Internalization of Components of the Host Cell Plasma Membrane During Infection by *Trypanosoma cruzi*

TMU Carvalho+, W De Souza, ES Coimbra*

Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21944-970 Rio de Janeiro, RJ, Brasil *Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos, RJ, Brasil

Epimastigote and trypomastigote forms of Trypanosoma cruzi *attach to the macrophage surface and are internalized with the formation of a membrane bounded vacuole, known as the parasitophorous vacuole (PV). In order to determine if components of the host cell membrane are internalized during formation of the PV we labeled the macrophage surface with fluorescent probes for proteins, lipids and sialic acid residues and then allowed the labeled cells to interact with the parasites. The interaction process was interrupted after 1 hr at 37ºC and the distribution of the probes analyzed by confocal laser scanning microscopy. During attachment of the parasites to the macrophage surface an intense labeling of the attachment regions was observed. Subsequently labeling of the membrane lining the parasitophorous vacuole containing epimastigote and trypomastigote forms was seen. Labeling was not uniform, with regions of intense and light or no labeling. The results obtained show that host cell membrane lipids, proteins and sialoglycoconjugates contribute to the formation of the membrane lining the PV containing epimastigote and trypomastigote* T. cruzi *forms. Lysosomes of the host cell may participate in the process of PV membrane formation.*

Key words: *Trypanosoma cruzi* macrophage-parasite interaction - parasitophorous vacuole - fluorescence microscopy

Trypanosoma cruzi is a parasite which causes Chagas disease in humans, a prominent health problem in Latin America (Dias 1934, Tanowitz et al. 1992). As an obligate intracellular pathogen that infects a wide variety of nucleated cells, the parasite gains access to the host cell and is initially found inside an endocytic vacuole. After 2 hr it disrupts the membrane lining the vacuole and begins to transform into the amastigote form which is able to divide. Subsequently, amastigotes transform back into trypomastigotes and after rupture of the host cell the parasites are released into the intercellular space infecting neighbouring cells or reaching the bloodstream from where they can infect new cells in various tissues or can be ingested by the insect vector and complete the life cycle (Brener 1973, De Souza 1984).

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+Corresponding author. Fax: +55-21- 260.2364. E-mail: tecia@leao.biof.ufrj.br
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Host cell entry by protozoa involves the formation of a membrane-bounded parasitophorus vacuole (PV). In the case of *T. cruzi* evidences exist that in phagocytic and non phagocytic host cells lysosomes fuse with the PV (Milder & Kloetzel 1980, Carvalho & De Souza 1989). However, only trypomastigote forms are able to disrupt the membrane lining the vacuole while epimastigotes are destroyed within the PV (Nogueira & Cohn 1976, Carvalho & De Souza 1989, Andrews et al. 1990).

There are very few studies on the biogenesis of the PV of phagocytic and non professional phagocytic host cells infected by *T. cruzi*. Cytochemical studies showed that plasma membrane enzymes such as Na^{+}-K^{+}-ATPase, Mg^{+2}-ATPase, 5'-nucleotidase and adenylyl cyclase (Meirelles & De Souza 1896) and anionic sites detected using cationized ferritin particles (Meirelles et al. 1984) are not internalized during ingestion of *T. cruzi* by macrophages. However, concanavalin A-binding sites are internalized and found in the luminal face of the PV membrane (Meirelles et al. 1983). Hall et al. (1991) demonstrated by immunofluorescence and intracellular radioiodination that the membrane lining the endocytic vacuole containing *T. cruzi* differs depending on the developmental stages of the parasite.

One approach to analyse the participation of components of the host cell plasma membrane in

the biogenesis of the PV is to label the host cell membrane before interaction and to follow the fate of the labeled probes during the interaction process. In the present communication we will review the use of non permeant fluorescent probes to label lipids, proteins and sialoglycoconjugates and follow their fate, during interaction of epimastigote and trypomastigote forms of *T. cruzi* with macrophages as well as, the participation of host cell lysosomes during PV formation.

FORMATION OF THE PARASITOPHOROUS VACUOLE

Macrophages can ingest and kill invading microorganisms using microbicidal mechanisms that are activated once they attach to the cell surface and that exist within a parasitophorous vacuole formed by fusing of a phagosome with lysosomes (Sadick 1992). However, the infective trypomastigote forms of *T. cruzi* is able to disrupt the membrane lining the PV and replicate as amastigotes in the cytosol (Nogueira & Cohn, 1976, Carvalho & De Souza 1989). In contrast, the epimastigote form is killed and degraded within the vacuole, so it is of interest to study the origin and composition of the PV membrane containing the different stages of *T. cruzi*.

