A novel role for the peritrophic matrix in protecting *Leishmania* from the hydrolytic activities of the sand fly midgut

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SUMMARY

The role of the peritrophic matrix (PM) in the development of *Leishmania major* infections in a natural vector, *Phlebotomus papatasi*, was investigated by addition of exogenous chitinase to the bloodmeal, which completely blocked PM formation. Surprisingly, the absence of the PM was associated with the loss of midgut infections. The chitinase was not directly toxic to the parasite, nor were midgut infections lost due to premature expulsion of the bloodmeal. Most parasites were killed in chitinase-treated flies within the first 4 h after feeding. Substantial early killing was also observed in control flies, suggesting that the lack of PM exacerbates lethal conditions which normally exist in the blood-fed midgut. Early parasite mortality was reversed by soybean trypsin inhibitor. Allosamadin, a specific inhibitor of chitinase, led to a thickening of the PM, and also prevented the early parasite mortality seen in infected flies. Susceptibility to gut proteases was extremely high in transitional-stage parasites, while amastigotes and fully transformed promastigotes were relatively resistant. A novel role for the PM in promoting parasite survival is suggested, in which the PM creates a barrier to the rapid diffusion of digestive enzymes, and limits the exposure of parasites to these enzymes during the time when they are especially vulnerable to proteolytic damage.

Key words: chitinase, *Leishmania*, peritrophic membrane, sand fly, allosamidin.

INTRODUCTION

In blood-feeding Diptera, including sand flies, the peritrophic membrane or matrix (PM) is secreted by the midgut epithelium, and within the first 1–4 h it forms a cylindrical sheet that completely envelopes the food in the abdominal midgut (Gemetchu, 1974; Blackburn *et al*. 1988; Walters *et al*. 1993). It consists of a network of chitin in a matrix composed of proteins and proteoglycans. The major roles ascribed to the PM include preventing damage or clogging of microvilli by the luminal contents (Richards & Richards, 1977; Berner, Rudin & Hecker, 1983), compartmentalization of digestive events by acting as a permeability barrier for digestive enzymes (Terra, 1990), and protection against pathogenic microbes by providing a barrier to their development (Peters, 1992; Miller & Lehane, 1993). The ability of the PM to serve as a physical barrier to parasites is well described. Lewis (1953) showed that most microfilariae of *Onchocerca volvulus* become trapped within the peritrophic matrix of *Simulium damnosum* and are subsequently lost. The development of malaria parasites within unnatural vectors was shown to be limited by the inability of ookinetes to cross the PM (Stohler, 1957; Billingsley & Rudin, 1992); and 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 (Shahabuddin *et al*. 1993). Similarly, in sand flies, loss of *Leishmania* infection in some unnatural vectors was found to be due at least in part to the failure of the parasite to escape from the PM prior to bloodmeal excretion (Feng, 1951; Walters *et al*. 1992), an event which is thought normally to be facilitated by the action of a parasite-derived chitinase (Schlein, Jacobsen & Schlomai, 1991).

In studies designed to define more conclusively the role of the PM in controlling the striking specificity of some *Leishmania*/sand fly interactions,

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sand flies were fed bloodmeals containing exogenous chitinase in order to disrupt PM formation *in vivo*. A novel role for the PM in protecting *Leishmania* from the proteolytic activities of the blood-fed midgut is further explored and discussed.

MATERIALS AND METHODS

Infection of sand flies

A colony of *Phlebotomus papatasi* (Israeli strain) was maintained in the Department of Entomology at Walter Reed Army Institute of Research. Three to 5-day-old sand flies were infected through a chick skin membrane on heparinized mouse blood seeded with $2–10 \times 10^6$ ml cryopreserved amastigotes of *L. major* (MHOM/IL80/Friedlin) clone V1. Amastigotes were isolated from BALB/c mouse footpad lesions as previously described (Saraiva *et al*. 1983). For sand fly infections using promastigotes, the mouse red blood cells (RBC) were washed twice in 0·86 % saline and added back to the plasma which had been heat inactivated at 56 °C for 45 min. Promastigotes were grown in Medium 199 supplemented with 20 % (v/v) heat-inactivated foetal calf serum, penicillin (100 U/ml) streptomycin (50 μg/ml), and 12·5 mM L-glutamine (all from ABI, Columbia, MD), 40 mM Hepes, pH 7·4, 0·1 mM adenine, and 0·0005 % haemin (M199/S). Procyclic promastigotes were harvested in the logarithmic phase (1–2 days) and added to the heparinized mouse blood at 10^7 promastigotes/ml. To assay the effect of chitinase on PM formation and parasite development, chitinase from *Streptomyces griseus* (Sigma Chem. Co., St Louis, MO) was added to bloodmeals at a final concentration of 1 U/ml. Allosamidin, a potent inhibitor of various chitinases (Sakuda *et al*. 1986), was obtained from A. Isogai, University of Tokyo, and was added to the bloodmeal at a final concentration of 1·0 mM. In some experiments, soybean trypsin inhibitor (Sigma) was also added to the bloodmeal (1 mg/ml). Blood-fed female flies were separated and maintained on 30 % sucrose solution at 27 °C, 80 % relative humidity. At various times after feeding, the flies were killed in 10 % detergent solution, their midguts were dissected and the number of midgut promastigotes determined by placing individual guts into a 1·5 ml capacity microcentrifuge tube containing 30–100 μl of phosphate-buffered saline (PBS), pH 7·4. Each gut was homogenized using a Teflon-coated micro-tissue grinder, and motile promastigotes were counted in a haemocytometer.

Effect of chitinase on parasite transformation and growth in vitro

An identical bloodmeal preparation as was used for the membrane feeds, containing heparinized mouse blood plus 10^7 ml *L. major* amastigotes, was dispensed in a volume of 100 μl into 96-well flat-bottomed microtitre plates. *S. griseus* chitinase was serially diluted in PBS, and 5 μl of each dilution was added to the blood such that the highest final concentration of the chitinase was 2·5 U/ml. Transformation to and growth of promastigotes at 26 °C was determined daily by counting parasites in diluted blood under a haemocytometer.

