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Abortive infection of *Lutzomyia longipalpis* insect vectors by aflagellated *Ld*ARL-3A-Q70L overexpressing *Leishmania amazonensis* parasites

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Summary

Leishmania donovani **ADP-ribosylation factor-like protein 3A (*Ld*ARL-3A) is a small G protein isolated from the protozoan parasite *L. donovani* with no defined physiological function. Previously [Cuvillier, A., Redon, F., Antoine, J.-C., Chardin, P., DeVos, T., and Merlin, G. (2000) J Cell Sci 113: 2065–2074] we have shown that overexpression in *L. amazonensis* promastigotes of the mutated protein *Ld*ARL-3A-Q70L, which remains constitutively associated with GTP, leads to the disappearance of the flagellum but does not impair cell viability or growth. Here we report that parasites overexpressing *Ld*ARL-3A-Q70L can invade *in vitro* cultivated macrophages to the same extent as control cells, demonstrating that the flagellum is not necessary for attachment to or engulfment into macrophages. These infections are productive because amastigotes differentiate and multiply. However, aflagellated *Ld*ARL-3A-Q70L-overexpressing *Leishmania* promastigotes could not survive in experimentally infected *Lutzomyia longipalpis* insect vectors, in contrast to untransfected or native *Ld*ARL-3A-overexpressing cells. Overexpression of the native and mutated proteins did not modify *in vitro* procyclic to metacyclic lipophosphoglycan maturation or differentiation from procyclic to metacyclic promastigotes, nevertheless there is a block in transmission of *Leishmania*. Better understanding of *Ld*ARL-3A pathways, notably those regarding flagellum biogenesis, may lead to the future development of *Leishmania*-specific drugs, which may stop parasite transmission in nature without affecting other species.**

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Introduction

Leishmania are protozoan parasites of the Mastigophora subphylum, Kinetoplastida order and Trypanosomatidae family. They are responsible for considerable suffering and morbidity among humans: more than 12 million individuals are infected and two million new cases are estimated to occur every year (Choi and Lerner, 2001; WHO/OMS, 2001). Treatment with pentavalent antimony-based drugs has been used for decades despite toxicity and loss of efficiency with the emergence of resistant strains. Alternative therapies, novel concepts and more adequate financial means are urgently needed to combat this illness (Guerin *et al.*, 2002). As a prerequisite, considerable effort in basic research is mandatory for a better understanding of the complex relationships between hosts and parasites.

Leishmania exists alternatively as a flagellated extracellular promastigote inside the alimentary tract of female sandfly vector or as an aflagellated intracellular amastigote within acidic parasitophorous vacuoles of the mammalian host mononuclear phagocyte (Molyneux and Killick-Kendrick, 1987). Passage from one host to another occurs when a female sandfly bites a mammal for a bloodmeal.

Differentiation from amastigotes to promastigotes in the insect gut is associated with considerable morphological (from ovoid aflagellated to an elongated flagellated form) and physiological (33°C–37°C to ambient temperature, acidic to neutral environment) changes. Amastigotes escape from lysed ingested macrophages and divide once or twice before transforming to actively dividing procyclic promastigotes which display an inherent capacity to attach to the sandfly midgut epithelial cells. This attachment allows the parasites to avoid elimination during excretion of the digested blood meal (Molyneux and Killick-Kendrick, 1987). The final stage of differentiation is to non-dividing metacyclic promastigotes (Lawyer *et al.*, 1990), allowing detachment from the epithelium and migration to the anterior part of the gut, thus available for injection into a mammal at the next sandfly bloodmeal. This whole process takes between one to two weeks.

Several intermediate developmental forms have been described during the differentiation of promastigotes, from the non-infective procyclic stage to the infective metacyclic stage, both morphologically (Molyneux *et al.*, 1975) and physiologically (Sacks and Perkins, 1984; 1985; Lainson *et al.*, 1987). Of these forms, only procyclic (well established by *in vitro* cultivation) and metacyclic (recently partially characterized biochemically) (Howard *et al.*, 1987; Lira *et al.*, 1998; Sacks and Melby, 1998; Courret *et al.*, 1999) promastigotes are able to be cultured for several *Leishmania* species.

Promastigote attachment to and detachment from the epithelial midgut has been clearly related to developmental changes in the chemical nature of the lipophosphoglycan (LPG) that constitutes the main outer component of the *Leishmania* membrane (McConville *et al.*, 1992; Pimenta *et al.*, 1992; Sacks, 1992; Sacks *et al.*, 1995; Saraiva *et al.*, 1995; Mahoney *et al.*, 1999). Lipophosphoglycan is also involved in the specific interactions between particular sandfly and *Leishmania* species (Borovsky and Schlein, 1987; Pimenta *et al.*, 1994; Sacks *et al.*, 1994; 2000; Sacks, 2001; Kamhawi *et al.*, 2000).

However, early electron microscopic observations showed that another factor is probably involved in the attachment of promastigotes to the sandfly midgut and foregut epithelia. Indeed, the flagellum was seen to adhere tightly to epithelial cells and form hemidesmosomes and sometimes penetrate between the microvilli and into the cytoplasm itself, also showing morphological changes in the adherence zones (Killick-Kendrick *et al.*, 1974a,b; 1977; Molyneux *et al.*, 1975; Walters *et al.*, 1987; 1989a,b).

ADP-ribosylation factor-like proteins (ARLs) are small G proteins whose function is still not well characterized (Boman and Kahn, 1995; Lin *et al.*, 2000; Oloumi *et al.*, 2002; Rosenwald *et al.*, 2002; Schurmann *et al.*, 2002; Sharer *et al.*, 2002). In a previous work (Cuvillier *et al.*, 2000) we described *Ld*ARL-3A, which is expressed only in the promastigote form of *Leishmania* cells. We overexpressed either native or mutated *Ld*ARL-3A in *L. amazonensis* promastigotes; the Q70L mutant GTP-constitutively bound form led to the disappearance of the flagella, in contrast to native protein overexpression. There was no apparent impact on parasite viability in culture or infectivity, because cutaneous lesions developed in a similar proportion for all tested clones in Balb/c mice footpads after experimental infection. However, the flagellum is a rather prominent feature of *Leishmania* morphology and it would be surprising that evolution had selected for it without an advantage at some step of the parasite life cycle. Experimental conditions are far different from those found in nature, and it appeared of significant interest to examine in detail macrophage infection and the more rarely investigated insect vector infection. Here we show that while macrophage infection appears normal, aflagellated *Leishmania* cells do not survive in insects and thus transmission is blocked.

