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Spontaneous cystogenesis in vitro of a Brazilian strain of *Toxoplasma gondii*

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ABSTRACT

Conversion of *Toxoplasma gondii* tachyzoites to the bradyzoite stage and tissue cyst formation in the life cycle of the parasite have crucial roles in the establishment of chronic toxoplasmosis. In this work we investigated the in vitro cystogenesis and behavior of the EGS strain, isolated from human amniotic fluid. We observed that tachyzoites of the EGS strain converted to intracellular cysts spontaneously in LLC-MK_2 epithelial cells, HSFS fibroblasts and C6 glial cell lineage. The peak of conversion occurred in the LLC-MK_2 cells after 4 days of infection, when 72.3 ± 15.9 of the infected cells contained DBA positive cysts. Using specific markers against bradyzoite, tachyzoite and cyst wall components, we confirmed stage conversion and distinguished immature from mature cysts. It was also observed that the deposition of cyst wall components occurred before the total conversion of parasites. Transmission electron microscopy confirmed the fully conversion of parasites presenting the typical characteristics of bradyzoites as the posterior position of the nucleus and the presence of amylopectin granules. A thick cyst wall was also detected. Besides, the scanning microscopy revealed that the intracyst matrix tubules were shorter than those from the parasitophorous vacuole intravacuolar network and were immersed in a granular electron dense material. The EGS strain spontaneously forms high burden of cysts in cell culture without artificial stress conditions, and constitutes a useful tool to study this stage of the *T. gondii* life cycle.

1. Introduction

Toxoplasma gondii, the causative agent of toxoplasmosis, is an obligate intracellular protozoan parasite spread throughout the world [1]. One of the main features of this protozoan is its ability to infect a large range of animals, from birds to mammals, including humans. The main infective stages of *T. gondii*: bradyzoites, tachyzoites and sporozoites, rapidly penetrate into all nucleated cells. On the other hand, under some conditions, such as the presence of the host's immune response, tachyzoites localized within a parasitophorous vacuole (PV), convert into the long term resistant form, the bradyzoites, which divide slowly inside a modified PV with a cyst wall [2]. Certainly, the conversion of tachyzoites into bradyzoites plays a fundamental role in the maintenance of *T. gondii* in nature, especially with the transmission of the parasite through carnivorism between intermediate hosts [3].

Bradyzoites remain hidden inside intracellular cysts, protected from destruction by the immune system and are the main component of the chronic phase of toxoplasmosis [4]. Under natural conditions, *Toxoplasma* cysts are preferentially found in neurons and muscle cells [5]. This stage can persist for the whole life of the host [6], without further consequences. However, there is a risk of reactivation of the acute phase of the disease, especially in situations in which the immune system is compromised, as occurs in HIV infected patients and during treatment with radiation, corticoids or antineoplastic drugs [7–9].

In infected mice, it has been shown that the ability of forming cysts is associated with virulence factors [10]. A classification based on genetic studies of circulating strains isolated in Europe and North America splits the *T. gondii* population into three different clonal lineages: I, II and III. Type I is considered the most virulent and lethal, while types II and III are considered avirulent and result in a chronic infection [11]. However, strains isolated in Asia and South America display a different pattern, showing recombinant genotypes that join both features: the virulence and the cystogenic competence in mice [12]. Ferreira et al. [13] showed that 20 isolates from Brazil are recombinant genotypes.

The initial studies of *Toxoplasma* cysts used cysts isolated from mouse brains [14]; however, the factors that lead to cyst formation and the role of the parasite and the host in this scene started to be elucidated only when this conversion could be induced in vitro. The

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first to succeed in inducing the conversion of tachyzoites to bradyzoites in vitro was Hogan [15]. Subsequently, several authors have shown that different strains of *T. gondii* could form cysts in cell cultures from different origins [16–22].

Experiments carried out in vitro have shown that it is possible to obtain bradyzoites by spontaneous conversion of tachyzoites as well as by induction of such process by changes in the culture conditions such as (a) alkalization from 7.2 to 8.0 [23], (b) the use of IFN-γ and NO inductors [24,25], (c) increase in the temperature from 37 °C to 42 °C, and (d) chemical stress with sodium arsenite [23]. Under stress conditions differentiation rates of up to 70% were attained, whereas, in a non stressed environment, only about 10% of conversion was obtained with the RH strain [23].

In this work we describe results obtained with the EGS strain of *T. gondii*, which was isolated from the human amniotic fluid of a patient with toxoplasmosis and corresponds to a recombinant I/III strain [26,27]. Our experiments using the epithelial cell line LLC-MK_2, HFSF fibroblasts and C6 glial cells show that this strain is an interesting model for further studies on cyst formation and reactivation of infection by *Toxoplasma*. The EGS strain presents in vitro high rates of spontaneous conversion of tachyzoites into bradyzoites. Infected cells presented both tachyzoite and bradyzoite infective forms, maintaining the behavior of a virulent strain.

2. Methodology

2.1. Ethics statement

This study (Protocol n. IBCCF 099/100) was approved by the Ethics Committee for Animal Experimentation of the Health Sciences Centre, Federal University of Rio de Janeiro. All animals received humane care in compliance 5 with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences, USA.

