Experimental infection of *Leishmania (L.) chagasi* in a cell line derived from *Lutzomyia longipalpis* (Diptera:Psychodidae)

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The present work describes the in vitro infection of a cell line Lulo, derived from Lutzomyia longipalpis *embryonic tissue, by* Leishmania chagasi *promastigotes. This infection process is compared with a parallel one developed using the J774 cell line. The* L. chagasi *MH/CO/84/CI-044B strain was used for experimental infection in two cell lines. The cells were seeded on glass coverslips in 24-well plates to reach a final number of $2x10^5$ cells/well. Parasites were added to the adhered Lulo and J774 cells in a 10:1 ratio and were incubated at 28 and 37ºC respectively. After 2, 4, 6, 8, and 10 days post-infection, the cells were extensively washed with PBS, fixed with methanol, and stained with Giemsa. The number of internalized parasites was determined by counting at least 400 cultured cells on each coverslip. The results showed continuous interaction between* L. chagasi *promastigotes with the cell lines. Some ultrastructural characteristics of the amastigote forms were observed using transmission electron microscopy. The highest percentage of infection in Lulo cells was registered on day 6 post-infection (29.6%) and on day 4 in the J774 cells (51%). This work shows similarities and differences in the* L. chagasi *experimental infection process in the two cell lines. However, Lulo cells emerge as a new model to study the life-cycle of this parasite.*

Key words: *Leishmania chagasi* - Lulo cell line - J774 cell line - infection percentages - parasitophorous vacuoles

Leishmania chagasi is a causative protozoan parasite of American visceral leishmaniasis (Grimaldi & Tesh 1993). The disease is endemic in some regions of Colombia (Corredor et al. 1989, 1990) and throughout all the South and Central America (Grimaldi & Tesh 1993, Tanner 1996); the parasite has been isolated from hosts, reservoirs and sand fly vectors. This species, as well as other species belonging to the *Leishmania* genus, develops through a digenetic life cycle consisting of an extracellular promastigote form that develops and multiplies in the alimentary tract of the sand fly vector and an intracellular amastigote form that resides and multiplies inside the infected mammal host's macrophages. Clinical and experimental evidence indicates that vector, parasite, and host factors influence the evolution and outcome of the infection caused by *Leishmania* parasites (Grimaldi &Tesh 1993).

Promastigotes are routinely cultured in vitro using cell free medium; such easily obtained media have also been used to induce promastigote transformation to amastigote-like forms in response to elevated temperature and acid pH (Hendrick et al. 1978, Hunter et al. 1982, Pan 1984, Zilberstein et al. 1991, Bates et al. 1992, Bates & Tetley 1993, Zilberstein & Sahapira 1994, Bates 1994, León et al.

1995, Hodgkinson 1996, Saar et al. 1998). It has been shown that these axenic amastigotes closely resemble animal-derived amastigotes. *Leishmania* in vitro differentiation, maturation, and replication has also been achieved in both macrophage cell cultures (Chang & Dwyer 1978, Chang 1979, Pearson et al. 1981, Aikawa et al. 1982) and non-macrophage cell lines, mainly in fibroblast cultures (Mattock & Petters 1975, Chang 1978, Dedet et al. 1983, Schwartzman & Pearson 1985, Corte-Real et al. 1995, Hervás Rodrigues et al. 1996, Pessotti et al. 2004). However, in spite of current support, cell lines and cell free media being available for transforming the life cycle of diferent *Leishmania* species, there is no information available regarding this process in cell cultures obtained from phlebotomine sand fly vectors.

The present study describes the in vitro infection of Lulo cell line (Rey et al. 2000) by *L. chagasi* promastigotes and some ultrastructural characteristics regarding the parasite's interaction with these cells were determined. This cell line was derived from *L. longipalpis* embryonic tissue, which is the main vector of *L. chagasi* in its natural environment. The experimental infection of Lulo cell line was compared to infection of J774 cell (Ralph et al. 1975).

