Stage-Specific Variations in Lectin Binding to *Leishmania donovani*

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Visceral leishmaniasis is caused by the dimorphic protozoan *Leishmania donovani*, which exists as an aflagellar amastigote within mammalian mononuclear phagocytes and as a flagellated extracellular promastigote in its sandfly vector. We have identified four plant lectins that bind to the *L. donovani* surface, and through these we have documented stage-specific differences in exposed surface carbohydrates. Concanavalin A bound to both promastigotes and amastigotes; binding was inhibited by mannose or α-methyl-mannoside, implying a mannose-containing residue on the surface of both parasite stages. *Ricinus communis* agglutinin, which binds to galactose-containing residues, also bound to both stages and was inhibited by lactose, implying a galactose-containing glycoconjugate on the parasite surface. Two other lectins, wheat germ agglutinin (WGA) and peanut agglutinin (PNA), exhibited stage specificity in their binding characteristics. Amastigotes bound WGA but not PNA. During the process of conversion from the amastigote to the promastigote stage, the WGA-binding glycoconjugate was lost, and a PNA-binding residue was newly displayed. WGA binding was inhibited by N-acetyl-D-glucosamine and was not altered by neuraminidase treatment, suggesting the presence of an exposed N-acetyl-D-glucosamine moiety on the amastigote surface. The PNA binding site is known to accommodate the oligosaccharide β-D-galactose-(1→3)-N-acetyl-D-galactosamine; in our system, PNA may have identified an internal rather than a terminal galactose on the promastigote surface. Localized binding of WGA and PNA to the surface of intermediate phases of the parasite suggested inhomogeneous and changing surface characteristics during conversion from amastigote to promastigote stages. This evolution of *L. donovani* surface glycoconjugates may be important in the adaptation of the organism to its divergent mammalian host and arthropod vector environments.

Visceral leishmaniasis, or kala azar, is a well-recognized cause of mortality in many parts of the world including Asia, Africa, Europe, and South America. The causative protozoan, *Leishmania donovani*, has a biphasic life cycle. In humans it is found exclusively in mononuclear phagocytes throughout the reticuloendothelial system as an amastigote, a spherical or ovoid form 2 to 3 μm in diameter. Upon ingestion by the sandfly vector, the protozoan converts to its extracellular stage, the promastigote, a spindly-shaped flagellated form 10 to 15 μm in length. The promastigote is subsequently inoculated by the sandfly back into the mammalian host, whereupon it converts to the amastigote stage, probably within mononuclear phagocytes. Once the process is initiated, parasites spread to adjacent mononuclear phagocytes, eventually leading to clinical disease.

There remain numerous questions concerning the life cycle and mechanism of infection by this important pathogen. The widely divergent arthropod vector and mammalian host environments require an extreme adaptability on the part of the organism. In particular, the promastigote must be able to survive from the point of inoculation by the insect into mammalian skin, evading serum factors that are potentially lethal to this stage of the organism (22), and must contact and enter the protective environment of the mononuclear phagocyte. Being an obligate intracellular parasite in mammals, it is of teleological advantage for *Leishmania* sp. to be recognized and ingested by the appropriate phagocyte. A number of authors have inferred, based on in vitro studies, that the recognition of the parasite by mononuclear phagocytes may depend on a glycoconjugate on the parasite surface (2, 5, 19, 27–29); however, the critical determinant has yet to be identified. In addition, whether amastigote and promastigote are recognized by the same mechanism is unclear. Dwyer and collaborators have isolated the pellicular membrane of *L. donovani* and found it to include a large carbohydrate fraction, both glycoprotein and glycolipid (9). Because surface glycoconjugates are likely to be important in the pathogenesis of visceral leishmaniasis, we have explored the surface properties of *L. donovani* by using lectins as probes. Stage-specific differences between lectin-binding molecules were followed ultrastructurally during in vitro conversion from amastigote to promastigote. The localization of evolving surface components may have implications concerning the differing roles of amastigotes and promastigotes.

