Studies on the Extracellular Environmental Factors that Induce *Toxoplasma gondii* to Egress from Tissue Cysts

(トキソプラズマの組織シストからの脱出を誘導する細胞外環境因子に関する研究)

2021

The United Graduate School of Veterinary Science, Gifu University
(Gifu University)

SAITO, Taizo

Studies on the Extracellular Environmental Factors that Induce *Toxoplasma gondii* to Egress from Tissue Cysts

(トキソプラズマの組織シストからの脱出を誘導する細胞外環境因子に関する研究)

SAITO, Taizo

CONTENTS

CHAPTER 3

Specific sodium-potassium ratio and the presence of calcium triggers excystation

ABBREVIATIONS

A AIC: Akaike's information criterion

B BFD1: Bradyzoite-formation deficient 1

C CI: confidence interval

cGMP: Cyclic guanosine monophosphate

CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeated/ CRISPR-Associated Protein 9

CytoD: Cytochalasin D

D DBA: Dolichos biflorus agglutinin

DHFR-TS: Dihydrofolate reductase-thymidylate synthase

DMSO: Dimethyl sulfoxide

E EC: Extracellular buffer

EGTA: Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

F FCS: fetal calf serum

G GRA2: Dense granule protein 2

H HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

I IC: Intracellular buffer

INF-γ: Interferon gamma

i.p.: intraperitoneal injection

P PBS: Phosphate-buffered seline

p.i.: post infection

PV: Parasitophorous vacuole

R ROC: Receiver operating characteristic

S SAG1: Surface antigen 1

T TgGC: Guanylate cyclase

T. gondii: *Toxoplasma gondii*

TgPKG: Protein kinase G

V VGCCs: Voltage-gated cation channels

GENERAL INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan belonging to the phylum Apicomplexa. It has a vast host range, being capable of infecting nearly all mammals including humans, and birds, and is one of the most common zoonotic parasites worldwide, infecting about one-third of the world's human population and about 20% of the world's pig population (21, 61). In humans, the parasite causes encephalitis in immunocompromised patients, miscarriages in pregnant women, and fetal abnormalities (46). In dogs, pigs, and cats, similar pathogenesis to that in humans has been reported (7, 15). In addition, it causes neurological diseases in dogs, hepatitis and inflammatory bowel disease in cats, and death of young animals, respiratory symptoms due to lung lesions, and necrosis of the liver in pigs (7, 15).

Toxoplasma gondii infects the Felidae as a definitive host, differentiates into dioecious gametocytes, reproduces sexually in the intestine, and excretes oocysts in the feces (3). The oocysts mature outside the body of the host, forming sporocysts, and sporozoites are formed inside the sporocysts (3). When mammals, including humans, and birds, which are intermediate hosts, ingest mature oocysts, sporozoites are released from the oocysts, and the parasites disseminate and multiply asexually in almost all of the tissues in the body in a form called tachyzoite during the acute phase immediately after infection (3). Since tachyzoites are characterized by high motility and rapid proliferation, they are thought to be advantageous for systemic dissemination and increase in absolute numbers. However, tachyzoites are eliminated by host immunity and therapeutic agents (16).

Later, it enters the chronic phase: tachyzoites change to a form called bradyzoite, mainly in the brain and muscles, forming a structure called latent cyst, which is composed of many bradyzoites enclosed in a shell (16). When tachyzoites differentiate into bradyzoites and form cysts, bradyzoites are not eliminated by the host's immune system. Instead, bradyzoites lose motility and proliferative efficiency. Bradyzoites are also resistant to therapeutic agents (16, 31). Thus, the change to bradyzoites and cyst formation are necessary for establishing and maintaining latent infection. Cysts are maintained inside host cells for a long time, possibly several years (49). However, once they are ingested by a new host, for example, via the intake of cyst-containing organs/tissues of meat products, the zoites inside the cyst escape from the cyst ("excystation") in the intestine of the new host, thereby establish the infection (13). This is accompanied by the differentiation of bradyzoites into tachyzoites (27), although it is unclear whether this differentiation occurs before or after excystation. In addition to the differentiation induced by ingestion, excystation and differentiation from bradyzoites to tachyzoites within infected tissue occurs when the host becomes immunocompromised during chronic infection, which can be lethal (17, 26, 33).

Thus, the "stage conversion" of *T. gondii* between tachyzoites and bradyzoites occurs with timely manner in its life cycle, in response to drastic changes in the surrounding environment, e.g., the predation of host animal by carnivores, immune response, and the immunosuppression of the host. Regarding the stage conversion from asexual tachyzoites to sexual gametocytes in the intestine of a definitive host, it was recently reported that the linoleic acid composition in the intestinal tract of

cats induces stage-conversion as an important environmental trigger (34). In the cat intestine, the enzyme activity of delta 6 desaturase, which synthesizes fatty acids using linoleic acid as a substrate, is deficient, resulting in the accumulation of linoleic acid from the diet (45, 53). It is thought that *T. gondii* recognizes this accumulation as a trigger for sexual reproduction. However, the mechanism by which *T. gondii* recognizes the change in the environment to induce the stage conversion from bradyzoite to tachyzoite is unknown, despite its importance in the pathogenicity of toxoplasmosis.

In contrast to the environmental factor that induces stage-conversion, *i.e.* environmental cue, from bradyzoite to tachyzoite, the signal cascade in *T. gondii* cells during the stage conversion and molecules involved, such as second messengers, kinases, and histone remodeling enzymes, has been well studied. Recently, Waldman *et al.* identified a transcription factor, bradyzoite-formation deficient 1 (BFD1), which is homologous to the human transcription factor, c-Myb, as the master regulator of stage conversion. This transcription factor binds to the promoters of many stage-specific genes, and the deletion of BFD1 prevents stage conversion and suppresses the expression of most of the bradyzoite-specific genes (64). In addition to the signal cascade in *T. gondii* cells, the stage-specific gene expression patterns of each stage, tachyzoites, bradyzoites, oocysts, and sporocysts (24, 64), have been well studied, and the databases are open to the public (https://toxodb.org/toxo/app).

As mentioned above, an environmental cue to start stage conversion between

tachyzoites and bradyzoites is not yet known. The differentiation of tachyzoites into bradyzoites in *in vitro* culture systems can be induced by stresses such as high temperature (43 °C), high alkali environment (pH 8.0), and depletion of arginine and pyrimidine (5, 22, 54, 55, 65), although it is unlikely that such changes occur in the host body since they are artificial stresses that usually do not exist *in vivo* conditions. In addition, the treatment of macrophages and astrocytes infected with *T. gondii* with interferon gamma (INF-γ) also induced stage conversion from tachyzoites into bradyzoites inside these cells *in vitro* (4, 30). This stage conversion is thought to be triggered by NO induced by IFN-γ (29). Thus IFN-γ and NO is considered a possible environmental cue for stage conversion (29). However, bradyzoites are mainly observed in skeletal muscles and neurons of experimentally infected animals, but not in macrophages or astrocytes (14, 35). It has also been reported that tachyzoites infecting cultured skeletal muscle cells autonomously convert into the bradyzoite form without any special treatment (20, 58). Therefore, it is likely that IFN-γ is not essential for this stage conversion *in vivo*. To the best of our knowledge, no candidate of environmental cues for inducing stage conversion from bradyzoites into tachyzoites has been reported.

In *T. gondii* life cycle, excystation of latent cysts and stage conversion of bradyzoites into tachyzoites occur at two time points: predation of cyst-containing tissues and immunodeficiency of the cyst-infecting host. Many studies have been reported on the cyst reactivation in immunodeficient hosts, because it can lead to lethal conditions such as serious encephalitis. The depletion of CD4 and CD8 positive cells eventually leads to the appearance of large numbers of tachyzoites in systemic

tissues, resulting in a lethal condition (26). However, it is not yet known whether such immunosuppression is a direct environmental cue to start the signal cascade leading to the stage conversion. It is possible that sporadic cyst activation occurs regardless of immune status, with CD4 and CD8 positive cells merely contributing to the elimination of the resulting tachyzoites. In fact, a small but certain number of cysts excystations and the appearance of tachyzoites are observed in the tissues of immunocompetent mice. Thus direct environmental cues to induce stage conversion in immunodeficient hosts are not yet known. Such cues that induce the stage conversion upon predation of cyst-containing tissues are also not yet known.

Considering the survival strategy of the parasite, the trigger of stage conversion from bradyzoite to tachyzoite, in an immunocompromised host may vary depending on occasions and may not be definitive, since the conversion is not essential for the parasite survival. In contrast, rapid excystation and stage conversion from bradyzoites into tachyzoites at the time of predation is essential for the parasite for its transmission, systemic dissemination and number increase in the new host. Therefore, I decided to focus on the stage conversion upon cyst ingestion.

In this study, I searched for an environmental cue for induction of cyst-excystation and stage conversion from bradyzoites into tachyzoites, thus clarifying the temporal orders of excystation and differentiation from bradyzoites to tachyzoite.

CHAPTER 1

T. gondii actively induces excystation in response to extracellular efflux

1. INTRODUCTION

In the chronic phase, *T. gondii* differentiates from tachyzoites to bradyzoites and forms cysts, which are numerous bradyzoites covered with walls, mainly in nerve cells and muscle cells, and undergoes latent infection (44, 56). However, when the infected animals are preyed, the parasites differentiate from bradyzoite to tachyzoite and immediately to infect the predator. Upon predation, the cyst-containing tissue is subject to digestion, and cysts that were immersed in intracellular fluid in the host cell, become immersed in digestive and intestinal fluids as cysts are shed from the host cell. The excystation must occurs during the period. It is unclear whether the excystation is the results of physical and biochemical destruction of the cyst wall by digestive fluids, or the process actively caused by the parasites upon sensing the changes in the surrounding environment. In this chapter, I examined whether *T. gondii* can actively induce excystation.

2. MATERIALS AND METHODS

2.1. Cells, mice, and parasites

The Vero (African green monkey kidney) cell line was cultured in RPMI 1640 medium containing 7.5% FCS at 37 ℃ in a 5% CO_2 incubator. C57BL/6J and Crlj:CD1(ICR) mice were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). ICR mice were used in most of the experiments, because they can produce a larger number of cysts. However, for some experiments, C57BL/6J was used. Infected mice were maintained in 225 × 338 × 140 mm cubic cages with 4 to 6 mice per cage. The bedding and drinking water were changed and food pellets were added approximately every 3 days. All experiments using mice were conducted according to the guidelines issued by Gifu University, Japan (permission no. 17059). In this study, transgenic *T. gondii* TgCatJpGi1/Taj/GRA Red, which expresses a red fluorescent protein (DsRed Express) in both the tachyzoite and bradyzoite stages, was used (1). Tachyzoites were maintained in Vero cell cultures. TgCatJpGi1/Taj/GRA Red strains used was derived from the TgCatJpGi1/Taj strain. The TgCatJpGi1/Taj strain is one of the cystogenic strains (59). Among *T. gondii* strains, the number of cysts in the experimentally infected mouse brain highly varied. In order to obtain enough number of cysts, TgCatJpGi1/Taj strain was used in this study.

2.2. Buffer composition

Extracellular buffer (EC) consisted of 141.8 mM NaCl, 5.8 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–NaOH, and 7.5% FCS. The acidity of

EC was adjusted to pH 7.2 with HCl. Intracellular buffer (IC) consisted of 5 mM NaCl, 142 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–KOH, 2 mM Ethylene glycol bis(2-aminoethyl Ether)-N,N,N',N'-tetraacetic acid (EGTA), and 7.5% FCS. The acidity of IC was adjusted to pH 7.2 with HCl.