Limiting dilution analysis of midgut parasites

In order to determine the number of viable parasites in each midgut during the time preceding their transformation to promastigotes, individual midguts were asceptically dissected and homogenized, and the gut contents were serially diluted in 96-well flatbottomed microtitre plates containing biphasic medium, prepared by plating 50 μl of NNN medium containing 30 % defibrinated rabbit blood, and overlaying with 50 μl M199/S. The number of viable parasites in each gut was determined from the highest dilution at which promastigotes could be cultured following up to 7 days incubation at 26 °C.

Determination of the rate of bloodmeal excretion and erythrocyte digestion

The rate of bloodmeal excretion was compared by estimating the total haemoglobin content in isolated midguts using a haemoglobin assay kit (Sigma Chemicals) as described by the supplier. Midguts from individual flies were homogenized in 1 ml of Drabkin's solution and the optical density of the mixture was measured immediately at 450 nm. Relative concentrations of haemoglobin in blood-fed midguts were determined over 98 h post-feeding. The rate of erythrocyte digestion was compared by determining the number of intact RBC in isolated midguts homogenized in PBS and counted in a haemocytometer.

Assay for trypsin activity

Chromozyme TRY (Boehringer-Mannheim, Indianapolis, IN) is a tripeptide linked to a chromogenic substrate. The release of the chromogen was measured spectrophotometrically as follows: 185 μl of 0·2 M Tris–HCl, pH 8·0, 10 μl substrate (1 mg/ml chromozyme TRY) and 5 μl enzyme preparation, either gut homogenate or bovine pancreas trypsin standard (Sigma), appropriately diluted, were mixed thoroughly in flatbottomed 96-well plates, and the product formation measured continuously at 405 nm. The dilution of midgut homogenate which produced chromogen linearly for at least 20 min was used. Total trypsin activity in each midgut, expressed in enzyme units (1 EU is defined as 1 mM of

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substrate hydrolysed/min) was calculated from the V_{max} of each reaction (slope of the linear curve) applied to a standard curve of V_{max} values generated using known amounts of bovine pancreas trypsin.

Assay for parasite susceptibility to lysates from blood-fed midguts

A suspension of purified tissue amastigotes (10^6/ml) was exposed to transformation conditions by incubation in M199/S for up to 20 h at 26 °C. At various times during transformation, a 1·0 μl aliquot of the parasite suspension was added to a frozen-thawed lysate of a single midgut, which had been obtained aseptically from flies membrane-fed 24 h earlier on uninfected mouse blood, and stored in individual microcentrifuge tubes at −40 °C until use. Parasites were exposed to the midgut lysate for either 1 or 2 h at 26 °C. The suspension was diluted to 200 μl in M199/S, and the number of viable parasites was determined by limiting dilution in biphasic medium.

Microscopical examination of infected midguts

Sand fly midguts were fixed in 2·5 % glutaraldehyde in 0·1 M cacodylate buffer with 4 % sucrose and 5 mM $CaCl_2$ for 1 h at room temperature. The guts were washed in PBS and post-fixed in a 1 % osmium tetroxide solution containing 0·8 % potassium ferrocyanide and 5 mM $CaCl_2$ (Pimenta & De Souza, 1986). Tissues were dehydrated in acetone and embedded in Epon. Histological sections of 1 μm thickness from the Epon/embedded midguts, were warmed in a hot plate, stained with 0·01 % toluidine blue and observed with a light microscope. Thin sections of the same samples were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope.

RESULTS

Effect of exogenous chitinase and chitinase inhibitor on PM formation in P. papatasi

Light microscopical examination of histological sections of infected, control midguts revealed a fully developed PM by 24 h (Fig. 1A). The PM enclosed the entire bloodmeal just above the gut epithelium. The enclosed bloodmeal was confined to the abdominal midgut. In contrast, no PM was seen in the infected, chitinase-treated flies, and the bloodmeal remained dispersed throughout the hindgut and the abdominal and thoracic midgut, and was in direct contact with the midgut epithelium (Fig. 1B).

Electron microscopical (EM) observations of infected control midguts 24 h after feeding confirmed the PM as an amorphous, laminated structure that segregates the bloodmeal contents from the midgut epithelium (Fig. 1D). In addition, the micrograph reveals a region between the epithelial microvilli and the PM, lacking in either RBC or parasites. Parasites, including some degenerate forms, can be seen in the periphery of the bloodmeal adjacent to the PM. EM observations of infected, chitinase-treated flies confirmed the absence of PM at 24 h (Fig. 1E) or 48 h after feeding (Fig. 1F). The bloodmeal contents are in direct contact with the microvilli. Parasites seen towards the periphery of the bloodmeal are in close proximity to the microvilli. Their lack of intracellular ultrastructure is characteristic of dead or dying cells.

A lower power light-micrograph of a toluidine blue-stained, histological section of a midgut from an infected, allosamidin-treated fly is shown in Fig. 1C. The PM is 2 to 2·5-fold thicker and far more densely stained than the PM in control, infected flies. The bloodmeal is confined to a smaller intraperitrophic space. These effects of allosamidin on PM structure were observed even in uninfected bloodfed flies (not shown), suggesting that a sand fly chitinase is normally produced which modifies the PM during its early formation.

Effect of exogenous chitinase on the survival of L. major *in* P. papatasi

All flies membrane-fed on bloodmeals containing 10^7 amastigotes/ml had heavy midgut infections when examined at 36 h, with an average of $1{\cdot}1 \times 10^5$ promastigotes/midgut (Fig. 2). Inclusion of *S. griseus* chitinase in the bloodmeal resulted in the complete absence of infection in 90 % of blood-fed flies. When the infections in chitinase-treated flies were initiated using promastigotes, parasites were lost in only 20 % of flies, and the average number of promastigotes per midgut after 36 h was not significantly reduced. In another experiment, the effect of chitinase on fly infections initiated using purified amastigotes was compared with infections initiated using infected mouse peritoneal macrophages (Fig. 3). The chitinase-treated flies membrane-fed on purified amastigotes again either lost their infections entirely or showed poor parasite growth when examined at 40 h. All of the chitinase-treated flies infected via parasitized macrophages contained viable parasites at 40 h; however, the mean number of midgut promastigotes was reduced by 70 %, and this difference was highly significant ($P < 0{\cdot}01$).