**MATERIALS AND METHODS**

**Parasites.** A Sudanese strain of *L. donovani* was maintained by intracardiac injection of 6-week-old Syrian hamsters. After 4 to 6 weeks, heavily infected hamsters were sacrificed, and their spleens and livers were removed and aseptically homogenized in a tissue grinder and suspended in phosphate-buffered saline (pH 7.4) (PBS). The homogenate was centrifuged at 150 × g for 20 min to remove debris, and the supernatant containing amastigotes was centrifuged at 1,200 × g for 15 min. The pellet was washed twice in 30 ml of PBS and either purified further for study as amastigotes or allowed to convert to promastigotes. Before amastigote studies, organisms were suspended in 100 ml of Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) with 3% heat-inactivated (56°C, 30 min) fetal bovine serum (GIBCO), 100 U of penicillin per ml (GIBCO), and 50 μg of gentamicin per ml (Sigma Chemical Co., St. Louis, Mo.). They were then applied to a column of CF-11 Whatman cellulose (Whatman, Inc., Clifton, N.J.) as described by Brazil (3). The filtrate was centrifuged at 1,200 × g for 15 min.
and washed twice in 50 ml of PBS. Amastigotes were counted in a Petroff-Hauser chamber. To allow conversion to promastigotes, organisms were suspended in a modified minimal essential medium (GIBCO) with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and gentamicin (50 μg/ml) (22). After 3 to 15 days in culture, promastigotes were harvested and washed twice in 50 ml of PBS before use. Converting parasites were isolated from culture medium after 1 to 2 days and washed twice in 50 ml of PBS in preparation for lectin experiments.

**Lectin-mediated agglutination.** Lectins tested with and without specific inhibitory monosaccharides were: concanavalin A (ConA) with D-mannose or D-glucose, peanut agglutinin (PNA) with D-galactose, wheat germ agglutinin (WGA) with N-acetyl-neuraminic acid (NANA) or N-acetyl-d-glucosamine (GlcNAc), lectin from *Ricinus communis* (RCA) with D-galactose or lactose, soybean agglutinin (SBA) with D-galactose lectin from *Ulex europaeus*, with L-fucose, and lectin from *Lotus tetragonolobus* with L-fucose. All lectins and oligosaccharides were obtained from Sigma.

Assessment of agglutination at low magnification was found to be unreliable; therefore, the following microagglutination assay was used. Lectins were adjusted to final concentrations of 500, 250, 125, 63, 31, 16, 8, 4, 2, 1 μg/ml in PBS and studied alone or with 10 mM inhibitory sugar. Control parasites were studied in PBS without lectin or sugar. Promastigotes were added to produce a final concentration of 10^7 per ml in a 100-μl volume. Samples were allowed to agglutinate for 30 min at 4°C or room temperature. After gentle mixing, the concentration of single promastigotes (per milliliter) was determined by using a hemocytometer at ×400 magnification. The activity of lectins that did not agglutinate promastigotes, SBA, lectin from *Ulex europaeus*, and lectin from *L. tetragonolobus*, was verified by their ability to hemagglutinate 1.5% (vol/vol) type O human erythrocytes in PBS at 4°C. It was not possible to reliably assess lectin-mediated agglutination of amastigotes.

**Fluorescein-labeled lectin binding.** Fluorescein isothiocyanate (FITC)-labeled lectins were assayed for binding to promastigotes and to amastigotes. FITC-labeled ConA, PNA, WGA, SBA, RCA, and *L. tetragonolobus* lectin were obtained from Sigma. Inhibitory saccharides were the same as those listed above, with the addition of α-methyl-mannoside or lactose tested as inhibitory sugars for ConA or PNA, respectively. Amastigotes were studied 5 to 6 h after isolation from infected spleens because of previous electron microscopic studies showing that hamster cell membranes are initially adherent to the parasite surface, but are largely lost by 3 h after harvesting from an infected spleen (10).

Lectin concentrations were the same as for the agglutination experiments. Washed promastigotes at 1.0 × 10^7 per ml, column-purified amastigotes at 5.0 × 10^7 per ml, or washed converting parasites at 5.0 × 10^7 per ml were incubated with FITC-labeled lectins at 4°C or room temperature for 30 min. Wet mounts of each sample were then examined at 1,000× magnification, using a Zeiss microscope with phase and epifluorescence optics. Samples were called positive when all live organisms (assessed by flagellar motility) in several fields of view exhibited fluorescence; at least 100 organisms were examined. Negative samples had no live organisms positive for fluorescence.

**Neuraminidase treatment of amastigotes.** WGA can bind to either sialic acid or GlcNAc-containing residues (1, 12, 13, 24). To investigate which saccharide was being identified by WGA on the amastigote surface, amastigotes were treated with neuraminidase (type X from *Clostridium perfringens*; Sigma; EC 3.2.1.18), 0.1 U/ml for 60 min at 37°C. Organisms were then washed in PBS and added to serial dilutions of FITC-labeled WGA. Lectin binding was assessed as described above. The activity of the neuraminidase was demonstrated by the ability of normal human serum to hemagglutinate neuraminidase-treated (0.03 U/ml, 37°C, 60 min) type O human erythrocytes (1.5% [vol/vol], 4°C), as compared with untreated erythrocytes.

