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MOLECULAR ASPECTS OF PARASITE-VECTOR AND VECTOR-HOST INTERACTIONS IN LEISHMANIASIS[1]

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■ **Abstract** *Leishmania*-sand fly interactions are reviewed in the context of the potential barriers to the complete development of the parasite that exist within the midgut environment of phlebotomine flies and the molecular adaptations that the parasite has evolved that permit the development of transmissible infections to proceed. Cell surface and secreted phosphoglycans protect the parasite from the proteolytic activities of the blood-fed midgut, mediate attachment to the gut wall in order to maintain infection during excretion of the bloodmeal, and contribute to the formation of a biological plug in the anterior gut that may promote transmission by bite. The importance of vector saliva in modulating the host response to transmitted parasites is also reviewed.

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INTRODUCTION

Leishmania are pathogenic protozoa of the order Kinetoplastida and the family Trypanosomatidae. They have a dimorphic life cycle consisting of extracellular promastigotes that multiply and develop within the alimentary tract of the sand fly vector and intracellular amastigotes that reside and multiply within the phagolysosomal vacuoles of their host macrophages. Depending mainly on the species of *Leishmania*, human infection can display a spectrum of clinical manifestation from localized cutaneous involvement to late destruction of mucous membranes to generalized systemic disease with fatal outcome. Distribution of the more than 20 species and subspecies of *Leishmania* and the diseases they produce are determined by the availability of competent vectors. As far as is known, natural vectors of all *Leishmania* species belong to the subfamily Phlebotominae (sand flies) of the family Psychodidae. The greatest concentration of phlebotomine species is in the forests of South America, where the genus *Lutzomyia* predominates. *Phlebotomus* is one of two large genera found in the Old World and is distributed largely in arid and semi-arid zones. *Lutzomyia* and *Phlebotomus* spp. account for virtually all known transmissions of *Leishmania* to humans, comprehensively reviewed by Killick-Kendrick (54). Moreover, even the vector-competent species often display remarkable specificity for the *Leishmania* species they transmit in nature (reviewed in 53). In many instances, these restrictions cannot be accounted for by the absence of sympatric (colocalized) sand flies or by host blood-meal preferences. Thus, many if not most phlebotomine species appear to be inherently refractory to the full development of *Leishmania*. A summary of the major *Leishmania* species infecting humans, their clinical associations, geographic distribution, and proven or suspected sand fly vectors is provided in Table 1.

Understanding the basis of molecular interactions at the sand fly–*Leishmania* interface is fundamental to any study of vector competence and disease transmission. This review focuses on those studies that have identified parasite- or sand fly–derived molecules that play a role in the development of transmissible infections and on those studies that have investigated the role of sand fly saliva in modulating the outcome of infection in the mammalian host.

Development of Transmissible Infections

The first experimental evidence of transmission of leishmaniasis by the bite of sand flies was by Shortt et al in 1931 (108), who achieved the transmission of *Leishmania donovani* to hamsters by the bite of *Phlebotomus argentipes*. Similar studies involving other species of *Leishmania* and sand flies (3, 62, 78, 126)

TABLE 1 Proven or suspected vectors of the major *Leishmania* spp. infecting humans, including their clinical associations and geographic distributions

Parasite	Clinical forms[a]	Geographic distributions	Proven or suspected vectors
L. donovani	AVL; PKDL	China	*P. alexandri*
		India, Nepal, Bangladesh	*P. argentipes*
		East Africa	*P. martini, P. orientalis*
L. infantum	ZVL; ZCL	Southern Europe	*P. ariasi, P. perniciosus*
		Eastern Mediterranean	*P. perniciosus, P. langeroni*
		China	*P. chinensis, P. major*
L. major	ZCL	Middle East, Southwest Asia	*P. papatasi*
		Africa	*P. papatasi, P. duboscqi*
L. tropica	ACL; LR	Middle East, Southwest Asia	*P. sergenti*
		Africa	*P. sergenti, P. saevus*
L. aethiopica	CL; MCL; DCL	East Africa	*P. longipes, P. pedifer*
L. chagasi	ZVL; ZCL	Central and South America	*Lu. longipalpis*
L. mexicana	ZCL; DCL	Central America	*Lu. olmeca*
L. amazonensis	ZCL; DCL	Central and South America	*Lu. flaviscutellata*
L. V. braziliensis	CL; MCL	Central and South America	*Lu. wellcomei, Lu. carrerai*
L. V. guyanensis	CL	Central and South America	*Lu. umbratilis, Lu. anduzei*
L. V. panamensis	CL	Central and South America	*Lu. trapidoi*

[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.

indicate that while the life cycle of *Leishmania* within their phlebotomine vectors varies depending on the parasite-vector pairs involved, some general aspects regarding the development of suprapylarian *Leishmania* species appear to be consistent [reviewed in (52)]. Suprapylarian *Leishmania* species include all members of the genus, with the exception of members of the *Vianna* subgenus, e.g., *Leishmania braziliensis*; these species are distinguished by the fact that they do not enter the hindgut but confine their development to the midgut of their natural vectors.

Three general stages of parasite development in the vector are depicted in Figure 1: (*a*) The infective bloodmeal containing *Leishmania* amastigotes is passed

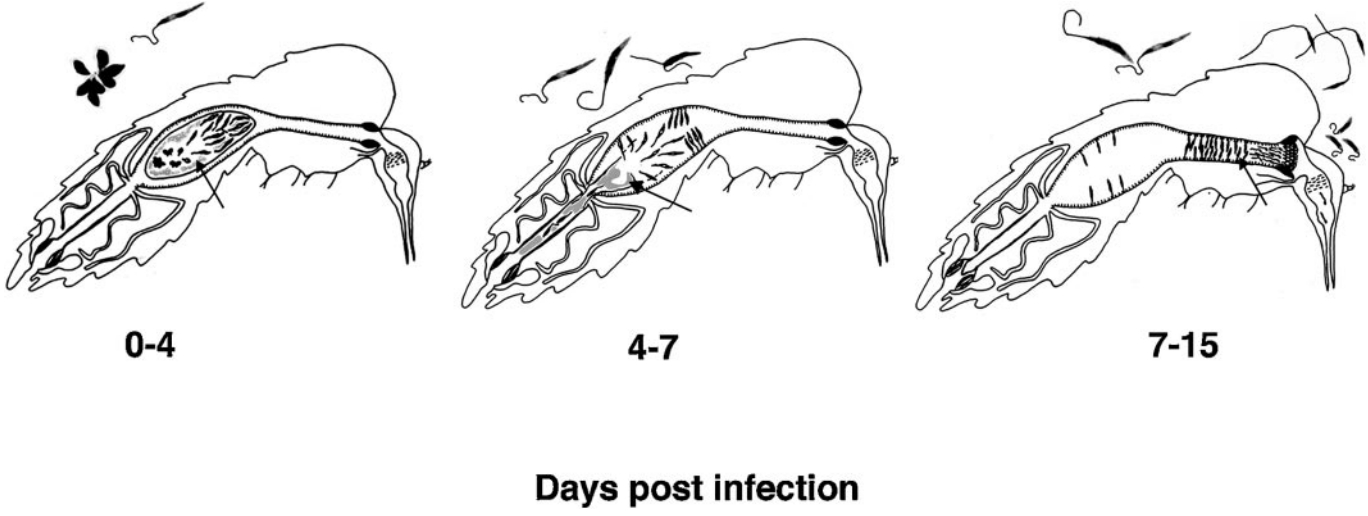

Figure 1 Stages in the development of *Leishmania* infection in the sand fly, including the sequential appearance of promastigote morphological forms and their location within the different aspects of the midgut and foregut.

into the abdominal midgut, where water is removed and the blood is retained inside a well-structured peritrophic membrane (PM). The transformation of amastigotes to promastigotes occurs within 12–18 h. These initially transformed promastigotes are termed procyclics and remain short, ovoid, and only slightly motile. Intense multiplication of these forms begins at approximately 18–24 h. Dividing promastigotes are found in rosettes with flagella directed toward the center. During 36–60 h, rapid multiplication within the digesting bloodmeal (arrows) continues, accompanied by the transformation of promastigotes to a long, slender, highly motile form termed nectomonad. (*b*) By 60–72 h, tremendous numbers of nectomonads are found packed in the anterior portion of the abdominal midgut, with many attached via their flagella to the epithelial cell microvilli. Rupture of the peritrophic membrane is first visible in the anterior parts of the envelope, and small amounts of digested blood may be visible in the hindgut. (*c*) By day 7, the passage of the digested bloodmeal is complete, and the anterior migration of promastigotes to the region of the cardia (thoracic midgut) and stomodeal valve proceeds until a massive accumulation of parasites behind the valve is achieved. This migration is associated with the transformation of nectomonads into short, broad forms termed haptomonads, which are occasionally seen in division, and into metacyclic promastigotes, which are short, slender, highly active forms with a flagellum at least twice the length of the cell body and which are never seen in division. Haptomonads attach via hemidesmosomes to the cuticular intima of the stomodeal valve or to each other by a viscous gel-like matrix that restricts their motility. The valve itself appears to become degenerated. Some parasites, mainly metacyclics, migrate beyond the stomodeal valve into the esophagus, pharynx, and proboscis. It is believed that metacyclic promastigotes, derived from the foregut or from behind a degenerating stomodeal valve, are inoculated during blood feeding and initiate infection in the mammalian host.

