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Research Brief

Flagellar attachment of *Leishmania* promastigotes to plastic film in vitro

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Abstract

Trypanosomatid parasites are able to use their flagella for attachment to cuticular surfaces within their arthropod hosts. In this study the attachment mechanism of *Leishmania* promastigotes was investigated using a new and quantifiable in vitro assay system. The results showed that hemidesmosomal flagellar attachment to three different plastic substrates occurred (Melinex, Polyvinyl, Thermanox). Attachment density was increased by scratching the surface of the substrate or by coating with the hydrocarbons n-octacosane and paraffin. Variation in attachment density was observed, depending on the culture medium and the parasite isolate used. All four species examined, *L. braziliensis, L. donovani, L. major* and *L. mexicana*, were capable of flagellar attachment in vitro. Collectively, these data indicate that flagellar attachment is mediated by a non-specific hydrophobic interaction in *Leishmania* species.

Index Descriptors and Abbreviations: *Leishmania*; Flagellum; Attachment; Promastigote

Parasitic protozoa within the Order Kinetoplastida, Family Trypanosomatidae employ a unique mechanism by which they attach themselves to the gut wall of their arthropod hosts. These organisms are all flagellates, possessing a single anteriorly located flagellum, which in addition to providing motility within the gut of their arthropod hosts, also functions as an attachment organelle (Vickerman and Tetley, 1990). The trypanosomatids include species that are confined to arthropods, for example *Crithidia* species, as well as organisms of medical and veterinary importance that are transmitted from arthropods to mammalian hosts, for example *Leishmania* and *Trypanosoma* species. In the latter group, interest has also been stimulated by the proposition that flagellar attachment is required for the subsequent development of mammal-infective forms (Bonaldo et al., 1988; Hendry and Vickerman, 1988; Kleffman et al., 1998).

Upon contact with cuticle-lined surfaces of the gut (i.e., hindgut and/or foregut) in their arthropod hosts, the tip of the trypanosomatid flagellum can bind and expand into an attachment plaque (Brooker, 1971; Killick-Kendrick et al., 1974; Killick-Kendrick et al., 1988; Kollien et al., 1998; Molyneux, 1969; Molyneux, 1975; Thevanez and Hecker, 1980; Vickerman, 1973). In these various studies the flagellar surface membrane was underlaid with an electron-dense zone coincident with the region of attachment, and fibres were seen running from this structure towards the axonemal microtubules. These structures have been called hemidesmosomes because they superficially resemble one half of vertebrate epithelial desmosomes. In *Leishmania* such attached forms are known as haptomonad promastigotes (Killick-Kendrick et al., 1974). In contrast to their ultrastructure, the biochemical composition of hemidesmosomes is less understood, and only one study on *Trypanosoma congolense* has been published to date (Beattie and Gull, 1997). This identified a 70 kDa protein as a major component of the attachment plaque. Similarly, the nature of the interaction between the flagellar surface membrane itself and the substrate is not fully understood. However, it has been shown that some trypanosomatids can attach via their flagella to plastic surfaces in vitro (Gray et al., 1981; Hommel and Robertson, 1976; Maraghi et al., 1987), suggesting that attachment is not dependent on specific receptor–ligand interaction. Further, it was recently shown for *Trypanosoma cruzi* that attachment to the rectal cuticle in *Triatoma infestans* is mediated by a hydrophobic interaction between the flagellar plaque and superficial hydrocarbons and lipids (Schmidt et al., 1998).