Results

Wild-type exponentially growing *L. amazonensis* promastigotes display a flagellum about the length of the cell body (Fig. 1, P-1). We have already shown that overexpression of the *Ld*ARL-3A small G protein, using an episomal pTEX-based vector, does not change this phenotype (Fig. 1, P-2), whereas the flagella become very short with the constitutively GTP-binding form of the protein *Ld*ARL-3 A-Q70L (Fig. 1, P-3) (Cuvillier *et al.*, 2000). 3A1-purified metacyclic promastigotes display a much longer flagellum for wild-type (Fig. 1, M-1) and native *Ld*ARL-3A-overexpressing cells (Fig. 1, M-2) but not in *Ld*ARL-3A-Q70L-overexpressing cells (Fig. 1, M-3).

*Ld*ARL-3A as well as *Ld*ARL-3 A-Q70L transformed *Leishmania* clones could be divided in two classes (Cuvillier *et al.*, 2000), those infectious to Balb/c mice generating footpad lesions of at least 1 cm diameter in about two

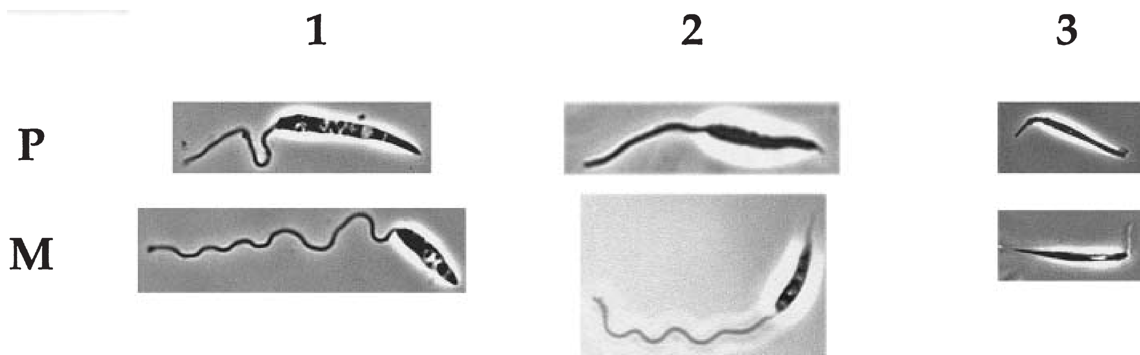

Fig. 1. Morphology of procyclic and metacyclic promastigotes. Exponentially growing procyclic and 3A1-purified metacyclic promastigotes were observed under phase-contrast light microscopy. Approximate size of a procyclic cell body: 10 μm; same scale for every picture. 1: *L. amazonensis* BA125 parental line; 2: *Ld*ARL-3A overexpressing cells; 3: *Ld*ARL-3A-Q70L overexpressing cells. P, procyclic promastigotes; M, metacyclic promastigotes.

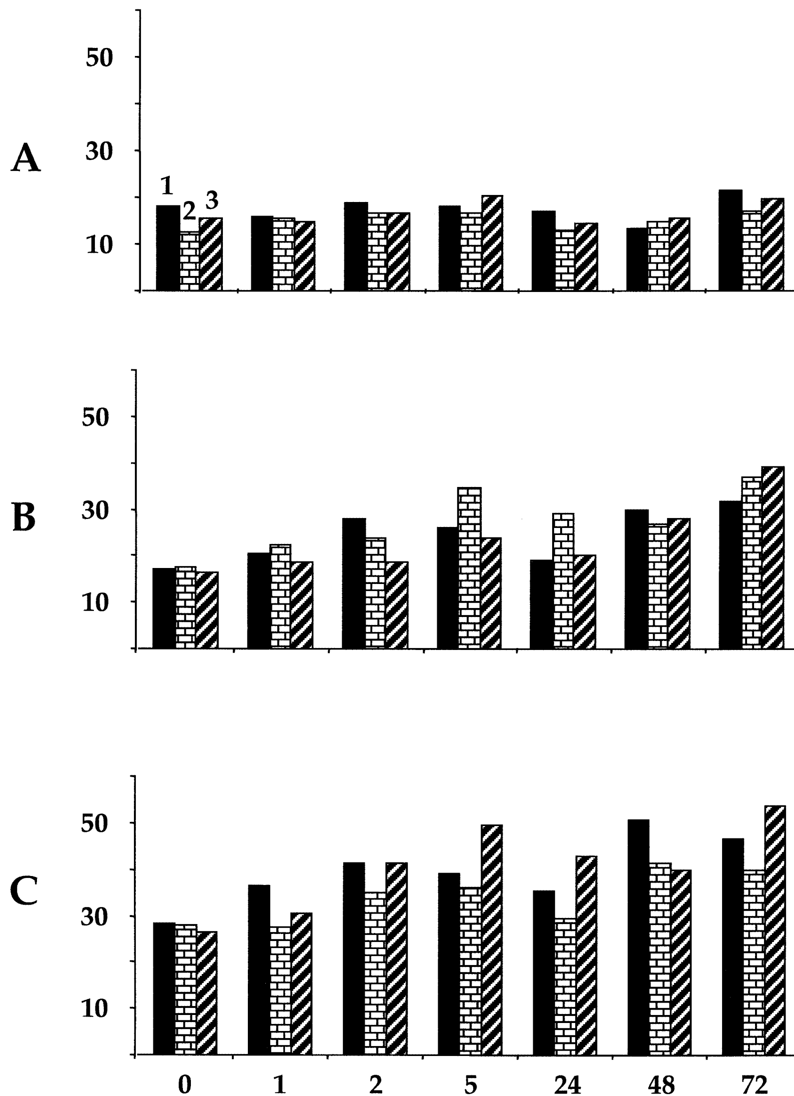

Fig. 2. *In vitro* infection of murine bone marrow macrophages. Macrophages were isolated, cultivated, infected and the cultures processed as described in *Experimental procedures*. 1: *L. amazonensis* BA125 parental line; 2: *Ld*ARL-3A overexpressing cells; 3: *Ld*ARL-3A-Q70L overexpressing cells. Ordinate: per cent of infected macrophages (total count: 200 macrophages).

months (as did the parental line) and those who did not generate lesions until much later (4–5 months) if at all. In this study, we chose two equivalent infectious clones of each transformed *Leishmania* line with similar results obtained for both.