DTAF forms covalent bonds with surface-exposed membrane proteins and consequently labels the cell surface (Edidin 1989). The cells were washed once with PBS, pH 7.2, and with PBS/200 mM borate, pH 8.5, at a 1:1 proportion. After that the cells were incubated with DTAF (0.5 mg/ml) in the dark for 15 min at 4ºC. Then cells were washed once in PBS + 20% FCS for 5 min at 4ºC, washed in PBS once and incubated in 199 medium. Subsequentley unlabeled parasites were put to interact with those labeled cells (10:1 parasite-host cell ratio) for 1 hr at 37ºC in a 5% CO_2 atmosphere. The cells were then washed twice with PBS to remove non aderent and extracellular parasites, fixed in 4% freshly prepared formaldehyde solution in 0.1M phosphate buffer, pH 7.2, mounted and analyzed by CLSM. Initially an intense labeling at the region of attachment of the parasites to the macrophage surface was observed. In parasites inside the host cell we observed the labeling of the membrane lining the parasitophorous vacuole (Fig. 1a,b). As with PKH26, the labeling of the PV was not uniform. We also observed labeling of small vesicles of the host cell.

PKH-26, a non-permeant fluorescent lipophilic probe that binds to cellular membrane, has been used to label several types of cells (Samlowski et al. 1991, Ward et al. 1993). Macrophages were washed three times in PBS and incubated with PKH26 (100 µg/ml) in PBS for 30 s at 4ºC. Then 500 µl of fetal calf serum (FCS) was added. The cells were washed twice with PBS and put to interact with non labeled parasites (10:1 parasite-host cell ratio) for 1 hr at 37ºC in a 5% CO_2 atmosphere. After this the cells were washed twice with PBS to remove those parasites that were not strongly adered to the host cell, and processed as described for DTAF. During attachment of the parasites to the macrophage membrane we observed an intense labeling. In parasites inside macrophages we observed labeling of the membrane lining the parasitophorous vacuole containing epimastigote and trypomastigote forms (Fig.1 c, d). However, this membrane labeling was not uniform. Some points showed an intense labeling, while in others no labeling was observed. Some small endocytic vesicles of the host cell was also labeled. Our present observations show clearly that lipids and proteins of the plasma membrane of macrophages are internalized during the process of cell infection by *T. cruzi*. It is important to point out that the probes used did not bind to any specific membrane macromolecule. There are cytochemical evidence that plasma membrane enzymes are excluded from the membrane lining the PV (Meirelles & De Souza 1986), thus suggesting the existence of mechanisms to select which membrane components are internalized. We did not observe differences in the behaviour of the probes when epimastigote or trypomastigote forms were used.

Recent reports (Ward et al. 1993, Pouvelle et al. 1994, Lingelbach & Joiner 1998), have shown that erythrocyte membrane lipids, but not proteins, are incorporated into the PV containing *Plasmodium*. In the case of *T. gondii* both proteins and lipids are internalized during formation of the PV (Pacheco-Soares & De Souza 1998, Lingenlbach & Joiner 1998). Both probes described have been used to label the surface before interaction and to follow their fate during formation of the PV containing *Plasmodium* and *Toxoplasma* (Ward et al. 1993, Pacheco-Soares & De Souza 1998, Lingelbach & Joiner 1998). Our observations showed an initial concentration of the labeled probes at the site of parasite attachment and their subsequent incorporation, together with the parasites, concentrating in the membrane lining the PV. Using the same approach it was shown previously that when tachyzoites of *T. gondii* attach to the host cell surface transfer of the probe to the parasite surface took place (Pacheco-Soares & De Souza 1998). This phenomenon was not observed with *T. cruzi*.

