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Evidence that the vectorial competence of phlebotomine sandflies for different species of *Leishmania* is controlled by structural polymorphism in the surface lipophosphoglycan (LPG).

Phlebotomine vectors can in some instances transmit only certain species of *Leishmania*. Comparison of a large number of vector/parasite pairs revealed that species differences in vectorial competence were in case directly correlated with the ability of promastigotes to attach to the sandfly mid-gut, the variable outcomes of which were controlled by structural polymorphism in the surface lipophosphoglycan (LPG) of the parasite. The ability of the *Phlebotomus papatasi* to transmit only *Leishmania major* could be attributed to the unique, highly substituted nature of the *L. major* LPG that provides for multiple terminally exposed B-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 potentially vector for every *Leishmania* species examined. The data suggest that at least some of the 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 so to provide a selective evolutionary drive for LPG structural polymorphism.
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 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 suprapylarian Leishmania species within their natural or permissive vectors of different Leishmania species do in some cases display interspecies polymorphisms (5). Adhesion of 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 a sheds form of LPG (9). In all species studied to date, the LPGs comprise 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 P(β-1,4Galβ1-4Manα1, which the 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. a. donovani (14) and L. tropica (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. McConville, 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 phosphoglycan 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 VI (MHOM/IL/80/Friedlin), and L. major strain LRC-137, clone VI21 (MHOM/IL/67/Jericho-II), each isolated from patients with cutaneous leishmaniasis in Israel; L. major Nacl strain (MRHO/SU/59/P), isolated from a geikel gerbil in the former Soviet Union; L. major strain L119 (MTAT/KE/00/T4), an LPG-deficient strain isolated from a patient with cutaneous leishmaniasis in Kenya (15); L. donovani strain IS from Sudan (MHOM/SD/00/IS-2D) and L. donovani Mong strain from India (MHOM/IN/83/Mong-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

*ABSTRACT* LPG, lipophosphoglycan.

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in Afghanistan: *L. amazonensis* Josefa strain MHOM/BR. 90 Josefa, isolated from a patient with cutaneous leishmaniasis in Brazil; and RBD2, a variant cell line of *L. donovani* IS selected for insect resistance and defective in the synthesis of LPG (16). To select an LPG mutant of *L. donovani* strain, parasites were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (16, 17), and $7 \times 10^8$ 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 C7AE, which is specific for the disaccharide phosphate repeat of LPG (18). The single PNA-negative, ricin-positive, C7AE-positive colony was plated once more, yielding a clonal mutant designated KIRK, which was maintained in medium with PNA at 50 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 Biotechnologies, Columbia, MD). 40 mM Hepes (pH 7.4), 0.1 mM adenine, and 0.0005% hemin. Procytic promastigotes were harvested in logarithmic phase (1-2 days) and washed with Hanks' balanced salt solution containing 1 mM MgCl$_2$ and 0.15 mM CaCl$_2$. Promastigotes were purified from macerated mouse footpad lesions or from hamster spleens (19) and stored at -70°C.

Sand-Fly Infection and Dissection. *P. papatasi* and *P. erreyni* 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 heparinized mouse blood containing 10$^6$ promastigotes or 10$^6$ procyclic promastigotes per ml. For infections using procytic 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 CO$_2$ 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 Teflon-coated 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 $\times$ 10$^5$ 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 procytic 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 antibodies containing monoclonal antibody 4G12, an IgG 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 procyclic promastigotes of the various *Leishmania* species, including *L. tropica*, for which the lack of animal models made it difficult to assess the role of 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).

Procyclic-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 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. 3. Outcome of infections in *P. argentipes* 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 procyclic promastigotes. Day 2: solid bar; day 5: hatched bar. Fifteen to 20 flies were examined at each time point per group.

detective *L. major* strains L119 and KIRK. In contrast, significant and comparable levels of binding were observed for each species of *Leishmania* incubated with *P. argentipes* midguts (Fig. 4B). The average number of procyclic 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. argentipes* 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 IS strain (P < 0.01).

*P. argentipes* midguts are ~30% smaller than those from *P. papatasi*.

Survival of *Leishmania* Species in *P. argentipes*. Two days after infection of *P. argentipes* 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. argentipes* 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 Procyclic Promastigotes. Procyclic 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* procyclics of VI, L137, and Neal, respectively, remained bound per gut (Fig. 4A). Procyclics of all the other species bound poorly (<2900), as did promastigotes of the LPG-
promastigotes of various competence observed and subsequent development. Differences in midgut adhesion lactose residues for stable binding to occur. combinations would result in the survival of midgut infections on recognition of multiple, densely configured terminal ga-

Differences in the binding of procyclic promastigotes of galactose residues are either absent entirely or restricted to ground level seen in midguts incubated with antibody alone. Incubated with the major LPG, prepared from the Fnedlin strain. Midguts less LPG. and its side chains include highly unusual mannose substitutions terminating in P-linked galactose. While its monoclonal antibody 45D3, which recognizes an epitope ences which affect its survival in the fly. The KIRK mutant, opened midguts of either sand-fly vectors were in every case predictive of which the putative receptors on papatasi, and this may account at least in part for the relatively lower level of binding to P. argentipes 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 IS 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. argentipes, followed by washing and immunofluorescent staining with monoclonal antibody 45D3, which recognizes an epitope common to all LPGs. P. argentipes 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 PO4-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.

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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 egression. 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 permissive vectors. Surprisingly, P. argenipes, which is the proven vector of L. donovani in India, was permissive not only to the Indian L. donovani strain but also to all of the other species tested. In vitro binding assays using P. argenipes 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. argenipes midguts was substantiated by the lack of binding of the R2D2 strain, which is a specific LPG-deficient mutant derived from L. donovani 1S (16). The data suggest that P. argenipes 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. argenipes 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. argenipes has resulted in L. tropica establishing itself as a co-endemic agent of visceral leishmaniasis (D.L.S., unpublished work).

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