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Influence of *Leishmania* infection on blood-meal digestion in the sandflies *Phlebotomus papatasi* and *P. langeroni*

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Abstract. The presence of amastigote-initiated infections of *Leishmania major* parasites caused a significant suppression in alkaline protease, trypsin and aminopeptidase activity during the first 30 h after ingestion of the infected bloodmeal in *Phlebotomus papatasi*, the natural vector of *L. major*. Protease levels were significantly higher in infected flies after 72 h than in the control group, where digestion had ceased. Evidence for the suppression of protease activity in infected *P. langeroni*, a sympatric but un-natural vector of *L. major*, was less clear; there was no difference in alkaline protease activity between control and infected groups in the first 24 h. However, protease, trypsin and aminopeptidase activities were elevated after 72 h in infected *P. langeroni*, indicating a delay in the time to the end of digestion and passage of the bloodmeal. The potential advantages for parasite development in suppressing protease activity and extending the period of bloodmeal digestion are discussed.

Phlebotomine sandflies transmit *Leishmania* parasites, which inhabit the gut of the insect during their entire development in the vector. The ability of *Leishmania* to survive and develop during bloodmeal digestion is the first stage in the successful establishment of the parasite population in the vector (Molyneux and Killick-Kendrick 1987). During this period a variety of physiological changes take place within the gut, including the release of proteases for digestion of bloodmeal proteins (Lehane 1991).

The possibility that insect-derived proteolytic enzymes are important in influencing the development of medically important protozoans has been recognized for some time (Gooding 1972). *Plasmodium* ookinetes probably evade deleterious effects of proteases by penetrating the gut wall before enzyme secretion has become maximal (Gass and Yeates 1979; Ponnadurai et al. 1988); enhanced levels of aminopeptidase activity in *Anopheles stephensi* might be one factor that prevents infection by *Plasmodium falciparum* (Feldmann et al. 1990). Trypsin activity in the midgut of *Glossina morsitans morsitans* might be important in the transformation of *Trypanosoma brucei brucei* (Yabu and Takayanagi 1988; Imbuga et al. 1992a); in vitro preparations of *T. brucei* partially inhibited trypsin-like activity in midgut preparations of *G. morsitans* (Imbuga et al. 1992b). In contrast, digestive proteases do not affect the development of the epimastigotes of *T. cruzi* in their vector *Rhodnius prolixus* (Garcia and Gilliam 1980).

As long ago as 1938, Adler suggested that factors induced by the ingestion of rabbit serum prevented the development of those *Leishmania* not specific to a host species of sandfly. Borovsky and Schlein (1987) proposed that it was trypsin-like activity that prevented the survival of *L. donovani* in its unnatural host *Phlebotomus papatasi*.

Within 10 h of bloodmeal ingestion, *P. papatasi* and *P. langeroni* produce significant amounts of trypsin and aminopeptidase (Dillon and Lane 1993). Activity peaks during the 2nd and 3rd day, respectively, for *P. papatasi* and *P. langeroni*. *P. papatasi*, the vector of *L. major* that causes cutaneous leishmaniasis, and *P. langeroni*, the vector of the visceralizing parasite *L. infantum*, are sympatric species. Both parasite species are capable of developing in *P. langeroni*, but *L. infantum* (a member of the *L. donovani* group) is incapable of developing in *P. papatasi* (El Sattar et al. 1991).

Herein we describe the effect of amastigote-initiated *L. major* infections on the proteolytic activity in the midgut of the natural host *P. papatasi* and in *P. langeroni*, an experimental host.

Materials and methods

Sandflies

Phlebotomus papatasi (originally from Cyprus) and *P. langeroni* (from El Agamy, Egypt) were kept as described by Killick-Kendrick (1987).

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Infection of sandflies

Flies (3–4 days old) were infected by membrane feeding on heparinized human blood containing parasites to a final concentration of 5×10^5/ml as described previously (Davies et al. 1990). Bloodfed females were maintained with males at 26°–28° C and >90% humidity and were given access to 15% (w/v) sucrose solution. All infection experiments used cryopreserved amastigotes of *Leishmania major* (LV39). Control samples of sandflies were fed on the same batch of blood but without parasites (i.e., uninfected).

Enzyme assays. Sandflies were dissected at fixed periods after bloodfeeding and the midguts were stored in saline at −20° C (Dillon and Lane 1993). Alkaline protease, trypsin and aminopeptidase were estimated as previously described (Dillon and Lane 1993); controls containing saline instead of enzyme preparation were used for each assay.

