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Atypical virulence in a type III *Toxoplasma gondii* strain isolated in Japan

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ABSTRACT

The virulence of a type III *Toxoplasma gondii* strain isolated in Japan and designated here as TgCatJpGi1/TaJ was examined in mice and micro minipigs in this study. Despite its type III genotype, oral or intraperitoneal inoculation of cysts from it resulted in severe virulence in C57BL/6J and BALB/c mice. In contrast, mice inoculated with a high dose of TgCatJpGi1/TaJ tachyzoites showed no obvious clinical signs of infection, and all of them survived for > 21 days post-inoculation. Furthermore, no clinical signs of infection were seen when micro minipigs were inoculated with 900 cysts. Interestingly, our allelic type screening of the virulence-related *rop5*, *rop16*, *rop17*, *and rop18* genes, as based on restriction fragment length polymorphism analysis (RFLP), revealed that the RFLP patterns for TgCatJpGi1/TaJ were identical to those from nonvirulent type III parasites. These results suggest that TgCatJpGi1/TaJ possesses an unknown virulence factor or factors.

1. Introduction

Toxoplasma gondii, a zoonotic protozoan parasite, causes widespread infections in humans and other animals. Infection with *T. gondii* is mainly acquired when the tissue cysts from this species in undercooked or raw meat are ingested, or by ingesting the sporulated oocysts excreted in the feces from infected cats. Although most *T. gondii* infections in humans and animals are generally asymptomatic, severe disease, encephalitis, myocarditis and pneumonitis can occur in immune-compromised individuals. Moreover, infection of *T. gondii* during pregnancy can cause severe damage to the fetus and induce abortions and stillbirths.

In North America and Europe, the population structure of *T. gondii* is remarkably clonal. Restriction fragment length polymorphism (RFLP) analysis of six loci from 106 strains isolated in North America and Europe led to the reclassification of *T. gondii* into 3 lineages: types I, II and III [1]. Type I and-III parasites display different virulence patterns in mice, with type I being highly virulent and exhibiting a 100% lethal dose (LD_{100}) profile in mice upon injection with 10^0 parasites. In contrast, type II has low virulence ($LD_{50} > 10^3$) and type III is avirulent ($LD_{50} > 10^5$) [2]. The “3-clone hypothesis” is supported widely by the findings from the strains isolated in North America and Europe, with rare exceptions. In South America, Africa and Asia, however, many distinct clones of *T. gondii*, from which RFLP patterns do not correspond to types I or III, have been isolated [3–5]. These strains are designated as ‘atypical’ or ‘exotic’ [6]. For example, the Chinese type 1 strain, which dominates in China, displays a significant phenotypic variation within same genotype group [7]. This indicates that the current knowledge gained from genotyping studies on *T. gondii* is insufficient for understanding the current worldwide situation for this parasite.

It has been reported that some polymorphic rhoptry proteins (ROPs) including ROP18, ROP5, ROP16 and ROP17 are responsible for virulence in *T. gondii*, and that rhoptry-related virulence is associated with *rop18* and *rop5* allelic types in particular [8]. This pair of parasite effectors target host immunity by disrupting immunity-related GTPases (IRGs) [9]. IRGs require IFN-γ for the control of toxoplasmosis in mice

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[10,11]. It is also known that ROP16 and ROP17 are involved in the pathogenicity of *T. gondii*. ROP16 is important for down-regulating the host innate immune response by activating STAT3/6 [12,13], while ROP17 plays a role in the avoidance of parasite clearance by host cells [14].

In this study, we examined two *T. gondii* strains isolated from cats in Japan and identified a type III strain that showed atypical virulence in mice.

2. Materials and methods

2.1. Animals

C57BL/6J and BALB/c (female, 8 weeks of age) mice were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). Micro minipigs (male, 8 month of age) were purchased from Fuji Micra Inc. (Fujinomiya, Japan). All the experiments using animals were performed in accordance with the Gifu University Animal Care and Use guidelines from the Committee (Permission Nos. 15,005 and 15,078).

2.2. Parasites

The first *T. gondii* strain, which was isolated from the lung biopsy sample from a cat with lymphoma that was treated at Gifu University Animal Hospital, Japan, is designated here as TgCatJpGi1/TaJ. The second *T. gondii* strain was isolated from a cat in Tokyo a few decades ago after which it was maintained in the laboratory and is designated TgCatJpTy1/k-3. These strains and a type III strain called CTG [2] were maintained by serial passage in mice by brain cyst inoculation. Mice were inoculated with a few cysts (< 10) and the cysts for further passage were obtained from the surviving mice. PLK strain [2], a type II strain, was maintained in the Vero cell cultures as tachyzoites.

