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Infection and characterization of *Toxoplasma gondii* in human induced neurons from patients with brain disorders and healthy controls

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Abstract

Toxoplasma gondii is a protozoan parasite capable of establishing persistent infection within the brain. Serological studies in humans have linked exposure to *Toxoplasma* to neuropsychiatric disorders. However, serological studies have not elucidated the related molecular mechanisms within neuronal cells. To address this question, we used human induced neuronal cells derived from peripheral fibroblasts of healthy individuals and patients with genetically-defined brain disorders (i.e. childhood-onset schizophrenia with disease-associated copy number variations). Parasite infection was characterized by differential detection of tachyzoites and tissue cysts in induced neuronal cells. This approach may aid study of molecular mechanisms underlying individual predisposition to *Toxoplasma* infection linked to neuropathology of brain disorders.

Keywords

Toxoplasma gondii; induced neuronal cells; cysts; tachyzoites; schizophrenia; brain disorders

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Conflict of interest

The authors declare that they have not competing interests for this specific manuscript.

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1. Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite (Apicomplexa phylum) that infects nucleated cells from humans and other vertebrate species. Serological studies indicate that the infection is found in almost 25 % of the world's human population [1].

T. gondii exposure has been proposed to be a risk factor for many brain disorders, including schizophrenia, bipolar disorder, obsessive-compulsive disorder, mania, anosmia, encephalitis, dementia in immunosuppressed HIV patients [2–9]. For example, individuals with first-episode, recent onset, and chronic schizophrenia show a higher percentage of antibodies against *T. gondii* than do control groups [10–12]. Additional serological data that link *T. gondii* and psychiatric disorders have been reported in studies of individuals with bipolar disorder [13,14]. Recently, possible association of *T. gondii* infections with Alzheimer's and Parkinson's diseases has been shown by the detection of increased levels of antibody against *T. gondii* in patient sera [15,16]. Although these studies indirectly suggest a possible relationship between *T. gondii* infection and the risk for brain disorders, it remains elusive how *T. gondii* may affect the human brain, or neuronal networks, consequently resulting in these disease conditions. Building an *in vitro* cell model for *T. gondii* infection would be an important step in attempting to define such mechanisms.

The life cycle of *T. gondii* is divided into sexual (feline infection) and asexual (non-feline infection) stages. The asexual component consists of two distinctive growth phases: rapidly replicating tachyzoites and slower growing bradyzoites. The tachyzoites replicate inside the host cells until they egress to infect surrounding cells or lyse the cell [1,4]. When threatened by elimination by the host's immune response, tachyzoites differentiate into bradyzoites and form cysts, which can persist for life, typically in the host's brain, skeletal or cardiac tissue [17].

In the present study, we used a cellular model to characterize *T. gondii* infection in human neurons derived from healthy controls and patients with brain disorders. Several groups, including our group, have recently reported human immature neurons that are directly converted from peripheral fibroblasts by introducing three or four genetic factors [18–24]. These converted cells are termed induced neuronal cells (iN cells) and their key neuronal features, have been demonstrated [18–22,24]. Specifically, our group had already demonstrated that iN cells have sodium channels, express the excitatory neuron markers β III-tubulin, neuronal nuclei (NeuN), calcium/calmodulin-dependent protein kinase II (CaMKII), vesicular glutamate transporter 1 (VGLUT1), and voltage-gated, type I alpha subunit (SCN1A), and can generate action potentials [20]. Here we report the successful *T. gondii* infection of iN cells derived from both healthy controls and patients with brain disorders. We also present the basic characterization of tachyzoites and cyst formation in these cells.

2. Materials and methods

2.1. Fibroblasts from human subjects

To directly address the effects of *Toxoplasma* infection on brain disorders in patient cells, we conducted upper arm skin biopsies and generated fibroblasts from patients and age-matched controls. Samples from adult normal controls and patients with childhood-onset schizophrenia carrying either the 16p11.2 duplication or the 22q11.21 deletion were collected at the Johns Hopkins Schizophrenia Center and the lab of Dr. JL Rapoport (NIMH, Bethesda, MD), respectively (Table 1). Fibroblasts were maintained in MEM (Minimum Essential Medium) (11095, Invitrogen) supplemented with 10 % fetal bovine serum (26140-079, GIBCO), 1 % non-essential amino acids (11140-050, GIBCO), 1 % sodium pyruvate (11360-070, GIBCO), and 1 % penicillin-streptomycin (1540-122, GIBCO) in a 5 % CO₂ incubator at 37 °C.

