1,3-galactosyltransferase activity. The impact of this lesion, an impaired *Leishmania*-sand fly interaction, provides genetic evidence for previously published data that specific LPG oligosaccharides mediate the binding of *Leishmania* promastigotes to the sand fly midgut and that this trait alone can affect the ability to produce transmissible infections.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained as follows: medium 199, Dulbecco's modified Eagle's medium, glutamine, penicillin/streptomycin, and

* This work was supported in part by Grant R37 AI20491 (to S. J. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 606-323-6693; Fax: 606-323-1037; E-mail: turco@pop.uky.edu.

[1] The abbreviations used are: LPG, lipophosphoglycan; PG, phosphoglycan; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

HEPES from Life Technologies, Inc.; phenyl-Sepharose CL-4B, octyl-Sepharose CL-4B, UDP-galactose, GDP-mannose, *N*-methyl-*N*-nitroso-*N′*-nitroguanidine, adenine, and alkaline phosphatase (*Escherichia coli*) from Sigma; UDP-[^{3}H]galactose and [^{3}H]galactose from Amersham Corp.; [^{3}H]mannose from American Radiolabeled, Inc.; Sephadex G-150 from Pharmacia Biotech Inc.; AG-1 and AG-50 from Bio-Rad; peanut agglutinin and *Ricin communis* agglutinin from Vector Laboratories (Burlingame, CA); and phycoerythrin-conjugated goat anti-mouse IgG from Molecular Probes, Inc. Phosphatidylinositol-specific phospholipase C was purified from *Bacillus thuringiensis* (8).

Strains—L. major promastigotes (World Health Organization designation MHOM/IL80/Friedlin, clone V1) were cultivated in medium 199 supplemented with 20% (v/v) fetal calf serum, 20 mM HEPES, 100 μM adenine, 2 mM glutamine, penicillin (1000 units/ml), and streptomycin (50 units/ml). Promastigotes were passed daily to ensure exponential growth.

Generation of LPG Mutants—Procyclic parasites were mutagenized according to the protocol of Iovannisci *et al.* (11). Approximately 10^8 parasites (10^6/ml) were incubated in DMEM containing 8 μg/ml *N*-methyl-*N*-nitroso-*N′*-nitroguanidine (4 h, room temperature). The parasites were washed twice with fresh DMEM and cultured for 1 week in complete medium before selection. Parasites mutant in lipophosphoglycan side chain biosynthesis were negatively selected by agglutination with the monoclonal antibody WIC79.3 (12). Promastigotes from log phase cultures were washed twice with cold DMEM and resuspended at 10^7/ml DMEM containing a 1:100 dilution of WIC79.3 ascites fluid. The suspension was incubated for 0.5 h at room temperature, and the agglutinated parasites were pelleted by low speed centrifugation (1000 rpm, 10 min). The supernatant was collected and centrifuged (3000 rpm, 10 min) to pellet non-agglutinated (*i.e.* WIC79.3 negative) parasites that were then placed into complete medium and further subjected to 10 rounds of selection. The WIC79.3 negative parasites were cloned by limiting dilution and analyzed by FACS for WIC79.3 reactivity.

FACS Analysis of LPG Expression—Log phase promastigotes were washed once with FACS buffer (phosphate-buffered saline, pH 7.2, 5% fetal calf serum). Washed parasites (10^6/ml) were incubated (0.5 h, on ice) with 3 μg/ml WIC79.3 Fab′ fragments in round-bottom microtiter plates. The parasites were washed twice with FACS buffer and incubated (0.5 h, on ice) with fluorescein-conjugated goat anti-mouse IgG Fab′ (Jackson ImmunoResearch Laboratories). After incubation with the second stage antibody, the parasites were washed twice and resuspended in FACS buffer containing 2% formaldehyde. Stained parasites were analyzed for fluorescence using a Becton Dickinson FACScan (San Jose, CA) and CellQuest software.

Agglutination Assays—Antibodies or lectins (50-μl aliquots) were serially diluted with DMEM containing 2% bovine serum albumin into flat-bottomed Immulon-2 microtiter plates. Washed parasites were added in equal volume (final density, 2–3 × 10^6 cells/ml) and gently mixed. The plates were incubated for 0.5 h at room temperature, and the percentage of free parasites compared to control wells (without lectin or antibody) was determined.

Metabolic Labeling and Extraction of LPG—Exponentially growing cells (1–3 × 10^9) were metabolically labeled with [^{3}H]galactose (100 μCi) in Dulbecco's modified Eagle's medium for 2 h at room temperature. LPG was extracted and solubilized in Solvent E (H_2O:ethanol/diethylether/pyridine/NH_4OH, 15:15:5:1:0.017) as described elsewhere (13). The LPG extract was evaporated to dryness under N_2, resuspended in 3 ml of 0.1 M NaCl, 0.1 M acetic acid, and applied to a 1-ml phenyl-Sepharose column equilibrated in the same buffer. The column was washed sequentially with 3 ml of equilibration buffer, 2 ml of 0.1 M acetic acid, and 1 ml of H_2O. LPG was eluted with Solvent E and evaporated to dryness (13).

Gel Filtration of LPG—The lipid anchor of LPG was removed by resuspending lyophilized LPG in CHAPS buffer (50 mM HEPES, 5 mM EDTA, 0.2% CHAPS, pH 7.4) and incubating with phosphatidylinositol-specific phospholipase C (37 °C, 24 h). The resultant phosphoglycan (PG) was dialyzed against water, lyophilized, and resuspended in 40 mM NH_4OH/1 mM EDTA. The PG was applied to a Sephadex G-150 column (1 × 85 cm) and eluted with 40 mM NH_4OH, 1 mM EDTA. Fractions were collected (0.6 ml) and counted by liquid scintillation. Void and retention volumes were determined with blue dextran and [^{3}H]mannose, respectively.