2.3. Preparation of TgCatJpGi1/TaJ and TgCatJpTy1/k-3 tachyzoites

The brains of chronically infected C57BL/6 mice were harvested and homogenized in 1 ml of phosphate-buffered saline (PBS). The brain emulsion was passed through a 21 G needle twice. Pre-warmed (37 °C) acid pepsin solution (pepsin, 0.05%; NaCl, 5%; HCl, 8.5 μM; and distilled water up to 4 ml) was added to the brain emulsion followed by incubation at 37 °C for 10 min. After incubation, a 2.7 ml aliquot of $NaHCO_3$ solution (1.2%) was added and the emulsion was incubated at room temperature for 10 min. The emulsion was then filtered through a 5-micrometer filter (Merck Millipore Ltd., Ireland) and centrifuged at 600 $\times g$ for 10 min. The sediment was suspended in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) and then added to a Vero cell culture in RPMI-1640 medium containing 7.5% fetal bovine serum and 2.0% gentamicin sulfate at 37 °C with 5% CO_2 present. After sufficient numbers of tachyzoites had proliferated in the Vero cell culture, they were isolated for use in the following experiments.

2.4. Phylogenetic tree analysis

We obtained the sequence data for uracil phosphoribosyl transferase (UPRT) intron 1, UPRT intron 7, hypothetical protein (HP) intron 2, dense granule protein 6 (GRA6), dense granule protein (GRA7), and surface antigen gene 1 (SAG1) in the representative haplogroups from the NCBI database (http://www.ncbi.nlm. nih.gov/) or ToxoDB (http://toxodb.org/toxo/) with which to build the phylogenetic trees. Each sequence was aligned using MAFFT ver. 7 [15]. Phylogenetic analysis of these sequences (3700 base pairs; bps) was conducted in RAxML ver. 8.1.5 [16] using the maximum-likelihood method and the GTR + Γ + I model. This model follows the IQ-TREE ver. 1.4.4 (http://www.iqtree.org/) model test. To evaluate the reliability of the phylogenetic tree, bootstrap analysis was conducted 1000 times.

2.5. Cyst infections

C57BL/6 mice were infected intraperitoneally with 1×10^5–1×10^7 tachyzoites or with 10–20 cysts. After 14 days post-infection at least, the brains from the surviving mice were harvested and homogenized in 5 ml of PBS. The number of cysts in five 50 μl aliquots of the brain emulsion was counted and the brain emulsion was diluted to the required concentration in PBS. The diluted emulsion containing 100 cysts (500 μl aliquot) was injected intraperitoneally into each C57BL/6 mouse. C57BL/6 or BALB/c mice were inoculated orally with 200 μl of the diluted emulsion containing 10, 50 or 100 cysts. The micro minipigs were given 900 TgCatJpGi1/TaJ cysts mixed in with their food.

2.6. Tachyzoite infections

To purify the tachyzoites from the Vero cell cultures, the infected Vero cells were broken by passage through a 27 G needle three times and then centrifuged at 2000 rpm for 10 min. To remove the host cell debris, the pellet was suspended in 10 ml of RPMI medium and filtrated using a 5-micrometer filter. Parasite numbers were counted using a cell counter, and 1×10^5 tachyzoites were injected intraperitoneally into each C57BL/6 mouse.

2.7. Typing rop18, rop5, rop16, and rop17 genes

The polymorphic regions of *rop18*, *rop5*, *rop16*, and *rop17* genes were PCR-amplified as described previously, with the minor modifications set out below [6,17]. The sequences of the amplified (*rop5, rop16* and *rop17*) gene fragments were determined, but instead of digesting the PCR products with restriction enzymes, the RFLP profile for each ROP gene was estimated from its sequence.

2.8. Western blot analysis

A crude tachyzoite antigen solution was prepared from PLK strain tachyzoites. The tachyzoites purified as mentioned above were lysed using Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and an equal volume of electrophoresis sample buffer (Santa Cruz Biotechnology Inc., Dallas, TX, USA) was added and heated at 95 °C for 5 min. A 15 μl aliquot of the crudely purified tachyzoite antigens was loaded into each well of 5–20% SDS gel. After electrophoresis, the antigens were transferred to a nitrocellulose membrane using the Trans-blot@SD Semi-dry Transfer cell (Bio-Rad, Hercules, CA, USA) over 90 min followed by incubation in 3% skimmed milk overnight. The membrane was then incubated in a micro minipig serum sample (diluted 1:1000 in 3% skimmed milk) at 37 °C for 1 h. The membrane was washed six times (5 min each wash) in PBS plus Tween 20 (PBS-T). The membrane was incubated with a rabbit anti-pig IGG-HRP secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) (diluted 1:20000 in 3% skimmed milk) at 37 °C for 1 h, and finally washed in PBS-T six times (5 min per wash). Amersham ECL™ Prime western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, England, UK) was used to visualize the reactive bands, according to the manufacturer's instructions.

2.9. Statistical analysis

Statistical differences in the survival rates of the experimental groups were analyzed by the log rank test. Statistical test was performed by Statcel 4 software (OMS publication, Saitama, Japan) with $p < .05$ being considered significant.