Evidence that the vectorial competence of phlebotomine sand flies for different species of Leishmania is controlled by structural polymorphisms in the surface lipophosphoglycan

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ABSTRACT Phlebotomine vectors can in some instances transmit only certain species of Leishmania. Comparison of a large number of vector/parasite pairs revealed that species-specific differences in vectorial competence were in every case directly correlated with the ability of promastigotes to attach to the sand-fly midgut, the variable outcomes of which were controlled by structural polymorphisms in the surface lipophosphoglycan (LPG) of the parasite. The ability of Phlebotomus papatasi to transmit only Leishmania major could be attributed to the unique, highly substituted nature of L. major LPG that provides for multiple terminally exposed β-linked galactose residues for binding. While the relatively unsubstituted LPGs of other Leishmania species were unable to mediate promastigote attachment to P. papatasi, they could mediate binding to midguts of Phlebotomus argentipes, which was found to be a potentially competent vector for every Leishmania species examined. The data suggest that at least some phlebotomine vectors differ with respect to the parasite recognition sites which they express and that midgut adhesion is a sufficiently critical component of vectorial competence as to provide the evolutionary drive for LPG structural polymorphisms.

Leishmania parasites are transmitted to their mammalian hosts via the bite of an infected female sand fly. There is much evidence that some Leishmania species can be transmitted only by certain phlebotomine species and not by others (reviewed in ref. 1). The life cycle of supranyelarian Leishmania species within their natural or permissive vectors suggests a number of potential barriers to complete development that might occur in vector-incompetent species, including susceptibility to the proteolytic enzymes in the gut, which might inhibit the growth of parasites in the digesting bloodmeal (2); inability to escape the peritrophic matrix, which behaves as an early barrier to parasite migration out of the abdominal midgut (3); inefficient binding of promastigotes to midgut epithelial cells, which is thought to prevent the loss of infections during excretion of the digested bloodmeal (4, 5); and defective differentiation to and forward movement of metacyclic promastigotes, which is required for positioning of infective-stage parasites for efficient transmission by bite (6, 7).

Of all these events, the attachment of promastigotes to midgut epithelial cells might be most likely to explain, at least in part, species-related differences in vectorial competence, since it is controlled by molecules which display extensive interspecies polymorphisms (5). Adhesion of Leishmania major promastigotes to midguts of their natural vector, Phlebotomus papatasi, was found to be an inherent property of logarithmic-phase (procyclic) promastigotes controlled by the expression of terminally exposed galactose residues on the lipophosphoglycan (LPG), the major promastigote surface molecule. Among the earliest methods used for species identification was serotyping of spent-medium excreted factor (EF) (8), which is now known to contain the shed form of LPG (9). In all species studied to date, the LPGs comprise linear chains of phosphorylated oligosaccharide repeats which are anchored to the membrane via a glycosylphosphatidylinositol anchor (reviewed in refs. 10 and 11). The lipophosphoglycan moieties share a common backbone consisting of repeating disaccharide units of PO4·6Galβ1-4Manα1, where the 3 position of the Gal residue can either be unsubstituted [as in East African isolates of L. donovani (12)], partially substituted [as in L. mexicana (13) and in L. amazonensis and Indian isolates of L. donovani (S.J.T. and D.L.S., unpublished work)], or almost completely substituted with a variety of saccharide side chains [as in L. major (14) and L. tropica (S.J.T. and D.L.S.; M. McConvilie, unpublished work)]. Additional interspecies polymorphisms may also occur in the structure of the predominant neutral, mannose-containing oligosaccharides that cap the nonreducing terminus of the lipophosphoglycan chain.

We have compared the vectorial competence of P. papatasi plus another important vector of leishmanial disease in the Old World, Phlebotomus argentipes, for various species of Leishmania. We report that promastigotes and their LPGs of different Leishmania species do in some cases display inherently different binding capacities for the midguts of different vectors, and the extent of binding in each case forcefully predicts in which parasite/sand fly combinations the development of transmissible infections can occur.

MATERIALS AND METHODS

Parasites. The following cloned lines of Leishmania promastigotes were used: NIH/Friedlin strain of L. major, clone V1 (MHOM/IL/80/Friedlin), and L. major strain LRC-137, clone V121 (MHOM/IL/67/JerichBio-II), each isolated from patients with cutaneous leishmaniasis in Israel; L. major Neck strain (MRHO/SU/59/P), isolated from a giant gerbil in the former Soviet Union; L. major strain LL19 (MTAT/KE/00/ T4), an LPG-deficient strain isolated from a patient with cutaneous leishmaniasis in Kenya (15); L. donovani strain 138 from Sudan (MHOM/SD/00/138-2D) and L. donovani Mongi strain from India (MHOM/IN/83/Mongi-142), each isolated from bone marrow biopsies of patients with visceral leishmaniasis; L. tropica Azad strain (MHOM/AF/83/Azad), isolated from a patient with cutaneous leishmaniasis acquired.