In order to rule out a direct effect of exogenous chitinase on *Leishmania* viability, the same amastigote-seeded bloodmeals used for the membrane feeds in Fig. 2 were distributed in 96-well culture plates, and dilutions of the chitinase were added such that the starting final concentration of chitinase in the

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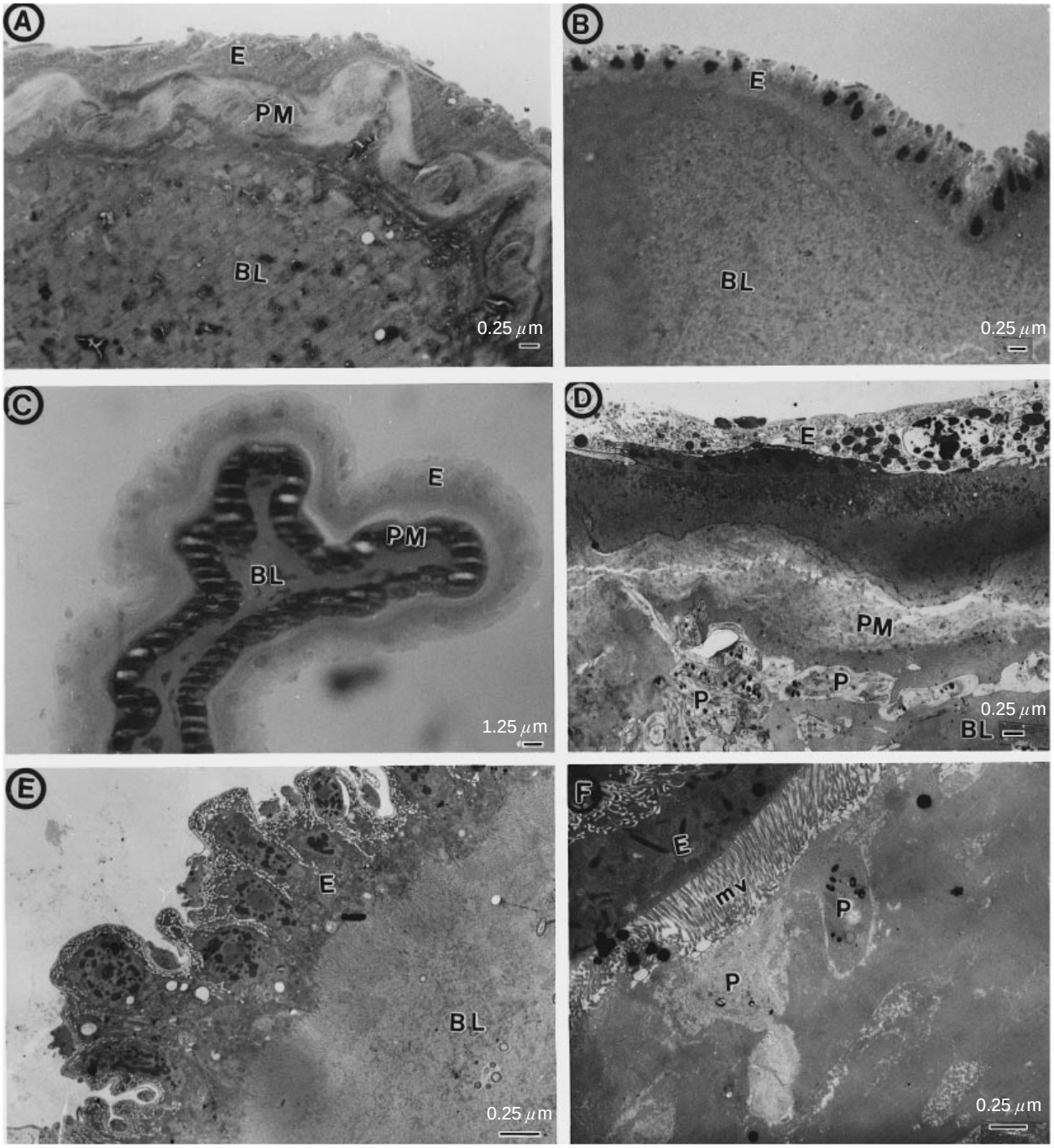

Fig. 1. (A) Light micrograph of a histological section of a midgut from an infected, control fly 24 h after feeding. The peritrophic membrane (PM) is clearly visible and separates the midgut epithelium (E) from the bloodmeal (BL). (B) Histological section of a midgut from an infected, chitinase-treated fly 24 h after feeding. No PM is present, and the epithelium is in direct contact with the bloodmeal. (C) Lower power light micrograph of a sectioned midgut from an infected, allosamidin-treated fly 24 h after feeding. The PM is intensely stained and thickened. (D) Electron micrograph of a midgut from an infected, control fly 24 h after feeding. The PM separates the bloodmeal containing several parasites (P) from the epithelial cells. (E) Electron micrograph of a midgut from an infected, chitinase-treated fly 24 h after feeding. There is no PM and the blood contents are in direct contact with the microvilli. (F) Electron micrograph of a midgut from an infected, chitinase-treated fly 48 h after feeding. Parasites are in direct contact with the microvilli (mv), and present ultrastructural aspects of dying cells.

blood was 2·5 U/ml. Chitinase did not inhibit parasite transformation or growth even at the highest concentrated tested (not shown). Thus the effect of the enzyme on parasite survival *in vivo* is due to some alteration in the midgut environment.

Effect of exogenous chitinase on bloodmeal excretion and digestion

The absence of the PM might alter the gut physiology such that the bloodmeal contents are

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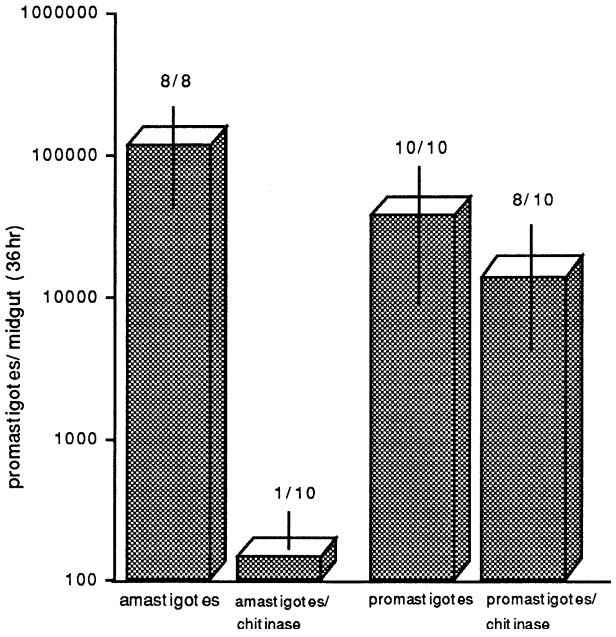

Fig. 2. Effect of chitinase on *Phlebotomus papatasi* infections initiated with 10^7/ml *Leishmania major* amastigotes or promastigotes. Values represent the mean number ± 1 S.D. of viable promastigotes/midgut 36 h after feeding. Numbers above each bar refer to the number of flies positive for parasites over the total number of flies examined in each group.

