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ORIGINAL PAPER

Endothelial cell invasion by *Toxoplasma gondii*: differences between cell types and parasite strains

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Abstract *Toxoplasma gondii* disseminates and causes congenital infection by invasion of the endothelial cells. The aim of this study was to analyze the ability of two strains to invade two endothelial cell types. Tachyzoites of the RH and ME49 strains were expanded in Balb/c and C57BL6-RAG2−/− mice, respectively. Tachyzoites were harvested from 72 h Vero cell cultures and incubated for 30 min to 4 h at 10:1 parasite/cell ratio in 24-well plates, containing monolayers of either HMEC-1 line or human umbilical cells (HUVECs). The number of infected cells and parasitic vacuoles per infected cell were counted in Wright stained slides. A slow increase in the proportion of infected cells occurred but varied according to cell type–parasite strain combination: ME49 tachyzoites invaded up to 63 % HMEC-1 cells, while RH parasites infected up to 19 % HUVECs. ME49 and RH tachyzoites invaded 49 and 46 % HUVECs and HMEC-1 cells, respectively. Reinvasion and formation of new parasitophorous vacuoles of infected cells was more frequent than invasion of noninfected cells. The results support that the factors influencing invasion, and thus dissemination and vertical transmission, are parasite type, host cell type/subtype, and activation state. Interestingly, *T. gondii* virulence does not seem to relay on its invasion efficiency, but probably on replication speed.

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Introduction

Toxoplasma gondii is a protozoan of the phylum Apicomplexa, together with numerous pathogens of significant medical and veterinary importance (Dubey 2010). The tachyzoites replicate inside nucleated cells making invasion an essential step of the life cycle, which it is a complex multistage process involving initial attachment to the host cell, followed by sequential discharge of specialized secretory organelles, and active formation of the parasitophorous vacuole (Carruthers and Boothroyd 2007). Replication and egression are other two processes that may determine parasite virulence and dissemination (Kafsack et al. 2009).

Transplacental passage of the protozoan may occur, causing congenital toxoplasmosis (Elsheikha 2008). It is known that strain virulence may influence generation of clinical problems, migration across epithelial/endothelial barriers, and vertical transmission efficiency (Ortiz-Alegría et al. 2010; Saeij et al. 2005). Paradoxically, less virulent strains seem to be more frequent among humans and domestic animals, with either acquired or congenital infection, at least in some parts of the world (reviewed in Ortiz-Alegría et al. 2010). Infection of endothelial cells lining the placental blood vessels is thought to be among the major transmission routes to the fetus; therefore, the study of their invasion is of great interest (Dimier and Bout 1993).

Actually, it is known that *T. gondii* tachyzoites invade, replicate, and traverse endothelial cells (Benedetto et al. 1997; Cortez et al. 2005; Dimier and Bout 1993, 1996; Smith et al. 2004; Zamora et al. 2008). Available data suggest that infection and proliferation depends on the endothelial cell tissue origin (Smith et al. 2004) and the parasite strain/virulence, but these

aspects have been scarcely studied and only at one time during invasion of this cell type, i.e., before replication starts (Lachenmaier et al. 2011). The kinetics of invasion has not been analyzed comparing two endothelial cell types and two *T. gondii* strains of different virulence.

Materials and methods

Ethics and bioethics The results of the present work originated from project 037/2007, approved by the research and animal care revision boards of the Instituto Nacional de Pediatría, SSA, Mexico, which lie in national as well as international regulations for research with humans and animals.

Endothelial cells isolation and culture HUVECs were obtained by the procedure reported before by Paez et al. (2005). Briefly, term cords were treated with 0.2 % type II collagenase (Roche, Hertfordshire, UK) and cultured until confluence at 37 °C in a 7 % CO_2 atmosphere, using M199 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B (Gibco/BRL, Grand Island, NY, USA); 10 % heat-inactivated fetal calf serum (FCS, Hyclone Logan, Utah, USA); 40 μg/mL bovine endothelial cell growth factor (bECGF; Roche, IN, USA); 5 IU/mL porcine heparin, 10 mM HEPES, and 2 mM L-glutamine (Sigma, St. Louis, Missouri, USA). The HUVECs used in all experiments were of the third pass being >95 % cells positive for CD105, endothelial specific marker (BD PharMingen San Diego, CA USA; not shown).

The HMEC-1 (CDC/EU HMEC-1) is a semi-immortalized and stable cell line obtained from human microvasculature (Bonnefoy et al. 2001; Xu et al. 1994). These cells were cultured according to Ades et al. (1992) and Unger et al. (2002) at 37 °C, with 7 % CO_2 in MCDB131 medium, supplemented with 1.0 μg/mL hydrocortisone, penicillin G (100 IU/mL), streptomycin (100 μg/mL), and amphotericin B (0.025 μg/mL) (Gibco, Invitrogen, Gran Island, USA); 10 % FCS (Hyclone, Utah, USA), 10 mM L-glutamine, and 20 μg/mL endothelial cell growth factor bovine (bECGF; Roche, IN, USA).