**Electron microscopic assay.** The distribution of WGA and PNA binding during conversion from amastigote to promastigote stages was assessed by electron microscopy. Peroxidase-labeled lectins (Sigma) at 250 μg/ml in PBS were incubated with PBS-washed organisms on day 0, 1, 2, or 3 of culture after being harvested from an infected spleen. Organisms were then fixed in 2% glutaraldehyde in PBS and stored for up to 3 days at 4°C. Fixed samples were reacted with diaminobenzidine in the presence of H2O2 as described by Graham and Karnovsky (14). Samples were rinsed twice in PBS (pH 7.3) with 3.5% sucrose at 4°C. They were preincubated for 30 min at room temperature in 0.075% diaminobenzidine in PBS (pH 6.0) and incubated for 45 min at room temperature in 0.075% diaminobenzidine–0.01% H2O2 in PBS (pH 6.0). Samples were rinsed twice in PBS (pH 7.3) and reincubated at 4°C in 2% glutaraldehyde. Control organisms with no lectin added were similarly fixed. Organisms were not postfixed in osmium because of possible confusion between osmii and positive and peroxidase positivity. Samples were then washed twice in 0.07 M phosphate buffer and sequentially dehydrated in 40, 60, 80, and 100% ethanol. Subsequent washes were 50% and then 100% propylene oxide (in ethanol), then in 50% Epon (in propylene oxide). After the final wash in 100% Epon (standard Epon 812 mixture), capsules were allowed to harden. Sections were cut and mounted for examination and photography on a JEOL 100-S electron microscope.

**RESULTS**

**Lectin-mediated agglutination.** Several exposed carbohydrate residues on the *L. donovani* surface were detected by using lectins of differing specificity. Shown in Fig. 1 are results of agglutination experiments with promastigotes incubated at room temperature with each of six different lectins. Agglutination, defined as a decreased number of free promastigotes compared with control, occurred with ConA, PNA, and RCA at lectin concentrations of ≥8 μg/ml, with 50% agglutination achieved between 2 and 4 μg/ml for ConA, between 4 and 8 μg/ml for PNA, and between 2 and 4 μg/ml for RCA. Because oligosaccharides at low concentration should compete with exposed saccharide residues on the parasite surface for binding to lectin if the two saccharides are similar in structure (4, 23), mono- and disaccharides were tested for their ability to inhibit lectin-mediated promastigote agglutination. Agglutination was specifically inhibited by 10 mM mannose in the case of ConA, and by 10 mM galactose with PNA. RCA-mediated agglutination was not altered by galactose but was inhibited by 10 mM lactose. In the case of all three lectins, agglutination occurred between all parts of the organisms, i.e., body-to-flagellum, body-to-body, and flagellum-to-flagellum, with no preference as to the pole agglutinated, implying that the carbohydrates identified coated the entire surface of the organism. A number of parasites appeared to agglutinate within themselves body-to-flagellum, although these were counted as free parasites in our assay. There was no difference in agglutination at 4 or 26°C. The three lectins mediating promastigote agglutination...
have binding sites complementary to d-mannose and d-glucose (ConA), β-d-galactose (1→3)-N-acetyl-d-galactosamine (PNA), and β-galactose (RCA) residues.

**Fluorescein-labeled lectin binding.** Binding of FITC-labeled lectins to both stages of *L. donovani* at room temperature and at 4°C is summarized in Table 1. With all experiments performed a minimum of 3 times, and minimal detectable concentrations of lectins listed. Lectins listed as negative were tested at concentrations up to 500 μg/ml without detectable binding.

Examples of the typical FITC-labeled lectin binding pattern are pictured in Fig. 2, showing organisms incubated with FITC-labeled RCA (50 μg/ml) at room temperature. In Fig. 2a is shown a promastigote with a fluorescent rim along its surface and flagellum and apparently inside the flagellar pocket (arrow), typical of both RCA and PNA binding. The ConA pattern was similar but without entry into the flagellar pocket. RCA binding to amastigotes also exhibited a rim pattern (Fig. 2b), as did ConA and WGA. In the case of RCA, however, there was a localized area of increased fluorescence, possibly indicating either locally increased external RCA-binding carbohydrate or RCA within the flagellar pocket.

Fluorescent lectin binding to promastigotes corresponded well with agglutination. ConA, PNA, and RCA binding were detected and were completely and specifically inhibited by the carbohydrates listed in Table 1 at a 10 mM concentration, but not by unrelated saccharides. Binding of ConA was not inhibitable by β-glucose but was undetectable in the presence of 10 mM β-mannose.