Alkaline protease assay. A 125 µl volume of enzyme preparation (supernatant from homogenates of two midguts) and 125 µl of azoalbumin substrate (15 mg/ml in 0.1 *M* TRIS-HCl buffer, pH 8.5) were incubated for 1 h at 30° C. The reaction was stopped by the addition of 250 µl of 10% (w/v) cold trichloroacetic acid. The endpoint absorbance of the supernatant (after removal of the precipitate) was read at 450 nm using a microplate reader. Control readings, from preparations to which substrate was added after the trichloroacetic acid, were subtracted from the experimental readings to account for changes in absorbance due to spontaneous breakdown of the substrate. One unit of protease activity is defined as the amount of enzyme required to produce an absorbance change of 1 under the conditions of the assay.

Trypsin assay. Benzoyl-arginine-*p*-nitroanilide (BApNA) was prepared as 2 m*M* in 7% dimethylformamide (DMF) in 0.1 *M* TRIS buffer (pH 8.5). In all, 100 µl of enzyme preparation (supernatant from one gut homogenized in 120 µl of saline, prepared as described above) was mixed with 100 µl of substrate (final concentration, 1 m*M*) in buffer in the well of a microtitre plate at room temperature (23° C) and the rate of reaction in each well was automatically calculated by linear regression for the first 5 min of the reaction. The change in optical density per minute was converted into the micromolar amount of substrate hydrolysed per minute by reference to a standard curve for *p*-nitroanilide.

Aminopeptidase assay. Leucine-*p*-nitroanilide (LpNA) was dissolved in 3% dimethylsulphoxide in 0.05 *M* TRIS buffer to a final concentration of 1 m*M*. The assay was completed using the same procedure described for the trypsin assay.

The chromogenic substrates used in the present study were previously found to be specific for trypsin and aminopeptidase activity (Dillon and Lane 1993). The results are expressed as enzyme-activity units (EU), being the amount of enzyme required to hydrolyse 1 µmol of substrate per minute under the conditions of the assay. All substrates and buffers were obtained from Sigma Ltd.

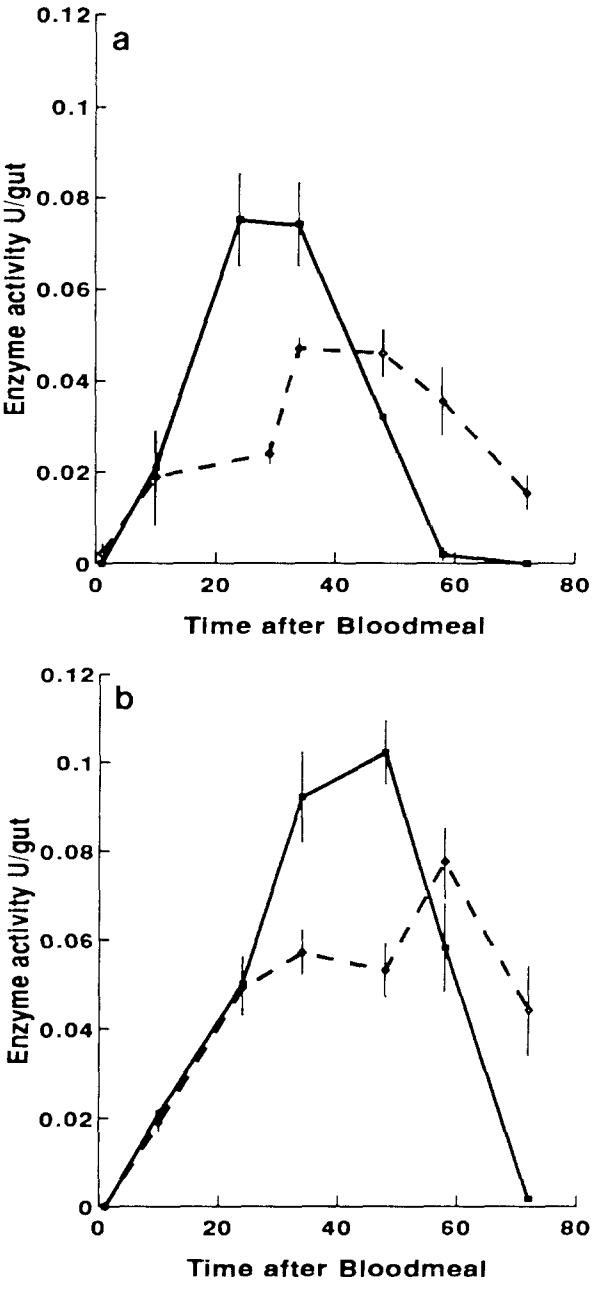

Fig. 1 a, b. Alkaline protease activity in midgut extracts of **a** *Phlebotomus papatasi* and **b** *P. langeroni* after a human bloodmeal containing amastigotes of *Leishmania major*. Enzyme activity from control flies (bloodmeal only) is represented by a *continuous line* and that from infected flies, by a *dashed line*. *Vertical bars*, ±SE ($n=6$)