Potential Barriers to Complete Development

The life cycle of suprapylarian *Leishmania* species within their natural or permissive vectors suggests a number of potential barriers to complete development that may have provided the evolutionary drive for expression of specific parasite molecules required for successful development in the fly. These barriers include the digestive enzymes in the gut, which may inhibit the early growth of parasites in the bloodmeal; the peritrophic membrane, which may behave as a physical barrier to parasite migration out of the abdominal midgut; the excretion of the digested bloodmeal, which may result in loss of infection from the gut; and the anatomy and physiology of the anterior gut, which may prevent the forward movement of metacyclic promastigotes and their egestion during blood feeding. The study of the molecular basis of these various parasite-vector interactions has been facilitated by the fact that there are species, and in some cases, intraspecific differences among *Leishmania* in their infectivity for a particular sand fly vector. Reciprocally, there are species differences among sand flies for a particular *Leishmania*. Heroic efforts at establishing *Leishmania major*–resistant and -susceptible lines of *Phlebotomus*

papatasi have confirmed that these phenotypes are under genetic control, which at least in the case of *P. papatasi* is polygenic (132, 133). Unfortunately, these lines were not maintained, and the sand fly molecules controlling susceptibility or resistance in these crosses have not been identified. Furthermore, molecular comparisons involving different species of sand flies that are resistant or susceptible to a particular *Leishmania* have not been pursued. Thus, it has been the identification of species- or strain-specific parasite molecules and the generation of *Leishmania* mutants specifically deficient in these molecules that have led to most of the advances in our understanding of the molecular basis of vector competence.

SURVIVAL OF *LEISHMANIA* IN THE BLOOD-FED MIDGUT

Susceptibility to Digestive Enzymes in the Blood-Fed Midgut

The ingestion of the bloodmeal induces the synthesis and secretion of digestive enzymes, including trypsin, chymotrypsin, aminopeptidase, carboxypeptidase, and alpha-glucosidase, into the lumen. These enzymes are released into ectoperitrophic space and pass across the PM to digest the bloodmeal. In the midgut of unfed *Phlebotomus langeroni* and *P. papatasi* there is little baseline protease activity. Following bloodmeal ingestion, significant levels of proteases are produced in 10 h and peak levels are reached between 18 and 48 h, depending on the sand fly species and the source of the bloodmeal (10, 22, 25). Adler (1) was the first to investigate how bloodmeal digestion during the early stages of infection in *P. papatasi*, which normally transmits *L. major*, might explain its natural resistance to other *Leishmania* species. He found that by decreasing the percentage of rabbit serum in the bloodmeal, the infection rate in flies infected with an inappropriate species (presumably *Leishmania tropica*) was significantly enhanced. He suggested that products of serum digestion destroy the incompatible *Leishmania* species and that lowering the concentration of the serum protein components of the bloodmeal lowered the level of induced proteases. It was not specified in this brief report when the loss of infections involving the inappropriate strain occurred, although it is presumed to have occurred early, during the peak of digestive activies within the blood-fed midgut. Reduced parasite numbers and even dead or damaged parasites have been observed in the midguts of sand flies infected with noncompatible *Leishmania* strains 2–3 days following blood feeding (62, 97, 106). Pimenta et al (82) observed that the midgut environment, in the first few hours after blood feeding, is harmful even for a strain of *L. major* that is capable of complete development in the fly, because roughly 50% of the initial parasite inoculum was killed during this time. Adler's idea that these harmful conditions are due to the high concentrations of proteolytic enzymes has been validated in more recent studies in which the enhanced survival of *L. donovani* in *P. papatasi* following meals devoid of serum (erythrocytes, saline, and amastigotes) was correlated with delayed timing and decreased levels of peak protease activities (97). Other treatments that reduced proteolytic activity in the gut, such as the addition of soybean trypsin inhibitor

to the bloodmeal, also promoted the early survival of *L. donovani* in *P. papatasi* (10, 82).

Inhibition of Midgut Digestion in Infected Sand Flies

Implied in these results is that the relative survival of a compatible *L. major* strain within *P. papatasi* is due either to their intrinsic resistance to digestive enyzmes in the blood-fed midgut or to their ability to reduce the levels of enzymes to which they are exposed. Differences in the susceptibility of *Leishmania* strains or species to proteolytic enzymes has not, so far as we are aware, been investigated to date. There is experimental support for the later suggestion regarding the inhibitory effects of parasites on digestion. Schlein et al (10, 100) reported that the proteolytic enzymes produced by *P. papatasi* during digestion of a rabbit bloodmeal were inhibited or delayed on infection with *L. major* promastigotes but not *L. donovani* promastigotes. Similarly, Dillon & Lane (26) reported that inclusion of amastigotes of *L. major* in a human bloodmeal delayed and depressed the peak levels of protease activity in the *P. papatasi* midgut. The effects of inappropriate *Leishmania* species were not compared. It should be noted that the survival of *L. major* in *P. papatasi* has also been observed in the absence of any significant inhibition of or delay in peak protease activities during infection (82). Moreover, the significance of the *P. papatasi* data would be reinforced were the inappropriate *Leishmania* species found to be capable of inhibiting bloodmeal digestion in their natural or permissive vectors. The only other vector species for which the effect of *Leishmania* on protease actvities has been studied is *P. langeroni*, which is considered a potential vector for *Leishmania infantum* and an unnatural vector for *L. major*. Blood meals consisting of human or canine blood mixed with *L. infantum* resulted in reduced enzyme activity and a slower rate of blood-protein digestion compared with normal blood or blood mixed with *L. major* (21, 22). There was no correlation, however, between the level of the proteolytic activity and the survival of promastigotes in the gut. In each case, survival and growth of promastigotes was observed throughout the period of bloodmeal digestion and up to 16 days postinfection.

The idea that parasite survival depends, at least in part, on modulating the proteolytic activities in the gut would be strengthened if parasites were shown to be damaged or killed by exposure to these enzymes. The only study, so far as we are aware, to address this point directly involved a 1–2 h exposure of *L. major* to lysates of a single blood-fed *P. papatasi* midgut in vitro (82). It is interesting that fresh tissue amastigotes and fully differentiated promastigotes were relatively resistant to killing during this exposure, whereas parasites within early stage amastigote-to-promastigote transition (2–8 h) became highly susceptible to killing, with greater than 95% reduction in numbers of viable parasites compared with controls. These findings are consistent with those of Dillon & Lane (26), who indicated that fully differentiated promastigotes thrive in the presence of trypsin in culture. These findings suggest that species- or strain-related differences in parasite survival within the early blood-fed midgut, particularly when experimental infections are initiated using amastigotes, might be due to differences in the kinetics

of stage transformation. A delay of only a few hours might expose the vulnerable transitional forms to lethal concentrations of proteolytic enzymes.

