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Microreview

Leishmania–sand fly interactions controlling species-specific vector competence

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Summary

Leishmaniasis is caused by a wide range of parasites that are transmitted by an even wider range of sand fly vectors. The phlebotomine vectors of Leishmaniasis are in some cases only permissive to the complete development of the species of *Leishmania* that they transmit in nature. The parasite–sand fly interactions that control this specificity are related to differences in the ability of the parasite to inhibit or to resist killing by proteolytic enzymes released into the mid-gut soon after blood feeding, and/or to maintain infection in the mid-gut during excretion of the digested blood meal. In each case, surface expressed or released phosphoglycan-containing molecules appear to promote parasite survival. The evidence that the surface lipophosphoglycan (LPG) mediates promastigote attachment to the mid-gut epithelium so as to prevent their loss during blood-meal excretion is especially strong based on the comparison of development in sand flies using LPG-deficient mutants. LPG displays interspecies polymorphisms in their phosphoglycan domains that in most cases can fully account for species-specific vector competence.

Introduction

Protozoan parasites of the genus *Leishmania* are transmitted through the bite of infected phlebotomine sand flies. *Leishmania* produce a spectrum of diseases in their human hosts, the cutaneous and visceral forms of which are determined in large part by the species of transmitted parasite. The distribution of the more than 20 species and subspecies of *Leishmania* and the diseases they produce are determined by the availability of competent vectors. In a comprehensive review of phlebotomine vectors of leishmaniasis (Killick-Kendrick, 1990a), it was concluded that of the 81 sand fly taxa, at least 19 have been incriminated as vectors. A summary of Old World *Leishmania* species, their clinical associations, geographical distribution and proven or suspected sand fly vectors is provided in Table 1.

Based on field investigations, there appears to be a close evolutionary fit between a *Leishmania* species and the sand fly species that transmits it in nature, i.e. certain sand flies are able to transmit only certain species of *Leishmania* (Killick-Kendrick, 1985). There is, for example, no evidence that *Phlebotomus papatasi* is involved in the natural transmission of any species other than *Leishmania major*, despite the fact that this sand fly has a wide distribution in regions endemic for other species of *Leishmania*. Similarly, *P. sergenti* is a proven vector of only *L. tropica*, again despite the fact that it is found in biotopes containing other *Leishmania* species. These specific associations have been reproduced in the laboratory; *P. papatasi*, fed on either experimental lesions or through a membrane, will support the full growth and development of *L. major*, but not of any other *Leishmania* species (Adler, 1927, 1938; Adler *et al*., 1938; Heyneman, 1963; Pimenta *et al*., 1994). *Phlebotomus sergenti* has shown a high specificity for *L. tropica* strains, which are able to develop mature, potentially transmissible infections, whereas *L. major and L. donovani* fail to develop (Killick-Kendrick *et al*., 1995; Kamhawi, 2000). This review will focus on those studies that have attempted to define the *Leishmania*–sand fly interactions that control species-specific vector competence.

Some general aspects of the complete development of *Leismania* in sand flies involving Old World parasite/vector combinations appear to be consistent (Molyneux and Killick-Kendrick, 1987; Killick-Kendrick, 1990b; Lawyer *et al*., 1990). The blood meal containing the ingested amastigotes is taken into the abdominal mid-gut where it is rapidly enveloped by the peritrophic membrane; a chitinous matrix that is secreted by gut epithelial cells. The peritrophic membrane begins to break down at about 3 days, accelerated perhaps in infected flies by the action of a parasite-derived chitinase (Schlein *et al*., 1991). Procyclic promastigotes, which appear as short, ovoid, slightly motile forms, develop in the abdominal mid-gut

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Table 1. Proven or suspected vectors of Old World *Leishmania* spp., their clinical associations and geographical distribution.