2.2. Neuronal cell generation

iN cells for *T. gondii* infection were generated by direct conversion of fibroblasts. Fibroblasts were plated onto four-well plastic chamber slides (Lab-Tek chamber slides, Permanox, C6932, Sigma) coated with extracellular matrigel (E1270, Sigma), that was diluted 1:40 in Dulbecco's modified Eagle Medium (DMEM) (11995073, GIBCO), and maintained in fibroblast medium. On the following day, cells were infected with Achaete-Scute Complex-like 1 (*ASCL1*), POU domain, class 3, transcription factor 2 (*POU3F2*), Myelin Transcription factor 1-like (*MYT1L*) lentiviruses [20] at 4:1 multiplicity of infection (MOI) in the presence of polybrene (8 µg/mL, H9268, Sigma). Infected fibroblasts were maintained in fibroblast medium for 48 h, after which the medium was replaced by neuronal medium containing 1:1 ratio Neurobasal medium (21103, GIBCO) and DMEM/F12 (11320, GIBCO), supplemented with B27 supplement (17504, Invitrogen) and N2 supplement (17502, Invitrogen). Further medium changes for the generation of iN cells were half media changes. For induction, neuronal medium contained the small molecule compounds SB-431542 (10 µM, S4317, Sigma), human recombinant noggin (500 ng/ml, 6057, R&D Systems), CHIR99021 (2 µM, 04-0004, Stegmet), and db-cAMP (200 µM, D0627, Sigma), for 2 weeks after infection. Valproic acid (VPA) (2 mM, P4543, Sigma) was added to the neuronal medium in combination with the small molecule compounds for the first week after infection. For maintenance 2 weeks past infection, medium was changed to neuronal medium containing Brain-Derived Neurotrophic Factor (BDNF) (10 ng/ml, 248-BD, R&D Systems), Glia cell line-Derived Neurotrophic Factor (GDNF) (2 ng/ml, 21-GD, R&D Systems), Neurotrophin-3 (NT3) (10 ng/ml, 267-N3, R&D Systems), and db-cAMP (200 µM, D0627, Sigma). In all experiments, cells were maintained at 37 °C in a 5 % CO₂ incubator.

2.3. *T. gondii* preparation

T. gondii strain RH-RFP, a recombinant Type I strain that constitutively expresses red fluorescent protein in the parasite cytosol, was generously provided by Isabelle Coppens (Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD) [25–27]. Parasite tachyzoites were grown and maintained on HFF (ATCC).

2.4. *T. gondii* infection

At the time of infection, the human iN cells were inoculated with RH-RFP tachyzoites at an MOI of 1, 2, 3, 5, or 10 (parasite:cell::1, 2, 3, 5, or 10:1) in neuronal medium containing BDNF (10 ng/ml, 248-BD, R&D Systems), GDNF (2 ng/ml, 21-GD, R&D Systems), NT3 (10 ng/ml, 267-N3, R&D Systems), and db-cAMP (200 mM, D0627, Sigma). Tachyzoites were added on culture day 16 together with the half media change. Inoculated cells were maintained in culture for up to 5 more days at 37 °C in a 5% CO₂ incubator. Since iN cells showed almost no cysts with lower MOIs or shorter incubation times, quantitative measurement was conducted in cells with 10 MOI at 5 days after infection (see below).

