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Toxoplasma gondii Strains Defective in Oral Transmission Are Also Defective in Developmental Stage Differentiation[▽]

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Toxoplasma gondii undergoes differentiation from rapidly growing tachyzoites to slowly growing bradyzoites during its life cycle in the intermediate host, and conversion can be induced in vitro by stress. Representative strains of the three clonal lineages showed equal capacity to differentiate into bradyzoites in vitro, as evidenced by induction of bradyzoite antigen 1, staining with *Dolichos biflorus* lectin (DBL), pepsin resistance, and oral infectivity in mice. We also examined several recently described exotic strains of *T. gondii*, which are genetically diverse and have a different ancestry from the clonal lineages. The exotic strain COUG was essentially like the clonal lineages and showed a high capacity to induce bradyzoites in vitro and in vivo, consistent with its ability to be efficiently transmitted by the oral route. In contrast, exotic strains MAS and FOU, which are defective in oral transmission, showed a decreased potential to develop into bradyzoites in vitro. This defect was evident from reduced staining with DBL and the cyst antigen CST1, failure to down-regulate tachyzoite antigens, such as tachyzoite surface antigens 1 and 2A, and decreased resistance to pepsin treatment. Despite normal in vitro differentiation, the exotic strains CAST and GPHT also showed decreased oral transmission, due to formation of smaller cysts and a lower tissue burden during chronic infection, traits also shared by MAS and FOU. Collectively, these findings reveal that the limited oral transmission in some strains of *T. gondii* is due to inefficient differentiation to the bradyzoite form, leading to defects in the formation of tissue cysts.

The protozoan *Toxoplasma gondii* is one of the most successful parasites of vertebrates, capable of infecting a wide range of warm-blooded hosts, including humans. It is estimated that more than one quarter of the human population worldwide is infected (26). The ingestion of food or water contaminated with oocysts from cat feces or ingestion of tissue cysts in undercooked meat are the two major routes of human infection (12, 19). In North America and Europe, *T. gondii* has a highly clonal population structure comprised of three widespread and similar lineages referred to as types I, II, and III (2, 11, 25, 35). More than 95% of the strains from these regions can be classified into one of these highly clonal lineages. This marked clonality is evidence of a rapid expansion following a severe genetic bottleneck that is estimated to have occurred circa 10,000 years ago (23, 39).

Following infection of intermediate hosts with *T. gondii*, the parasite initially multiplies in the fast-growing tachyzoite stage before differentiating into the slowly replicating form called the bradyzoite (20). Unlike tachyzoites, bradyzoites are relatively resistant to gastric digestion (i.e., pepsin) (17) and thus are infectious orally both to cats, the definitive host, and to other intermediate hosts (12). Cats are very sensitive to infection by bradyzoites, while generally $\geq 1,000$ bradyzoites are needed for consistent infection of mice by the oral route (14). Bradyzoite transmission between intermediate hosts may be important for spread through the food chain via carnivorous feeding or scavenging, thus bypassing the need for the cat.

Direct oral transmission between intermediate hosts is unique to *T. gondii* and is not present in related parasites such as *Neospora* spp. and *Hammondia* spp. (18).

Direct oral transmission between intermediate hosts is a common feature of the clonal lineages of *T. gondii*, and this may have contributed to their rapid expansion and successful spread (39). While clonality is the predominant pattern in *T. gondii* in North America and Europe, occasionally recombinant strains or those with more exotic genotypes are encountered (2, 25). It has been suggested that such exotic lineages represent the genetic makeup of strains isolated from geographically restricted areas (1). Examination of several exotic lineages previously indicated that some of them are defective in oral transmission between intermediate hosts (39). However, the underlying molecular basis of the defect in oral transmission of these exotic strains has not been previously defined.

In vitro models of tachyzoite-to-bradyzoite differentiation have been established using a variety of stress conditions that mimic the stresses of the host immune response. These conditions include treatment with gamma interferon (5), mitochondrial inhibitors (6), alkaline pH (pH 8.1) (38), and high temperature (37). The stress response is controlled in part by eIF-2 α kinase, which is well characterized as a stress response in eukaryotic cells (41), and differentiation also involves cyclic nucleotide kinases (21). Microarray studies (9), large-scale sequencing of stage-specific cDNA (28, 31), and serial analysis of gene expression tags (34) reveal that stage conversion involves changes in the expression of a large number of genes, although the regulatory mechanism(s) involved in this switch is poorly understood.

A number of markers have been used to follow stage conversion, including the decreased expression of tachyzoite sur-

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TABLE 1. Primers used for qPCR in this study

Gene	Gene identification no. ^a	Forward primer	Reverse primer
<i>ACT1</i>	25.m00007	TCCCGTCTATCGTCGGAAAG	CCATTCCGACCATGATACCC
<i>SAG1</i>	44.m00009	ATCGCCTGAGAAGCATCACTG	CGAAAATGGAAACGTGACTGG
<i>BAG1</i>	55.m00009	GACGTGGAGTTCGACAGCAA	ATGGCTCCGTTGTCGACTTCT
<i>SAG2A</i>	59.m00008	TGTTTCAGGTTGCTGCAGTGA	CAAGCAGCGCTCGTTTCTT
<i>LDH2</i>	80.m00010	GAAGTGTGCACGCTTTGCAA	CAGCCAGTGATGATGCAGCT

^a *Toxoplasma gondii* Genome Resource (<http://toxodb.org>).

face antigen 1 (SAG1) (8) and the concomitant induction of bradyzoite antigen 1 (BAG1) (45) and lactate dehydrogenase 2 (LDH2) (43). One of the hallmark features of the cyst wall is positive staining with *Dolichos biflorus* lectin (DBL) (27), which recognizes *N*-acetylgalactosamine. The major DBL-binding component of the cyst wall has been described as a 116-kDa glycoprotein termed CST1 (44). While the cyst may present a permeability barrier to drugs and aid in immune evasion, the composition of the wall is not well understood. Treatment with acid-pepsin, as occurs in the stomach, dissolves the cyst wall and liberates bradyzoites, which remain infectious following this treatment (17).

In the present report, we defined conditions that allow in vitro development of fully mature bradyzoites as defined by antigen expression, cell wall composition, pepsin resistance, and oral infectivity. We then compared the induction of bradyzoite development in clonal and several exotic strains of *T. gondii* in order to define the basis of their defects in oral transmission.