Abbreviation: LPG, lipophosphoglycan.

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in Afghanistan; L. amazonensis Josefa strain (MHOM/BR/00/Josefa), isolated from a patient with cutaneous leishmaniasis in Brazil; and R2D2, a variant line cell of L. donovani IS selected for ricin resistance and defective in the synthesis of LPG (16). To select an LPG mutant of L. major Friedlin strain, parasites were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (16, 17), and 7 × 10⁸ promastigotes were resuspended in 20 ml of M199 medium to which peanut agglutinin (PNA) was added at 100 µg/ml. Agglutinated cells were allowed to settle overnight, the parasites in the supernatant were grown to stationary phase, and the PNA selection was repeated. The PNA-negative population was plated on M199 agar, yielding 32 colonies. Of these, none showed agglutination with PNA and 31 failed to agglutinate with either ricin agglutinin or the monoclonal antibody CA7AE, which is specific for the disaccharide phosphate repeat of LPG (18). The single PNA-negative, ricin-positive, CA7AE-positive colony was plated once more, yielding a clonal mutant designated KIRK, which was maintained in medium with PNA at 30 µg/ml.

All promastigotes were grown in medium 199 supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum, penicillin (100 units/ml) streptomycin (50 µg/ml), and 12.5 mM l-glutamine (all from Advanced Biotecnologies, Columbia, MD), 40 mM Hepes (pH 7.4), 0.1 M adenine, and 0.0005% hemin. Procylic promastigotes were harvested in logarithmic phase (1–2 days) and washed with Hanks’ balanced salt solution containing 1 mM MgCl₂ and 0.15 mM CaCl₂. Amastigotes were purified from macerated mouse footpad lesions or from hamster spleens (19). Amastigotes were stored at −70°C.

Sand-Fly Infection and Dissection. P. papatasi and P. argentipes sand flies were reared and maintained in the Department of Entomology, Walter Reed Army Institute of Research. Three- to 5-day-old female sand flies were fed through a chick skin membrane (20) on a mixture of hepaturinated mouse blood containing 10⁶ amastigotes or 10⁶ procylic promastigotes per ml. For infections using procylic promastigotes, the red blood cells were washed twice in 0.86% NaCl and added back to the plasma, which was heat-inactivated at 56°C for 45 min. Blood-engorged sand flies were separated and maintained at 28°C with 30% sucrose. At various times after feeding, the flies were anesthetized with CO₂, and their midguts were dissected and examined microscopically for promastigotes. In some experiments 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 (PBS), pH 7.4. Each gut was homogenized with a Tefloncoated microtissue grinder, and released promastigotes were counted in a hemocytometer.

In Vitro Assay for Promastigote Binding to Sand-Fly Midgut. Binding of promastigotes to sand-fly midguts was quantitated by a modification of an in vitro technique (5). Three- to 5-day-old nonfed female sand flies, maintained on 30% sucrose, were dissected in PBS. 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 × 10⁷ per ml) 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 PBS. Guts were homogenized and released promastigotes were counted as described above. P values were obtained from Student's t test for paired samples.

Fluorescent Staining of LPG-Infected Midguts. LPGs from each species of Leishmania were purified from procylic promastigotes by specific extraction of LPG followed by affinity chromatography using octyl-Sepharose (21). Opened, dissected midguts were fixed with 2% formaldehyde in PBS at 4°C for 20 min. After several washes in PBS they were incubated for 45 min with LPG at 10 µg/ml. After several washes the guts were incubated in a 1:200 dilution of ascites containing monoclonal antibody 45D3 (an IgG1 which recognizes LPGs of all species studied to date; D.L.S., unpublished work) followed by incubation with fluoresceinated anti-mouse IgG. Stained guts were examined microscopically under ultraviolet and bright field exposures.

RESULTS

Survival of Leishmania Species in P. papatasi. Two days after membrane feeding on mouse blood containing different species of Leishmania amastigotes, midgut promastigotes were found in 100% of P. papatasi fed on L. major or L. donovani IS, in 75% of flies fed on L. amazonensis, and in 55% of flies fed on L. donovani Mongi (Fig. 1). On day 4, shortly after the bloodmeals had been digested and passed, the infection rate remained 100% in P. papatasi infected with L. major, but 0% in flies infected with each of the other species. Similar results were observed when flies were infected with procylic promastigotes of the various Leishmania species, including L. tropica, for which the lack of animal models made it difficult to obtain amastigotes for use in fly infections. The infections were scored quantitatively by homogenization of individual midguts and counting of released parasites. Infection rates in flies on day 2 were 90–100%, and there was no significant difference in the number of midgut promastigotes when flies infected with the different species were compared (Fig. 2). On day 8, infections were retained only in flies infected with L. major (90%), compared with L. donovani IS (16%), L. donovani Mongi (0%), L. amazonensis (9%), and L. tropica (0%) (Fig. 2).