Parasite	Clinical associations[a]	Geographical distributions	Proven or suspected vectors
L. donovani	AVL; PKDL	China	*P. alexandri*
		Indian subcontinent	*P. argentipes*
		East Africa	*P. martini*
		East Africa	*P. orientalis*
L. infantum	ZVL; ZCL	Southern Europe	*P. ariasi*
		Southern Europe; Eastern Medit	*P. perniciosus*
		Eastern Mediterranean	*P. langeroni*
		China	*P. chinensis*
		China; Eastern Medit	*P. major*
L. major	ZCL	Africa, Middle East, South-west Asia	*P. papatasi*
		Africa	*P. dubosqi*
L. tropica	ACL; LR	Africa, Middle East, South-west Asia	*P. sergenti*
		Kenya	*P. saevus*
L. aethiopica	CL; MCL; DCL	East Africa	*P. longipes*
		East Africa	*P. pedifer*

a. AVL, anthroponotic visceral leishmaniasis; PKDL, post kala-azar dermal leishmaniasis; ZVL, zoonotic visceral leishmaniasis; ZCL, zoonotic cutaneous leishmaniasis; ACL, antroponotric cutaneous leishmaniasis; LR, Leishmaniasis recidivans; MCL, mucocutaneous Leishmaniasis; DCL, diffuse cutaneous leishmaniasis.

after 18–24 h, and undergo rapid division prior to their transformation to long, slender promastigotes, termed nectomonads, 3–4 days post-feeding. These forms fill the anterior abdominal mid-gut, with many becoming attached by their flagella to the microvillar lining. By 4–5 days, most of the digested blood meal is excreted and nectomonads can be found in the thoracic mid-gut. This forward migration is accompanied by transformation to shorter, broader haptomonads and rounded paramastigotes, as well as short, slender, highly active metacyclic promastigotes. These forms establish a massive infection of promastigotes at the stomodeal valve, which is in some cases accompanied by the invasion of the foregut, including the pharynx, cibarium and proboscis. It is believed that metacyclic promastigotes, derived from the foregut, or from behind a degenerating stomodeal valve (Schlein *et al*., 1992), are inoculated during blood feeding and initiate infection in the mammalian host. Although the average number of promastigotes delivered into the skin by an infected sand fly remains unknown, data from forced feeding experiments suggest that as few as 100–1000 parasites is not an underestimate (Warburg and Schlein, 1986). The sequential morphological development of promastigotes and the regions of the alimentary tract in which they are commonly found are summarized in Fig. 1.

Susceptibility and resistance of sand flies to the complete development of *Leishmania* appear to be under genetic control, which at least in the case of *P. papatasi*, is polygenic (Wu and Tesh, 1990a, b). While the genes controlling these phenotypes have not been identified, their functional associations are suggested by the barriers to complete development that have been identified in refractory flies. These barriers include: (i) the digestive enzymes that are released into the mid-gut after blood feeding that can inhibit early parasite survival and growth; (ii) the peritrophic membrane surrounding the blood meal in the abdominal mid-gut that can act as a physical barrier to the anterior migration of the parasite; and (iii) the excretion of the mid-gut contents after blood-meal digestion that can result in the removal of parasites from the gut. Of these potential barriers to the development of fully transmissible infections, differences in the ability of the parasite to survive within the early blood-fed mid-gut and/or to persist in the gut after excretion of the digested blood meal have received the most experimental support.

Early susceptibility to mid-gut proteases

Proteolytic enzymes are secreted into the lumen of the mid-gut in response to ingestion of blood, and a maximal level is reached in many blood-sucking diptera, including sand flies, 18–32 h after blood intake (Dillon and Lane, 1993; Schlein and Jacobson, 1998). Adler (1938) was the first to investigate how blood-meal digestion during the early stages of infection in *P. papatasi* might explain its natural resistance to certain parasites. He found that by decreasing the percentage of serum in the blood meal, the infection rate in flies infected with an inappropriate species (presumably *L. tropica*) was significantly enhanced. These studies in *P. papatasi* have been recently reproduced using another inappropriate *Leishmania* species, *L. donovani*, with the added finding that the enhancement of infection in blood meals devoid of serum was associated with decreased proteolytic activity in the mid-gut (Schlein