Parasite Molecules Controlling Early Survival

Taken together, the data indicate that the early blood-fed midgut is a potentially lethal environment for the developing parasite and that expression of stage- and species-related molecules promotes parasite survival and growth during this critical time. The identification of molecules that might play a role in defending the parasite against proteolytic damage has focused on a family of glycoconjugates, the phosphoglycans, that incorpororate the common structure PO_4-6Gal(β1-4)Manα1 (46, 72, 77; Figure 2). These molecules are either attached to the cell surface through glycosylphosphatidylinositol (GPI) lipid anchors, including the lipophosphoglycan (LPG) and the proteophosphoglycan (PPG), or they are secreted as protein-containing phosphoglycans, including the secreted proteophosphoglycan (sPPG) and a secreted acid phosphatase (sAP). All *Leishmania* species express at least two of these characterized phosphoglycans.

LPG is the major glycoconjugate on the surface of *Leishmania* promastigotes. It is expressed on the entire surface, including the flagellum and is organized as

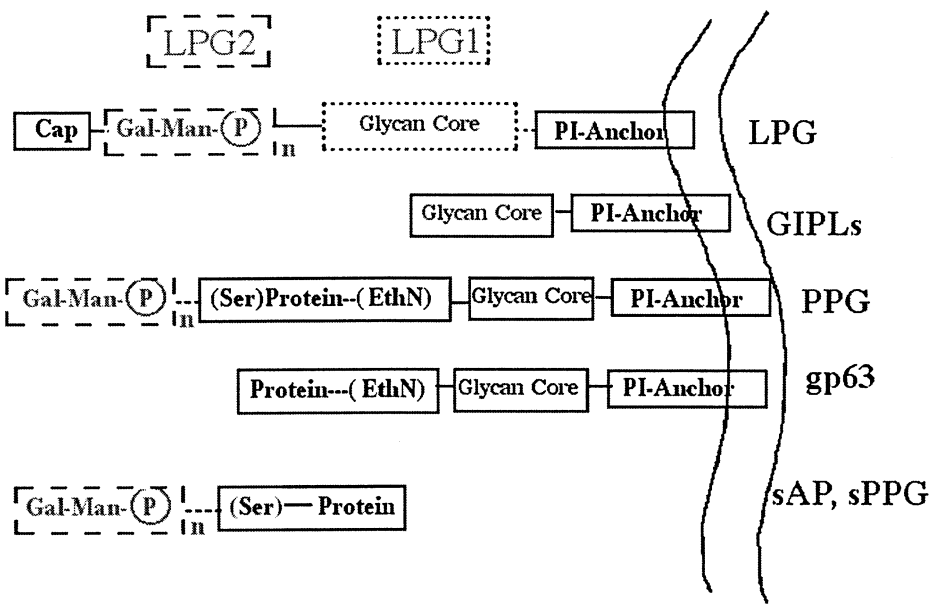

Figure 2 The major glycoconjugates of *Leishmania* promastigotes depicting the shared structures of cell surface and secreted molecules. Structures depicted as either *LPG1* (dotted-lined box) or *LPG2* (dashed-lined box) designate those domains that are specifically affected by mutations in these respective genes.

a densely packed filamentous glycocalyx. LPG is a tripartite molecule consisting of a backbone of multiple repeat phosphodisaccharide units of -6Galβ1,4Manα1-PO_4- that are either unsubstituted or variably substituted with a variety of side chain oligosaccharides (see discussion of interspecies polymorphisms below). The phosphoglycan chain is linked via a hexasaccharide glycan core to a 1-*O*–alkyl-2-*lyso*-phosphatidylinositol lipid anchor, and it is capped by a nonphosphorylated oligosaccharide. The sAP is serine- and threonine-rich, and many of these residues are modifed with phosphoglycans (112). *Leishmania* promastigotes synthesize sAP and release it from the flagellar pocket as monomeric or oligomeric molecules (29, 105). sPPG is released as a filamentous macromolecule that forms a network and appears to be associated with cell aggregation (45). The overall structure of sPPG displays many similarities to mammalian mucins, in which the peptide backbone comprises only a small proportion of the weight (4%), whereas the phosphoglycan modifications of the serine residues comprise the rest (45). Recently, a gene encoding *L. major* PPG has been cloned, the product of which is predominantly membrane associated and is expressed on the promastigote cell surface anchored via a putative GPI (44). Finally, *Leishmania* produce a related class of GPI-anchored molecules that do not incorporate the nominal phosphoglycan. These molecules include the glycophosphatidylinositol lipids (GIPLs) (30) and GPI-anchored proteins such as the metalloproteinase, gp63 (15, 32). The major glycoconjugates of *Leishmania* promastigotes, including their unique and common structures, are depicted in Figure 2.

Analysis of Parasite Mutants

The use of specific mutants deficient in the biosynthesis of one or more of these molecules has been a powerful approach to defining their function. The biosynthetic genes studied to date were identified by genetic complementation of LPG-deficient lines. *LPG1* was identified by transfection of the *L. donovani* R2D2 mutant and is involved in addition of galactofuranose to the glycan core of LPG (42, 89). As such, the defective expression of phosphoglycan containing molecules in R2D2 or in mutants generated by targeted deletion of the *LPG1* gene is restricted to LPG. These mutants continue to assemble and secrete other protein-linked phosphoglycans (110). In contrast, *LPG2*, identified by complementation of another *L. donovani* LPG-deficient mutant called C3PO, encodes a transporter for the uptake of GDP-Man into the Golgi apparatus, and as such, C3PO or mutants generated by targeted deletion of *LPG2* are deficient in the synthesis of all phosphoglycans (24, 69). Study of the interaction of R2D2 with *P. argentipes* or of the *L. major-LPG1*-deficient mutant with *P. papatasi* revealed that, in each case, promastigote growth was only slightly reduced within the early blood-fed midgut (92). In contrast, C3PO or a *L. donovani* LPG2-targeted null mutant were completely killed during their first 24-h exposure to the blood-fed midgut. Transfection of C3P0 and Ld*lpg2*$^-$ with *LPG2* restored their surface LPG expression, their ability to assemble other phosphoglycans, and their capacity to survive the conditions in the digesting bloodmeal. Taken together, the results with the *LPG1* and *LPG2*

mutants suggest that one or more of the other released phosphoglycan-containing structures, e.g., sAP or PPG, play important roles in protecting the parasite during its early exposure to digestive enzymes in the gut. Released molecules bearing the phosphoglycan epitope were detected in high abundance in midguts of *P. papatasi* infected with *L. major* as early as day 2 (23), and the fibrous network of secreted PPG and sAP produced by some *Leishmania* species in vitro may correspond to a similar matrix observed in the abdominal midguts of infected sand flies (111). 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. This would be analogous to the capacity of gastric mucins to protect intestinal epithelial cells in humans.

These data are consistent with an earlier observation by Schlein et al (101), which suggests that released glycoconjugates from cultured *L. major* promastigotes, but not similarly released material from *L. donovani*, could promote early survival of a glycoconjugate-deficient *L. major* strain in the *P. papatasi* midgut. The glycoconjugates were not characterized, but it is likely that sAP and/or PPG were present in the released material. In addition to promoting the survival of the glycoconjugate-deficient *L. major* strain, the released material caused a delay in the digestion of the bloodmeal, which suggests that the amount or activities of the proteolytic enyzmes in the gut had been reduced. Modulation of enzyme activity in the midgut ascribes a different role to these molecules than that suggested for the mucin-like structures, which are thought to protect the parasite surface against the proteolytic enzymes to which they are exposed. Certain secreted enzymes such as sAP may be capable of dephosphorylating and thus inhibiting the activity of fly digestive enzymes. This would not explain why the released glycoconjugates from *L. donovani* failed to achieve the same effect. It is also not known how the interspecies polymorphisms in phosphoglycan domains (see below) could affect the inhibitory activities that these molecules might have for proteolytic enzymes in the gut. Clearly, further studies using well-defined glycoconjugates directly assayed for inhibitory effects on gut digestive enzymes would help to clarify the essential role that these molecules play during the parasite's earliest encounter with the invertebrate host.

The other major class of parasite molecules for which a role in *Leishmania*–sand fly interactions has been explored is gp63. Also referred to as leishmanolysin, it is a 63-kDa zinc metalloproteinase containing a GPI anchor and is expressed on the surface of promastigotes of diverse species of *Leishmania* (11). The presence of gp63-like proteinase and gp63 gene-homologues in monoxenous trypanosomatids (e.g., Crithidia) (31) has led to the speculation that gp63 may play a role in parasite survival within the insect gut. Targeted deletion of the *L. major* gp63 genes did not, however, significantly alter the growth and development of the parasite within the phlebotomine vector (47). The mutants grew normally in the blood-engorged midgut of both *P. argentipes* and *P. papatasi*. Thus, gp63 does not appear to be needed to confer resistance to proteolytic enzymes in the gut, nor does it appear

to be required for nutrient utilization in the bloodmeal during the early stages of development in the fly.