MATERIALS AND METHODS

Parasite strains. We compared representative strains of the clonal lineages using well-characterized prototypic strains. The GT-1 strain (type I) was isolated from skeletal muscle of a goat (16). The ME49 strain (type II) (American Type Culture Collection ATCC 50611) was isolated from the diaphragm of a sheep (30). The CTG strain (type III) (ATCC 50842) was isolated from oocysts shed from an infected cat (33). To assure that the full biological capability to differentiate was retained by these representative strain types, isolates were used within 10 to 20 passages following transmission through a cat. In addition, we examined a number of exotic strains that do not fit within the clonal lineages, including MAS (ATCC 50870), isolated from a congenital infection in France (11); CAST (ATCC 50868), isolated from a patient with HIV in the United States (35); FOU (ATCC 50869), isolated from a human transplant in France (11); GPHT, isolated from a human congenital infection in France (10); and COUG, an isolate from a cougar in British Columbia, Canada (3). Parasites were propagated in human foreskin fibroblast (HFF) cells grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), 1 mM glutamine, 10 µg/ml gentamicin, under 5% CO₂ at 37°C. While these other isolates were not passaged through cats, they were used at the lowest available passage number in vitro, generally less than 10 passages following infection in rodents.

In vitro differentiation of strains of *Toxoplasma gondii*. In vitro bradyzoite induction was performed using alkaline treatment of confluent monolayers of HFF cells grown in 6-well plates. The plates were infected with tachyzoites of each strain and differentiation was induced by culture in sodium bicarbonate-free RPMI 1640 containing 1% FBS, HEPES (pH 8.1) at 37°C without CO₂ (38). Cultures were incubated for 3 to 9 days, with replacement of the medium every 2 to 3 days.

Acid-pepsin digestion. Acid-pepsin solution from porcine stomach (biological activity, 1:1,000) (Sigma Chemical Co., St. Louis, MO) was used to quantify bradyzoites produced in cell culture (13). Cysts were treated with 0.26 mg/ml pepsin, 0.170 M NaCl (pH 1.4) at 37°C for 45 min and then neutralized with 1.2% sodium bicarbonate (pH 8.3). Pepsin-resistant parasites were quantified by plating dilutions on HFF monolayers and counting foci or plaques at 7 to 9 days postinfection.

RNA extraction and cDNA synthesis. Total RNA was isolated from freshly harvested parasites using TRIzol (Invitrogen Corp., Carlsbad, CA). The total RNA (1 µg per sample) was transcribed into cDNA using 50 µM oligo(dT)₂₀ and 200 units of SuperScript III reverse transcriptase (RT) (Invitrogen) in a volume of 20 µl following the manufacturer's protocol. For negative controls, water was added instead of RT.

Real-time RT-PCR. PCR primers were designed using Primer Express software, version 1.0 (Applied Biosystems, Foster City, CA). Real-time quantitative PCR (qPCR) was performed using a SmartCycler (Cepheid, Sunnyvale, CA) with a reaction mixture volume of 25 µl containing SYBR greenER qPCR SuperMix universal (Invitrogen), 400 nM of each primer, and 1 to 2 µl of cDNA. The reaction conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Nontemplate controls (water blank or host cell total RNA) were run with every assay and all determinations were performed in duplicate to ensure reproducibility. Data analysis was conducted using SmartCycler software (Cepheid). The relative gene expression levels were calculated as the fold change using the formula $2^{-\Delta\Delta C_T}$, where ΔC_T = threshold cycle (C_T) of actin - C_T of the target gene (shown in Table 1) and $\Delta\Delta C_T$ = ΔC_T of the tachyzoite stage RNA - ΔC_T of the bradyzoite stage RNA (29). The housekeeping gene encoding actin (ACT1) was used as a reference control.

Immunostaining. HFF cells were grown to confluence on chamber slides (Labtek, Campbell, CA), infected with parasites, and examined at different times, 5, 7, and 9 days after bradyzoite induction. For immunofluorescence assays, parasites were fixed for 15 min in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, permeabilized for 10 min in 1% Triton X-100, and blocked for 10 min in 5% FBS and 5% normal goat serum. Samples were incubated for 1 h with monoclonal antibody (mAb) 8.25.8 against BAG1 (42), mAb DG52 against SAG1, or mAb 73.18 to CST1 (44) diluted 1:1,000 in PBS in 1% FBS. The samples were washed three times and primary antibodies were detected with goat anti-mouse immunoglobulin G (IgG) conjugated to Alexa 594 (Invitrogen Molecular Probes, Carlsbad, CA) diluted 1:1,000. After being washed three times, the samples were incubated with fluorescein isothiocyanate-conjugated DBL diluted 1:1,000 (Sigma, St. Louis, MO). The slides were rinsed in PBS, mounted with Vectashield (Vector, Burlingame, CA), and analyzed with a Zeiss AxioSkop wide-field epifluorescence microscope equipped with an AxioCam charge-coupled-device camera. Images were captured using AxioVision v3.1 (Carl Zeiss Inc., Thornwood, NY). Cells were examined at $\times 40$ magnification and the average numbers of cysts were determined by counting 10 fields from duplicate samples. The sizes of the cysts formed in vitro were estimated using the measurement tool in AxoVision to trace the perimeter and calculate the area. The experiments were repeated two or more times, and a representative example is shown.

Oral infection of mice with bradyzoites induced in vitro. Eight-week-old female CD-1 outbred mice (Charles River Laboratories, Wilmington, MA) were used for experimental infections. Mice were infected by oral administration of 100, 1,000, or 10,000 cysts of *T. gondii* developed in vitro (five animals/dose). At the end of the 30-day observation period, blood samples were collected from surviving mice, and sera were tested by Western blotting for antibodies against *T. gondii* RH strain lysate. Mouse sera were diluted 1:1,000 in PBS containing 1% nonfat dry milk. Horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ) was diluted 1:10,000 and used as the secondary antibody. Antibody binding was detected by using enhanced chemiluminescent substrate (Pierce Biotechnology Inc., Rockford, IL). The percent cumulative mortality was defined as the number of animals that succumbed to infection/the total number of infected animals (i.e., deaths plus seropositive survivors), as described previously (40).

Production of cysts in vivo and challenge in naive mice. Tachyzoites of *T. gondii* strains were propagated in vitro and used to induce chronic infections in mice or rats by intraperitoneal (i.p.) injection. At 1 to 2 months postinfection, the animals were sacrificed and the brains were removed and homogenized in PBS.

