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Review Article

"Vector-Host-Parasite" Inter-relationships in Leishmaniasis: VI. The metacyclic stage versus the paramastigote stage in *Leishmania* transmission

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ABSTRACT

Recent developments in study of leishmaniasis has spurred an increasing interest in research on the interaction between *Leishmania* and their vectors. These studies on the "vector-parasite" interactions have been wide-ranging but often suffer from difficulties in manipulating *in vitro* vector systems. Despite the progress on many fronts, little is known about the conditions that actually induce transformation of the parasitic protozoa in their natural environment. The digestive tract of an arthropod and the extracellular milieu of the mammalian host are subject to physiological, nutritional, hormonal, immunological and other changes, any of which could be a candidate for investigation. Advances in molecular biology and protein analysis techniques allow more effective study of interactions among vector, host, and parasite with a view to identification and characterization of molecules which control parasite infectivity. Therefore, this paper will comment on recent studies which provide a basis for future work, summarize recent knowledge about the infective stage of leishmaniasis and emphasize aspects deserving more extensive study.

KEYWORDS: *Leishmania*, differentiation, metacyclic stage, paramastigote stage, infective stage.

INTRODUCTION

Leishmania parasites occur in two main morphogenic forms: promastigotes and amastigotes. The former are found and develop through various morphological stages in the gut of female phlebotomine sand flies, while the latter are found and multiply within phagolysosomal system of vertebrate macrophages.

Various adaptations are required for the parasite to complete a full cycle in the vector (Killick-Kendrick 1979). Studies on *Leishmania* spp. interaction with various species of sand fly vectors (Molyneux & Killick-Kendrick 1987) have been unsuccessful in demonstrating the complete life-cycle of *Leishmania infantum* in *Phlebotomus ariasi* (natural vector in France) in the laboratory, inspite of the presence of massive infections of the stomodaeal valve (Rioux *et al.* 1972). Also, transmission attempts using *Phlebotomus papatasi* (natural vector) infected with *Leishmania major* from infected hamsters were unsuccessful, despite the presence of parasite within the mouthparts (Shehata *et al.* 1988). The above researchers concluded that there is a gap in the combinations between sand fly and *Leishmania* parasites and transmission of *Leishmania 'in vitro'* is questionable. However, transmission in nature is likely more efficient than implied from laboratory experiments. Many factors which may influence laboratory infections can be controlled by feeding of wild populations of flies on a natural reservoir host under isolated field condition (Lewis & Ward 1987). Some researchers have postulated that mammalian blood meal contains a transformation blocking factor, and the change of amastigote to promastigote is inhibited until this factor is degraded by the

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digestive enzymes of the sand fly (Brun *et al.* 1976). However, early transformation of amastigotes to promastigotes can take place within the blood meal of many different haematophagous arthropods including most phlebotomine species (Adler & Theodor 1927a,b; Sacks *et al.* 1994). The stimulus for the transformation of amastigotes to promastigotes is currently thought to be the fall in temperature from the vertebrate host to the invertebrate vector (Hommel 1978).

Differentiation of *Leishmania* stages: The opinion that there is a *Leishmania* form within the fly uniquely adapted for life in the vertebrate host was offered by some of the earliest investigators (Adler & Theodor 1930; Adler & Ber 1941). This idea has been reinforced by the finding that there is sequential differentiation of promastigotes from a non-infective to an infective stage (Sacks & Perkins 1984). The differentiation of *Leishmania* involves both qualitative and quantitative changes in various biochemical parameters (Chang *et al.* 1990). Mechanisms which control such changes and their effect on leishmanial differentiation remain to be defined (Joshi *et al.* 1993).

Previous reports have recorded changes in size as well as in shape of *Leishmania major* promastigotes during culture *in vitro* in different culture conditions (Greenblatt *et al.* 1985). These researchers noted that size and shape variations in cultures of cloned parasites suggests that the phenomenon is likely due to culture conditions. Other research (Daba *et al.* 1997a) demonstrated that both the size and the shape of promastigotes *in vivo* of either *L. major* or *L. infantum* were affected by the kind of blood meal present in the *Phlebotomus langeroni* gut. These two investigations point to the importance of the surrounding feeding medium as a factor affecting parasite differentiation.