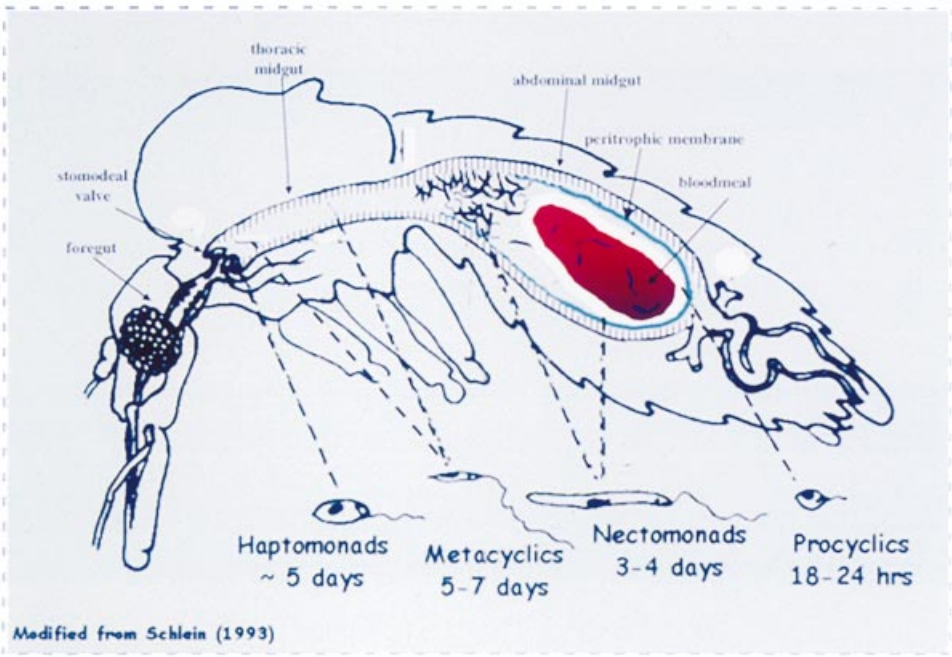

Fig. 1. Diagram of the sand fly alimentary tract containing an infective blood meal. The figure depicts a partially digested blood meal with dividing promastigotes in the abdominal mid-gut, a partially degenerated peritrophic membrane, and escaped promastigotes with some attached to the microvilli via their flagella. The sequential morphological development of promastigotes and regions of the gut in which these forms are typically found are also shown.

and Jacobson, 1998). Other treatments that reduced proteolytic activity in the gut, such as the addition of soyabean trypsin inhibitor to the blood meal (Borovsky and Schlein, 1987), also promoted the early survival of inappropriate species.

These findings on their own do not account for the differential survival of *Leishmania* species within the mid-gut. Such a mechanism was proposed by Schlein *et al*. (Schlein and Romano, 1986; Borovsky and Schlein, 1987) who reported that the proteolytic enzymes produced by *P. papatasi* during blood-meal digestion were inhibited or delayed by infection with *L. major* but not by other species. Proteolytic activity could be similarly modified by released glycoconjugates, with the survival of a glyco-conjugate-deficient strain enhanced by addition of material released by *L. major* but not *L. donovani* (Schlein, Schnur *et al*., 1990). These glycoconjugates represent a class of phoshoglycan-containing molecules that are either attached to the cell surface through phosphatidylinositol (PI) lipid anchors (lipophosphoglycan, LPG) or secreted as protein-containing phosphoglycans (proteophosphoglycan, PPG, and an acid phosphatase, sAP) (Ilg *et al*., 1994; Mengeling and Turco, 1998). Parasite mutants deficient in these molecules were killed within the first 24 h after blood feeding and could be rescued by restoration of phosphoglycan expression after transfection with the appropriate gene (Sacks, *et al*., 2000). Released molecules bearing the phosphoglycan epitope were detected in high abundance in *L. major*-infected *P. papatasi* mid-guts as early as day 2 (Davies *et al*., 1990), and the fibrous network of secreted PPG and sAP produced by some *Leishmania* species *in vitro* has been suggested to correspond to a similar gel-like matrix observed in infected sand flies (Walters *et al*., 1987; Lawyer *et al*., 1990). In addition to inhibiting the levels of proteolytic enzymes in the gut, an abundance of these secreted phosphoglycan-containing products, by virtue of their negative charge, might protect the promastigote by acting as a transient barrier against digestive enzymes in the vicinity of the parasite. The surface LPG, which is organized as a densely packed glycocalyx structure, might further protect the cell surface from proteolytic attack. Because the membrane-bound and released glycoconjugates display interspecies differences in their phospoglycan structures (detailed below), these polymorphisms might account for differences in the ability of these molecules to inhibit the levels of digestive enzymes in the gut or to protect the parasite surface from proteolytic damage.

