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Toxoplasma gondii invasion and replication within neonate mouse astrocytes and changes in apoptosis related molecules

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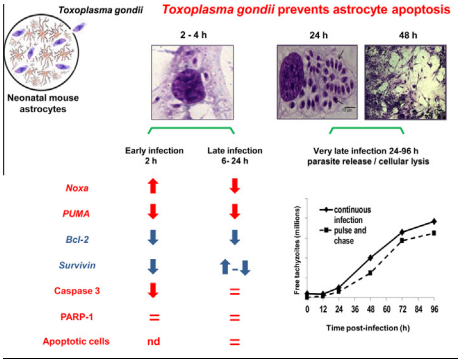
HIGHLIGHTS

- *Toxoplasma gondii* tachyzoites totally invade murine astrocytes in four hours.
- Early *T. gondii* infection induces both pro-apoptotic and anti-apoptotic signals.
- Late infection regulates Survivin, Noxa, Caspase 3/7 and PARP-1.
- *T. gondii* prevents apoptosis in neonatal mouse astrocytes.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxoplasma gondii invades any nucleated cell, but different replication speed and effects on survival/apoptosis processes have been found depending on cell type. There are scarce and controversial results regarding the effect of this parasite on host cell apoptosis within the brain. The invasion and replication of *T. gondii* RH strain within newborn mouse astrocytes were evaluated in the present work. At 4 hpi > 90% cells were infected and harbored one to three parasitophorous vacuoles with one tazchyzoite/vacuole. Cell culture massive destruction started after 24 h of exposure, when the parasite already replicated, with a duplication time of around 5 h. The effect of *T. gondii* infection on apoptosis was also evaluated by changes in some anti- and pro-apoptotic markers. At early infection times decreased *Bcl-2*, *Survivin* and *PUMA* and increased *Noxa* expression was found, although *Survivin* and *Noxa* mRNA levels reverted towards an anti-apoptotic phenotype after 6 h. Caspases 3/7 activity decreased three hours after infection, although it returned to normal levels thereafter. This enzymatic activity was strongly stimulated by Cisplatin (anti-neoplasic drug) but it was inhibited by previous *T. gondii* infection. Likewise, parasite invasion prevented PARP-1 fragmentation and cell apoptosis induced by the same drug. In

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conclusion, astrocytes seem to activate some apoptosis signals shortly after infection, but the parasite takes control of the cell and inhibits programmed death for up to 24 h, until it replicates, egresses and generates cellular destruction.

1. Introduction

Toxoplasma gondii is an intracellular parasite that invades and replicates in all nucleated cells, including the central nervous system (CNS, Carruthers and Suzuki, 2007). This protozoan may produce neurological problems in congenitally infected newborns such as hydrocephalus, deafness, blindness and mental retardation (Jones et al., 2003). Likewise, it may cause toxoplasmic encephalitis in immunocompromised adults, mainly due to reactivation of a chronic infection (Carruthers and Suzuki, 2007). In immunocompetent individuals the infection does not result in clinical disease, although it may cause psychiatric and neurological disorders (Flegr et al., 2000; Hamidinejat et al., 2010). It also provokes behavioral changes and coordination/sensory deficits in mice (Vyas et al., 2007; Gulinello et al., 2010; Webster and McConkey, 2010). Currently it is unknown if these changes are due to modification of signaling processes leading to cell death, with the resulting reduction in CNS cell numbers (Shen et al., 2001; Takahashi et al., 2001; el-Sagaff et al., 2005; Laliberté and Carruthers, 2008). Alternatively, the parasite may alter neuronal functions, as it has been recently reported for Ca^{2+} signaling upon glutamate stimulation, which could explain behavioral changes in the infected host (Haroon et al., 2012).

Astrocytes are the most abundant glial cells within the mammalian brain. They support neurons and perform nutrition and waste clearance functions; they also release "gliotransmitters" and participate in intercellular communication (Volterra and Meldolesi, 2005; Giaume et al., 2007). *In vitro* studies have demonstrated that astrocytes support *T. gondii* invasion and replication. Paradoxically, infection may induce cytokines and chemokines, which recruit and activate inflammatory cells, and thus help parasite clearance or control (Halonen et al., 1996; Fischer et al., 1997; Creuzet et al., 1998; Lüder et al., 1999; Wilson and Hunter, 2004).