In this study, we describe a convenient quantifiable in vitro attachment assay for *Leishmania* promastigotes, investigation of various factors that influenced attachment, and present data that flagellar attachment in *Leishmania* is non-specific and can be enhanced by hydrophobic interaction. Eight different *Leishmania* isolates were used: two of *L. mexicana* (MNYC/BZ/62/M379; MHOM/GT/94/U276); two of *L. braziliensis* (MHOM/BR/84/LTB300; MHOM/BR/75/M2903, LV436); two of *L. major* (MHOM/IL/80/Friedlin, FV-1; MRHO/SU/59/P, LV39); and two of *L. donovani* (MHOM/ET/67/HU3, LV9; MHOM/SD/62/1S-2D). Promastigotes were routinely maintained at 26 °C in medium M199 (Life Technologies 22350) supplemented with 10–20% (v/v) heat-inactivated foetal calf serum (FCS; Life

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Technologies 10108), BME vitamins (Life Technologies 21040), and 25 μg gentamicin sulphate/ml (Sigma G1272). Three different plastic substrates were used: Melinex film (Agar Scientific), Thermanox (Miles Laboratories) and Polyvinyl (Henleys Medical). Plastic sheets were either used as supplied (''smooth''), or scratched by thorough abrasion with Emery paper (''rough''). In either case 15 mm × 15 mm squares were cut from sheets, sterilised by immersion in 70% ethanol, air-dried, and placed individually into wells of 12-well tissue culture plates (Costar). Promastigotes cultured as above were washed and resuspended in fresh medium at 10^7/ml and 2 ml volumes placed into each well. Plates were covered with lids and sealed with Parafilm (Merck) to prevent drying out and incubated at 26 °C for varying periods of time. Cultures were fed every 2–4 days or as required by the removal of the 2 ml of culture and replacement with 2 ml of fresh medium. To assay attachment of promastigotes, plastic squares were removed with forceps, washed in Hank's balanced salt solution (Life Technologies), fixed in methanol and stained with 10% (v/v) Giemsa's stain in phosphate buffer, pH 7.2. The number of attached promastigotes in 10 fields under 1000× oil immersion light microscopy was determined for each square.

First, to verify that hemidesmosomal-type attachment was occurring in this system the morphology and ultrastructure of attached promastigotes was investigated. SEM revealed clusters of attached promastigotes on the surface of the substrate (Fig. 1A). These were concentrated over scratches where ''rough'' substrates were used.