The numbers of cysts were estimated by counting aliquots of 10 μ l from a total volume of 1 ml (mice) or 4 ml (rats). The sizes of the cysts were estimated using the measurement tool in AxioVision software to determine the diameter, which was then used to calculate volume using the standard geometric formula for a sphere. To establish oral transmissibility, cysts were administered orally by a gastric feeding tube. In parallel, separate groups of animals were injected i.p. with an equal number of cysts. Survival of the mice was monitored for 30 days, after which the animals were tested for seroconversion as described above. Cumulative infectivity was defined as the number of animals succumbing to infection plus survivors that were seropositive/the total number of animals used per group (typically 5).

Fluorescent staining of cysts with DBL. Chronically infected animals were sacrificed ~1 month postinfection, and the brains were removed and homogenized in 1 ml of PBS (mice) or 4 ml of PBS (rats). An aliquot of the brain suspension was fixed in 5% formaldehyde containing 0.2% Triton X-100 in PBS for 20 min. Cysts were washed in PBS, blocked using a solution of 10% normal goat serum in PBS, and stained with fluorescein isothiocyanate-labeled DBL. The suspension was examined using wide-field epifluorescence microscopy, and the cysts were imaged as described above.

Transmission electron microscopy. For ultrastructural analysis, cysts were induced for 9 days as described above and fixed in 2% paraformaldehyde–2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM phosphate buffer (pH 7.2) for 1 h at room temperature. Following three washes in phosphate buffer, the cells were postfixed in 1% osmium tetroxide (Polysciences Inc., Warrington, PA) for 1 h at room temperature. The samples were then rinsed extensively in distilled water prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h at room temperature. Following several rinses in distilled water, the cells were dehydrated in a graded series of ethanol dilutions and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 70 to 80 nm were cut, stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA, Inc., Peabody, MA).

Animal care and housing. The animals were cared for by the Division of Comparative Medicine and all procedures conducted here were approved by the Animal Studies Committee at Washington University.

Statistics. Triplicate experiments were performed and the means and standard deviations calculated for statistical comparisons. The differences between the means were compared using Student's *t* test.

RESULTS

Induction of bradyzoite or cyst wall antigens following in vitro culture under stress conditions has previously been used to define stage conversion in *T. gondii* (38, 45). Previous studies have primarily used the type II strain ME49 or clones from this strain, which are extremely efficient at cyst production in vitro and in vivo. To determine the relative contribution of the parasite's genotype to stage conversion, we evaluated several prototypical strains of the three clonal lineages of *T. gondii*. In addition to evaluating the expression of bradyzoite or cyst wall antigens, we also tested whether fully mature bradyzoites form in vitro under stress conditions. Bradyzoite infectivity was evaluated based on resistance to acid-pepsin treatment or oral infection of naive mice. Finally, we compared the responses of the well-studied clonal lineages to those of several exotic lineages that have previously been shown to be defective in oral transmission in the mouse model. Collectively, these studies define the molecular and cellular basis for the defect in transmission of these naturally occurring strains.

In vitro development of cysts by clonal strains of *T. gondii*. We tested the induction of bradyzoites following alkaline pH stress in vitro by representative strain types of *T. gondii*. Cyst conversion was identified by staining with fluorescent DBL (27) and antibodies specific to the bradyzoite antigen, BAG1 (42). A representative example of the staining for DBL and BAG1 is shown in Fig. 1A. The numbers of DBL⁺ BAG1⁺ cysts were determined and the average sizes of the cysts were

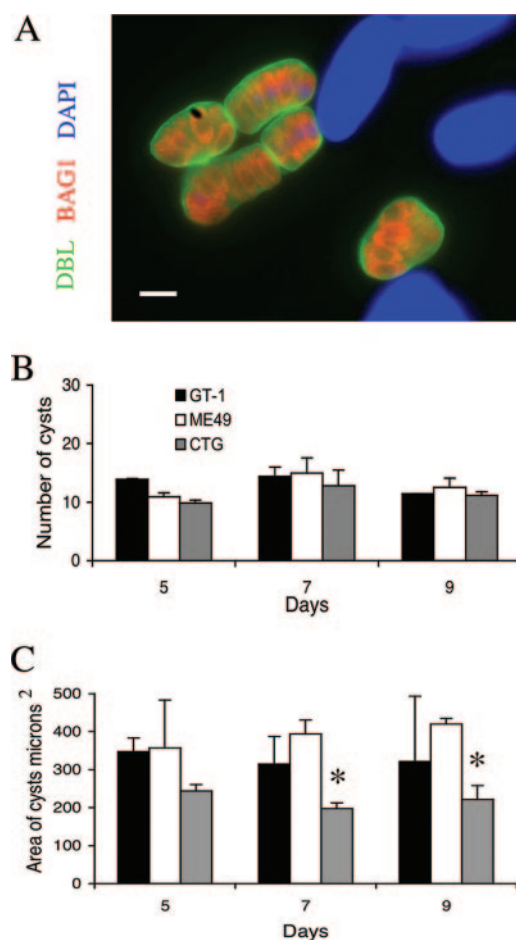


FIG. 1. In vitro differentiation to bradyzoites by clonal lineages. Representative strains for type I (GT-1), type II (ME49), and type III (CTG) strains were induced to differentiate by high-pH culture for 5, 7, and 9 days. Cyst development was detected by staining with fluoresceinated DBL and by using the mAb 8.25.8 to BAG1 followed by secondary antibodies conjugated to Alexa 594. (A) Representative example of BAG1 staining (red channel) and DBL-positive staining (green channel) for in vitro-derived cysts of *T. gondii*. Nuclei are stained in blue (DAPI). Scale bar = 10 μ m. (B) The average numbers of cysts per $\times 40$ magnification field were similar among all three lineages at all time points. (C) Average sizes (area in μ m²) of cysts formed by *T. gondii* strains from the three lineages. ME49 formed the largest cysts, while cysts formed by GT-1 and CTG were somewhat smaller. The differences between ME49 and CTG were statistically significant at day 7 and day 9 (*, $P \leq 0.05$). Error bars show standard deviations.

measured at 5, 7, and 9 days after induction (Fig. 1). The average numbers of cysts were similar for the three strains at all three time periods of in vitro development (Fig. 1B). Strain ME49 formed larger cysts than CTG, and the differences were statistically significant ($P \leq 0.05$) at day 7 and day 9 (Fig. 1C). Cysts formed by GT-1 also tended to be smaller than those of ME49, but due to the higher variance in this group, the differences were not significant (Fig. 1C). These results reveal that conversion to cysts, as defined by DBL staining and BAG1 expression, occurs rapidly after in vitro induction among all three clonal lineages.