EC + dimethyl sulfoxide buffer (EC + DMSO) consisted of 141.8 mM NaCl, 5.8 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–NaOH, 56 mM DMSO and 7.5% FCS. The acidity of EC + DMSO was adjusted to pH 7.0 with HCl. EC + Cytochalasin D buffer (EC + CytoD) consisted of 141.8 mM NaCl, 5.8 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–NaOH, 1 μM Cytochalasin D, 56 mM DMSO and 7.5% FCS. The acidity of EC + CytoD was adjusted to pH 7.0 with HCl.

2.3. Isolation of *T. gondii* cysts

To propagate *T. gondii* cysts, mice were infected i.p. with $10^{2\sim3}$ tachyzoites of TgCatJpGi1/Taj/GRA Red. More than 3 weeks p.i., their brains were excised after euthanasia by cervical dislocation. Each brain was homogenized and suspended in 5 ml of solution for the subsequent experiment (RPMI 1640 medium containing 7.5% FCS, EC, IC, EC＋DMSO and EC＋CytoD buffer). The cysts in the buffer solutions were observed microscopically and collected with a disposable micropipette. The isolated cysts were used in the subsequent experiments.

2.4. Observation of *T. gondii* excystation

To observe cyst excystation in Vero cell culture, a single cyst of TgCatJpGi1/Taj/GRA

Red was added into each well of a 96-well culture plate in which Vero cells had been cultured in 150 µl of RPMI 1640 medium containing 7.5% FCS. Each experimental group contained 40 ± 5 cysts. The culture plate was incubated at 37 °C in a 5% CO_2 incubator. Excystation of the cyst in each well was observed under fluorescence microscopy every 24 h for 7 days. It was confirmed that acidity of RPMI 1640 medium in the Vero cell culture reached pH 7.2 within 12 h from the start of incubation.

To observe cyst excystation in buffer solution without host cells, a single cyst of TgCatJpGi1/Taj/GRA Red was added to each well of a 96-well plate containing 150 µl of the test buffer. Each experimental group contained 10–12 cysts. The plate was incubated at 37 °C in a 5% CO_2 incubator. After 3 days, excystation was observed with fluorescence microscopy. In some experiments, non-excysted cysts were collected after incubation for 3 days and incubated in EC for another 3 days. After incubation for 3 days in EC, excystation was observed again. In all experiments in this study, when we observed both the disappearance of the cyst wall under a phase-contrast microscope and the diffusion of tachyzoites around the location of the cyst, we decided that the excystation had occurred.

2.5. Statistical analysis

The Fisher's exact test was used for comparisons of the rate of excystation (50).

3. RESULTS

3.1. Excystation occurs without host digestive enzymes

Firstly, I examined whether the physical and/or chemical digestion of the cyst wall by host-derived digestive enzymes is required for *T. gondii* excystation. To release *T. gondii* cysts from the host cell and obtain "naked cysts", I physically homogenized the brains of experimentally infected mice containing latent cysts. The homogenate including the naked cysts was added to a Vero cell culture in RPMI 1640 medium, with no digestive enzymes. Within 3 days, although exact quantification was not performed, the number of cyst-like structures was drastically reduced, while many parasites growing inside the Vero cells were observed (n=10), indicating that the naked cysts released from the host cells had excysted autonomously, without digestive enzyme treatment. To confirm this autonomous excystation, the naked cysts were isolated from the brain homogenates, added to the Vero cell cultures, and observed over time (Fig. 1). The naked cysts cocultured with Vero cells began excystation within 2 days and the excysted parasites invaded the Vero cells surrounding the released cyst (Fig. 1 and 2). Most excystation occurred 2–4 days after cyst isolation (Fig. 2). I thus confirmed that neither digestive enzymes nor brain tissue is required for *T. gondii* excystation.

3.2. Extracellular ionic composition induces excystation

The ionic composition of RPMI 1640 medium is similar to that of the extracellular environment. Therefore, we hypothesized that the ionic composition of the extracellular environment triggers *T. gondii* excystation. Naked cysts were

incubated for 3 days in two different buffers without host cells, EC and IC buffer solutions, mimicking the ionic compositions of the extracellular and intracellular environments, respectively, which differ in the Na^+, K^+ and Ca^{2+} concentrations. The excystation of the naked cysts occurred in EC buffer, but didn't occur at all in IC buffer (Fig. 3). These results indicate that the specific ionic composition of the microenvironment surrounding the cyst is a trigger for excystation and that no host-cell derived factor is required. The naked cysts maintained in IC buffer were viable because the parasites in these cysts were released after the cysts were transferred into EC buffer. Among cysts that transferred from IC buffer to EC buffer, the number of excysted cysts was as follows (no. of excysted cysts/no. of total transferred cysts); 3/5, 2/4, and 1/5, in three independent experiments.

3.3. Excystation failure in EC buffers is not due to genetic background of *T. gondii*

As shown in Fig. 4, although excystation frequently occurred in EC buffer, naked cysts occasionally fail to excyst excystation even in EC buffers. To examine the possibility that subclones without the ability of excystation were generated during the parasite maintenance in the laboratory, I conducted the experiments using the three parasite subclones derived from a single cyst, whose excystation in EC had been confirmed (Fig. 4A). Three single zoites released from an excysted cyst were isolated and cultured as subclones. The tachyzoites of the three subclones were inoculated into three mice per subclone separately. Then, cysts were obtained from each mouse brain and cultured in EC (Fig. 4A). The frequency of excystation of the cysts derived from three subclones varied from 0 % to 92 % (Fig. 4B).

3.4. Inhibition of cytoskeleton function inhibits excystation

In addition, when Cytochalasin D, an inhibitor of actin polymerization, was added to EC, the excystation was inhibited (Fig. 5). In this study, enough concentration of cytochalasin D for the inhibition of tachyzoite motility, 1 μM (10), was used.

4. DISCUSSION

In this chapter, I showed that *T. gondii* cysts excystation was induced by be released from host cell and cultured in extracellular environment, and *T. gondii* is thought to egress from the host cell by recognizing the difference in the ionic compositions of the intracellular and extracellular environments. No chemical digestion of the cyst wall by host digestive enzymes is required for excystation. Moreover, as shown in Fig. 5, excystation and following release of parasites from the cyst were not observed in the presence of cytochalasin D, which is an inhibitor of motility of *T. gondii* (9). These results indicate that the excystation of *T. gondii* is not the result of cyst-wall disruption by host-derived digestive enzymes but is an active response of the parasite to the change from intracellular to extracellular microenvironment surrounding the parasites. Although the release of the inner zoites from cysts had been thought to be a passive phenomenon caused by the digestion of cyst walls by the host digestive juice (13), it was revealed that *T. gondii* has an active excystation mechanism independent of host digestive juice.

Toxoplasma gondii cysts are normally located within the host cell, but it is thought that the cysts are released from the host cell during digestion and are immersed in intestinal fluid in the digestive tract. The intestinal fluid is a typical extracellular fluid composition with high sodium, low potassium, and calcium (36), which is similar to the in ionic composition of the EC buffer used in this study. This suggests that *T. gondii* parasites in the cysts sense the conditional change along predation via the change of the ionic composition of the surrounding environment and induces

excystation.

In this study, it was shown that cytochalasin D, at a concentration that inhibits the motility of tachyzoite (10), inhibit the excystation (Fig. 5). Although the possibility that cytochalasin D killed parasites in the cysts cannot be completely ruled out, it was known that a mutant *T. gondii* strain with a point mutation in the single copy actin gene, which codes a target protein of cytochalasin D, can survive in the presence of cytochalasin D (10). Therefore, it is unlikely that cytochalasin D acted on molecules other than actin to kill the parasite. The motility of the parasites inside the cyst may be essential for excystation.

Although EC buffer induced excystation of *T. gondii*, excystation did not occur even in EC buffers in some cases. In this study, I demonstrated that the lack of excystation in EC buffer in some cases was not due to the difference in the genetic background of the parasites (Fig. 4). Therefore, although the composition of the substances contained in EC buffer is the main inducer of excystation, there may be some unknown environmental cues affecting excystation efficiency. In addition, excystation rate varied between independent experiments in which excysted cysts were observed. The efficiency also might be affected by unknown factors.

5. FIGURES

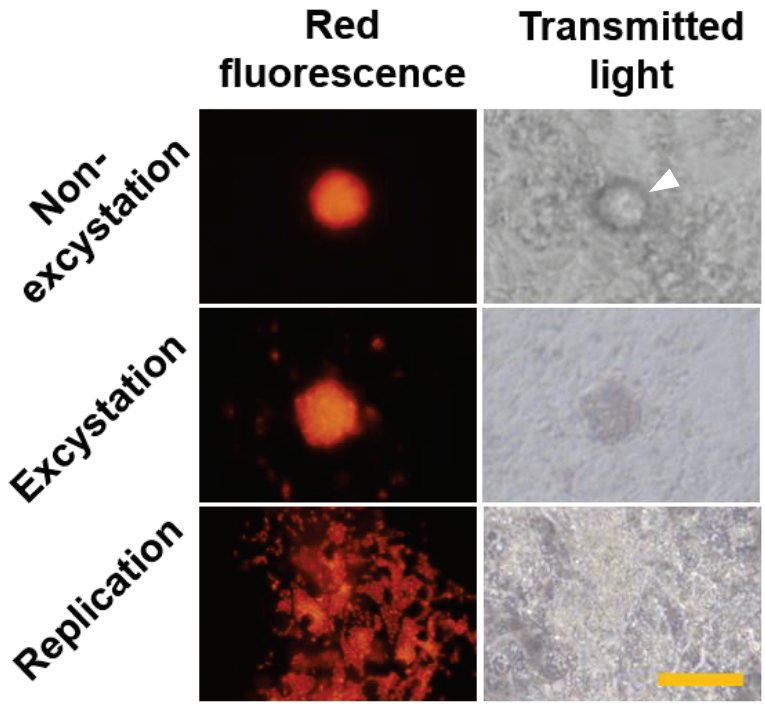

Fig. 1 Typical examples of excystation.

Upper panels: A non-excysted cyst of TgCatJpGi1/Taj/GRA Red strain. Middle panels: A Cyst in excystation. The cyst wall is obscured, and a large number of parasites were observed around the cyst. Lower panels: replicating parasites in Vero cells. White arrowhead: cyst wall. Scale bars represent 100 μm.

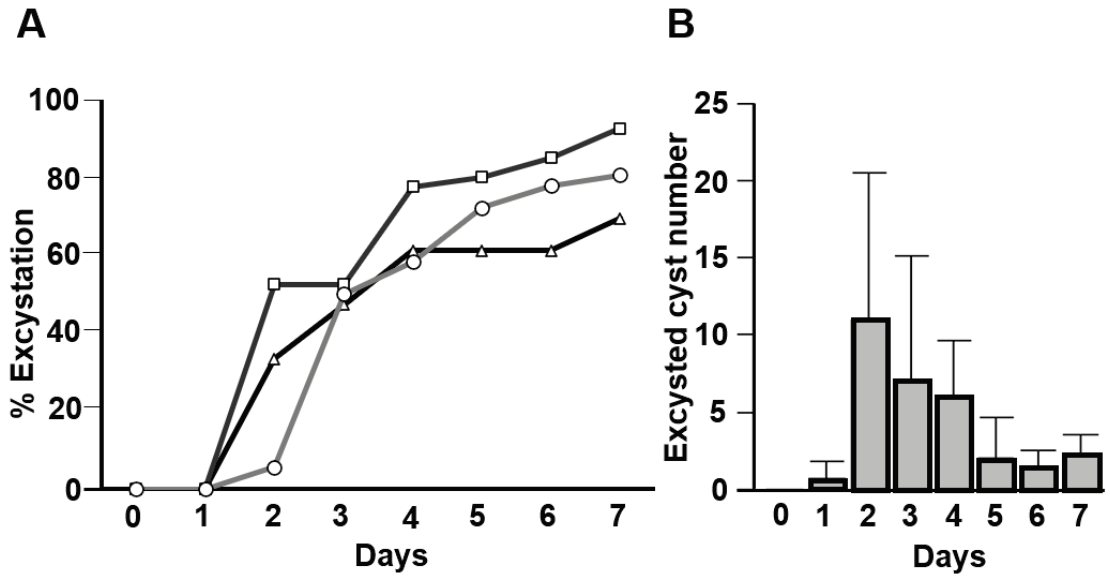

Fig. 2 Naked cysts released from the host cells excysted autonomously.