Separation and Detection of LPG Oligosaccharides—Purified LPG was depolymerized by mild acid hydrolysis (0.02 N HCl, 5 min, 100 °C), and the aqueous-soluble fragments were treated with 0.25 units of *E. coli* alkaline phosphatase (37 °C, 18 h). The dephosphorylated fragments were labeled (37 °C, 18 h) with 8-aminonaphthalene-1,3,6-trisulfate and subjected to electrophoresis using the *O*-linked oligosaccharide profiling gel according to the manufacturer's instructions (GLYKO-FACE electrophoresis products, GLYKO, Inc. Novato, CA). The fluorophore-labeled fragments were visualized with a GLYKO UV imager.

Quantification of Repeat Units per LPG—Purified LPG (0.5 mg) was deaminated by incubation (18 h, 37 °C) with 300 μl of 0.5 M $NaNO_2$ and 300 μl of 0.25 M sodium acetate to remove the phosphatidylinositol anchor and generate the phosphoglycan derivative containing 2,5-anhydromannose at the reducing end. The 2,5-anhydromannose residue was reduced to 2,5-anhydromannitol with the addition of 2 mg/ml $NaBH_4$ in 1 M NH_4OH, and then the PG was hydrolyzed with 2 N trifluoroacetic acid to generate phosphorylated and neutral monosaccharides. Phosphorylated monosaccharides (primarily galactose 6-phosphate) were removed by anion exchange chromatography, and the neutral monosaccharides were subjected to alditol acetate derivatization in preparation for gas chromatography-mass spectroscopy. Briefly, monosaccharides were reduced with 2 mg/ml $NaBH_4$ in 1 M NH_4OH (2 h, room temperature), and borate was removed by the repeated addition of methanol:acetic acid (9:1, v/v) and evaporation. Dried samples were *O*-acetylated by incubation (20 min, 121 °C) with 0.1 ml of acetic anhydride and 0.1 ml of pyridine and dried under nitrogen. The derivatized monosaccharides were dissolved in 1 ml of dichloromethane and extracted three times with water. The lower phase was dried under nitrogen at room temperature and resuspended in methanol for gas chromatography using an SP2380 column.

In Vitro LPG Biosynthesis—Parasites were harvested at a density of 3–5 × 10^7 cells/ml, and membranes were prepared as described previously (14). Standard incubation mixtures (400 μl) contained 0.4 mg of membrane protein, 50 mM HEPES (pH 7.4), 25 mM KCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 1 μg/ml leupeptin, 1 mM ATP, 0.5 mM dithiothreitol, and 4 μCi UDP-[^{3}H]galactose (specific activity, 15.3 Ci/mmol). For experiments in which LPG was used as an exogenous acceptor for side chain addition, 200 μg of purified LPG from either *L. donovani* or Spock was added. To determine the ability of isolated membranes to synthesize complete LPG, 10 μM GDP-mannose was included in the assay. The mixture was incubated for 1 h at 26 °C. The reaction was terminated with the addition of 3 volumes of distilled water, and the membranes were pelleted in a microcentrifuge. LPG was released from the membrane pellet and depolymerized by hydrolysis with 40 mM trifluoroacetic acid (100 °C, 8 min). The hydrolysate was lyophilized to remove trifluoroacetic acid, and samples were resuspended in 15 mM Tris (pH 9) and dephosphorylated with alkaline phosphatase. The samples were desalted over Bio-Rad AG1-X8 and AG50-X12 columns and resuspended in MeOH:H_2O (1:1) for application to a Whatman 3-mm chromatography paper and run for 36 h in a descending paper chromatography tank pre-equilibrated with 1-butanol:pyridine:H_20 (6:4:3). The paper was cut into 1-cm segments and counted by liquid scintillation. Migration distances were compared to oligosaccharide standards, which were visualized using the periodic acid/silver nitrate method (15).

Sand Fly Infection and Dissection—P. papatasi sand flies were reared and maintained in the Department of Entomology, Walter Reed Army Institute of Research. 3–5-day-old female sand flies were fed through a chick skin membrane (16) on a mixture of heparinized mouse blood containing 10^6 procyclic promastigotes per milliliter. For infections using procyclic promastigotes, the red blood cells were washed twice in 0.86% NaCl and added back to heat-inactivated (56 °C, 45 min) plasma. Blood-engorged sand flies were separated and maintained at 28 °C with 30% sucrose. At various times after feeding, the flies were anesthetized with CO_2, and their midguts were dissected. The number of midgut promastigotes in infected flies was determined by placing individual midguts into a microcentrifuge tube containing 30 μl of phosphate-buffered saline, pH 7.4. Each gut was homogenized with a Teflon-coated microtissue grinder, and released promastigotes were counted in a hemocytometer.

In Vitro Assay for Promastigote Binding to Sand Fly Midguts—Binding of promastigotes to sand fly midguts was quantitated by a modification of an *in vitro* technique (6). 3–5-day-old nonfed female sand flies, maintained on 30% sucrose, were dissected in phosphate-buffered saline. Heads, crops, hindguts, and Malpighian tubules were removed, and the isolated midguts were opened along the length of the abdominal segment with a fine needle. Midguts (7–10 per group) were placed in the concave wells of a microscope chamber slide. *Leishmania* promastigotes (2.5 × 10^7) in 40 μl were added to the guts and incubated for 45 min at room temperature. The guts were then individually washed by placing them in successive drops of phosphate-buffered saline. Guts were homogenized, and released promastigotes were counted as described above.

