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Does *Leishmania infantum* Use Degenerating Midgut Cells of The Vector as A Nutrient Source?

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ABSTRACT

Ultrastructural evidence from *Phlebotomus langeroni* experimentally infected with *Leishmania infantum* was found from day 4 postinfection to support the hypothesis that shed midgut cells act as an important nutrient source for the parasite. Thirteen areas of degenerating cells occurred in 12 sections of infected flies compared to none in complementary controls 8 days post-infection. Parasites penetrated degenerating cells in 7 of 30 flies sectioned (days 8-11 post-infection), and were frequently found packed around shed cells in the gut lumen. No melanisation was observed, suggesting that although higher cell turnover was probably induced by parasites they did not destroy intact cells.

INTRODUCTION

Much attention has been given to the development of *Leishmania* in the gut of their sand fly vectors, but little attention has been given to the nutrient source for parasites after the bloodmeal has been digested. *Leishmania* develop entirely within the gut lumen of their sand fly vectors during the adult stage of the life cycle. Immediately after ingestion in a bloodmeal the amastigotes transform into promastigotes and rapid multiplication takes place. After rupture of the peritrophic matrix the remains of the digested bloodmeal are voided and suprapylarian *Leishmania* parasites attach to the microvilli of the midgut (Pimenta *et al.*, 1992). The attached parasites transform into metacyclics which are released and move freely in the lumen before transmission to a new host by bite. For the period following digestion of the bloodmeal, there is no obvious source of nutrients except the sugar meal of sand flies. It is well known that sugar meals are essential for the development of mature infections in sand flies (Shortt *et al.*, 1931; Molyneux and Kellick-Kendrick, 1987) but it is unclear whether this carbohydrate source is a nutrient source alone or contributes to the process of metacyclogenesis. It would be necessary to demonstrate the survival of the parasite beyond the bloodmeal passage.

Like most metazoans, insects have a turnover of the epithelium cells lining the gut (Fain-Maurel *et al.*, 1973; Thomas and Gouranton, 1973). This has been studied in another group of bloodsucking insects such as mosquitoes (Weaver and Scott, 1990) but not for sand flies.

In sand flies, the only study of the quantitative histological changes associated with bloodmeal digestion is that of Rudin and Hecker (1982) but they did not examine the guts of infected sand flies or measure cell turnover.

The parasites are not passive within the sand fly gut, they are able to modulate the sand fly-derived protease activity of bloodmeal digestion (Dillon and Lane, 1993; Pruzinova *et al.*, 2015) and even modify the insect's behavior (Beach *et al.*, 1985).

The objective of this study is to test the hypothesis that parasite growth in the crucial period following bloodmeal digestion utilizes senescent cells released into the gut lumen. This was achieved by ultrastructural examination of infected and uninfected sand flies at fixed periods post-bloodmeal using a natural vector-parasite combination *Phlebotomus langeroni* and *Leishmania infantum*.

MATERIALS AND METHODS

Insect and parasite cultures:

Phlebotomus langeroni (origin; El-Agamy, Alexandria, Egypt) was reared in the laboratory according to the technique described by Schmidt (1964).

Leishmania infantum (MCAN/EG/87/RTC-3, LEM-1213 MON-98) originally isolated from a dog in El Agamy, was grown in NNN medium.

Infection of sand flies:

Flies (5 days old) were infected by membrane (chick-skin) feeding on fresh defibrinated rabbit blood containing parasites to a final concentration of 2×10^6 /ml. Fully engorged females were maintained with males at 24-26°C, at 60-70%rh and given access to 30% sucrose solution.

Electron microscopy:

Females were dissected and examined for infection daily from 3-10 days post-infection (d. p. i.). Suitable guts attached to the head were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C overnight then washed in the same buffer for 20h. Post fixation was in 1% osmium tetroxide in cacodylate buffer at 4%

for 1.5h, after rinsing in distilled water followed by staining in 0.5% uranyl acetate at room temperature (22°C) for 30 min. The fixed guts were dehydrated in an ethanol/propylene oxide series, followed by embedding in fresh TAAB resin overnight at room temperature. Blocks were polymerized at 60°C for 12-24h. Sections stained with 0.1% lead citrate were examined with a JEOL CX100 Trans-mission electron microscope at 80KV. The midguts from uninfected control insects maintained under the same conditions were also examined using the same procedure.