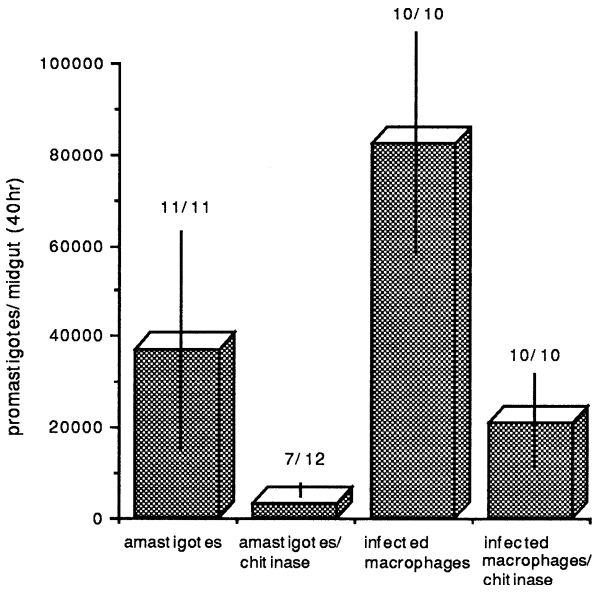

Fig. 3. Effect of chitinase on *Phlebotomus papatasi* infections initiated with 2×10^6/ml purified *Leishmania major* amastigotes or 2×10^6/ml intra-macrophage amastigotes. Values represent the mean number ± 1 S.D. of viable promastigotes/midgut 40 h after feeding. Numbers above each bar refer to the number of flies positive for parasites over the total number of flies examined in each group.

digested and/or excreted more rapidly than is normal. As a relative measure of bloodmeal loss over time, the haemoglobin concentration of whole dissected midguts was determined. There was no significant difference in blood retention in midguts from blood-fed flies compared with blood-fed infected and/or chitinase-treated flies when examined immediately after engorgement (Fig. 4A). Slight increases in haemoglobin concentrations were measured over the next 24 h. We believe this is due to the fact that prior to containment by the PM, some of the bloodmeal is more readily lost during dissection. By 48 h, bloodmeal loss appeared greater in the chitinase-treated, infected flies compared with each of the other groups, although these flies still retained greater than 80% of the initial bloodmeal content. For each group, the largest decline in haemoglobin concentration occurred between 48 and 72 h, with almost complete loss of the remnant

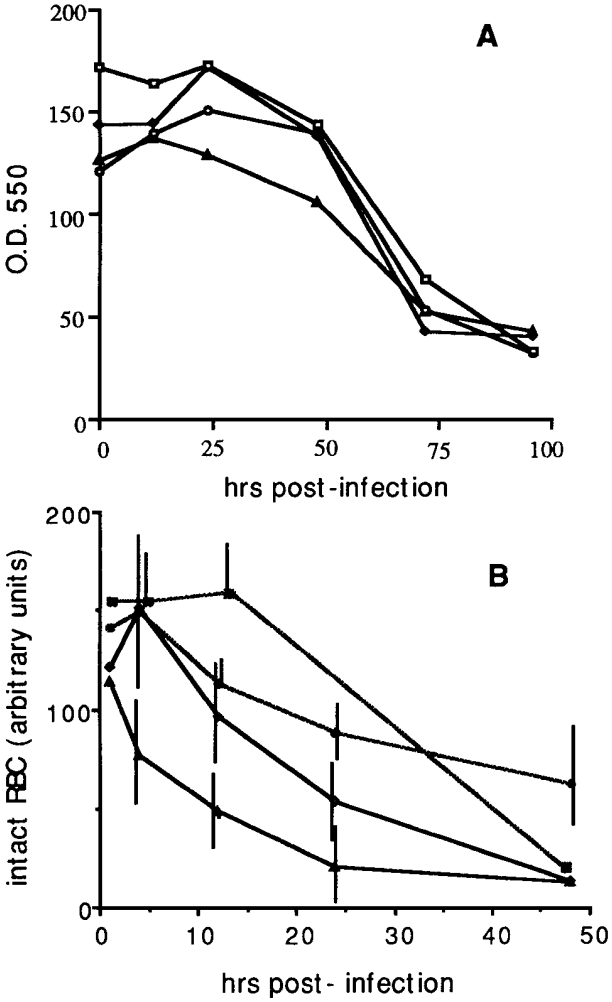

Fig. 4. (A) Relative haemoglobin concentration in blood-fed midguts from uninfected (□); infected (◇); chitinase-treated (○) and infected, chitinase-treated (△) flies. Values at each time-point represent the mean O.D. 550 nm readings of 8–10 flies/group. (B) Relative concentration of intact RBC in blood-fed midguts from infected (◇); infected and chitinase-treated (△); infected, chitinase and trypsin inhibitor-treated (●); infected, allosamidin treated flies (□). Values at each time-point represent the mean ± 1 S.D. of 10–12 flies/group.