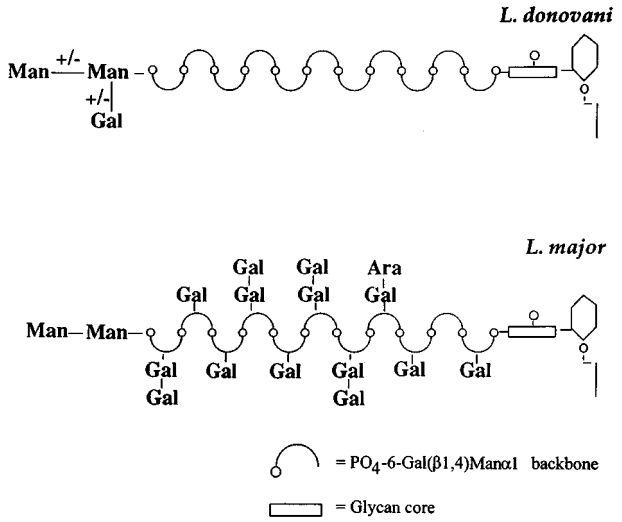

FIG. 1. **Schematic diagram of structures of LPG from *L. donovani* and *L.major*.** The structures shown are modified from Thomas *et al.* (3) and McConville *et al.* (4). The structure of the glycan core is Gal(α1,6)Gal(α1,3)Gal$_f$(β1,3)[Glc(α1-PO$_4$)→6]Man(α1, 3)Man(α1,4)GlcN(α1,6) and is linked to a 1-*O*-alkyl-2-*lyso*-phosphatidylinositol anchor. The galactose residues of the side chains of the *L. major* LPG are in β1,3-linkage.

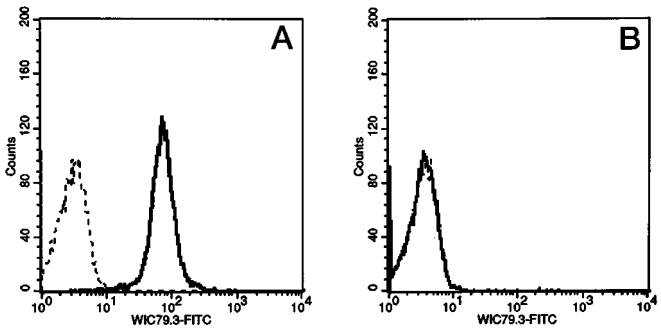

FIG. 2. **Expression of the WIC79.3 epitope by wild-type and Spock promastigotes.** Wild-type (*A*) and Spock (*B*) promastigotes were incubated with control antibody (*dotted lines*) or WIC79.3 (*solid lines*). Primary antibody binding was revealed using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG.

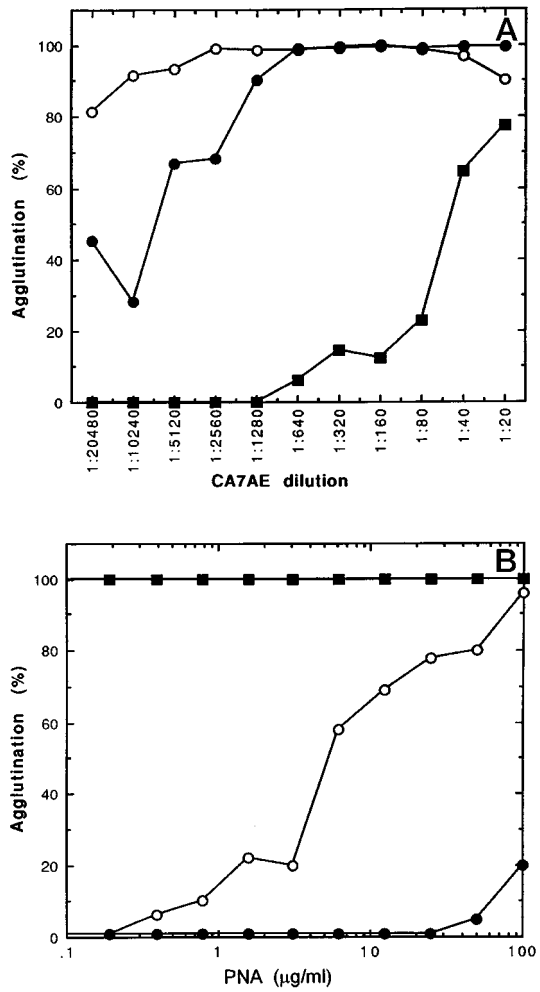

FIG. 3. **Agglutination profiles of wild-type, Spock, and *L. donovani* promastigotes.** CA7AE (*A*) or peanut agglutinin (*B*) was serially diluted into microtiter wells, and wild-type (*closed squares*), Spock (*closed circles*), or *L. donovani* (*open circles*) were added to a final concentration of 2×10^6 cells/ml. After incubation at room temperature, percent agglutination was determined by counting free parasites in each well compared to the control well containing no antibody or lectin.

RESULTS

Generation of LPG-defective Mutants—*L. major* procyclic promastigotes were mutagenized with nitrosoguanidine and subjected to several rounds of negative selection with the monoclonal antibody WIC79.3. This antibody recognizes the mono- and di-β1,3-galactose side chains of wild-type LPG (Fig. 1) (12, 17). Selection enrichment was monitored by FACS analysis, and WIC79.3 negative parasites were cloned by limiting dilution. Fig. 2 compares by FACS analysis the WIC79.3 reactivity of the wild-type *L. major* (Fig. 2*A*) to that of one mutant, Spock (Fig. 2*B*), which was chosen for further analysis. Spock has maintained its WIC79.3 negative phenotype through daily passage in laboratory culture. Species typing analyses by polymerase chain reaction amplification of the mini-exon repeats and by isoenzymes validated that Spock was derived from the parental *L. major* strain.

Lectin and Antibody Agglutination—Spock's lack of WIC79.3 reactivity could be explained either by a complete absence of LPG or by the loss of the specific WIC79.3 epitope on LPG. To discriminate between these possibilities, an agglutination assay with the monoclonal antibody CA7AE was performed (Fig. 3*A*). CA7AE was raised against *L. donovani* LPG (Fig. 1) and recognized phosphorylated disaccharide repeats that were unmodified by side-chain oligosaccharides (18). As expected, *L. donovani* promastigotes were agglutinated at all CA7AE dilutions tested. Appreciable agglutination of wild-type *L. major* parasites did not occur because the abundant, β1,3-galactose-containing side chains of its LPG mask the CA7AE epitope. In contrast, Spock is significantly agglutinated by high dilutions (1:5000) of CA7AE ascites. This result indicates that Spock synthesizes LPG that is relatively unmodified by side-chain oligosaccharides, similar to that of *L. donovani* LPG.