(A) Cumulative ratio of excysted cysts (% excystation) in RPMI 1640 medium. Cysts were incubated in RPMI 1640 medium for 7 days, and the number of excysted cysts counted. ○, △, and □ indicate the results of each independent experiment. Thirty to 40 cysts were used in each experiment. (B) The number of cysts excysted each day. Bars represent the means of the actual measurements of three independent experiments, and error bars represent S.D.

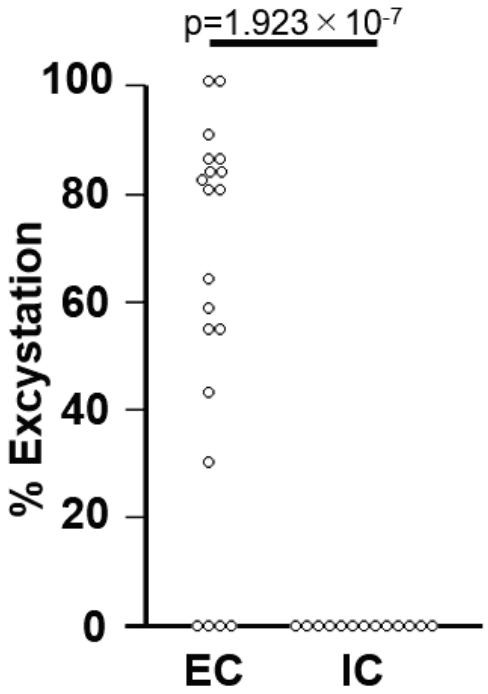

Fig. 3 Inducing excystation with extracellular (EC) buffer.

Cysts were incubated in EC or intracellular (IC) buffer for 3 days, and the rate of excysted cysts was measured. Ten to 12 cysts were used for each group in each experiment. ○ indicates the result of each independent experiment. Fisher's exact test was used.

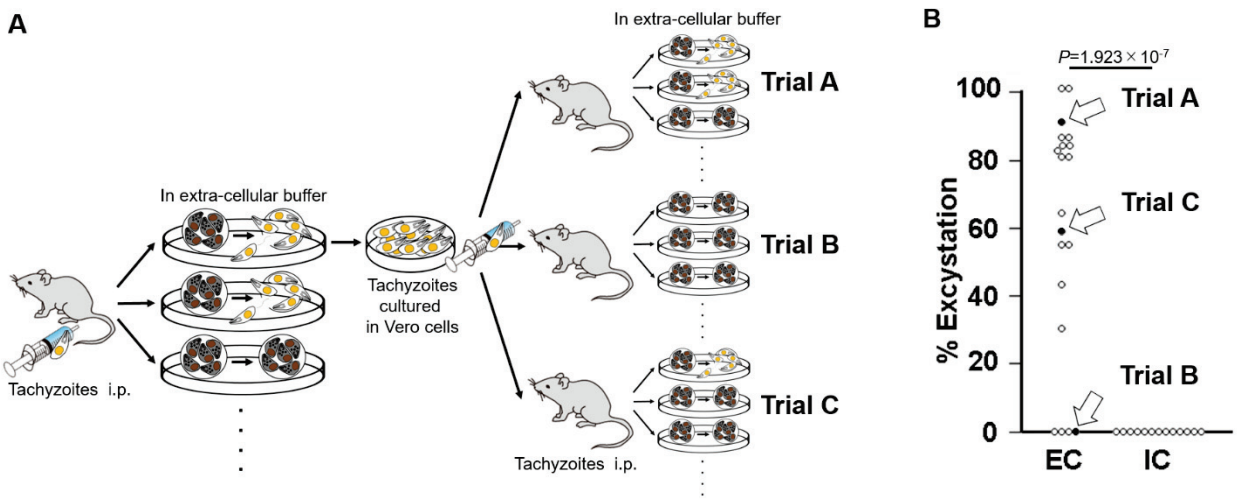

Fig. 4 Excystation frequencies of *Toxoplasma gondii* subclones derived from one clone.

(A) A mouse was injected i.p. with 10^3 tachyzoites of TgCatJpGi1/Taj/GRA Red strain. At 2 months p.i., cysts were isolated from the brain and cultured in extracellular buffer. Tachyzoites from an excysted cyst (subclone) were cultured in Vero cells. Three mice were injected with the subcloned tachyzoites. At 2 months p.i., cysts were isolated from the three mice, cultured in extracellular buffer, and the excystation frequencies calculated. (B) Frequencies of excystation of cysts derived from one clone passaged in three mice. ○ and ● indicates the rate of excysted cysts in each independent experiment. ● shows the results of Trial A, Trial B, and Trial C.

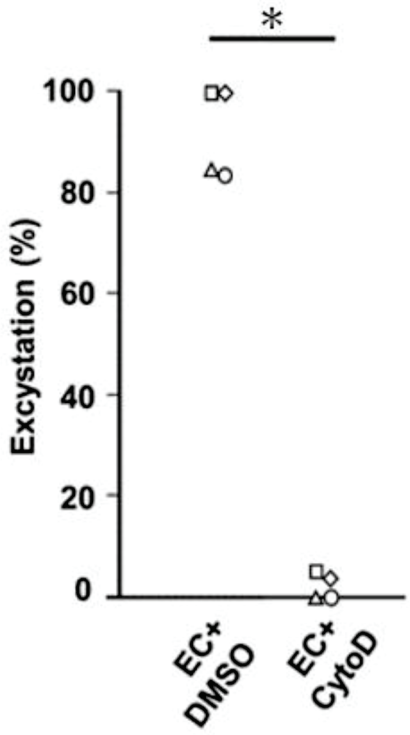

Fig. 5 Inhibition of excystation by Cytochalasin D, an actin polymerization inhibitor.

Cysts were incubated for 3 days in EC in the presence of Cytochalasin D. As a control, cysts were also incubated in EC with the solvent, DMSO. Twelve to twenty cysts were used for one group in each experiment. ○, △, □, and ◇ indicate the results of each independent experiment. Mann–Whitney U test was used; $^{*}P < 0.05$.

CHAPTER 2

Differentiation from bradyzoite to tachyzoite occurs prior to excystation

1. INTRODUCTION

In Chapter 1, I showed that the change of the ionic composition was the major factor that induced excystation and this induction was dependent on polymerization of actin filaments which is the driving power of mechanical movement of *T. gondii*. This suggests that excystation is caused by mechanical motion of the parasites within the cyst.

Tachyzoites possess high motility and they use these motile abilities to enter and egress host cells when needed (23, 28). Although bradyzoites within latent cysts, are often thought to have low motility, it was reported that bradyzoites also move within and between host cells (17). Bradyzoites within the cysts use the motility to form daughter cysts (17). Free bradyzoites egressed from the cysts invade into host cells using the mechanical movement (17). At this moment, it is not clear whether, once exposed to the extracellular environment, bradyzoites within cysts acquire moving ability and cause excystation, or stage-conversion from bradyzoite to tachyzoite occurs prior to the excystation and tachyzoites egress out from the cyst. Thus, I examined the timing of stage conversion among these, in Chapter 2.

2. MATERIALS AND METHODS

2.1. Cells, mice, and parasites

The Vero cell line was cultured in RPMI 1640 medium containing 7.5% FCS at 37 °C in a 5% CO2 incubator. C57BL/6J mice were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Infected mice were maintained in 225 × 338 × 140 mm cubic cages with 4 to 6 mice per cage. The bedding and drinking water were changed and food pellets were added, approximately every 3 days. All experiments using mice were conducted according to the guidelines issued by Gifu University, Japan (permission no. 17059). Transgenic *T. gondii*, TgCatJpGi1/Taj/SAG-Timer, which expresses a fluorescent protein (DsRed-E5) only in the tachyzoite stage, was established as described in next section. DsRed-E5 is a fluorescent timer protein in which the fluorescent signal changes from green to red over time (62). Tachyzoites of the transgenic parasites were maintained in Vero cell cultures.

2.2. Construction of transfer vector

The DsRed-E5 gene was obtained from the plasmid, pTimer-1 (Takara, Japan), by digestion with NotI and BamHI, followed by filled in 3′-recessed ends with a Klenow fragment (Takara, Japan). The obtained fragment was ligated into the BamHI-digested and Klenow fragment-treated plasmid pUC/SAG-GRA (60). The resulting plasmid was designated pUC/SAG-timer. DsRed-E5 coding sequence, under the control of the SAG1 promoter, was obtained from pUC/SAG-timer by digestion with EcoRI and HindIII, followed by Klenow fragment treatment, and was then ligated into the SpeI-digested and Klenow fragment-treated plasmid

pDHFR-Tsc3 (11). The resulting plasmid was designated pDHFR-SAG-timer. Tachyzoites of *T. gondii* TgCatJpGi1/Taj strain were transfected with plasmid pDHFR-SAG-timer and the tachyzoites stably expressing DsRed-E5 were cloned as described previously (39). The stable transgenic parasite expressing DsRed-E5 was designated TgCatJpGi1/Taj/SAG-Timer.

2.3. Immunofluorescence staining

In vitro stage conversion of TgCatJpGi1/Taj/SAG-Timer was induced with SB202190, which is a p38 MAPK inhibitor and known to induce differentiation from tachyzoite to bradyzoite, as previously described (42). After the induction, cultures of infected Vero cells were washed with PBS, fixed in 4% paraformaldehyde in PBS for 20 min, and washed once with PBS. The washed cell monolayers were then blocked for 5 min with 1 M glycine in PBS, and washed once with PBS. The fixed cells were then permeabilized and blocked for 30 min in PBS containing 3% BSA and 0.2% TritonX-100 (base solution), and washed once with PBS. Then, the fixed and permeabilized cell monolayers were incubated in a base solution containing 1:250 dilution of Dolichos biflorus agglutinin (DBA) Biotin Conjugate (Vector Biolabs, PA, USA) for 1 h, and then washed twice with a base solution. The cell monolayers were then incubated in a base solution containing 1:250 dilution of fluorescent probe Streptavidin AMCA Conjugate (Vector Biolabs, PA, USA) for 1 h, and then washed twice with a base solution.

For immunostaining of tachyzoites, Vero cells infected with TgCatJpGi1/Taj/SAG-Timer were fixed, permeabilized and stained as above, except

that 1:100 dilution of Toxoplasma gondii SAG1 Monoclonal Antibody (D61S) (ThermoFisher, MA, USA), which specifically recognizes tachyzoite stage parasites, and 1:50 dilution of AMCA-conjugated AffiniPure Alpaca Anti-Mouse IgG (H + L) (Jackson Immunoresearch, PA, USA) were used as primary and secondary antibodies, respectively.

