

THE LIFE-CYCLE OF *LEISHMANIA* IN THE SANDFLY WITH SPECIAL REFERENCE TO THE FORM INFECTIVE TO THE VERTEBRATE HOST

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SUMMARY

A review is given of recent advances in studies on the life-cycles of leishmaniae in phlebotomine sandflies including observations on the morphology and attachment of the parasites, putative genetic exchange in *Leishmania*, newly found factors affecting the deve-

lopment of the parasite in the fly, and the characteristics of metacyclic promastigotes related to their preadaptation for life in the vertebrate host.

RÉSUMÉ : Cycle évolutif de *Leishmania* chez le phlébotome. Mise au point sur la forme infestante pour l'hôte vertébré.

L'auteur fait une mise au point sur les progrès récents obtenus dans l'étude du cycle évolutif de leishmanies chez le phlébotome en particulier sur la morphologie et l'attachement du parasite dans le tube digestif, les facteurs récemment découverts modifiant le

développement intra-vectoriel du parasite, les échanges génétiques putatifs chez *Leishmania*, et les caractéristiques des formes promastigotes métacycliques en rapport avec leur pré-adaptation à la survie chez l'hôte vertébré.

INTRODUCTION

Advances in the colonisation of sandflies over the past twenty years led to new findings on the life-cycles of leishmaniae in the sandfly many of which have been summarized by Killick-Kendrick (1979) and Molyneux and Killick-Kendrick (1987). In the present paper, a brief review is given of recent advances with special reference to the form of promastigote now believed to be preadapted for life in the vertebrate host (the so-called metacyclic promastigote).

MORPHOLOGY OF *Leishmania* IN THE SANDFLY

Confirmation that at least some amastigotes ingested by sandflies divide one or more times before transforming into promastigotes comes from a study on the life-cycle of *L. i. chagasi* in *Lu. longipalpis* (Lainson *et al.*, 1987). Dividing amastigotes were seen in stained smears of the infected midguts of sandflies which had fed through a membrane on suspensions of amastigotes 24, 36 and 43 h before dissection. It may be that a mammalian bloodmeal contains

a transformation blocking factor (Brun *et al.*, 1976), and that the change from amastigote to promastigote is inhibited until the factor is degraded by the digestive enzymes of the sandfly.

Promastigotes of varying morphology are the predominant form in the thoracic and abdominal midguts and the stomodaeal valve. Killick-Kendrick *et al.* (1974) adopted the terms nectomonad and haptomonad to describe two forms of promastigotes of *L. amazonensis* seen by the electron microscope in the midguts of experimentally infected *Lu. longipalpis*. They defined nectomonads as long, slender, electron-dense unattached promastigotes and haptomonads as broad, electron-lucid promastigotes with modified flagella capable of producing electron-dense plaques (hemidesmosomes) inside the sheath, associated with attachment to the cuticular intima of the stomodaeal valve. The same terms have been used for similar forms seen in other infections (Warburg *et al.*, 1986; Lawyer *et al.*, 1987; Walters *et al.*, 1987, 1989a, b). The definitions of nectomonads and haptomonads were challenged by Walters *et al.* (1989a) who preferred them simply to denote either free-swimming or attached promastigotes with no consideration of morphology. A separation into nectomonads and haptomonads was not seen in preliminary observations on the ultrastructure of *L. i. infantum* in *P. ariasi* (Killick-Kendrick *et al.*, 1977c). Haptomonads seen by Lawyer *et al.* and Walters *et al.* were sometimes in division, whereas divi-

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ding haptomonads of *L. amazonensis* were not seen in *Lu. longipalpis* (Killick-Kendrick *et al.*, 1974; Molyneux *et al.*, 1975).

Division of promastigotes in sandflies is principally by binary fission. The sequence of events in the division of *L. i. infantum* in *P. ariasi* and *L. i. chagasi* in *Lu. longipalpis* (both natural pairs of parasite and vector) is (i) the production of a daughter flagellum, (ii) division of the nucleus into two, (iii) division of the kinetoplast into two and (iv) separation into two individuals by longitudinal fission beginning at the anterior end (Molyneux and Killick-Kendrick, 1987; Walters *et al.*, 1989b).