If the species specificity of vector competence is related to differential inhibition of or susceptibility to digestive enzymes in the blood-fed mid-gut, then this should be reflected by differences in parasite survival and growth during their early exposure to these enzymes in the gut. Reduced parasite numbers and even dead or damaged parasites have been observed in the mid-guts of

refractory sand flies 2–3 days after blood feeding (Shatova *et al*., 1984; Pimenta *et al*., 1994; Schlein and Jacobson, 1998).

Loss of infection associated with blood-meal excretion

The majority of studies that have followed the development of various *Leishmania* species within inappropriate vectors have not observed an early inhibition of parasite survival and growth. Instead, the loss of infection occurs later, and is associated with the excretion of the digested blood meal. The studies summarized below are grouped according to the Old World *Leishmania* species that they concern.

Early studies compared the development of strains responsible for visceral leishmaniasis (*L. donovani and L. infantum*) within susceptible and refractory sand flies. Hindle (1931) observed that compared with the natural vector species (*P. major*), *P. sergenti* seems to be an equally favourable host for the early development of a Chinese strain of *Leishmania* (presumably *L. infantum*), but that the persistence of flagellates in the mid-gut was dependent on the presence of undigested blood meal. When the alimentary canal no longer contained any food material, then the flagellates disappeared. Heyneman (1963) examined the development of a newly isolated strain of *L. donovani* from Sudan in two laboratory-reared colonies of *P. papatasi* and found uniformly high-intensity mid-gut infections at days 1 and 2, a moderate reduction in parasite numbers at days 3 and 4, followed by rapid loss of most parasites through the hindgut on day 5. Killick-Kendrick (1985) examined the development of *L. infantum* in wild-caught *P. sergenti* and in a natural vector, *P. perniciosus*. Both sand fly species became initially infected in similar proportions; however, the parasites in *P. sergenti* were lost with the faeces passed on days 3–4. In more recent studies (Pimenta *et al*., 1994; Kamhawi, 2000), the survival and growth of *L. donovani* strains from Sudan and India in either *P. papatasi* or *P. sergenti* were found to be similar to that observed for the appropriate *L. major* and *L. tropica* species, respectively, during the first 2–3 days after feeding. On days 4–7, shortly after the blood meals had been digested and passed, the infection rate remained $>90\%$ in *P. papatasi* infected with *L. major*, and $>70\%$ for *P. sergenti* infected with *L. tropica*, but 0% in flies infected with *L. donovani*.

A number of studies have compared the development of *L. major* in vector competent and refractory flies. Infection rates for *L. major* in a refractory sand fly (*Sergentomyia schwetzi*) were similar to the rates within a natural vector (*P. duboscqi*) for the first 3 days post-feeding, after which time infections in *S. schwetzi* were completely lost (Lawyer *et al*., 1990). Identical findings were reported when *L. major* amastigotes were used to infect *P. sergenti* or *P. papatasi* (Kamhawi, 2000). At days 3 and 4 after the ingestion of blood, the mean parasite load was high in both flies. Whereas mature mid-gut infections developed in a high proportion of *P. papatasi* on days 7–13, the complete loss of *L. major* in *P. sergenti* followed closely with the passage of the digested blood meal in these flies (days 4–7). The inability of certain substrains of *L. major* to produce mature infections in *P. papatasi* or *P. dubosqi* was also associated with the rapid loss of heavy mid-gut infections immediately after defecation of the digested blood meal (Cihakova and Volf, 1997).