Lectin binding to the amastigote revealed surface characteristics differing from the promastigote stage. PNA binding to amastigotes was not detectable, whereas WGA binding was. Because of the dual specificity of the WGA binding site, with affinities for both polymers of GlcNAc and sialic acid residues (1, 12, 13, 24), inhibition studies were performed with both GlcNAc and NANA. Binding was found to be decreased in the presence of GlcNAc but not with NANA. In addition, treatment of amastigotes with neuraminidase, which cleaves off sialic acid residues (25), did not alter binding of WGA to the organism.

The stage specificity of lectin binding to *L. donovani*

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**FIG. 1.** Lectin-mediated *L. donovani* promastigote agglutination. The percentage of free promastigotes, calculated by the formula (number of free promastigotes in the sample/number of free promastigotes in the control) × 100, is plotted along the ordinate. Lectin concentration is plotted along the abscissa. Curves indicate representative experiments; each agglutination experiment was performed in triplicate. UEA₁, *U. europaeus*.

### Table 1. FITC-labeled lectin binding to *L. donovani*: stage specificities

<table>
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<tr>
<th>Lectin</th>
<th>Promastigotes</th>
<th>Amastigotes</th>
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<td>Inhibitory carbohydrate a</td>
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<td>ConA</td>
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a +, all live organisms exhibited fluorescence; –, no live organisms were fluorescent. At least 100 organisms were examined. Experiments were performed in triplicate.

b Minimal lectin concentrations at which binding was detectable (micrograms per milliliter).

c Carbohydrate concentration was 10 mM.

d α-MM, α-methyl-mannopranose; d-mannose did not inhibit binding.

e LTA, *L. tetragonolobus*.

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amastigotes and promastigotes was further investigated during in vitro conversion of the organism from its amastigote to its promastigote stages. FITC-labeled lectin binding patterns (Table 2) revealed binding of ConA and RCA throughout conversion, inhibited by α-methyl-mannopyranoside (but not D-glucose) and lactose, respectively. PNA binding commenced on day 2 of conversion, with initial lectin binding seen in the flagellar pocket and subsequently along the entire parasite surface. Interestingly, PNA binding on day 3 was positive on a larger percentage of organisms at 4°C than at 26°C, and a fraction of the parasites were observed to change from weakly positive to negative when warmed from 4°C to room temperature. All organisms were positive at both temperatures on day 5, suggesting not only a change in surface carbohydrate pattern as the organism matured, but also a change in the ability of the organism to cap and shed this lectin on day 3 as compared with day 5. WGA binding, which was homogenous on amastigotes, was lost over days 1 and 2 of conversion, with converting forms characteristically positive at their flagellar pole. As quantified by Dwyer et al. in 1974, many converting parasites spontaneously form rosettes in culture; however, smaller numbers of rosettes are present after the conversion to the promastigote stage is complete (10). We noted that a large number of these rosetting intermediate forms were WGA positive at the pole facing toward the rosette, the flagellar pole. WGA positivity was not seen to extend into the flagellar pocket.

Electron microscopic assay. The PNA and WGA binding patterns observed with FITC-labeled lectins during the in vitro conversion from amastigote to promastigote were verified by electron microscopy, using peroxidase-labeled lectins. Examples are shown in Fig. 3 and 4. WGA binding (Fig. 3) was homogenous around the amastigote surface (Fig. 3a). This was lost inhomogeneously during stage conversion (Fig. 3b) and was undetectable in the mature promastigote (Fig. 3c). Notably, WGA binding was never seen within the flagellar pocket. PNA binding, however, was undetectable on the amastigote (Fig. 4a), but during stage conversion was seen first inside the flagellar pocket (Fig. 4b) and bound homogeneously to the entire promastigote surface, including the flagellum, in the mature form (Fig. 4c). Agglutination of promastigotes with PNA, and of amastigotes with WGA, was observed with this assay, although quantitation was not feasible.

**DISCUSSION**

Lectins, defined by Goldstein and Hayes as carbohydrate-binding proteins or glycoproteins (13), have been used in the identification of exposed surface glycoconjugates because of their ability to noncovalently and relatively specifically bind to saccharide residues (4, 13). In this study, we have identified four plant lectins that bind to the *L. donovani* surface, and through these we have documented similarities and stage-specific differences between exposed surface carbohydrates on amastigotes and promastigotes. ConA was found to bind to both parasite stages and to agglutinate promastigotes. The carbohydrate-binding site of the ConA molecule is known to accommodate both D-mannose and D-glucose residues (13); inhibition of binding of D-mannose in the case of promastigotes, and by α-methyl-mannoside in the case of amastigotes, implies residues containing or resembling mannose on the surface of both parasite stages. Similarly, RCA, which binds to galactose-containing saccharides (13), also bound to both parasite stages, implying a galactose-containing glycoconjugate on the parasite surface.