MATERIALS AND METHODS

Parasites - The strain of *L. chagasi*, MH/CO/84/Cl-044B, was isolated from a human case of visceral leishmaniasis in Quipile, Cundinamarca, Colombia. This strain has been maintained by periodic inoculations of promastigotes in Syrian golden hamsters (*Mesocricetus auratus*) in the parasitology laboratory at the National Institute of Health in Colombia. *L. chagasi* amastigotes were recovered from the spleen of infected hamsters as previously described (Duque et al. 1988). Promastigotes were obtained by in

vitro conversion of amastigotes at 24ºC in *Drosophila* Schneider medium, suplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg/ml of streptomycim. Some *L. chagasi* promastigotes cultures were stored in liquid nitrogen. The promastigotes used for infecting J774 and Lulo cells were day-7 stationary phase cultures from the second passage in culture. These promastigotes were washed in phosphate-buffered saline (PBS), pH 7.2, before being used in cell-parasite coculture experiments.

Cell lines - The Lulo cell line, obtained from embryonic tissue of *L. longipalpis* and previously established (Rey et al. 2000), was cultured in a 1:1 mix of L15 (Leibovitz 1963) and Grace media (Grace 1962) supplemented with 10% FBS, pennicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were incubated at 28ºC, without CO_2. The J774 cell line (Ralph et al. 1975), isolated from reticulated cells from Balb/c mice (kindly provided by Dr Marcela Camacho del Centro Internacional de Física de la Universidad Nacional de Colombia) was maintained in RPMI medium, 1640 (Sigma), with FBS (15%) and gentamycin (1%) and incubated at 37ºC with 5% CO_2. The confluent monolayers, in both cell lines, were mechanically removed with the help of rubber policemen. The cells were harvested in log phase of growth. To prepare cells for infection, J774 and Lulo cells were put separately into 15 ml centrifuge tubes to prepare them for infection and were then washed by centrifuging for 10 min at $400 \times g$ with each culture media mentioned above.

Cell line infection - Cells from each cell line were seeded on glass coverslips inside 24 well-plates (Corning) to a final number of 2×10^5 cells per well. Twenty-four hours after adhesion and growth on the coverslips, the semiconfluent monolayers were infected with promastigotes in stationary phase using 10 parasites per cell. Infected Lulo and J774 cells, separately in different plates, were incubated at 28ºC without CO_2 and at 37ºC with 5% CO_2, respectively. Some coverslips were removed in early assays after 1, 2, and 3 h of incubation in coculture; the preparation was fixed with methanol and stained with giemsa. The number of promastigotes adhered per cell in both cell lines was determined by counting 200 cells per coverslip in duplicate cocultures. Following 12 h incubation, non-internalized promastigotes were removed by washing with PBS and incubated using the same culture media. The coverslips with the attached infected cells were removed after 2, 4, 6, 8, and 10 days post-infection, washed with saline solution, fixed and stained as described earlier. Cells were observed and photographed using a light microscope with an Olympus microphotographic system, 400× to 1000× magnification. These assays were repeated three times to determine the experimental data's reproducibility. The number of infected cells and the average number of promastigotes per cell was calculated by counting at least 400 cells per coverslip. Results were expressed as the percentage of infected cells and infection index. This last parameter was obtained by multiplying the infection percentage by the average number of intracellular amastigotes per cell (Lonardoni et al. 2000). Differences between experimental groups were determined by using

the Student's t-test. Differences at $P < 0.05$ were regarded as significant.

Amastigote to promastigote differentiation assays - Lulo cells (final number of 2×10^6 cells/ml) were seeded in 12.5 cm^2 flasks, previously containing 4 ml of fresh L-15/ Grace medium and supplemented as described above. These cells were incubated at 28ºC; semiconfluent monolayers were reached after of subculturing for 3 days. The Lulo cells were then infected with *L. chagasi* promastigotes in stationary phase at a 10:1 an parasite:cell ratio and incubated again in the same conditions for 24 h. The monolayers were washed with PBS to remove non-interiorized promastigotes and fresh medium was added. However, to assure total removal of non-internalized promastigotes, new washes were carried out each day with PBS and fresh medium was added. The infected monolayers were gently scraped on day 6 post-infection and the content of the flasks was vigorously pipetted to dissociate the cells. These cells were then seeded in NNN medium and incubated to 26ºC. The cocultures were observed daily with an inverted microscope to identify transformed promastigotes. The previous technique was used for the J774 cells; however, the maintenance conditions described above were for these cells.