Apoptosis is a form or programmed cell death, activated by several stimuli, like intracellular parasites. It is a physiological response that mediates elimination of pathogens and restores homeostasis. However, parasites have evolved strategies to modulate host cell apoptosis in order to evade immunity and enhance intracellular survival. *T. gondii* is able to alter the cell apoptotic machinery, promoting or inhibiting it, depending on its virulence and load, as well as on the host cell type (Nash et al., 1998; Carmen and Sinai, 2007; Laliberté and Carruthers, 2008).

The mechanisms by which *T. gondii* interferes with apoptosis signals are not fully understood. In various cell types it inhibits caspases-3, 7, 8 or 9 (Goebel et al., 2001; Payne et al., 2003; Gais et al., 2008; Angeloni et al., 2009). It may also act on the enzyme poly-ADP ribose polymerase (PARP-1) and modulates expression of anti-apoptotic members of the Bcl-2 family (Goebel et al., 2001; Molestina et al., 2003; Kim et al., 2006; Gais et al., 2008; Hwang et al., 2010). Likewise, the parasite can act upon the transcription factor NF-κβ involved in survival regulation (Molestina et al., 2003; Molestina and Sinai, 2005; Laliberté and Carruthers, 2008). Within the murine CNS *T. gondii* may induce neuronal apoptosis, although the panorama is still unclear (Shen et al., 2001; Takahashi et al., 2001; el-Sagaff et al., 2005).

The study of parasite effects on astrocytes can give light to pathological events within the CNS. Thus, the aim of this work was to study *T. gondii* tachyzoite invasion and replication kinetics in astrocyte primary cell cultures, as well as the dynamic changes of molecular and cellular markers of apoptosis.

2. Methods

The present study was authorized by the Animal Care Ethics Committee of the National Institute of Public Health of Mexico. Adult Balb/c female mice were housed in the pathogen-free animal house in normal light/dark cycle and provided with food and water *ad libitum*. They were left to breed with male mice for up to 48 h. Neonatal mice, up to 6 h old, were sacrificed by decapitation and the brain was removed under sterile conditions. The mothers were not sacrificed.

2.1. Astrocyte cultures

The dissected brain was put in sterile Hanḱs solution. Tissue disaggregation was achieved with 0.1% trypsin and type I DNAse (Sigma, St. Louis, MO) in agitation for 10 min at 37 °C. Fetal calf serum (FCS) was added to inhibit trypsin. Cells were washed by centrifugation and resuspended in DMEM supplemented with 10% FCS, 1 mM glutamine, MEM vitamins and antibiotic–antimycotic mixture (Invitrogen, Carlsbad, CA). Cells were cultured at 37 °C in 5% CO_2/95% air at a density of 100,000/35 mm Petri dish or 10,000/well in Lab-Teck-8 well-plates (Nunc, Roskilde, Denmark) pre-treated overnight with 0.5 mg/mL poly-D-lysine (Sigma, St. Louis, MO). After 8 days in culture, 98% astrocyte-2% neuron population was obtained with an average density of 1×10^6 cells per Petri dish or 1×10^5 cells per Lab-Teck-8 well. Identification of the cell types was done by immunofluorescence with mouse polyclonal anti-GFAP (astrocyte specific marker) and mouse monoclonal against the neuron specific marker, B tubulin III (Millipore, Billerica, MA) as described previously (Suárez-Rodríguez and Belkind-Gerson, 2004).

2.2. T. gondii invasion and replication in astrocytes

Tachyzoites of the RH strain (virulent, type I) were harvested from the peritoneal cavity of Balb/c mice inoculated 2–3 days before. The tachyzoites were then seeded on Vero cells cultured in DMEM medium, supplemented with 5% FCS and 1× antibiotic–antimycotic mixture and followed for 7–10 days. After spontaneous host cell lysis, the tachyzoites were obtained by 2000 rpm centrifugation.

For invasion and replication assays astrocytes were exposed to *T. gondii* at 10:1 parasite:host cell. This dose was chosen on the basis of preliminary experiments (not shown) and scarce data on the literature (Contreras-Ochoa et al., 2012). Cultures were followed for 30 min to 96 h (continuous exposure). Pulse and chase experiments were also done incubating the cells with the parasites for two hours, washing with sterile PBS and culturing for 94 h more. Free parasites were collected from the supernatant, centrifuged, resuspended in 1 mL of PBS and counted in a Neubauer camera.

Tachyzoite invasion and replication, as well as astrocyte culture destruction were evaluated by examination of Wright stained slides under an optical microscope (Leica, Wetzlar, Germany). Then, infection percent and number of parasitophorous vacuoles (PV) per astrocyte and of tachyzoites/PV were determined per