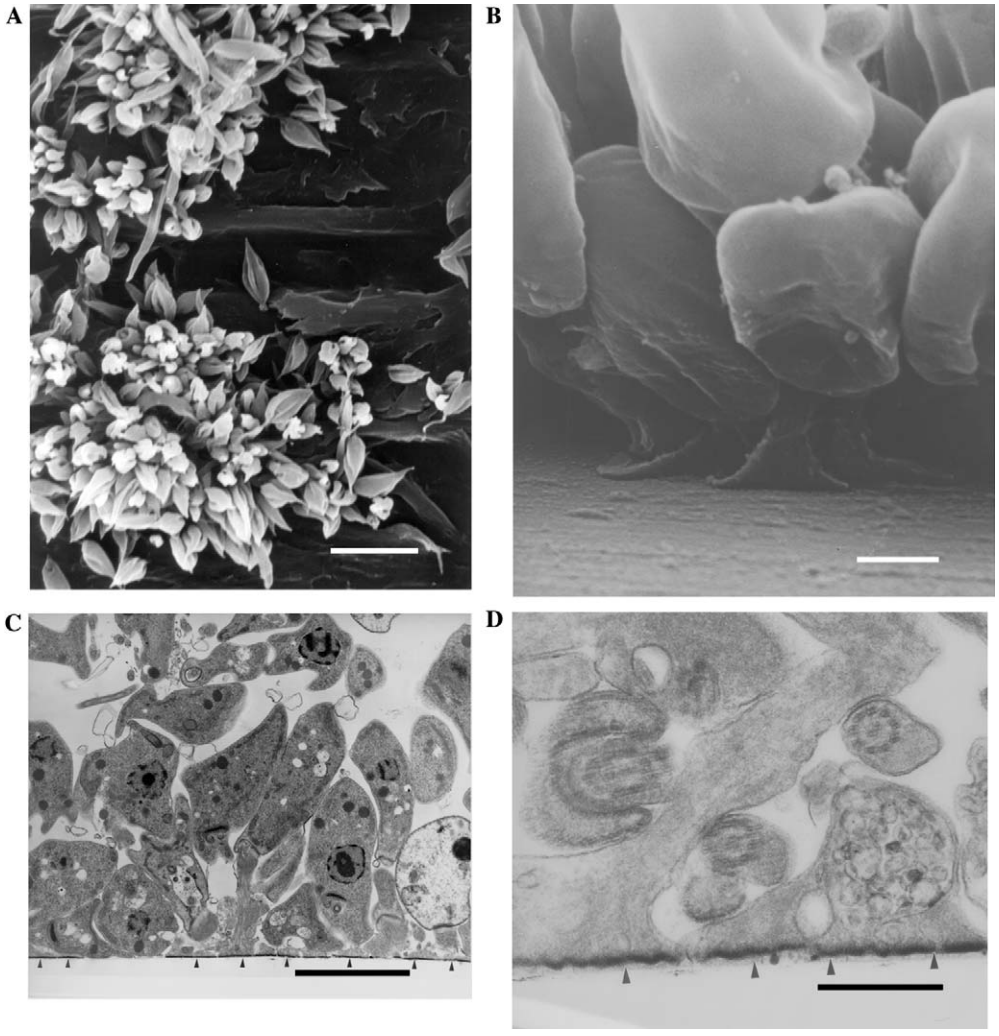

Fig. 1. Analysis of flagellar attachment by electron microscopy. (A) Example of SEM low power view showing *L. mexicana* promastigotes attached to Melinex. Bar represents 10 μm. (B) SEM high power view of *L. mexicana* promastigotes attached to Melinex. Bar represents 1 μm. (C) Example of TEM low power view showing *L. mexicana* promastigotes attached to Melinex. The arrowheads indicate hemidesmosomal attachement plaques; bar represents 5 μm. (D) TEM high power view of *L. mexicana* promastigotes attached to Melinex. Bar represents 0.5 μm. Individual samples were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 45 min at 26 °C, fixative discarded and replaced with 0.1 M cacodylate buffer, pH 7.4, and stored at 4 °C until being processed further. For scanning electron microscopy (SEM) samples were dehydrated through 70 and 90% ethanol for 15 min each followed by three changes in absolute ethanol for 15 min each. These were transferred to amyl acetate and critical point dried with CO_2. A 20 nm layer of gold was deposited on the cells, which were observed using a Hitachi S-520 scanning electron microscope. For transmission electron microscopy (TEM) samples were post-fixed for 15 min in 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, at room temperature, then washed in buffer for 15 min. These were then dehydrated through an ethanol series as above, transferred to propylene oxide for 15 min, into propylene oxide/Epon–Araldite resin (1:1) for 30 min, resin alone for 1 h, and resin again for 3 h. Samples were then embedded in fresh resin and polymerised at 80 °C for 48 h. Ultra-thin sections were cut at 90 nm, picked up on 200 mesh hexagonal copper grids and stained for 20 min with uranyl acetate, washed in distilled water, then stained for 5 min in lead citrate, finally washed with 0.02 M NaOH, then distilled water, and dried. Sections were observed using a Philips CM10 transmission electron microscope.

Higher magnification showed attachment of individual promastigotes was mediated by the flagellum, which was expanded into a foot-like projection in close contact with the substrate (Fig. 1B). The morphology of these parasites was indicative of haptomonad promastigotes. Further examination by TEM revealed the internal ultrastructure of attached flagella, which possessed a typical hemidesmosomal appearance, with an electron-dense zone just beneath the plasma membrane of the attached flagellar surface (Figs. 1C and D). These results demonstrated that the desired hemidesmosomal type of attachment was occurring in this in vitro system.