In vitro *murine bone marrow macrophage infection*

In vitro cultivated mouse bone marrow macrophages were infected for 4 h at 4°C with 5-day-old stationary phase promastigotes of the parental BA125, *Ld*ARL-3A- (flagellated) or *Ld*ARL-3A-Q70L (aflagellated)-overexpressing *L. amazonensis* cells. At different times thereafter (0, 1, 2, 5, 24, 48 and 72 h), the cultures were fixed and inspected visually.

In the experiments shown in Fig. 2, infections were done at a various infection ratios, 1 promastigote per macrophage (panel A), 4/1 (panel B) and 10/1 (panel C). Early after infection (up until 5 h), the percentage of macrophages with attached or internalized promastigotes varied from 15–20% to 40–50% depending on the infectivity ratio; in some experiments, as many as 100% of macrophages were infected (not shown). However, within the same experiment, these percentages were never significantly different between the three types of *Leishmania* cells. In addition, the orientation of the promastigotes in contact with the macrophages was apparently random. Thus, the flagellum does not seem to be essential for these steps as previously reported (Mosser and Brittingham, 1997).

At later times (24–72 h), amastigotes became visible within individualized parasitophorous vacuoles. The percentage of infected macrophages did not change much, whereas the number of amastigotes per macrophage (Table 1) increased from 1 to 2 at 24 h to a mean of 4–6 at 72 h, with many macrophages harbouring only 1

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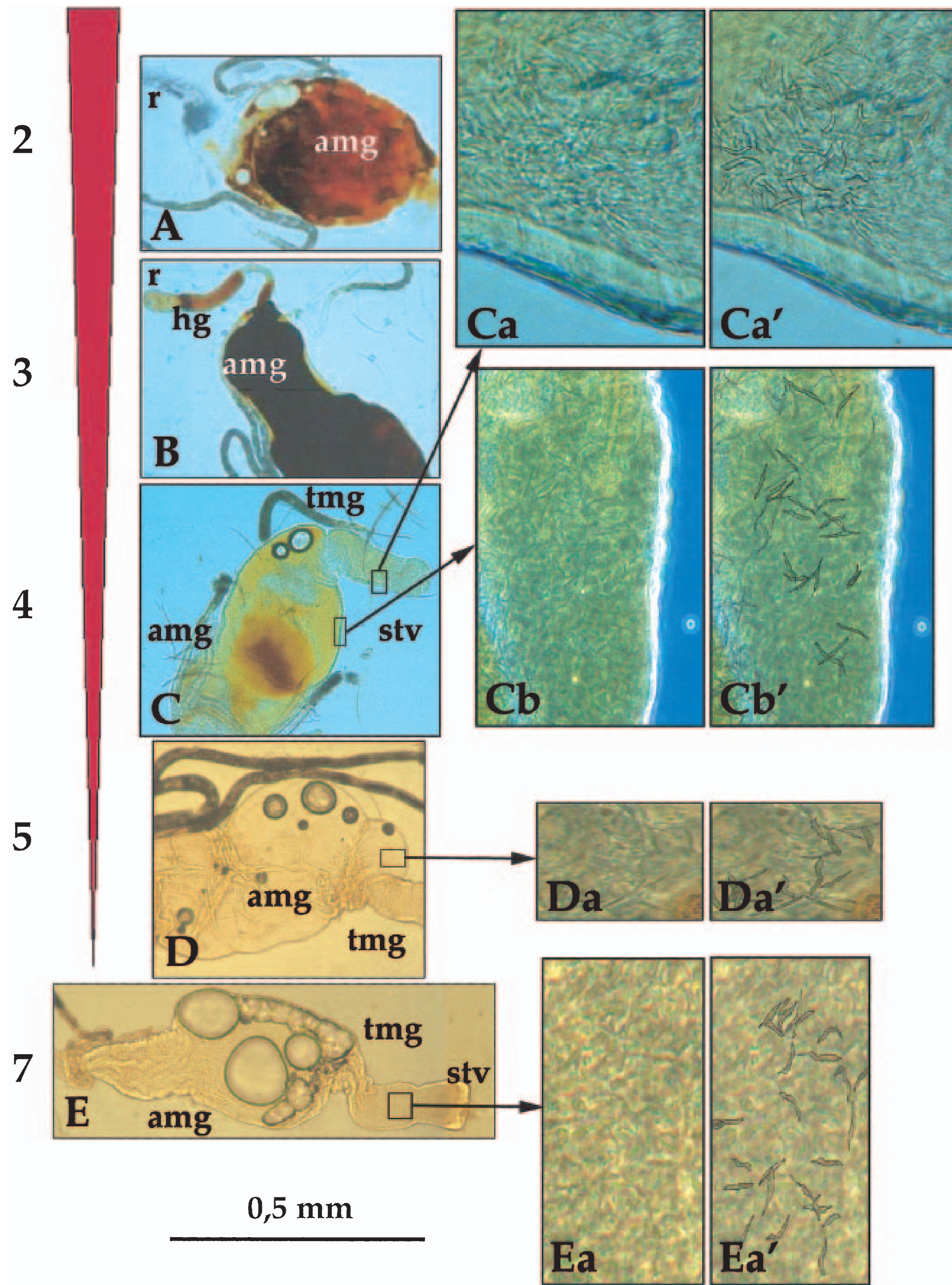

Fig. 3. Follow up of an experimental infection of *Lu. Longipalpis* by wild-type *L. amazonensis* 2–7 days after an experimental bloodmeal containing BA125 *L amazonensis* amastigotes, female insects were killed and their guts observed under a light microscope. The left side vertical red arrow symbolizes the amount of blood present in the guts, and the numbers 2–7, the days after the bloodmeal. From day 4, with the blood digestion, *L. amazonensis* promastigotes become visible by transparency (Ca is an enlargement and Ca′ the same enlargement where *Leishmania* promastigotes have been highlighted by hand; same for Cb/Cb′, Da/Da′ and Ea/Ea′). Amg, abdominal midgut; hg, hindgut; r, rectum; stv, stomodeal valve; tmg, thoracic midgut. Total length of the gut: approximately 0.5 mm.