2.4. Observation of stage conversion from bradyzoites to tachyzoites

Single cysts of TgCatJpGi1/Taj/SAG-Timer were incubated in 200 µl of RPMI 1640 medium containing 7.5% FCS in which Vero cells had been cultured in each well of a 96-well culture plate at 37 °C in a 5% CO_2 incubator. Seven to 10 cysts were used for each experiment. The culture plate was incubated for 3 days and excystation and fluorescence were observed with fluorescence microscopy every 24 h. The appearance of green fluorescence was considered to indicate the expression of DsRed-E5 under the control of the tachyzoite-specific SAG1 promoter, and therefore the stage conversion from bradyzoite to tachyzoite.

3. RESULTS

3.1. Establishment of recombinant *T. gondii* strains with tachyzoite-specific fluorescence

To visualize the timing of the bradyzoite–tachyzoite stage conversion, a transgenic *T. gondii* with fluorescent protein DsRed-E5 gene driven under the tachyzoite-stage specific promotor SAG1 was constructed and designated as TgCatJpGi1/Taj/SAG-Timer strain. The color of this fluorescent protein changes from green to red over time, in the course of a few hours (62), with which both the red and green signals are observed until the red signal fully developed. The plasmid vector for the construction of this strain contains mutated dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene (11) in addition to the DsRed-E5 gene with SAG1 promotor and confers resistance to pyrimethamine (Fig. 6). DHFR-TS in this plasmid possess 5'-end of the original DHFR-TS promoter. According to the database, this promoter is considered to be active in both tachyzoite and bradyzoite stages (https://toxodb.org/toxo/app/record/gene/TGGT1_249180). Therefore, the parasites transfected with this plasmid, acquire pyrimethamine resistance in tachyzoite and bradyzoite stages. This plasmid vector was used to transfect tachyzoites of *T. gondii* TgCatJpGi1/Taj strain, and the parasite in which these genes were integrated into its genome was selected and cloned in pyrimethamine supplemented medium. The resulting recombinant parasite, TgCatJpGi1/Taj/SAG-Timer strain, was cultured in tachyzoite growing condition or bradyzoite inducing condition. These tachyzoites and bradyzoites were specifically stained using SAG1-specific antibodies or cyst

wall-binding lectin, Dolichos Biflorus Agglutin (DBA), respectively (Fig. 7). As shown in Fig. 7A, almost all tachyzoites, with the SAG1 signal (blue color) showed green fluorescence (green fluorescence parasites/SAG1 positive parasites = 279/295, 95% CI: 91.3–96.8%). Considering that small portion of tachyzoites differentiate into bradyzoites in cultured cells (42), a few non fluorescent parasites were considered bradyzoites. Green fluorescence intensity varied among SAG1 positive tachyzoites (Fig. 7A). However, the parasites in parasitophorous vacuoles (PVs) that were strongly stained by DBA lectin did not show green fluorescence (Fig. 7B). The parasites within weakly stained PVs which seemed to be under the stage-conversion between tachyzoite and bradyzoite, showed no or weak green fluorescence (Fig. 7B). Among the 58 observed vacuoles clearly stained by DBA lectin, the intensities of green fluorescence of 50 vacuoles were under detectable levels. These results confirmed that the gene-modified parasite, TgCatJpGi1/Taj/SAG-Timer, displays green fluorescence only in the tachyzoite phase and that bradyzoites in matured cysts does not display any florescence.

3.2. Activation of the SAG1 promotor prior to the excystation

C57BL mice were infected with TgCatJpGi1/Taj/SAG-Timer strain tachyzoites, and latent cysts were obtained after more than 15 days from the infection. The obtained cysts were co-cultured with Vero cells in RPMI medium to induce excystation. As shown in the upper panels of Fig. 8A, the TgCatJpGi1/Taj/SAG-Timer cysts showed no fluorescence at the beginning of culture. During the excystation process, both green and red fluorescence appeared before the cyst wall was lost at day 2 in all examined cysts (Fig. 8A middle and Table 1). Among 26 cysts examined, all cysts

showed fluorescence before excystation and 18 cysts showed excystation by the third day. No cyst showed excystation before the appearance of fluorescence (Fig. 8B and Table 1). These results indicate that the stage conversion from bradyzoite to tachyzoite occurred before excystation. At the time of appearance of fluorescence, all zoites in cysts became fluorescent and all examined cysts became fluorescent by the second day (Fig. 8 and Table 1), suggesting the synchronized stage conversion of the zoites in cysts.

4. DISCUSSION

In a previous report, when *T. gondii* cysts were placed in the pepsin solution that mimicked gastric juice, the cyst walls were digested and the internal bradyzoite were released (13). Also, when the bradyzoites were released from the cysts with the artificial digestion and fed orally to mice, infection of the small intestinal cells with the intact bradyzoites was observed (14), suggesting that excystation follows destruction of pouch-like structure enclosed by cyst wall, due to digestion by the host digestive juice, then the internal bradyzoites are released, infect the small intestinal wall, and differentiate into tachyzoites.

However, in this chapter, the tachyzoite-specific SAG1 promoter was activated prior to the excystation when the cysts were exposed to extracellular condition. Tachyzoites are characterized by higher motility than bradyzoites, and this motility allows tachyzoites to pass through the host cellular membrane during egress (23). Considering the facts that excystation was inhibited by cytochalasin D which inhibits *T. gondii* motility, stage-conversion from bradyzoite to tachyzoite starts prior to the excystation, and high motility of the stage-converted tachyzoites within the cyst may be used to mechanically break through the cyst wall during the egress from the host cell. In summary, there are two types of excystation: “passive excystation” caused by the digestive juices of the host and “spontaneous excystation” caused by the mechanical motion of the parasites within the cysts. Until this study was conducted, the concept of “spontaneous excystation” had not been recognized. In this study I could advocate the new concept, “spontaneous excystation”.

In a series of experiments, it takes 2 to 4 days for the excystation from the first exposure to extracellular environment. Considering that the food mass passes through the digestive tract and excreted within 48 hours after ingestion (43), it seems that the time when cysts spent in the gastrointestinal tract is too short for an orally ingested cyst to cause a “spontaneous excystation” in the gastrointestinal tract. In addition, the ingested cyst wall can be digested in gastric juice (13, 14). Excystation of orally ingested cysts might be mainly “passive” form.

On the other hand, in the case of the excystation of latent cyst within the tissue of immunodeficient host, there is no mechanism to digest cyst wall. Under these conditions, it is unlikely that a "passive excystation" that requires digestive juices will occur. Therefore, rupture of the latent cyst in the host tissues is most likely to be caused by “spontaneous excystation”. After the cyst rupture, many immune cells accumulate around the ruptured cysts (19). It is reasonable for the parasite survival to induce the stage-conversion prior to the excystation and obtain high motility so that egressed parasites can immediately invade into new host cells and escape from the host’s immune attack.

5. FIGURES AND TABLE

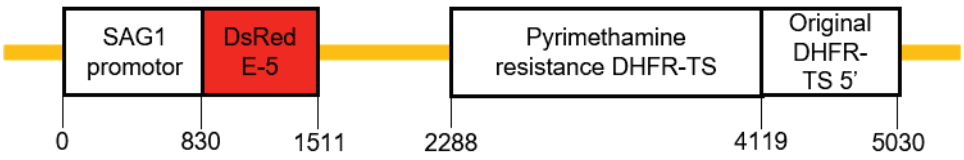

Fig. 6 Overview of the expression cassette used for gene transfer to TgCatJpGi1/Taj/SAG-Timer strain.

The expression cassette construct contains pyrimethamine resistance mutated DHFR-TS expressing cassette bound with 5' region of original DHFR-TS, and DsRed E-5-expressing cassette bound with promotor region of SAG1.

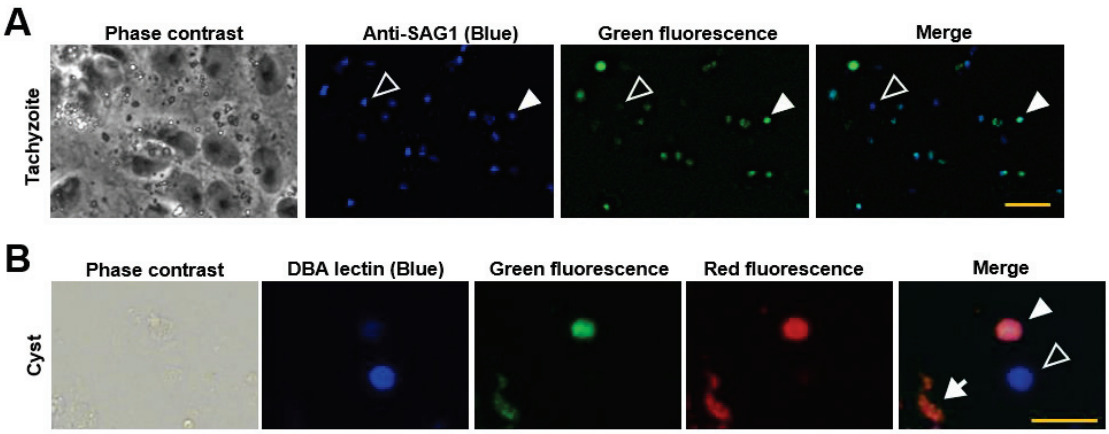

Fig. 7 Verification of the stage-specific expression of green fluorescence of TgCatJpGi1/Taj/SAG-Timer in tachyzoites.

(A) Tachyzoite: parasite-infected Vero cells were immunostained with anti-SAG1 antibody (blue). Solid and open arrowheads indicate parasites with strong green and faint green fluorescence, respectively. (B) Cyst: SB202190, a cell-permeable inhibitor of p38 MAP kinase was used to induce cyst formation in vitro, followed by staining of the cyst wall using Dolichos Biflorus Agglutinin (DBA) lectin (blue). White arrow indicates parasites which were not stained by DBA lectin. Solid and open arrowheads indicate parasitophorous vacuoles weakly or strongly stained by DBA lectin, respectively. All scale bars indicate 50 μm.

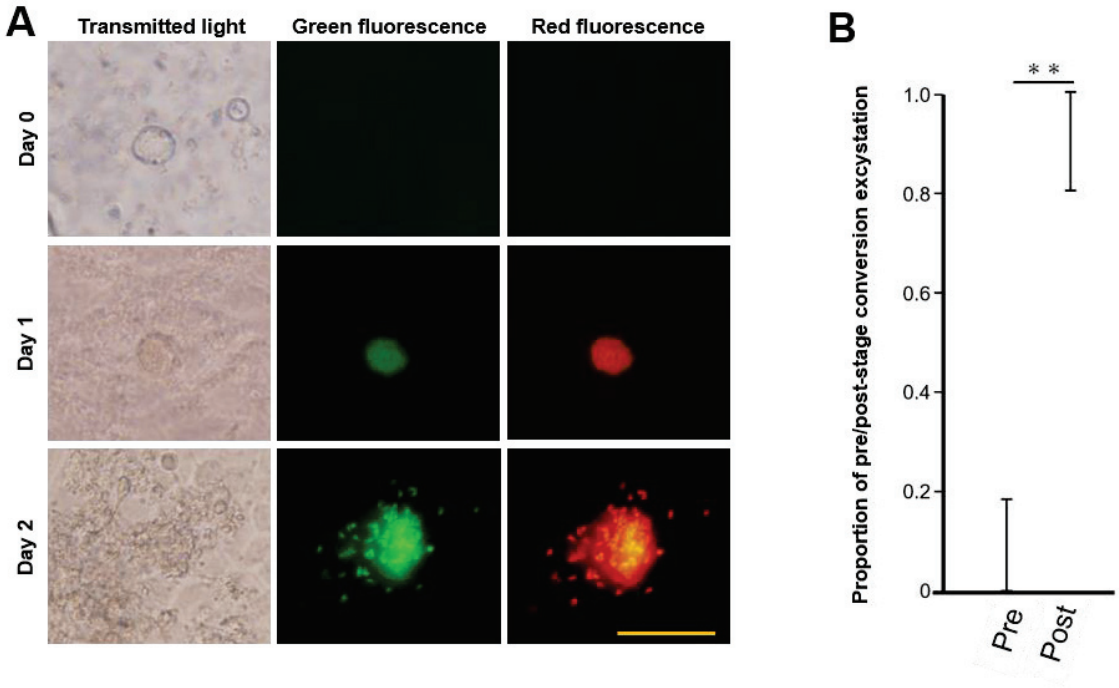

Fig. 8 *T. gondii* bradyzoite–tachyzoite stage conversion before excystation.