To investigate the influence of physical properties of the substrate, attachment to three different types of plastic substrate (Melinex,

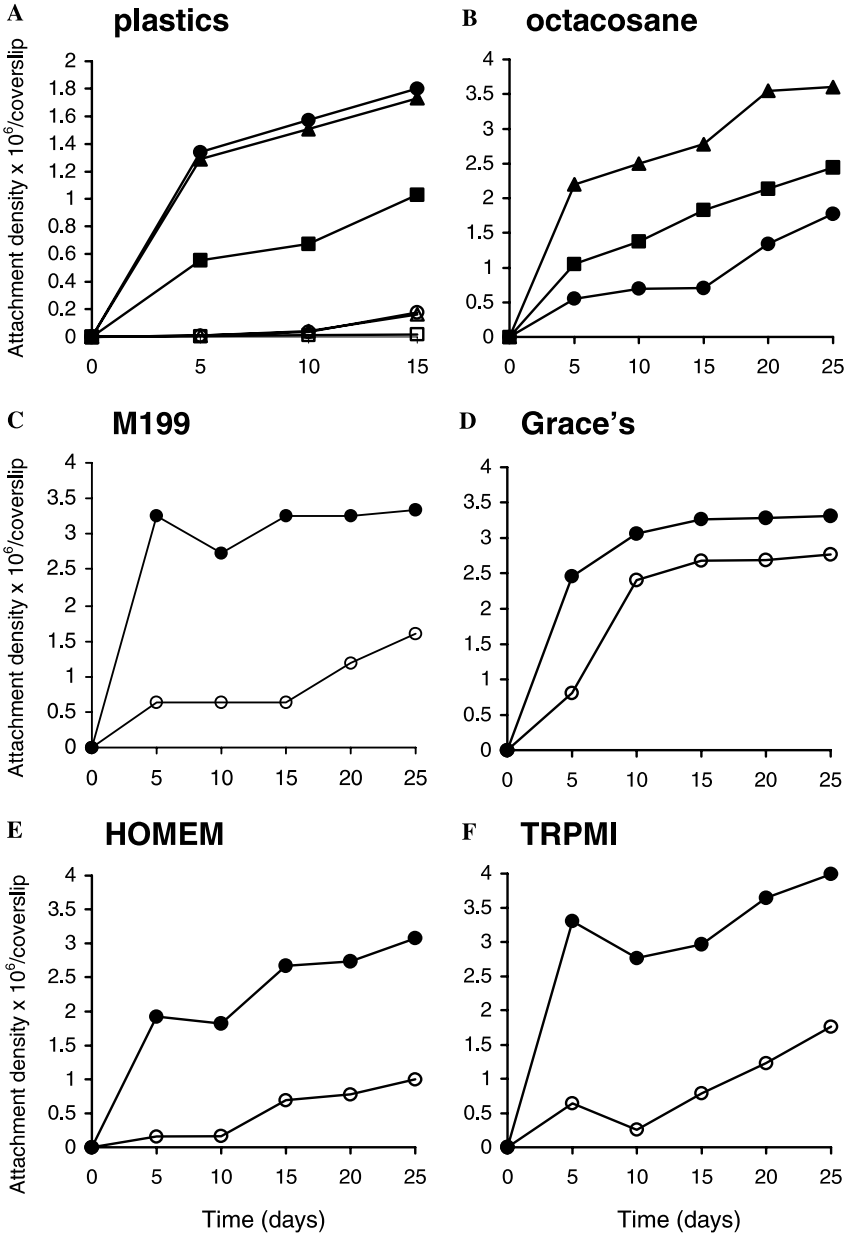

Fig. 2. Influence of various factors on attachment to plastic substrates. (A) Example of an experiment in which *L. mexicana* promastigotes were cultured in M199, 10% FCS and attachment to different substrates was assessed: smooth Melinex (○-○), rough Melinex (●-●), smooth Thermanox (△-△), rough Thermanox (▲-▲), smooth Polyvinyl (□-□), and rough Polyvinyl (■-■). Results are the average of 10 counts per time point. (B) Effect of hydrophobic coating on attachment. *L. mexicana* promastigotes cultured in M199, 10% FCS were offered untreated smooth Melinex (●-●), or Melinex coated with n-octacosane (■-■), or rough Melinex (▲-▲). Results are the average of 10 counts per time point. In each case plastic squares were briefly immersed in the relevant solution, then removed and allowed to drain and air dry before use. The two hydrocarbons tested (*n*-octacosane, Sigma O-2126; paraffin wax, Merck 36114) were both melted, plastic squares briefly immersed, then removed and the hydrocarbons allowed to solidify before sterilising prior to use. (C–F) Influence of culture media on attachment to Melinex substrate. *L. mexicana* promastigotes were cultured in (C) M199, (D) Graces, (E) HOMEM, or (F) TRPMI, each supplemented with 10% FCS. Promastigotes attached either to smooth Melinex (○-○) or rough Melinex (●-●) in each culture medium. Results are the average of 10 counts per time point. Grace's medium was used as supplied by the manufacturers (Life Technologies 21590); HOMEM was prepared and RPMI (Life Technologies 52400) supplemented with tryptose (TRPMI) as described (Berens et al., 1976, 1981). Base media were supplemented with foetal calf serum and gentamicin sulphate.

Thermanox, and Polyvinyl) was compared, both as supplied by the manufacturers (''smooth'') or after abrasion with Emery paper (''rough''). Promastigotes could attach to all three substrates, but was better to Melinex and Thermanox than to Polyvinyl (Fig. 2A). However, in all cases attachment was significantly better to rough substrates than to smooth substrates (Fig. 2A; t test $p < 0.01$ all time points), which confirmed earlier reports on the effects of scratching and supports the idea that binding is via a non-specific interaction. Hydrophobic interactions have been shown to play a role in the non-specific attachment of *T. cruzi* epimastigotes. To address this possibility in *Leishmania*, flagellar attachment was investigated using smooth Melinex coated with *n*-octacosane or paraffin (not shown), and comparing the density of promastigotes that could attach to smooth controls. Both hydrocarbons