Proteins are of the utmost importance in biological processes (Bell *et al.* 1970). Amino acids and small peptides are the principal growth substrates for *Leishmania* spp. and some amino acids play an important part in cellular physiology (Krassner & Flory 1971; Lehninger 1975; Mukkada & Simon 1977; Law & Mukkada 1979).

Leishmania sp. utilize their nutritional requirements from the digested blood present inside the sand fly gut. Protein electrophoresis of blood from different mammalian spp. has demonstrated major differences in blood proteins among different vertebrate species (Daba *et al.* 1977b). These differences in blood proteins may in turn affect the molecular processes which lead to stage differentiation in *Leishmania*. This leads to a logical inference that the kind of blood meals *in vivo* affect the biology and physiology of the leishmanial species inside the sand fly gut and serve as principal growth substrate responsible for production of infective or non-infective organisms within a single life-cycle.

Generation of the infective stage as reported by previous authors Sacks & Perkins (1984) found that the generation of infective stage (metacyclic promastigote stage) *in vitro* occurs as the organisms approach the stationary phase of growth, or *in vivo* from 7-10 days after sand fly infection. These promastigotes were highly virulent and could produce lethal infection in BALB/C mice. The researchers concluded that the generation of the infective stage was growth cycle-dependent and restricted to non-dividing organisms.

Additionally, generation of the infective stage (metacyclic promastigote stage) from rapidly dividing avirulent populations can occur as early as day 3 and is well under way by day 4 (Sacks & Perkins, 1985). The researchers postulated that the progressive increase in promastigote infectivity might be due to depletion of blood meal nutrient components by both the fly and dividing parasites. On the other hand, organisms of logarithmic phase (non-metacyclic forms) infective to hamsters are present in the midgut of experimentally infected sand flies from hours after infective blood meal (Lainson & Shaw 1987; Lainson *et al.* 1987).

The infective form can clearly be present in both the logarithmic phase and the stationary phase of growth. Usage of infective clones are responsible for the presence of the infective stage in logarithmic phase culture or in the sand fly midgut immediately after

infective blood meal. With high percentage of infective clones recently isolated, from animal stocks or from promastigotes cryopreserved in liquid nitrogen (within 2 passages after primary isolation (Jaffe *et al.* 1984)), may persist some of these infective stage to the stationary phase in culture media or to late infection in sand fly guts even in the presence of unsuitable blood meal. Possibly, the quantity of unsuitable blood in the midgut of experimentally infected sand flies is insufficient to convert all highly virulent clones to avirulent ones as occurred in culture media. *In vitro*, the loss of infectivity with prolonged subpassaging may be due to overgrowth by the non-infective population as a result of utilization of unsuitable blood (Daba *et al.* in press). If the sand flies take fewer number of amastigotes (as in case of natural infection) or the culture contains a lower percentage of the virulent clones, the amount of unsuitable blood meal could be enough to convert sufficient numbers of virulent (infective) clones to an avirulent (non-infective) ones such that transmission would not occur.

Dividing and non-dividing paramastigotes: Walters *et al.* (1989b) found that some paramastigotes are in dividing forms, while Killick-Kendrick (1979) believed that paramastigotes do not divide. These contrasting findings may be explained as the early transformation of aflagellated form (amastigotes), from the infected animal, to flagellated form in the sand fly gut or the infective clones from virulent strain culture. This could lead to the appearance of infective forms (paramastigotes) able to transmit disease within few hours (Lainson & Shaw 1987; Lainson *et al.* 1987) before starting blood meal digestion that could affect parasite differentiation (Daba *et al.* 1997a). These paramastigotes have an ability to divide and convert to other forms in the presence of blood meal nutrient components. In late infection (stationary phase), paramastigotes are unable to divide due to the depletion of blood meal nutrient by continuous utilization of both the fly and dividing parasites.