Cysts of TgCatJpGi1/Taj/SAG-Timer were used in this experiment. (A) Stage-conversion and excystation. The same cyst was continuously observed on day 0, day 1 and day 2. A representative example is shown. Scale bar indicates 50 μm. (B) Confidence interval (95%) of the population in which excystation occurred pre- or post-stage conversion. Fisher's exact test was used for comparison the population of excystation between pre- and post- stage conversion; $^{**}P < 0.001$.

	No. of stage-converted cyst/No. of examined cyst [a]			No. of excysted cyst/no. of stage-converted cysts [b]
	day 0	day 1	Day 2	
Mouse 1	0/10	0/10	10/10	7/10
Mouse 2	0/9	0/9	9/9	4/9
Mouse 3	0/7	0/7	7/7	7/7

[a]No. of excystations that were observed during the 2 day observation.

[b]Excystation and/or following zoite spreading was confirmed at day 3.

Table 1 Timing and frequency of stage conversion.

CHAPTER 3
Specific sodium-potassium ratio and the presence of calcium triggers excystation

1. INTRODUCTION

In Chapter 1, I found that excystation of *T. gondii* cyst is triggered by exposure to the extracellular fluid. However, it was not known what kind of component in the extracellular fluid induces excision and stage-conversion from bradyzoite to tachyzoite. Among the compounds in EC buffer used in chapter 1, sodium, potassium, and calcium are widely used for intracellular signal transduction in various scenes of life phenomena. Sodium and potassium are important compounds to generate cellular membrane potential and calcium is an important intracellular messenger molecule in various organisms (8). In the case of *T. gondii*, it was reported that decrease in concentration of potassium in the host cell induces the egress of the tachyzoites from the host cell (37). Calcium was reported to contribute to the induction of microneme secretion, the acquisition of tachyzoite motility, the invasion in host cell, and escape from host cells (2, 6, 32). Thus, these electrolytes are physiologically important for *T. gondii*, but the contribution of these electrolytes to excystation has not been known yet.

Although the majority of *T. gondii* cysts in the brain are seen inside the neurons (35), the presence of intact cysts in the intercellular space of the brain was also reported (40). This indicates that exposure to the extracellular fluid under the physiological

conditions does not necessarily lead to the excystation. Actually, as shown in chapter 1, excystation is not induced occasionally even in the EC buffer. These facts indicate that the efficiency induced by extracellular fluid can be inhibited sometimes by unknown cues, which could not be controlled in the experiments in chapter 1.

I adjusted the EC buffer to pH7.2 in the study shown in chapter 1. The acidity of intestinal fluid varies between pH 5.7-7.4 (18) which is lower than the acidity of tissue fluid including the brain (8, 38). In addition, slight change of pH in the EC buffer is possible during the course of the experiment. Therefore, it is necessary to investigate the effect of pH difference on excystation of the parasite.

In this chapter, I determined the ion concentration that triggers the excystation of *T. gondii* cyst and revealed that a slight change in pH in the tissue fluid drastically affects the efficiency of excystation.

2. MATERIALS AND METHODS

2.1. Cells, mice, and parasites

The Vero (African green monkey kidney) cell line was cultured in RPMI 1640 medium containing 7.5% FCS at 37 °C in a 5% CO2 incubator. ICR mice were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). Infected mice were maintained in 225 × 338 × 140 mm cubic cages with 4 to 6 mice per cage. The bedding and drinking water were changed and food pellets were added approximately every 3 days. All experiments using mice were conducted according to the guidelines issued by Gifu University, Japan (permission no. 17059). In this chapter, transgenic *T. gondii* TgCatJpGi1/Taj/GRA Red was used (1). Tachyzoites were maintained in Vero cell cultures.

2.2. Buffer composition

Five different buffer solutions containing discontinuous gradient concentrations of Na^+/K^+ were prepared as follows. Buffer Ⅰ ($Na^+/K^+ > 30$) consisted of 142 mM NaCl, 5.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–NaOH, and 7.5% FCS. Buffer Ⅱ ($Na^+/K^+ = 2$) consisted of 100 mM NaCl, 47.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 16.7 mM HEPES–NaOH, 8.3 mM HEPES–KOH, and 7.5% FCS. Buffer Ⅲ ($Na^+/K^+ = 1$) consisted of 73.8 mM NaCl, 73.8 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 12.5 mM HEPES–NaOH, 12.5 mM HEPES–KOH, and 7.5% FCS. Buffer Ⅳ ($Na^+/K^+ = 1/2$) consisted of 47.5 mM NaCl, 100 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 8.3 mM HEPES–NaOH, 16.7 mM HEPES–KOH, and 7.5% FCS. Buffer Ⅴ (Na^+/K^+

< 1/30) consisted of 5.5 mM NaCl, 142 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–KOH, and 7.5% FCS. The acidity of all five buffers was adjusted to pH 7.0 with NaOH or HCl. The maximum amount of NaOH added was 5×10^{-5} mol/l, and the maximum amount of HCl added was 3×10^{-4} mol/l.

Extracellular (EC) buffer consisted of 141.8 mM NaCl, 5.8 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–NaOH, and 7.5% FCS. The acidity of EC was adjusted to pH 7.0, pH 7.2 or pH 7.4 with HCl.

EC + 1,2-bis(2-aminophenoxy)ethane-n,n,n′,n′tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM) buffer (EC + BAPTA-AM) consisted of 141.8 mM NaCl, 5.8 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–NaOH, 20 μM BAPTA-AM, 56 mM DMSO and 7.5% FCS. The acidity of EC + BAPTA-AM was adjusted to pH 7.0 with HCl.

EC + EGTA buffer (EC + EGTA) consisted of 141.8 mM NaCl, 5.8 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–NaOH, 10 mM EGTA, and 7.5% FCS. The acidity of EC + EGTA was adjusted to pH 7.0 with HCl. EC–Ca buffer (EC–Ca) consisted of 141.8 mM NaCl, 5.8 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 25 mM HEPES–NaOH, 10 mM EGTA, and 7.5% FCS. The acidity of EC–Ca was adjusted to pH 7.0 with HCl.

2.3. Observation of *T. gondii* excystation

To observe cyst excystation in buffer solution, a single cyst of TgCatJpGi1/Taj/GRA Red was added to each well of a 96-well plate containing 150 μl of the test buffer. Each experimental group contained 10–12 cysts. The plate was incubated at 37 °C in a 5% CO_2 incubator. After 3 days, excystation was observed with fluorescence

microscopy. In all experiments in this study, when we observed both the disappearance of the cyst wall under a phase-contrast microscope and the diffusion of tachyzoites around the location of the cyst, we observed that the excystation had occurred. In this chapter, I tested excystation in several types of buffers with a variety of ionic compositions, using the excystation frequency in EC buffer within 3 days as the positive control. Although the frequency of excystation in EC buffer varied across the trial (0%–100%), more than half the cysts used for each experiment excysted in the majority of trials (Fig. 4). Therefore, in subsequent experiments, only conditions under which the excystation frequency exceeded 50% in EC buffer were considered successful, and only the data from the successful trials were analyzed.

2.4. Statistical analysis

Steel's multiple comparison test was used for comparisons of the rate of excystation between single groups and a control group. Logistic regression analysis of the processed data was performed with the statistical software R (50). The explained variable is occurrence of excystation or not. Explanatory variables are Na+/K+ ratio and a dummy variable to evaluate separately the first, second, and third experiments: (Vd1, Vd2) = (0, 0), (1, 0), and (0,1), respectively (Table 2).

The created model was:

$$logit\ P = \alpha + \beta_1 (Na^+/K^+) + \beta_2\ Vd1 + \beta_3 Vd2$$

In the model, the probability of coefficient values of Vd1 and Vd2 was not significant (Table 3). Therefore, the dummy variables Vd1 and Vd2 were eliminated to create

the new model:

$$logit\ P = \alpha + \beta_1 (Na^+/K^+)$$

The predictive performance and model fit were assessed with Akaike's information criterion (AIC) and the area under the receiver operating characteristic (ROC) curve (AUC). AUC and its 95% confidence interval (CI) were calculated with the R "pROC package" (47). We used the second model because AIC and AUC were similar for the first and second models (Table 3).

3. RESULTS

3.1. Extracellular Na^+/K^+ ratio induces excystation

The difference in Na^+/K^+ ratio between extracellular and intracellular environments causes a variety of physiological events. Therefore, I firstly examined the effect of Na^+/K^+ ratio in the microenvironment surrounding *T. gondii* cysts on the induction of excystation. Five different buffers contained the same concentration of Ca^{2+}, Mg^{2+} and Cl^- as EC buffer but with various Na^+/K^+ ratio with the same ionic strength (Table 4). Buffers Ⅰ and Ⅴ had the same Na^+/K^+ ratios as the EC and IC buffers, respectively. As shown in Fig. 9, buffer Ⅰ, in which the Na^+/K^+ ratio was the highest, induced excystation most effectively. To confirm the effect of Na^+/K^+ ratio on excystation, I performed a logistic regression analysis, which showed that Na^+/K^+ ratio was significantly associated with the frequency of excysted cysts (Table 2 and 3). According to the logistic regression model, excystation percentages were significantly higher in the higher Na^+/K^+ ratio environment, such as that of the extracellular fluid (Fig. 9).

3.2. Calcium is essential for excystation

To examine whether the Ca^{2+} in EC buffer is necessary for the induction of excystation, *T. gondii* cysts were incubated in EC buffer containing EGTA, a chelator of Ca^{2+} (EC + EGTA) or in EC buffer lacking Ca^{2+} (EC–Ca). Significantly lower percentage of excystation occurred in the EC–Ca or EC + EGTA (Fig. 10A). BAPTA-AM, an intracellular calcium-specific chelator, also inhibited excystation

(Fig. 10B). These results indicate that the induction of excystation requires Ca^{2+}.

3.3. Physiological pH inhibits excystation

To examine the effect of pH on the excystation of *T. gondii*, naked cysts were incubated in EC buffer at pH 7.0, 7.2, or 7.4. As shown in Fig. 11, excystation was inhibited at pH 7.4.