significantly enhanced attachment, with *n*-octcosane giving the highest density (Fig. 2B; $p < 0.05$ all time points), although this was not as effective as using rough Melinex (Fig. 2B; $p < 0.01$ all time points). In addition to these hydrocarbons, Melinex squares were coated with a range of other substances to test for enhanced attachment, but none of these caused an increase in attachment rate over controls (not shown). The substances tested included: various lectins (Vector Laboratories: wheat germ agglutinin, peanut agglutinin, soybean agglutinin, concanavilin A all at 1 mg/ml for coating); human plasma fibronectin and bovine vitronectin (Life Technologies; 1 mg/ml and 100 μg/ml, respectively); poly-L-lysine (Sigma; 0.1% w/v); and glycol-chitin (Trudel and Asselin, 1989; 1% w/v).

To examine the possible effect that growth in different culture media might have on attachment, promastigotes were exposed to smooth and rough Melinex substrate in four different culture media: M199, TRPMI, Grace's and HOMEM, each supplemented with 10% FCS. In each culture medium attachment was again significantly better to rough than to smooth substrates (Figs. 2C–F), extending the observations made above. In M199, TRPMI, and HOMEM the difference was highly significant ($p < 0.01$ all time points). However, attachment to smooth Melinex was better in Grace's compared to the other media, rough Melinex showing a statistically significant difference at two time points only (5 and 10 days).

The flagellar attachment of only a limited number of *Leishmania* species has been examined to date and inter-isolate variation has not been previously addressed. Therefore, the attachment of eight different isolates of *Leishmania* was examined: two isolates of *L. mexicana*, two of *L. braziliensis*, two of *L. major*, and two of *L. donovani* (Fig. 3). This revealed considerable inter-isolate and inter-species variation in the degree of attachment, ranging from *L. braziliensis* LTB300, which showed the highest level, to *L. major* LV39, which showed the lowest level, with a peak density 30-fold lower than that observed for LTB300. Nevertheless flagellar attachment was observed in all of the isolates examined and was consistently higher to rough Melinex than to smooth Melinex. There was no evidence for a systematic difference in attachment between species, although this possibility cannot be excluded. Thus although it may appear that *L. major* is relatively poor at attaching, it should be noted that there was considerable variation between the two isolates of *L. braziliensis* and between the two isolates of *L. donovani* examined. This indicates that different isolates can vary in attachment, irrespective of the species concerned. Other factors such