2.5. Immunofluorescence staining

iN cells were fixed on culture day 21, five days post-infection with 4% paraformaldehyde (15713, Electron Microscopy Science) for 10 min at room temperature. Fixed iN cells were permeabilized with 0.3 % Triton X-100 (9002-93-1, Sigma) in PBS (21-031, CORNING) for 10 min at room temperature. Cells were soaked in blocking solution [PBS, 10 % normal goat serum (G6767, Sigma), 1 % bovine serum albumin (A9647, Sigma)] for 2 h at room temperature and then exposed to primary antibodies diluted in blocking solution at 4 °C overnight. The primary antibodies used were: a mouse anti-MAP2 (Microtubule-Associated-Protein) (1:500, M2320, Sigma) to identify the iN cells, a rabbit SAG1 (Surface-Antigen-1) (1:1,000, AbD Serotec, 9070-0556) for tachyzoites, and DBA (Dolichos Biflorus Agglutinin)-biotinylated (1:100, Vector Lab, B-1035) for cysts. Primary antibodies were combined with the following secondary antibodies: respectively, Alexa-488 (1:400, Life Technology), Alexa-594 (1:400, Invitrogen), and DyLight 649-Streptavidin (1:300, Vector Lab, SA-5649). Cells were incubated in secondary antibodies for 3 h at room temperature. Lastly, cells were directly mounted with Prolong Gold anti-fade mounting medium with DAPI (P-36931, Life Technology) onto cover glass. Cells were visualized using a Zeiss epifluorescence microscope at 40x magnification. Of note, as the red signal from RH RFP was not strong enough to clearly visualize tachyzoites, we conducted immunostaining for SAG1.

2.6. Quantification and statistical analysis

Quantitative analyses of iN cells infected with tachyzoites or cysts were based on three independent experiments. Specifically, the percentage of iN cells carrying the tachyzoites was calculated by determining the ratio of MAP2- and SAG1-positive iN cells to MAP2-positive iN cells. The percentage of iN cells infected with cysts was defined as the ratio of MAP2- and DBA-positive iN cells to MAP2-positive iN cells. The average number of tachyzoites or cysts per infected iN cells was calculated by dividing the total number of intracellular tachyzoites or cysts by the total number of iN cells infected with tachyzoites or cysts in a given visual field. Our definition of “cyst” takes into account the DBA immunoreactivity and the additional morphological criterion of cyst size measured by its diameter. The diameter was measured by an epifluorescent microscope following its calibration. The average size (diameter) of cysts was calculated by dividing the sum of the diameter in all cysts by the total number of cysts. Statistical analyses were performed using

the Kruskal-Wallis test followed by Dunn's post hoc test. A value of $p < 0.05$ was considered significant.

3. Results and discussion

T. gondii infection in iN cells was carried out at 16 days after genetic cell conversion when cells express the representative neuronal marker, MAP2. *T. gondii* infection of iN cells was first validated by the identification of tachyzoites by immunostaining for SAG1, which is the major parasite surface protein belonging to the related sequence family involved in the invasion of the parasite into the cells. Tachyzoites were found to be localized in the soma of iN cells (Fig. 1A). We observed that 45.9 % of iN cells maintained tachyzoites 5 days after infection (Fig. 1B). We did not find any robust differences in the percentage of tachyzoite-positive cells among individuals (controls and individuals with psychiatric disorders). Nonetheless, the number of tachyzoites per infected iN cells was higher in cells with 22q11 deletion compared to those from the other two groups (Fig. 1C).

T. gondii differentiation within the neurons was investigated by examining spontaneous cyst formation 5 days after infection. Cyst formation was assessed by the presence of *N*-acetylgalactosamine moieties, specific to the cyst wall, which is labeled by DBA lectin [28]. We observed 1–2 round-shaped cysts per iN cell (although a small fraction of iN cells carried 3–8 cysts), which were located adjacent to the nucleus (Fig. 2A and Supplementary Fig. S1). We found cyst formation in 57.0 % of iN cells (Fig. 2B). In contrast, the iN cells' parental fibroblasts were completely lysed by parasite infection. Therefore, the change in cell phenotype from fibroblasts to neurons seems to have enabled the cyst formation in iN cells. Although we cannot completely rule out the possibility that cysts were formed in host fibroblasts prior to this 5-day timepoint, it is likely that predominating tachyzoites killed the fibroblasts before forming cysts. We did not find any differences in the number of cysts per cell among the three groups of iN cells (Fig. 2C). Of note, the diameter of the cysts was smaller in cells from patients, in particular in the individual carrying the 16p11.2 duplication (Fig. 2D). Cyst formation was assessed after 5 days post-infection. If neuronal cell viability is improved even under *T. gondii* infection, one might observe the formation of bigger cysts after a longer infection time.