RESULTS

Only sand flies with a large number of parasites, when examined by TEM, were included in the infected group. Parasite burdens in these specimens were typical of those seen in the field, i.e. naturally infected. Flies exposed to an infected bloodmeal but found to be without parasites after dissection were rejected. Degenerating cells in the midgut epithelium were readily recognized by their electron lucid and highly vacuolated appearance and general intracellular disorganization. In the later stages of this process, either the cells were, ejected, into the gut lumen (Fig. 1) or the cell membrane was disintegrated completely, releasing the cell contents into the gut lumen (Fig. 2a). In many cases it appeared that the microvilli were lost from the disintegrating midgut cells. Even at day 4 p.i., parasites were seen inside degenerating midgut epithelial cells, with flagella ramifying the sand fly cells and numerous parasites within the cell envelope (Fig. 2 a, b, c). An important feature of these inclusions is the absence of an epithelial cell membrane surrounding the parasite, indicating that the parasite is moving within the compromised cell. This observation excludes the possibility that the parasites are wrapped in convoluted cell membranes. We did not observe any difference in the ultrastructure of promastigotes within the degenerating cells compared to those in the gut lumen indicating that there is no loss of structural

integrity in the intracellular parasites. In the gut lumen the sloughed cells were the centre of what can only be described as a feeding frenzy, of parasites (Fig. 2 a, b, c).

Of 30 infected females examined between 8 and 11 days p.i., 7 contained numerous areas with parasites in degenerating epithelial cells. Given that only 6 thin sections were examined from each infected sand fly, this is a very high proportion exhibiting direct evidence for parasites in degenerating midgut cells. For the remaining 23 specimens it cannot be assumed that there was no involvement of parasites with senescent cells, even though a large number of sections was processed in this study, six sections is nevertheless only a small subsample of the total midgut.

In several sections of heavy infections, numerous flagella were packed into the base of folds in the gut epithelium close to the basement membrane (basal labyrinth). It was noticeable that the cells in this location were devoid of microvilli compared to uninfected controls (Figs. 3 & 4). In some cases bundles of flagella were seen within the basement labyrinth of disrupted cells (Fig. 5).

When infected flies were compared to uninfected controls at the same period post bloodmeal, there was a noticeable increase in the number of degenerating cells in the infected group. For example, at day 8, in 12 sections (2 flies x 6 sections) there were a total of 13 areas with degenerating epithelial cells but none in the complementary control sample.

DISCUSSION

In this study we have clearly demonstrated the association of *Leishmania* and degenerating cells of the midgut in the period after digestion of the bloodmeal. This is based on both the presence of parasites and their flagella in midgut cells as well as the increased frequency of cell death in infected flies compared to uninfected flies.

The relatively large number of flies examined and the use of uninfected controls give weight to these observations. Parasites flagella of different lengths were observed

crammed into the base of folds in the gut epithelium and no doubt this affords the parasite a site away from the main stream of the gut lumen. According to Wilson *et al.* (2010) *Leishmania* binding to the sand fly midgut is stage- dependent and is related to those forms found in the middle phase of development, but is absent in the earlier procyclic promastigote stage. Wheeler *et al.*, (2011) in their description of morphological events during the *L. mexicana* cell cycle *in vitro* showed that at division *L. mexicana* promastigotes are first formed with a new flagellum that is significantly shorter than the existing older flagellum, whose length is variable and continues to increase over multiple cell cycles. The regulation of flagellum length (Casanova *et al.*, 2009) and diversity of flagellum function (Gluenz *et al.*, 2010) are interesting areas of *Leishmania* biology.

Two general questions need to be addressed: do parasites directly penetrate cells and cause their death, and can promastigotes survive in the absence of the protein and lipid resources provided in the vector by epithelial cells?

Epithelial cell degeneration in insect guts is a normal process which occurs in the absence of parasites. In the present study midgut cell degeneration pattern was similar to that observed in *Petrobium maritimus* (Fain-Maurel *et al.*, 1973), the cell becomes compressed, distally bulges out into the lumen, and finally, the membrane ruptures and the cell contents discharge into the gut lumen. Cellular turnover was observed in the posterior midgut of the mosquito *Culiseta melanura* 7 days post-bloodmeal by Weaver and Scott (1990). They suggested that a cytoplasmic projection into the gut lumen was part of a process to reduce the excess accumulation of epithelial cell volume associated with the onset of bloodmeal digestion.