The cell surface carbohydrate of *Leishmania* promastigote stages: Prior studies have noted changes in surface properties of *Leishmania* promastigotes during culture *in vitro* (Doran & Herman 1981; Franke *et al.* 1985; El Amin El Roufaie *et al.* 1987). Sacks *et al.* (1985) demonstrated that the development of infective stage *L. major* promastigotes is accompanied by changes in surface carbohydrates, as determined by agglutination with plant lectins. The ability of both two lectins, [peanut agglutinin (PNA) and Ricinus communis (RCA120)], to agglutinate promastigotes decreased as cells progressed from the logarithmic to the stationary phase during growth. The absence of agglutination of approximately 50% of stationary-phase promastigotes by PNA and RCA120 might reflect a decrease in the expression of subterminal D-galactose residues on these organisms. The investigators concluded that promastigotes of *L. major* lacking galactose are infective forms since the generation of infective stage promastigotes *in vitro* occurs as organisms approach the stationary phase of growth (Sacks & Perkins 1984). These changes in surface carbohydrates were restricted to all of the *L. major* strains studied. However, they did not find any differences in the agglutinability of logarithmic and stationary phase of *Leishmania donovani* promastigotes by PNA. Apparently, the nature of the specific changes in lectin binding varies between *Leishmania* species, and some alterations in surface carbohydrates accompany the development of promastigotes into an infective stage. Notably, the investigators suggested that manipulation of the culture conditions might additionally enhance the conversion process.

Jacobson & Schnur (1990) showed that promastigotes derived from one clone of *L. major* exhibit different surface carbohydrate configurations and excrete different amounts, and possibly different "types" of glycoconjugate depending on the type of media. Stationary phase promastigotes grown from diphasic NNN cultures lost their ability to bind lectins specific for galactose, N-acetylgalactosamine and fucose. Promastigotes grown in defined media lost their ability to bind to fucose-specific lectin, but bound with higher affinity to

lectins specific for galactose and N-acetylgalactosamine during the stationary phase than during the logarithmic phase. The researchers suggested that infectivity of the virulent strain was not likely to be dependent on the expression of a single surface carbohydrate, since the surface carbohydrate configurations of the promastigotes vary according to the nutrients in the external environment. The researchers also found that both quantitative and qualitative variations were seen in the antigenic glycoconjugates released into the media. They concluded that the genetic difference between isolates may arise from the difference in culture media between different laboratories.

Furthermore, Dell & Engel (1994) reported that the biochemical and morphological changes in *Leishmania* parasites during its life-cycle are likely to result from programmed changes in gene expression in response to the changes in the external environment of the parasite. In addition, chromosomal rearrangements may occur when parasites are subject to nutrient stress. This genetic variability of chromosomes are responsible for the production of several different types of polymorphisms (Rovai *et al.* 1992) and reflects expression of stage-specific proteins (Bates 1994).

In vivo, the external environment of the parasite inside the sand fly gut is the kind of blood meal. Different kinds of blood may have different effects on both the cell surface receptors and the gene expression of *Leishmania* parasites as mentioned above in case of *in vitro* cultures. Therefore, the kind of blood meal might be responsible for the ability of parasites to adapt to either the digestive tract of the sand fly (i.e. responsible for the mechanisms of either the attachment or the detachment of *Leishmania* flagella to sand fly gut epithelium) and for the preadaptation of parasites (infective stage) for life in the vertebrate host(s). This explanation may help to clarify the nature of "vector-host-parasite" interactions *in vivo*, which had not understood before.

The characteristic features of both the metacyclic stage and the paramastigote stage: The striking features of metacyclic stage in the sand fly are the slender body, small size, the free flagellum about twice the length of the body, apposition between the kinetoplast and the nucleus, high motility and lack of attachment to sand fly tissues (Killick-Kendrick 1986; 1990; Lawyers *et al.* 1990). Paramastigotes are short, broad, with pointed posterior end and longer flagellum (< 2-3 times the body length), and large juxtaposed kinetoplast and nucleus. Sometimes the kinetoplast and the nucleus are almost equal in size and can be closely associated, possibly masking one another (Daba *et al.* 1977a). The paramastigote features of juxtaposed kinetoplast and nucleus, size and shape are identical to that of the amastigotes, except for the presence of flagellum in the former. Paramastigotes (the flagellated form in the invertebrate vector) can easily convert to amastigotes (aflagellated form in the vertebrate host) by losing their flagellum. These similarities between paramastigotes and amastigotes support the notion that the paramastigotes have an ability to adapt to and survive within the vertebrate host macrophages.