TABLE 2. Infection of mice challenged by feeding of in vitro-produced cysts of *T. gondii*

Strain and no. of days cultivated ^a	No. of mice infected/no. of mice challenged by indicated dose of cysts ^b		
	100	1,000	10,000
ME49			
5	ND	0/5	2/5
7	0/5	1/5	2/5
9	0/5	3/5	5/5
GT-1			
9	0/5	4/5†	2/5†
CTG			
9	1/5	2/5	3/5

^a Days of in vitro cultivation to induce cysts before infection.^b Infection was determined at 30 days postinoculation by positive Western blotting of serum against whole tachyzoite antigen. The estimated dose of cysts is based on DBL-positive staining. ND, not done; †, infected animals died during the 30-day period.

Oral infection of CD-1 mice with in vitro-produced bradyzoites. To determine whether in vitro-derived cysts were capable of causing oral infection, we challenged CD-1 mice with cysts produced by alkaline pH stress. CD-1 mice were orally challenged with different doses of DBL⁺ cysts from the ME49 strain induced in vitro for 5, 7, and 9 days of culture. At 30 days postinoculation, the number of infected animals was determined by Western blotting for the detection of antibodies specific to *T. gondii*. While the overall oral infectivity of in vitro-produced cysts was low, it increased approximately 10-fold between day 5 and 9 (Table 2). By day 9 of culture, more than half of the mice became infected with a challenge dose of 1,000 cysts (Table 2). Examination of these cysts by 4',6'-diamidino-2-phenylindole (DAPI) staining revealed that they contained from 10 to 20 nuclei (data not shown). Hence, the oral dose of 1,000 cysts likely contained $\sim 10^4$ total parasites. To determine the extent of conversion to mature bradyzoites, we treated in vitro-derived cysts with acid-pepsin for ~ 45 min and tested their viability by plaquing on host cell monolayers. These experiments indicated that between 5 and 10% of total parasites were resistant to acid-pepsin at 9 days postinfection (Table 3 and data not shown). Collectively, these studies establish that, following in vitro culture for 9 days, a dose of

$\sim 1,000$ cysts containing $\sim 10^3$ acid-pepsin-resistant parasites is capable of causing oral infection in the mouse.

To determine whether cysts produced by different *T. gondii* strains differed in oral infectivity, cysts developed during 9 days of induction were used to challenge CD-1 mice by the oral route. All three clonal types showed similar levels of oral transmissibility in the mouse model (Table 2). Collectively, these studies show that rapid induction of bradyzoite antigens occurs following alkaline pH culture and that fully mature bradyzoites, as defined by oral transmission, are produced by all three clonal lineages.

In vitro development of cysts by exotic strains of *T. gondii*.

The previous finding that some exotic strains have reduced oral transmission in the mouse model (39) suggests that they may have defects in the development of mature bradyzoites. These exotic strains were tested in vitro for induction of bradyzoite antigens following culture at alkaline pH and staining for the bradyzoite markers BAG1 and DBL. Similar to the clonal lineages, exotic strains COUG, CAST, and GPHT expressed BAG1 at high levels by day 5 of culture (Table 3). However, the strains MAS and FOU displayed much lower levels of expression of BAG1 (Table 3). A more profound difference was observed in the staining patterns with DBL. Even among cysts where BAG1 staining was positive, the exotic strains MAS and FOU failed to stain appreciably with DBL, while the remaining exotic and clonal lineages stained strongly (Fig. 2 and Table 3). The exotic strain MAS also stained very weakly for the cyst wall antigen CST1, even in cysts expressing BAG1, while the remaining strains stained strongly with this marker following the induction of bradyzoite development (Fig. 2, Table 3). The tachyzoite antigen SAG1 is gradually shut off during in vitro differentiation (38), and consistent with this, a majority of the vacuoles formed by the clonal lineages failed to stain with antibodies to SAG1 by day 9 of culture (Table 3). In contrast, exotic strains MAS and FOU continued to express high levels of SAG1 (Table 3).

We also compared the induction of bradyzoite differentiation by exotic and clonal-lineage strains using several reference genes that undergo dramatic changes in gene transcription during differentiation (4, 9, 43). The tachyzoite-specific genes *SAG1* and *SAG2A* showed 10- to 100-fold repression during conversion to bradyzoites in the clonal strain ME49 and the exotic strain COUG (Fig. 3). In contrast, there was almost no

TABLE 3. Comparisons of in vitro-derived cysts of clonal and exotic strains of *T. gondii*

Strain	Type	Host	Location	% BAG1 positive ^a	% SAG1 positive ^b	DBL positive ^c	CST1 positive ^d	Pepsin resistant (%) ^e
GT-1	I	Goat	United States	100	34.1	+++	+++	9.40
ME49	II	Sheep	United States	100	34.4	+++	+++	3.40
CTG	III	Cat	United States	100	36.7	+++	+++	4.10
COUG	Exotic	Cougar	Canada	93.3	35.0	+++	+++	6.40
CAST	Exotic	Human	United States	100	31.7	+++	+++	5.03
GPHT	Exotic	Human	France	100	43.9	+++	+++	2.75
MAS	Exotic	Human	France	70.4	75.0	Weak	Weak	0.07
FOU	Exotic	Human	France	66.1	100	Weak	+++	0.03

^a Percentage of positive vacuoles at day 5 of induction, as detected with mAb 8.25.8 to BAG1.^b Percentage of positive vacuoles at day 9 of induction, as detected with mAb DG52 to SAG1.^c + + +, strongly positive vacuoles at day 5 of induction, as detected with fluorescent DBL.^d + + +, strongly positive vacuoles at day 5 of induction, as detected with mAb 73.18 to CST-1.^e Percentage of total parasites resistant to acid-pepsin treatment at day 9 of induction, as determined by plaquing on HFF monolayers.