Several studies have shown that glycoconjugates located on the surface of *T. cruzi* or the macrophage are involved on the process of parasite-host cell interaction (Meirelles et al. 1983, Araujo-

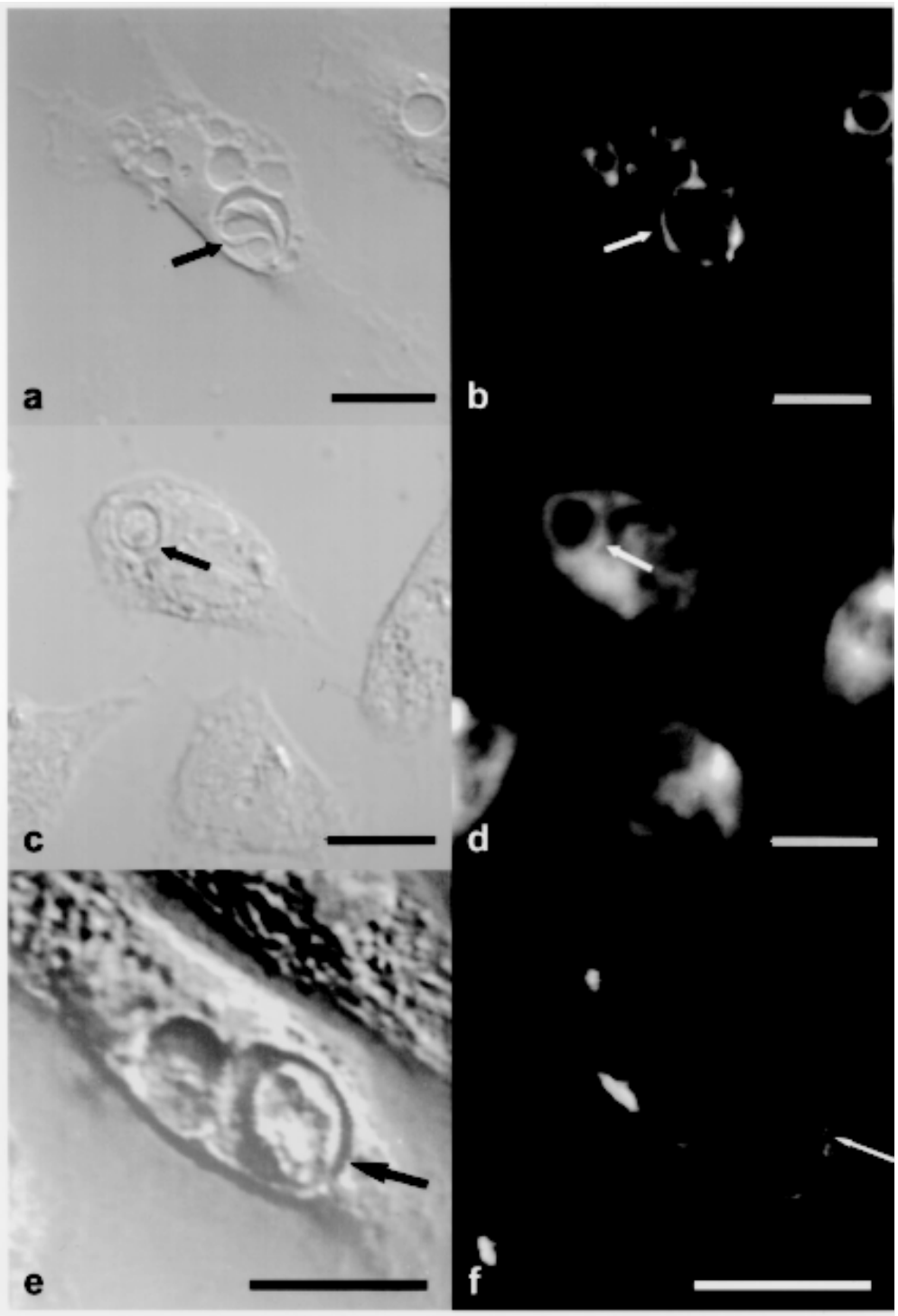

Fig.1- a, b: DTAF labeled macrophages were incubated with trypomastigote forms of *Trypanosoma cruzi* for 1 hr at 37ºC. Observation by confocal laser scanning microscope (CLSM): Dic (a) and corresponding fluorescence (b) images. Observe the labeling of the membrane lining the parasitophorous vacuole containing a trypomastigote form (arrow); c, d: macrophages labeled with PKH-26 were put to interact with trypomastigote forms of *T. cruzi* for 1 hr at 37ºC. Observation by CLSM: Dic (c) and corresponding fluorescence images. Observe the fluorescent labeled plasma membrane lipids in the parasitophorous membrane containing a trypomastigote form (arrow); e, f: fluorescein tiosemicarbazide labeled macrophages incubated with trypomastigote forms of *T. cruzi* for 1 hr at 37ºC. Observation by CLSM: Dic (e) and corresponding fluorescence (f) images. We can observe labeling of the membrane lining the parasitophorous vacuole containing a trypomastigote form (arrow). Bar: 10 mm