PERSISTENCE OF *LEISHMANIA* FOLLOWING BLOODMEAL EXCRETION

Timing of the Loss of Infection in Refractory Sand Flies

The majority of studies that have carefully 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 bloodmeal. Most of these studies pertain to the development of unnatural *Leishmania* species in *P. papatasi*. Heyneman (39) 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 midgut 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. In more recent studies (83), the survival and growth of *L. donovani* strains from Sudan and India in *P. papatasi* were found to be comparable to that observed for the appropriate *L. major* species during the first 2–3 days after feeding. On days 4–7, shortly after the bloodmeals had been digested and passed, the infection rate remained >90% in *P. papatasi* infected with *L. major* but 0% in flies infected with *L. donovani*. 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 (55). Since 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, the growth of a strain of *L. tropica* in *P. papatasi*, both originating from the Jordan Valley, was heavy during the first 2–3 days following the infective feed, and the loss of midgut promastigotes was clearly associated with defecation of the bloodmeal remnants during days 4–7 (50, 83). The early development of *Leishmania panamensis* in *P. papatasi* was generally similar to its development in a natural host, *Lutzomyia gomezi*, with intense multiplication of procyclic promastigotes and generations of elongate nectomoads. While 86% of the flies were infected during bloodmeal digestion, only 34% remained infected following bloodmeal excretion (125). Finally, the inability of certain substrains of *L. major* to produce mature infections in *P. papatasi* or *Phlebotomus duboscqi* was also associated with the rapid loss of heavy midgut infections immediately following defecation of the digested bloodmeal (19). It should be added that even when the early survival of *L. donovani* within the *P. papatasi* midgut appeared to be compromised (5% of flies infected on day 3) and could be rescued by addition of trypsin inhibitor (90% of flies infected on day 3), there was still a substantial loss of parasites associated with bloodmeal excretion (26% of flies infected on day 6) that is not typically observed when flies are infected with a compatible strain of *L. major* (97).

The inability of *Phlebotomus sergenti* to support the complete development of any species other than the one it transmits in nature, *L. tropica*, is consistently associated with bloodmeal excretion. Hindle (40) observed that compared with the natural vector species (*Phlebotomus major*), *P. sergenti* seems to be an equally favorable host for the early development of a Chinese strain of *Leishmania* (presumably *L. donovani*), but that the persistence of flagellates in the midgut was dependent on the presence of a bloodmeal. When the alimentary canal no longer contained any food material, the flagellates disappeared. Killick-Kendrick (53) examined the development of *L. infantum* in wild-caught *P. sergenti* and in a natural vector, *Phlebotomus perniciosus*. Both species were initially infected in similar proportions; however, the parasites in *P. sergenti* were lost with the feces passed on days 3–4. Identical findings were reported when *L. major*, *L. donovani*, or *L. tropica* amastigotes were used to infect *P. sergenti* (50). At days 3 and 4, following the ingestion of blood, the mean parasite load was high in each case. While mature midgut infections developed in a high proportion of *L. tropica*-infected flies on days 7–13, the complete loss of *L. major* and *L. donovani* in *P. sergenti* closely followed the loss of the digested bloodmeal (days 4–7). Persistence of *L. major* and *L. donovani* in *P. papatasi* and *P. argentipes*, respectively, was confirmed in parallel infections.

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 midgut, particularly since the peak concentration of these enzymes is thought to occur at 18–36 h postfeeding. The strong correlation between the loss of bloodmeal and the rapid loss of promastigotes suggests that the inappropriate strains are not being killed but are passed out of the midgut along with the digested meal. Two general mechanisms have been proposed to explain the inability of promastigotes to persist in the gut during this time: (*a*) failure to escape from the enclosed peritrophic membrane and (*b*) failure to atttach to midgut epithelial cells.

Escape from the Peritrophic Membrane

In blood-feeding Diptera, including sand flies, the peritrophic membrane or matrix (PM) is secreted by the midgut epithelium., Within the first 1–4 h, the PM forms a cylindrical sheet that completely surrounds the food in the abdominal midgut (9, 34, 124). It consists of a network of chitin in a matrix composed of proteins and proteoglycans. One of the major roles ascribed to the PM is the compartmentalization of digestive events by acting as a permeable barrier for digestive enzymes (113). In *P. papatasi*, addition of exogenous chitinase to the bloodmeal, which completely blocked PM formation, resulted in greater killing of developing parasites within a few hours following the infective bloodmeal (82). It was suggested that the PM creates a barrier to the rapid diffusion of digestive enzymes and limits the exposure of parasites to these enzymes when they are especially vulnerable to proteolytic damage. While *Leishmania* may exploit PM formation for its early survival, at later stages it can clearly act as a physical barrier to development, as

has been described for other arthropod vectors. Lewis (65) showed that most microfilariae of *Onchocerca volvulus* become trapped within the peritrophic matrix of *Simulium damnosum* and are subsequently lost. In some instances, the development of malaria parasites was limited by the inability of ookinetes to cross the mosquito PM (8). Addition of allosamidin, a specific inhibitor of chitinase, completely blocked oocyst development in a natural vector by preventing ookinete secretion of a chitinase required for their penetration of the PM (102). There are at least two examples in which the loss of *Leishmania* infection in unnatural vectors appeared to be due, at least in part, to the failure of the parasite to escape from the PM. In each of these studies, the first involving *L. donovani* infections in *Phlebotomus mongolensis* (33) and the second involving *L. panamensis* infections in *P. papatasi* (125), the membrane did not break down during blood digestion, and the trapped parasites were excreted from the gut. In comparisons of different *L. major* strains in *P. papatasi*, the strain that developed most successfully was the one that escaped from the peritrophic membrane most rapidly (19). Studies involving a chitinase inhibitor have provided the most direct evidence that the PM can be a physical barrier to parasite development in the fly (82). In *P. papatasi* fed on bloodmeals containing 1 mM allosamidin, the PM appeared thicker and was sustained as an intact structure for longer (up to 7 days). The effect of allosamidin on *L. major* development was to prevent their escape from the bloodmeal. So long as the bloodmeal was retained within the abdominal midgut, the promastigotes remained viable and the flies remained infected. In every case when the bloodmeals in the allosamidin-treated flies were finally passed, the parasites were also lost.

These data indicate that the actions of a parasite and/or sand fly-derived chitinase are required for the parasites to escape containment by the PM. Schlein et al (99) were the first to identify chitinolytic enzymes in *Leishmania* and to suggest that they are responsible for the observed break up of the anterior part of the membrane in infected flies. The intact membranes that have been observed in some infected flies raise the possibility that there are species or strain-related differences in *Leishmania* chitninases, or else differences in the substrate components of the PM in different flies. A secretory chitinase has since been cloned in *L. donovani* and was found to be antigenically and ezymatically conserved in diverse *Leishmania* species (103, 104). Deletion of this gene would provide the clearest indication of its role in vector interactions, especially because sand flies possess their own chitinolytic system that might be exploited by the parasite for escape from the PM.

