AN ULTRASTRUCTURAL STUDY ON THE EARLY DEVELOPMENT OF *LEISHMANIA CHAGASI* (KINETOPLASTIDA: TRYPANOSOMATIDAE) IN ITS VECTOR *LUZOMYIA LONGIPALPIS* (DIPTERA: PSYCHODIDAE)

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SUMMARY

Development of *Leishmania chagasi* in the blood-meal and the midgut of its vector *Lutzomyia longipalpis* was studied by light and transmission electron microscopy. It was demonstrated, for the first time, that macrophages are ingested intact and remain so until, at least, 12 hours after the infective blood-meal. The amastigote and promastigote forms appearing between 12-96 hours

after infection are described. The previously suggested probability of direct transformation of small unchanged amastigotes into metacyclic promastigotes (Lainson and Shaw, 1988) was not confirmed. All amastigotes disappeared from the midgut before the appearance of any metacyclic promastigote.

RÉSUMÉ: Ultrastructure de la phase précoce du développement de *Leishmania chagasi* (Kinetoplastida : Tripanosomatidae) dans son vecteur *Lutzomyia longipalpis* (Diptera : Psychodidae).

Le développement de *Leishmania chagasi* dans le repas sanguin et l'intestin moyen du vecteur *Lutzomyia longipalpis* a été étudié en microscopie optique et électronique. Il a été montré, pour la première fois, que les macrophages sont ingérés intacts et qu'ils restent intacts pendant au moins 12 heures après le repas sanguin. Les formes amastigotes et promastigotes, trouvées entre 12 et

96 heures après l'infection, sont décrites. L'hypothèse proposée par Lainson et Shaw (1988) d'une transformation directe des petits amastigotes originaux en promastigotes métacycliques, n'a pas pu être confirmée par nos observations. Il a été montré que tous les amastigotes disparaissent de l'intestin avant l'apparition des premiers promastigotes métacycliques.

INTRODUCTION

Investigations on the development of *Leishmania* in sandflies are important for elucidation of the transmission mechanism of leishmaniasis and the description of infective parasite form(s) (Killick-Kendrick 1979; Molyneux and Killick-Kendrick, 1987; Killick-Kendrick, 1990). Light and Electron microscopy studies have demonstrated that the forms found in the sandfly are the amastigote, the nectomonad, haptomonad, and metacyclic promastigote and the paramastigote (Killick-Kendrick *et al.*, 1974; Killick-Kendrick, 1979, 1990).

Due to problems with histological preparations, there are, however, no previous reports on the first 24 hours ultrastructural development of *Leishmania* in the midgut of the vector. Light microscopy investigations have shown that in the blood-meal, the amastigote enlarges and divides, at least once, before transforming into a dividing promastigote (Shortt, 1928; Strangeways-Dixon and Lainson, 1966;

and Lainson and Shaw, 1988). Recently, Lainson and Shaw (1988) suggested that the ingested amastigotes of *Leishmania chagasi* take two lines of development in its vector *Lutzomyia longipalpis*. Most amastigotes enlarge, divide and give rise to large promastigotes which fail to divide any further. A smaller number of amastigotes remain unchanged until about 36-43 hours when they transform directly into small metacyclic promastigotes.

In the present paper, we describe results of an ultrastructural and light microscopy investigation on the early (0-96 h) development of *Leishmania chagasi* in the midgut of its vector *Lu. longipalpis*. Particular emphasis was placed on the first 24 hours development of the parasite in the vector and no attempts were made to follow the migration of the parasite to the foregut of the fly.

MATERIALS AND METHODS

The parasite used was a Brazilian strain of *L. chagasi* isolated from a patient with visceral leishmaniasis in Maranhão (MHOM/BR/150406), Brazil, and maintained by intraperitoneal passage in hamsters (*Cricetus auratus*) and cotton rats (*Sigmodon hispidus*). The *Lu. longipalpis* sandflies were from a closed labo-

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ratory colony which originated from females collected from L'Aguila (Tolima, Columbia).