4. DISCUSSION

In this chapter, I shown that *T. gondii* responds to difference in Na^+ /K^+ ratio and Ca^{2+} in the environment surrounding the cyst, induces cyst excystation. The concentrations of Na^+, K^+, and Ca^{2+} in the host intestinal fluid were 123.4, 7.6, and 0.6 mM, and gastric fluid were 72.2, 7.8, and 0.15 mM, respectively (36), which are close to the composition of ECs that induced the excystation in this study (Na^+, K^+, and Ca^{2+} = 167.0, 5.5, and 1.0 mM, respectively). Therefore, sensing the concentration of these electrolytes in the intestinal fluid is the important factor for the excystation induction.

In the acute phase, tachyzoites are surrounded by the PV membrane, which is known to be permeable for the molecules smaller than 1500 Da (51). On the other hand, there are no previous studies on the permeability of cyst wall. However, considering that cytochalasin D, of which molecular weight is 507.6 Da, affected bradyzoites inside the cysts, much smaller ions likely permeate the cyst wall freely. It was reported that guanylate cyclase (TgGCs), which was suggested to sense K^+ and H^+, are expressed on the parasite surface in tachyzoites (63, 66). Same or similar molecules might be expressed on the surface of bradyzoites and sense electrolytes passed through the cyst wall.

In Chapter 1, excystation occasionally did not occur even in the EC buffer (Fig. 3). As discussed in Chapter 1, it was unlikely that genetic predisposition was the cause of excystation because the parasite populations were derived from the same clone

(Fig. 4). Since the pH of EC was standardized to 7.2 in the experiment, it was not due to the change in acidity at the start of these experiments, either. These facts indicate that there are still unknown cues that inhibit excystation. However, in this study, I have not verified the change in pH over time during the experiment. Although HEPES was added to stabilize the pH of the solution in this study, it remains possible that the solution turned more acidic in the CO_2 incubator. The effect of changes in the acidity of the solution over time on the excystation efficiency is for further study. As shown in Fig. 11, around the acidity of the host tissue flid, pH 7.0 to 7.4, the excystation efficiency was higher as the pH was lower. However, it was not yet examined the effect of extremely low acidity conditions, as like gastric juice below pH 6.0. The effect of extremely low pH of gastric juice on excystation of *T. gondii* cysts in the intestine should be also verified in future.

In the mouse brain, most cysts occur intracellularly in neurons or glial cells (35). This is consistent with our finding that the *T. gondii* cysts were maintained as intracellular inclusions, and majority of the excystation occurred in the majority of cysts released from the host cells. However, previous studies reported the observation of extracellular cysts in mouse brain and in the *in vitro* culture (30, 40). In this study, I showed that subtle differences in the acidity of the surrounding environment affected the efficiency of excystation and physiological or lower pH (>pH 7.4) inhibited excystation. The excystation of a small number of cysts accidentally released from neurons or glial cells may be partially inhibited by the physiological pH of the host tissue. This might be the reason why extracellular cysts without excystation were observed in the brain. Although excystation in RPMI1640

medium, with the original pH of pH 7.8, immediately after opening a package, was observed (Fig. 1 and 2), the acidity of the medium under the experimental condition was approximately pH7.2. Therefore, the results suggest that the excystation is suppressed by the physiological pH.

5. TABLES AND FIGURES

Table 2 Model formulae used in this study.

Model 1
Logit P(X) = – 2.1849 + 1.3516 C_{Na^+/K^+} + 1.0289 Vd1 + 0.1122 Vd2
Model 2
Logit P(X) = – 1.8384 + 1.2909 C_{Na^+/K^+}

The association between cyst excystation and Na^+/K^+ ratio (C_{Na^+/K^+}) was analyzed by logistic regression analysis based on data obtained from three independent experiments. In Model 1, the first, second and third experiments were separately evaluated using dummy variables; (Vd1, Vd2) = (0, 0), (1, 0) and (0,1), respectively. In Model 2, the difference between each experiment was not evaluated.

C_{Na^+/K^+}: Na^+/K^+ ratio
Vd1: dummy variable 1, Vd2: dummy variable 2

Table 3 Regression co-efficient value, the model fitting, Akaike's Information Criterion (AIC) and Area Under the Curve (AUC), of two models used in this study.

	C_{Na^+/K^+}		Vd1		Vd2		AIC	95% CI of AUC
	Value	*P*	Value	*P*	Value	*P*		
Model 1	1.3516	< 0.001	1.0289	0.0889	0.1122	0.842	113.904	0.7371 - 0.9052
Model 2	1.2909	< 0.001					114.119	0.6902 - 0.8732

CI: Confidence interval

Table 4 Ionic ratio, concentration (mM) and pH of each buffer used in this study.

	I	II	III	IV	V
Na^+/K^+	30	2	1	1/2	1/30
Na^+	167.0	116.7	86.3	55.8	5.5
K^+	5.5	55.8	86.3	116.7	167.0
Ca^{2+}			1.0		
Mg^+			1.0		
Cl^-			151.5		
pH			7.0		

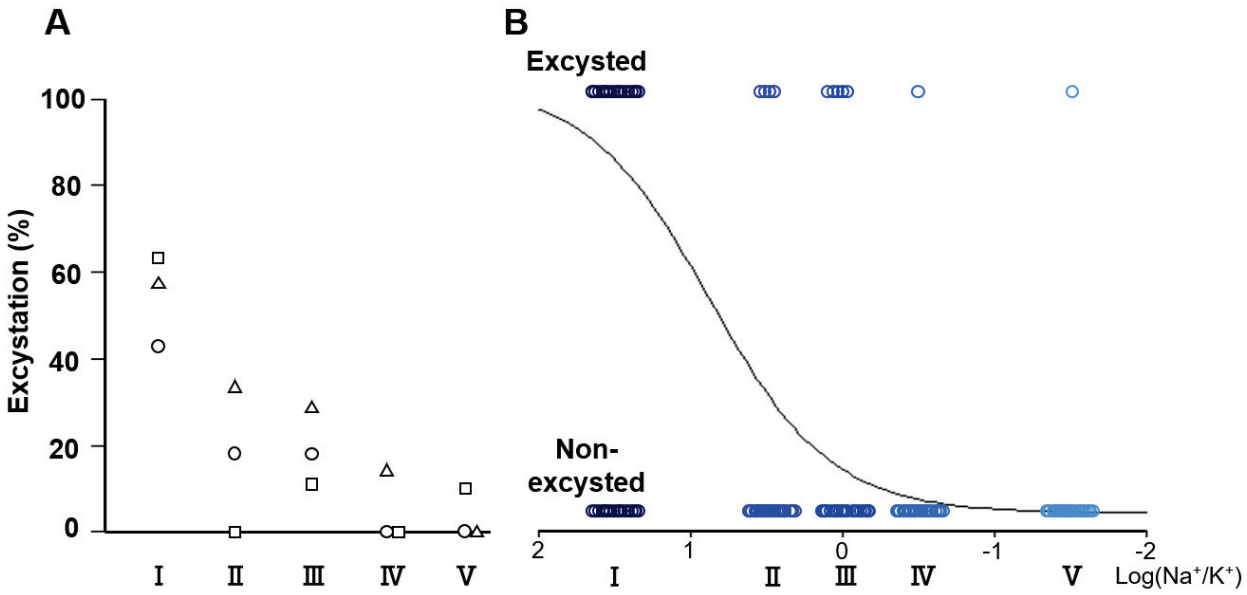

Fig. 9 A specific Na^+/K^+ ratio induces *Toxoplasma gondii* excystation.

(A) Effect of Na^+/K^+ ratio on excystation. Cysts were incubated in five different buffers (I – V) containing gradient concentrations of Na^+/K^+ for 3 days, and the rate of excysted cysts was measured. Three independent experiments were performed. Eight to 12 cysts were used for each group in each experiment. ○, △, and □ indicate the results of each independent experiment. (B) The relationship between the possibility of excystation and the Na^+/K^+ ratio was analyzed with a logistic regression analysis. X- and Y-axes indicate the Na^+/K^+ ratio and speculated excystation probability, respectively. Dots indicate all the events observed in three independent experiments. To avoid the overlapping of dot images, the events observed in experiments using a particular solution are shown as dot images distributed with a certain width along the X-axis.

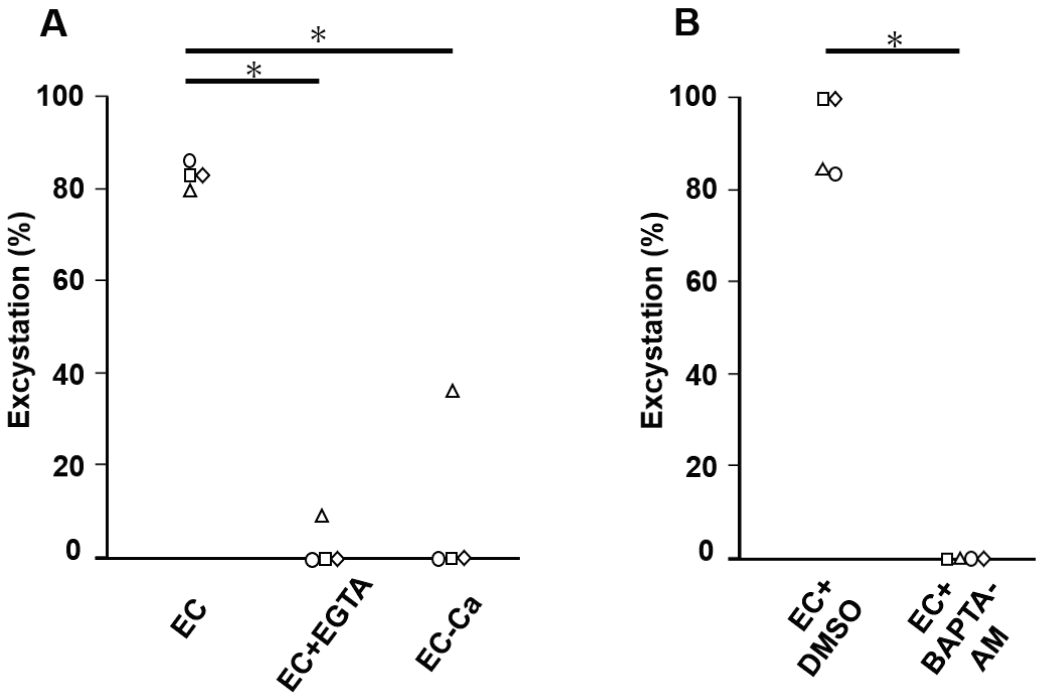

Fig. 10 Ca^{2+} induces *Toxoplasma gondii* excystation.

(A) Effect of calcium on excystation. Cysts were incubated with extracellular (EC) buffer, EC+EGTA buffer, or EC−Ca buffer for 3 days, and the rate of excysted cysts evaluated. Four independent experiments were performed. Ten to 12 cysts were used for each group in each experiment. ○, △, □, and ◇ indicate the results of each independent experiment. (B) Inhibition of excystation by 1,2-bis(2-aminophenoxy)ethane-n,n,n',n'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM). Cysts were incubated for 3 days in EC in the presence of BAPTA-AM. As a control, cysts were also incubated in EC with the solvent, DMSO. Twelve to twenty cysts were used for one group in each experiment. ○, △, □, and ◇ indicate the results of each independent experiment. Mann–Whitney U test was used; $^{*}P < 0.05$.