Results and discussion

Infection by amastigotes of *Leishmania major* in a human bloodmeal delayed and depressed the peak levels of protease activity in the midgut of *Phlebotomus papatasi*. The maximal alkaline protease activity that occurred at 34 h post-bloodmeal was significantly lower (Fig. 1 a, $t=2.6$, $P<0.05$) in infected *P. papatasi* than the maximal activity that occurred after 24 h in uninfected insects. Similarly, significantly lower peak levels of activity towards the trypsin substrate occurred after 50 h (Fig. 2 a, $t=3.34$, $P<0.005$) in infected insects as compared with the peak activity observed after 30 h in the control *P. papatasi*.

In contrast, although there was a delay in the time taken to reach peak activities in infected *P. langeroni* (the unnatural host of *L. major*), differences between maximal activities were less apparent. There was no significant difference in maximal alkaline protease activity (Fig. 1 b, $t=1.1$), but maximal trypsin activities were significantly higher in the control group of insects (Fig. 2 b, $t=2.8$, $P<0.05$).

Trypsin and aminopeptidase were significantly depressed as early as 10 h (trypsin: $t=5.38$, $P<0.002$;

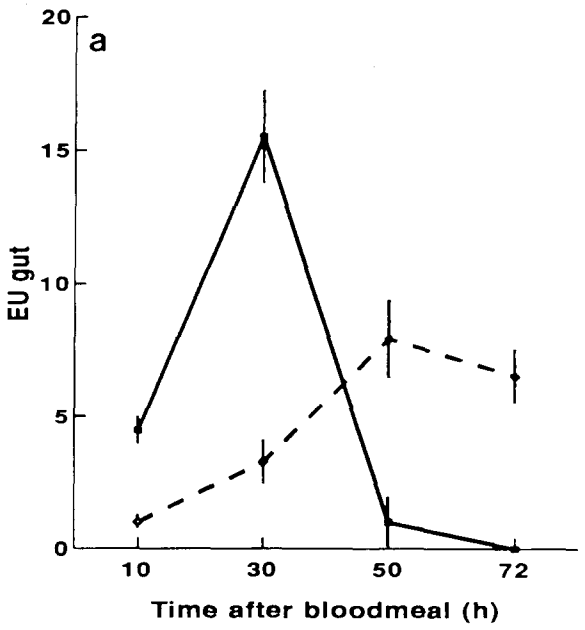

Fig. 2a, b. Trypsin activity in midgut extracts of **a** *P. papatasi* and **b** *P. langeroni* after a human bloodmeal containing *L. major* amastigotes. *Continuous line*, control flies; *dashed line*, infected flies; *vertical bars*, ±SE ($n=8$)

