Resistance of *Phlebotomus papatasi* to infection with *Leishmania donovani* is modulated by components of the infective bloodmeal

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SUMMARY

The circumstances which permit the establishment of *Leishmania* infections in sandflies were investigated by altering the growth conditions for *L. donovani* parasites in the unsuitable vector *Phlebotomus papatasi*. Only 5·0% of the sandflies harboured a few parasites 3 days after feeding on promastigotes in defibrinated blood. Heparinized blood or the addition of trypsin inhibitor to the meals allowed persistence of infections (day 6) in 9·9% and 25·8% of the flies respectively. Meals of erythrocytes, saline and amastigotes produced 44·4% fly infection on day 6, while similar promastigote-initiated infections remained in 70·3% of the flies. Proteolytic activities in the guts of sandflies fed on the above meals without parasites, were the highest after defibrinated bloodmeals. Erythrocytes with saline decreased the maximal alkaline protease level from 20·8 U to 13·5 U/fly; that of trypsin from 3·9 U to 1·8 U/fly and that of the aminopeptidase from 5·5 U to 3·9 U/fly. After meals of heparinized blood, the maximal alkaline protease activity (12·0 U/fly) was also much lower than after defibrinated blood-feeding. The different diets which resulted in comparatively low enzymatic activities, including blood with trypsin inhibitor, also promoted the survival of infections. This implies that the proteolytic activity in the sandfly gut modulates the vector susceptibility.

Key words: *Phlebotomus papatasi*, *Leishmania*, vector-specificity, proteolytic enzymes, bloodmeal.

INTRODUCTION

The cycle of *Leishmania* in the gut of the sandfly is suited to cope with various conditions. Infections begin when the sandfly bites and ingests blood and amastigotes. The intake of blood elicits the secretion of proteolytic enzymes and the formation of a peritrophic membrane (PM) which surrounds the meal. The parasites multiply extensively during the blood digestion and the amastigotes change to promastigotes which exit from the PM. Afterwards, in the medium of the sugar meals, they spread to the cardia and many of them attach their flagella between the microvilli, or to the cuticular lining of the cardiac valve. Further transformations then lead to the formation of short haptomonads and infective promastigotes which are characteristic for mature infections (Killick-Kendrick, 1979; Molyneux & Killick-Kendrick, 1987; Walters, 1993; Schlein, 1993).

It has been shown several times that *Leishmania* infections often fail at the early stage in sandflies other than the natural vector (summarized by Killick-Kendrick, 1985). These observations led to studies of the reasons for the vector specificity: Adler (1938) observed that the plasma in the artificial infective bloodmeals of *Phlebotomus papatasi* prevented the growth of *L. tropica* infections. Later studies indicated that the high levels of the proteolytic enzymes in the gut of *P. papatasi* are apparently a barrier for infection since they were lowered by the natural *L. major* parasites (Schlein & Romano, 1986; Borovsky & Schlein, 1987). Alternatively, it was suggested that the persistence of infections depends on the parasites binding by their surface lypophosphoglycan (LPG) to the gut cell microvilli (Pimenta *et al*. 1994).

In the present study we also investigated the reasons for the vector specificity and correlated the survival of *L. donovani* infections in *P. papatasi* with the proteolytic activities in the sandfly guts.

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MATERIALS AND METHODS

Cells

Leishmania donovani (MHOM/SD/??/Khartoum) parasites were originally obtained from Dr J. Keithly, Cornell Medical School, Ithaca, NY. Parasites were routinely isolated from Balb/c mice infected i.v. with 10^7 stationary-phase promastigotes. Amastigotes were purified from liver and spleen tissue 6 weeks post-infection, according to the method described by Glaser *et al*. (1990). Axenic amastigotes were produced by culturing at 37 °C in a 95% air:5% CO_2 humid chamber in RPMI–MES medium as described by Joshi, Dwyer & Nakhashi (1993). Amastigote viability was tested by the erythrosin B exclusion test (Jaffe, Grimaldi & McMahon-Pratt, 1984). Promastigotes were

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routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose content (Biological Industries, Beth Haemek, Israel) supplemented with 10 % (v/v) foetal calf serum (FCS), 4 mM L-glutamine, streptomycin at 200 μg/ml, and penicillin at 200 I.U./ml (Teva, Jerusalem).