Table 1. Number of *Leishmania* parasites per infected macrophage.

Hours after infection	*L. amazonensis* BA125			*L. amazonensis* BA125/pTEX-*Ld*ARL-3A			*L. amazonensis* BA125/pTEX-*Ld*ARL3A-Q70L		
	1/1	4/1	10/1	1/1	4/1	10/1	1/1	4/1	10/1
0	1.0 ± 0.0	1.1 ± 0.2	1.3 ± 0.7	1.0 ± 0.0	1.1 ± 0.2	1.2 ± 0.5	1.1 ± 0.3	1.1 ± 0.3	1.2 ± 0.4
1	1.0 ± 0.2	1.4 ± 0.7	1.4 ± 0.8	1.2 ± 0.4	1.3 ± 0.7	1.6 ± 1.1	1.1 ± 0.3	1.1 ± 0.3	1.6 ± 1.0
2	1.3 ± 0.6	1.1 ± 0.3	1.4 ± 0.7	1.1 ± 0.4	1.2 ± 0.6	1.3 ± 0.7	1.2 ± 0.6	1.4 ± 0.7	2.0 ± 1.5
5	1.1 ± 0.2	1.4 ± 0.7	1.6 ± 1.0	1.3 ± 0.7	1.8 ± 1.2	1.7 ± 1.3	1.2 ± 1.3	1.8 ± 1.1	2.1 ± 1.5
24	1.6 ± 1.1	1.5 ± 1.2	1.5 ± 0.8	1.7 ± 1.5	2.1 ± 2.0	2.0 ± 1.7	1.4 ± 1.3	1.9 ± 1.2	2.1 ± 1.5
48	1.7 ± 0.9	1.7 ± 1.0	3.9 ± 3.4	2.1 ± 1.6	2.0 ± 1.2	2.5 ± 2.0	1.8 ± 1.5	2.1 ± 1.6	2.9 ± 3.1
72	2.7 ± 1.7	3.6 ± 2.4	6.7 ± 7.0	3.7 ± 4.3	2.8 ± 3.8	3.9 ± 4.5	2.7 ± 3.2	3.1 ± 2.5	4.0 ± 4.5

In the experiment depicted in Fig. 2, *Leishmania* cells attached to (first five hours) or internalized (24 h and later) into macrophages were counted. The ratio between the numbers of promastigotes and macrophages is indicated on top of each column. The mean number for 200 infected macrophages and the standard deviation are indicated.

amastigote – probably representing secondary infections – but many also with up to 25. Amastigotes could be observed inside parasitophorous vacuoles of macrophages up to 8 days after infection (not shown).

In conclusion, neither *Ld*ARL-3A and *Ld*ARL-3A-Q70L overexpression nor the absence of flagellum impaired the promastigote infectivity toward macrophages *in vitro*.

Infection of sandflies with amastigotes isolated from infected macrophages

Murine bone marrow macrophages were cultivated *in vitro* and infected with stationary phase promastigotes of the three types of parasites, i.e. wild-type BA125, *Ld*ARL-3A and *Ld*ARL-3A-Q70L overexpressing *L. amazonensis*. Two days later, amastigotes were isolated and 10^7 amastigotes of each type were used for *Lu. longipalpis* female infection by artificial blood feeding. For each experiment, a total of 150–200 female flies were infected. About 60–70% survived after 7 days depending on the experiment. Every day following infection until day 7, 12–15 live flies were sacrificed. Digestive tracts were dissected from the stomodeal valve to pylorus, and either observed under a light microscope or macerated before counting *Leishmania* cells.

The *Lu. longipalpis* gut can be divided into three parts, the foregut inside the head, comprising the proboscis, cibarium, pharynx and oesophagus; the midgut from the stomodeal valve to the pylorus, itself divided into thoracic and abdominal midgut; and the hindgut from the pylorus to the rectum. In the course of a *L. amazonensis* infection subsequent to a bloodmeal (Fig. 3), blood is concentrated inside a peritrophic membrane in the abdominal midgut. From day 2–3, the peritrophic membrane is under digestion and undigested products are excreted (Fig. 3, visible on top-left of the picture on day 3) whereas the parasites, which have differentiated from non-motile amastigotes to motile promastigotes, escape, divide actively, and attach to the midgut epithelium thus avoiding elimination with undigested products. Although they are visible and motile on day 4, it is not possible to differentiate attached from free promastigotes at this magnification. At later times, metacyclic promastigotes migrate near the stomodeal valve (visible for example on day 7 on Fig. 3), where they secrete plug-like proteophosphoglycans (Stierhof *et al.*, 1999; Ilg, 2000) which probably block the digestive tract, possibly forcing the insect to expel it with the *Leishmania*, thus making a new bloodmeal possible and initiating a new infection cycle.

Several experiments were done using wild-type *L. amazonensis* and two sets of virulent and independent *Ld*ARL-3A- and *Ld*ARL-3A-Q70L-overexpressing clones; the results were the same. To quantify the extent of insect infections, individual midguts were dissected, macerated and the *Leishmania* cells released in the surrounding medium were counted. For the whole course of the experiment, the percentage of infected guts was not significantly different for wild-type BA125 (Fig. 4A, left part) and *Ld*ARL-3 A overexpressing (Fig. 4A, middle part) *L. amazonensis*. On days 2–3, dissection and maceration of the guts (the presence of blood in all guts did not allow visual localization of the parasites) with subsequent parasite counts revealed that 100–97% and 100–75% of the flies were infected, respectively; the number of parasites – with the majority still at the amastigote stage – per midgut was high (Day 2: 44 000 ± 10 000 and 40 000 ± 6000 per gut, respectively; Day 3: 18 000 ± 7000 and 20 000 ± 8000 per gut). Later, few remaining amastigotes but more and more promastigotes became visible, concomitant with the disappearance of blood; however, this disappearance was not completely synchronous for all guts so that on day 5, 35% of them still contained blood and were all infected (9000 ± 3500 and 7000 ± 1500 per gut, respectively), and among the 65% without blood, 51% (9000 ± 3500 per gut) and 89% (2500 ± 600 per gut), respectively, were infected. By day 7, a time when the blood has been completely digested, 47% and 75% of the flies remained infected and the mean number of promastigotes per gut remained high