Our studies indicate that human neuronal cells (iN cells) can be infected by *T. gondii*. Most previous studies of *T. gondii* growth in neurons have been conducted in animal models. We propose a new system to directly investigate the growth of *T. gondii* tachyzoites and their conversion to bradyzoites *in vitro* in human postmitotic neurons. In the current study, we have demonstrated that the RH strain forms cysts in iN cells to further validate the importance of our human iN cell system in studying *T. gondii* infections.

Using iN cells has several advantages over using neuroblastoma cell lines as an *in vitro* model to study human neuronal infection. First, we can generate iN cells from peripheral fibroblasts from patients with various brain disorders and/or genetic mutations, and can thus assess the effects of these factors on neuronal susceptibility to *T. gondii*. Second, iN cells display key neuronal traits, such as action potentials that are not observed in less differentiated cells, including neuroblastoma cells. While there are a few reports of *T. gondii*

infection in human primary neuronal cultures derived from retina of cadavers [29] or aborted fetuses [30], iN cells have a major advantage over these earlier cell culture that neuronal cells can be obtained from living subjects with defined genetic and clinical characteristics at the individual levels.

The present report has generated several interesting questions to be addressed in future studies. First, it will be important to systematically observe tachyzoite growth, considering primary or secondary infections, as well as parasite invasion or replication. Second, future studies should modify culture conditions to keep the cells alive for longer, such as by co-culturing with astrocytes and/or including additional trophic factors, to allow further investigation of cyst formation in human neurons.

Indeed, since human cells are used, this model is more relevant to human infection than models that employ neuronal cells from other species, such as mice and rats [31]. In addition, our cell model allows for the assessment of neuronal mechanisms directly influenced by *T. gondii* infection, independent of the host immune response. Further applications of iN cell technology may lead to an increased understanding of host factors operant in *T. gondii* infection in humans, as well as individual variations in the neuronal response to *T. gondii* infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

T. gondii infection in iN cells with tachyzoites. (A) Immunostaining of infected iN cells (MAP2, green) with tachyzoites (SAG1, red), the high replicative form of *T. gondii*. Tachyzoites were located in the soma. Nuclei were stained with DAPI (blue). Nuclei are outlined by dashed white lines. Scale bar, 10 μ m. (B) Percentage tachyzoites-positive iN cells over total infected iN cells. Healthy control, circles; 16p11.2 duplication, pluses; 22q11.21 deletion, triangles. (C) Average number of tachyzoites in each iN cell. Healthy control, circles; 16p11.2 duplication, pluses; 22q11.21 deletion, triangles. A total of 118 iN cells were counted. * $p < 0.05$.

Fig. 2.

T. gondii infection in iN cells with cysts. (A) Immunostaining of infected iN cells (MAP2, green) carrying cysts (DBA, pink), the dormant form of *T. gondii*. Cysts in the soma pushed the nucleus (DAPI, blue) to the side. Nuclei are outlined by dashed white lines. Scale bar, 10 μm. (B) Percentage of cysts-positive iN cells. Healthy control, circles; 16p11.2 duplication, pluses; 22q11.21 deletion, triangles. (C) Average number of cysts in each iN cell. Healthy control, circles; 16p11.2 duplication, pluses; 22q11.21 deletion, triangles. A total of 149 iN cells were counted. (D) Average diameter (μm) of cysts. Healthy control, circles; 16p11.2 duplication, pluses; 22q11.21 deletion, triangles. A total of 69 cysts were measured.

**p<0.01.

Table 1

Demographic information

	Age	Sex	Race	Age of onset
Control	25	Female	Caucasian	
	27	Female	Caucasian	
16p11.2 duplication	28 *	Female	Caucasian	10
	16	Female	Caucasian	8
22q11.21 deletion	24	Female	Caucasian	10
	23	Female	Caucasian	9

* This subject also carries the 22q13.3 duplication.