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***Leishmania* sand fly interaction: progress and challenges**

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Summary

Complex interactions occur between *Leishmania* parasites and their sand fly vectors. Promastigotes of *Leishmania* live exclusively within the gut, possess flagella and are motile, and kinesins, kinases and G proteins have been described that play a role in regulating flagellar assembly. Movement within the gut is not random: promastigotes can detect gradients of solutes via chemotaxis and osmotaxis. Further, they use their flagella to attach to the fly midgut using surface glyconjugates, a key step in establishment of the infection. Differentiation of mammal-infective stages is characterised by significant biochemical and cellular remodelling. Further, the parasites can manipulate the behaviour of the vector to their maximise transmission and flies may even deliver altruistic apoptotic forms to aid transmission of infective stages.

Introduction

The vectors of leishmaniasis are female phlebotomine sand flies (Diptera: Psychodidae, subfamily Phlebotominae). Approximately 1000 species of sand fly have been described to date, and of these about 70 are proven or suspected vectors. The *Leishmania* vectors are restricted to two genera: *Phlebotomus* species in Africa, Asia and Europe; and *Lutzomyia* species in Central and South America. The 20 or so *Leishmania* species of medical importance are usually transmitted by only one or two particular sand fly species. Thus, there is a fairly selective relationship between parasite and vector, which is typically the result of both ecological and molecular factors. The parasite has two main morphological forms: amastigotes, 3–5 μm ovoid cells found in mammalian hosts and responsible for disease pathology; and promastigotes, 5–15 μm elongated flagellated cells found in sand fly hosts [1]. In this article I will review some of the more important recent developments in this field from the past 2–3 years, drawing on both direct and indirect studies of *Leishmania*-sand fly interaction. For more detailed background on the life cycle of *Leishmania* the reader is recommended to consult references 1 and 2.

Flagellum and motility

Leishmania and their trypanosome cousins are sometimes informally known as haemoflagellates. As this name implies they possess flagella and exhibit motility for some or all of their life cycle (depending on the particular haemoflagellate). Even amastigotes, which are non-motile, still possess a rudimentary flagellum. However, motility plays a crucial part in the life cycle of *Leishmania* in the sand fly vector. The flagellum is a remarkable organelle: in addition to providing a means of locomotion it is also involved in two, possibly three, different mechanisms of attachment to the sand fly gut (discussed below). One of the first parasite developmental events that occur in sand flies following uptake of amastigotes is the growth of a functional flagellum, as these forms differentiate into procyclic promastigotes. The flagellum is positioned at the anterior end of the cell body (think propeller not tail), and drags the organism through its environment. The length of the flagellum varies between different promastigote forms and is a dynamic structure. For example, metacyclic promastigotes, the mammal-infective stages, have relatively long

flagella and are highly motile. A proteomic analysis of the *Trypanosoma brucei* flagellum revealed a complement of at least 300 proteins [3], and we can expect a similar complexity for the leishmanial counterpart. Consequently the growth, maintenance and disassembly of the flagellum will require a sophisticated set of interacting mechanisms. Microtubule elongation/shortening occurs at the tip of the flagellum, and it follows from this that intraflagellar transport (IFT) that brings tubulin and other components to the tip is potentially important in regulating flagellar length. Direct evidence for such a role of IFT in *Leishmania* comes from a recent study on a new member of the kinesin-13 family, KIN13-2 [4]. Kinesins are motor proteins, and *Leishmania* possess an unusually high number of these proteins. KIN13-2 localised predominantly to the flagellum in *L. major*, and showed microtubule binding and de-polymerising activity. When transfected into promastigotes, overexpression led to flagellar shortening, whereas knockdown (assayed in *T. brucei*) led to elongation. Interestingly, a bioinformatics search for potential IFT components [5], whilst revealing several good candidates, did not identify KIN13-2, illustrating that both bioinformatic and experimental studies are essential in order to assign function with confidence.