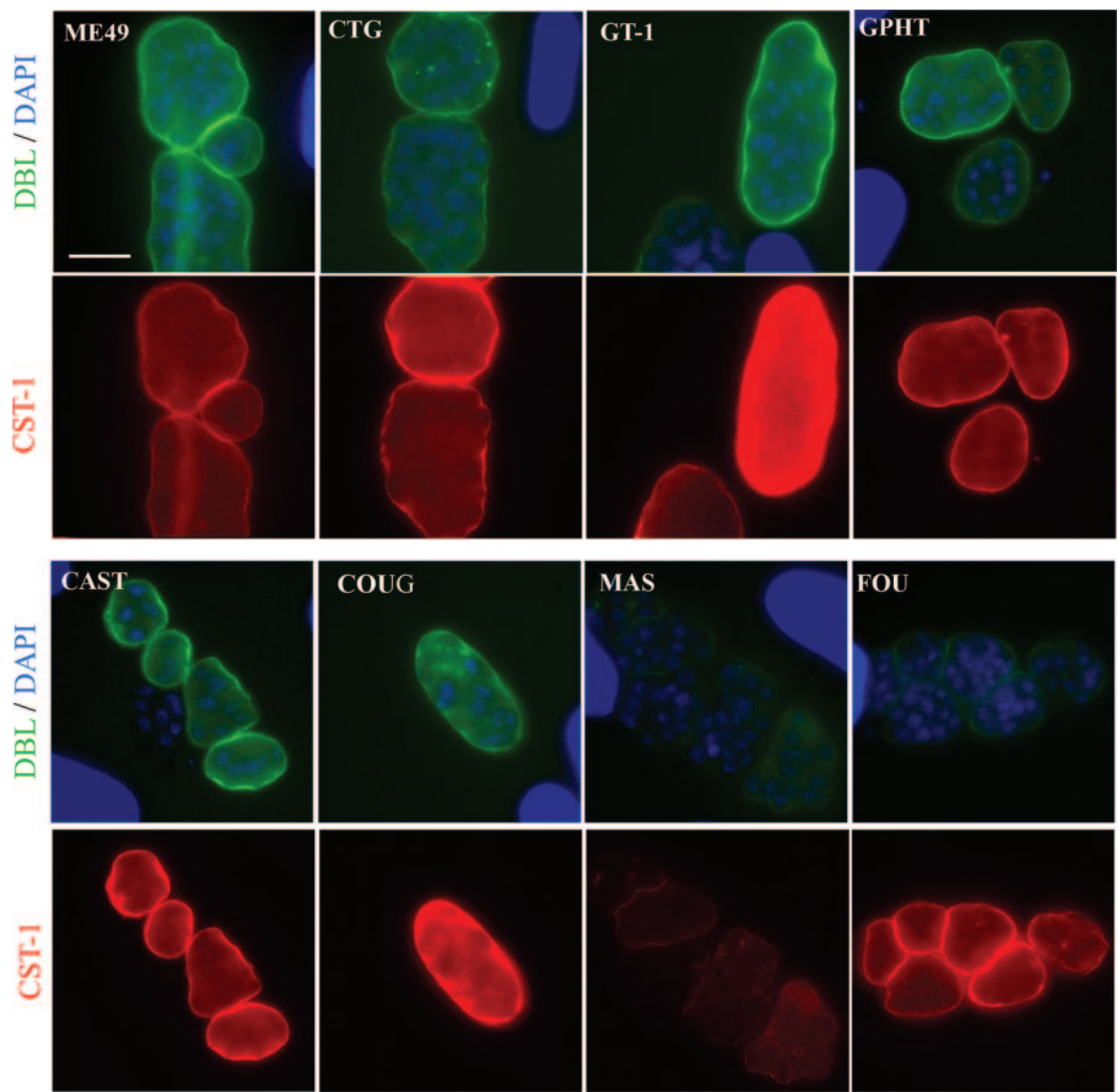


FIG. 2. Expression of cyst antigens following in vitro differentiation of *T. gondii* strains. The majority of strains strongly express both the cell wall epitope detected by DBL (green) and the CST-1 antigen (red). However, MAS expressed both markers poorly and FOU failed to stain with DBL, while staining positively for CST-1. At 5 days postinduction, the cells were fixed and stained with fluoresceinated DBL (green) and antibody to CST-1 followed by goat anti-mouse IgG conjugated to Alexa 594 (red). Nuclei are stained in blue. Scale bar = 10 μ m. All pictures shown were recorded under similar optical conditions, imaged with the same exposure time, and processed identically.

down-regulation of these tachyzoite-specific genes in exotic strains MAS and FOU following culture under bradyzoite-inducing conditions (Fig. 3). All of the strains turned on the bradyzoite gene *BAG1* strongly under inducing conditions, while only FOU failed to induce the bradyzoite-specific gene *LDH2* (Fig. 3). In summary, these results indicate that, while exotic strains are able to induce some bradyzoite markers, they are defective in down-regulating tachyzoite genes following culture under conditions that normally induce differentiation.

In vitro development of pepsin-resistant parasites. The relative resistance to acid-pepsin was used as a criterion to evaluate the in vitro production of bradyzoites (17). The number of resistant parasites was determined by plaquing on host cell monolayers after pepsin treatment and neutralization. The

clonal lineages expressed pepsin-resistant parasites at levels that ranged between 3 and 9% of all viable parasites (Table 3). Similar levels were observed for the exotic strains GPHT, CAST, and COUG (Table 3). However, the exotic strains MAS and FOU proved very inefficient at producing pepsin-resistant forms in vitro (Table 3). The failure of MAS and FOU to express pepsin resistance is consistent with their impaired ability to express bradyzoite antigens and suggests they are defective in cyst production in vitro.