Groups of about three hundred 3-4 days old females were infected by feeding them through a chick-skin membrane (Ward *et al.*, 1978) on a *L. chagasi* infected hamster spleen triturated in Locke's solution and suspended in defibrinated heat-inactivated rabbit blood in the ratio of 1:1 (final amastigote count = 10^5 /ml). The engorged females were then kept, in 20³ cm cage, in an incubator at 26° C, 95 % RH and offered fresh saturated sucrose solution continuously. At 12, 24, 48, 72 and 96 hours after the infective feed, midguts of 13 infected females were dissected and either prepared as Giemsa-stained smears or processed for EM. The Giemsa's-stained midgut smears were prepared as described by Wallace (1979) and the morphological characteristics of the parasites were examined using an Olympus microscope at $\times 1,125$ magnification. Measurements were made with an Olympus camera-lucida, at the same magnification, and calculated as mean $\pm$ SD. Proportions of different morphological forms were estimated by counting the numbers of individuals of each form and all the parasites in 5 microscopic fields (magnification = $\times 1,125$) of Giemsa-smears of 6 separate flies (*i. e.* total of 30 microscopic fields) and calculating the respective percentages as described by Lainson and Shaw (1988).

The EM processing techniques used in this study were based on those of Killick-Kendrick *et al.* (1974) with the following modifications. 1) After post fixation in 1 % osmium tetroxide, the guts were washed in 0.1 M cacodylate buffer and then stained *en bloc* with 2 % (aq.) uranyl acetate (Ryder and Kellenberger, 1958), for 20 minutes. 2) The embedding of the dehydrated specimens was in Spurr's resin (Spurr, 1969) which was then polymerized at 70° C for 9 hours. 3) Ultra-thin sections were cut on a diamond knife at a thickness of 90 nm and picked up on 300 mesh hexagonal thin-bar copper grids. 4) The stained grids were examined in a Phillips (CM10) transmission electron microscope (Philips Analytical, Cambridge, UK) operated at 80 kV. Images were recorded on Ilford EM film (Ilford, Moberley, Cheshire, UK). Guts distended with fresh-blood-meals were processed for EM as described above except for the following modifications. 1) The initial fixation was in 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.4). 2) The dehydration process

in the graded series of ethanol was lengthened by a further 3 hours immersion in absolute ethanol. 3) Embedding was in LR White resin (London Resin Co-Ltd., Basingstoke).

RESULTS

The time of appearance and the proportions of the different morphological forms of *L. chagasi* during the first 72 h of their course of infection in the fly are illustrated in Figure 1.

12 HOURS AFTER THE INFECTIVE FEED

At 12 hours after the infective feed, the midgut lumen of the fly was distended with the blood-meal which appeared as electron dense material consisting of variable size patches (Fig. 2). Distorted and lysed RBCs were abundant in the cellular constituents of the blood and the splenic tissues. The blood-meal appeared to be surrounded by a diffuse peritrophic membrane which was of variable dimensions in different parts of the gut (Fig. 2) and appeared as patches of electron dense material interspaced by pale areas. Between the membrane and the microvillus lining of the gut wall, a peritrophic space was filled by small scattered particles. *L. chagasi* amastigotes were either lying freely between the constituents of the blood-meal (Fig. 2a) or confined within ingested macrophages which were observed to be intact and showed clear large nuclei and phagolysosomes (Fig. 3).

Two amastigote forms were observed. The first, was similar in dimensions and structure to the amastigotes encountered in the vertebrate host and is referred to as amastigote (I) (or unchanged amastigote, Fig. 4A). The

FIG. 1. — Time of appearance and proportions of different morphological forms of *Leishmania chagasi* in the midgut of *Lutzomyia longipalpis*, 0-72 h after an infective feed; AI = ingested amastigotes; AII = enlarged amastigote; PI = promastigote (I); PII = promastigote (II); PIII = promastigote (III) (nectomonad); PIV = promastigote (IV) (haptomonad); total number of parasites = total number/5 microscopic fields for each fly/6 flies = 177 parasites at 12 h, 558 parasites at 24 h, 423 parasites at 48 h and 176 parasites at 72 h.

FIG. 2. — Electron micrograph of a peritrophic membrane of *Lutzomyia longipalpis*, 12 h after a blood-meal infected with *Leishmania chagasi* amastigotes; a = enlarged amastigote; bm = blood meal; ge = midgut epithelial wall; mv = microvilli border of the gut epithelium; pm = peritrophic membrane; ps = peritrophic space.

FIG. 3. — Electron micrograph of a spleen macrophage infected with *L. chagasi* amastigotes lying in the midgut lumen of *Lutzomyia longipalpis*, 12 h after an infective feed; a = a small amastigote; k = amastigote kinetoplast; m = amastigote mitochondrion; n = amastigote nucleus; MN = macrophage nucleus; pv = parasitophorous vacuoles surrounding the amastigotes.

second was enlarged and elongated measuring $3.8 \pm 0.4 \times 1.8 \pm 0.4 \mu\text{m}$ and is referred to as amastigote (II) (or enlarged amastigote, Fig. 4B). The main ultrastructural difference between the two forms was that the enlarged amastigote showed a denser ribosomal content than the small one. In most individuals, the flagellum was situated internally. However, in one amastigote of the small form and a few others of the large form, it extended to the most anterior part of the parasite and protruded a small distance ($0.242 \mu\text{m}$) outside the body.