Attachment to Midgut Epithelial Cells—The Role of LPG

Killick-Kendrick et al (58) first confirmed by electron microscopy that promastigotes (*Leishmania amazonensis*) anchor themselves to the epithelial lining of the midgut (*Lutzomyia longipalpis*) by inserting their flagella between the microvilli. Extensive and ordered attachment of promastigotes to the epithelium of the abdominal and thoracic midgut has been consistently observed in light and electron microscopic studies performed on *Leishmania*-sand fly pairs in which mature

infections are seen (48, 62, 78, 126–128). There is general agreement that this attachment serves to anchor the parasite against the peristaltic action of the gut. A series of studies has investigated the role of the surface LPG in midgut adhesion. In initial studies confined to *L. major*, the binding of promastigotes to *P. papatasi* midguts in vitro could be completely inhibited in a dose-dependent manner using LPG (84). Purified LPG was also shown by immuno-gold labeling to bind directly to dissected midguts, with localization to the microvillar lining. Similar in vitro analyses have been extended to demonstrate LPG-mediated binding of *L. donovani* to *P. argentipes* midguts (93), and of *L. tropica* to *P. sergenti* midguts (50). Again, the most powerful evidence implicating a role for LPG in midgut adhesion is the data obtained using LPG-deficient mutants (92). As mentioned above, *L. donovani* and *L. major* mutants deficient solely in LPG expression owing to a loss of *LPG1* displayed only a slight reduction in the survival and growth of promastigotes within the early blood-fed midgut. Their ability to persist in the midgut following bloodmeal excretion was completely lost, however, and this defect was correlated with the inability of the mutant promastigotes to bind to midgut epithelial cells in vitro. When LPG expression was restored to wild-type levels by reintroduction of *LPG1*, the ability of the mutant to bind to and persist in the midgut was also restored.

The timing of the loss of the mutant parasites in vivo, as well as the loss of their capacity to bind to vector midguts in vitro, certainly provides strong correlative evidence that the main function of LPG in the fly is to mediate attachment to the gut wall to prevent loss of infection during elimination of the digested bloodmeal. Nonetheless, because the timing of the loss of infection would also be consistent with the failure of parasites to escape from a PM enclosed bloodmeal, consideration should be given to the possiblity that the LPG, perhaps by mediating parasite binding to lectin-like proteins in the matrix (81), may be required to facilitate or localize the action of the parasite chitinase. The binding of LPG to matrix material should be investigated, and the status of the PM in infections involving LPG-deficient lines should be more carefully observed.

Species-Specific Midgut Attachment

While the core-PI domains of LPGs from different *Leishmania* species are conserved, remarkable interspecies differences are found in the repeating units and cap structures of the phosphoglycan domain (Figure 3). The backbone repeat units of -6Galβ1,4Manα1-PO_4- can either be unsubstituted, as in Sudanese strains of *L. donovani*, *L. braziliensis*, and *Leishmania chagasi* (80, 119); completely substituted at the C-3 position of the Gal, as in *L. major* and *L. tropica* (73, 74); partially substituted at the C-3 position of the Gal, as in Indian *L. donovani* strains and *Leishmania mexicana* (43, 70); or partially substituted at the C-2 position of the Man, as in *Leishmania aethiopica* (73). The nonreducing capping sugars contain mannose, galactose, or glucose, which vary quantitatively and qualitatively among species. In *L. major* LPG, the repeating units of the

phosphoglycan chain are completely substituted with a variety of galactose-containing side chains (74). The contribution of these specific sugars to midgut binding has been investigated by depolymerization of the phosphoglycan chain, separation of the various oligosaccharides repeats by HPLC, and by comparison of their abilities to inhibit promastigote binding to *P. papatasi* midguts in vitro. The major phosphorylated trisaccharide fragment, which contains the terminal side chain sugar Gal(β1−3), and the major phosphorylated tetrasaccharide fragment formed by side chain substitution with Gal(β1-3) Gal(β1-3) were as inhibitory as the intact phosphoglycan (84). In contrast, the unsubstituted phosphorylated disaccharide, PO_4-6Gal(β1-4)Man, was a poor inhibitor. The role of these side chain oligosaccharides has been confirmed using a different class of *L. major* LPG mutants, which express normal levels of LPG but are deficient in LPG side chain biosynthesis (14). One mutant, termed Spock, was generated by negative selection with a mAb specific for the galactose terminated tri- and tetrasaccharide repeats and is specifically deficient in the galactosyl transferase required for the β1-3 addition of galactose to the disaccharide repeat. The lack of these side chains eliminated Spock's ability to bind significantly to *P. papatasi* midguts in vitro, and Spock was unable to maintain infections in sand flies beyond passage of the digested bloodmeal. A West African strain of *L. major* (NIH Seidman) was found to be naturally deficient in the expression of Gal side chains, and accordingly this strain failed to persist following bloodmeal excretion in *P. papatasi* but did mature in a closely related vector species, *P. duboscqi* (70).

The behavior of *L. major* Seidman strain and the mutant Spock in *P. papatasi* was similar to that of *L. donovani*, whose LPG normally lacks side chains terminating with galactose. These data suggest that the inter- and intraspecies-specific polymorphisms in the phosphoglycan domains of LPG might result in species- and strain-restricted midgut attachment and thereby determine species- and strain-specific vector competence. The experience with a relatively large number of parasite-vector pairs examined indicates that the extent to which the parasite is able to attach to the sand fly midgut forcefully predicts the outcome of fly infection in vivo. When promastigotes of *L. major* strains, *L. donovani* strains from Sudan and India, an *L. tropica* strain, and an *L. amazonensis* strain were incubated with *P. papatasi* midguts, only the *L. major* promastigotes remained bound after washing (83). Identical differences in binding were observed when midguts were stained with LPGs purified from each of these strains. Reciprocal specificities were recently observed using midguts prepared from *P. sergenti*, in which case only *L. tropica* promastigotes and LPG showed significant binding (50). In contrast, midguts of either *P. argentipes* or *Lu. longipalpis* bound promastigotes and/or LPGs from most *Leishmania* species tested. This correlates with the fact that, in contrast to *P. papatasi* and *P. sergenti*, both *P. argentipes* and *Lu. longipalpis* are able to maintain infections with a number of different *Leishmania* species (79, 83, 123, 126). The laboratory-based vector competency studies involving a large number of parasite-vector pairs are summarized in Figure 3. Two general conclusions can be drawn: (*a*) For *Leishmania* species that express highly branched,

species-restricted LPG structures, their natural vectors display a high degree of species-specific vector competence and (*b*) for *Leishmania* species that express unsubstituted or poorly substituted LPGs, their natural vectors are more broadly permissive to the full development of diverse *Leishmania* species.

Midgut Receptors for Parasite Attachment

The fact that significant differences in LPG-mediated binding were observed when different vector species were compared argues that the molecules that serve as parasite attachment sites can vary between different phlebotomine species and may therefore provide the evolutionary pressure for LPG structural polymorphisms. The selection for the highly branched and distinctive LPG structures expressed by *L. major* (74) and *L. tropica* (73) strains occurred, in this view, in order for the parasite 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. With respect to intraspecies variations, the expression of terminal glucose residues on both the LPG capping and side chain domains of Indian *L. donovani* strains (70) compared with the mannose capped and unsubstituted LPGs expressed by Sudanese strains (119) was selected by their ability to potentiate binding to *P. argentipes*. Presumably these side chains are not needed to promote attachment in the phlebotomine species available to *L. donovani* strains in East Africa (e.g., *Phlebotomus orientalis*). Similarly, the existence of *L. major* strains in some geographic regions, such as West Africa, that express unsubstituted forms of LPG occurred because their transmission cycle is effectively maintained by a vector, presuambly *P. duboscqi*, that can accommodate parasites bearing the nonbranching backbone phosphoglycan repeats. Vectors such as *P. duboscqi* and *Lu. longipalpis* may express binding sites absent in *P. papatasi* or *P. sergenti* that recognize shared or related structures, e.g., the terminally exposed neutral hexoses in the neutral capping domains, and thus are more broadly permissive to many species and strains of *Leishmania*.

These findings suggest that gut-associated lectins or lectin-like molecules, which have been described for sand flies (120–122), serve as parasite attachment sites. Preliminary information regarding a microvillar protein from *P. papatasi* midguts that binds to *L. major* LPG on Western ligand blots has recently been reported (27). Recently, a gene encoding a homologue of galectin, a galactose-binding protein found on mammalian cells, has been identified in midgut libraries from *P. papatasi* (J. Valenzuela and S. Kamhawi, unpublished data). There may be an additional receptor lining the gut that is involved in binding of the parasite via the flagellum. In an early study, the binding of an *L. major* promastigote flagellar preparation to frozen sections of *P. paptasi* midguts was inhibited by a monoclonal antibody that recognizes a membrane protein in the flagellum (131). Although the inhibition observed was only partial and may have been due to steric interference of LPG-mediated binding, it is certainly possible that the flagellar protein contributes to the flagellum-oriented attachment to microvilli that has been typically described. On the other hand, because LPG also covers the flagellum and the flagellum is an anterior organelle, the oriented attachment might simply be

explained by the greater probability that the flagellar LPG ligands encounter the gut lining first. Furthermore, the insertion of the flagellum between the microvilli, which presumably maximizes the number of binding sites, might be precluded by the parasite's much larger cell body (4).