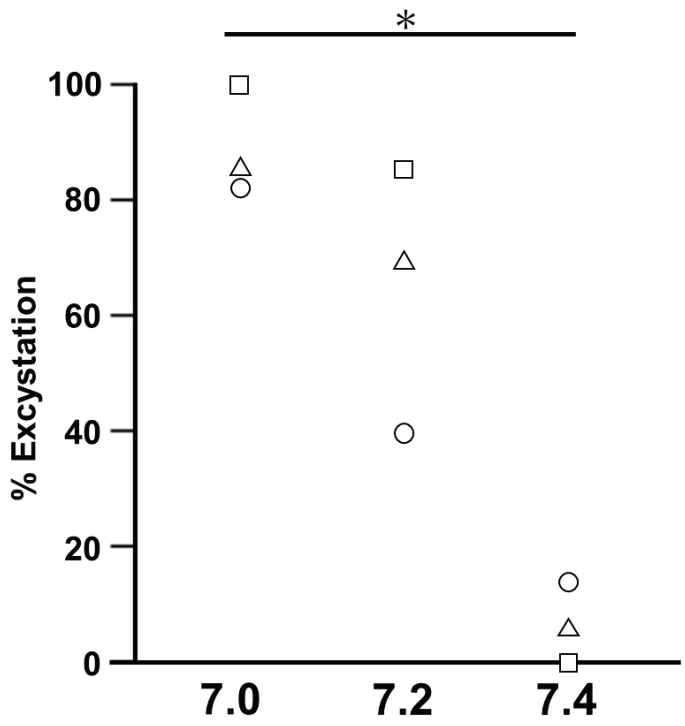

Fig. 11 Effect of pH on *Toxoplasma gondii* excystation.

Cysts were incubated for 3 days with extracellular (EC) in which the acidity was adjusted to pH 7.0, pH 7.2, or pH 7.4, and the percentage of excysted cysts was measured. Three independent experiments were performed. Ten to 12 cysts were used for each group in each experiment. ○, △, and □ indicate the results of each independent experiment. Steel's multiple comparison test was used; $* P < 0.05$.

GENERAL DISCUSSION

Many species of parasites, including *T. gondii*, have complex life cycles, and the differentiation events occur at the time of their invasion of a host, their egress from the host body, or their transmission across multiple hosts. Thus, to complete their complex life cycles, parasites must undergo cellular differentiation at the appropriate time in their life cycles. Although the transformations of many species of parasites during their life cycles have been well described, only a few studies have sought to identify the environmental cues that trigger parasite stage conversion (67). As stage conversion is a phenomenon that adapts to the surrounding environment and changes its morphology and motility, it is importance to first detect changes in the surrounding environment. Signal transduction, which senses changes in the surrounding environment and stage-specific gene expression in parasites, is accompanied by the phenomenon of environmental cue recognition. Therefore, elucidation of the molecular mechanisms that effects parasite's stage conversion needs to start from the identification of the environmental cue, which is the switch that activates the molecular mechanism.

In this study, I revealed that the Na^+/K^+ ratio and the presence of Ca^{2+} in the surrounding environment are environmental cues that trigger the stage conversion of *T. gondii* bradyzoites to tachyzoites. There is no evidence that the environmental cues identified in this study are also used in actual excystation in the host body. However, considering that latent cysts are maintained within the host cells and released from the host cells at the moment of the predation, it is possible that the

same trigger is also used in the host body.

Although environmental cues to induce a stage conversion between tachyzoites and bradyzoites were found in this study, the receptor molecule(s) of the parasite cells for the cues are still unknown. Considering that both the Na^+/K^+ ratio and the presence of intracellular Ca^{2+} are necessary to induce stage conversion, cation channel(s) expressed in bradyzoite may be a candidate for the receptor molecules. The need for a particular Na^+/K^+ ratio suggests that the membrane voltage of bradyzoites may be related to the stage conversion and excystation processes. Among a variety of cation channels, voltage-gated cation channels (VGCCs) are candidates for this process. VGCC homologues have been identified in the genome of *T. gondii* (41), although their biological functions have not yet been elucidated. To verify whether VGCCs are the receptor molecules that recognize environmental cues, it is necessary to construct *T. gondii* strains lacking these molecules, and then verify whether these strains conserved the ability of excystation.

Sidik *et al.* generated gene-deletion mutant library of *T. gondii* tachyzoites using Clustered Regularly Interspaced Short Palindromic Repeated / CRISPR-Associated Protein 9 (CRISPR/Cas9) system and guide-RNA library targeting for all annotated protein-coding genes in *T. gondii* genome and confirmed that tachyzoites lacking TGME49_222060, one of the homologs of VGCCs genes, existed in the gene-deletion mutant library one week after the genetic modification (52). It strongly suggested that TGME49_222060 is not essential for tachyzoite and TGME49_222060 deleted tachyzoites can survive at least one week. However, I could not construct

TGME49_222060 knock out parasite using homologous recombination (data not shown). TGME49_222060 might be essential for long-term survival of tachyzoites.

The environmental cues that were identified to induce excystation in this study, including pH and K^+ concentration in the surrounding environment, intracellular Ca^{2+} concentration, and the actin-based contractile motility of the parasite, are known to be the triggers of tachyzoite egress from the host cells (2, 37, 48, 63). The excystation of bradyzoites beyond the cyst wall and egress of tachyzoites from the host cells might share some of the same mechanisms. Specifically, TgGC which has been suggested to synthesize cGMP by sensing H^+ and K^+ in the tachyzoite stage (63, 66), protein kinase G (TgPKG) which is activated by cGMP (63), various signaling pathways dependent on TgPKG (63), and glideosome which is an organelle that controls the mechanical motion of the parasite body and is regulated by these signaling pathways (23, 63), may also be involved in excystation.

In immunosuppressed patients, cyst excystation in tissues and the stage conversion from bradyzoites to tachyzoites ultimately results in toxoplasmosis, including life-threatening encephalitis (33). However, even in the brains of latently infected asymptomatic immunocompetent mice, small numbers of spontaneous excystation events have been observed (19). This spontaneous excystation may be triggered by the release of cysts from the host cell, such as accidental death of the host cell. However, when the host's immunity is intact, the excysted parasites must be immediately eliminated and the spontaneous excystation might not be noticed. In contrast, in immunocompromised cases, the parasites egressed from the cysts are

rarely eliminated and cause symptoms. It might be a reason why reactivation of latent cyst appears to occur only in immunocompromised host. If so, understanding the mechanism of the spontaneous excystation in *T. gondii* may allow the reactivation of latently infected cysts to be controlled in immunocompromised patients. In addition, latent cysts in the muscles of livestock used for meat production, are important as a source of infection for humans. Considering that tachyzoites egressed from cysts by spontaneous excystation must be immediately eliminated in immunocompetent hosts, in future, it may be possible to remove latent cysts from livestock tissues by artificially inducing a spontaneous excystation. The new concept of "spontaneous excystation" presented in this study helps to understand and control toxoplasmosis.

ACKOWLEDGMENTS

I owe my deepest gratitude to my supervisor Assoc. Prof. Yasuhiro Takashima (Gifu Univ.) for intellectual guidance, constructive criticism and encouragement during the period of study. I would like to express my gratitude to Prof. Katsuya Kitoh (Gifu Univ.) for his perennial supervision and valuable comments during undergraduate and Ph.D. research. I am deeply grateful to Prof. Naoaki Yokoyama (Obihiro Univ of Agr. & Vet. Med.) for his valuable suggestion and teaching me profundity of biology.

My special thanks to Prof. Tadashi Itagaki (Iwate Univ.), Prof. Naoaki Yokoyama (Obihiro Univ. of Agr. & Vet. Med.), Prof. Tetsuya Furuya (Tokyo Univ. of Agr. & Tech.) and Prof. Hideto Fukushi (Gifu Univ.) for their valuable suggestion and their patience in revising this dissertation.

I would like to show my appreciation to Dr. Tatsunori Masatani (Gifu Univ.) for his technical support and valuable comments in the gene recombination and optical observation of this work.

REFERENCES

1) Appiah-Kwarteng, C., Saito, T., Toda, N., Kitoh, K., Nishikawa, Y., Adenyo, C., Kayang, B., Owusu, E.O., Ohya, K., Inoue-Murayama, M., Kawahara, F., Nagamune, K., Takashima, Y., 2019. Native SAG1 in *Toxoplasma gondii* lysates is superior to recombinant SAG1 for serodiagnosis of *T. gondii* infections in chickens. *Parasitol. Int.* **69**: 114–120.

2) Arrizabalaga, G., Boothroyd, J.C., 2004. Role of calcium during *Toxoplasma gondii* invasion and egress. *Int. J. Parasitol.* **34**: 361–368.

3) Attias, M., Teixeira, D.E., Benchimol, M., Vommaro, R.C., Crepaldi, P.H., De Souza, W., 2020. The life-cycle of *Toxoplasma gondii* reviewed using animations. *Parasites Vectors* **13:** 588.

4) Bohne, W., Heesemann, J., Gross, U., 1993. Induction of bradyzoite-specific *Toxoplasma gondii* antigens in gamma interferon-treated mouse macrophages. *Infect. Immun.* **61:** 1141–1145.

5) Bohne, W., Roos, D.S., 1997. Stage-specific expression of a selectable marker in *Toxoplasma gondii* permits selective inhibition of either tachyzoites or bradyzoites. *Mol. Biochem. Parasitol.* **88:** 115–126.

6) Caldas, L.A., de Souza, W., Attias, M., 2007. Calcium ionophore-induced egress of *Toxoplasma gondii* shortly after host cell invasion. *Vet. Parasitol.* **147:** 210–220.

7) Calero-Bernal, R., Gennari, S.M., 2019. Clinical toxoplasmosis in dogs and cats: an update. *Front. Vet. Sci.* **6:** 54.

8) Costanzo, L.S., 2018. Physiology 6th edition. Elsevier, UK

9) Djurkovi, O. Vladimir M., 2001. Murine model of drug-induced reactivation of

Toxoplasma gondii. *Acta Protozool.* **40:** 99-106.

10) Dobrowolski, J.M., Sibley, L.D., 1996. Toxoplasma invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* **84:** 933–939.

11) Donald, R.G., Roos, D.S., 1993. Stable molecular transformation of *Toxoplasma gondii*: a selectable dihydrofolate reductase-thymidylate synthase marker based on drug-resistance mutations in malaria. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 11703–11707.

12) Dubey, J.P., 2007. Bradyzoite-induced murine toxoplasmosis: stage conversion, pathogenesis, and tissue cyst formation in mice fed bradyzoites of - different strains of *Toxoplasma gondii*. *J. Eukaryot. Microbiol.* **44:** 592–602.

13) Dubey, J.P., 1998. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. *Vet. Parasitol.* **74:** 75–77.

14) Dubey, J.P., 1997. Tissue cyst tropism in *Toxoplasma gondii*: a comparison of tissue cyst formation in organs of cats, and rodents fed oocysts. *Parasitology* **115:** 15–20.

15) Dubey, J.P., 1986. A review of toxoplasmosis in pigs. *Vet. Parasitol.* **19:** 181–223.

16) Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin. Microbiol. Rev.* **11:** 267–299.

17) Dzierszinski, F., Nishi, M., Ouko, L., Roos, D.S., 2004. Dynamics of *Toxoplasma gondii* differentiation. *Eukaryot. Cell* **3:** 992–1003.

18) Fallingborg, J., 1999. Intraluminal pH of the human gastrointestinal tract. *Dan. Med. Bull.* **46:** 183–196.

19) Ferguson, D.J.P., Hutchison, W.M., Pettersen, E., 1989. Tissue cyst rupture in

mice chronically infected with *Toxoplasma gondii*: An immunocytochemical and ultrastructural study. *Parasitol. Res.* **75:** 599–603.