Transmission electron microscopy - Infected J774 cells and Lulo cells (each kept separate 6-well plates) were fixed in situ for 1 h in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 and sucrose. The cells were rinsed with the same buffer, postfixed for 1 h with 1% osmium tetroxide diluted in the same buffer, washed again, dehydrated in a graded series of ethanol and embedded in epon-araldite resin. Ultra-thin sections were obtained using an ultramicrotome LKB and the sections were stained with aqueous uranyl acetate and lead citrate. The grids were observed by transmission electron microscope (Zeiss EM110).

RESULTS

Light microscopy - The Lulo cells showed higher number of promastigotes adhered compared with values obtained from J774 cells (Fig. 1). Moreover, with time of incubation tested (1 to 3 h) there was an increase in number of promastigotes adhered per cell in both cell lines. Twelve hours post-infection, we detected a strong attachment of *L. chagasi* promastigotes and Lulo cells, evidenced by parasite resistance becoming detached during PBS washes. New PBS washes were performed during the following days until complete removal of the parasites on the 3rd day post-infection. By contrast, promastigotes were easily removed from the J774 cells during the same interval of time, no additional washes being necessary. During the time taken for these experiments, observations on days 2, 4, 6, 8, and 10 post-infection showed amastigote forms of the parasite and completely stained structures inside the parasitophorous vacuoles (Fig. 2A). However, motile parasites (promastigote-like forms) were observed during days 2, 4, and 6 in some Lulo cells vacuoles (Fig. 2B). Non-promastigote-like forms were observed in J774 cells. The Table shows the values obtained in vitro infection with *L. chagasi* in the selected cell lines. Percentages and infec-

tion index demonstrated that Lulo cells presented a gradual increase from day 2 to day 6, where they reached their maximum peak (29.6%), with a decrease around day 8 and lower numbers around day 10 (Fig. 3). The percentages and index of infection in the J774 cell line were higher compared to those for the Lulo cells (Table). However, the maximum value was registered on day 4 (51%).

Amastigote to promastigote differentiation - Promastigotes began to appear in the cocultures after three days post-infection of Lulo cells, amastigotes to promastigotes differentiation was completed within 7 days. J774 cell differentiation was slower, beginning 6 days post-infection.
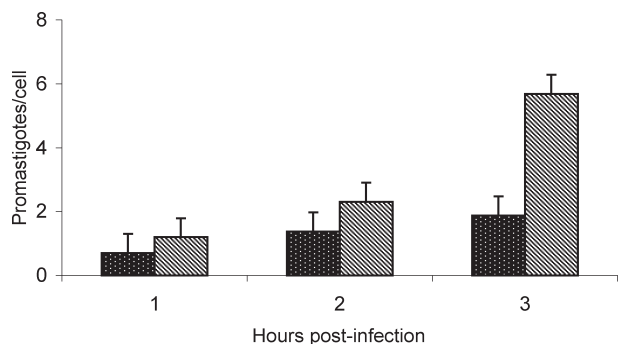

Fig. 1: interaction between promastigotes of *Leishmania chagasi* with cell lines tested: Lulo (bars with diagonal lines) and J774 (dark bars with clear points). Adhered cells were incubated with *L. chagasi* at 1:10 ratio at 28ºC (Lulo) and 37ºC (J774) for 3 h. Results are expressed as number of attached promastigotes per cell. Mean ± SD of three separate experiments done in duplicate.
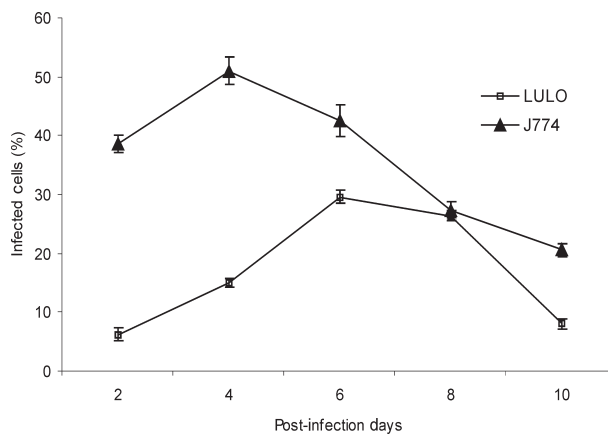