Experiments with amastigotes in culture

Cultures were initiated with 3×10^4/ml viable, tissue-derived amastigotes and grown in DMEM with 20 % of one of the following additives: defibrinated and inactivated (at 56 °C for 30 min) whole rabbit blood; inactivated rabbit plasma; rabbit erythrocytes washed 3 times in PBS (pH 7·2) and resuspended 1:1 in saline. The cultures were maintained at 28 °C, daily samples were taken, diluted with 0·01 % saponin in 168 mM ammonium chloride and the parasites were counted in quadruplicate. The experiments were repeated twice.

Sandfly infective meals and the assessment of infections

Experimental *P. papatasi*, 2–4 days old, were from a colony that had been started with flies from Kfar Adumim (10 km east of Jerusalem). Maintenance of the colony and method of artificial infection were as described by Schlein *et al.* (1983). Infective meals consisted of 1×10^6 amastigotes or promastigotes/ml (100–200 parasites/fly meal) in different media including: defibrinated rabbit blood with or without 0·025 % soybean trypsin inhibitor (Sigma, USA); heparinized rabbit blood (166 U/ml) or washed, packed rabbit erythrocytes diluted 1:1 in saline. After the infective meal, the sandflies were maintained on 20 % sucrose and water at a temperature of 24 ± 1 °C and 80 % RH. The guts of the flies were dissected in saline and examined under a phase-contrast microscope at different times up to 10 days post-infection. The number of parasites in the sandflies was scored from 0 (no parasites seen in the gut or cardia); I = 1–10; II = > 10–100; III = > 100–1000; IV = > 1000. Infections in the abdominal midgut and the cardia were scored separately. Experiments were repeated at least 3 times to produce sufficient sample populations. Parasite mortality was estimated by the proportions of cells with disintegrating organelles in sandfly gut smears stained with Giemsa's stain. Such preparations were also used to define the morphotypes of the parasites at different infection stages. Fly samples of the series that had been fed on promastigotes and erythrocytes in saline, were fixed with Bouins' fluid 6 or 10 days post-infection. They were embedded in paraffin, cut in sagittal serial sections and stained in Mayer's haemalum according to Culling, Allison & Barr (1985).

Assays for proteolytic enzymes

Proteolytic enzyme assays were carried out with series of 2 to 4-day-old sandflies that had been fed through membranes on one of the following diets: defibrinated and inactivated rabbit blood, heparinized rabbit blood or washed rabbit erythrocytes diluted 1:1 in saline. The protein content of defibrinated blood and erythrocytes in saline was determined by the method of Bradford (1976). After feeding, the sandflies were maintained on water only. Batches of 10 flies were taken from each series at specific time-intervals and immediately frozen at −70 °C. The fly samples were homogenized (10/100 μl) in the enzyme buffer (50 mM Tris–HCl, 20 mM $CaCl_2$, pH 8·2) in a mini-homogenizer (Jencons, Hemel Hempstead) and the homogenates were centrifuged for 10 min at 12000 ***g***. The substrates used in the assays for specific enzyme activity were the synthetic peptides benzoyl-arginine-*p*-nitroanilide (BApNA) for trypsin and leucine-*p*-nitroaniline (LpNA) for aminopeptidase (Erlanger, Kokovsky & Cohen, 1961; Gooding & Rolseth, 1976; Dillon & Lane, 1993*a*). Reaction mixtures were either 2 mM BApNA in alkaline buffer (7 % dimethylformamide in 50 mM Tris–HCl, 20 mM $CaCl_2$, pH 8·2) or 2 mM LpNA (3 % dimethylsulfoxide in 50 mM Tris–HCl, 20 mM $CaCl_2$, pH 8·2) that were freshly prepared. In these assays, 10 μl of enzyme preparation mixed with 100 μl of reaction mixture and 90 μl of enzyme buffer were incubated for 20 min at room temperature. The reaction was stopped and the interference of the haemoglobin was avoided by adding 50 μl of 30 % glacial acetic acid. The mixture was then centrifuged for 5 min at 12000 ***g***, the supernatant (200 μl) was transferred to a 96-well microtitre plate and the reactions read at $\lambda = 405$ nm in a Titertek® Multiscan ELISA buffer. Enzyme buffer was substituted for the homogenate as a control. The change in optical density was converted to μmols of substrate hydrolysed by reference to a standard curve of *p*-nitroanaline. One enzyme unit of activity (U) is the amount of enzyme required to hydrolyse 1 μmol of substrate/min under the conditions of the assay. The substrates azocasein (4 mg/ml) and azoalbumin (15 mg/ml) in Tris–HCl buffer, pH 8·4, were used to assay for general alkaline protease activity (Tomarelli, Charney & Harding, 1949; Dillon & Lane, 1993*a*). The reaction mixture that contained 10 μl of enzyme in 115 μl of Tris–HCl buffer and 125 μl of substrate was incubated for 2 h at 37 °C, and the reaction stopped with an equal volume of 10 % (w/v) cold trichloroacetic acid. After 15 min the preparations were centrifuged at 12000 ***g*** for 5 min to remove the precipitate. The clear supernatants (200 μl) were transferred to microtitre plates and the reactions read as described above. Enzyme buffer was substituted for the homogenate as a