Another study has also revealed an interesting phenotype affecting flagellar length, this time from the manipulation of a thymidine kinase [6]. Several *Leishmania* protein kinases have been previously shown to influence flagellar length [7] (by currently unknown mechanisms), and their substrates could perhaps include kinesins such as the one described above. However, the influence of a putative thymidine kinase (TK1), ostensibly part of the pyrimidine salvage pathway, on flagellar length was unexpected. A TK1 single knock-out (*Leishmania* are diploid, so a single knock-out retains one copy of TK1) showed increased flagellar length, whereas intriguingly a double knock-out (null mutant) showed reduced flagellar length. It is possible that these are indirect phenotypic effects, for example, because knock-out is perturbing general intracellular nucleotide pools. However, another possibility is that the TK1 plays an unexpected role in a signalling/cytoskeletal organisation pathway. More direct evidence of a role for signalling pathways in regulation of flagellar length comes from a further study on the small G-protein ARL-3A (ADP-ribosylation factor-like protein 3A) [8]. Site-directed mutagenesis showed that continuous recycling between GTP and GDP-bound forms of ARL-3A was necessary to maintain correct flagellar length. Mutation of critical residues and blockade of either the GTP or GDP binding site led to flagellar shortening. The protein partners of the *Leishmania* ARL-3A and downstream cascade are not known yet. In summary, these recent publications that have revealed various flagellar effects are continuing to provide pieces of the jigsaw regulating flagellar assembly. Clearly there is some way to go before the full picture will emerge.

Assuming a functional flagellum is present, do we know any more about how it functions? Motility of promastigotes is certainly important in a general way, since the organisms must find their way to different parts of the sand fly gut in order to complete their life cycle [1,2]. They begin their journey in the abdominal midgut within a bloodmeal, and with some variations, depending on whether they go through a hindgut phase, will eventually end up in the anterior midgut/foregut in a position suitable for transmission by bite. Previous reports that this movement is not random but is directed by taxic responses have received further recent support [9]. Promastigotes responded positively to both chemotactic and osmotactic stimuli, that is they were capable of swimming towards low concentrations of certain substances such as sugars (positive chemotaxis) in the absence of a significant increase of osmolarity, whereas higher concentrations of additional substances such as simple salts induced osmotaxis. Interestingly promastigotes also displayed a positive taxic response to sand fly saliva. The response to sugars, taken up by flies as a supplementary energy source after bloodfeeding, and saliva both indicate that promastigotes can orient their motility to take them forward in the gut, a key adaptation for transmission, since both signals originate

from the anterior. In addition to being a directional cue the presence of sugars might influence differentiation of the parasite, since different promastigote forms synthesise complex glycoconjugates and differ in their surface glycocalyx [1]. Also saliva was previously suggested to be a possible stimulator of differentiation [10], and this suggestion is supported by the recent observation that saliva is ingested during sugar feeding by sand flies [11]. The taxic responses described above were all positive in nature. Curiously, in another study [12], promastigotes exhibited negative chemotaxis to ATP, a response apparently mediated by a surface membrane adenine nucleotide transporter functioning as a receptor. The survival value of this response is not immediately obvious, the authors speculate this may be helping the infection process in the mammalian host, but some unknown function in the vector is also possible. Regarding the osmotactic response of promastigotes, some interesting observations have been reported indicating a role for aquaglyceroporins (AQPs) in the process [13]. AQPs are part of the larger family of aquaporins, one subgroup comprised of water-specific channels found in membranes (aquaporins), whereas the AQP subgroup has a wider specificity transporting glycerol, and other small uncharged solutes in addition to water. The main role proposed for AQPs to date in parasitic protozoa has been in the regulation of cell volume, for example in response to hypo-osmotic stress. Therefore, it was interesting to find that the *Leishmania* AQP1 was localised to the flagellum in promastigotes. Further, overexpression of the protein led to an increased sensitivity to osmotactic stimuli. Thus, AQP appears to have a potential dual function both as a sensor and in mediating a homeostatic response to osmotic gradients.