Fig. 3: comparison of the in vitro infection between Lulo cells and J774 cells by *Leishmania chagasi*.
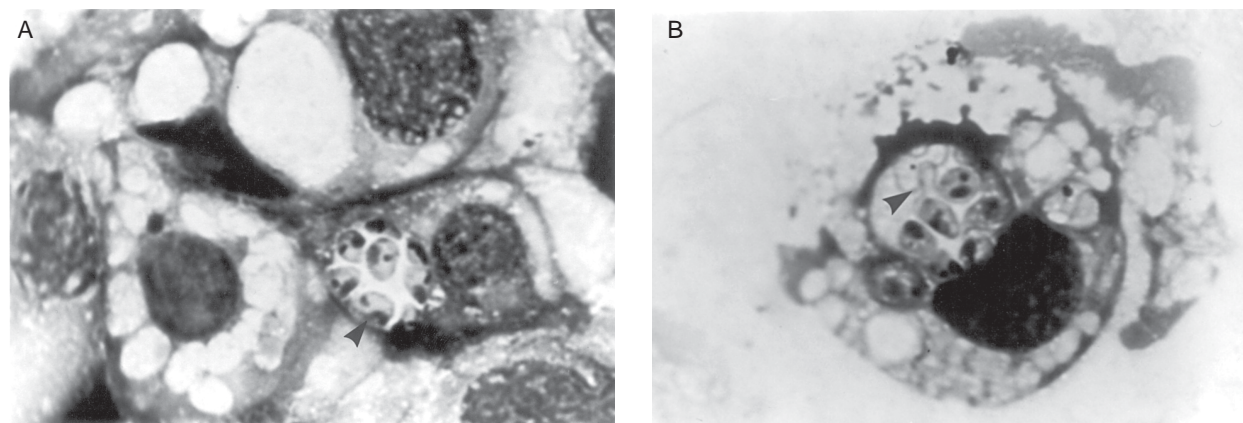

Fig. 2: light micrographs of Lulo cells infected with *Leishmania chagasi*. A: amastigotes (arrowhead) within a parasitophorous vacuole in Lulo cell cytoplasm 2 days after infection, showing the nucleus and kinetoplast in each of them. (× 1000); B: promastigote-like forms inside a parasitophorous vacuole in a Lulo cell, 4 days post-infection. The flagellums of one of them can be seen in detail (arrowhead). (× 1000).

TABLE

Mean data obtained of the *Lesihmania chagasi* experimental infection process of the two cell line

Day	Lulo			J774		
	Infection percentage	Amastigotes/cell	Infection index	Infection percentage	Amastigotes/cell	Infection index
2	6.2 ± 1.1	2.6	16.12	38.6 ± 1.5	5.4	208.4
4	15 ± 0.7	4.6	69	51 ± 2.3	10.0	510
6	29.6 ± 1.1	6.4	189.44	42.6 ± 2.7	18.6	792.36
8	26.4 ± 0.8	9.3	245.52	27.4 ± 1.3	13.8	378.12
10	8 ± 0.8	1.9	15.2	20.6 ± 1.1	6.1	125.66

Each value is the mean of three experiments done in duplicate; ± standard error; differences between Lulo cells and J774 cells were significant at $p < 0.05$, except for day 8 when there were no significant differences in infection percentage. There was differences among days in each cell lines.