Regenerative cells are found in the basal region of the gut epithelium in the sand fly *Lutzomyia longipalpis* (Rudin and Hecker, 1982) but the role of these cells as replacements is unknown.

In our investigation we never saw parasites or their flagella within intact and apparently healthy cells but only in cells showing some signs of internal disruption. Similarly, flagella of *L. braziliensis* were also observed in a degenerating midgut cell of its natural vector *Lutzomyia wellcomei* (Killick-Kendrick *et al.*, 1974). The report of flagella of *L. amazonensis* in the midgut cell of an unnatural vector *Lutzomyia longipalpis* (Molyneux *et al.*, 1975) did not show that parasites had actually invaded healthy cells because the flagella were surrounded by plasma membranes. This indicates that the flagella could have been located in an invagination of the cell membrane.

Moreover we do not have any evidence that parasites break through the basal membrane of the gut and hence into the haemolymph. In some cases we even found bundles of flagella within the basal labyrinth of disrupted cells and opposed to cells of the overlying muscle layer but here there was always a plasma membrane between the flagella and the muscle cells (Fig. 5).

An interesting finding was the absence of any melanisation of parasites or the affected epithelial cells despite the common phenomenon of melanin deposition around parasites and pathogens in the guts of insects. For example, melanisation of the oocyst of *Plasmodium* adjacent to the basal membrane takes place in *Anopheles gambiae* (Collins *et al.*, 1986) and of the fungus *Culicinomyces clavispurus* invading *Culex* larvae (Sweeney *et al.*, 1983). Melanisation is known in sand flies for the entomopathogen *Ascogregarina* in *Lutzomyia longipalpis* (Warburg and Ostrovoska, 1989) and from preliminary data on phenoloxidase activity in *Phlebotomus papatasi* (Dillon, unpublished). The absence of an immune response to *Leishmania* midgut may be due to the lack of recognition of the

parasite, or alternatively, cell death prior to parasite scavenging does not activate the phenoloxidase cascade. The lack of melanisation supports the hypothesis that the cell is already dead before parasites enter. Whether parasites can survive in the gut without shed epithelial cells as a nutrient source is not clear. Sand flies take sugar meals between bloodmeals and this is important in the development of mature infections. However, the optimum carbon source for, log phase, promastigotes cultivated *in vitro* are amino acids, (Mukkada, 1985) rather than carbohydrates.

Nutrient depletion has been cited as a trigger for differentiation to the metacyclic form (Sacks and Perkins, 1985), but there is no direct evidence that depletion is required for differentiation (Howard *et al.*, 1987). Indeed, metacyclic promastigotes are partly distinguished by their high motility. The fact that a protein additive to a sugar meal improved *Leishmania major* transmission (Warburg and Schlein, 1986) possibly by increasing the parasite population, suggests that any opportunity for the parasite to exploit alternative nutrient sources might enhance parasite transmission.

This study has shown that *Leishmania* promastigotes are able to exploit degenerating midgut epithelial cells and probably increase the cell turnover to provide themselves with a valuable source of their amino acids, lipids and carbohydrates in the nutrient limited periods between bloodmeals.