In the cuticular-lined parts of the foregut other than the mouthparts (oesophagus, pharynx and cibarium), the commonest morphological form is the paramastigote which differs from the promastigote by having the kinetoplast beside the nucleus. Its shape is oval or round and it is much less motile than the promastigote. Like haptomonads on the stomodaeal valve, paramastigotes attach to the cuticular intima of the foregut of the fly by the tip of the flagellum within which hemidesmosomes are formed (Killick-Kendrick *et al.*, 1977a, 1988; Walters, 1989a, b). Whereas Killick-Kendrick (1979) believed paramastigotes in the foregut did not divide, Walters *et al.* (1989b) interpreted their electronmicrographs of some paramastigotes of *L. i. chagasi* in the foregut of *Lu. longipalpis* as dividing forms. An equally plausible interpretation is that a parasite or a flagellum had been cut across a fold and a similar interpretation is possible with electronmicrographs of paramastigotes of *L. panamensis* in the foregut of *Lu. gomezi* believed by Walters *et al.* (1989a) also to be in division.

Paramastigotes were the principal morphological form of *L. braziliensis* recognised in electronmicrographs of the pylorus and ileum of experimentally infected *Lu. longipalpis* (Killick-Kendrick *et al.*, 1977b). Unlike paramastigotes of other species in the foregut of the sandfly, the hindgut forms of *L. braziliensis* were commonly in division and attachment, by hemidesmosomes, was at any part of the flagellum, not only the tip. Similar studies by Walters *et al.* (1989a) on another species of the Neotropical subgenus *Viannia* Lainson and Shaw, 1987, *L. panamensis*, in *Lu. gomezi*, a sandfly assumed to be a natural vector (see Killick-Kendrick, 1990), showed that the predominant form attached to the intima of the hindgut was not a paramastigote, but a « spatulate-shaped promastigote ». This form was described for the first time in the gut of the sandfly. Paramastigotes were « comparatively scarce » and there was little evidence of proliferation of promastigotes in the hindgut, particularly in the pylorus and ileum. Other unusual features were the presence of nectomonads attached by hemidesmosomes (which is as a contradiction) and a heavy infection of the whole of the hindgut, from pylorus to anus, with the greatest densities of parasites in the

pylorus, ileum and rectum; the last site was not previously considered to be colonised by species of *Viannia*.

The differences in the observations by Walters *et al.* (1989a) and other workers show that further studies of the ultrastructure of parasites of this subgenus in proven vectors are needed before the life-cycles of species of the subgenus *Viannia* can be fully compared with those of the suprapylarian parasites.

ATTACHMENT OF LEISHMANIAE IN THE SANDFLY

With one exception, all accounts of the ultrastructure of leishmaniae in sandflies have shown attachment of promastigotes to the microvilli of the abdominal midgut by the insertion of the flagella of the parasites (Killick-Kendrick *et al.*, 1974; Kaddu & Mutinga, 1981; Molyneux *et al.*, 1986; Lawyer *et al.*, 1987; Walters *et al.*, 1987, 1989b). The exception is the only report, referred to above, on the ultrastructure of a species of *Viannia*, *L. panamensis*, in the midgut of a sandfly by Walters *et al.* (1989a) who suggested that, because the parasites moved quickly from the hindgut to the anterior of the thoracic midgut, they perhaps had no « need to stabilize themselves in the midgut by association with the microvilli ».

Warburg *et al.* (1989) devised an *in vitro* assay to investigate attachment of *L. major* and *L. panamensis* to the midgut epithelium of *P. papatasi*. Both parasites exhibited a much stronger affinity for the internal surface of the midgut than to other sandfly tissues. Tests with 13 sugars to see if the attachment was lectin-mediated gave negative or inconsistent results. A monoclonal antibody to a protein which occurs on the tip and localized patches along the length of leishmanial flagella substantially inhibited adhesion. The authors concluded that the recognition of the microvilli was probably mediated biochemically and that the recognition of cuticle was probably by a different means.