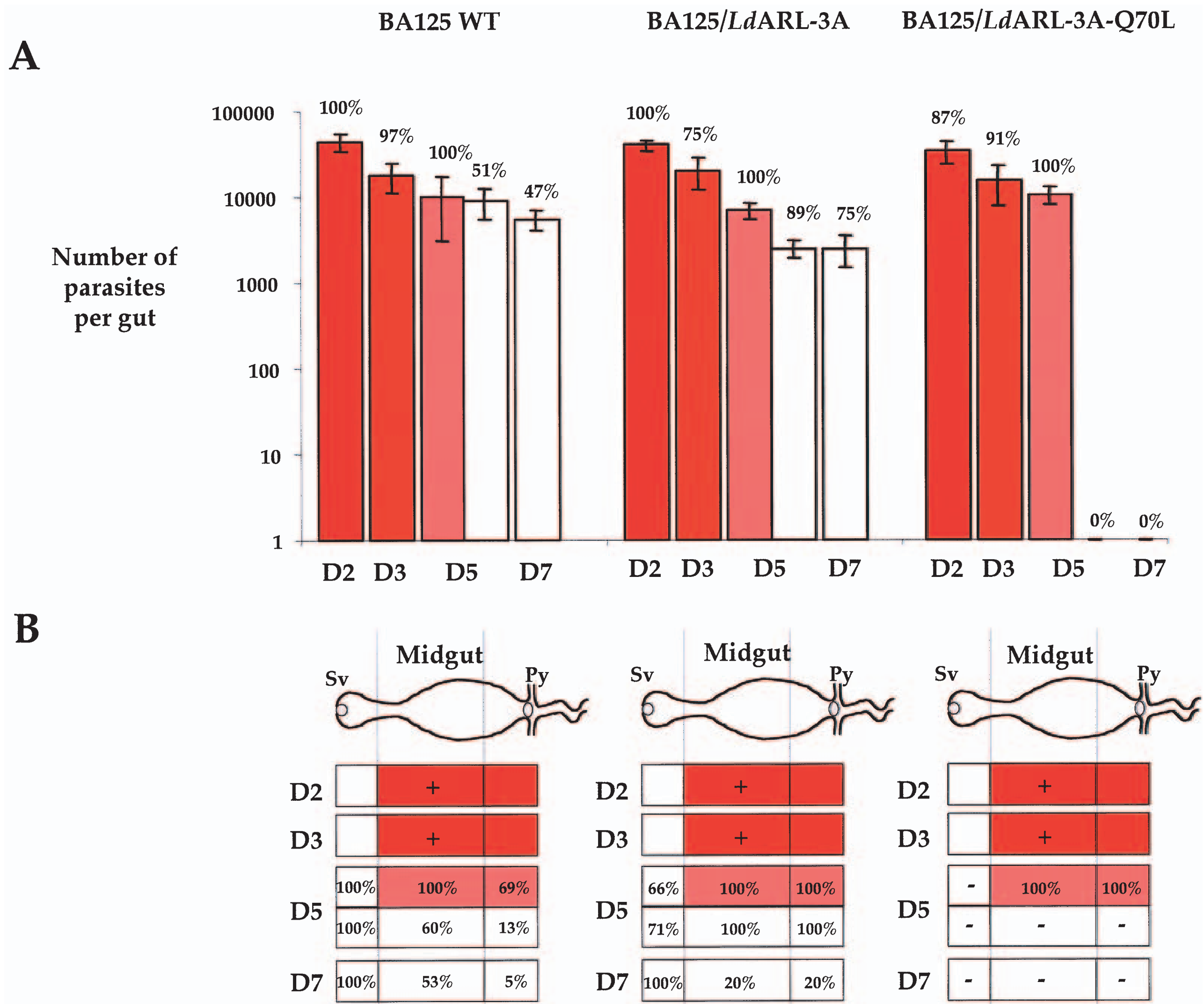

Fig. 4. Survival of *L. amazonensis* lines inside *Lu. Longipalpis* intestinal tract.
A. Number of parasites present in the guts during the course of the infection. Insects were bloodfed and infected with wild-type (WT) BA125 *L amazonensis* (left), LdARL-3A- (middle part) or LdARL-3A-Q70L- (right) overexpressing parasites; 10–12 individuals were killed from day 2 to day 7 (D2-D7), guts isolated, observed and macerated before parasite counting. More or less intense red colour symbolizes the presence of more or less blood in the gut. On day 5, guts with and without remaining blood were observed separately. The percentage of infected guts is indicated on top of the histograms.
B. Localization of parasites within the guts. The percentages represent the proportion of infected midguts harbouring parasites in the vicinity of the stomodeal valve (Sv), the midgut and around the pylorus (Py). Plus sign means presence of parasites, minus sign, absence.

(5500 ± 1500 and 2500 ± 1000 per gut). Now differentiated promastigotes were concentrated in the thoracic midgut near the stomodeal valve (Fig. 4B, left and middle part) whereas they had almost completely disappeared from the pyloric area and abdominal midgut.

The results were significantly different when amastigotes from *Ld*ARL-3A-Q70L-overexpressing clones were used. First, infections appeared similarly on days 2 and 3, with 87% and 91% of the flies infected (Fig. 4A right), but again because of the blood, localization within the gut was not possible. Counting of the amastigotes within the homogenates on days 2 and 3 revealed the presence of 34 000 ± 10 000 and 15 500 ± 7700 parasites per gut. On days 4, 5, 6 and 7, however, when the blood had disappeared after digestion/excretion, *Leishmania* cells also disappeared (Fig. 4B right). On day 5, the only parasites that could be found were in blood-containing guts, 30% of all guts, 100% of those guts being still infected with 10 500 ± 2500 parasites per gut. Only once on day 6 was a fly found with about 100 aflagellated immotile promastigotes located exclusively in the abdominal midgut, but it was not possible to tell whether they were attached to the epithelium or not. However, at this time interval, flagellated promastigotes of the other *Leishmania* strains were all

located near the stomodeal valve, their obligatory location for a successful transmission to a mammalian host. On day 7, neither blood in any gut nor *Leishmania* was found in all flies examined.