Whereas amastigote (I) was not observed dividing, a few individuals of amastigote (II) were seen undergoing multi-

plication. It was clear that the division sequence started by production of a second flagellum followed by nuclear fission and then the division of the kinetoplast and the cytoplasm.

The enlarged amastigote form was more abundant ($111/177 = 62.7\%$) than the smaller form ($66/177 = 37.3\%$).

24 HOURS AFTER THE INFECTIVE FEED

At 24 hours after infection the peritrophic membrane appeared more compact and less electron dense than at

FIG. 4. — Ultrastructure of small (unchanged) and enlarged amastigotes (amastigotes (1) and (2) of *Leishmania chagasi* amastigote lying freely between the blood-meal constituents of *Lutzomyia longipalpis*, 12 h after an infective feed: A = amastigote (1); A = amastigote (2); bb = basal bodies; e = endosome (nucleolus); f = flagellum; fcs = flagellum central singlets; fr = flagellar reservoir; k = kinetoplast; m = mitochondrion; n = nucleus; pl = plasma-lemma; r = ribosomes; v = vacuole; sm = subpellicular microtubules.

FIG. 5. — Ultrastructure of the first and the second promastigote forms (promastigote (I) and (II)) of *Leishmania chagasi* in the midgut lumen of *Lutzomyia longipalpis*, 24 h after an infective feed; A = promastigote (1); B = promastigote (2); e = endosome (nucleolus); f = flagellum; fr = flagellar reservoir; k = kinetoplast; m = mitochondrion; n = nucleus; sm = subpellicular microtubules; v = vacuole; note electron lucid cytoplasm in promastigote (1).

12 hours. Some amastigote (II) parasites continued their enlargement reaching $6.1 \pm 0.9 \times 1.7 \pm 0.5 \mu\text{m}$. At this stage, two other forms of the parasite also appeared in the blood-meal. These forms were an oval promastigote measuring $5.5 \pm 1.1 \times 3.1 \pm 0.7 \mu\text{m}$ and referred to as promastigote (I) (Fig. 5A) and a slender promastigote measuring $10.2 \pm 1.5 \times 1.5 \pm 0.2 \mu\text{m}$ and referred to as promastigote (II) (Fig. 5B).

The ultrastructure of the two amastigote forms remained similar to those described for 12 hours after the infective feed. Promastigote (I) was exclusively electron lucid and promastigote (II) included some electron lucid individuals and some highly electron dense parasites.

Although amastigote (II) and promastigote (I) were multiplying actively, none of the amastigote (I) and promasti-

gote (II) individuals was dividing. The sequence of division of the multiplying forms was similar to that observed for amastigote (II) at 12 hours after the infective feed. It appears that, whereas the division of amastigote (II) resulted in more amastigotes, multiplication of some promastigote (I) produced the slender promastigote (II) parasites. From the body sizes, proportions and the anterior position of the kinetoplast in many amastigote (II) parasites, it was clear that this form was transforming into the promastigote (I) form.

48 HOURS AFTER THE INFECTIVE FEED

The blood-meal and the parasites were still surrounded by the peritrophic membrane which started to disintegrate

anteriorly and posteriorly. Most parasites were nectomonads (Killick-Kendrick, 1979) and accounted for 77.8 % (329 out of 423 counted parasites). Other forms present at this time were: promastigote (II) (72 out of 423 = 17.0 %), amastigote (II) (10 out of 423 = 2.4 %), amastigote (I) (2 out of 423 = 0.5 %), and a haptomonad promastigotes (Killick-Kendrick, 1979) (10 out of 423 = 2.4 %). The only dividing forms observed at 48 hours after the infective feed were the promastigote (II) and the nectomonad. The sequence of division of most individuals of both forms started by transverse fission of the nucleus followed by production of a second flagellum and division of the kinetoplast. It seemed that the resulting daughter nuclei were then displaced laterally and the cell divided longitudinally.

72-96 HOURS AFTER THE INFECTIVE FEED

At 72 hours after the infective feed, the proportion of the nectomonads decreased to 54.0 % (95/176) and the haptomonad proportion increased to 35.8 % (63/176) (Fig. 1). The promastigote (II) was still present, although at a low proportion (18/176 = 10.2 %). The peritrophic membrane was broken and the blood-meal was almost digested.