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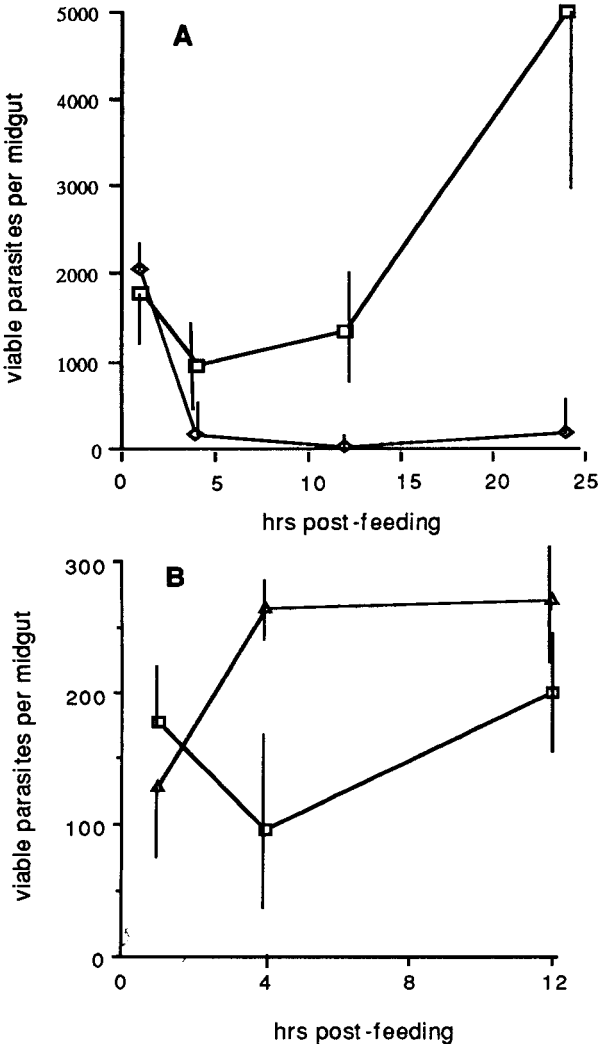

Fig. 5. Early kinetics of parasite killing or growth in control (□), chitinase-treated (◇), and allosamidin-treated flies (△). Flies were fed on bloodmeals containing (A) 10^7 amastigotes/ml or (B) 2×10^6 amastigotes/ml. Values represent the geometric mean ± 1 S.D. of 6–10 flies/group.

bloodmeal occurring by 96 h. Thus the bloodmeal appears to be retained for a sufficient period of time within midguts lacking a PM to permit the transformation and growth of the parasite prior to expulsion of the digested bloodmeal.

In comparison with bloodmeal excretion, bloodmeal digestion was significantly altered by the presence of the chitinase (Fig. 4B). Control flies showed a slight increase in the numbers of intact RBC at 4 h, and a lag time of 12–24 h before substantial haemolysis began. In flies lacking a PM, a 30% decrease in numbers of intact RBC had already occurred by 4 h, over 50% were lysed by 12 h, and by 24 h few intact RBC remained. The addition of a soybean trypsin inhibitor prevented the early (0–4 h) digestion of erythrocytes seen in chitinase-treated flies. RBC digestion was delayed in these flies even when compared with controls. The presence of the chitinase inhibitor, allosamidin, also

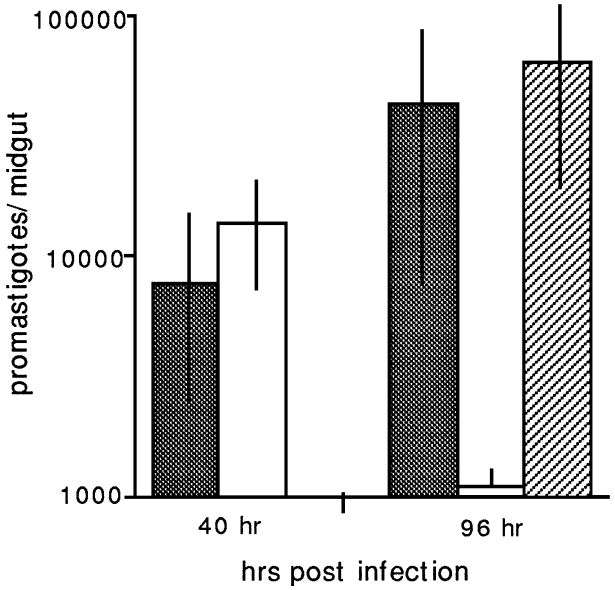

Fig. 6. Effect of allosamidin on *Phlebotomus papatasi* infections initiated with 2×10^6/ml *Leishmania major* amastigotes. Values at each time-point represent the mean number ± 1 S.D. of viable promastigotes/midgut, 12–24 flies/group. Control flies (■), allosamidin-treated flies (□), allosamidin-treated flies in which bloodmeals were absent (⊡) or still present (▨) at 96 h.

caused a significant delay in erythrocyte digestion, with little or no haemolysis detectable within the first 12 h.

Time-course of L. major *killing in blood-fed midguts*

The kinetics of parasite killing in chitinase-treated sand flies was investigated by determining the numbers of viable midgut parasites during the first 24 h after feeding. Midguts were dissected using asceptic techniques, and the contents assayed for numbers of viable parasites by limiting dilution in biphasic medium. Control or chitinase-treated flies fed on bloodmeals containing 10^7 amastigotes/ml contained approximately 1800 viable amastigotes per midgut when examined within 1 h after feeding (Fig. 5A). By 4 h, over 90% of the amastigotes in the chitinase-treated flies had been killed, with virtually complete killing seen by 12 h. Interestingly, there was also substantial reduction (50%) in numbers of viable amastigotes observed in the control flies at 4 h, although in this case no further decline was observed, and division of the surviving parasites was apparent by 24 h.

The early killing of parasites in control flies was confirmed in another experiment in which flies were fed on a bloodmeal suspension containing 2×10^6 amastigotes/ml (Fig. 5B). Again in the control, infected flies there was roughly a 50% reduction in numbers of viable parasites at 4 h. Included in this experiment was a group of flies treated with allosamidin, in which the number of viable parasites