Jorge & De Souza 1984, 1986). Special emphasis has been given to the role played by sialoglycoconjugates due to the influence of sialic acid residues on the interaction process (Meirelles et al. 1984, Vermelho & Meirelles 1994). It has been shown that cells containing few or no residues of sialic acids, either due to removal of the residues by neuraminidase treatment (Araújo-Jorge & de Souza 1984), blockage of the residues using lectins (Araújo-Jorge & De Souza 1986) or their absence due to mutation (Ciavaglia et al. 1993) are much less infected by *T. cruzi* than control cells. In addition, experiments carried out in CHO cells deficient in the glycosilation process showed that *T. cruzi* disrupts faster the PV membrane of these cells as compared with control cells (Hall et al. 1992). Based on those information we decided to analyze the fate of sialoglycoconjugates during the *T. cruzi*-macrophage interaction process. For this we used Fluorescein-5-thiosemicarbazide (Ward et al. 1993) as a probe for labeling of surface-exposed sialoglycoconjugates of macrophages. The cells were washed in PBS twice and incubated in a freshly prepared solution of sodium m-periodate (5 mM in PBS) for 30 min at 4ºC. The cells were then washed three times with PBS at 4ºC and incubated in PBS containing 0.66 mg/ml of fluorescein-5-thiosemicarbazide for 30 min at 4ºC, washed with 199 medium twice and incubated with unlabeled parasites (10:1 parasite-host cell ratio) for 1 hr at 37ºC in a 5% CO_2 atmosphere. After that time the cells were washed twice with PBS to remove non adherent and extracellular parasites and processed as described above. As described before for lipids and proteins we observed labeling in the region of adhesion of epimastigote or trypomastigote forms of *T. cruzi* to the macrophage surface and subsequently labeling of the membrane lining the PV (Fig.1 e, f). Previous studies in which the macrophage surface was labeled with cationized ferritin, which binds to anionic sites, showed internalization of these sites and their concentation in endocytic vacuoles but not in the PV (Meirelles et al. 1984). The presence of sialoglycoconjugates in the membrane lining the PV could in some way explain the fact that only after a few hours the membrane is disrupted (Hall et al. 1992). Studies carried out with mutant cells which do not present surface-exposed sialoglyco-conjugates have shown that *T. cruzi* disrupts the PV membrane faster than in control cells (Hall et al. 1992). In addition it was shown that removal of sialic residues enhances the activity of the parasite hemolysin (Hall et al. 1992).

The participation of lysosomes during formation of the parasitophorous membrane was also analyzed. Some reports showed that after internalization of *T. cruzi* by macrophages lysosomes fused with phagosomes (Milder & Klotzel 1980, Carvalho & De Souza 1989). We have quantified the process of phagosome lysosome fusion in macrophages (Carvalho & De Souza 1989) observing that fusion occurred only in 41% for trypomastigote and 37% for epimastigote forms after 1 hr of interaction. In non phagocytic cells Tardieux et al. (1992) showed a recruitment of lysosomes in the early step of *T. cruzi* interaction. So we used macrophages as phagocytic and Vero as non professional phagocytic cells to vizualize this early process of lysosome fusion. We labeled secondary lysosomes of host cells with 1mg/ml of Lucifer Yellow. After 2 hr at 37°C, the cells were washed twice in 199 medium, and incubated again for 30 min at 37°C. The labeled cells were put to interact with the parasite for times varyng from 15 to 60 min. Then the cells were washed with 199 medium, mounted and analyzed by CLSM. In Vero cells we easily observed the concentration of lysosomes at the region of parasite adhesion after 15 min of interaction. This process was not observed in all parasites attached to Vero cells. With macrophages we also observed a concentration of lysosomes at the region of parasite attachment. We could not observe clearly, as in Vero cells, the process of lysosome fusion in this early step of interaction.

In conclusion, our results indicate that the host cell membrane contributes in proteins, lipids and sialoglyoconjugates to the formation of the *T. cruzi*-containing PV independently if infective trypomastigotes or non-infective epimastigotes are used. Mainly in non phagocytic cells, lysosomes participate in the early formation of PV membrane. Further biochemical studies are necessary in order to know in more detail the composition of the membrane lining the PV in order to understand better the evasion strategies used by *T. cruzi* to disrupt the membrane, a fundamental step for parasite multiplication within the host cell.

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