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Table 1. The development of *Leishmania donovani* infections in *Phlebotomus papatasi* following feeding on different infective meals

Infective meal	Days post-infection	Number of flies with different infection levels*					Proportion and (%) infected
		0	I	II	III	IV	
Promastigotes in defibrinated blood	3	95	5	0	0	0	5/100 (5·0)
Promastigotes in heparinized blood	2	8	2	10	13	2	27/35 (77·1)
	3	30	3	8	8	3	22/52 (42·3)
	6, 7	73	0	2	5	1	8/81 (9·9)
Promastigotes in blood with trypsin inhibitor	3	7	2	9	18	37	66/73 (90·4)
	6	46	1	3	3	9	16/62 (25·8)
Promastigotes and erythrocytes in saline	2	1	1	7	9	1	19/20 (95·0)
	3	11	2	5	8	8	23/34 (67·6)
	4	10	2	2	8	9	21/31 (67·4)
	6, 7	27	9	16	24	15	64/91 (70·3)
	10	18	2	6	8	14	30/48 (62·5)
Amastigotes and erythrocytes in saline	2	6	3	8	9	17	37/43 (86·0)
	6	30	1	3	6	14	24/54 (44·4)

* Infection levels, parasites/sandfly gut: I = < 10; II = 10–100; III = 100–1000; IV = < 1000.

control. All assays were carried out in duplicate, repeated with 2 samples including at least 20 flies, and the data presented are the averages of the combined results.

Statistical analysis

The data from the sandfly infection experiments were analysed by arcsine transformation of proportional data and using Fisher's exact test for significant differences, χ^2 and McNemar's test for light (classes I and II) and heavy (classes III and IV) infection level differences (Zar, 1984). The total enzymatic activity was calculated from the area under the curve of the different time-points using the GraphPad Prism program version 2.0.

RESULTS

The development of infections in different fly series

Three days after a meal of defibrinated blood and promastigotes, only 5/100 of the flies retained a few parasites and in 2 of them the parasites were dead. The use of heparinized blood for infective meals promoted the survival rate to 77·1, 42·3 and 9·9 % in in days 2, 3 and 6–7 post-infection respectively. The survival of infections was also increased by addition of soybean trypsin inhibitor to the sandfly meal. Parasites were found in 90·4 % of the flies 3 days post-infection; however, estimated mortality of > 50 % was observed in 31/73 of the infected flies. The infection rate in this series was 25·8 % three days later (Table 1). Infective meals of erythrocytes and saline permitted the establishment of infections in a high proportion of flies (Table 1). The infection rate in flies that had been fed promastigotes was 95·0 % two days later and between days 3 and 10 it ranged between 70·3 and 62·5 %. After similar infection with amastigotes 86·0 % of the flies contained parasites on day 2 but, as observed in stained preparations, many of them were dead. Estimated mortality of > 50 % was observed in 18/43 of the infected flies. Infection rate of 44·4 % was found in this series 6 days post-infection.