Fig. 3*B* shows the results of an agglutination assay with peanut lectin (peanut agglutinin). Peanut agglutinin recognizes terminal galactose residues in a β1,3-linkage to the penultimate sugar (galactose, in the case of *L. major* LPG) (19). While wild-type *L. major* parasites were completely agglutinated at all lectin concentrations tested, Spock parasites remained unagglutinated at concentrations as high as 50 μg/ml, indicating the absence of terminally exposed β-galactose residues. This is in contrast to the sensitivity of *L. donovani* to peanut lectin, which agglutinates the parasite at concentrations above 6 μg/ml, reflecting the presence of a terminal β-galactose residue in the neutral cap.

Characterization of in Vivo Labeled LPG from Spock—The relative ability of Spock to synthesize LPG was examined by metabolic labeling of the cells with [^{3}H]galactose and [^{3}H]

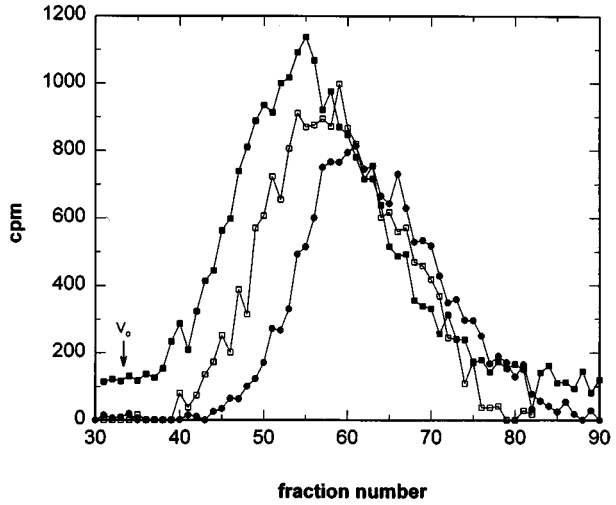

FIG. 4. **Sephadex G-150 chromatography of wild-type, Spock, and *L. donovani* PG.** PG was purified from wild-type *L. major* (*closed squares*), Spock (*open squares*), and *L. donovani* (*closed circles*) and chromatographed on Sephadex G-150.

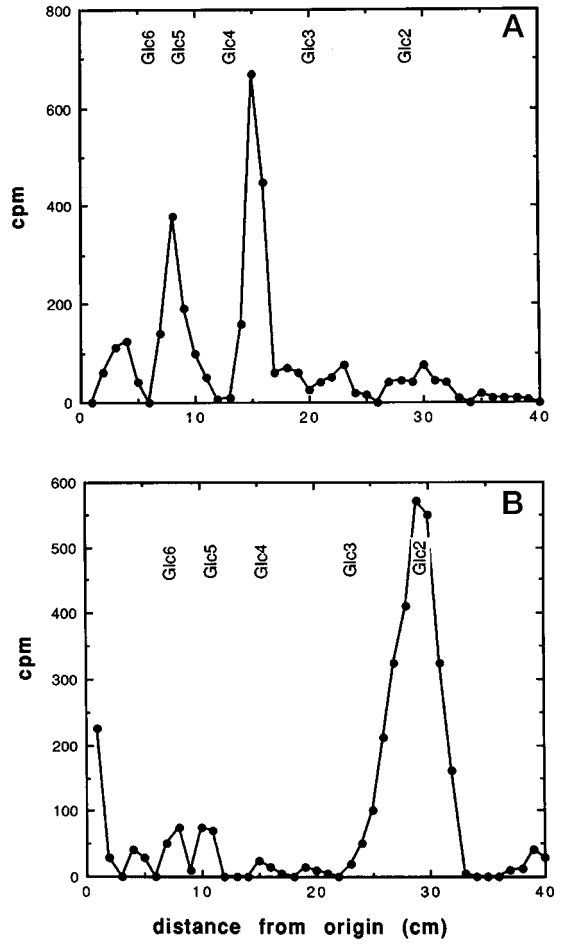

FIG. 5. **Paper chromatography of LPG recovered from *in vivo* [^{3}H]galactose labeling of promastigotes.** Wild-type (*A*) or Spock (*B*) parasites were incubated with [^{3}H]galactose, and LPG was extracted as described under "Experimental Procedures." The LPG was subjected to mild acid hydrolysis and alkaline phosphatase treatment prior to paper chromatography of the oligosaccharide fragments. *Glc6* to *Glc2* represent the migration distance of oligoglucose standards.

mannose, extraction of LPG by organic solvents, and purification of the glycoconjugate on a column of phenyl-Sepharose. Incorporation of radioactive galactose and mannose precursors

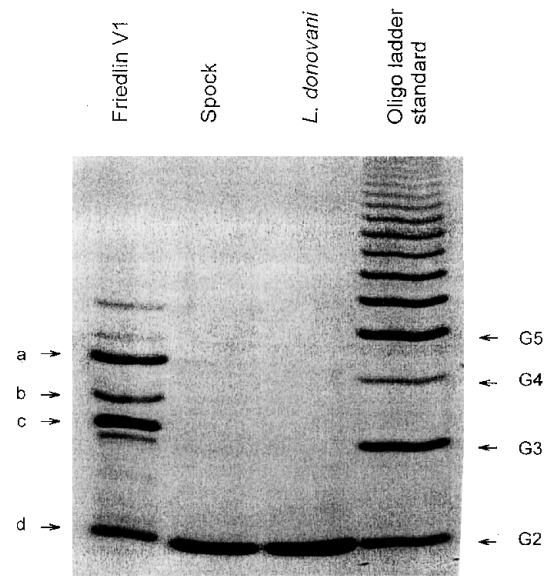

FIG. 6. **Glyko-FACE analysis of oligosaccharide fragments.** LPG-isolated wild-type *L. major* (*lane 1*), Spock (*lane 2*), and *L. donovani* (*lane 3*) were depolymerized with mild acid, derivatized with 8-aminonaphthalene-1,3,6-trisulfate, and electrophoresed. *Lane 4* contains oligoglucose standards. *Arrows* indicate the migration of oligosaccharide standards: *a*, Gal-Gal-Gal-Man; *b*, Ara-Gal-Gal-Man; *c*, Gal-Gal-Man; *d*, Gal-Man. *G5* to *G2* represent the migration distance of oligoglucose standards.