In conclusion, there was a clear correlation between the presence of a flagellum and the sustainability of sandfly infection by *Leishmania* promastigotes: as long as blood remained in the guts, all types of parasites remained there also; but once the blood had been digested and/or expelled, only wild-type and *Ld*ARL-3A-overexpressing parasites remained whereas *Ld*ARL-3A-Q70L-overexpressing ones disappeared.

Is the absence of the flagellum solely responsible for parasite disappearance from the insect gut?

Overexpression of *Ld*ARL-3 A-Q70L might modify the differentiation process from amastigotes to promastigotes and metacyclogenesis. We tried to address this question with the available tools at our disposal.

Concerning amastigote to promastigote differentiation, amastigote and promastigote forms were separately counted after maceration of blood-containing guts up to day 5, based on a morphological appearance: round and small versus elongated cell body. According to this criterion, there was no difference in the kinetics of promastigote appearance (data not shown) and the same numbers of promastigotes were found in the blood-containing guts on days 3–5 for the 3 types of *Leishmania.* From the pioneering work of Sacks and co-workers (Sacks, 2001), surface LPG is known to be an essential factor for parasite gut attachment. We examined the LPG antigenicity of the promastigotes *in vitro* with the parental strain *L. amazonensis* BA125, *Ld*ARL-3 A and *Ld*ARL-3 A-Q70L-GTP overexpressing clones maintained for 5 days in stationary phase at maximal density; the 3A1 monoclonal antibody directed against *L. amazonensis* procyclic LPG (Courret *et al*., 1999) was used for agglutination of procyclic promastigotes and 80–90% of the cells were agglutinated in all cases, demonstrating that surface LPG is probably unaffected by the overexpression. Differentiation thus appears comparable for all types of cells.

Concerning metacyclogenesis inside guts, it was not possible to directly examine aflagellated cells since they had disappeared from the guts. *In vitro*, mAb 3A1 non-agglutinated, i.e. metacyclic promastigotes of the above experiments were obtained in the same proportions for the three types of parasites, varying from 4 to 15% between experiments. Light microscopy observations showed practically homogeneous metacyclic populations for the parental line BA125 and the *Ld*ARL-3A overexpressing cells, with a typical morphology, i.e. a very long flagellum and a short cell body (Fig. 1, lanes 1 and 2, bottom) compared to a shorter flagellum and a longer cell body for procyclics (Fig. 1, top). In the absence of flagellum however, no obvious morphological difference between procyclic and metacyclic promastigotes could be observed for *Ld*ARL-3 A-Q70L overexpressing cells (Fig. 1, lane 3, top and bottom).

Western blots showed that the 27 kDa CPB-2 cysteine protease, which is preferentially expressed in metacyclics (Mottram *et al*., 1997), was equally detectable in the 3A1-purified metacyclic promastigotes with (Fig. 5, 1-M and 2-M) and without (Fig. 5, 3-M) flagellum, while it was expressed at much lower levels by procyclic promastigotes (Fig. 5, 1-, 2-, 3-P).

Thus, *Ld*ARL-3A- and *Ld*ARL-3 A-Q70L-overexpressing cells both display apparently procyclic LPG, which is recognized by the mAb 3A1, and both clones seem to differentiate to metacyclic promastigotes as evidenced by the expression of the metacyclic-specific marker CPB-2 cysteine protease.

These data are supportive, if not definitive, to correlate the absence of the *Leishmania* flagellum and the disappearance of parasite from the guts.

Discussion

All three kinds of parasites – wild type, native and mutant *Ld*ARL-3A overexpressing *L. amazonensis* – were able to generate 1 cm diameter cutaneous lesions about 2 months after injection of 5.10^6 cells into Balb/c mice footpads (Cuvillier *et al*., 2000). However in this assay, such a high innoculum does not mimic natural infections where a sandfly injects only 100–1000 cells during a

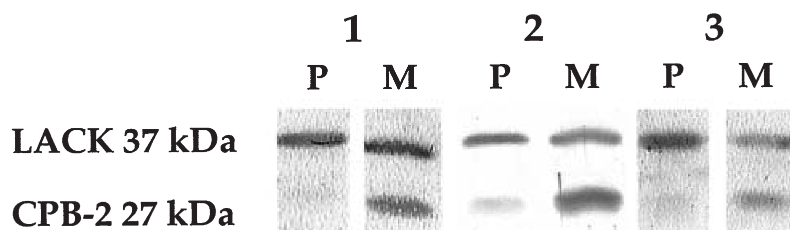

Fig. 5. Characterization of procyclic and metacyclic promastigotes. 3×10^6 cells were lysed, submitted to SDS-PAGE; Western blots were developed with anti-LACK (Mougneau *et al*., 1995) (to show that equal amounts of extracts were loaded on the gels) and anti-CPB 2.8 immune sera which cross-reacts with metacyclic-specific CPB-2 (Mottram *et al*., 1997). 1: *L. amazonensis* BA125 parental line; 2: *Ld*ARL-3A overexpressing cells; 3: *Ld*ARL-3A-Q70L overexpressing cells. P, procyclic promastigotes; M, metacyclic promastigotes.

bloodmeal (Belkaid *et al*., 2000), and in addition may not directly reflect the actual infectivity of the clones. Although *Lu. longipalpis* sandfly experimental infections with *L. amazonensis* can be performed from mouse footpad lesions, we have observed that among the amastigotes present in 2-month-old lesions, only half remained Neomycin-resistant, indicating that the other half had lost the pTEX episomes in the absence of Neomycin selection pressure and did not overexpress the recombinant proteins (Cuvillier *et al*., 2000). Alternatively, transformed promastigotes can be used to infect macrophages *in vitro* where amastigotes are available for sandfly infection after only a few days, i.e. only after a very limited number of cell divisions with no expected episomal loss. Subsequently, recombinant protein overexpression resumes as soon as the cells re-differentiate to promastigotes within the insect gut, with the short flagellum phenotype restored for all appropriate cells. Thus, it was necessary to check whether the transformed clones used for sandfly infections were also infectious toward macrophages.