The course of infections

In successful infections the parasites multiplied and changed to long nectomonads which were the dominant form towards the end of the blood digestion in most of the flies. After the exit of the parasites from the peritrophic membrane, they generally migrated to the cardia and a large proportion of them were attached by their flagella to the epithelial cells. The parasites remained in the abdominal midgut only in 12 of the lightly infected flies (classes I and II, Table 1). Parasite attachment to the cuticular cover of the cardiac valve and significant degeneration of the valve were seen in only a few of the flies in the histological preparations. Various short haptomonad forms and typical infective promastigotes were found in gut smears of some infected flies 6 days post-infection and they were common in most of them 4 days later. In most of the infected flies, a lump of a gel-like matrix entwined with parasites, was formed at that time in the anterior part of the cardia.

Proteolytic activities in the gut of sandflies fed on different meals

Following feeding on defibrinated blood (control) the alkaline protease activity in the flies rose to a

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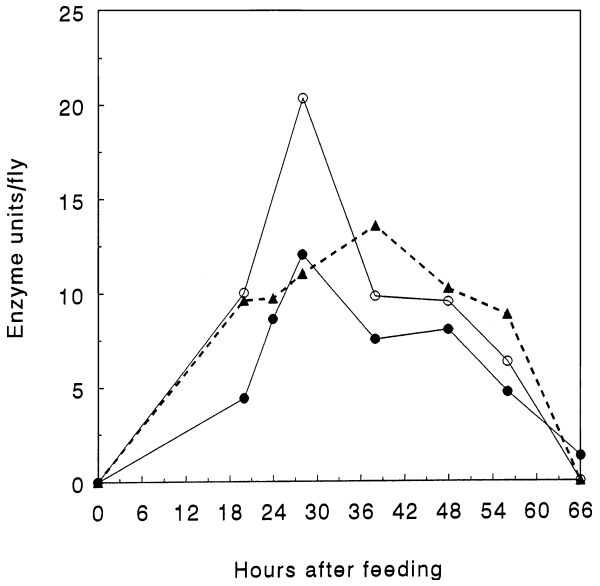

Fig. 1. Alkaline protease activity (as measured with azocasein) of *Phlebotomus papatasi* after feeding on the following meals: defibrinated whole blood (○); washed erythrocytes in saline (▲); heparinized blood (●); (n = 20 flies for each time-point).

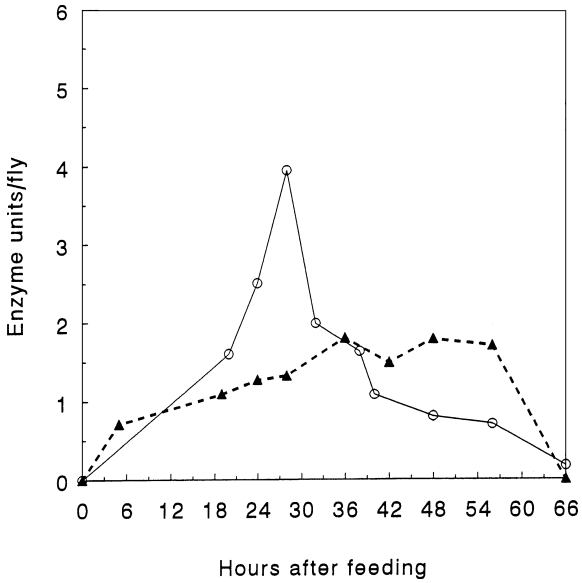

Fig. 2. Trypsin activity (as measured with BApNA) of *Phlebotomus papatasi* after feeding on the following meals: defibrinated whole blood (○); washed erythrocytes in saline (▲); (n = 20 flies for each time-point).

peak of 20·3 U/fly on the 28th hour (h) and decreased towards the 66th h. A similar synchrony at a lower level, with a peak of 10·0 U/fly was recorded after feeding on heparinized blood. The calculated total activity of the control was 646·6 U/fly and in the other group it was 371·5 U/fly. In flies fed on erythrocytes in saline the peak alkaline protease activity (13·5 U/fly) was also lower than that of controls, it was delayed to the 38th h, and the total activity was 537 U/fly (Fig. 1). The trypsin activity induced by control meals rose to a peak of 3·9 U/fly