showed a slight decrease and increase, respectively, into Spock LPG compared to wild type (data not shown). To obtain information regarding the relative sizes of the radioactive LPGs, wild-type *L. major*, Spock, and *L. donovani* LPGs were delipidated by nitrous acid deamination and then were subjected to gel filtration on Sephadex G-150 (Fig. 4). Wild-type *L. major* PG eluted first, followed by Spock and then *L. donovani*, indicating that the wild-type *L. major* synthesizes a slightly larger glycoconjugate than either the mutant or *L. donovani*. In addition, the length of each PG was assessed by determining the ratio of mannose:2,5-anhydromannose (27.4 (wild-type *L. major*), 20.0 (Spock), and 16.4 (*L. donovani*)). These ratios reflect the relative number of repeats in LPG assuming a similar number of mannose residues in the glycan core and cap domains.

Characterization of the Repeating Units of Spock LPG—To confirm that Spock produces an LPG defective in the synthesis of side chain sugars recognized by WIC79.3 and peanut agglutinin, [^{3}H]galactose-labeled LPGs from wild-type and Spock cells were depolymerized with mild acid to selectively cleave the sugar-1-phosphate bonds of the repeating units of LPG, generating phosphooligosaccharides. After dephosphorylation of the sugars with alkaline phosphatase, the neutral oligosaccharide fragments were resolved by descending paper chromatography along with appropriate standards (Fig. 5). Based on published data (3, 20–22), the major ^{3}H-labeled fragments (*i.e.* repeat units) from the wild-type LPG migrated in a manner consistent with the trisaccharide Gal-Gal-Man (Fig. 5*A*, *fractions 14–16*) and a mixture of tetrasaccharides, Gal-Gal-Gal-Man and Ara-Gal-Gal-Man (Fig. 5*A*, *fractions 7–10*). There were lesser amounts of other oligosaccharides. In contrast, Spock LPG revealed a single major [^{3}H]galactose-labeled fragment that co-eluted with the standard Gal-Man disaccharide (Fig. 5*B*, *fractions 25–32*).

Confirmatory information about the repeating units was obtained by FACE analysis. LPGs from wild-type *L. major*, *L. donovani*, and Spock cells were subjected to mild acid hydrolysis. Aliquots of the hydrolysate were dephosphorylated with