Indeed this was the case: aflagellated *Ld*ARL-3A-Q70L overexpressing *L. amazonensis* cells were able to infect mice *in vivo* (Cuvillier *et al*., 2000) and macrophages *in vitro* (present study) to the same extent as flagellated promastigotes. *In vivo* and *in vitro* infections were done using 5-day stationary phase promastigotes, i.e. 8–10 days after last passage in culture – a period long enough to allow effective metacyclic maturation confirmed by the fact that macrophage infections lasted more than 7–8 days and were productive. Finally, our data showed that aflagellated *Ld*ARL-3A-Q70L-overexpressing cells failed to establish a durable infection within the insect vector *Lu. longipalpis,* indicating that they are no longer able to complete the entire cycle of the infection and to infect new human hosts.

*Ld*ARL-3A-Q70L overexpression, when compared to *Ld*ARL-3A overexpression, might lead to physiological changes in addition to flagellum length, that could be relevant for survival in insects. We tried to address this question given the current knowledge of the process. First, secreted acid phosphatase, a polymeric enzyme which is abundantly secreted by *L. amazonensis* promastigotes in culture and which might have some yet unknown role for *Leishmania* survival in the insect gut (Ilg, 2000), has previously (Cuvillier *et al*., 2000) been found secreted in the culture medium in similar levels by clones overexpressing the mutated and the native protein. Second, within the insect gut, *Leishmania* cells undergo several differentiation events from amastigote to procyclic promastigote which divide actively and attach to the epithelial cells, and then differentiate to metacyclic promastigote which stop dividing, detach and migrate to the anterior parts of the gut. From *in vitro* cultures, we have observed (Cuvillier *et al*., 2000) that footpad lesion amastigotes originating from all clones could differentiate to dividing promastigotes and reinfect mice in experimental infections. Here, we observed the kinetics of morphological differentiation from amastigote to promastigote as extracted from the insect guts daily following amastigote infection of the sandflies and there was no specific difference among the different clones examined (except for the presence or absence of flagellum). Moreover, the formation and disappearance of the peritrophic membrane, which is protective for *Leishmania* inside the insect gut (Pimenta *et al*., 1997), seemed to follow the same kinetics according to microscopic observations for all kinds of *Leishmania* infections.

However, one essential element for *Leishmania* cell survival within the insect gut is the surface LPG coat, responsible for the attachment to and detachment from the gut epithelium, depending on developmental changes in its composition during metacyclogenesis (Sacks and Perkins, 1985; Saraiva *et al*., 1995; Sacks *et al*., 2000; Sacks, 2001). We used a specific antiprocyclic *L. amazonensis* LPG (Courret *et al*., 1999) which equally agglutinated *in vitro* cultivated native and mutated protein overexpressing cells. Non-agglutinated cells probably displayed another kind of LPG on their surface. To ascertain that the LPG found in both types of cells is the same would require further work including extensive purification and structure analysis (Ilg *et al*., 1992), and attachment studies to dissected insect guts (Pimenta *et al*., 1992). It should be noted that in the latter studies, a failure of aflagellated *Ld*ARL-3 A-Q70L overexpressing cells to attach to the guts would not prove an intrinsic inability to attach, as it has never been shown that wild-type *Leishmania* cells devoid of flagella by any means (although such cells are not available) can still attach without ‘grabbing’ the epithelium by their flagellum.

The closest human homologues of *Ld*ARL-3A are ARL-3 and ARL-2 with 58 and 52% identity, respectively (Cuvillier *et al*., 2000), although the functional homologue of *Ld*ARL-3 A remains unknown (A. Sahin, G. Lemercier, E. Tetaud, B. Espiau, *et al*., in prep.). What is known of ARL-3 to date in various organisms is its interaction with rod cGMP phosphodiesterase delta (Linari *et al*., 1999) and *retinitis pigmentosa-2* protein (Bartolini *et al*., 2002). ARL-2 interacts with BART – a protein of unclear function (Sharer and Kahn, 1999; Sharer *et al*., 2002), with phosphodiesterase delta (Hanzal-Bayer *et al*., 2002), and also with tubulin-folding Cofactor D (Bhamidipati *et al*., 2000); it has a role in microtubule biogenesis (Radcliffe *et al*., 2000) and cytoskeleton stability (Antoshechkin and Han, 2002). These proteins might at least be involved in the control of tubulin polymerization. This latter putative function is not easily reconciled with an effect on the structure of complex glycolipids like LPG in *Leishmania*, although it is not yet possible exclude this possibility.

Flagellated *Leishmania* cells not agglutinated by the 3A1 monoclonal antibody displayed the typical morphology of metacyclic promastigotes with a very long flagellum and short cell body, a feature not visible in aflagellated cells; these latter cells expressed a metacyclic marker cysteine protease CPB-2 (Mottram *et al*., 1997) in equal levels to flagellated metacyclics. More complete answers may be obtained in future studies, when all stage-specific *Leishmania* genes become known and their expression patterns are monitored both *in vitro* and within guts.

These data suggest that aflagellated cells did not lose the potential to differentiate to metacyclic promastigotes, and that the cause of their loss from the insect gut lies in the absence of the flagellum rather than in a defect of their differentiation. Flagella are indispensable for *Leishmania* cells to escape from the blood clot and attach to the midgut as procyclic promastigotes, or to migrate to the anterior part of the gut after differentiation to free metacyclic promastigotes. Because the cells disappear early after day 3, it is more likely that they disappear because they cannot attach, but if they cannot migrate at a later stage of differentiation, they will also disappear. Experiments are now in progress to determine whether similar effects can be obtained using other *Leishmania* species.

Our study is the first to report stable aflagellated *Leishmania* cells and provide the initial evidence that a small G protein is involved in flagellum biogenesis. Unlike haploid *Chlamydomonas* which can be mutagenized and thus allow the isolation of motility-/flagellum morphogenesis-deficient mutants identified by complementation of the genes involved (Pazour and Witman, 2000), *Leishmania* are diploid and such a strategy has not yet been possible.

A significant difference with the other genetic approaches lies in the fact that instead of eliminating a gene and its function, we have modified a gene and its function. Therefore, it may be possible to try and block this function in a similar fashion as the induced mutation by exogenously providing well-designed molecules, targeting this gene product and/or its effectors when discovered in such a way so *Leishmania* transmission is blocked at the insect stage. A good candidate for this would be a specific yet unknown GAP (GTPase activating protein) for whom inhibitors should be designed. However, several targets must be considered together within this pathway or in combination with other pathways, in order to avoid the selection of *Leishmania* resistance.