Transmission electron microscopy - Electron micrographs demonstrated *L. chagasi* promastigote form interaction with Lulo cell membrane 2 days post-infection, without a preferential orientation. The parasites adhered by the flagellar tip or the posterior pole. The parasites' lateral adherence to the Lulo cell surface was also observed (Fig. 4A). Samples taken from Lulo cells 3 days post-infection, presented amastigotes in a typical narrow parasitophorous vacuole (Fig. 4B). Some cells' organelle ultrastructure (n: parasite nucleus, N: cell nucleus, and f: flagellum) was clearly visible in this parasite (Fig. 4B). Promastigote-like forms were also observed inside the parasitophorous vacuoles following 4 days interaction with Lulo cells (Fig. 4C). Parasites inside of parasitophorous vacuoles were also visible in J774 cells 2 days post-infection (Fig 4D).
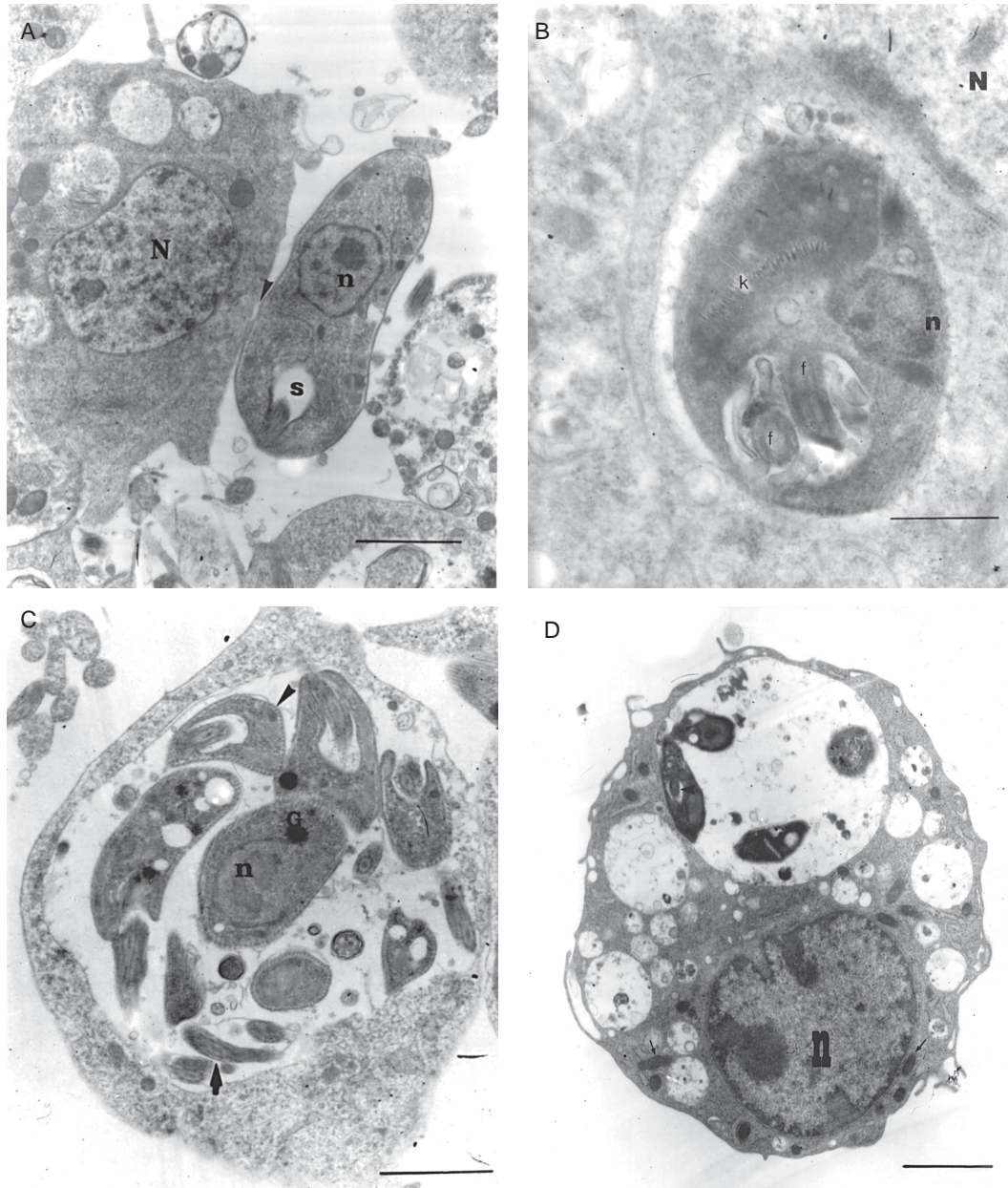