The infective stage in *Leishmania* transmission: The evidence that the metacyclic promastigotes are adapted for life in a vertebrate host comes from studies on cultured promastigotes, although the behavior of metacyclic promastigotes in culture may differ from that in the midgut of the sand fly (Mallinson & Coombs 1989a). The carbohydrate configurations on the surface membrane of the promastigotes are extremely variable depending on the nutrients in the external environment (Jacobson & Schnur 1990). Furthermore, changes in the biochemistry and morphology of *Leishmania* parasites during its life-cycle are likely to be the result of programmed changes in gene expression in response to changes in the external environment of the parasite (Dell & Engel 1994). Therefore, each kind of blood meal has a particular effect on *Leishmania* differentiation and the reservoir host(s) blood being the key factor for development of parasites to the infective stage (Daba *et al.* 1997a). Alteration of the growth media from reservoir host blood to other media for

Leishmania species leads to misinterpretation that the metacyclic form (short promastigotes) is the infective form. It is clear that the metacyclic form is the end stage of differentiation of parasites in presence of unsuitable media or unsuitable blood (Daba *et al.* 1997a). Previous authors have consistently highlighted the risks of extrapolating such laboratory findings to *Leishmania* species in the field. Thus, the significance of such developmental infective form has remained ambiguous. The authors therefore, consider the paramastigote as a terminal infective stage in *Leishmania* transmission.

The paramastigotes form is a significant stage of *Leishmania* development (Molyneux & Killick-Kendrick 1987). In natural infections, rounded forms are found in the foregut of sand fly and inoculation of these produce infections in hamsters (Molyneux 1977). The forward movement of parasites in the lumen of the oesophagus to the pharynx of sand flies is typically accompanied by a reduction in the size of the parasite, which changes from elongate promastigotes to round or oval paramastigotes (Killick-Kendrick 1979; Sacks *et al.* 1994). The consistent findings of paramastigote in sand flies infected with suprapylaria and peripylaria indicates that this form is a significant stage of development (Molyneux & Killick-Kendrick 1987). The variety of forms of promastigote and paramastigote observed in the life-cycle of *Leishmania* indicates that changes in parasite antigens may occur in sand fly (Molyneux & Killick-Kendrick 1987). The ultrastructure of paramastigotes suggests that they may be infective form due to their similarities with amastigotes (Walters *et al.* 1989a).

Differentiation of paramastigote forms according to the type of blood meal The research done by Daba *et al.* (1997a) demonstrated that the paramastigote forms of *L. major* has two different phenotypes depending on the type of blood in which *L. major* develops inside the *P. langeroni* gut. This visible characters of an organism may result from the interaction of genetic characteristics of both the parasites and the host blood that may lead to changes in the genotype of the organism, since, it was found that the phenotype and/or genotype of a pathogen can change dramatically following culture in an artificial system (Randolph & Nuttall 1994). There are two assumptions for explanation of such phenomenon. The first one is that both paramastigotes may be either real infective stage, i.e. each one responsible for transmission of one kind of *Leishmania* disease and in this case either host' blood can be considered reservoir hosts for the same parasite (*L. major*). The second assumption is that, one of these paramastigotes is real infective stage, while the other is unreal (non-infective), i.e. unable to transmit the disease due to the difference in their phenotype and/or genotype from the real state and in this case, one of the host' blood could be considered reservoir host and the other one is considered accidental host for the same *Leishmania* species. Evidence for the first hypothesis comes from Costa Rica where it was found that *L. chagasi* causes cutaneous leishmaniasis (Zeledon *et al.* 1989). In South America, infections with the same organism are almost exclusively associated with visceral leishmaniasis (Grimaldi *et al.* 1989). Parasite isolates from cutaneous cases proved indistinguishable from isolates from visceral infections (Lanzaro & Warburg 1995). The researchers describing this phenomenon proposed that the variation in clinical manifestations of one *Leishmania* species (*L. chagasi*) may be due to the genetic variability in sand fly species. They also mentioned that variability in clinical manifestations is not unique to *L. chagasi* infections. In North Africa and Southern Europe, *L. infantum* (a parasite arguably identical with *L. chagasi*) causes visceral infections in some areas, but primarily cutaneous disease in other geographical regions (Ben-Ismail *et al.* 1992).