Both cell types present many of the activities of endothelial cells (Pauly et al. 1992; Bouïs et al. 2001).

Parasites and invasion experiments Tachyzoites from the virulent RH and nonvirulent ME49 strains of *T. gondii* were maintained by intraperitoneal passage in Balb/c and C57BL6/RAG2−/− knockout mice, respectively. The parasites were collected in RPMI-1640 medium (Gibco/BRL, Grand Island, NY, USA) from the peritoneal cavity 96 h after infection, counted, inoculated on confluent Vero cell cultures, and left for 72 h in DMEM medium (Gibco/BRL, Grand Island, NY, USA), supplemented with 10 % FCS and L-glutamine. The tachyzoites were harvested from the supernatant by low speed centrifugation, counted, and used to infect endothelial cells. Parasites were utilized only if their viability was superior to 95 %, as assessed by Trypan blue exclusion.

For invasion experiments, cells were cultured on round plastic coverslips located inside 24-well plates (Nunc, Inc., Naperville, FL), at a density of 100,000 per well in their respective complete culture media and left 72 h to allow monolayer formation. Afterwards, tachyzoites of either RH or ME49 strain were added at a dose of infection (doi) of 10:1 parasite/cell for 0.5, 1, 2, 3, or 4 h in the respective media added with 10 % FCS. This doi was chosen from preliminary experiments, in which 1:1, 2:1, 4:1, and 10:1 parasite/cell ratios were tested (not shown). We chose incubations of up to 4 h, since it has been reported that the intracellular duplication time of RH strain in human fibroblasts is around 4 h (Radke et al. 1998, 2001). After washing off the free parasites, the slides were fixed with absolute methanol and stained with Wright dye. Examples of cells infected with different numbers of vacuoles are shown in Fig. 1. Percent infected cells and number of vacuoles/infected cell were determined using an optical microscope (Carl Zeiss, Munich, Germany) at ×100 magnification, completing at least 100 cells per slide and 4 fields. Three experiments, each in triplicate, were performed.

Statistical analysis Differences along time and among groups were evaluated by Kruskal–Wallis ANOVA, followed, when appropriate, by Mann–Whitney *U* test, using the SPSS V18.0 software.

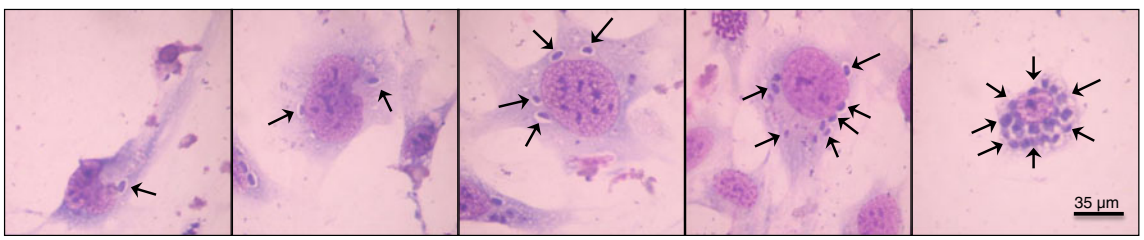

Fig. 1 Examples of cell invasion by *T. gondii*. In the slides are HMEC-1 cells infected with ME49 strain at different times: 30 min, 1, 2, 3, and 4 h. *Arrows* indicate tachyzoites in their parasitophorous vacuoles

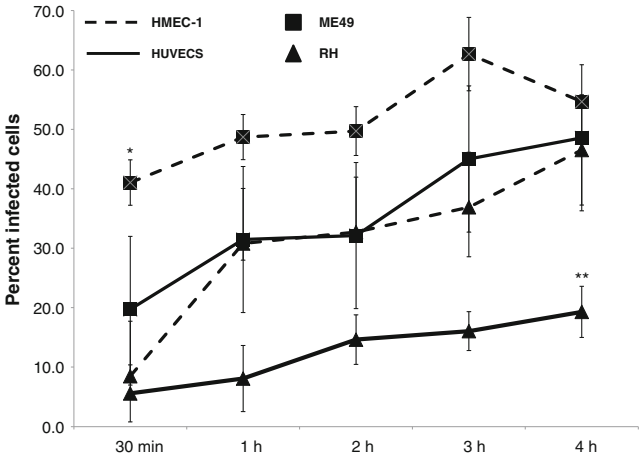

Fig. 2 *Toxoplasma gondii* RH and ME49 invasion kinetics in two cells types, HMEC-1 and HUVECS. Parasite/cell ratio used was 10:1. Values are mean±SD of three experiments in triplicate. (*Asterisk*) significantly different to the other three combinations at 30 min only (Mann–Whitney *U* test, $P<0.05$). (*Double asterisk*) significantly different from the other three groups along time (Kruskal-Wallis, $P<0.01$), except for HMEC-1 infected by RH strain at 30 min