2.2. Parasites

The experimental protocols to maintain the EGS strain were established by Ferreira et al. [28]. The EGS strain was maintained in CF1 mice, infected orally, with 10 to 20 cysts obtained from brains of previously infected mice. After 48 h post infection the mice were treated with 0.5 mg/ml of sulfadiazine (Sigma Chemical Co.) dissolved in the drinking water, for 10 days, in order to control the acute infection and allow the cyst formation.

Tachyzoites of the EGS strain were obtained from CF1 mice intraperitoneally infected with 100–200 cysts. After 5–7 days of infection, parasites were collected from peritoneal fluid and allowed to interact with a confluent monolayer of LLC-MK2 cells at a ratio of 10:1 parasites per cell. The parasites used in all experiments were obtained from the supernatant of the routinely infected cultures.

The maintenance of the LLC-MK2 cultures presenting cysts was performed by trypsinization of the monolayers and expansion to new flasks. The infected cultures were kept for a maximum of 2 months. It was essential to keep free space in the culture flasks to allow the expansion of the culture and to collect the parasites from the supernatant frequently in order to avoid high burden of parasites in the cultures.

Tachyzoites from the RH strain were collected from CF1 mice intraperitoneally infected with 10^7 parasites/ml for 48 h, whereas bradyzoites of Me-49 strain were obtained from chronically infected CF1 mice, as described elsewhere [29].

2.3. Host cells

LLC-MK2 cells (ATCC©) (epithelial kidney cells from *Macaca mullata*), HFSF human fetal skin fibroblast (kindly donated by Dr. Renato Damatta-UENF, BR), C6 glial cell (from *Rattus novergicus* brain) (ATCC©), epithelial embryonic kidney cell HEK-293 (from *Homo sapiens*) (ATCC©) and L6 (muscle cell line from *R. novergicus* skeletal muscle) (ATCC©) were maintained in the appropriated medium supplemented with 10% FBS and 2 mg/ml gentamicin (RPMI (Invitrogen) for LLC-MK_2 and HFSF, DMEM F-12 (Invitrogen) for C6 and DMEM High Glucose (Invitrogen) for HEK-293 and L6). Serial passages were conducted by trypsinization when the cell density approached confluence in a monolayer. One day before the experiments, all the different cell lineages were seeded into 25 cm^2 flasks or onto 13 mm rounded cover slips and maintained at 37 °C in 5% CO_2.

2.4. Host cell infection assays

EGS parasites collected from the supernatant of a 10 day old infected culture were allowed to interact for 45 min with fresh LLC-MK_2 monolayers in a ratio of 5:1 or 10:1 parasite per host cell. The monolayers were washed twice with RPMI in order to remove remaining extracellular parasites and incubated for 5, 12, 18, 24, 30, 48, 72, 96, 192 and 288 h at 37 °C in 5% CO_2. Afterwards, infected cells were processed for transmission electron microscopy (TEM) or immunofluorescence (IFA) as described below.

RH tachyzoites and Me-49 bradyzoites were allowed to interact with LLC-MK_2 monolayers in ratios of 1:1 and 10:1, respectively for 24 to 72 h and were processed for IFA as described below.

2.5. Immunofluorescence microscopy

Immunofluorescence assays were performed using a specific monoclonal antibody against bradyzoites (7E5 anti-BAG-1 kindly provided by Dr. Louis Weiss) and polyclonal rabbit antibody against tachyzoites (anti-SAG-1) (kindly provided by Dr. John Boothroyd). The cultures were fixed for 20 min at room temperature in 4% freshly prepared formaldehyde diluted in phosphate buffered saline (PBS) pH7. The samples were permeabilized with 0.5% Triton X-100 for 20 min and incubated in a solution containing 1.5% bovine serum albumin (BSA), 0.025% fish gelatin (Sigma Chemical Co.) in PBS pH 7.2, for 1 h at room temperature, followed by incubation for 1 h with anti-BAG-1 (1:200) and anti-SAG-1 (1:300), consecutively. Afterwards, the coverslips were incubated with goat anti-mouse antibody conjugated with Alexa 546 or with goat anti-rabbit antibody conjugated with Alexa 488 (Molecular Probes) in 1:400 dilution. Controls were performed omitting the primary antibodies. After that, the coverslips were incubated with 0.5 μg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma Chemical Co.) for 5 min. To label the cyst wall, the cover slips were incubated for 1 h with 10 μg/ml *Dolichus biflorus* lectin conjugated to FITC (DBA-FITC) (Sigma Chemical Co.), which recognizes *N*-acetyl-galactosamine. The coverslips were then mounted on slides with Prolong Gold Antifade (Invitrogen). The samples were examined under a Zeiss Axioplan microscope and Leica SP5 LSCM. The excitation wavelength for Alexa 488 stained samples was argon laser at 20%, and emission was collected between 506 and 517 nm. Alexa 546 staining was imaged with the excitation laser HeNe 546 and the reflection signal was collected between 576 and 596 nm.

2.6. Evaluation of cystogenesis

The evaluation of the cystogenic behavior of the EGS strain was carried out by counting 300 cells per coverslip and discriminating the following parameters: non-infected cell, infected cell, cells with DBA positive cysts, cells with SAG-1 positive vacuoles. The percentages of infected cells positive for DBA or SAG-1 were calculated in three different experiments. Anti-SAG-1 labeled vacuoles were counted as parasitophorous vacuoles containing tachyzoites, while vacuoles