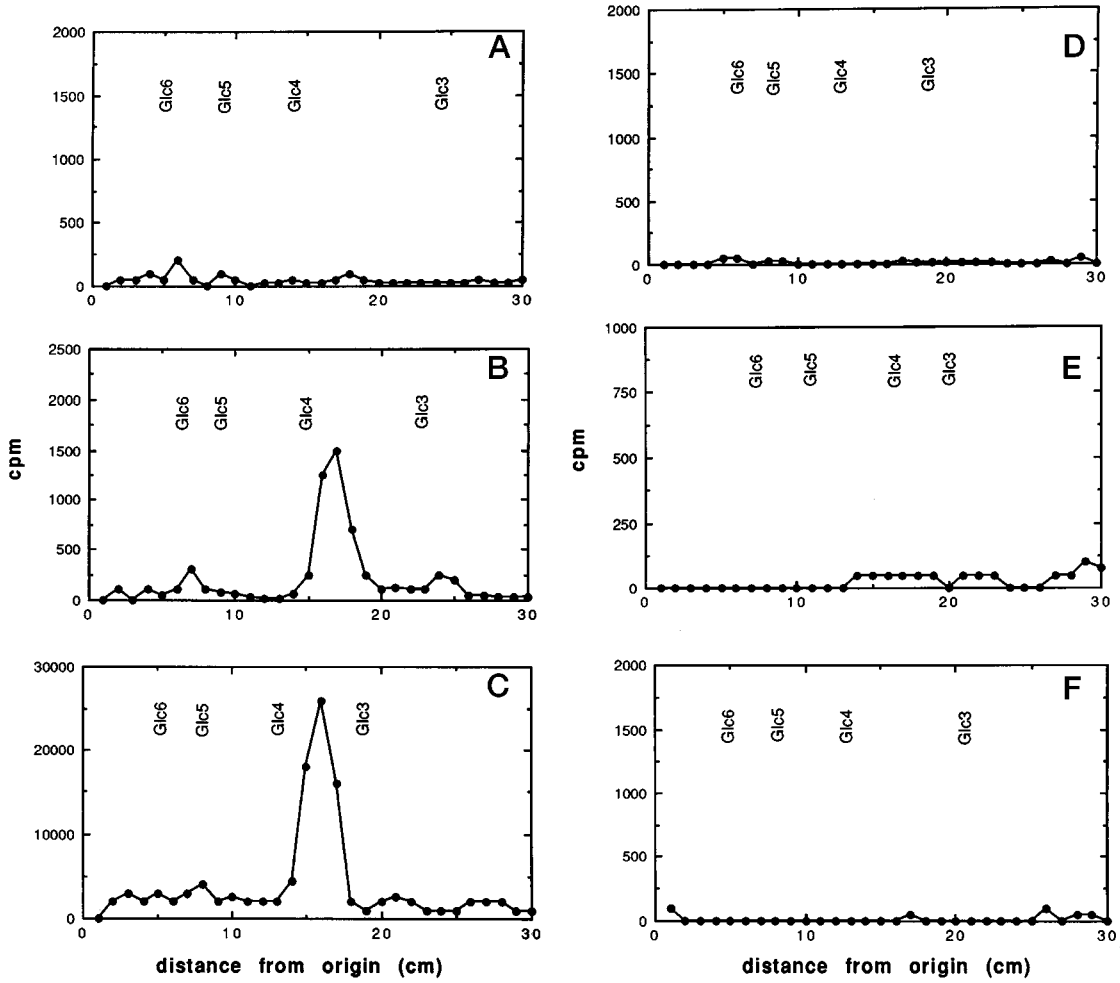

FIG. 7. **Ability of wild type and Spock to add side chains to exogenously supplied LPG.** Membranes isolated from wild-type *L. major* (*A–C*) or Spock (*D–F*) promastigotes were incubated in the absence (*A*, *D*) or presence of exogenously added *L. donovani* LPG (*B*, *E*) or Spock LPG (*C*, *F*). LPG was extracted from the membranes, depolymerized, and alkaline phosphatase-treated prior to paper chromatography. *Glc6* to *Glc3* indicate migration distance of oligoglucose standards.

alkaline phosphatase. The fragments were derivatized at the reducing ends with a fluorophore and subjected to electrophoresis. Fluorescent bands were visualized by digital UV imaging (Fig. 6). In *lane 1*, the most prominent fragment is the trisaccharide from the wild-type *L. major* LPG, with lesser amounts of two tetrasaccharides and the Gal-Man disaccharide. The characteristic disaccharide repeating unit (Gal-Man) of the *L. donovani* LPG is shown in *lane 3*. The pattern of fragments from Spock LPG (*lane 2*) is virtually identical to that of *L. donovani*, indicating the lack of side chains. There was no change in the pattern of Spock LPG fragments as a function of the growth phase (data not shown).

Analysis of the Defect in a Cell-free System of LPG Biosynthesis—The results from experiments using intact Spock cells suggested that the LPG from these mutants did not have the characteristic side chain sugars initiated by a β1,3-galactose addition to the PO_4-Gal-Man backbone units. To investigate if the defect was due to the absence of β1,3-galactosyltransferase activity or a possible modification of the LPG that precludes side chain addition, a cell-free glycosylation assay was used (14). Membranes from wild-type *L. major* or Spock were incubated with exogenous Spock or *L.donovani* LPG and UDP-[^{3}H]galactose. The repeating units of the prelabeled LPG were analyzed for the presence of oligosaccharide side chains by subjecting the material to mild acid hydrolysis and dephosphorylation with alkaline phosphatase. The fragments generated were resolved by descending paper chromatography.

When wild-type or Spock membranes were incubated in the absence of exogenous LPG (Fig. 7, *A* and *D*, respectively), no incorporation of [^{3}H]galactose from UDP-[^{3}H]galactose was observed. This experiment confirms the lack of *de novo* LPG biosynthesis in the absence of exogenously added GDP-mannose. Analysis of fragments from both exogenously supplied *L. donovani* and Spock LPG (Fig. 7, *B* and *C*, respectively) that had been incubated with wild-type *L. major* membranes revealed the presence of trisaccharide fragments, indicating that both LPGs are competent acceptors for β1,3-galactose addition. In contrast, analysis of LPG fragments generated from the incubation of Spock and *L. donovani* LPG with Spock membranes showed no trisaccharides, indicating that the Spock membranes lack the enzyme for side chain addition (Fig. 7, *E* and *F*, respectively). However, Spock membranes are enzymatically active and capable of *in vitro* assembly of the PO_4-Gal-Man repeat units when both UDP-[^{3}H]galactose and GDP-mannose are included in the incubation medium. Incorporation of the radiolabel into wild-type and Spock LPG was similar (44,600 cpm *versus* 66,680 cpm, respectively). Analysis of the wild-type LPG fragments showed the presence of tri- and tetrasaccharides (Fig. 8*A*), while fragments generated with Spock membranes were predominantly disaccharides (Fig. 8*B*).

In Vitro Binding of Spock to Sand Fly Midguts—Isolated *P. papatasi* midguts were incubated with wild-type *L. major* or Spock procyclic promastigotes. After washing, the guts were homogenized, and released parasites were counted. The results

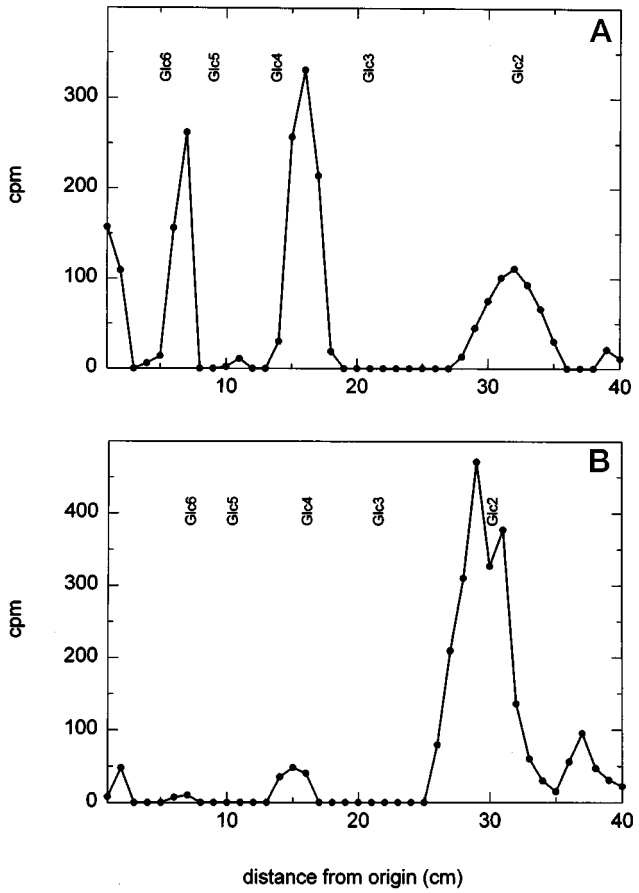

FIG. 8. **Ability of wild-type and Spock membranes to synthesize LPG *in vitro*.** LPG was extracted from wild-type (*A*) and Spock membranes (*B*), depolymerized, and alkaline phosphatase-treated prior to paper chromatography. *Glc6* to *Glc2* indicate migration distance of oligoglucose standards.