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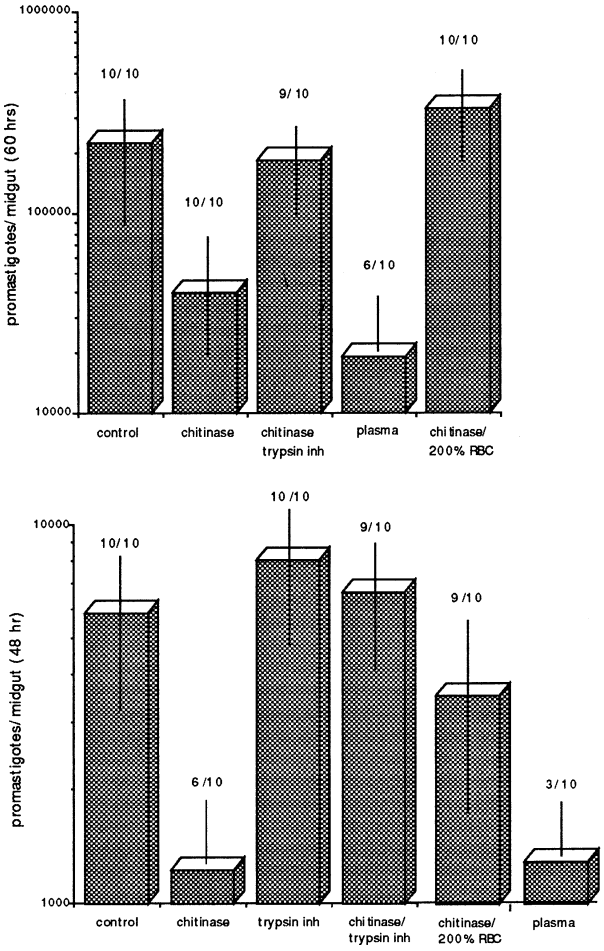

Fig. 7. Effect of chitinase, soybean trypsin inhibitor and RBC concentration on *Leishmania major* infections in *Phlebotomus papatasi*. Flies were infected with 10^7 amastigotes/ml and examined at 60 h (A); or infected with 2×10^6 amastigotes/ml and examined at 48 h (B). Values represent the mean $\pm$ 1 S.D. of 10–12 flies/group.

in the midgut was actually higher at 4 h. The number of promastigotes in control and allosamidin-treated flies at later time-points, when midgut promastigotes could be directly counted, is shown in Fig. 6. There were nearly twice as many promastigotes in the allosamidin-treated flies at 40 h compared to controls, although this difference was not significant. By 96 h, the digested bloodmeals had been passed in 12 of 12 control, infected flies and an average of 42000 ± 36000 promastigotes were retained in the midgut. By 96 h in the allosamidin-treated flies (Fig. 6), bloodmeals had been passed in only 33% (8 of 24), and in these flies few parasites remained (1100 ± 800). In flies retaining a bloodmeal at 96 h, the PM remained intact, and the blood contained an abundance of active promastigotes (64000 ± 44300).

The role of trypsin in the killing of L. major *in blood-fed and chitinase-treated sand flies*

A soybean trypsin inhibitor was included in the bloodmeals to determine the role of midgut proteases

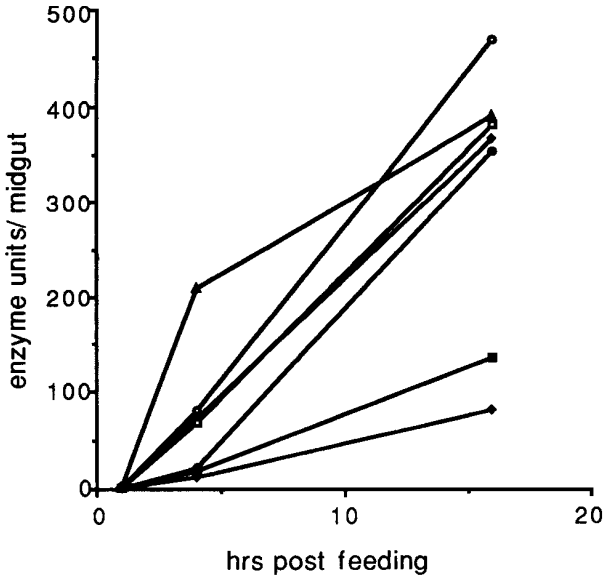

Fig. 8. Early kinetics of trypsin activity in blood-fed uninfected midguts (●), blood-fed, infected midguts (◆); blood-fed, infected midguts containing either chitinase (○); chitinase and trypsin inhibitor (◇); chitinase plus 200% RBC (▲); 100% plasma, no RBC (■) or allosamidin (□). Values represent mean enzyme units/midgut at each time-point, 10–12 midguts/group.

in the early parasite mortality. In separate experiments in which the infections were initiated using 10^7 or 2×10^6 amastigotes/ml, chitinase treatment was again found to significantly reduce the numbers of parasites in the midgut, by 85% and 82%, respectively (Fig. 7A). In each experiment, the reduction in midgut promastigotes in the flies treated with chitinase was completely reversed by the trypsin inhibitor. It is important to note that the trypsin inhibitor did not affect the ability of the chitinase to disrupt PM formation *in vivo* (data not shown). The addition of trypsin inhibitor to the bloodmeal also increased the mean number of parasites surviving in control, infected flies, although this difference was not significant (Fig. 7B).

The trypsin activity in blood-fed/treated midguts was assayed using a chromogenic substrate. Early levels of enzyme activity were determined since parasite killing in chitinase-treated and control flies appeared to be initiated within the first few hours after feeding. Trypsin activity induced by the bloodmeal was detectable by 4 h, and rose sharply by 16 h in the blood-fed, infected and chitinase-treated flies (Fig. 8). No significant difference in total midgut-trypsin activity was observed between infected and uninfected flies, or between control infected flies and those treated with either chitinase or allosamidin. Thus, the lack of PM did not appear to alter the timing or amount of trypsin induced by the bloodmeal. In flies treated with both chitinase and trypsin inhibitor, no trypsin activity was detectable at 4 h, and only low levels were seen at 16 h. The doubling of RBC concentration in the blood-

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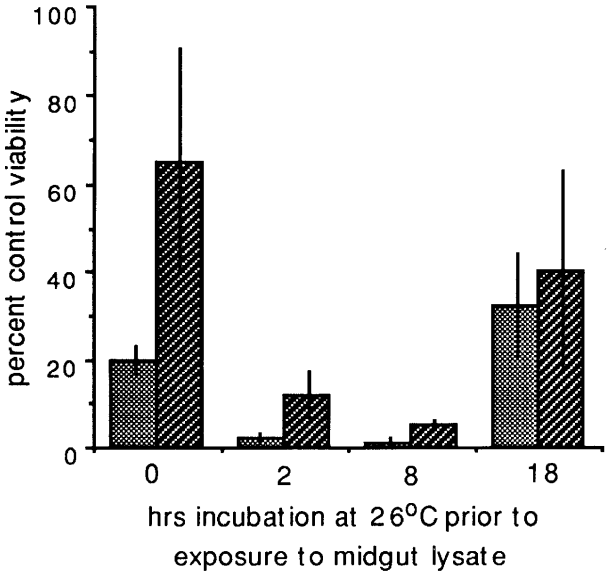