Because the kind of blood meal in which the *Leishmania* species develop is responsible for the phenotypic variability in the infective stage (paramastigotes), the authors propose that the variation in clinical manifestations of the same *Leishmania* species may be due to the genetic variability of host bloods in different geographical regions (i.e. the source of sand fly infection in nature) that have an effect on the phenotype and/or genotype of a

pathogen (Randolph & Nuttall 1994). On the other hand, the infection of vertebrate host, *in vitro,* may occur directly from virulent clones taken from culture media without the need for invertebrate vector. So, sand fly species have no role on parasite infectivity inside its gut and subsequently, has no role on the phenotype of the parasite.

Conclusion: The presence of viable *Leishmania* parasites inside the sand fly gut does not connote transmission of pathogenic parasites will occur unless the infective stage is present. The infective stage must have an ability to adapt to and survive within the vertebrate host macrophages. Although paramastigotes may be present in the sand fly gut, it does not necessarily follow that it is a real infective form. It may differ in its phenotype from the infective stage, hence unable to adapt and survive in the vertebrate host macrophages and unable to cause infection. Transmission may occur from reservoir host to either reservoir host or to accidental host, but transmission may not occur from accidental hosts at all. Thus, the presence of *Leishmania* parasite inside the vertebrate host(s) does not imply that it is a reservoir host. The reservoir host(s) of one *Leishmania* species may be considered an accidental host(s) for another. The failure of many laboratory trials to transmit some *Leishmania* species by bites of laboratory infected sand flies could be attributed to "vector-host-parasite" specificity.

Finally, it is important to clarify that there is more than one parasite form present in most the artificial or natural culture media or sand fly gut. All these forms are inoculated into the animal without discrimination. It is difficult, practically, to decide which form is responsible for the infection. More effort is needed to define the real infective stage, to restore this important issue. The question naturally arises, "Is the metacyclic promastigote stage the infective stage in *Leishmania* transmission ?". This question is left to be answered.

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الملخص العربى

العلاقة التداخلية بين العائل الوسيط والعائل الخازن والطفيل لمرض الليشمانيا ٠ ٦- طور الميتاسيكليك مقابل طور البارامستيجود فى نقل مرض الليشمانيا

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أشتملت الدراسات الحديثة عن طفيل *الليشمانيا* على أبحاث شتى لمعرفة العلاقة بين طفيل *الليشمانيا* والعائل الناقل للمرض. ولكن لا تزال هذه العلاقة يكتنفها الغموض. فمثلا فى بعض الحالات وجد طفيل *الليشمانيا* داخل معدة الحشرة بعد عدواها معمليا، ولكن لم تستطيع هذه الحشرة نقل المرض بعد أغتذائها على العائل. وقد أظهرت بعض الدراسات أن بأختلاف نوع الوسط الغذائى الذى ينمو فيه الطفيل سواء فى المعمل أو داخل معدة الحشرة له تأثير واضح على شكل وحجم طفيلف *الليشمانيا*. كما وجد أنه له تأثير أيضا على التركيب السطحى والجينى للطفيل. هذه الدراسات تقودنا الى أهمية نوع الدم الذى يغتذى عليه الحشرة الناقلة كوسط غذائى للطفيل فى الطبيعة. وقد وجد أن التركيب البروتينى للدم يختلف بأختلاف نوع العائل، وكل نوع من أنواع الدم له دوره الهام فى تعدد أشكال الطفيل داخل معدة الحشرة حيث أنه يهضم داخل معدة الحشرة الى جزئيات أصغر يغتذى عليها الطفيل وبذلك يؤثر على نموه وتعدد أشكاله. مما سبق يتضح لنا أن كل نوع من أنواع *الليشمانيا* له متطلباته الخاصة من الوسط المحيط به ليتحول الى الطور المعدى الذى يكون له القدرة على نقل المرض. هذه الدراسة تفتح المجال أمام الباحثين لدراسة العلاقة بين العائل الناقل والعائل الوسيط والطفيل لمعرفة العناصر المطلوبة لكل نوع من *الليشمانيا* حتى يتكون الطور المعدى المسبب للعدوى.