Promastigote-initiated infections were also compared for two other virulent strains of L. major (Neal and L137) and two attenuated L. major strains (L119 and KIRK), which express deficient amounts and atypical forms of L. major LPG. Excellent growth and survival of midgut promastigotes were observed for the two virulent strains, confirming the competence of P. papatasi for L. major strains, even of diverse geographical origin. In contrast, midgut infections with L119 and KIRK were completely lost, suggesting that the ability of L. major strains to persist in P. papatasi after bloodmeal passage is dependent on their expression of an appropriate amount and form of LPG. The KIRK mutant also showed significantly less growth than the wild-type strain on day 2, suggesting that LPG might play a role in the growth and

Fig. 1. Outcome of infections in P. papatasi membrane-fed on bloodmeals containing amastigotes of various species or strains. Day 2, solid bar; day 4, hatched bar. Twenty to 30 flies per group were examined at each time point. Lm, L. major; Ld, L. donovani; La, L. amazonensis; Men, Mongi; Jos, Josefa.
Fig. 2. Outcome of infections in *P. papatasi* membrane-fed on bloodmeals containing promastigotes of various species or strains. (Upper) Number of promastigotes in midguts of individual flies on day 2. (Lower) Percent of flies still infected on days 6–8 (15–20 flies per group). Lt., *L. tropica*; other abbreviations are as in Fig. 1.

Survival of *Leishmania* Species in *P. argenteipes*. Two days after infection of *P. argenteipes* with different species of *Leishmania* amastigotes, transformation to and excellent growth of midgut promastigotes were found in 100% of the flies in each group (Fig. 3). On day 5, again after the bloodmeals had been fully digested and passed, the infection rates remained 100% in flies fed on *L. donovani* 1S and *L. amazonensis* and were still 80% in the flies fed on *L. major* and *L. donovani* Mongi. Heavy anterior midgut infections were found in a high proportion of flies in all groups when examined on day 12 (data not shown). *P. argenteipes* also appeared competent for the full development of *L. tropica*. Infections in this case were initiated with promastigotes, and 100% of the flies were infected both before and after bloodmeal passage. In contrast, promastigote-initiated infections using the LPG-deficient *L. donovani* mutant R2D2 were relatively low on day 2 (55%) and were completely absent on day 5.

Midgut Binding of Procylic Promastigotes. Procylic promastigotes of each strain were incubated with *P. papatasi* midguts which had been cut open in the posterior segment to allow parasites to penetrate freely into the lumen. After washing, an average of 34,400, 26,000, and 34,000 *L. major* procylics of *Vl*, *L137*, and *Neal*, respectively, remained bound per gut (Fig. 4A). Procylics of all the other species bound poorly (<2900), as did promastigotes of the LPG-

![Fig. 3](image-url)  
**FIG. 3.** Outcome of infections in *P. argenteipes* membrane-fed on bloodmeals containing various species or strains of *Leishmania*. All infections were initiated with amastigotes except for Azad (*L. tropica*) and R2D2, which were initiated with procylic promastigotes. Day 2, solid bar; day 5, hatched bar. Fifteen to 20 flies were examined at each time point per group.

defective *L. major* strains L119 and KIRK. In contrast, significant and comparable levels of binding were observed for each species of *Leishmania* incubated with *P. argenteipes* midguts (Fig. 4B). The average number of procylic promastigotes attached after washing varied from 5400 to 11,000 per gut. While the strain with the greatest binding was the Indian *L. donovani* strain, for which *P. argenteipes* is a natural vector, this difference was not significant when compared with the binding observed for each of the other species. The binding of the R2D2 mutant, 600 per gut, was significantly less than that of the wild-type 1S strain (*P* < 0.01). *P. argenteipes* midguts are ≈30% smaller than those from *P.*
papatasi, and this may account at least in part for the relatively lower level of binding to P. argentinae midguts, even when the two natural parasite/vector combinations are compared.