Fig. 9. Susceptibility of *Leishmania major* amastigotes, promastigotes, and transitional stages to killing by digestive enzymes in blood-fed midguts. A suspension of purified *Leishmania major* amastigotes was incubated for up to 18 h at 26 °C, and at various times during transformation to promastigotes aliquots were incubated with lysate from a single blood-fed midgut. Percent control viability determined from the geometric mean number of viable parasites (5–9 assays/group) after 2 h (⊡) or 1 h (▨) incubation with midgut lysates over the number of viable parasites in identical aliquots incubated alone.

meals increased the level of early trypsin release, whereas plasma alone induced no detectable activity until 12 h, at which time the levels were still significantly less than in controls. Peak trypsin activity was observed in most groups at 30 h (data not shown), consistent with prior reports (Borovsky & Schlein, 1986; Dillon & Lane, 1993).

Effect of RBC concentration on parasite survival in blood-fed and chitinase-treated midguts

The PM might moderate exposure of parasites to gut proteases by creating a physical barrier to enzyme diffusion. In an attempt to mimic the effect that PM containment of the bloodmeal within the intraperitrophic space has on blood cell density, flies were fed on a bloodmeal containing chitinase plus a 2-fold increase in normal RBC concentration. Significant recovery of early parasite survival was observed in the experiments shown in Fig. 7A and B ($P < 0{\cdot}02$ and $P < 0{\cdot}05$, respectively, compared with chitinase-treated, normal blood). The absence of PM formation in these flies was confirmed. In contrast, in flies membrane-fed on plasma alone (no RBC, no chitinase), the infections were sharply reduced to the levels seen in the chitinase-treated flies fed on normal blood. The *in vitro* transformation and growth of parasites in 200 % and 0 % RBC was identical to their growth in normal blood (data not shown). However, the PMs observed in flies fed on plasma alone remained very thin and never fully enveloped the bloodmeal.

Stage specificity of parasite killing by lysates from blood-fed midguts

Since the effect of the chitinase on parasite mortality was far greater when infections were initiated with amastigotes compared with promastigotes, this suggested that susceptibility of *Leishmania* to midgut digestion is stage specific. This question was explored further by comparing the susceptibility of fresh tissue amastigotes, fully transformed promastigotes, and cells within amastigote to promastigote transition, to killing by digestive enzymes present in lysates prepared from 24 h blood-fed, uninfected midguts. Fresh tissue amastigotes and 18 h promastigotes were relatively resistant to killing during a 1 or 2 h exposure to the midgut lysates (Fig. 9). In contrast, parasites within early-stage transition (2–8 h) became highly susceptible to killing (greater than 95 % reduction in numbers of viable parasites compared with controls). Many more amastigotes were killed after the 2 h compared with the 1 h exposure to the midgut lysates. This was likely due, at least in part, to their having initiated their transformation to a more susceptible form during the 2 h incubation with the midgut lysate.

DISCUSSION

The development of transmissible infections within phlebotomine vectors is thought to depend, at least in part, on access of midgut promastigotes to adhesion receptors on the gut wall (Pimenta *et al.* 1994), and to their colonization of the anterior gut (Killick-Kendrick, 1979). Thus, disruption of a presumed barrier to promastigote migration, the bloodmeal-induced PM, was expected to facilitate the development of infection in these flies. The current studies show that addition of exogenous chitinase to the bloodmeal can successfully prevent PM formation in sand flies, extending the original observations made in mosquitoes (Shahabuddin *et al.* 1993). Surprisingly, the absence of a PM resulted in the loss of infection in these flies. The loss of parasites in chitinase-treated flies was confined to flies infected with tissue amastigotes, or with macrophages containing intracellular amastigotes; promastigote-initiated infections were relatively unaffected. The loss of infections was not due to a direct toxicity of the chitinase for the parasite, since transformation and growth of *L. major* amastigotes *in vitro* was not affected by high concentrations of the enzyme. Nor was the loss of infection due to premature excretion of the bloodmeal, since most of the blood was still present in the midgut following

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the time when parasite loss had already occurred. The number of viable parasites in chitinase-treated flies was already diminished by 90% 4 h after bloodfeeding. Most of the remaining parasites were killed within the next few hours. Interestingly, substantial killing of parasites in control, blood-fed flies was also seen by 4 h, suggesting that the absence of a PM exacerbates lethal conditions that exist normally in the blood-fed midgut. The finding that significant parasite mortality occurs during the development of *Leishmania* in a natural vector is novel, and was made possible by enumerating viable midgut parasites within the first few hours after engorgement.

The possibility that parasites were killed by the digestive enzymes produced by the blood-fed midgut was explored based on previous observations implicating gut proteases in the killing of inappropriate *Leishmania* species within *P. papatasi* (Adler, 1938; Schlein & Romano, 1986; Borovsky & Schlein, 1987). In the study by Borovsky & Schlein (1987) the addition of soybean trypsin inhibitor to the bloodmeal enhanced the midgut survival of *L. donovani*. Similarly, we found that inclusion of a trypsin inhibitor prevented much of the early killing of *L. major* seen in control, and especially in the chitinase-treated flies. There was, however, no indication that the effect of the chitinase was to increase the total amount of trypsin activity in the gut; nor did the timing of trypsin release appear to be altered compared with control, infected flies. The trypsin activity measured at 4 h was relatively low in each group, especially when compared with the peak levels seen at 30 h. This early trypsin release appears, nonetheless, to have been sufficient to damage the parasite and to initiate bloodmeal digestion.