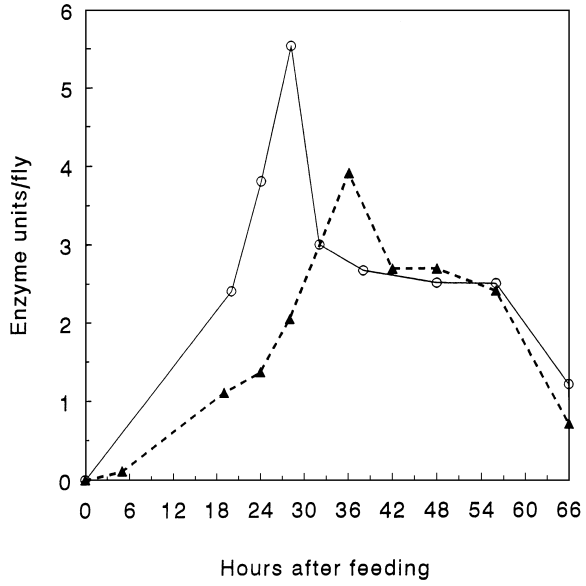

Fig. 3. Aminopeptidase activity (as measured with LpNA) of *Phlebotomus papatasi* after feeding on the following meals: defibrinated whole blood (○); washed erythrocytes in saline (▲); (n = 20 flies for each time-point).

28 h after feeding, decreased to 2·0 U/fly at 51 h and phased out by the 66th h. After meals of erythrocytes in saline this activity reached its highest levels, below 2·0 U/fly, between the 36th to the 56th h (Fig. 2). The total trypsin activity was 80·1 U/fly whereas in the control it was 80·4 U/fly.

Aminopeptidase activity after control bloodmeals also reached its peak after 28 h and rose to 5·5 U/fly. It decreased to a level of about 2·6 U/fly between the 33rd and the 56th h and then continued to decline. This activity was also postponed and lowered to a peak of 3·9 U/fly after 36 h in flies that had been fed on erythrocytes in saline (Fig. 3). The total activity of 117·1 U/fly in this group was lower than the 153·2 U/fly in controls. The mean protein content of inactivated rabbit blood was $254 \pm 15{\cdot}2$ mg/ml and that of erythrocytes in saline was $191{\cdot}7 \pm 16{\cdot}2$ mg/ml.

The transformation of amastigotes in different media

In medium with whole blood or with plasma there was no transformation in the first day and on day 2 there were $1{\cdot}5 \times 10^3$ and 2×10^3 promastigotes/ml respectively. Whereas, after 24 h there were already 8×10^3 promastigotes/ml in medium with erythrocytes. The highest number of promastigotes within a week (1×10^7/ml) was in the medium with erythrocytes and in the other media it was 5×10^6/ml or less. The amastigotes decreased from 3×10^4/ml to 1×10^3/ml on day 6, in medium with blood or erythrocytes and on day 7 with plasma (Fig. 4A–C).

Statistical analyses

There were significant differences in the proportions of infected flies (PI) and heavy infections (PH)

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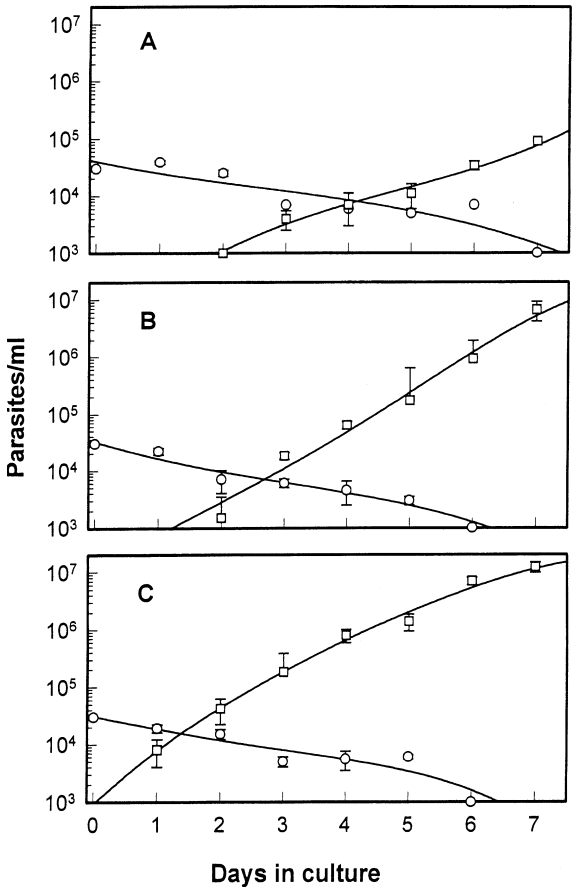

Fig. 4. The decrease in amastigotes (○) and growth of promastigotes (□) of *Leishmania donovani* (Khartoum strain) in DMEM culture medium supplemented with (A) 20% rabbit plasma; (B) 20% defibrinated rabbit blood or (C) 20% washed erythrocytes in saline (average ± S.E.).