The selection for specific LPG ligands by midgut attachment sites presupposes that this binding is a required condition for the complete development of transmissible infections. It should be noted that while mature infections, when appropriately examined, have consistently been associated with promastigotes attached to midgut epithelial cells, the conclusion that this is an essential event remains based only on correlative data. Particularly for certain vectors species (e.g., *Lu. longipalpis*) for which bloodmeal excretion is rarely accompanied by the loss of infection, regardless of the parasite strain, consideration must be given to the possibility that differences in their gut physiology (e.g., more gradual, less forceful peristalsis) may permit parasites to persist in the gut during expulsion of the meal even in the absence of attachment to the gut wall. Thus, there was no need for these parasites to significantly modify their LPGs, not because their available vectors display midgut receptors for unmodified structures, but rather because midgut attachment is of little consequence to the maintenance of infection in these flies. Alternatively, lower affinity and less-specific interactions, mediated by the shared cap structures and/or flagellar proteins, might be sufficient for the parasite to resist the expulsive force to which it is exposed in these flies.

MATURATION OF TRANSMISSIBLE INFECTIONS

Stage-Specific Midgut Attachment

Following passage of the bloodmeal, the maturation of infection presumably involves the release of large numbers of parasites from the midgut, which may or may not be preceded by their differention to metacyclic promastigotes. In contrast to nectomonads and other dividing forms, metacyclic promastigotes have never been seen in attachment but remain free in the lumen to migrate anteriorly. This behavior might be explained, at least in part, by their loss of intrinsic binding potential (90). Whereas dividing promastigotes from culture display an inherent capacity to attach to midgut epithelial cells of an appropriate vector, metacyclic promastigotes purified from stationary-phase culture can no longer bind. Developmentally regulated modifications in LPG structure control the stage-specificity of midgut adhesion (84). In the transition of *L. major* promastigotes from procyclic to metacyclic forms, LPG repeating units approximately double in number and terminate with Arabinose, which masks the Gal-binding moiety (75, 91). For *L. donovani* strains from Sudan, the terminal α-Man- and β-Gal-containing cap structures that mediate binding of procyclic promastigotes become cryptic as a possible consequence of the elongation and clustering of the phosphoglycan chains during metacyclogenesis (93). For the LPG of Indian strains of *L. donovani*, metacyclogenesis is associated with both chain elongation and the downregulation of glucose side chain biosynthesis (70). Thus, three general structural modifications depicted in Figure 4 have

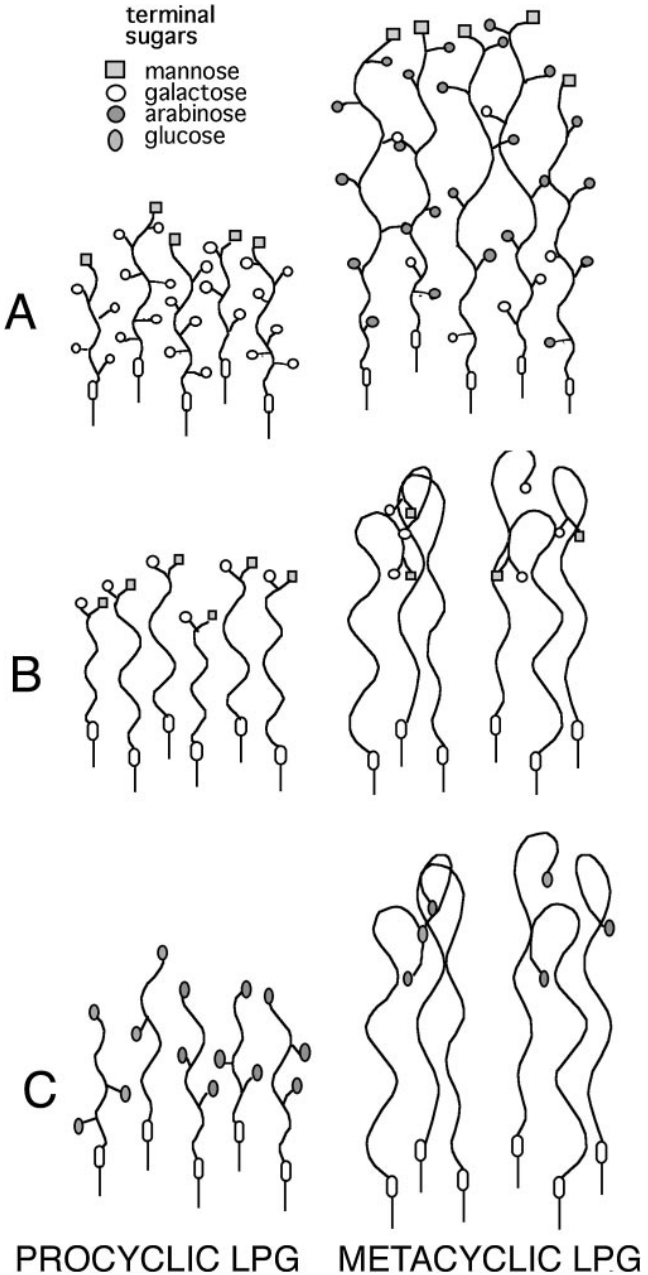

Figure 4 Comparison of the species- and stage-specific LPG structures of procyclic and metacyclic promastigotes depicting three general mechanisms of developmental modification resulting in loss of the terminally exposed sugars involved in midgut attachment.

been proposed to explain, depending on the species or strain, the loss of the terminally exposed sugars that are involved in midgut attachment. Even though the metacyclic LPGs of other *Leishmania* species, such as *L. tropica* and *L. amazonensis*, have not been characterized in detail, it is known that they are similarly modified because monoclonal antibodies that recognize procyclic forms of their LPGs no longer can bind (20, 68), and more importantly, the metacyclic LPGs no longer stain their appropriate vector midguts in vitro (D. Sacks et al, unpublished data). The developmental modifications in LPG are in fact the basis for identifying and purifying metacyclic promastigotes from culture using stage-specific lectins and antibodies. That similar modifications accompany metacyclogenesis in vivo has been supported by studies in *P. papatasi*, in which a monoclonal antibody specific for *L. major*-metacyclic LPG stained metacyclic forms were recovered from the guts (95) and stained sections of infected flies most strongly in the foregut and in the region behind the stomodeal valve (23).

The detachment of parasites from the midgut during development might also be explained by the shedding of the LPG involved in binding or saturation of binding sites by released phosphoglycans. The ultrastructure of infected midguts immunogold-labeled with anti-LPG antibodies prior to sectioning revealed heavily labeled promastigotes bound to the microvilli, which were themselves poorly labeled (95). This is in agreement with electron microscopy (EM) studies of Lang et al (60) and argues against active shedding of LPG in the midgut. The proof that sequential attachment and release are largely controlled by changes in the intrinsic binding properties of the surface LPG will depend on the generation of mutants that are defective in stage-regulated biosynthetic processes.

Stage-Differentiation and Anterior Migration

So long as *Leishmania* can generate and maintain high parasitic loads in the midgut during bloodmeal digestion and excretion, there has been little evidence that the final phase of development in the fly will vary according to species or strain. It is known, for example, that if the deficient growth and/or persistence of *L. donovani* in *P. papatasi* is overcome by inhibiting the early killing (97) or increasing the initial inoculum of parasites in the bloodmeal (1, 2), then these infections will mature normally, including differentiation to infective promastigotes and accumulation of large numbers of parasites behind the stomodeal valve. Thus, for the terminal stages of development in the fly, it has not been possible to compare permissive and refractory parasite-vector combinations in order to help identify the molecules involved in, for example, cues for stage differentiation or anterior migration. The few in vitro studies that have addressed these points are mentioned below.