Since the amount of trypsin released appears unaffected by the chitinase, an alternative explanation for the exacerbation of parasite killing is that PM disruption affects the rate at which released enzymes are distributed through the bloodmeal. Studies in mosquitos have revealed a temporal and spatial pattern of bloodmeal digestion that seems especially relevant to the early killing of *Leishmania* observed in infected sand flies. The mosquito studies showed that the bloodmeal is digested in the abdominal midgut from the periphery towards the center of the bolus (Staubli, Freyvogel & Suter, 1966; Briegel & Lea, 1975). Trypsin immunoreactivity was localized at the earliest time-point examined (8 h) along the PM; the immunoreactivity did not extend deep into the bolus until 16–24 h (Graf *et al*. 1986). Thus the *Leishmania* cells which are killed early on in blood-fed sand flies might be expected to lie in the periphery of the bloodmeal. The PM might moderate the early exposure of parasites to gut proteases by acting itself as a permeability barrier, or by creating a dense, cellular barrier to enzyme diffusion through its containment of the bloodmeal within the intraperitrophic space. Diuresis, which accompanies PM formation as one of the earliest midgut responses to bloodmeal ingestion (Williams, Hagedorn & Beyenbach, 1983), might add a further constraint to the circulation of released enzymes. Erythrocyte digestion was found in our studies and others (Vaughan & Azad, 1993) to be normally delayed for up to 12 h. The fact that substantial erythrocyte digestion occurred in chitinase-treated flies by 4 h, supports a role for the PM in minimizing the early exposure of the bloodmeal contents to digestive enzymes.

The relationship between blood cell density and parasite survival was addressed directly; parasite survival in midguts lacking a PM could be restored to near control levels by doubling the initial concentration of erythrocytes in the bloodmeal, and in flies fed on plasma alone, very few parasites survived. In these flies, the lack of correlation between the amount of early trypsin induced by the bloodmeal and the extent of parasite killing is especially striking, and emphasizes the importance of blood cell barriers to enzyme distribution. The circulation of digestive enzymes throughout the bolus in the flies fed on plasma alone might have been especially rapid, since these flies also failed to form an intact, sustainable PM. Adler (1938) was the first to note an effect of increasing serum concentration on parasite mortality. In his study, the killing was attributed not to a reduction in RBC concentration, but to an inhibitory effect of the serum itself; and was meant to explain why an incompatible parasite strain, *L. donovani*, failed to survive in *P. papatasi*. Species differences in bloodmeals have been observed to influence the success of even *L. major* infection in *P. papatasi* (Schlein & Jacobson, 1994, 1996). Consistent with the model proposed here, this might be explained by inherent species differences in blood cell density affecting the rate of enzyme diffusion.

Since conditions that moderate the early exposure of amastigotes to midgut digestive enzymes seem to be critical for parasite survival, it was important to address the possibility that intra-macrophage amastigotes, the presumed source of fly infections in nature, might avoid lethal exposure by remaining confined inside host cells until their differentiation into protease-resistant promastigotes. Infection of chitinase-treated flies using infected macrophages instead of purified amastigotes still resulted in substantial early parasite mortality (70%), and suggests that intracellular amastigotes are rapidly released from their host cells after engorgement.

The streptomycete antibiotic, allosamidin, is a potent competitive inhibitor of insect, plant, and parasite chitinases. Although it weakly inhibits *S. griseus* chitinase, it was shown to be a potent inhibitor of the chitinase made by *P. gallinaceum* ookinetes (Shahabuddin *et al*. 1993). In sand flies fed on

bloodmeals containing 1 mM allosamidin, the PM appeared thicker, and was sustained as an intact structure for longer. In comparison with control, infected flies, both erythrocyte digestion and parasite killing was significantly delayed. This suggests that the matrix itself provides a barrier to enzyme diffusion. At later time-points, the effect of allosamidin on parasite development was to prevent their escape from the bloodmeal. So long as the bloodmeal was retained within the abdominal midgut, the promastigotes remained viable. In every case, when the bloodmeals in the allosamidin-treated flies were finally passed, the parasites were also lost, indicating that the action of a parasite and/or sand fly-derived chitinase (Schlein *et al*. 1991) is required in order for the parasites to escape containment by the PM and for the infections to persist after bloodmeal excretion.

The apparent difference between the sensitivity of amastigotes and promastigotes to midgut digestion *in vivo* was further investigated *in vitro* by controlled exposures of tissue amastigotes, fully transformed promastigotes, and transitional extracellular forms, to lysates from a single blood-fed midgut. Parasites within a transitional stage of amastigote to promastigote differentiation were extremely susceptible to killing in this assay. We believe that the relative resistance of differentiated promastigotes to midgut digestion is related to their acquisition of lipophosphoglycan (LPG), a protein-free glycosylated phosphatidylinositol (GPI) which forms a densely organized glycocalyx on the promastigote surface (Turco & Descoteaux, 1992). This conclusion is based on the observation that LPG minus mutants of *L. donovani* were completely killed by blood-fed *P. argentipes* within 48 h, and that each of 3 transfected mutants, rescued for LPG expression by genetic complementation, displayed at least partial recovery of early midgut survival (Pimenta *et al*. 1994; Sacks, Beverley & Turco, unpublished observations). Our data do not support the previous suggestion that LPG released by the parasite inhibits the level of protease activity in infected midguts (Schlein, Schnur & Jacobson, 1990). Infection did not diminish the amount of trypsin activity in the gut over any time-frame that the enzyme was produced, and prior observations by us and others have failed to detect released forms of LPG in the infected midgut (Saraiva et al. 1995; Lang *et al*. 1991).

Although LPG is present in very low or undetectable levels in amastigotes, this stage does express an abundance of another class of surface localized, protein-free GPIs, the low-molecular mass glycoinositolphospholipids (GIPLs), which might perform a similar function to LPG by protecting the plasma membrane from both insect and mammalian hydrolases (McConville, 1991). The susceptibility of transitional forms is not understood. The integrity of the glycocalyx might be compromised during early transformation due to the delay in synthesis and surface expression of LPG, which may be required to complement the GIPLs in masking the increased cell surface area of promastigotes. During transformation *in vitro* and *in vivo*, surface expression of LPG is delayed for at least 14–16 h (Sacks, unpublished observations; Saraiva, 1995).

In summary, these studies provide direct evidence that the PM, while clearly a potential barrier to the development of infection, also promotes early parasite survival by moderating the exposure of parasites to the hydrolytic activities of the bloodfed midgut prior to their differentiation into protease-resistant forms.

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