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Microscopic analysis of calcium ionophore activated egress of *Toxoplasma gondii* from the host cell

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ABSTRACT

Toxoplasma gondii invades and destroys nucleated cells of warm blooded hosts in a process which involves several steps: recognition, adhesion, penetration, multiplication inside a parasitophorous vacuole (PV) and egress. The last one is the least understood. Parasite egress from LLC-MK2 cells infected with the RH strain of *T. gondii* was artificially triggered with 4BrA23187 calcium ionophore. The combination of videomicroscopy, field emission scanning electron microscopy (FESEM), and transmission electron microscopy (TEM) showed that egress does not result from host cell rupture due to overloading with tachyzoites. Videomicroscopy showed that upon calcium ionophore administration parasite rosettes disassemble, the contour of the parasitophorous vacuole disappears and each tachyzoite takes a separate route to the extracellular medium. FESEM and TEM showed the fragmentation of the intravacuolar network, the fragmentation of parasitophorous vacuole membrane and individual tachyzoites with extruded conoids migrating through the cytosol, tightly surrounded by remnants of parasitophorous vacuole membrane or free in the cytosol. Both videomicroscopy and FESEM showed that a single parasite can cross the host cell membrane without disrupting it, while a large number of parasites, egressing simultaneously, rupture the membrane and the cell as a whole. These data suggest that invasion and egress share less similarities than previously believed.

1. Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is capable of infecting virtually any nucleated cell of warm blooded animals and epidemiological data indicates that nearly one-third of the world's population is infected, fortunately, most in asymptomatically hosts (Montoya and Liesenfeld, 2004). Toxoplasma can cause foetus abortion or severe neurological damage to newborns. Retinocoroiditis, leading to sight impairment is the most common consequence in adults, frequently as a consequence of the ingestion of undercooked beef, pork or other meats (Han et al., 2008). The infective form of this parasite is characterized by the presence of secretory organelles (micronemes, rhoptries and dense bodies) which discharge their content during the process of interaction of the protozoan with the host cell. It is well known that host cell recognition and active invasion with secretion of micronemes and rhoptries lead to the formation of a non-fusogenic parasitophorous vacuole (PV) inside which a single parasite goes through several division cycles (Jones and Hirsch, 1972). As tachyzoites divide, the PV grows until parasites are released from the host cell.

Abbreviations: PVM, parasitophorous vacuole membrane; IVN, intravacuolar network.

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Invasion includes a sequence of interdependent events: attachment, which depends mainly on recognition between SAG-s and surface molecules of the host cell, followed by microneme secretion and conoid extrusion, both dependent on intraparasitic Ca^{2+} release (Carruthers et al., 1999; Carruthers and Boothroyd, 2007; Mondragon and Frixione, 1996; Monteiro et al., 2001). These steps precede the release of rhoptry contents, which target the membrane of nascent PV (Carruthers, 2002; Dubremetz et al., 1998). Once the PV containing a single parasite is formed, successive endodyogenic division cycles take place, leading to the formation of a rosette of parasites linked by the posterior end to a residual body and entangled in a network of tubular membranes that connect neighboring parasites and the whole assembly to the parasite vacuolar membrane (PVM) (Magno et al., 2005).

Parasite egress usually takes place 24–48 h after infection. A current interpretation of the egress, elegantly sustained by Moudy et al. (2001), argues that the mechanical stress exerted upon the plasma membrane by one or more large PVs causes a leakage of intracellular K^+ that leads, via phospholipase C (PLC) cascade, to an increase of intraparasitic $[Ca^{2+}]$, which finally results in the egress of parasites. Lavine and Arrizabalaga (2008) share this point of view, and reinforce it, concluding that motility is not essential for egress to occur.

According to Hoff and Carruthers (2002), invasion and egress share similarities in relation to morphology, motility and the participation of Ca^{2+}. In view of this, calcium ionophores such as A23817 and nigericin have been used to induce both invasion and egress related phenomena, such as microneme release and conoid extrusion (Mondragon and Frixione, 1996; Monteiro et al., 2001; Fruth and Arrizabalaga, 2007). However, an invading parasite does not cross the plasma membrane barrier but is engulfed by the forming PVM. On the other hand, egress requires that parasites assembled in a rosette separate from each other and cross the PVM, the cytosol and cytoskeleton meshwork of the host cell and, finally, its plasma membrane, to reach the extracellular space.

A three-step model for signaling of ionophore induced egress (IIE) proposed that a rise in calcium around the parasite, and, possibly also within it – resulting from the release of internal stores – is followed by a parasite-dependent permeabilization of host cell plasma membrane and PV, leading to the third signal to egressing parasites (Black et al., 2000). Crossing of host plasmalema was previously described as a very traumatic event, which would eventually lead to host cell lysis (Black and Boothroyd, 2000). The fate of the intravacuolar network (IVN) and PVM upon egress of the parasite remains to be elucidated. In this respect the relevance and the information that morphological studies at the ultrastructural level can provide has been underestimated. Invasion, in its morphological, molecular and biochemical aspects, has been frequently addressed in the literature (Boothroyd and Dubremetz, 2008). On the other hand, knowledge on the same issues of egress is very scarce. In this report, we trigger the release of *T. gondii* from 24 h infected cells using calcium ionophore A23817 (Endo et al., 1982) in order to follow the morphologic dynamics of parasite release. The results obtained from observations carried out by video-microscopy, transmission and field emission scanning electron microscopy, indicate that from the morphological point of view, invasion and egress are quite distinct processes. These observations agree with the recent description by Kafsack et al. (2009) of a porine-like protein secreted by micronemes on egress.

2. Materials and methods

2.1. Chemicals

Egress of *T. gondii* was induced with 10 μM of 4BrA23187 calcium ionophore (Sigma Chemical Company, St. Louis, MO, USA).

2.2. Parasites and host cell culture

T. gondii tachyzoites of the RH wild-type were obtained by peritoneal wash of 2–3-day-infected mice. The cell suspension was centrifuged at $1000 \times g$ for 10 min to remove cell debris and peritoneal leukocytes and the number of parasites in the supernatant determined in a Neubauer chamber. The parasites were resuspended in Dulbecco's modified Eagle's medium (DMEM). Swiss mice were bred at the Universidade Estadual do Norte Fluminense animal facility. The experimental protocol was approved by the Instituto de Biofísica Carlos Chagas Filho (Universidade Federal do Rio de Janeiro) Ethics Committee for animal experimentation.

Macaca mulatta monkey kidney cells (LLC-MK2) were maintained *in vitro* in DMEM supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. Cellular viability test was determined using neutral red and absorbance values were determined in an ELISA reader. For light microscopy, cultures were fixed and stained with Panotic Solution Kit®.

2.3. In vitro infections

Confluent monolayers of LLC-MK2 cells were infected with 5 or 10 parasites per cell, depending on the assay. After the interaction period of 40–50 min at 37 °C in a 5% CO_2 atmosphere, the supernatant containing parasites that had not entered a cell was aspirated and replaced by fresh medium and the infection was allowed to proceed for 24 or 48 h, after which the cells were rinsed with DMEM. Then 10 μM calcium ionophore diluted in DMEM free of serum was added and 15 min later the monolayers were fixed for light or electron microscopy. All experiments included uninfected monolayers of LLC-MK2 cells as control. In initial experiments we varied both the concentration of ionophore and the time of incubation. Using our experimental conditions the concentration of 10 μM and an incubation time of 15 min were considered the most suitable.

2.4. Videomicroscopy

Confluent monolayers of LLC-MK2 cells previously adhered to a glass coverslip were infected with *T. gondii*,