Metacyclics of *L. mexicana* were induced in vitro by culture at low pH (5). Although promastigotes certainly acidify their growth media in vitro, the pH conditions of a sand fly midgut during infection are not known. Another extrinsic factor influencing metacyclogenesis in the fly may be sand fly saliva. Exposure of the parasite to saliva could occur if infected sand flies feeding on sugar meals

or additional bloodmeals, re-ingest some of the saliva that is secreted in order to facilitate feeding (see below). The possible effect of this direct exposure on the parasite differentiation and virulence has been investigated in vitro. Multiplication of *L. amazonensis* was arrested by the addition of salivary gland homogenate of *Lu. longipalpis* (16). The sensitivity of the promastigotes to the homogenate increased during development from the logarithmic to the stationary phase of growth. Hemin, a product of blood digestion, appeared to inhibit the static effects of saliva by maintaining the promastigotes in division (18). This observation is consistent with the finding that hemoglobin inhibited the generation of infective stage promastigotes of *L. major* in culture (96). The data suggest that exposure to saliva, in conjunction with the removal of the bloodmeal, might provide exogenous signals for the differentiation of dividing noninfective forms into nondividing infective metacyclics. Demonstrating that salivary molecules are actually present in the midgut following blood or sugar feeding would enhance the significance of these observations.

The anterior migration of unattached promastigotes to the thoracic midgut and cardiac valve has generally been attributed to promastigotes following a sugar concentration gradient, formed as the sugar meals are gradually spilled from the crop into the anterior gut. The capacity of promastigotes to migrate chemotactically in the presence of sugars has been demonstrated in vitro (13). Moreover, early studies of laboratory transmission of *L. donovani* by *P. argentipes* was shown to be facilitated by the provision of raisins as a source of sucrose (107, 108). Anterior migration has also been seen, however, in the absence of sugar meals (130).

Transmission by Bite

Because actual transmission by bite has rarely been included as an endpoint in the analysis of *Leishmania*-sand fly interactions, the molecules controlling this ultimate event in the life cycle in the vector are essentially unknown. The accumulation of large numbers of metacyclic promastigotes in the anterior regions of the gut, including their presence in the proboscis, may not in themselves be sufficient conditions for transmission by bite. The prevailing view is that in addition to the presence of infective stage promastigotes in the anterior gut, efficient transmission involves the formation of a biological plug that impairs the intake of blood (6, 56, 57). This is thought to promote regurgitation of infective promastigotes from the foregut or behind the stomodeal valve as the fly attempts to dislodge the plug from the feeding apparatus.

One element of the plug is undoubtedly the mass of parasites themselves, either attached to the cuticular lining of the stomodeal valve, or stacked up behind the valve embedded in a gel-like matrix that reduces their motility and leads to massive swelling of the cardia. The attached and embedded parasites are typically haptomonad forms, whereas the metacyclics appear to remain unattached and highly motile (62, 128, 130). Using monoclonal antibodies that do not

cross-react with LPG, Stierhof et al. (111) were able to show by immuno-EM that in *Lu. longipalpis* infected with *L. mexicana* or in *P. papatasi* infected with *L. major*, the gel-like matrix that is formed is morphologically and immunologically identical to the filamentous PPG that is produced by these parasites in vitro. The molecules involved in the attachment of parasites to the cuticular lining are not known; ultrastructurally, they are seen to be held in place by the flagellum whose distal end expands to form a disc-like attachment organelle. Damage to the valve itself has been described, due perhaps to the action of *Leishmania* chitinases, causing the valve to remain open and facilitate the regurgitation and egestion of metacyclic promastigotes from the fly (98). Maintaining the flies on bloodmeals as opposed to sugar meals inhibited transmission, possibly because hemoglobin inhibits the release of chitinases by the promastigotes (96). The generation of parasite mutants selectively deficient in chitinase or PPG in conjunction with studies of actual transmission by bite will be required to determine what role, if any, these molecules play in the final stage of development in the vector.

VECTOR-HOST INTERACTIONS THAT MODULATE LEISHMANIASIS: THE ROLE OF SAND FLY SALIVA

The relationship of vector sand flies with the leishmanial diseases they transmit does not end with the deposition of parasites into the skin of the mammalian host. Because infected sand flies will also inoculate small amounts of saliva, recent studies examined how the modification of the inoculation site by salivary components can influence the outcome of infection. Sand flies probe the skin creating a hemorrhagic pool on which to feed, salivating in the process (reviewed in 86, 87). Their salivary proteins are endowed with an array of pharmacologic activities designed mainly to induce vasodilation and prevent blood clotting. In addition to these pharmacologic activities, sand fly saliva has immunosuppressive or immunogenic properties that in each case modify the host response to Leishmaniasis.

Disease Enhancing and Immunomodulatory Effects of Sand Fly Saliva

For several different species of *Leishmania*, the co-injection of parasites with salivary gland homogenates of either *Lu. longipalpis* or *P. papatasi* produced a substantial increase in lesion size and/or parasite burden compared with controls injected with parasites alone (7, 28, 66, 71, 94, 115, 118). The exacerbative effect in C57BL/6 mice was so powerful that the inoculation of 10^3 purified *L. major* metacyclics with *P. papatasi* salivary homogenate into the mouse ear, a dermal site, converted the mice from a healing to a nonhealing phenotype (7). The exacerbative effect of the salivary homogenate of both *Lu. longipalpis* and *P. papatasi* was

associated with the induction of IL-4 and was abrogated in mice treated with anti-IL-4 monoclonal antibodies and in IL-4-deficient mice (7, 66, 71). Moreover, the frequency of epidermal cells producing Th2 cytokines, mainly IL-4 and IL-5, 6 h following infection was significantly increased in the presence of the salivary gland sonicate (7). Other immunomodulatory activities of saliva may contribute to these in vivo outcomes, including the inhibition of several macrophages related functions: antigen presentation, IFN-γ-induced iNOS gene expression and NO production, and the induction of proliferation in primed parasite reactive T cells (35, 51, 116).

With one exception, the molecules present in the salivary gland homogenates that are responsible for exacerbation of *Leishmania* infection have not been identified. Preliminary findings have been reported (117) regarding the disease enhancing effects of maxadilan, which is a powerful vasodilatory peptide found in the salivary glands of *Lu. longipalpis* (64). Differences in the amount of maxadilan present in the saliva of sibling species of *Lu. longipalpis* were suggested to influence their capacity to enhance Leishmaniasis (129). Maxadilan is known to have immunomodulatory properties, including inhibition of T cell activation and DTH response (85), and inhibition of TNF-α, but induction of IL-6, IL-10, and prostaglandins E_2 in macrophages (12, 61, 109). Instead of maxadilan, *P. papatasi* possesses pharmacologically active levels of vasodilatory adenosine and 5′AMP (88). Adenosine is an established anti-inflammatory molecule and inhibits the production of IL-12, IFN-γ, TNF-α, and NO and enhances the production of IL-10 (37, 38, 63, 67). Other salivary molecules with known immunomodulatory properties are hyaluronidase and adenosine deaminase. Hyaluronidase, identified from the salivary glands of both *Lu. longipalpis* and *P. papatasi*, generates hyaluronan fragments that downregulate the production of IFN-γ and induce chemokine and iNOS gene expression in macrophages (41, 76). Adenosine deaminase, found in *Lu. longipalpis* but not in *P. papatasi*, prevents T cell apoptosis caused by the accumulation of adenosine (17). Inosine, the by-product of adenosine degradation by adenosine deaminase, inhibits the production of inflammatory cytokines including IL-12 and INF-γ (36). The relationship of these various salivary molecules and their potential immunomodulatory activities to the IL-4-dependent, exacerbative effects of whole salivary gland homogenate in vivo remains to be demonstrated.

Recently, Kamhawi et al. (49) demonstrated that the outcome of infection and host immune response of mice following the transmission of *L. major* by bites of its natural vector *P. papatasi* was significantly different from the needle-inoculation models described above. The healing phenotype of C57BL/6 mice was maintained, and the same severity of disease was observed in wild-type and IL-4-deficient mice. Moreover, in contrast to the co-inoculation by needle of parasites and salivary gland sonicate, the epidermal cell response to the bites of infected flies showed a low expression of IL-4 and an absence of IL-5. Because salivation is undoubtedly an obligatory part of the sand fly probing and feeding process, it may not be possible to remove saliva as a component of transmission by bite in order to

assess its role in promoting infection. Nevertheless, it is reasonable to conclude that salivary secretions do not induce IL-4, which may be an artifact of whole salivary gland homogenate preparation used in the co-injection studies. It should be noted, however, that Theodos et al (115) demonstrated an exacerbative effect of *Lu. longipalpis* saliva by the subcutaneous inoculation of *L. major* at a site probed by *Lu. longipalpis* 1 h earlier. The data imply that saliva, at least of this vector species, contains secreted molecules that enhance *Leishmania* infection and that similar experiments involving *P. papatasi* might help to clarify the conflicting results involving this fly.