All attachment to the cuticular intima of both the hind and foreguts is mediated by hemidesmosomes. Similarly, if parasites invade the diverticulum, attached forms again display hemidesmosomes in the flagellar sheath (Molyneux *et al.*, 1986). The molecular mechanism of the attachment is not known.

METACYCLIC PROMASTIGOTES

Perhaps the greatest recent advance in studies of leishmaniae in the sandfly is the confirmation of the production of so-called metacyclic promastigotes, forms in the sandfly which appear to be already adapted for life in the vertebrate host (Sacks and Perkins, 1984; see reviews by Sacks, 1988, 1989). Stages of *Leishmania* assumed to be the stage infective to the vertebrate were seen in the mouthparts of sandflies many years ago and gave rise to Adler's

assumption, now contested, that transmission by bite required that such parasites were in the proboscis. The general view now prevailing is that, while transmission could readily take place when parasites are in the mouthparts, regurgitation of infective promastigotes from more posterior parts is no less important (Killick-Kendrick, 1986; Warburg & Schlein, 1986; Jefferies *et al.*, 1986). Nevertheless, promastigotes in the proboscis are not uncommon having been recorded in more than twenty combinations of parasite and fly.

Striking features separating metacyclic forms from others in the sandfly are high motility, lack of attachment to sandfly tissues, the small size of the body and the presence of a long free flagellum with a length about twice that of the body. Measurements of 50 randomly encountered metacyclic promastigotes of *L. major* in a stained smear made from the proboscis of an experimentally infected specimen of *P. papatasi* revealed that they were monomorphic with a mean body length of $10.1 \pm \text{SD } 0.89 \mu\text{m}$ and flagellum length of $20.5 \pm \text{SD } 2.16 \mu\text{m}$. The posterior end was sharply pointed and the flagellar pocket was relatively deep ($1.5\text{-}2.5 \mu\text{m}$) (Killick-Kendrick, 1986). It has been suggested that paramastigotes of *L. i. chagasi* and *L. panamensis* might also be infective to the vertebrate host because of similarities in the ultrastructure of paramastigotes and amastigotes (Walters, 1989a, b). This evidence may not be considered enough in the absence of observations, discussed below, showing modifications to the surface membrane and changes in enzyme activity which are, apparently, related to an ability to resist complement and the respiratory burst of macrophages.

Sacks and Perkins (1985) sequentially assayed the infectivity of a standard number of promastigotes of *L. major* harvested from the guts of *P. papatasi*, and of *L. mexicana* from *Lu. longipalpis*, and showed a progressive increase with time. This suggests that metacyclic promastigotes are produced in increasing numbers as the infection develops in the alimentary tract of the sandfly.

With *L. amazonensis* in *Lu. flaviscutellata* and *L. i. chagasi* in *Lu. longipalpis*, Lainson *et al.* (1987) showed that at least some parasites were infective to hamsters 15 h after an infective bloodmeal. In studies on the morphology of *L. i. chagasi* in *Lu. longipalpis*, Lainson and Shaw (1988) concluded that from 15-120 h after an infective bloodmeal there were two lines of parasite development. One was the transformation of amastigotes directly into metacyclic promastigotes which then divided producing more metacyclic promastigotes. The other began with large, vacuolated amastigotes which, having divided at least twice, gave rise to large promastigotes which did not themselves divide and produced no obvious product. Lainson and Shaw's observations may explain the increase in infectivity with time reported by Sacks and Perkins (1985). The role of the « unproductive » line remains unexplained, nor is it known how long the non-dividing promastigotes can live, but

Lainson and Shaw (1988) suggested they might play a role in creating suitable conditions for the line of metacyclic promastigotes. The report by Lainson and Shaw is the only one of division of the forms infective to the vertebrate. According to Mallinson and Coombs (1989a) metacyclic promastigotes of *L. major* do not divide *in vitro* but they can change to multiplicative promastigotes over a period of 24-48 h. However, the behaviour of metacyclic promastigotes in culture may not be the same as in the midgut of the sandfly.