Fig. 4: transmission electron micrographs showing interaction between *Leishmania chagasi* parasites with J774 and Lulo cells, some parasite and cell ultrastructural characteristics were also observed. A: point of contact between promastigote membrane and the Lulo cell membrane after 48 h incubation. Parasite adhesion to cell was through the cell body. n: promastigote nucleus; S: flagellar sack; N: Lulo cell nucleus. Bar: 1.5 µm; B: ultrastructure of an amastigote localized inside narrow parasitophorous vacuole in a Lulo cell, 3 days post-infection. Two flagella can be seen (f); k: kinetoplast; n: parasite nucleus; N: cell nucleus. Barr: 0.3 µm; C: promastigote-like forms inside of a large parasitophorous vacuole in a Lulo cell after 4 days of interaction. The arrow indicates a segment of the flagellum. n: nucleus; arrowhead: subpellicular microtubules; G: granules. Bar: 1.5 µm; D: J774 cell with three parasites inside a large parasitophorous vacuole, 2 days post-infection. kinetoplast (k) can be observed in an amastigote and a transversal section shows a flagellum (arrowhead) surrounded by the flagellar sack in another parasite. n: cell nucleus; mitochondria (arrow). Bar: 2.0 µm.

DISCUSSION

We have demonstrated that *Leishmania* promastigotes can invade cells derived from embryonic tissues obtained from the phlebotomine sand fly vector and transform into amastigote-like forms inside these cells. *L. chagasi* presented morphological characteristics when developed inside Lulo cells that allowed parasitic forms to be differentiated during their development in both cell lines, even though J774 cells present macrophage properties. This comparison also revealed some similarities.

Light microscopy revealed that promastigotes present a high affinity for the surface of Lulo cells. Such an observation has been recorded previously (Rey et al. 2000). It was also possible to identify relatively high levels of promastigotes attached to the Lulo cells as well as was demonstrated that the adhesion of the parasite to this cells was maintained during a long period of time. This process was probably enhanced by the favorable conditions provided by the L-15/Grace medium where simultaneous and intense promastigotes replication was observed. The Lulo cells also presented characteristics typical of mixed cultures having pluripotential cells. It is probable that the Lulo cell line may simulate some of the in vitro interaction events that occur between the parasite and the epithelial cells inside the insect's midgut. This observation could explain the parasite's strong adhesion to the Lulo cells. It is possible that this process is an indication of the presence of carbohydrates on the cell surface of the promastigote, similar to those molecules responsible for the adherence of the parasite to sand fly midguts (Sack et al. 1985, 1994, Pimenta et al. 1991, Saraiva et al. 1995, Sacks & Kamhawi 2001, Fampa et al. 2003). Although, promastigotes adherence to the J774 cell surface was observed, this phenomenom was present in less proportion, persisting only until removal of the parasites with PBS washes, 12 h post-inoculation. However, the levels of infection were higher when compared with the values obtained from the Lulo cells. The difference in the adhesion could be attributed to the fact that there exists mechanisms of adhesion caused by similar molecules to the integrins, between the parasite and the Lulo cells, which would explain the necessity of successive washings to remove the adhered parasites. This strong interaction between the parasite and Lulo cells, that would be similar to which happens in the digestive tract of the insect, could lead to the acquisition of the insect molecules which could act like opsonins (Prina et al. 2004), later recognized by the cells of the immune system of the mammalian host. On the other hand, in the case of the J774 cells, due to its characteristics as phagocitic cells, the interaction of parasite-host cell could be similar interactions to those that would happen between molecules presents in the pathogens surface denominated pathogen-associated molecular patterns (PAMPs) and receptors present in the phagocitic cells denominated pattern-recognition receptors (PRRs), during phagocitosis, which would favor the penetration of the parasite to the cells. In previous works, basics and applied (Aikawa et al. 1982, Looker et al. 1986, Bosque et al. 1998, Pelletier et al. 2003), where the interaction between *Leishmania* parasites and macrophages was evaluated, it was suggested that the adhesion of the parasites with these cells occurs early and persists for a relatively short time before the internalization. It is possible that a similar situation has happened with the infected J774 cells.