20) Ferreira-da-Silva, M. da F., Takács, A.C., Barbosa, H.S., Gross, U., Lüder, C.G.K., 2009. Primary skeletal muscle cells trigger spontaneous *Toxoplasma gondii* tachyzoite-to-bradyzoite conversion at higher rates than fibroblasts. *Int. J. Med. Microbiol.* **299:** 381–388.

21) Foroutan, M., Fakhri, Y., Riahi, S.M., Ebrahimpour, S., Namroodi, S., Taghipour, A., Spotin, A., Gamble, H.R., Rostami, A., 2019. The global seroprevalence of *Toxoplasma gondii* in pigs: A systematic review and meta-analysis. *Vet. Parasitol.* **269:** 42–52.

22) Fox, B.A., Gigley, J.P., Bzik, D.J., 2004. *Toxoplasma gondii* lacks the enzymes required for de novo arginine biosynthesis and arginine starvation triggers cyst formation. *Int. J. Parasitol.* **34:** 323–331.

23) Frénal, K., Dubremetz, J.-F., Lebrun, M., Soldati-Favre, D., 2017. Gliding motility powers invasion and egress in Apicomplexa. *Nat. Rev. Microbiol.* **15:** 645–660.

24) Fritz, H.M., Buchholz, K.R., Chen, X., Durbin-Johnson, B., Rocke, D.M., Conrad, P.A., Boothroyd, J.C., 2012. Transcriptomic analysis of toxoplasma development reveals many novel functions and structures specific to sporozoites and oocysts. *PLoS One* **7:** e29998.

25) Gazzinelli, R., Xu, Y., Hieny, S., Cheever, A., Sher, A., 1992. Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J. Immunol.* **149:** 175-80.

26) Gazzinelli, R.T., Hartley, J.W., Fredrickson, T.N., Chattopadhyay, S.K., Sher, A.,

Morse, H.C., 1992. Opportunistic infections and retrovirus-induced immunodeficiency: studies of acute and chronic infections with *Toxoplasma gondii* in mice infected with LP-BM5 murine leukemia viruses. *Infect. Immun.* **60:** 4394–4401.

27) Gregg, B., Taylor, B.C., John, B., Tait-Wojno, E.D., Girgis, N.M., Miller, N., Wagage, S., Roos, D.S., Hunter, C.A., 2013. Replication and distribution of *Toxoplasma gondii* in the small intestine after oral infection with tissue cysts. *Infect. Immun.* **81:** 1635–1643.

28) Håkansson, S., Morisaki, H., Heuser, J., Sibley, L.D., 1999. Time-lapse video microscopy of gliding motility in *Toxoplasma gondii* reveals a novel, biphasic mechanism of cell locomotion. *Mol. Biol. Cell.* **10:** 3539-3547.

29) Jeffers, V., Tampaki, Z., Kim, K., Sullivan, W.J., 2018. A latent ability to persist: differentiation in *Toxoplasma gondii*. *Cell. Mol. Life Sci.* **75:** 2355–2373.

30) Jones, T.C., Bienz, K.A., Erb, P., 1986. In vitro cultivation of *Toxoplasma gondii* cysts in astrocytes in the presence of gamma interferon. *Infect. Immun.* **51**: 147–156.

31) Konstantinovic, N., Guegan, H., Stäjner, T., Belaz, S., Robert-Gangneux, F., 2019. Treatment of toxoplasmosis: Current options and future perspectives. *Food Waterborne Parasitol.* **15:** e00036.

32) Lourido, S., Moreno, S.N.J., 2015. The calcium signaling toolkit of the Apicomplexan parasites *Toxoplasma gondii* and *Plasmodium* spp. *Cell Calcium* **57:** 186–193.

33) Luft, B.J., Remington, J.S., 1992. Toxoplasmic encephalitis in AIDS. *Clin. Infect. Dis.* **15:** 211–222.

34) Martorelli Di Genova, B., Wilson, S.K., Dubey, J.P., Knoll, L.J., 2019. Intestinal delta-6-desaturase activity determines host range for toxoplasma sexual reproduction. *PLoS Biology* **17:** e3000364.

35) Melzer, T.C., Cranston, H.J., Weiss, L.M., Halonen, S.K., 2010. Host cell preference of *Toxoplasma gondii* cysts in murine brain: A confocal study. *J. Neuroparasitology* **1:** 1–6.

36) Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Brodkorb, A., 2014. A standardised static in vitro digestion method suitable for food – an international consensus. *Food Funct.* **5:** 1113–1124.

37) Moudy, R., Manning, T.J., Beckers, C.J., 2001. The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*. *J. Biol. Chem.* **276:** 41492–41501.

38) Mutch, W.A.C., Hansen, A.J., 1984. Extracellular pH changes during spreading depression and cerebral ischemia: mechanisms of brain pH regulation. *J. Cereb. Blood Flow Metab.* **4:** 17–27.

39) Nishikawa, Y., Xuenan, X., Makala, L., Vielemeyer, O., Joiner, K.A., Nagasawa, H., 2003. Characterisation of *Toxoplasma gondii* engineered to express mouse interferon-gamma. *Int. J. Parasitol.* **33:** 1525–1535.

40) Pavesio, C.E.N., Chiappino, M.L., Setzer, P.Y., Nichols, B.A., 1992. *Toxoplasma gondii*: differentiation and death of bradyzoites. *Parasitol. Res.* **78:** 1–9.

41) Prole, D.L., Taylor, C.W., 2011. Identification of intracellular and plasma membrane calcium channel homologues in pathogenic parasites. *PLoS One* **6:** e26218.

42) Radke, J.R., Donald, R.G., Eibs, A., Jerome, M.E., Behnke, M.S., Liberator, P., White, M.W., 2006. Changes in the expression of human cell division autoantigen-1 influence *Toxoplasma gondii* growth and development. *PLoS Pathogen* **2:** e105.

43) Read, N.W., Miles, C.A., Fisher, D., Holgate, A.M., Kime, N.D., Mitchell, M.A., Reeve, A.M., Roche, T.B., Walker, M., 1980. Transit of a meal through the stomach, small intestine, and colon in normal subjects and its role in the pathogenesis of diarrhea. *Gastroenterology* **79:** 1276–1282.

44) Remington, J.S., Cavanaugh, E.N., 1965. Isolation of the encysted form of *Toxoplasma gondii* from human skeletal muscle and brain. *N. Engl. J. Med.* **273:** 1308–1310.

45) Rivers, J.P.W., Sinclair, A.J., Crawford, M.A., 1975. Inability of the cat to desaturate essential fatty acids. *Nature* **258:** 171–173.

46) Robert-Gangneux, F., Dardé, M.-L., 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin. Microbiol. Rev.* **25:** 264–296.

47) Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J.C., Müller, M., 2011. PROC: an open-source package for R and S + to analyze and compare ROC curves. *BMC Bioinformatics* **12:** 77.

48) Roiko, M.S., Svezhova, N., Carruthers, V.B., 2014. Acidification activates *Toxoplasma gondii* motility and egress by enhancing protein secretion and cytolytic activity. *PLoS Pathogen* **10:** e1004488.

49) Rougier, S., Montoya, J.G., Peyron, F., 2017. Lifelong persistence of toxoplasma

Cysts: A questionable dogma? *Trends Parasitol.* **33:** 93–101.

50) R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https:// www.R- proje ct. org/

51) Schwab, J.C., Beckers, C.J., Joiner, K.A., 1994. The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 509–513.

52) Sidik, S.M., Huet, D., Ganesan, S.M., Huynh, M.-H., Wang, T., Nasamu, A.S., Thiru, P., Saeij, J.P.J., Carruthers, V.B., Niles, J.C., Lourido, S., 2016. A genome-wide CRISPR screen in toxoplasma identifies essential apicomplexan genes. *Cell* **166:** 1423-1435.

53) Sinclair, A.J., McLean, J.G., Monger, E.A., 1979. Metabolism of linoleic acid in the cat. *Lipids* **14:** 932–936.

54) Soete, M., Camus, D., Dubrametz, J.F., 1994. Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH Strain of *Toxoplasma gondii in vitro*. *Exp. Parasitol.* **78:** 361–370.

55) Soete, M., Fortier, B., Camus, D., Dubremetz, J.F., 1993. *Toxoplasma gondii*: Kinetics of bradyzoite-tachyzoite interconversion *in vitro. Exp. Parasitol.* **76:** 259–264.

56) Sullivan, W.J., Jeffers, V., 2012. Mechanisms of *Toxoplasma gondii* persistence and latency. *FEMS Microbiol. Rev.* **36:** 717–733.

57) Suzuki, Y., Remington, J.S., 1989. A method for obtaining large numbers of trophozoites of avirulent strains of *Toxoplasma gondii* using an antibody to interferon-gamma. *J. Parasitol.* **75:** 174–176.

58) Swierzy, I.J., Lüder, C.G.K., 2015. Withdrawal of skeletal muscle cells from cell

cycle progression triggers differentiation of *Toxoplasma gondii* towards the bradyzoite stage: Toxoplasma differentiation in skeletal muscle cells. *Cell. Microbiol.* **17:** 2–17.

59) Taniguchi, Y., Appiah-Kwarteng, C., Murakami, M., Fukumoto, J., Nagamune, K., Matsuo, T., Masatani, T., Kanuka, H., Hoshina, T., Kitoh, K., Takashima, Y., 2018. Atypical virulence in a type III *Toxoplasma gondii* strain isolated in Japan. *Parasitol. Int.* **67:** 587–592.

60) Takashima, Y., Suzuki, K., Xuan, X., Nishikawa, Y., Unno, A., Kitoh, K., 2008. Detection of the initial site of *Toxoplasma gondii* reactivation in brain tissue. *Int. J. Parasitol.* **38:** 601–607.

61) Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. *Int. J. Parasitol.* **30:** 1217–1258.

62) Terskikh, A., Fradkov, A., Ermakova, G., Zaraisky, A., Tan, P., Kajava, A.V., Zhao, X., Lukyanov, S., Matz, M., Kim, S., Weissman, I., Siebert, P., 2000. "Fluorescent timer": Protein that changes color with time. *Science* **290:** 1585–1588.

63) Uboldi, A.D., Wilde, M., Bader, S.M., Tonkin, C.J., 2021. Environmental sensing and regulation of motility in toxoplasma. Mol. Microbiol. **115:** 916–929.

64) Waldman, B.S., Schwarz, D., Wadsworth, M.H., Saeij, J.P., Shalek, A.K., Lourido, S., 2020. Identification of a master regulator of differentiation in toxoplasma. *Cell* **180:** 359-372.

65) Weiss, L.M., Laplace, D., Takvorian, P.M., Tanowitz, H.B., Cali, A., Wittner, M., 1995. A cell culture system for study of the development of *Toxoplasma gondii* bradyzoites. *J. Eukaryot. Microbiol.* **42:** 150–157.

66) Yang, L., Uboldi, A.D., Seizova, S., Wilde, M.-L., Coffey, M.J., Katris, N.J.,

Yamaryo-Botté, Y., Kocan, M., Bathgate, R.A.D., Stewart, R.J., McConville, M.J., Thompson, P.E., Botté, C.Y., Tonkin, C.J., 2019. An apically located hybrid guanylate cyclase–ATPase is critical for the initiation of Ca^{2+} signaling and motility in *Toxoplasma gondii*. *J. Biol. Chem.* **294:** 8959–8972.

67) Zuzarte-Luís, V., Mota, M.M., 2018. Parasite sensing of host nutrients and environmental cues. *Cell Host Microbe* **23:** 749–758.