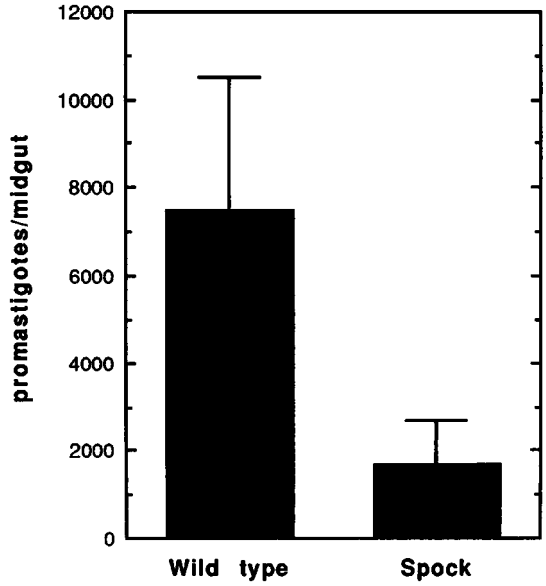

FIG. 9. **Attachment of wild-type and Spock promastigotes to *P. papatasi* midguts *in vitro*.** *P. papatasi* midguts were dissected and incubated with promastigotes, and binding was determined as described under "Experimental Procedures."

are shown in Fig. 9. The average number of wild-type promastigotes per midgut was approximately 7,500, while Spock exhibited significantly less binding (approximately 1,800 parasites/midgut).

Survival of Spock in P. papatasi—2 days after sand flies were membrane-fed on bloodmeals containing wild-type *L. major* or Spock, all flies in each group harbored similar numbers of viable promastigotes (data not shown). By day 5 postinfection, after passage of the digested bloodmeal, the parasite burden differed substantially in the three groups (wild type, 43,000 parasites/midgut; Spock, 800 parasites/midgut; *L. donovani*, 28 parasites/midgut), indicating that Spock, like *L. donovani*, is unable to maintain significant infection in *P. papatasi* vector (Fig. 10).

DISCUSSION

The ability of *Leishmania* parasites to bind to the sand fly midgut during excretion of the digested bloodmeal is essential for the development of transmissible infections. Sand fly vectors can, in some instances, transmit only certain species of *Leishmania*. Such species-specific differences in vectorial competence have been directly correlated with the ability of promastigotes to attach to the sand fly midgut, the variable outcomes of which are controlled by structural polymorphisms in LPG. The leishmanial receptor of *P. papatasi* is highly specific for the galactose-terminated oligosaccharide branches of *L. major* LPG (6). *L. major*, being the only Old World *Leishmania* to express abundant side chains terminating in galactose, appears to have yielded to a selective pressure that enables it to exploit a widespread phlebotomine species that is otherwise refractory to parasites expressing relatively unsubstituted LPGs.

The present report describes the interaction of a *L. major* LPG mutant, defective in β1,3-galactose addition, with the sand fly vector. This work furnishes genetic evidence for the previously published contention that the binding of *L. major* promastigotes to the midgut epithelium is mediated by the extensive side chain oligosaccharides (5, 6). The mutant, Spock, was generated by nitrosoguanidine treatment of wild-type *L. major* and negative selection with the monoclonal antibody WIC79.3. This monoclonal antibody is specific for the galactose-terminated oligosaccharide side chains of *L. major* LPG (7) and does not recognize this moiety on other glycoconjugates such as glycoproteins (23). Therefore, the use of WIC79.3 allowed us to specifically isolate parasites defective in LPG side-chain biosynthesis. The lack of these side chains diminished Spock's ability to bind to *P. papatasi* midguts in *in vitro* binding assays and to maintain infections in sand flies after passage of the bloodmeal. Thus, the mutant *L. major* Spock displayed a biological phenotype similar to that of *L. donovani*. The data provide strong evidence that the inability of *L. donovani* to utilize *P. papatasi* as natural vector can be explained solely on the basis of its LPG lacking appropriate side-chain additions, regardless of any other interspecies molecular differences that might exist.

Analysis of depolymerized, dephosphorylated LPG fragments from Spock indicated that the defect in Spock LPG biosynthesis lies in the β1,3 addition of galactose to the 3-hydroxyl of the galactose residue in the disaccharide repeats of the LPG backbone. Membranes from wild-type *L. major* but not from Spock were able to add galactose-initiated side chains to Spock LPG, demonstrating that the molecule is a competent substrate for oligosaccharide modification. It follows that the genetic defect of Spock involves the deficiency of a glycosylation enzyme (*i.e.* the absence of a β1,3-galactosyltransferase) rather than a covalent modification of the Gal-Man repeat unit, which would prevent side chain addition to the galactose residue. The lack of β1,3-galactosyltransferase activity in *L. donovani* has been demonstrated previously by Ng and co-workers (14).