Ultrastructure of cysts formed in vitro. The tissue cysts formed by *T. gondii* have a characteristic thin, convoluted wall underlain by granular material that also fills the lumen (20). Bradyzoites show several subtle morphological differences from tachyzoites, including the presence of amylopectin

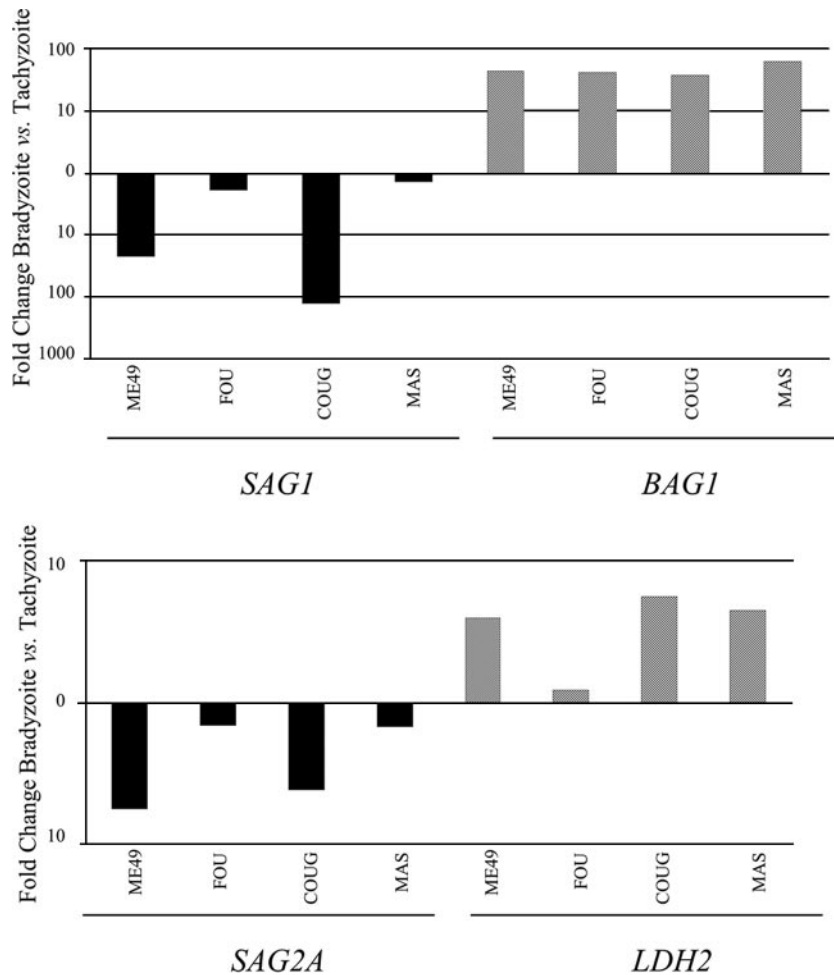


FIG. 3. qPCR analysis of stage-specific genes during in vitro differentiation of *T. gondii*. Parasites were cultured in vitro under conditions that induce differentiation. After 5 days, total RNA was harvested and analyzed by qPCR for the stage-specific genes shown. The ME49 and COUG strains showed strong repression of the tachyzoite-specific genes *SAG1* and *SAG2A*, while strains MAS and FOU continued to express these genes at levels typical of tachyzoites. All strains showed induction of the bradyzoite-specific gene *BAG1*, and all strains except FOU showed induction of *LDH2*. The values represent the fold change based on C_T comparison (see Materials and Methods).

granules, a posteriorly positioned nucleus, more abundant micronemes, and electron-dense rhoptries (20, 22). Consequently, we examined cysts formed in vitro for these diagnostic features. Following 5 to 9 days of in vitro culture at an alkaline pH, cysts were fixed and processed for conventional electron microscopy. Cysts of the ME49 strain showed a characteristic thin, convoluted cell wall containing a granular matrix (Fig. 4). Parasites within these cysts displayed the hallmark features of bradyzoites, including a posterior nucleus, solid-staining rhoptries, amylopectin granules, and an increased number of micronemes (Fig. 4A and B). Similar morphological features were exhibited by in vitro-derived bradyzoites of other clonal strains (i.e., GT-1 and CTG) and the exotic strains GPHT, COUG, and CAST (data not shown). In contrast, the exotic strains MAS and FOU exhibited features more similar to those of tachyzoites, including a centrally positioned nucleus, fewer micronemes, and rhoptries with a honeycombed appearance (Fig. 4C and D). Despite these tachyzoite-like features, the parasites were contained in a morphologically normal cyst wall

(Fig. 4). These features are consistent with a reduced ability to differentiate in vitro under stress conditions.

Strains of *T. gondii* produce very different sizes of cysts in vivo. The failure of several exotic strains to develop cysts in vitro suggested that they may also be deficient in vivo. Consequently, we infected mice or rats with different strains of *T. gondii* and examined the development of cysts in vivo (Table 4). In vivo-derived cysts were examined for average size and staining intensity with fluoresceinated DBL (Fig. 5). The clonal strain GT-1 stained strongly with DBL, although it exhibited a somewhat different pattern of staining, with the lectin-positive material often being found within the cyst rather than primarily on the wall (Fig. 4). MAS produced normal-looking cysts in vivo, and thus, the failure to develop mature bradyzoites in vitro is due to a reduced responsiveness to the stress induction conditions, rather than an inherent defect in bradyzoite formation. We failed to detect DBL-positive or BAG1-positive cysts in mice or rats that were infected with FOU, suggesting that it is highly defective in differentiation. While cyst sizes