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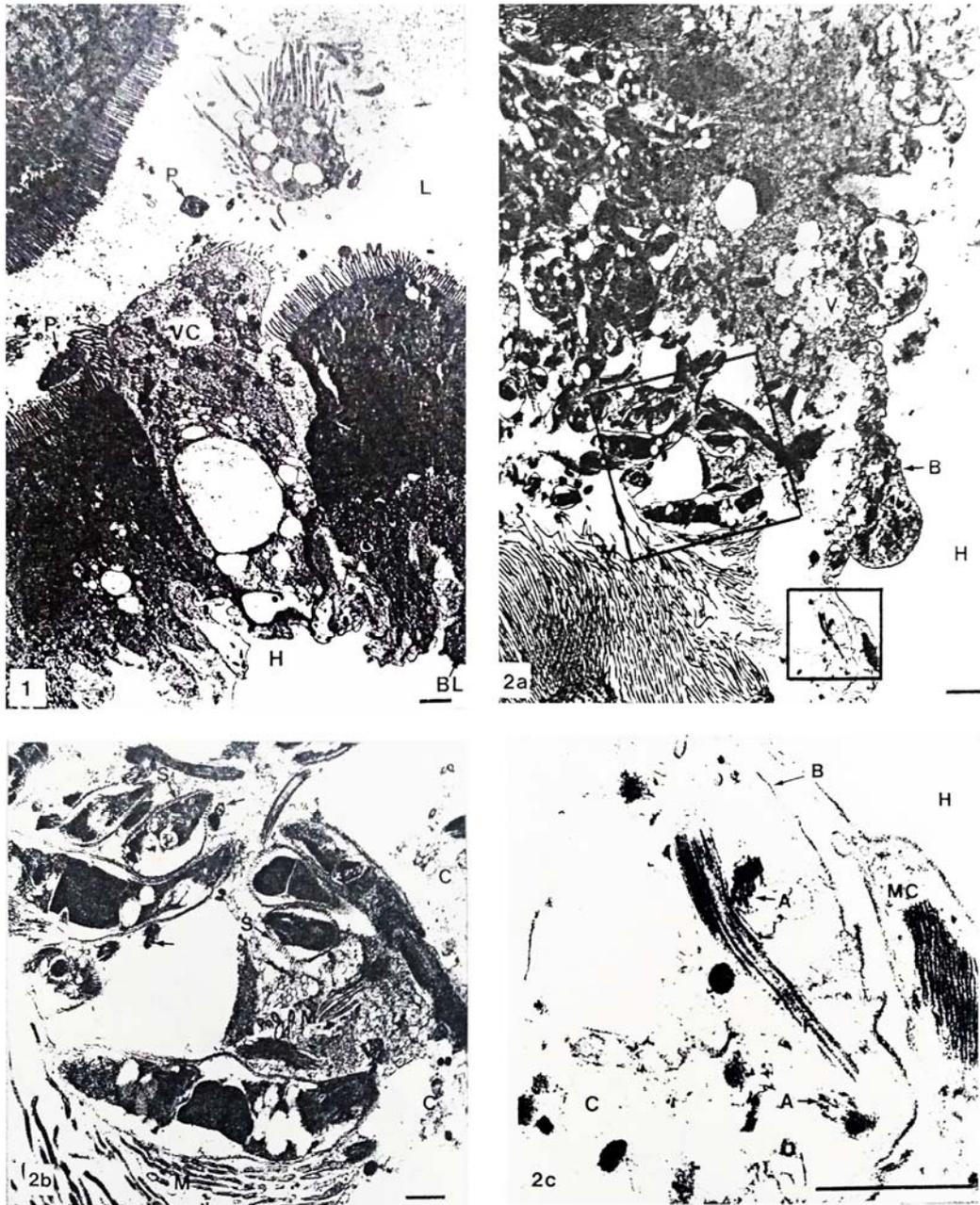


Fig. 1. Transverse TEM section of the midgut of *P. langeroni* 8 days post- infection with *L. infantum*. Vacuolated cell (VC). Parasites (P), Lumen (L), Haemocoel (H), Basal labyrinth (BL).

Fig. 2a. Transverse TEM section of the sand fly midgut 4 days post- infection with *L. infantum*. Disrupted cells with vacuole (V). Intact basal lamina (B).

Fig. 2b. Details of region within the large square on Fig. 2a. Transverse sections of flagella with axoneme are arrowed. Subpellicular microtubules (S) adjacent to parasite membrane. Microvilli (M), Midgut cell cytoplasm (C).

Fig. 2c. Detail of basement membrane of degenerating midgut cell for region within small square on Fig. 2a. flagella (F) ramifying throughout degenerating cell. Axoneme (A), Muscle cell (MC). All magnification bars are equivalent to 1 μm .