The metacyclic promastigotes started to appear in the midgut at 72-96 hours after the infective feed. This form showed a flagellum length of $15.9 \pm 2.9 \mu\text{m}$, a body length of $7.1 \pm 1.6 \mu\text{m}$ and a body width of $1.0 \pm 0.2 \mu\text{m}$. Its flagellum/body length ratio ranged between 1.8 and 3.3 μm .

DISCUSSION

DEVELOPMENT IN THE INFECTIVE BLOOD-MEAL

The EM processing techniques used has enabled us to observe, for the first time, some previously unknown aspects of the ultrastructural development of *L. chagasi* within the blood-meal of its vector *Lu. longipalpis*. The ingestion of intact macrophages contradicts the assumption that these cells are broken or lysed during the feeding process (Lewis, 1975) and *Leishmania* amastigotes are only taken freely into the midgut of the sandfly (Molyneux and Killick-Kendrick, 1987). These results support observations by Killick-Kendrick (pers. comm.), who noticed many uninfected macrophages in the midgut smears of *Phlebotomus ariasi* which had engorged on a dog infected with *L. infantum*.

The observations on the formation of the peritrophic membrane confirm the reports of Gemetchu (1974), Blackburn *et al.* (1988) and Walters *et al.* (1989). Similarly, the observations on the ultrastructure of the transforming amastigotes in the vector agrees with the findings on cultured parasites (Rudzinska *et al.*, 1964; Pan and Pan, 1986). It, however, seems that in the vector the amastigotes acquire most of the ultrastructural features of the promastigote

by 12 hours after the infective feed, except for the flagellar internal position and the subterminal ending of the subpellicular microtubules.

The presence of two amastigote forms at 12 hours after the infective feed, one enlarged dominant form and one maintaining the size of the ingested parasite supports the previous observations of Lainson and Shaw (1988). As observed by these authors, apart from the sizes, the morphological differences between the two forms appeared to be mainly in the cytoplasmic intensity; the larger form contained more concentrated ribosomes.

The division and transformation of some amastigotes at 12 hours after the infective feed is the earliest recorded time for *Leishmania* parasites. From the scarce number of dividing and transforming amastigotes, it, however, seems that division and transformation only start at this time. The timing of transformation of *Leishmania* parasites in the sandfly is thought to be influenced by many factors, including temperature and enzymatic degradation of a transformation inhibitory factor ingested from the vertebrate host (Brun *et al.*, 1976).

The presence of many enlarged multiplying amastigotes at 24 hours confirms the previous findings (reviewed by Killick-Kendrick, 1990) that the parasite enlarges and divides at least once before transforming into the promastigotes. Similarly, the presence of few dividing individuals showing a rudimentary protruding flagellum indicates simultaneous division and transformations, a result which supports the observations of Walters *et al.* (1989).

Although transformation was observed for both the large and the small forms, division appeared to be a criterion of the large form only. This may indicate that enlargement is a pre-requisite for the multiplication of the amastigote but not, necessarily so, for its transformation.

The observation that the first transformed promastigote to appear in the life cycle was a short oval promastigote (promastigote (I)) supports the report of Walters *et al.* (1989). Similarly, the observations on the division sequence of this promastigote form confirms the previous findings on different *Leishmania* life-cycles (Molyneux and Killick-Kendrick, 1987; Walters *et al.*, 1989b; Killick-Kendrick, 1990). In contrast, the multiplication sequence of the slightly elongated slender promastigote (promastigote (II)) and the nectomonad forms supports the observations of Walters *et al.* (1989) in that for these forms the nuclei divide before the flagella.

DEVELOPMENT OF THE METACYCLIC PROMASTIGOTE

Despite confirmation of the presence of the metacyclic promastigotes in midguts of infected sandflies (Killick-Kendrick, 1990), the precursors of these forms are not known. From the proportions of the different forms obtained in this study, it seems that the metacyclic pro-

mastigote could stem only from the haptomonad and/or the nectomonad form. These observations contradict the hypothesis of Lainson and Shaw (1988) that the metacyclic form may arise by direct transformation of small unchanged amastigotes. It seems unlikely that such direct transformation occurred, in the present study, because all amastigotes appeared to have disappeared from the midgut before any metacyclic promastigotes were observed. An alternative explanation to that of Lainson and Shaw (*loc. cit.*) is that the presence of unchanged amastigotes as late as 48 hours after the infective feed was due to an asynchrony in the development of the parasite.

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