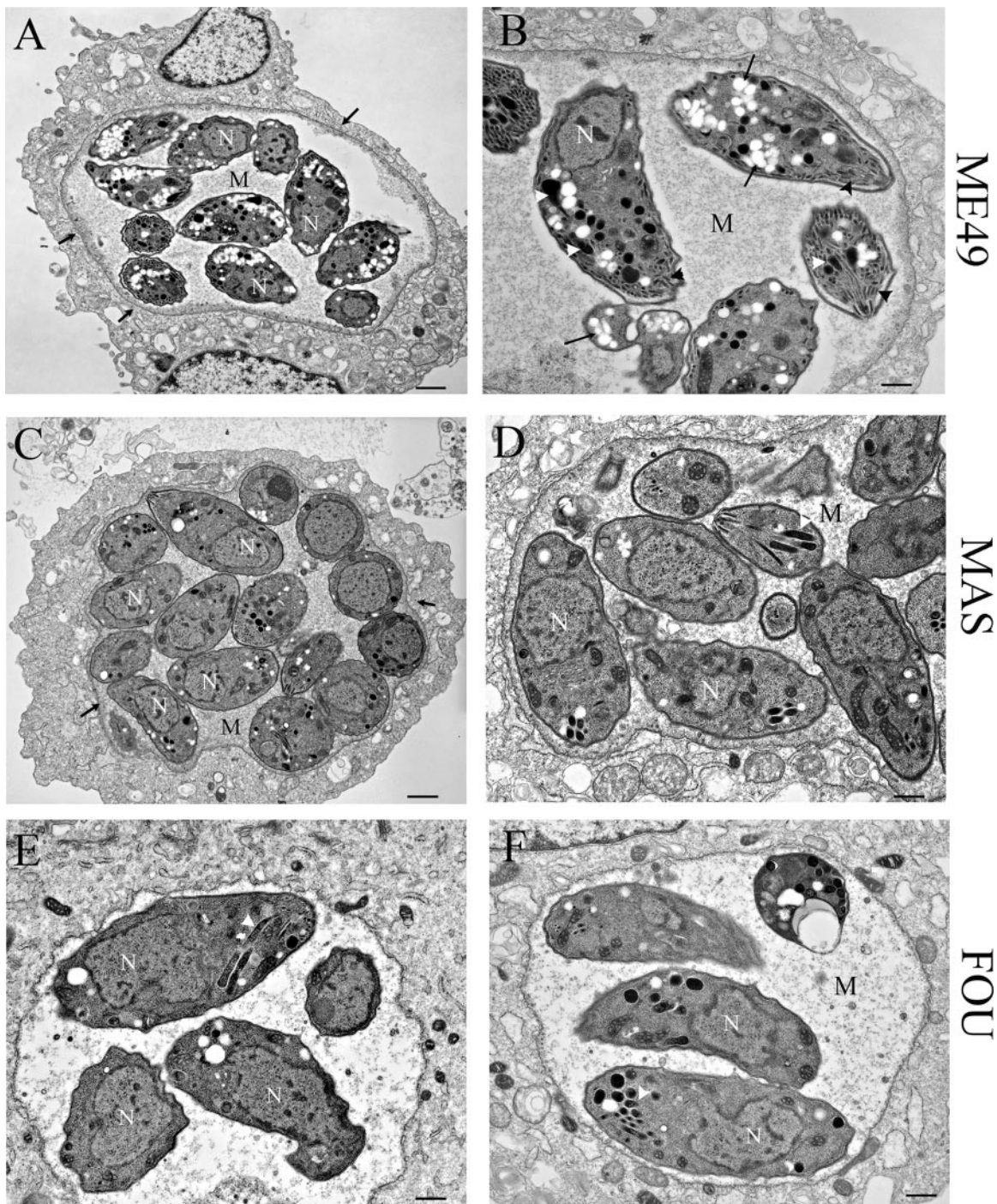


FIG. 4. Electron microscopy of *T. gondii* cysts formed after 9 days of induction in vitro. (A) Intracellular cyst of ME49 demonstrating the convoluted cell wall (arrows) and internal granular matrix (M). Parasite nuclei (N) are located posteriorly. (B) Enlargement of ME49 cyst demonstrating posterior nuclei (N), abundant micronemes (black arrowheads), amylopectin granules (arrows), and electron-dense rophtries (white arrowheads). (C) Cyst of MAS containing parasites that resemble tachyzoites. Although the cyst wall (arrows) and internal matrix (M) were similar, the parasites lacked features of bradyzoites. Parasite nuclei (N) were located centrally. (D) Enlargement of MAS cyst showing parasites with honeycombed rophtries (white arrowhead), centrally located nucleus (N), fewer micronemes, and fewer amylopectin granules. (E) Cyst of FOU showing tachyzoite-like features, including centrally located nucleus (N) and honeycombed rophtries (arrowhead). (F) Enlarged cyst of FOU showing characteristic tachyzoite-like features, including centrally located nuclei (N), fewer micronemes, and absence of amylopectin granules. Scale bars in A and C = 1 μ m; scale bars in B, D, E, and F = 500 nm.

ranged from 10 to >60 μ m in diameter for all strains, there were significant differences in the average sizes of the strains (Table 4). Notably, strains such as ME49 and COUG produced relatively large cysts, while MAS, GPHT, and CAST produced

much smaller cysts. When the different diameters were used to calculate the average volumes (assuming a spherical shape), the magnitudes of these differences in comparison to the average volume of ME49 were even more apparent (Table 4).

TABLE 4. Comparison of in vivo-derived cysts of clonal and exotic strains of *T. gondii*

Strain	Type	Rate of acute mortality of mice (%) ^a	Value for in vivo cysts		% of mice affected after oral cyst challenge ^d
			Diam ± SD (μm) ^b	Vol (%) ^c	
GT-1	I	100†	39 ± 18	38	50†
ME49	II	40	54 ± 18	100	100
CTG	III	40	41 ± 15	44	47
COUG	Exotic	73	59 ± 18	130	80
CAST	Exotic	100†	29 ± 11	16	10†
GPHT	Exotic	100†	23 ± 5.6	12	11†
MAS	Exotic	100†	17 ± 13	3	0
FOU	Exotic	100†	ND	ND	0

^a Cumulative rates of mortality of mice following i.p. challenge with tachyzoites. Shown are percentages of positive mice based on mortality (†) or seropositive survivors. The data in this column are summarized from reference 39, with the addition of new data for FOU and GPHT.

^b Measured from 10 to 15 separate cysts. ND, not done.

^c Determined by the standard geometric formula for a sphere based on the average diameter and normalized to the average volume of ME49. ND, not done.

^d CD-1 outbred mice were challenged orally with 5 to 10 cysts isolated from chronically infected mouse or rat brain. Shown are percentages of positive mice that died (†) or percentages of seropositive survivors. The data in this column are summarized from reference 39, with the addition of new data for GPHT.

Collectively, these results indicate that reduced oral transmission may be due to failure to induce proper cyst formation (e.g., FOU) or the production of small cysts containing fewer bradyzoites (e.g., MAS, CAST, and GPHT).

Oral transmission of strains of *T. gondii*. There was a strong correlation between the size of cysts produced in vivo and oral transmission (Table 4). As reported previously, oral challenge of mice with 5 to 10 cysts of the clonal lineages (ME49, GT-1, and CTG) led to ≥40% of animals becoming infected (Table 4). We have previously reported that oral challenge of mice with tissue cysts of the exotic strain MAS fails to result in infection (39). Consistent with that report, we observed here that this strain produced cysts in rats, yet these stages failed to cause oral infection when administered to mice (Table 4 and

data not shown). CAST is also impaired in oral transmission, although this defect can be overcome by greatly increasing the challenge dose (39). The exotic strains FOU and GPHT are highly virulent in mice; hence, rats, which are naturally resistant to *T. gondii*, were chronically infected (32). We were unable to produce cysts in vivo for strain FOU, as it appears to be defective in differentiation. Repeated subinoculation with brain homogenates from infected rats also failed to cause infection by either the oral or i.p. route in naïve mice, suggesting that the animals did not maintain a chronic infection despite being seropositive (Table 4). Mice challenged by the oral route with 5 to 10 cysts of GPHT became infected with very low efficiency, comparable to that of CAST (Table 4 and data not shown). These results indicate that GPHT is also deficient in direct oral transmission, likely due to the smaller sizes of cysts produced in chronically infected animals.