Binding of Purified LPGs to Phlebotomine Midguts. LPGs were purified from procyclic promastigotes of five strains of Leishmania known to differ in the structure of their phosphorylated oligosaccharide repeats. The structures for L. major and L. donovani 1S have been extensively characterized (12, 14) (Fig. 5). The characterization of the other LPGs remains preliminary. L. amazonensis LPG appears to be similar in structure to L. mexicana LPG (13) and is partially substituted with side chains containing one or two sugars. Indian L. donovani LPG is also partially substituted, whereas LPG from L. tropica appears to be almost completely substituted with larger oligosaccharide side chains.

The procyclic LPGs were incubated with dissected, opened midguts of either P. papatasi or P. argentinae, followed by washing and immunofluorescent staining with monoclonal antibody 45D3, which recognizes an epitope common to all LPGs. P. argentinae midguts incubated with each of the five LPGs were intensely stained throughout the abdominal and thoracic regions, with no difference in staining discernible between the groups (Fig. 6). In contrast, intense staining of P. papatasi midguts was observed only with L. major LPG, prepared from the Friedlin strain. Midguts incubated with the L. donovani, L. amazonensis, and L. infantum LPGs were stained only slightly above the background level seen in midguts incubated with antibody alone.

**DISCUSSION**

Differences in the binding of procyclic promastigotes of different Leishmania species to midgut epithelial cells of two sand-fly vectors were in every case predictive of which combinations would result in the survival of midgut infections and subsequent development. Differences in midgut adhesion, controlled by interspecies polymorphisms in LPG, might therefore explain the stage specificity of vectorial competence observed in nature. Most of the evidence sug-

Fig. 5. Basic structures of procyclic LPGs of L. major and L. donovani, depicting the PQ-Gal-Man backbone repeats with or without various oligosaccharide side-chain substitutions, as well as one of a number of mannose-containing neutral capping sugars.

Fig. 6. Fluorescent staining of midguts of P. argentinae (Upper) and P. papatasi (Lower) incubated with LPG purified from procyclic promastigotes of various Leishmania species. Control guts were incubated with primary and secondary antibodies only.
that by decreasing the percentage of serum in the bloodmeal, the infection rate in flies infected with an inappropriate species (presumably *L. tropica*) was significantly enhanced. Schlein et al. (26, 27) reported that proteolytic enzymes produced during bloodmeal digestion were inhibited by infection with *L. major*, as well as by *L. major* excreted factor (possibly LPG), but not by infection with other species. If the effect of the bloodmeal or digestive enzymes is to damage the parasites directly, then this should be reflected by differences in promastigote growth prior to bloodmeal excretion. The LPG-deficient mutants KIRK and R2D2 did in fact produce lower rates of infection and significantly lower numbers of parasites at 48 hr, supporting the view that the blood-engorged midgut is a potentially hostile setting for the parasite and that the LPG coat is somehow protective during this early stage of infection. This is not, however, the crucial point at which species-specific vectorial competence seems to be expressed in our studies, since in most cases, those promastigotes which produced normal amounts of LPG, regardless of species, sustained high infection rates and comparable levels of growth at 48 hr.

While only *L. major* may possess the appropriate ligands for binding to *P. papatasi* midguts, the LPGs of other species would be expected to mediate significant binding to the midguts of those sand flies which are known to be their natural or permiscuous vectors. Surprisingly, *P. argentipes*, which is the proven vector of *L. donovani* in India, was permiscuous not only to the Indian *L. donovani* strain but also to all of the other species tested. *In vitro* binding assays using *P. argentipes* midguts revealed significant and comparable levels of binding of promastigotes and LPGs for each species tested. The role of LPG in mediating attachment to *P.argentipes* midguts was substantiated by the lack of binding of the R2D2 strain, which is a specific LPG-deficient mutant derived from *L. donovani* 15 (16). The data suggest that *P. argentipes* midguts possess a receptor, lacking in *P. papatasi*, for a relatively conserved oligosaccharide on procyclic LPGs.

The comparison of promastigote-binding to the midguts of two Old World phlebotomine vectors indicates, not surprisingly, that the parasite recognition sites which they express are in some cases diverse and might therefore provide the evolutionary drive for LPG structural polymorphisms. The selection for the unusual, highly galactose-substituted LPG expressed by *L. major* strains occurred, in this view, to take advantage of a widely distributed sand-fly species which is inherently refractory to other Old World parasites expressing relatively unsubstituted forms of LPG (e.g., *L. donovani, L. infantum*) or an LPG lacking in terminally exposed galactose side chains (e.g., *L. tropica*). On the other hand, *P. argentipes* would be expected to serve as a common vector for the transmission of available sympatric parasite species. Such an outcome may have recently occurred in India, where it appears that the introduction of *L. tropica* into an area long endemic for transmission of *L. donovani* by *P. argentipes* has resulted in *L. tropica* establishing itself as a co-endemic agent of visceral leishmaniasis (D.L.S., unpublished work).

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