Finally, a few studies have compared the development of *L. tropica* in its natural vector, *P. sergenti*, with its development in resistant flies. When a laboratory colony of *P. papatasi* from Afghanistan was fed through a membrane on a high dose of *L. tropica* amastigotes from the same place, only 6% developed heavy, anterior infections (Killick-Kendrick *et al*., 1994). Because the flies were only examined at late time points (days 9–11) it is not possible to know at what stage the infections were lost. In more recent studies, however, the growth of a strain of *L. tropica* in *P. papatasi*, each originating from the Jordan Valley, was heavy during the first 2–3 days after the infective feed, and the loss of mid-gut promastigotes was clearly associated with defecation of the blood-meal remnants during days 4–7 (Kamhawi, 2000). The ability of the *L. tropica* strain to develop late, mature infections in a sympatric *P. sergenti* sand fly was confirmed in these studies.

The role of LPG in mediating mid-gut attachment

The abundance of apparently healthy promastigotes at relatively late time points in refractory flies argues against a role for killing by digestive enzymes in the blood-fed mid-gut, particularly because the peak concentration of these enzymes is thought to occur at 18–36 h post-feeding. The strong correlation between the loss of blood meal and the sudden loss of promastigotes suggests that the inability of *Leishmania* strains to persist in an inappropriate sand fly is related to their failure to remain anchored to the gut wall via specific attachment sites. Ultrastructural studies have consistently revealed promastigotes attached to the mid-gut epithelium via insertion of their flagella between the microvilli (Warburg *et al*., 1986; Molyneux, 1987; Walters *et al*., 1989; Lang *et al*., 1991). A role for LPG in mediating mid-gut attachment was predicted based on the fact that it is the major surface glycoconjugate on *Leishmania* promastigotes (McConville *et al*., 1992). It is expressed on the entire surface,