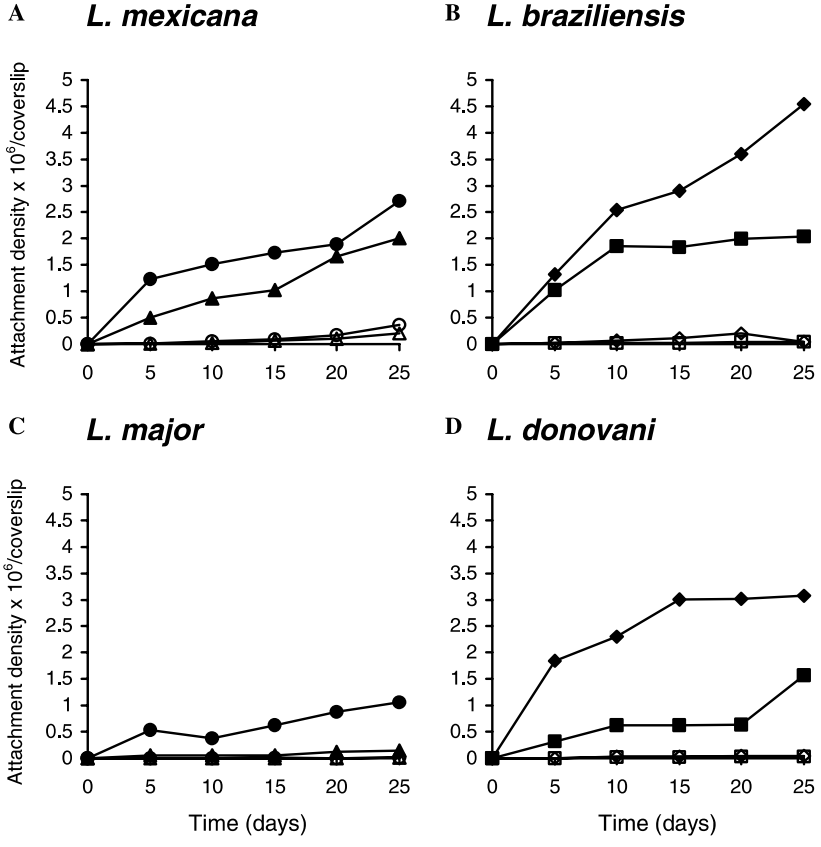

Fig. 3. Comparison of attachment in a range of *Leishmania* species and isolates. (A) *L. mexicana* M379 rough Melinex (●-●) or smooth Melinex (○-○); *L. mexicana* U276 rough (▲-▲) or smooth (△-△). (B) *L. braziliensis* LTB300 rough (◆-◆) or smooth (◇-◇); *L. braziliensis* 436 rough (■-■) or smooth (□-□). (C) *L. major* FV-1 rough (●-●) or smooth (○-○); *L. major* LV39 rough (▲-▲) or smooth (△-△). (D) *L. donovani* LV9 rough (◆-◆) or smooth (◇-◇); *L. donovani* 1S rough (■-■) or smooth (□-□). Results are the average of 10 counts per time point. Promastigotes were cultured in M199, 10% FCS, except *L. braziliensis* where 20% FCS was used.

as adaptation to in vitro culture may well play a role in generating such variation.

