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Trypanosoma cruzi invasion in non-phagocytic cells: an ultrastructural study

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Abstract: *Trypanosoma cruzi* is the causative agent of Chagas disease. This parasite requires the intracellular niche in order to proliferate and disseminate the infection. After invasion, *T. cruzi* resides temporarily in an acidic vacuole which is lysed by a not well-understood mechanism. Transmission electron microscopy was used to describe the process of *T. cruzi* escape from the parasitophorous vacuole over the time. Using HeLa (non-professional phagocytic cells) as host cell, we observed that recently internalized parasites reside in a membrane-bounded vacuole. A few hours later, the first sign of vacuole disruption appeared as membrane discontinuities. This observation was followed by a progressive vacuole swelling as evidenced by an electron-lucent halo between the parasite and the vacuole membrane. Apparently, the vacuole membrane remnants reorganized as small vesicles that eventually disappeared from the vicinity of the parasites. Finally, parasites reach the host cell cytosol where replication takes place. The thorough ultrastructural description of this process set the base for a comprehensive understanding of the parasite-host cell interaction and, thus open the possibility of new therapeutic intervention strategies.

Trypanosoma cruzi is a vector-borne parasite that causes Chagas disease. This disease was once confined to Latin American countries, but demographic factors have determined its occurrence in North America, Europe and other non-endemic areas (Schmunis and Yadon, 2010). The acute phase of the infection is frequently asymptomatic, but some cases develop into severe cardiac, esophageal or intestinal abnormalities, decades after the primary infection (Coura *et al.* 2014).

This parasite undergoes a complex developmental cycle, alternating between an insect vector and several mammalian hosts (Tyler and Engman, 2001, Barrias *et al*. 2013). In mammals, two parasite forms are recognized: trypomastigote and amastigote. Trypomastigotes are the non-replicative infective form that circulates through the bloodstream and can infect all nucleated cells, including professional and non-professional phagocytic cells (Andrade and Andrews, 2004). Once a trypomastigote infects a cell, it resides transiently in a membrane-bound compartment called the parasitophorous vacuole (PV). It is well established that PV interaction with lysosomes is essential to retain the parasite intracellularly and establish a productive infection (Andrade and Andrews, 2004). The resulting acidification of the PV allows the parasite to disrupt the vacuole membrane and reach the host cytoplasm (Ley *et al.* 1990), where it differentiates into amastigotes and begins intracellular proliferation. Following several rounds of division, they undergo a transition back into the trypomastigotes, leave the cell and reach the bloodstream, thereby disseminating the infection.

Exhaustive research has been done on the ultrastructural description of the entry process and the intracellular development of *T. cruzi* in professional phagocytes (Nogueira and Cohn, 1976; Milder and Kloetzel, 1980; Carvalho *et al.* 1981; Carvalho and Souza, 1989). However, despite its crucial role on the early dissemination of the infection, the morphological study of the infection process in epithelial cells has been largely disregarded. In this brief note, we present a comprehensive picture of the intracellular stage that occurs in mammalian epithelial cells only.