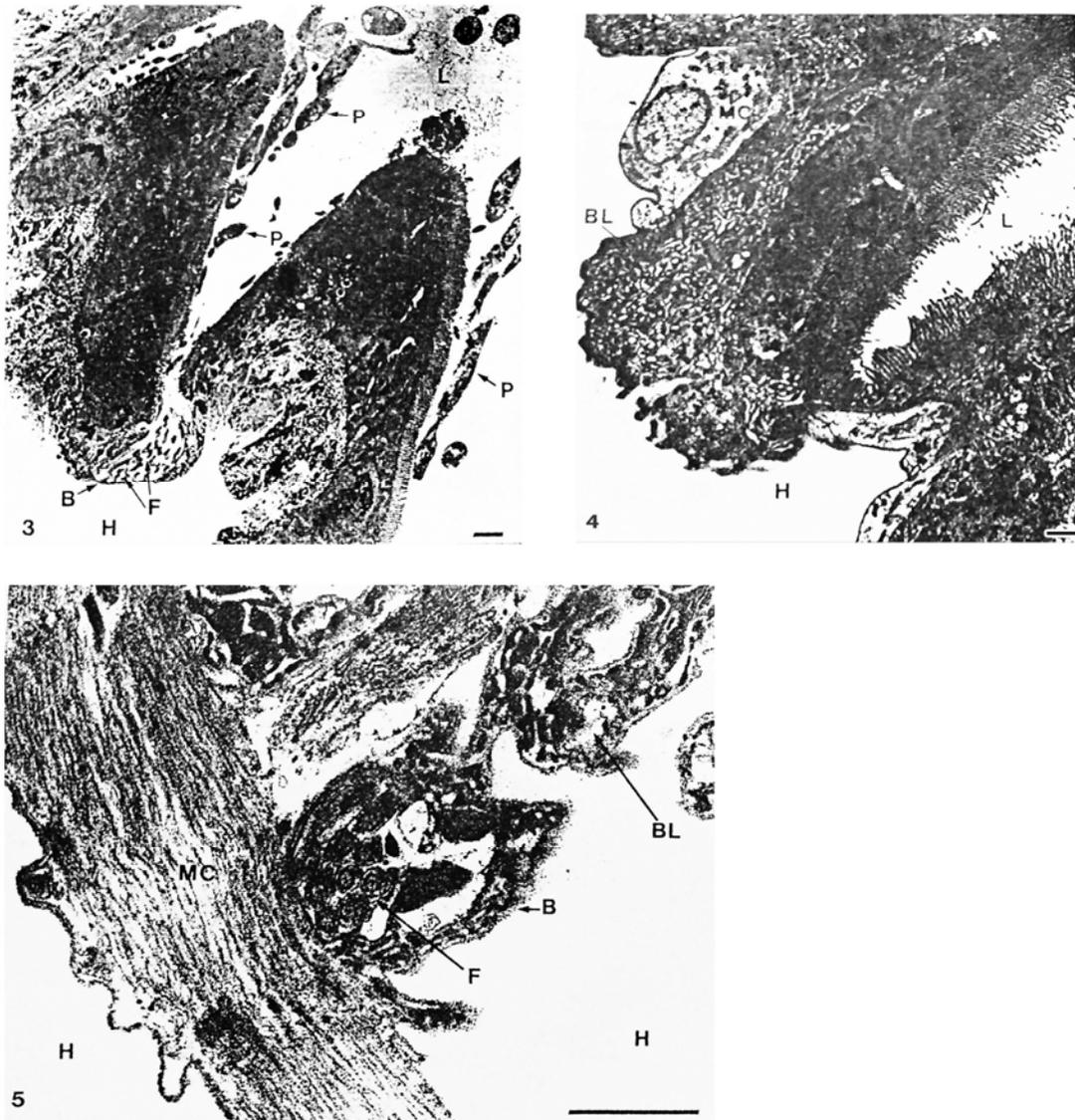


Fig. 3. Section of the midgut 8 days post-infection showing flagella (F) of the *Leishmania* parasites (P) anchored within a fold in the gut epithelium close to the basal lamina (B). Note absence of microvilli.

Fig. 4. Section of the midgut from an uninfected female taken 6 days post-bloodmeal. Note microvilli present within the fold of the gut wall adjacent to the basal labyrinth (BL).

Fig. 5. Transverse section of infected female showing parasites flagella closely applied to each other within the basal labyrinth (BL) adjacent to a muscle cell. All magnification bars equivalent to 1 μ m.

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