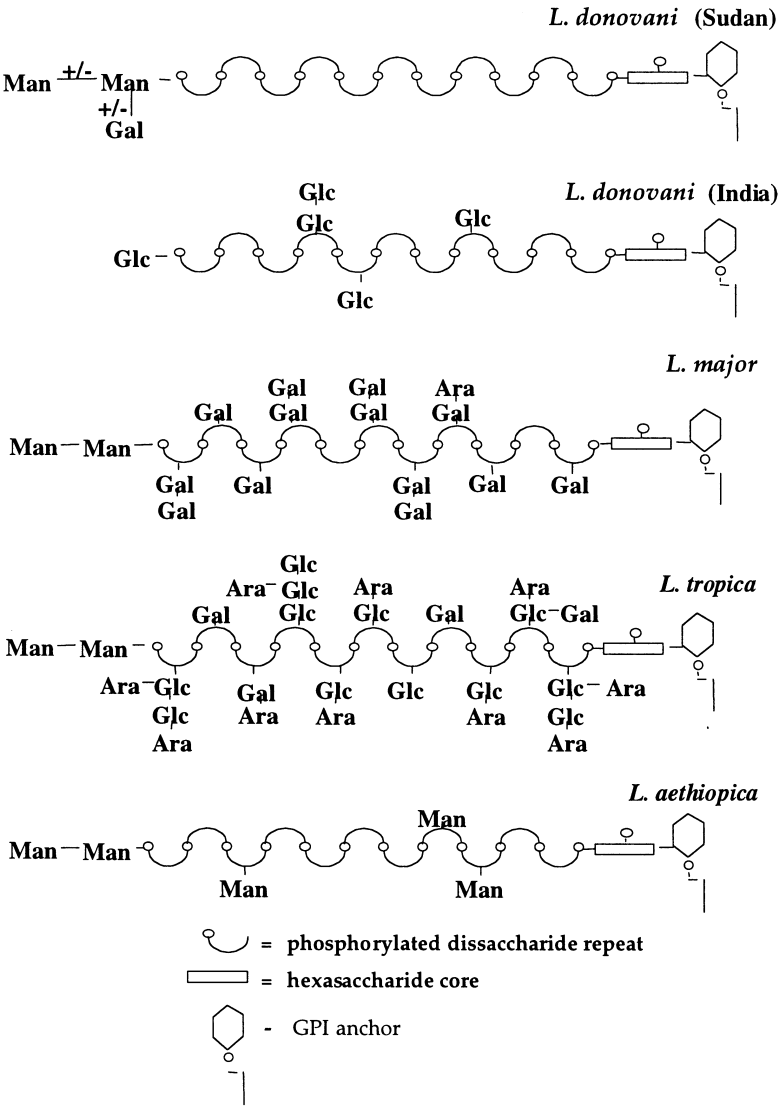

Fig. 2. Structural polymorphisms of LPGs from Old World *Leishmania* species. The oligosaccharide core and lipid anchor domains are conserved between species. The fine structures of the phosphoglycan domains are highly speculative, and reflect only the average proportion of each oligosaccharide repeat unit that has been shown to be expressed by the procyclic LPGs of each species.

including the flagellum, and is organized as a densely packed filamentous glycocalyx. LPG is a tripartite molecule, consisting of a phosphoglycan domain linked via a hexasaccharide glycan core to a 1-*O*-alkyl-2-*lyso*-phosphatidylinositol lipid anchor. The phosphoglycan moieties of all LPGs studied to date share a common backbone consisting of repeating disaccharide units of PO_4-6Gal(β1-4)Manα1, where the 3- position of the Gal residue can either be unsubstituted (*L. donovani,* Sudan) (Turco *et al*., 1987), partially substituted with glucose sidechains (Indian *L. donovani*) (Mahoney *et al*., 1999), or completely substituted with side-chain sugars that terminate primarily in galactose (*L. major*) (McConville *et al*., 1992) or in glucose and arabinose (*L. tropica*) (McConville *et al*., 1995). The polymorphic structures of LPGs from the clinically important Old World species of *Leishmania* are depicted in Fig. 2.

A role for LPG in mediating attachment to the mid-gut epithelium seems especially convincing based on a number of findings: (i) purified LPG binds to mid-guts *in vitro* (Pimenta *et al*., 1992, 1994; Kamhawi, 2000), (ii) LPG completely inhibits the binding of promastigotes to the gut in *in vitro*-attachment assays (Pimenta *et al*., 1992; Sacks *et al*., 1995), and (iii) LPG deficient mutants fail to attach to the mid-gut *in vitro* or to persist in the sand fly after blood-meal excretion *in vivo* (Sacks *et al*., 2000). The possibility that the polymorphic structures of the phosphoglycan domains of LPG might control species-specific mid-gut attachment, and by extension species-specific vector competence, has been investigated in both *P. papatasi* and *P. sergenti*. The ability of *P. papatasi* to transmit only *L. major* sp. has been attributed to the unique, highly substituted nature of *L. major* LPG that provides for multiple terminally exposed β-linked

galactose residues for binding. The LPGs of *Leishmania* species that lack side-chain substitutions, or express side-chains that do not terminate in β-linked galactose residues, fail to bind to *P. papatasi* mid-guts *in vitro*, and the parasites that bear these surface structures fail to persist in *P. papatasi* after blood-meal excretion (Pimenta *et al*., 1994). Furthermore, *L. major* mutants that express surface LPG devoid of galactose-containing side-chains lost their ability to attach to *P. papatasi* mid-guts *in vitro*, and also failed to persist in the mid-gut after blood-meal excretion (Butcher *et al*., 1996). In a reciprocal fashion, *L. major* LPG or unbranched LPGs from *L. donovani* failed to bind to *P. sergenti* mid-guts *in vitro*, whereas binding was readily observed using *L. tropica* promastigotes or purified *L. tropica* LPG (Kamhawi, 2000). The oligosaccharides that mediate *L. tropica* binding have not been investigated, but presumably involve the glucose-and arabinose-terminating side-chains that are unique to this *Leishmania* species.

