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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 suprapylarian *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 a 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 phosphoglycan moieties share a common backbone consisting of repeating disaccharide units of $\text{PO}_4\text{-6Gal}\beta\text{1-4Manal}$ 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., 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 V1 (MHOM/IL/80/Friedlin), and *L. major* strain LRC-137, clone V121 (MHOM/IL/67/Jericho-II), each isolated from patients with cutaneous leishmaniasis in Israel; *L. major* Neal strain (MRHO/SU/59/P), isolated from a giant 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* 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

in Afghanistan; *L. amazonensis* Josefa strain MHOM/BR/90/Josefa, isolated from a patient with cutaneous leishmaniasis in Brazil; and R2D2, a variant cell line of *L. donovani* 1S 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 7×10^8 promastigotes were resuspended in 20 ml of M199 medium to which peanut agglutinin (PNA) was added at 100 μ g/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 50 μ g/ml.

All promastigotes were grown in medium 199 supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum, penicillin (100 units/ml) streptomycin (50 μ g/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. Procyclic 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 $CaCl_2$. Amastigotes were purified from macerated mouse footpad lesions or from hamster spleens (19). Amastigotes were stored at $-70^\circ C$.

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 heparinized mouse blood containing 10^6 amastigotes or 10^6 procyclic promastigotes per ml. For infections using procyclic promastigotes, the red blood cells were washed twice in 0.86% NaCl and added back to the plasma, which was heat-inactivated at $56^\circ C$ for 45 min. Blood-engorged sand flies were separated and maintained at $28^\circ C$ 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 μ l 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 (2.5×10^7 per ml) in 40 μ l 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-Incubated Midguts. LPGs from each species of *Leishmania* were purified from procyclic 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 $4^\circ C$ for 20 min. After several washes in PBS they were incubated for 45 min with LPG at 10 μ g/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* 1S, 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 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* 1S (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

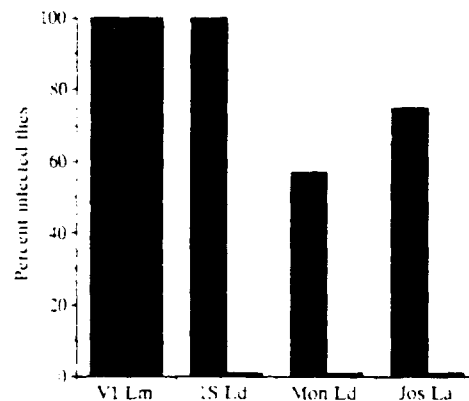


FIG. 1. Outcome of infections in *P. papatasi* membrane-fed on bloodmeals containing amastigotes of various species or strains. Day 2, solid bar; day 4, hatched bar. Twenty to 30 flies per group were examined at each time point. Lm, *L. major*; Ld, *L. donovani*; La, *L. amazonensis*; Mon, Mongi; Jos, Josefa.