Mathematical models tend to show that the best strategy to eliminate a parasitic disease is to stop transmission or to develop vaccines rather than to treat patients which leads to enhancement of parasite virulence and drug resistance (especially with only one or two different treatments available since many years) (Dye, 1996). Vaccines are being studied by a number of laboratories but are not yet available (Mauel, 2002). To stop transmission of insect-born diseases, insecticides like DDT have been widely used in the past with some success, but insects too become resistant. In addition to the toxicity and lack of specificity of insecticides, they last durably in the environment, and spread uncontrollably world-wide (by natural or human means), representing non-negligible threats for the environment and biodiversity (Simonich and Hites, 1995). The risk still remains that they will be used again to control parasite diseases given the lack of new effective products (Curtis and Lines, 2000). For leishmaniasis as for other parasitic diseases, new and well thought integrated strategies are needed (Shiff, 2002). A better targeting of insect vectors would certainly be beneficial; this is being done efficiently for the progressive elimination of *Trypanosoma cruzi* transmission over large areas of South America (Dias *et al*., 2002) by killing the insect vectors whose ecological niche is known and accessible. Phlebotomine vectors are less easily reached, but they might be attracted when relevant pheromones or chemoattractants are fully identified (O'Shea *et al*., 2002). Our work shows there may be another trail to follow to eliminate *Leishmania* without systematically eradicating the insects.

Experimental procedures

Parasites

The *Leishmania* strain used in this study was *L. amazonensis* MHOM/BR/1987/BA125 (BA125). Promastigotes were cultured at 24°C as previously described (Cuvillier *et al*., 2000). *Ld*ARL-3A- and *Ld*ARL-3A/Q70L-GTP-overexpressing *Leishmania* clones have been previously described (Cuvillier *et al*., 2000). In brief, amastigotes were recovered from mice footpad lesions, differentiated to promastigotes at 24°C for 2 days and amplified for 1 week before electroporation. Cells were spread onto agarose plates and incubated at 24°C for 3 weeks until colonies could be picked and clones amplified for 1–2 weeks before macrophage infection or frozen at −70°C. In all cases, the time spent in *in vitro* culture was limited to the minimum.

Metacyclic promastigote purification and Western blots

The 3A1 monoclonal antibody (mAb) (kindly provided by Dr E. Saraiva) was used for *L. amazonensis* metacyclic promastigote purification (Courret *et al*., 1999). It is directed against *L. amazonensis* non-infective procyclic promastigotes. Briefly, 5-day-old stationary-phase promastigotes were washed twice with PBS and resuspended at a density of 5×10^8 cells ml^{-1} in PBS plus a 1/1000th dilution of mAb 3A1 ascitic fluid. After a 30-minute incubation at room temperature, the cell suspension was centrifuged at 250 *g* for 5 min at 4°C. The unagglutinated metacyclic infective parasites remained in the supernatant, were washed twice with PBS, counted and used for macrophage infection. For Western blots, parasites were lysed with SDS in presence of an antiprotease cocktail (De Souza Leao *et al*., 1995) and extracts processed as previously described (Cuvillier *et al*., 2000). Rabbit anti-LACK immune serum (Prina *et al*., 1996) was kindly provided

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by Dr J-C Antoine, rabbit anti-*Lm*CPB 2.8 cysteine protease (Mottram *et al*., 1997) by Dr J. Mottram and they were used at 1/1000 dilutions.

Infected macrophages and amastigote preparation

Macrophages were prepared as described (Antoine *et al*., 1991). In brief, bone marrow cells were collected from tibias and femurs of Balb/c mice, resuspended in RPMI-1640 plus 10% heat-inactivated fetal calf serum, 50 units ml^{-1} penicilllin, 50 μg ml^{-1} streptomycin, 2 mM glutamine (Life Technologies) and 20% L-929 fibroblast-conditioned medium as a source of macrophage colony-stimulating factor, and incubated at 37°C in a humidified CO_2 incubator for 5 days. Adherent macrophages were washed with PBS and detached after a 10 min treatment at 37°C with 0.9% NaCl, 2 mg ml^{-1} glucose. Recovered macrophages were plated into 24-well clusters (Nunc) (with 12 mm round glass coverslips for infection monitoring or without coverslips for amastigote preparation) at a density of 2×10^5 cells per well and per ml of culture medium, plus 2.5% L-929 fibroblasts-conditioned medium. Adherent macrophages were incubated with stationary phase *L. amazonensis* promastigotes at various densities for 4 h at 4°C (determination of promastigote attachment to macrophages and infectivity) or 34°C (amastigote preparation). Free parasites were removed by two PBS washes. Infected macrophages were further incubated in culture medium at 34°C without L-929 fibroblast-conditioned medium for various times (infection monitoring) or 48 h (amastigote preparation). Cells on coverslips were fixed and stained with Giemsa solution (Kit RAL 555) before mounting with a drop of Eukitt and observation under a light microscope. For amastigote preparation, infected cells were scraped in PBS with a rubber policeman and homogenized by 10 strokes of a Teflon pestle in a Thomas potter. Debris were removed by a 5 min centrifugation at 20 *g*, amastigotes pelleted at 2500 *g* for 10 min.

Sandfly infection

A colony of *Lutzomyia longipalpis* (second to fourth generations) was maintained at the laboratory (Fiocruz Institute, Bahia, Brazil). Infections were performed as described (Tesh and Modi, 1984) with modifications. Three- to five-day-old sandflies were infected through a chick skin membrane on 300 μl of fresh heparinized Balb/c mouse blood/10^7 *L. amazonensis* amastigotes (prepared as described above) in culture medium (1:1, v:v). Blood-fed female sandflies were separated and maintained on 30% honey solution at 27°C, 80% humidity. At various times after feeding, the sandflies were examined for their blood meal status and were sacrificed by freezing at −20°C for 10 min. Midguts were excised from the stomodeal valve to the pylorus (Pimenta *et al*., 1994), transferred to a drop of PBS and examined under a Zeiss light microscope. Individual midguts were placed in microfuge tubes with 30 μl of NaCl 0.9%, and macerated using a Teflon-coated microtissue grinder. The number of promastigotes per midgut was determined by counting on a Malassez haemocytometer.

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