Midgut attachment

As mentioned above, the flagellum is also involved in attachment to the sand fly gut. One mechanism of attachment, shared with other trypanosomatids, is the formation of an expanded flagellar tip containing hemidesmosomal structures, and this is used to anchor promastigotes to cuticle-lined parts of the gut (foregut, stomodeal valve and hindgut) [14]. The ultrastructure of these hemidesmosomes has been known for many years, but this interesting flagellar modification remains to be biochemically described. A distinct mechanism is used by promastigotes to bind to the midgut, which is lined with an epithelium, the promastigotes inserting their flagella between the epithelial microvilli. There is no obvious ultrastructural modification of the flagellum associated with midgut binding, which is more like a receptor-ligand interaction. Also it remains unclear whether the involvement of the flagellum is essential per se, or merely a reflection that being at the anterior end the flagellum will contact the epithelium first and fits easily between the microvilli. An extensive body of evidence has accumulated demonstrating the role of the dominant promastigote surface molecule, a glycolipid called lipophosphoglycan (LPG), in this binding [1], and a sand fly galectin has been identified as the corresponding receptor [15]. Developmentally regulated modifications of LPG are important for attachment, release and preparation of promastigotes for transmission to a mammalian host, in particular the terminal sugar residues on LPG side chains. LPG is a unique molecule, so it is not surprising that the parasites contain a corresponding unique complement of enzymes to build this molecule. However, recent work has revealed an unexpected level of complexity, with the description of a large family of side chain galactosyltransferase (SCG) enzymes [16]. The *L. major* genome contains 14 *SCG* members, some corresponding to enzymes with a biochemically validated role in LPG biosynthesis, but the newest members with unknown substrates and function. The location of some of these genes close to telomeres together with evidence that the expansion of family members may have been created by gene conversion indicates a strong selective pressure driving diversity in this gene family. This could relate to LPG and its role in attachment, to other roles for LPG in the vector or mammalian host, or to other glycoconjugates. Further, in addition to unexplained role(s) of the SGC family, there is evidence now accumulating that indicates LPG may not be the exclusive parasite attachment

molecule. The use of mutants deficient in LPG biosynthesis was an important step in demonstrating the role of LPG in attachment, for example of *L. major* in its vector *P. papatasi*. However, the use of similar mutants now indicates that LPG-independent mechanisms of attachment may occur in certain sand flies, those that are permissive to a range of species [17, 18]. Caution must still be counselled until the identity of the postulated alternative receptor-ligand pair is definitively demonstrated, but if true will mean that promastigote attachment mechanisms are more varied than previously believed.

The existence of permissive vectors is itself of considerable epidemiological significance, whether LPG-independent attachment mechanisms are subsequently proven or not, as their presence may lead to the emergence of new foci of infection, for example in northern Israel [19, 20]. One of the most dramatic examples of this has occurred with *Lutzomyia longipalpis*, a permissive vector that appears responsible for the establishment of *L. infantum* in South America [21]. This parasite was formerly regarded as a separate species, but it is now clear that it is practically indistinguishable from Mediterranean *L. infantum* and at best could be regarded as a subspecies (*L. infantum chagasi*). Another interesting potential way in which new foci may emerge comes from the parasites, with the formation of genetic hybrids [22]. The existence of genetic exchange in *Leishmania* remains uncertain, and the particular circumstances described in this paper are unusual, recombination appearing to have occurred in an immunocompromised patient. Interestingly, the genetic hybrids formed were between *L. infantum*, typically transmitted by permissive vectors, and *L. major*, paradigm of specific parasite –vector combinations. The resulting hybrids expressed the *L. major* version of LPG and appear capable of transmission by *P. papatasi*. Thus the rather worrying prospect emerges of a new form of *L. infantum*, a parasite capable of causing visceral leishmaniasis, gaining access to a new vector species and considerably extending its range. Whether this happens in reality remains to be seen, since significant ecological barriers must also be overcome. Nevertheless what these various studies illustrate is that we may need to re-think our understanding of midgut attachment, and this has important epidemiological implications. They also re-emphasise the importance of the underlying phenomenon, the necessity for parasites to establish themselves by attachment beyond the bloodmeal phase of infection, a prerequisite for transmission. Much still remains to be learned about both *Leishmania* glycobiology and gut attachment mechanisms.

Metacyclogenesis and transmission

Metacyclogenesis is the differentiation process leading to the accumulation of mammal-infective metacyclic promastigotes and is the ultimate end-point of parasite development in the sand fly vector [2]. Not all promastigotes will inevitably become metacyclic forms, but a high proportion may do so, in the order of 50% in mature infections. Various biochemical changes occur as part of this pre-adaptation to life in the mammalian host and, not surprisingly, the properties and origin of metacyclic promastigotes is of considerable interest, not only in the area of parasite-vector interaction but also in understanding the critical early stages of the infection process in mammalian hosts. One interesting recent finding is that endosome sorting and autophagy are required for metacyclogenesis in *Leishmania* [23, 24]. In particular the presence of a functional late endosomal/autophagosomal pathway was required for the survival of starvation conditions, and nutrients are likely to be limiting at this stage of the life cycle in the vector, as well as for intracellular remodelling associated with metacyclogenesis itself. Another facet of metacyclogenesis was revealed by an investigation of sphingolipid metabolism [25]. This study revealed an unexpected and novel role for sphingolipids in ethanolamine biosynthesis. Previous results showing a loss in virulence for parasites defective in sphingolipid synthesis were explained by the use of this pathway for ethanolamine synthesis, which when supplied exogenously was able to complement mutants. Differentiation of metacyclic promastigotes

was affected because these forms contain elevated levels of phosphatidylethanolamine, which may be required in several ways, even possibly in the autophagy pathway mentioned above.