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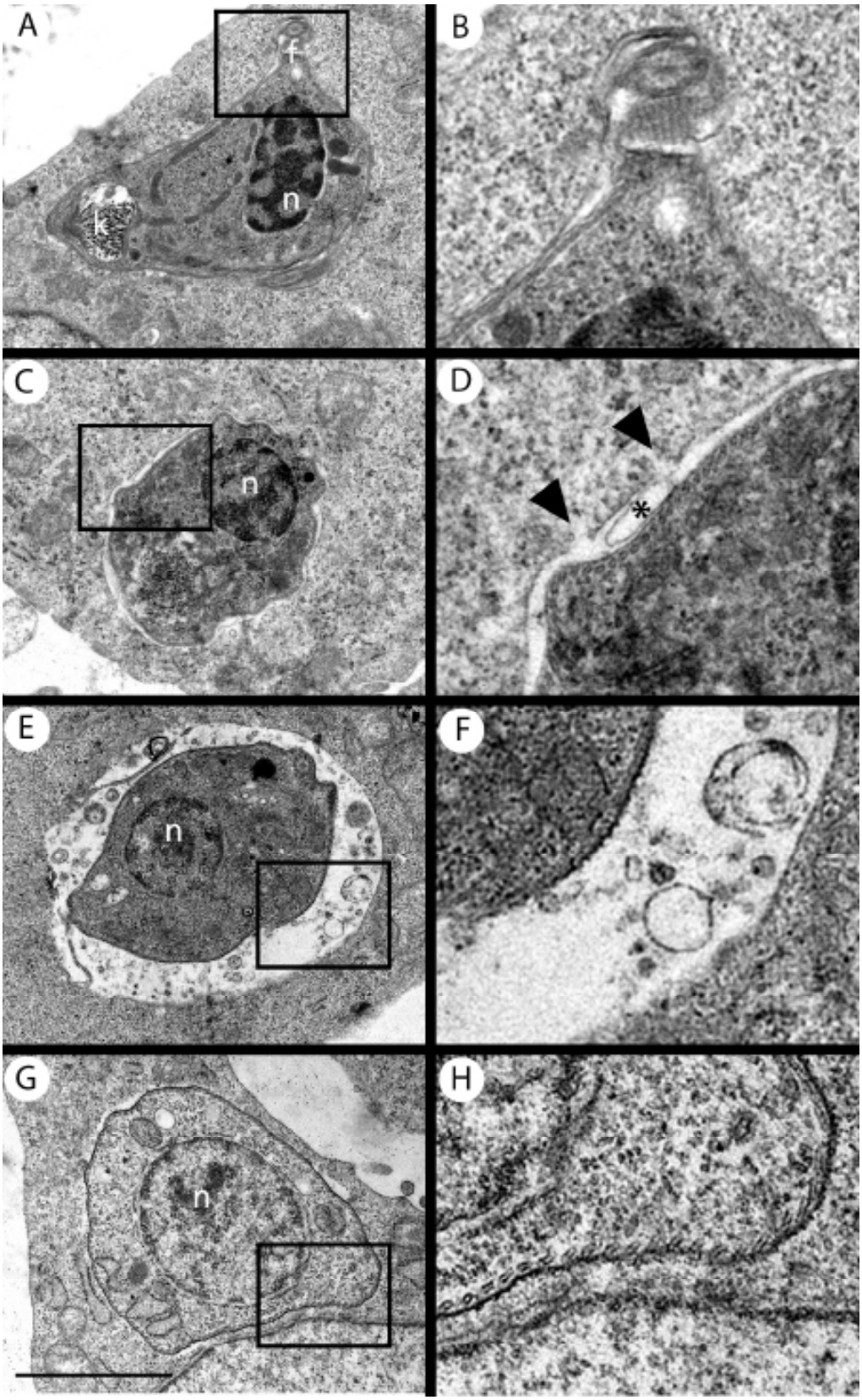

FIGURE 1. Micrographs showing HeLa cells infected with *Trypanosoma cruzi* trypomastigotes and then incubated for different time periods. (A) A trypomastigote inside an intact vacuole, 1 h after infection. (B) Enlargement of panel A, highlighting a cross-section of a flagellum and the PV's membrane. (C) A parasite inside a disrupted vacuole, 3 h after infection. (D) Enlargement of panel C. Arrowheads point to breaks in the PV's membrane. (E) Parasite inside a swollen vacuole, 6 h after infection. (F) Enlargement of panel E, showing the electron-lucent space with several small vesicles. (G) A parasite in direct contact with the cytosol. (H) Enlargement of panel G, showing the vacuole membrane is no longer present. Abbreviations; k, kinetoplast; n, parasite nucleus; f, flagellum. Scale bar represents 2 μm.

To carry out this study, HeLa cells were incubated with trypomastigotes from CL Brener strain for 1 hour at 37°C. After this period, extracellular parasites were removed by washing with PBS. One sample, corresponding to the first time point (1 hour), was immediately fixed with 2.5% glutaraldehyde, 4% formaldehyde in 1 M cacodylate sodium buffer for 10 minutes. The others were incubated for an additional period to complete a total assay time of 3, 6 and 12 hours before fixation. Then, cells were harvested by scraping, treated with 1% osmium tetroxide, dehydrated via graded ethanol solutions and embedded in Spurr's resin. Ultrathin sections were contrasted with aqueous uranyl acetate and lead citrate and examined in a Zeiss 900 electron microscope.

Intracellular trypomastigotes were easily recognized under the electron microscope through the identification of one or more of the characteristic ultrastructural features such as the flagellum, subpellicular microtubules and/or kinetoplast. One hour after infection, parasites were completely surrounded by a tightly apposed membrane-bound vacuole, mentioned above as the PV (Figs. 1A and 1B). After three hours of incubation, some PVs showed membrane discontinuities (Figs. 1C and 1D, black arrowhead). Also, it was noticeable the presence of an electron-lucent space between the PV's membrane and the parasite with some vesicles in it (Figs. 1C and 1D, asterisk). This space was even greater at six hours after infection, suggesting a progressive swelling of the vacuoles. At this time point, it was evident the PV breakdown since membrane remnants and several small vesicles could be seen in the electron-lucent space (Figs. 1E and 1F). As expected, trypomastigotes remained intact during the whole intravacuolar stage, indicating that the parasites were not being digested by lysosomal hydrolases. At 12 hours before the infection, parasites can be seen in direct contact with the cytosol where binary division takes place. No membrane remnants nor small vesicles were seen in close proximity of the parasites (Figs. 1G and 1H).