The comparison of promastigote binding to the mid-guts of different phlebotomine vectors indicates that the parasite recognition sites which these flies express are in some cases different, and might therefore provide the evolutionary drive for LPG structural polymorphisms. The selection for the highly branched and species-specific LPG structures expressed by *L. major* and *L. tropica* strains occurred, in this view, in order for these parasites to take advantage of widely distributed sand fly species, *P. papatasi* and *P. sergenti*, respectively, that are inherently refractory to *Leishmania* that express unsubstituted or inappropriately substituted forms of LPG. While little is known about the mid-gut receptors that are involved in these interactions, sand fly lectins that agglutinate *Leishmania* promastigotes *in vitro* have been described (Wallbanks *et al*., 1986; Svobodova *et al*., 1996; Volf *et al*., 1998), and preliminary information regarding a protein from *P. papatasi* mid-guts that binds to *L. major* LPG has recently been reported (Dillon and Lane, 1999). There may be a different receptor lining the gut that is involved in binding of the parasite via a flagellar protein that was identified using a monoclonal antibody that inhibited the binding of flagellar preparations to frozen sections of mid-guts *in vitro* (Warburg *et al*., 1989). While the inhibition observed was only partial and may have been due to steric interference of LPG-mediated binding, it is certainly possible that this protein contributes to the flagellum-oriented attachment to microvilli that has been typically described.

In comparison with the natural vectors of *L. major* and *L. tropica*, which demonstrate exquisite specificity for their respective parasite strains in the laboratory, the natural vectors of *L. donovani* and *L. chagasi*, which express non-branching or poorly substituted LPGs, appear to be broadly permissive to diverse *Leishmania* species. *Lutzomia longiplapis*, for example, which is the natural vector of *L. chagasi* transmission in the New World, has been used to study the complete development of *L. amazonensis* and *L. major* (Molyneux *et al*., 1975; Walters *et al*., 1993). *Phlebotomus argentipes*, which is the natural vector of *L. donovani* transmission in India, was also susceptible to the full development of *L. major, L. tropica* and *L. amazonensis*. (Pimenta *et al*., 1994; Kamhawi, 2000). The data suggest that *L. longipalpis and P. argentipes* mid-guts possess a receptor, lacking in *P. papatasi* and *P. sergenti*, for a conserved oligosaccharide on LPG, or else the binding is mediated by some other relatively conserved molecule on the promastigotes surface (e.g. the flagellar protein described above). Consideration should also be given to the possibility that the physiology of blood-meal digestion and expulsion differs in these species such that these flies exert little or no evolutionary pressure on the parasite to attach strongly to the mid-gut epithelium in order to maintain infection. The findings that certain sand flies are broadly permissive to multiple *Leishmania* species in the laboratory suggests that under appropriate field conditions these vectors might be involved in the transmission of more than one species of *Leishmania*. To date, however, there is no clear documentation that this has occurred.

In conclusion, phlebotomine vectors of leishmaniasis in some instances display striking species-restricted competency for the parasite strains that they transmit in nature. The specificity of the vector–parasite interactions appear to be controlled by phosphoglycan-containing molecules that have been implicated in the differential susceptibilty of *Leishmania* to digestive enzymes released into the blood-fed mid-gut, and/or to differences in LPG-mediated attachment to mid-gut epithelial cells. The species-specific oligosaccharides that these molecules display remain the clearest example of adaptive processes that are driven by the requirements for survival in the invertebrate rather than vertebrate host.

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