(Table 1, classes I, II versus III, IV) following the various infective meals: defibrinated blood and promastigotes (A); heparinized blood and promastigotes (B); defibrinated blood, trypsin inhibitor and promastigotes (C); erythrocytes, saline and promastigotes (D) or amastigotes (E).

Day 3 post-infection: series A compared to the other series PI $-P < 0{\cdot}001$, PH $-\chi^2 = 21{\cdot}7$, $P < 0{\cdot}001$. Series B compared to C, PI $-P < 0{\cdot}001$, PH $-\chi^2 = 8{\cdot}08$, $P = 0{\cdot}004$. Day 6 post-infection: Series B compared to C, PI $-P = 0{\cdot}038$, PH $-\chi^2 = 5{\cdot}77$, $P = 0{\cdot}016$. D compared to B, PI $-P = 0{\cdot}001$, PH $-\chi^2 = 31{\cdot}5$, $P < 0{\cdot}001$, compared to C, PI $-P = 0{\cdot}012$, PH $-\chi^2 = 26{\cdot}8$, $P = 0{\cdot}001$ and compared to E, PI $-P = 0{\cdot}002$, PH $-\chi^2 = 18{\cdot}02$, $P = 0{\cdot}001$.

DISCUSSION

The conditions which permit the development of *Leishmania* infections in a sandfly vector were investigated in this study. The experiments were carried out with a Sudanese strain of *L. donovani*, which is one of the *Leishmania* parasites that fail to grow in *P. papatasi* (Adler, 1947; Heyneman, 1963; Killick-Kendrick, 1985). A main criterion for the fitness of a *Leishmania* to a vector is the persistence of the parasites after the digestion of the infective bloodmeal (Killick-Kendrick, 1979). In our experiments this process was completed during the 4th day and the infections in the flies were monitored before and after this time.

The basic experiment exhibited the resistance of *P. papatasi* to the parasites. The flies were infected with promastigotes in defibrinated whole blood and only 5% of them harboured a few survivors after 3 days. Three changes in the composition of the infective bloodmeal drastically increased the initial infection rates and permitted the persistence of infections after the digestion of the meal. On days 6 and 7, the infection rate was 9·9% following meals containing heparinized, instead of defibrinated blood and it was further raised to 25·8% by the addition of soybean trypsin inhibitor. The highest infection rate resulted from the replacement of the plasma by saline. Amastigotes ingested with this medium produced an infection rate of 44·4% on day 6. While the proportion of promastigote-infected flies was 70·3% on day 6 and remained similar until day 10. This is almost as high as the 78·5% (139/177) infection rate of *P. papatasi* with the natural *L. major* parasites that was observed on day 7 after meals of promastigotes in rabbit blood (Schlein & Jacobson, 1996).

The high rate of early infections and the high numbers of parasites in these fly series indicated an initial, extensive parasite multiplication. However, a high proportion of the recorded parasites were dead, as observed for both the heparinized-blood and the amastigotes-infected series. This death of parasites during the blood digestion accounts for the decrease in the proportion of infected flies that was recorded later.

At the early phase of infection *Leishmania* parasites encounter the proteolytic gut enzymes which are secreted in response to the ingestion of blood (Dillon & Lane, 1993*a*). In *P. papatasi* infected with the vector-specific *L. major*, the peak of the proteolytic enzymes was delayed (Schlein & Romano, 1986; Borovsky & Schlein, 1987) or significantly decreased (Dillon & Lane, 1993*b*). Since infection with *L. donovani* did not have a similar effect it has been suggested that the manipulation of the enzymes by *L. major* affords protection for the parasites (Schlein & Romano, 1986; Borovsky & Schlein, 1987). We therefore monitored the levels of these enzymes to see whether they are influenced by the experimental sandfly meals which increased the infection rates.