The other two lectins of interest, WGA and PNA, exhibited stage specificity in their binding characteristics. WGA was found to bind exclusively to amastigotes, and PNA to promastigotes. The WGA binding site recognizes both (1→4)-linked GlcNAc units (1, 12) and NANA units (24). The failure of neuraminidase to alter WGA binding to the amastigote suggests that the WGA-binding glycoconjugate may not be a sialic acid, but may resemble or contain GlcNAc in its structure. This was verified by the inhibition of WGA binding by GlcNAc but not by NANA. During the process of stage conversion from amastigote to promastigote, the surface of the organism evolves such that the
FIG. 3. Electron micrographs of L. donovani parasites incubated with horseradish peroxidase-labeled WGA (250 μg/ml) on day 0 (a), day 1 (b), or day 3 (c) of in vitro conversion from the amastigote to the promastigote stage. Bars = 1 μm. Samples were not postfixed in osmium, so intracellular organelles are indistinct.

WGA-binding glycoconjugate is no longer exposed, and a PNA-binding residue is newly displayed. PNA has a binding site that is most complementary to the carbohydrate sequence β-D-galactose-(1→3)-N-acetyl-D-galactosamine with galactose in the penultimate position, and that binds less avidly to D-galactose (13, 20). Thus, in contrast to the RCA-binding glycoconjugate(s), PNA may identify an internal galactose rather than one in the terminal position. The pattern of WGA and PNA localization changed throughout stage conversion, implying inhomogeneity and alteration of surface glycoconjugates during the development of the organism. At this point, one can only speculate how the evolution of these glycoconjugates might affect the pathogenesis of the disease.

A number of authors have studied the surfaces of different Leishmania species according to their lectin-binding characteristics (6–8, 11, 15, 17, 26). Interestingly, promastigotes of nearly all species tested, with the exception of some strains studied by Jacobson et al. (17), were found to agglutinate in the presence of ConA, and almost all, with the exception of L. mexicana subsp. amazonensis (as reported by Ebrahimzadeh and Jones [11]), agglutinated in the presence of RCA. Of the L. donovani promastigote isolates reported, two of three strains tested by Schottelius agglutinated with PNA but not lectin from U. europeus; a third (Indian) strain did the reverse (26). Dwyer, using a cloned strain of L. donovani, found promastigote macroagglutination induced by ConA, L. tetragonolobus lectin, SBA, and WGA (7, 8), whereas in the present study, promastigotes agglutinated with only the first of these four lectins. Whether differences were due to different strains within the species is unclear.

The evolution of the parasite cell surface during in vitro stage conversion may have implications during the life cycle of the organism. It has been documented by Dwyer et al. that in vitro converting parasites are more often found in rosette formation than mature promastigotes (10); according to our observations, many of these converting forms bind WGA at their rosetting pole. In studies on wild and laboratory-
maintained Panamanian *Phlebotomus* sp. (sandfly vector for *Leishmania braziliensis*), similar rosettes of converting forms were found adherent to the hindgut and midgut walls of the sandfly in vivo as the *Leishmania* parasites ascended from the hindgut to the cardia and esophagus before inoculation into the mammalian host (16, 18, 21). It is conceivable that rosetting within the sandfly intestine is secondary to lectin-like associations between the parasites via their unipolar WGA-binding glycoconjugates, and possibly with the arthropod gut wall.

Finally, attachment and phagocytosis of *Leishmania* sp. organisms by mammalian macrophages, in both promastigote and amastigote forms, is known to occur in the absence of serum, and it is suspected that attachment of these organisms may occur via an exposed glycoconjugate on the parasite surface (2, 15, 19, 27–29). In the present study, we have surveyed such glycoconjugates on *L. donovani* by using lectins, documenting some surface characteristics that remain stable and some that evolve through the amastigote-to-promastigote stage conversion. Identification of the glycoconjugate(s) responsible for serum-free attachment to the human macrophage will be an important next step in understanding the immunobiology of visceral leishmaniasis.

FIG. 4. Electron micrographs of *L. donovani* parasites incubated with horseradish peroxidase-labeled PNA (250 µg/ml) on day 0 (a), day 2 (b), or day 3 (c) of in vitro conversion from the amastigote to the promastigote stage. Bars = 1 µm. Samples were not postfixed in osmium, so intracellular organelles are indistinct.

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LITERATURE CITED