Results

HMEC-1 cells were rapidly invaded by ME49 tachyzoites, i.e., more than 40 % of cells were already infected 30 min after exposure (Fig. 2). In contrast, less than 10 % HUVECs had been invaded by RH parasites at this time. In spite of this infection degree difference, the kinetics was similar, with no significant increase up to 4 h (Fig. 2). Except for HUVECs infected with RH strain, the number of vacuoles per cell significantly increased along the time, with more than 30 vacuoles per cell in some cases (Fig. 3).

Invasion of HMEC-1 cells by RH parasites and HUVECs by ME49 tachyzoites was of intermediate degree, i.e., around 30 % of cells were infected after 1 h of incubation. Nevertheless, the increase slope was not different among all groups, except for that of HMEC-1 cells infected with the RH strain, mainly because of a stepped increase from 30 to 60 min. A nonsignificantly different proportion of around 40 % was reached at the end of the incubation time, except in the case of HUVEC cells infected with RH strain, which remained very low (below 20 %) (Fig. 2).

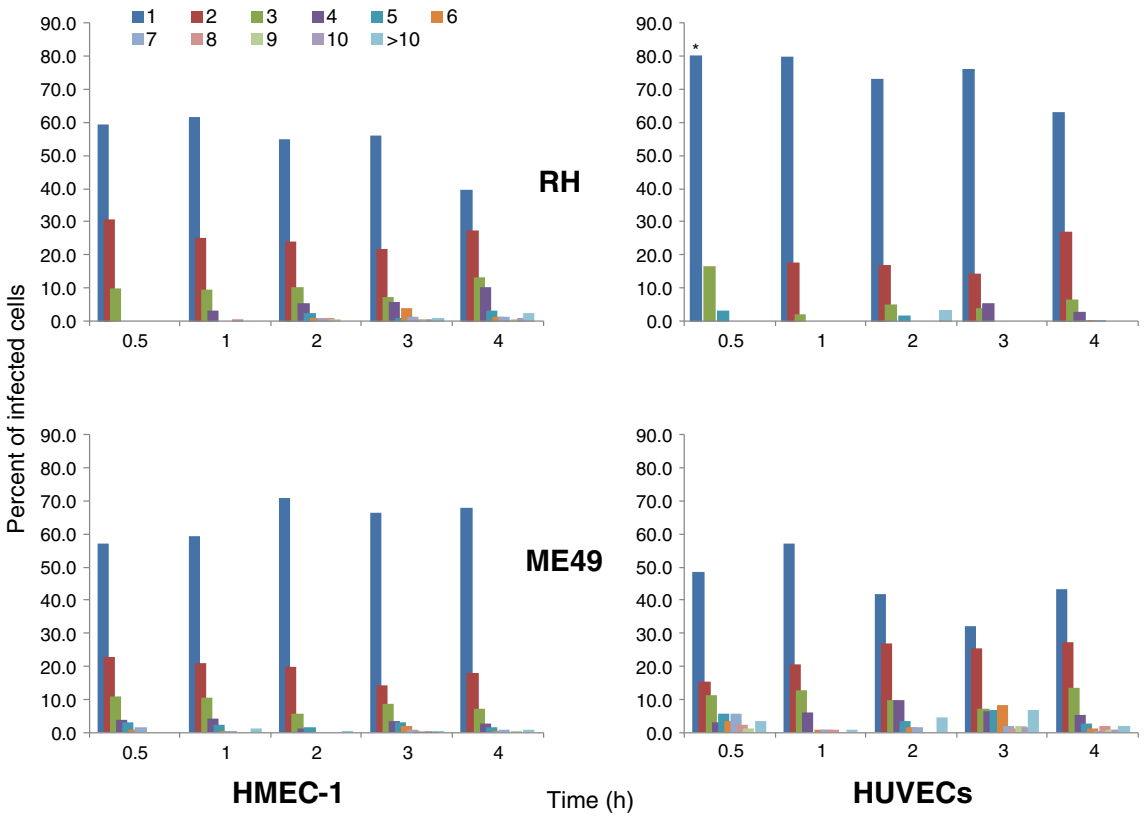

Fig. 3 Number of parasitophorous vacuoles per infected cell at different times of HMEC-1 and HUVECs exposition to RH and ME49 strains. The kinetics of infection was similar in HMEC-1 cells infected with the RH strain and HUVECs exposed to ME49 parasites; they statistically differed from the other two groups along the experiment (Kruskal-Wallis, $P<0.05$). (*Asterisk*) significantly different from the other three groups at 30 min of exposure only (Mann–Whitney *U* test, $P<0.05$)