The main conclusion of this report is that flagellar attachment binding in *Leishmania* promastigotes is non-specific, since it could be significantly enhanced by scratching the surface of the plastic substrate, and by coating the substrate with hydrocarbons. Although there was considerable variation in the attachment density, it was observed in all the isolates tested and was always enhanced by scratching. Previously only *L. major* was reported as attaching to the scratched surface of tissue culture flasks, whereas *L. donovani* and *Leishmania aethiopica* did not (Maraghi et al., 1987). Here, *L. donovani* was found to attach readily. Thus it appears that Melinex is a superior substrate for the attachment of *Leishmania* promastigotes, compared to tissue culture flasks. Melinex is commercially available and cultures can be readily scaled up for biochemical investigations. The current study also indicates that non-specific flagellar attachment is a property conserved across the genus as the isolates examined included members of both subgenus *Leishmania* (*Leishmania*), i.e., *L. mexicana*, *L. major*, and *L. donovani*, as well as *Leishmania* (*Viannia*), i.e., *L. braziliensis*.

Coating of plastic with the saturated hydrocarbon *n*-octacosane enhanced attachment as previously demonstrated in *T. cruzi* (Schmidt et al., 1998). Thus it appears that in *Leishmania* non-specific flagellar attachment is also enhanced by hydrophobic interaction, and, therefore, this seems likely to be the main mechanism of hemidesmosomal attachment in trypanosomatids. One possible exception is the attachment of *Trypanosoma brucei* epimastigotes to the salivary gland epithelium of *Glossina morsitans*, where a punctate type of hemidesmosome was described (Tetley and Vickerman, 1985). It seems very unlikely that an epithelial layer such as this is coated with hydrocarbons, as was shown to be the case for the rectal cuticle of *Tr. infestans* (Schmidt et al., 1998). Therefore, attachment to the salivary epithelium is likely to be mediated by a different mechanism. It should also be noted that a second and distinct mechanism of flagellar attachment is also known in *Leishmania*, but which does not involve formation of hemidesmosomes. In this case, promastigotes are able to bind to the microvillar surface of the midgut epithelium in phlebotomine sandflies via lipophosphoglycan, the major cell surface glycoconjugate (reviewed by Sacks and Kamhawi, 2001). Such binding helps to maintain the infection during bloodmeal excretion in the sandfly host.

Finally, it was shown that both the culture medium and isolate used could influence flagellar attachment. The reasons for these effects are not understood, but could be due to variation in the development and differentiation of haptomonad promastigotes in vitro. The precursors of haptomonad promastigotes in vivo are not certain (Rogers et al., 2002), but adaptation to in vitro culture and specific culture conditions are known to be able to influence differentiation of other life cycle stages such as the infective metacyclic promastigotes (Bates and Tetley, 1993; Zakai et al., 1998). Further studies on attached promastigotes are required to fully understand their place in *Leishmania* development, and their role in transmission of disease.

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References

Bates, P.A., Tetley, L., 1993. *Leishmania mexicana*: induction of metacyclogenesis by culture at acidic pH. Exp. Parasitol. 76, 412–423.

Beattie, P., Gull, K., 1997. Cytoskeletal architecture and components involved in the attachment of *Trypanosoma congolense* epimastigotes. Parasitology 115, 47–55.

Berens, R.L., Brun, R., Krassner, S.M., 1976. A simple monophasic medium for axenic culture of hemoflagellates. J. Parasitol. 62, 360–365.

Berens, R.L., Marr, J.J., LaFon, S.W., Nelson, D.J., 1981. Purine metabolism in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 3, 187–196.

Bonaldo, M.C., Souto-Padron, T., De Souza, W., Goldenberg, S., 1988. Cell-substrate adhesion during *Trypanosoma cruzi* differentiation. J. Cell Biol. 106, 1349–1358.

Brooker, B.E., 1971. Flagellar attachment and detachment of *Crithidia fasciculata* to the gut wall of *Anopheles gambiae*. Protoplasma 73, 191–202.

Gray, M.A., Cunningham, I., Gardiner, P.R., Taylor, A.M., Luckins, A.G., 1981. Cultivation of infective forms of *Trypanosoma congolense* at 28 °C from trypanosomes in the proboscis of *Glossina morsitans*. Parasitology 82, 81–95.