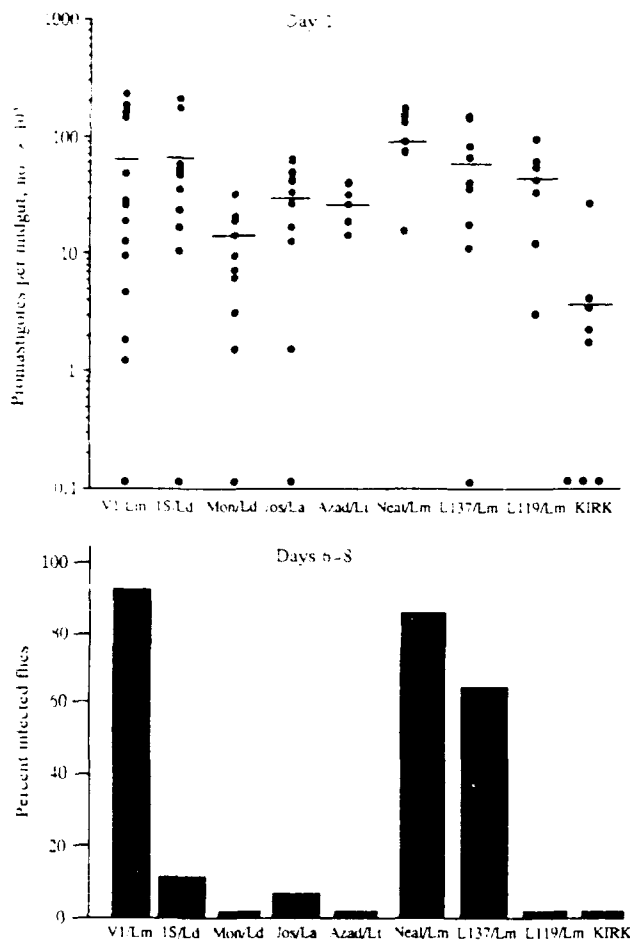


FIG. 2. Outcome of infections in *P. papatasi* membrane-fed on bloodmeals containing promastigotes of various species or strains. (Upper) Number of promastigotes in midguts of individual flies on day 2. (Lower) Percent of flies still infected on days 6–8 (15–20 flies per group). Lt, *L. tropica*; other abbreviations are as in Fig. 1.

survival of promastigotes in the bloodmeal even prior to its excretion.

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 V1, 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-

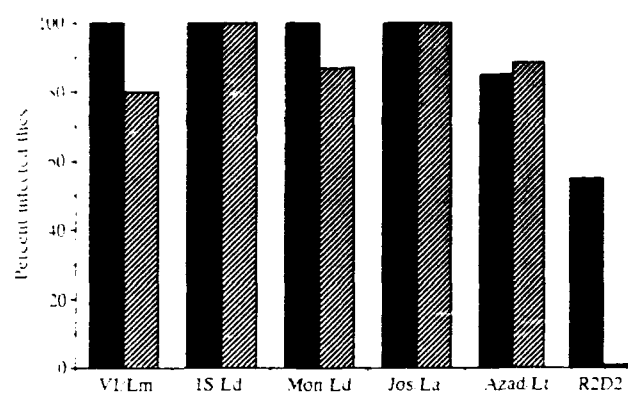


FIG. 3. Outcome of infections in *P. argentipes* membrane-fed on bloodmeals containing various species or strains of *Leishmania*. All infections were initiated with amastigotes except for Azad (*L. tropica*) and R2D2, which were initiated with procyclic promastigotes. Day 2, solid bar; day 5, hatched 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 1S strain ($P < 0.01$). *P. argentipes* midguts are $\approx 30\%$ smaller than those from *P.*

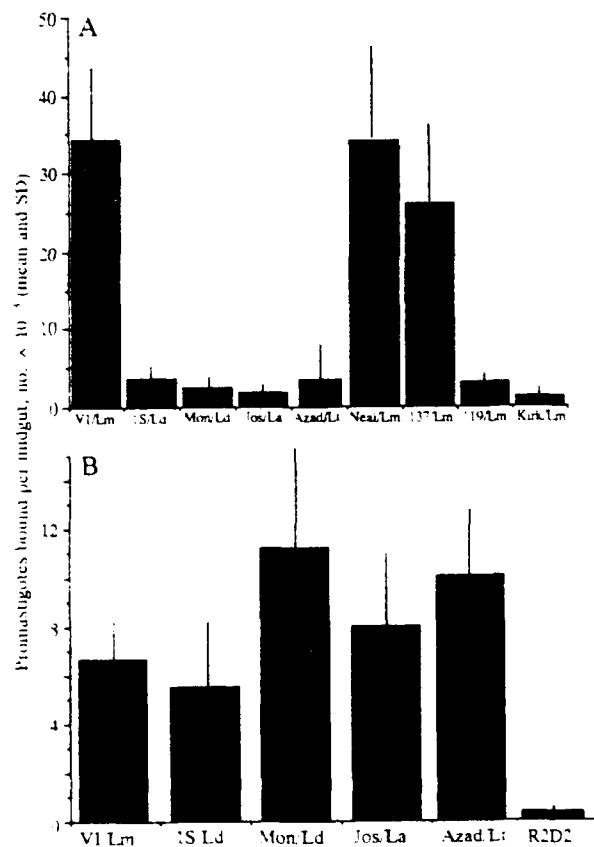


FIG. 4. Binding *in vitro* of *Leishmania* promastigotes to midguts (7–10 per group) of *P. papatasi* (A) or *P. argentipes* (B).