Discussion

Migration through epithelial and endothelial barriers is a major event during *T. gondii* migration and dissemination within the host body (Barragán et al. 2005; Saeij et al. 2005). Also, infection of the endothelial cells of the placental blood vessels of the decidua and within the chorionic villi is probably the main transmission route to the fetus. Thus, it is of relevance to study endothelial cell invasion. Published studies about this subject have mainly focused on the analysis of subcellular events that lead to vacuole formation or on the effect of various hormonal or immunological stimuli on tachyzoite replication (Benedetto et al. 1997; Cortez et al. 2005; Dimier and Bout 1993; 1996). Thus, there is little evidence on invasion susceptibility. For this reason, we compared the invasion kinetics of two *T. gondii* strains, RH (virulent) and ME49 (nonvirulent) in two endothelial cell types, HMEC-1 (skin microvasculature), and HUVECs (cord vasculature). Quite unexpected, the less virulent strain invaded a greater proportion of cells than the more aggressive RH strain. Lachenmaier et al. (2011) reported similar results, i.e., that RH tachyzoites invade a lower proportion of rat brain microvasculature endothelial cells in vitro than those of the ME49 strain. Even though, they only analyzed invasion at one time (2 h) and with low parasite dose (doi=1.0), which resulted in very low proportion of infected cells (<10 %). We decided to test different doses and times, and found that comparisons could not adequately be made at low doi and short times because results were largely variable among experiments. At 30 min to 4 h exposure and with a doi=10, clear differences could be seen, both in the proportion of infected cells and the number of parasitophorous vacuoles per cell. Lachenmaier et al. (2011) results were confirmed and extended regarding strain virulence influence on invasion and sustain the apparently paradoxical higher frequency of low virulence strains among subclinical acquired and congenital human cases of toxoplasmosis in some parts of the world (reviewed in Ortiz-Alegría et al. 2010).

As mentioned, the proportion of infected cells was unique for each cell type/parasite combination at 30/60 min exposure, with a slow and almost steady increase thereafter, partially due to further invasion of infected cells (the number of vacuoles per cell increased significantly along time). These data suggest that there are limiting amounts of superficial parasite or host cell mutual "receptors", which may vary within species (polymorphisms) among cell types/subtypes within an individual and among activation states of a given cell subtype. The influence of host cell type/subtype on *T. gondii* susceptibility has been documented: It is known for example that neurons, microglia, and astrocytes are differently invaded; also, human retinal endothelial cells seem more permissive to RH tachyzoites than those obtained from the aorta, the dermis, or the umbilical vein, although it is unclear if this is due to invasion or replication related phenomena (Contreras-Ochoa et al. 2012, 2013; Smith et al. 2004; Zamora et al. 2008). On the parasite side, *T. gondii* microneme protein 2 (MIC2) binds to the host adhesin ICAM-1, expressed by endothelial cells and the syncytiotrophoblast of the placenta (Abou-Bacar et al. 2004; Barragán et al. 2005). Variations among parasite strains in MIC2 expression could induce different invasiveness of adult or fetal endothelia. On the other hand, different levels of ICAM-1 expression might have an important impact on body dissemination and congenital toxoplasmosis occurrence.

The higher susceptibility of HMEC-1cells to *T. gondii* invasion in comparison to HUVECs might also be related to the host cell cycle: it has been reported that RH and especially the ME49 tachyzoites preferentially invade fibroblasts, trophoblasts, or HELA cells during S phase, and that the parasite may induce the invaded cell as well as the neighboring ones to enter S phase (Angeloni et al. 2009; Lavine and Arrizabalaga 2009; Youn et al. 1991). Thus, since HMEC-1 cells are more constantly replicating, the probability that the parasite finds them in this phase is higher than in the case of HUVECs. In this regard, it has been reported that glioblastoma cell lines seem to be more susceptible to infection by RH strain than primary cultured astrocytes (Brenier-Pinchart et al. 2004).

The results of the present work support the notion that not all endothelial cells are susceptible to invasion at a given moment of exposure, and their proportion will determine the extent of the parasite dissemination or the risk for vertical transmission. Factors influencing invasion are parasite type, as well as host cell type/subtype, and activation state. Interestingly, *T. gondii* virulence relies more on its replication speed than on its invasion efficiency.

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