Hendry, K.A.K., Vickerman, K., 1988. The requirement for epimastigote attachment during division and metacyclogenesis in *Trypanosoma congolense*. Parasitol. Res. 74, 403–408.

Hommel, M., Robertson, E., 1976. In vitro attachment of trypanosomes to plastic. Experientia 32, 464–466.

Killick-Kendrick, R., Molyneux, D.H., Ashford, R.W., 1974. *Leishmania* in phlebotomid sandflies. I. Modifications of the flagellum associated with attachment to the midgut and oesophageal valve of the sandfly. Proc. R. Soc. London Ser. B 187, 409–419.

Killick-Kendrick, R., Wallbanks, K.R., Molyneux, D.H., Lavin, D.R., 1988. The ultrastructure of *Leishmania major* in the foregut and proboscis of *Phlebotomus papatasi*. Parasitol. Res. 74, 586–590.

Kleffman, T., Schmidt, J., Schaub, G., 1998. Attachment of *Trypanosoma cruzi* epimastigotes to hydrophobic substrates and use of this property to separate stages and promote metacyclogenesis. J. Eukaryot. Microbiol. 45, 548–555.

Kollien, A.H., Schmidt, J., Schaub, G.A., 1998. Modes of association of *Trypanosoma cruzi* with the intestinal tract of the vector *Triatoma infestans*. Acta Trop. 70, 127–141.

Maraghi, S., Mohamed, H.A., Wallbanks, K.R., Molyneux, D.H., 1987. Scratched plastic as a substrate for trypanosomatid attachment. Ann. Trop. Med. Parasitol. 81, 457–458.

Molyneux, D.H., 1969. The fine structure of the epimastigote forms of *Trypanosoma lewisi* in the rectum of the flea, *Nosopsyllus fasciatus*. Parasitology 59, 55–66.

Molyneux, D.H., 1975. *Trypanosoma (Megatrypanum) melophagium*: modes of attachment of parasites to midgut, hindgut and rectum of the sheep ked, *Melophagus ovinus*. Acta Trop. 32, 65–74.

Rogers, M.E., Chance, M.L., Bates, P.A., 2002. The role of promastigote secretory gel in the origin and transmission of the infective stage of *Leishmania mexicana* by the sandfly *Lutzomyia longipalpis*. Parasitology 124, 495–507.

Sacks, D.L., Kamhawi, S., 2001. Molecular aspects of parasite–vector and vector–host interactions in leishmaniasis. Annu. Rev. Microbiol. 55, 453–483.

Schmidt, J., Kleffman, T., Schaub, G.A., 1998. Hydrophobic attachment of *Trypanosoma cruzi* to a superficial layer of the rectal cuticle in the bug *Triatoma infestans*. Parasitol. Res. 84, 527–536.

Tetley, L., Vickerman, K., 1985. Differentiation in *Trypanosoma brucei*: host–parasite cell junctions and their persistence during acquisition of the variable antigen coat. J. Cell Sci. 74, 1–19.

Thevanez, P., Hecker, H., 1980. Distribution and attachment of *Trypanosoma* (*Nannomonas*) *congolense* in the proximal part of the proboscis of *Glossina morsitans morsitans*. Acta Trop. 37, 163–173.

Trudel, J., Asselin, A., 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal. Biochem. 178, 362–366.

Vickerman, K., 1973. The mode of attachement of *Trypanosoma vivax* in the proboscis of the tsetse fly, *Glossina fuscipes*. J. Protozool. 20, 394–404.

Vickerman, K., Tetley, L., 1990. Flagellar surfaces of parasitic protozoa and their role in attachment. In: Bloodgood, R.A. (Ed.), Ciliary and Flagellar Membranes. Plenum Publishing Corporation, New York, pp. 267–304.

Zakai, H.A., Chance, M.L., Bates, P.A., 1998. In vitro stimulation of metacyclogenesis in *Leishmania braziliensis*, *L. donovani*, *L. major* and *L. mexicana*. Parasitology 116, 305–309.