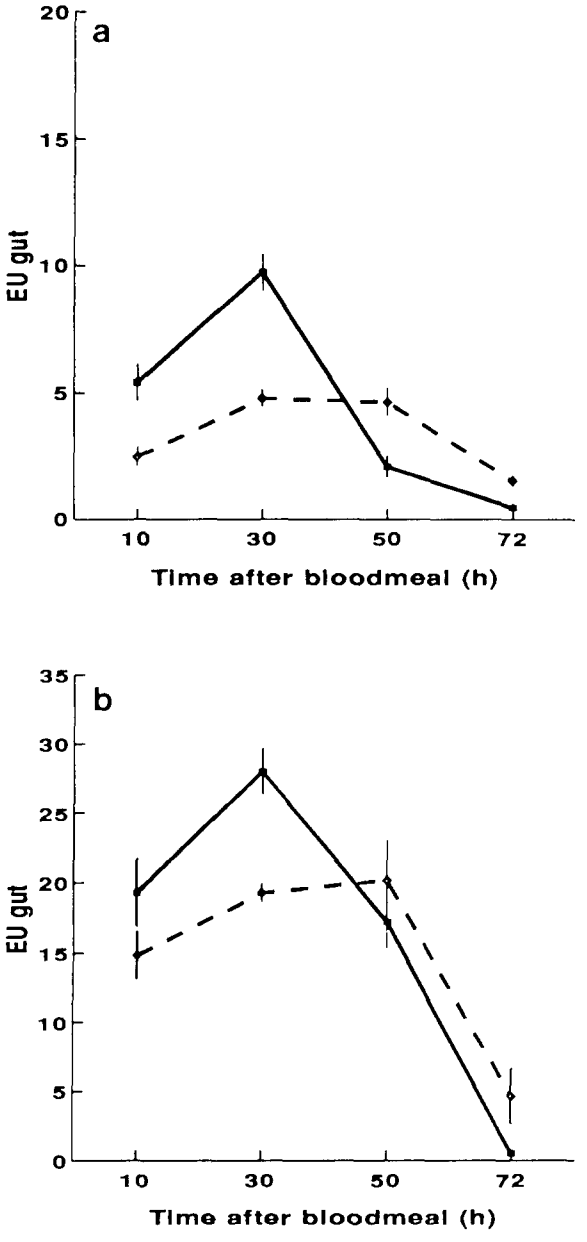

Fig. 3a, b. Aminopeptidase activity in midgut extracts of **a** *P. papatasi* and **b** *P. langeroni* after a human bloodmeal containing *L. major* amastigotes. *Continuous line*, control flies; *dashed line*, infected flies; *vertical bars*, ±SE ($n=8$)

aminopeptidase: $t=3.7$, $P<0.01$) and 30 h (trypsin: $t=7.1$, $P<0.002$; aminopeptidase: $t=6.1$, $P<0.002$) post-bloodmeal in infected *P. papatasi*. There was also a reduction in trypsin activity in midguts from infected *P. langeroni* at 10 and 30 h (Fig. 2b, 10 h: $t=3.67$, $P<0.002$; 30 h: $t=5.3$, $P<0.002$), but a reduction in aminopeptidase was apparent only at 30 h (Fig. 3b, $t=3.3$, $P<0.02$). The observation that protease activities were depressed within 30 h in infected sandflies from both species is interesting because the number of amastigotes ingested by an individual sandfly in these experiments was estimated to be <400, assuming that 0.8 µl of blood was ingested (Dillon and Lane, unpublished data, estimated by haemoglobin assay) and a uniform distribution of parasites of 5×10^5 ml^{-1}. The mechanism by which protease levels were suppressed at this early stage in bloodmeal digestion by relatively low numbers of parasites is unclear. Imbuga et al. (1992b) similarly observed that a low number of *Trypanosoma brucei* parasites (1500 ml^{-1}) was sufficient to cause a 50% reduction in trypsin activity in homogenates of midguts from *Glossina morsitans* after 5 min preincubation in vitro.

Suppression of proteolytic activity in infected *P. papatasi* was also described by Borovsky and Schlein (1987) within 30 h post-feeding, but they used sandflies infected with promastigotes in heat-treated blood. Pro-

mastigotes do not appear in significant numbers within the bloodmeal until >24 h after ingestion in amastigote-induced infections (Warburg et al. 1986; Elnaiem et al. 1992), and therefore the suppression of enzyme activity by promastigote-initiated infections shortly after infection could be misleading as compared with a 'natural infection'. The results of the present study indicate that enzyme suppression also occurs during amastigote-initiated infections.

Schlein et al. (1990) found that excreted glycoconjugates of cultured *L. major*, presumably including lipophosphoglycan, phosphoglycan and acid phosphatase (Turco and Descoteaux 1992), also caused a delay in bloodmeal digestion in *P. papatasi*. The extent to which glycoconjugates are released within the bloodmeal is not known; little free lipophosphoglycan (LPG) was found in the midgut after bloodmeal passage in *P. papatasi* using immunogold labeling and electron microscopy in contrast to immunoperoxidase staining of thicker (1–1.5 μm) sections (Lang et al. 1991).

One possibility would be that the proteases were inactivated or their secretion was reduced either by unidentified extracellular products or by surface components of the *Leishmania*. Crude membrane preparations of *T. brucei* inhibited *G. morsitans* midgut trypsin in a dose-dependent manner (Imbuga et al. 1992b). Studies with another parasite-host combination, the cestode *Hymenolepis diminuta* in the vertebrate gut (Schroeder et al. 1981) revealed that the parasite inhibited host trypsin. Trypsin activity was reduced against the whole-protein substrate azoalbumin but was retained fully against chromogenic peptides. The data suggested that inhibition was due to a small structural change in the molecule that partially inactivated the enzyme to whole-protein substrates but did not affect its catalytic activity against the chromogenic peptides. However, the mode of inhibition of insect proteases by *Leishmania* in the present study is likely to differ because enzyme activity was reduced towards the whole-protein and peptide substrates.