Evidence that metacyclic promastigotes are adapted for life in a vertebrate host comes from studies on cultured promastigotes. A major step was the finding that differences in the surface membrane of infective and non-infective forms could be exploited with lectins to separate the two forms (Sacks & Perkins, 1984, 1985; Sacks *et al.*, 1985; see also Ready & Smith, 1988). The surface carbohydrates of log-phase promastigotes differ from those of metacyclic promastigotes which are themselves broadly similar to those of amastigotes (Sacks *et al.*, 1985; Saraiva *et al.*, 1986). Sacks and Da Silva (1987) showed that the loss of agglutination to peanut agglutinin by metacyclic promastigotes of *L. major* was caused by the loss of expression of a glycolipid of the surface membrane of log-phase promastigotes. The different glycolipid of metacyclic promastigotes was a well-defined marker of these forms. Pimenta *et al.* (1990) then showed that the change from log-phase to metacyclic promastigotes was concomitant with the acquisition of novel carbohydrates which are associated with developmental modifications of lipophosphoglycan, the major surface glycoconjugate of these cells implicated in attachment to and survival in macrophages (see review by Turco, 1990). In studies on the ultrastructure of metacyclic promastigotes, Mallinson and Coombs (1989a) noticed an irregular, finely grained surface coat on metacyclic promastigotes of *L. major*. By a technique known as fracture-flip (Anderson Forsman & Da Silva, 1988), Pimenta *et al.* (1989) showed with the electron microscope that the surface of metacyclic promastigotes differs from that of log-phase parasites by the presence of a network of fusiform filaments 44 nm in length which, they thought, was probably the developmental lipophosphoglycan unique to the metacyclic forms. The same workers examined the two forms of promastigotes by transmission electron microscope and showed that metacyclic promastigotes of *L. major* have a glycocalyx of 17 nm thickness whereas that of log-phase parasites was only 7 nm.

From the time metacyclic promastigotes are deposited in the skin of the vertebrate host until they gain access to a macrophage, they are presumably exposed to the potentially lethal effects of complement which is, incidentally, exploited in gaining entry into the host cell (Da Silva *et al.*, 1989). It is surprising, therefore, that tests of serum resistance of log-phase and metacyclic promastigotes of different species have given contradictory results (refs in Mal-

linson & Coombs, 1989b). The possible influence of sandfly saliva on the survival of metacyclic promastigotes in the skin has not, however, been taken into account in these observations. Titus and Ribeiro (1988) described a striking enhancement of infectivity when minute amounts of sandfly saliva were added to inocula of cultured promastigotes of *L. major* and *in vitro* tests of complement sensitivity of metacyclic promastigotes should perhaps be done in the presence of saliva.

Mallinson and Coombs (1986) showed different levels of activity of a number of enzymes of infective and log-phase promastigotes and commented that the changes in metacyclic forms correlated with enzyme activities of amastigotes suggesting that the infective parasites were pre-adapted at a molecular level to enter and survive in macrophages. Mallinson and Coombs (1989a) confirmed these results and also reported that the content of some amino acids of metacyclic promastigotes of *L. major* and *L. mexicana* was more similar to that of amastigotes than of mid-log phase promastigotes. Furthermore, the endogenous and simulated respiration of the log-phase stages were appreciably higher than those of infective forms.

Mallinson and Coombs (1989b) studied the respiratory burst of macrophages after infection by promastigotes and found that, although this potentially lethal mechanism was not inhibited by metacyclic promastigotes (of *L. major*), the survival of the parasites was apparently good. Possibly these forms are adapted to resist the consequences of, rather than interfere with, the mechanism.

All the differences between infective and non-infective forms of promastigotes are obviously controlled genetically. Searle *et al.* (1989) reported observations on a gene which showed differential expression between infective and non-infective promastigotes and, by differentially screening a cDNA library constructed from stage-specific mRNA, Coulson and Smith (1990) isolated four clones encoding mRNAs which show elevated or unique expression in the metacyclic promastigotes of *L. major*. The same workers showed that there was no genomic rearrangement associated with changes in the morphology of promastigotes seen as infectivity to the vertebrate host is acquired.