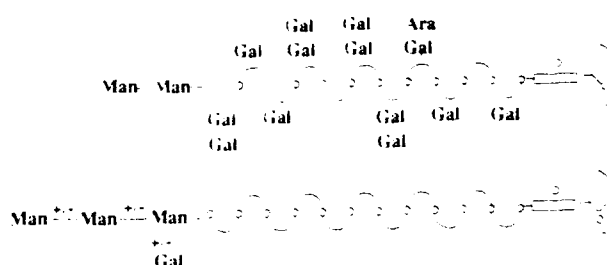


FIG. 5. Basic structures of procyclic LPGs of *L. major* and *L. donovani*, depicting the PO₄-Gal-Man backbone repeats with or without various oligosaccharide side-chain substitutions, as well as one of a number of mannose-containing neutral capping sugars.

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* 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. 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-

gesting a close evolutionary fit between *Leishmania* species and their vectors has been obtained from studies involving *P. papatasi* (1). As far as we are aware there is no evidence that *P. papatasi* is involved in the natural transmission of any *Leishmania* species other than *L. major*. This specificity was reproduced in the laboratory by our own studies, which confirmed several earlier accounts demonstrating that *P. papatasi*, fed on either experimental lesions or through a membrane, will support the full growth and development of *L. major* in high frequency, but not of any other *Leishmania* species (22–24). In experiments involving artificial meals, the resistance of sand-fly vectors to various species of *Leishmania* can be overcome by increasing the number of parasites ingested (22).

In our studies, the loss of promastigotes in *P. papatasi* infected with inappropriate *Leishmania* species coincided with the passage of the digested bloodmeal (days 3–4), suggesting a defect in their ability to anchor themselves to the gut wall. While promastigotes from the three virulent strains of *L. major* bound efficiently to *P. papatasi* midguts *in vitro*, the binding of promastigotes from each of the other species tested was relatively poor. An identical pattern of species-restricted binding was observed with LPG purified from procyclic promastigotes. The role of LPG in mediating the attachment of *L. major* promastigotes to *P. papatasi* midguts was demonstrated in our previous study, in which procyclic LPG from *L. major* or LPG repeat units with a terminal galactose residue were able to completely inhibit promastigote binding *in vitro* (5). That LPG is not only involved in, but required for, midgut binding is suggested in the present study by the low level of *in vitro* binding and lack of *in vivo* persistence of two LPG-deficient *L. major* strains, L119 and KIRK. Because L119 was not obtained by specific selection of an LPG-defective phenotype, it may express other differences which affect its survival in the fly. The KIRK mutant, in contrast, was specifically selected from the wild-type Friedlin V1 strain for deficient expression of LPG side-chain substitutions terminating in β -linked galactose. While its LPG has not been completely characterized, preliminary analyses reveal that the KIRK mutant expresses 6- to 10-fold less LPG, and its side chains include highly unusual mannose substitutions (S.J.T., unpublished observations). The high affinity that the wild-type *L. major* LPG has for the *P. papatasi* midgut is most likely explained by a unique aspect of its structure which provides for multiple β -linked terminal galactose residues per molecule for binding. For the LPG molecules of the other species examined, terminally exposed galactose residues are either absent entirely or restricted to the single neutral capping oligosaccharide. This suggests that the putative receptors on *P. papatasi* midguts are dependent on recognition of multiple, densely configured terminal galactose residues for stable binding to occur.

Adler (25) was the first to investigate factors expressed during the early stages of infection in *P. papatasi* that might explain its natural resistance to certain parasites. He found

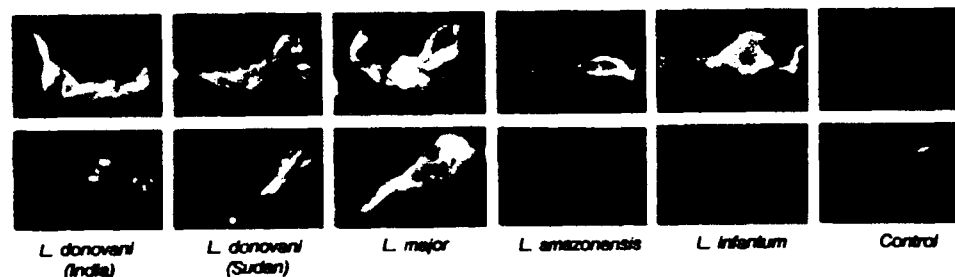


FIG. 6. Fluorescent staining of midguts of *P. argentipes* (Upper) and *P. papatasi* (Lower) incubated with LPG purified from procyclic promastigotes of various *Leishmania* species. Control guts were incubated with primary and secondary antibodies only.

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 permissive vectors. Surprisingly, *P. argentipes*, 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. 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* 1S (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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