Regarding the induction of metacyclogenesis, a variety of signals have been described in the literature, including low pH and nutrient depletion. Relatively little is known of the pH conditions in *Leishmania*-infected sand flies, but a V-ATPase of *Lu. longipalpis* has been recently identified that could play a role in gut acidification [26] and, therefore, the production of metacyclic promastigotes in mature infections. Another signal appears to be mediated by pteridine reductase (PTR1), originally proposed when a PTR1 knockout was found to have the interesting phenotype of hypervirulence [27]. Lack of PTR1 led to reduced levels of intracellular tetrahydrobiopterin, growth arrest and induction of metacyclogenesis. Recent work has revealed that PTR1 is degraded during the growth phase of promastigotes in culture co-incident with induction of metacyclogenesis [28]. Cause and effect have not been demonstrated, but this raises the possibility that degradation of PTR1 leads to reduced tetrahydrobiopterin and thereby acts as a signal stimulating metacyclogenesis. Interestingly, the degradation of PTR1 itself is proposed to act by ubiquitination and the proteosomal pathway, again emphasising the general role of proteases in differentiation [24].

Metacyclic promastigotes are essential for transmission, but their delivery to the mammalian host must be ensured. In that regard, it has been previously suggested that a parasite secretory chitinase plays a role in transmission either by helping to break down the peritrophic matrix or by damaging the stomodeal valve. The peritrophic matrix is a chitin/glycoprotein matrix that encloses the bloodmeal and is an early barrier to parasite establishment, whereas the stomodeal valve controls the junction between midgut and foregut, which must be navigated later. Evidence supporting both of these suggestions has been provided by using parasites over-expressing chitinase [29]. Compared to normal (wild-type) organisms, overexpressors were able to migrate forward earlier and were also transmitted more effectively by sand fly bite. The timing of delivery is also crucial to successful transmission, too early and the parasites will not have yet differentiated into metacyclic promastigotes in sufficient numbers, too late and the fly itself may not have survived the pathogenic effects of infection. Insights into this balance have been revealed in a study examining manipulation of vector behaviour [30]. When the time was optimal for transmission infected sand flies were found to display an increase in feeding persistence, they were more likely to resume feeding when interrupted than when the infection was immature. This feeding persistence led to increased parasite burdens in mice following fly bite and increased the probability of feeding on a second host. The precise mechanism behind the behavioural manipulation has not been determined, but the most likely explanation is that this is another manifestation of the “blocked fly hypothesis”, in which the sand fly gut is physically obstructed with promastigote secretory gel (PSG) [31]. The presence of PSG interferes with bloodfeeding and this is likely to lead to the persistent feeding behaviour observed. One final paper of interest regarding transmission concerns the possibility that apoptotic promastigotes accompanying viable metacyclic promastigotes aid the infection process [32]. Where it has been examined previously [e.g. 31] the vast majority of parasites egested by infected sand flies have been metacyclic promastigotes. However, when parasites were injected by needle it was found that apoptotic parasites enhanced the virulence of the inoculum by encouraging a silent form of phagocytosis [32]. Whether this is important in real fly bite transmission remains to be seen, but the data are certainly intriguing.

Conclusions

Advances continue to be made in understanding parasite-vector interactions and the basis of vector specificity, but much more remains to be done. Constraints on progress include the intrinsic challenge of simultaneously studying two organisms, and the compromises of laboratory investigations in comparison to the complexities of the real world. Additionally, sand flies have a reputation of being difficult to keep, and many species have never been colonised. It is certainly true that sand fly maintenance is a time consuming process requiring careful attention to detail. Maintaining *Leishmania* parasites also has its challenges, and in the current context it should be noted that access to tissue-derived amastigotes from experimental animal infections or to parasites that have been in culture for a limited period (weeks) is necessary. Parasites in long term culture (as promastigotes or amastigotes) will gradually lose their ability to infect and develop normally in sand flies. Therefore, the number of laboratories able to directly study *Leishmania*-sand fly interaction is limited. However, the relative ease of promastigote culture couple with reverse genetics means that progress in understanding the basic cellular and molecular biology of *Leishmania* can often, with careful interpretation, shed some light on parasite-vector interactions.

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