The inhibitory effect of heparin on mammalian serine proteases (Wu, Sheffield & Blajchman, 1994; Finotti & Manente, 1994) was also manifested in the sandflies. After meals of heparinized blood, the levels of the alkaline proteases at different times had a similar pattern, but was generally lower than that

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which followed control meals of defibrinated blood. The total decrease of the activity by the heparin was 30·7 %. Compared to the controls, meals of erythrocytes and saline delayed the rise of the alkaline proteases, trypsin and aminopeptidase activities by 8 to 10 h and decreased their maximal levels by 39·4, 53·8 and 35·0 % respectively. These meals were also followed by lower total amounts of alkaline proteases (17·0 %) and aminopeptidase (23·5 %). The reduction may have resulted from the 25 % decrease in the protein content and the lack of plasma albumin in the feeding solution (Lehane, Müller & Crisanti, 1996).

Altogether the experiments show that the different sandfly meals, including those with trypsin inhibitor, had a common effect on both the proteolytic enzymes and the survival of *L. donovani* in *P. papatasi*. These results imply that in natural conditions the proteolytic enzymes can determine the vector specificity of sandflies to *Leishmania*.

Whole blood, plasma or erythrocytes were added to cultures started with *L. donovani* amastigotes to observe their effects on the rate of promastigote formation and multiplication. In medium with whole blood or plasma only 7·4 % of the parasites were promastigotes after 48 h, while with erythrocytes, 73 % of the parasite population were promastigotes by this time. We presume that the lack of plasma in the infective meals of sandflies also accelerated the transformation of amastigotes and allowed the evasion of the rising proteolytic activity.

The observed events in the development of the infections were in agreement with the general descriptions of the *Leishmania* cycle (Killick-Kendrick, 1979; Molyneux & Killick-Kendrick, 1987) and to the growth of *L. major* in its specific vector *P. papatasi* (Warburg *et al.* 1986). Many of the parasites were attached to the microvilli of the epithelial gut cells in the histological sections. However, they were seldom seen attached to the cuticular lining of the cardiac valve and in most cases the valve appeared to be undamaged. The cardiac valve of *L. major*-infected *P. papatasi* atrophies subsequent to the attachment of the parasites (Schlein, Jacobson & Messer, 1992).

The specificity of sandfly vectors was recently studied by Pimenta *et al.* (1994) who used heparinized blood to infect *P. papatasi* and *P. argentipes* with their natural parasites *L. major* and *L. donovani* and with other *Leishmania* species or strains. Shortly after infection these parasites were found in a high proportion of sandflies, in all the series of both species. After the bloodmeals had been digested, most of the infections, except for those of *L. major*, were eliminated from the *P. papatasi*, while they generally remained in *P. argentipes*. Other *in vitro* experiments with the various promastigotes or their LPG, showed that they were indiscriminately bound to dissected guts of *P. argentipes*, and the affinity to *P. papatasi* guts was specific for *L. major*. Pimenta *et al.* (1994) therefore suggested that the attachment of the parasites to the epithelial cells prevents their excretion and sustains the infections.

Our results are in disagreement with this interpretation. We have shown that a high rate (up to 70·3 %) of late-stage *L. donovani* infections could persist in *P. papatasi*. The crucial period for the success of the infections was during blood digestion. Afterwards the attachment of *L. donovani* promastigotes to the epithelial gut cells was indistinguishable from that of *L. major*. Attachment defined as normal was also recorded for the infections of Indian *L. donovani*, *L. infantum* (Adler & Theodor, 1927, 1930) and *L. tropica* (Killick-Kendrick, Killick-Kendrick & Tang, 1994) which managed to succeed in *P. papatasi*.

Changes in the sandfly infection rates, that were caused in our study by different, artificial infective meals, were also observed in conditions that were closer to the situation in nature. It is plausible that the variations in the vector susceptibility in these experiments were also correlated to the proteolytic activity in the sandfly gut. The infective meals of different mammalian blood that modulated the *L. major* infections in *P. papatasi* (Schlein & Jacobson, 1996) may have had different effects on the secretion of these enzymes (Lehane, 1991). It was also observed that the infection rate of *Lutzomyia longipalpis* with *L. mexicana amazonensis* was drastically increased when the ambient temperature was lowered from 28 to 25 or 22 °C (Leaney, unpublished observations). This increase may have been in reverse correlation with the proteolytic activity since the metabolic processes of insects, including enzyme secretion, are temperature dependent (Chapman, 1984).

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