If aminopeptidases are secreted in response to peptide levels rather than proteins, then the suppression in levels of aminopeptidase seen in the present study could be a consequence of low titres of trypsin activity producing the necessary peptides rather than a direct effect by the parasite on the aminopeptidase.

The rapid fall in protease activity associated with the control group after 48 h, probably due to the passage of the bloodmeal remnants (Briegel 1975), did not occur in infected insects. There was a significant delay in peak enzyme activities in the infected guts such that as the levels were falling in uninfected guts of *P. papatasi*, the levels were elevated in infected guts after the 2nd day post-infection (protease, 58 h: $t=3.8$, $P<0.05$, Fig. 1; trypsin, 50 h: $t=3.44$, $P<0.01$, Fig. 2; aminopeptidase, 50 h: $t=3.9$, $P<0.002$, Fig. 3). After 72 h, the infected flies of both species continued to show substantial levels of protease activity in contrast to the virtual absence of enzyme activity in the control insects. Although some contribution to overall protease levels by the parasites cannot be discounted, most of the difference is presumably due to insect-derived proteases. The glycoprotein protease present on the surface of promastigotes (GP63) does not hydrolyse unblocked or *N*-terminal-blocked chromogenic peptides such as LpNA and BApNA (Bouvier et al. 1990) but the soluble parasite proteases are active towards BApNA (Pupkis and Coombs 1984). A preparation of lysed LV39 produced an activity of 0.95 $\mu M\ min^{-1}/10^{-7}$ promastigotes towards BApNA as compared with the activity observed in the midgut of infected flies after 72 h of 6.5 $\mu M\ min^{-1}$. The estimated population of $<10^5$ parasites in the midgut of *P. papatasi* at 3 days post-infection (Davies et al. 1990) would therefore provide only a small proportion of the total activity against the substrate.

It has been suggested that sandfly protease levels are actively depressed by *Leishmania* to enable parasite survival in appropriate insect hosts (Borovsky and Schlein 1987). Significantly lower peak levels of protease activity in *Leishmania* infected insects, as found in the sandfly species in the present study, might be important if proteases are harmful to parasite development. In an interesting experiment, Borovsky and Schlein (1987) included soybean trypsin inhibitor with a bloodmeal containing promastigotes of *L. donovani*, a species that normally fails to establish a successful infection in *P. papatasi*. The trypsin inhibitor successfully inhibited bloodmeal digestion and significantly more parasites were found surviving in these flies at 3 days post-infection. To demonstrate that this mechanism is a key event in vector-parasite specificity, it would be necessary to demonstrate the survival of *L. donovani* beyond bloodmeal passage and attachment to the midgut epithelium (Pimenta et al. 1992). As attractive as the host-specificity hypothesis for depressed gut-enzyme levels is, alternative hypotheses need to be explored.

One alternative interpretation of the trypsin inhibitor experiment is that a small proportion of the nutrients from the bloodmeal are being 'redirected' for use by the parasite population rather than being assimilated by the fly. Thus, the importance of insect protease suppression could be the extension in availability of the nutrient source for the growing parasite population rather than the harmful effects of proteases on the parasite. There are three lines of evidence to support this hypothesis. Schlein et al. (1990) recorded a correlation between undigested blood and the survival of *L. major* LRC119 (a strain deficient in LPG production) in the gut of *P. papatasi*. Moreover, the period of 3–4 days post-infection is a time of exponential multiplication of the parasites, when freely available supplies of exogenous nutrient are required; and finally parasites thrive in the presence of trypsin in culture (Dillon and Lane, unpublished data). Suppression of the digestive process during the first 2 days, resulting in a slight delay in the rate of digestion, could be sufficient for the parasite population to develop a large post-bloodmeal infection. The development of a large parasite population prior to bloodmeal passage might be important in the development of the metacyclic promastigote. High-density promastigote populations lead to inhibited cell division (Howard et al. 1987), possibly due to nutrient depletion after bloodmeal passage, and to the subsequent change

to stationary-phase infective metacyclic promastigotes (Sacks and Perkins 1985).

The length of the digestive period is also thought to be a determinant for establishment of *L. major* in the sandfly *P. duboscqi* (Lawyer et al. 1990). Digestion in the non-host vector *Sergentomyia schwetzi* was apparently faster than that in *P. duboscqi*, and an *L. major* infection failed to establish in the non-host vector after bloodmeal passage.

The mechanism by which protease activity is delayed and reduced in the midguts of the two species of sandflies infected with *L. major* remains obscure and is the subject of further investigation.

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