Light microscopy and transmission electron microscopy also revealed that Lulo cells presented amastigotes, individually or in groups within parasitophorous vacuoles. A significant average number of amastigotes per cell was also registered, indicative of the replication process experienced by parasites within these vacuoles, similar to that observed in vitro in other macrophage and fibroblast cell-lines (Mattock & Petters 1975, Chang 1978, Chang & Dwyer 1978, Pearson et al. 1981, Aikawa et al. 1982, Dedet et al. 1983, Schwartzman & Pearson 1985, Corte-Real et al. 1995, Hervás Rodrigues 1996, Pessotti et al. 2004). The increase in *L. chagasi* infection percentage and infection index in Lulo cells (using light-microscopy), became evident on days 2 and 4, reaching its peak on day 6, being more or less stable on day 8 (registering a non-significant reduction) and finally descending on day 10 (the lowest point reached in these data sets).

There was a concomitant increase in Lulo cells for the average number of amastigotes per cell over the period of time mentioned above, greatest average occurring on day 8. The greatest number of promastigotes had probably been internalized during the first 12 h of interaction. However, due to the fact that many of these parasites remained strongly adhered to the cells and the fact that they could not be removed by washing with PBS during this time, some of these parasites had probably become internalized during subsequent days until the rest of the promastigotes (both in culture medium and those still remaining adhered), had been completely removed by the mechanical action of washing on the 3rd day post-infection. There was a gradual loss of cells in the monolayer from each coverslip on day 4; however, this loss was higher on day 10, which could explain the strong descent registered in the infection percentage during this day.

A notable characteristic regarding Lulo cells was the presence of promastigote-like forms within parasitophorous vacuoles observed on days 2, 4, and 6 post-infection, similar to that recorded in vitro in P388D cells (Aikawa et al. 1982), when the parasite had been recently internalized inside cytoplasm and was being converted to an amastigote form inside the parasitophorous vacuole. However, the parasite was found as promastigote-like form during advanced days of infection in Lulo cells. Such a phenomenon can be explained by taking the following factors into account: (1) the temperature at which infected cells were maintained (28°C) was less than the temperature experienced by parasites in natural infection in vertebrate macrophages. This situation could eventually prolong the process of some promastigotes transforming themselves into amastigotes, even though such process was not simultaneously observed in all of them on the same days of infection as we observed within parasitophorous vacuole amastigote forms. This was probably due to the latter parasites being better adapted to cell-culture environmental conditions, thus being able to achieve the conversion; (2) it is probable that promastigote has invaded

host cell later on and the event had thus been registered, if we accept the argument that parasites were being continuously internalized during advanced days of infection as a valid argument according to the explanation stated above regarding late intracellular penetration of promastigotes strongly adhering to cells; (3) it is probable that this phenomenon was due to a combination of the previous factors.

Infection percentages were higher in J774 cells compared to Lulo cells, due to the former presenting macrophage characteristics with cells of phagocytic ability (Ralph et al. 1975, Papadimitriou & Ashman 1989). These cells were also physiologically adapted as parasite host cells. However, there was a significant decrease in infection in day 10 (same as in Lulo cells), probably caused by the same reasons indicated for this cell line. By contrast with Lulo cells, promastigote-like forms were not observed inside parasitophorous vacuoles in J774 cells, suggesting that these events were observed during stages following parasite transformation to amastigote form, after a relatively prolonged intracellular pathogen penetration time had passed. However, in spite of *L. chagasi* infection percentages being higher in the J774 line than in Lulo cells, it can be stated that reverse differentiation to promastigotes on seeding both cells in NNN medium and incubating them at 26°C was obtained in less time in Lulo than in J774 and thus final parasite population density did not reveal significant differences between the two cell lines. It is probable that maintaining some intracellular parasites as promastigote-like forms in Lulo facilitated their rapid exit from cells on being seed in NNN medium and parasite growth and replication thus occurred in less time.

The present work has shown *L. chagasi* interaction with cells derived from *L. longipalpis* (the main vector for this parasite in its natural environment) as well as the development of its biological cycle and intracellular replication, suggesting that these cells and (in the future) other cell cultures obtained from phlebotomine sand fly vectors could be considered as alternative models for studying multiple in vitro interaction events occurring between parasites from the *Leishmania* genus and sand fly vectors.

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