We have previously hypothesized that transition of trypomastigote to amastigote could initiate inside the vacuole (Cueto *et al.* 2017). Here, we did not find any change in the ultrastructure of the kinetoplast, a distinctive feature between trypomastigote and amastigote. Typically, trypomastigotes show a kinetoplast with basket-like structure with a dispersed kinetoplastid DNA while amastigotes are characterized by a kinetoplast with a rod-like structure, where the kinetoplastid DNA is highly condensed (Tomlinson *et al.* 1995). However, the change in the parasite's body shape, from the elongated shape of trypomastigotes to the spherical form of amastigotes, as we have observed here during the first hours after infection (Figs. 1A, 1C, 1E and 1G), supports our hypothesis. In this regard, *in vitro* studies have determined that the change in body shape occurred rapidly and preceded kinetoplast transformation (Tomlinson *et al.* 1995).

It is clear from our observations and the review of the literature that the time the parasite spends inside the PV varies depending on the cell type and the parasite strain. In our model, consisting of HeLa cells as host and CL Brener strain of *T. cruzi*, the entire process up to the vacuole lysis takes roughly 6 hours. However, Ley and col. (1990) showed that the parasite escapes from PV as early as 2 hours after infection when Y strain was incubated with human monocytes or Madin-Darby canine kidney epithelial cells. Conversely, Y strain incubated with mouse peritoneal macrophages seemed to take a longer time to escape from PV, since parasites were seen inside the vacuole as long as 8 hours after infection (Carvalho and Souza, 1989).

PV disruption is a key event in the *T. cruzi* intracellular cell cycle since parasites must reach the cytosol to start proliferation. An early study by Andrews and Whitlow (1989) presented evidence that a parasite porin-like protein, referred to as TcTox, would be responsible for vacuole disruption. TcTox is restricted to the luminal space of PV, where it is optimally active in the acidic milieu provided by the lysosomes. A *T. cruzi*'s trans-sialidase, an enzyme that is used to obtain sialic acid from host glycoconjugates, has also been implicated in PV exit (Rubin de Celis *et al.* 2006). We did not find any evidence of membrane having thickness, a feature that Nogueira and Cohn (1976) interpreted as a prequel of vacuole disruption. However, three hours after infection we observed the presence of vacuole membrane breaks such as those already described elsewhere (Carvalho and Souza, 1989; Ley *et al.* 1990). Our morphological evidence suggests that the membrane disrupting factor could be a pore-forming protein as proposed by Andrews and Whitlow (1989) or maybe a detergent-like molecule as melittin (Ladokhin and White, 2001).

Once the vacuole is disrupted, our data suggest that PV increases its volume (Fig. 1E), as revealed by the presence of an electron-lucent space between parasite surface and the PV's membrane. Although forces contributing to vacuole swelling are unknown, we can speculate that water influx through the pores could be accounted for by colloid osmotic effect, since the large diameters of these pores obviate ionic and osmotic effect. More important is that this phenomenon may contribute mechanically to the PV disruption, that ultimately leads to lysis. Membrane remnants resulting from lysis reorganize spontaneously into several small vesicles as those we observed inside the electron-lucent space. Eventually, vacuole membrane disintegrates completely, and the parasite can be seen in direct contact with the cytosol.

Based on our results and the available literature, we can conclude that the basic process we analyzed here occurs, although with a different timing, in all cells regardless of whether they are professional or non-professional phagocytes. Several essential questions remain to be answered: Which is the true nature of the parasite factor responsible for vacuole damage? Although, a TcTox was reported decades ago (Andrews and Whitlow, 1989; Ley *et al.* 1990), this paper did not result in a breakthrough since basic data as nucleotide and amino acid sequences are still lacking. Which are the signals that trigger the secretion of the PV lytic factor? The acidic nature of the PV is widely documented and has been linked with the TcTox secretion. However, the interaction with lysosomes also contributes with many other factors like proteases and glycosidases that also could be involved.

The detailed picture we described here provides a base for addressing these questions. The precise determination of the parasite (and host factors) involved in this process may lead to new strategies for the control of infection by inhibiting pathogen dissemination to new host cells.

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