Immunity Conferred by Presensitization to Sand Fly Saliva

The data discussed up until now concern the potential immunomodulatory effects of sand fly saliva on a naïve host. To the extent that salivary secretions also contain molecules that are immunogenic, the transmission of *Leishmania* by bite into a host that has been previously sensitized to sand fly saliva, including via the bites of uninfected sand flies, elicits an immune response at the site of the bite and potentially modifies the outcome of infection. The exacerbative effect of saliva on infection, seen when mice were co-inoculated with *L. major* and salivary glands sonicate of *P. papatasi*, was completely abrogated in mice pre-exposed to the salivary sonicate (7). This protection was reproduced following transmission of *L. major* by the bite of infective *P. papatasi* flies: Compared with naïve mice, mice pre-exposed to the bites of uninfected flies showed a reduction in lesion pathology (Figure 5), a reduction in parasite load, and a reduction in the ability to transmit *Leishmania* back to uninfected flies (49). The protection conferred by pre-exposure of mice to saliva was associated with a strong DTH response at the site of the bite. A DTH reaction, sometimes severe, is known to be elicited by *P. papatasi* bites in humans (59, 114). The protection in mice was also associated with a strong upregulation of INF-γ and IL-12 at the site of bite, which suggests that within this inflammatory setting infected macrophages might be activated for early killing of the parasites. The induction of a *Leishmania*-specific Th1 response might also be accelerated. Protection against *Leishmania* infections conferred by pre-exposure to sand fly bites might explain why in areas that are endemic for cutaneous leishmaniasis, the indigenous inhabitants, who are mostly bitten by uninfected flies, generally show attenuated infections compared with newcomers such as tourists or immigrants. Moreover, the powerful protection against cutaneous leishmaniasis that results from pre-exposure to saliva indicates that the immunogenic salivary molecules, which have yet to be identified, might be used as components of an antileishmanial vaccine.

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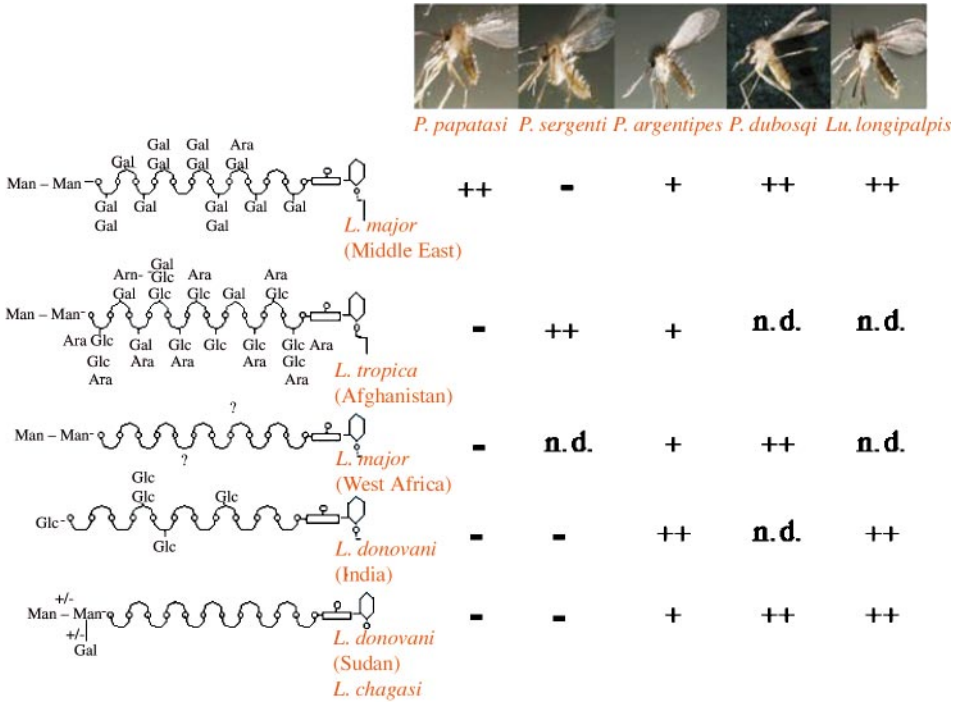

Figure 3 Summary of the vector competence of five phlebotomine species for various species and strains of *Leishmania*, based on the results of laboratory infections of flies fed through a membrane on mouse blood mixed with amastigotes. The relative outcomes refer to the intensity of infections persisting in the midgut following elimination of the bloodmeal. The species- and strain-related polymorphisms in LPG structure are represented, depicting the conserved core-anchor domains and phos-gal-man backbone repeats, the presence or absence of variable sidechain substitutions, and the variability in terminal capping sugars. N.D., not determined.

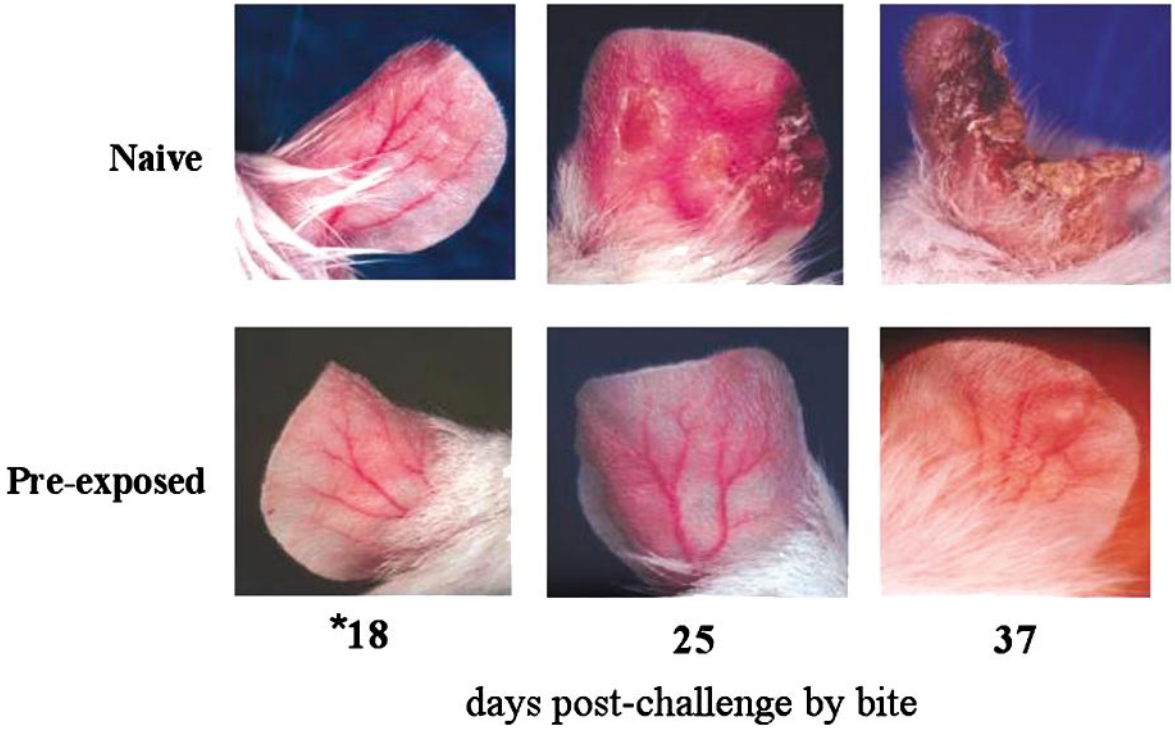

Figure 5 Progression of cutaneous lesions on ears of naive and pre-exposed BALB/c mice following the bite of *P. papatasi* infected with *L. major*. Mice were pre-exposed to the bites of 10–15 uninfected *P. papatasi* 2 and 4 weeks prior to their exposure to infected flies.

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