A mammalian β1,3-galactosyltransferase has been de-

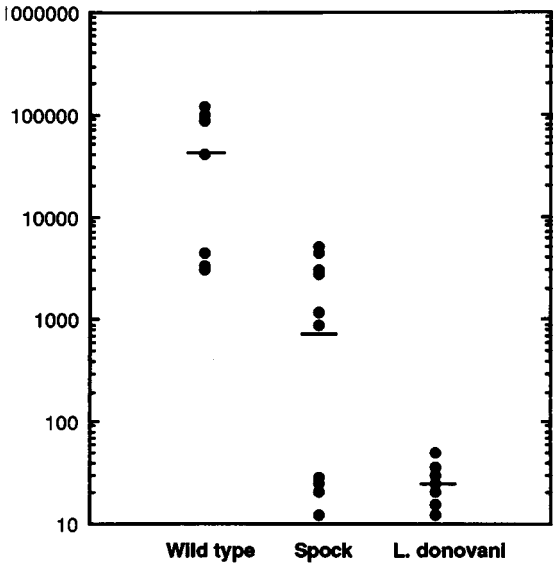

FIG. 10. **Growth of wild-type *L. major*, Spock, and *L. donovani* in the digestive tract of individual sand flies.** 5 days after membrane feeding *P. papatasi* bloodmeals containing promastigotes, midguts were removed and parasites were counted. *Bar* indicates average number of parasites per midgut.

scribed, the deficiency of which leads to Tn-syndrome (24, 25). When the Tn antigen (*O*-linked α*N*-galactosamine) or its sialylated derivative is not β1,3-galactosylated, hematopoietic cells bearing the antigen bind serum anti-α-GalNAc antibodies, causing the mild hemolytic anemia and thrombocytopenia observed in patients with this rare acquired disorder. Preliminary studies indicate that the transferase deficiency in Tn-syndrome results from the silencing of the galactosyltransferase gene or possibly its transcriptional control unit by DNA methylation. However, the enzyme has not been isolated nor has its gene been cloned, so molecular studies of the deficiency have not been performed.

Spock represents the first example of a *Leishmania* mutant defective in assembly of side chain sugars of LPG. Thus, Spock provides an important tool for the delineation of the biosynthetic pathway and functional significance of LPG. Experiments are currently underway to isolate the galactosyltransferase gene by functional complementation of Spock with wild-type *L. major* DNA using methods that enabled the isolation and characterization of the putative galactofuranosyltransferase gene of *L. donovani* (10).

Acknowledgments—We acknowledge Govind Modi for providing sand flies for this study, Dr. Herbert Avila and Ya Luo for species typing by polymerase chain reaction amplification, and Dr. R. Kreutzer for isoenzyme analysis.

REFERENCES

1. Turco, S. J., and Descoteaux, A. (1992) *Annu. Rev. Microbiol.* **46,** 65–94
2. McConville, M. J., Schnur, L. F., Jaffe, C., and Schneider, P. (1995) *Biochem. J.* **307,** 807–818
3. Thomas, J. R., McConville, M., Thomas-Oates, J. E., Homans, S. W., Ferguson, M. A. J., Gorin, P. A. J., Greis, K. D., and Turco, S. J. (1992) *J. Biol. Chem.* **267,** 6829–6833
4. McConville, M. J., Thomas-Oates, J. E., Ferguson, M. A. J., and Homans, S. W. (1990) *J. Biol. Chem.* **265,** 19611–19623
5. Pimenta, P., Turco, S. J., McConville, M. J., Lawyer, P., Perkins, P., and Sacks, D. L. (1992) *Science* **256,** 1812–1815
6. Pimenta, P. F. P., Saraiva, E. M. B., Rowton, E., Modi, G. B., Garraway, L. A., Beverley, S. M., Turco, S. J., Sacks, D. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9155–9159
7. Cappai, R., Morris, L., Aebischer, T., Bacic, A., Curtis, J. M., Kelleher, M., McLeod, K. S., Osborn, A. H., and Handman, E. (1994) *Parasitology* **108,** 397–405
8. Low, M. G., and Weglicki, W. B. (1983) *Biochem. J.* **215,** 325–334
9. Elhay, M., Kelleher, M., Bacic, A., McConville, M. J., Tolson, D. L., Pearson, T. W., and Handman, E. (1990) *Mol. Biochem. Parasitol.* **40,** 255–268
10. Ryan, K. A., Garraway, L. A., Descoteaux, A., Turco, S. J., and Beverley, S. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8609–8613
11. Iovannisci, D. M., Goebel, D., Allen, K., Kaur, K., and Ullman, B. (1984) *J. Biol. Chem.* **259,** 14617–14623
12. Kelleher, M., Bacic, A., and Handman, E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6–10
13. Orlandi, P. A., Jr., and Turco, S. J. (1987) *J. Biol. Chem.* **262,** 10384–10391
14. Ng, K., Handman, E., and Bacic, A. (1994) *Glycobiology* **4,** 845–853
15. Anet, E. F. L. J., and Reynolds, T. M. (1954) *Nature* **174,** 930–932
16. Tesh, R. B., and Modi, G. M. (1987) *Am J. Trop Med. Hyg.* **33,** 41–46
17. Kelleher, M., Curtis, J. M., Sacks, D. L., Handman, E., and Bacic, A. (1994) *Mol. Biochem. Parasitol.* **66,** 187–200
18. Tolson, D. L., Schnur, L. F., Jardim, A., and Pearson, T. W. (1994) *Parasitol. Res.* **80,** 537–542
19. Sacks, D. L., Heiny, S., and Sher, A. (1985) *J. Immunol.* **135,** 564–569
20. McNeely, T. B., Tolson, D. L., Pearson, T. W., and Turco, S. J. (1990) *Glycobiology* **1,** 63–69
21. Turco, S. J. (1989) *Exp. Parasitol.* **70,** 241–245
22. McConville, M. J., and Bacic, A. (1990) *Mol. Biochem. Parasitol.* **38,** 57–58
23. Handman, E., Hocking, R. E., Mitchell, G. F., and Spithill, T. W. (1984) *EMBO J.* **3,** 2301–2306
24. Thurnher, M., Rusconi, S., and Berger, E. G. (1993) *J. Clin. Invest.* **91,** 2103–2110
25. Berger, E. G., Thurnher, M., and Dinter, A. (1994) *Trends Cell Biol.* **2,** 103–108

Deficiency in β1,3-Galactosyltransferase of a *Leishmania major* Lipophosphoglycan Mutant Adversely Influences the *Leishmania*-Sand Fly Interaction

Barbara A. Butcher, Salvatore J. Turco, Beth Ann Hilty, Paulo F. Pimenta, Marialaura Panunzio and David L. Sacks

J. Biol. Chem. 1996, 271:20573-20579.
doi: 10.1074/jbc.271.34.20573