FACTORS AFFECTING THE LIFE-CYCLE OF LEISHMANIAE IN THE SANDFLY

Differences in the innate ability of different species of sandflies to support the growth of various species of *Leishmania* are well documented (refs in Killick-Kendrick, 1985). Schlein and Romano (1986) and Borovsky and Schlein (1987) studied the effect of *L. major* and *L. donovani* on the digestive enzymes of *P. papatasi*, a sandfly which supports a flourishing development of *L. major* but has an extremely low susceptibility to *L. donovani*. They showed that *L. major* depresses the enzyme activity but that

L. donovani enhances it, and suggested that low levels enabled *L. major* to thrive but high levels inhibited the growth of *L. donovani*. Earlier, Schlein *et al.* (1983) looked at the effect of turkey blood on the development of *L. major* in *P. papatasi* and found that the nucleated erythrocytes of the bird induced a high level of DNAase which was inimical to the parasite.

In recent work in which lines of *P. papatasi* susceptible and refractory to *L. major* were selected, variations in the susceptibility to *Leishmania major* by individual sandflies of the same species (*P. papatasi*) have been shown to be genetically controlled (Wu & Tesh, 1990b). The results of reciprocal crosses and crosses to the parent line suggested that neither character is dominant over the other and that different genes control susceptibility and refractoriness (Wu & Tesh, 1990a). A completely refractory line was not obtained, perhaps because this trait is controlled by more than one gene. From the infection rates of F₁ crosses of the two lines, it was concluded that the observations could not be explained by maternal inheritance. At the time of writing, no information on the way in which the genes act has been reported.

The gut of sandflies is normally sterile and the development of leishmaniae is generally inhibited by bacteria and fungi. Warburg and Ostrovska (1987) described a similar effect caused by a cytoplasmic polyhedrosis virus which, although not invading the parasite, caused such severe structural and functional aberrations in the gut that it made it unsuitable for the parasite.

GENETIC EXCHANGE IN *Leishmania*

Possible genetic exchange in *Leishmania* should be viewed against the backcloth of observations on the related genus *Trypanosoma* which is the more intensively studied of the two. The first firm evidence of genetic exchange in *Trypanosoma* came from the analysis of isoenzyme variations in stocks of *T. brucei* (Gibson *et al.*, 1980; Tait, 1980). Jenni *et al.* (1986) then cloned recombinant forms of *T. brucei* from mice which had been infected with metacyclic forms of *T. brucei* from tsetse flies given meals of two genetically defined clones. This was confirmed by other workers and, from crosses of four different pairs of strains of *T. brucei*, a total of 61 hybrid clones has now been isolated. Although both meiosis and syngamy are involved, the order remains unknown. It is believed that exchange is not obligatory (Tait and Turner, 1990).

The first evidence of genetic exchange in *Leishmania* also came from isoenzyme studies in which patterns were seen, firstly by Maazoun *et al.* (1981) and then by Le Blanc *et al.* (1986), which suggested hybridization. Two stocks of *Leishmania* out of 132 isolated from man, dogs, gerbils and sandflies in Al Ahsa, Saudi Arabia, were later found with isoenzyme profiles looking like hybrids of *L. major*

and *L. arabica* (Evans *et al.*, 1989). Karyotyping and studies with kDNA probes supported this conclusion (Evans *et al.*, 1987), although preliminary attempts to produce hybrids by cloning parasites from the midguts of *P. papatasi* which had been fed on mixtures of the two parasites met with no success (Evans *et al.*, 1989). Similarly, Gradoni *et al.* (1986) were unable to find hybrids in *P. perniciosus* which had been infected by two distinct zymodemes of *L. infantum*.

In spite of the difficulty of producing hybrids experimentally, a recent report by Lanotte and Rioux (1990) will doubtless encourage further attempts. These workers videotaped cultured promastigotes of, firstly, *L. infantum* and then of *L. tropica* apparently fusing into a single individual. No details of the nuclei or kinetoplasts were visible in the living, unstained parasites, but it seems probable that conjugation between individuals of the same species was actually witnessed. No similar observations have been reported with trypanosomes.

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