DISCUSSION

We compared the abilities of clonal versus exotic strains of *T. gondii* to differentiate in vitro into bradyzoites and to cause oral infection in the mouse, a model intermediate host. Members of all three clonal lineages demonstrated efficient induction of bradyzoite markers in vitro, including the expression of stage-specific antigens, ultrastructural changes, resistance to acid-pepsin, and oral transmission to mice. In contrast, a number of exotic lineages were partially defective in the formation of cysts, resulting in a reduced ability to transmit between intermediate hosts via the oral route. Defects in the exotic strains included the formation of fewer and smaller cysts and, in some cases, an inability to undergo efficient differentiation in vitro. Collectively, these studies reveal that efficient oral transmission of *T. gondii* may be due to rapid differentiation in response to stress leading to the production of larger and more numerous tissue cysts. While these features are shared by the clonal lineages and some exotic strains (i.e., COUG), they are lacking in several exotic strains studied here.

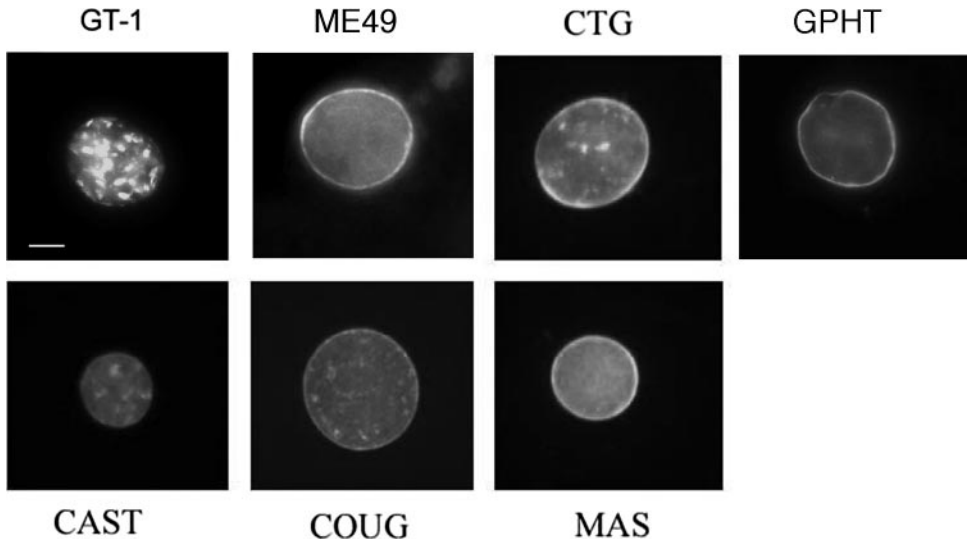


FIG. 5. In vivo-derived cysts of *T. gondii* stained with DBL. All strains of *T. gondii* produced cysts in vivo that stained strongly with lectin (white areas). Brain homogenates from chronically infected mice or rats were fixed and stained with fluoresceinated lectin. Scale bars = 10 μm.

Representative strains of all three clonal lineages demonstrated efficient expression of bradyzoite markers following in vitro induction with alkaline pH-induced stress. Stage conversion was evident from increased expression of the bradyzoite antigen BAG1 (42), with a corresponding decrease in the tachyzoite surface antigen SAG1 (8). This evidence was paralleled by the qPCR data showing the coordinate expression of tachyzoite genes (i.e., *SAG1* and *SAG2A*) in tachyzoites and the induction of bradyzoite genes (i.e., *BAG1* and *LDH2*) following the induction of cyst differentiation. The cysts stained positively with DBL, expressed the cyst wall antigen CST1 (44), and had ultrastructural features consistent with bradyzoites (14). These features are similar to previous reports of in vitro development of bradyzoites by the type II ME49 strain (5, 6, 37, 38). Our findings are notable, since they demonstrate a similar capacity for stage conversion among all three clonal lineages. We also demonstrate that in vitro-differentiated bradyzoites acquire pepsin resistance and are orally infectious when used to challenge mice. All three clonal strains tested here were comparable in their ability to form fully mature bradyzoites and induce oral infection in the mouse. These results may be dependent in part on the use of low-passage isolates, as we have observed that long-term maintenance of strains in in vitro culture results in the down-regulation of this differentiation pathway, especially in the type I lineage (unpublished data).

The relative infectivity of in vitro-derived bradyzoites compares favorably with bradyzoites found within tissue cysts that develop in the brains of chronically infected mice. Following in vitro culture for 9 days in alkaline pH medium, $\sim 1,000$ DBL⁺ cysts were required to reliably infect $\geq 50\%$ of mice by the oral route. Only a portion of the cysts formed in vitro contained pepsin-resistant parasites ($\leq 10\%$), and these cysts generally contained 10 to 20 bradyzoites, as judged by DAPI staining (data not shown). Hence, the infectivity of in vitro-derived bradyzoites compares favorably with the infectivity of bradyzoites derived from mouse brain cysts (15). In contrast, tachyzoites are sensitive to pepsin, and hence it requires $>10^6$ of these parasites to cause oral infection in the mouse (17). In comparing our results to those of these previous studies, we conclude that (i) a portion (i.e., $\sim 10\%$) of the bradyzoites within cysts formed in vitro are fully mature as defined by oral infectivity, (ii) maturation increases with the length of development, and (iii) in vitro-derived bradyzoites have approximately the same infectivity via the oral route as tissue cyst-derived bradyzoites. In vitro-derived bradyzoites provide an attractive alternative for studying transmission dynamics and gene expression during development and for screening for new inhibitors that may disrupt parasite chronicity.

The exotic strain COUG shares the efficient induction of bradyzoites in vitro with the clonal lineages, and cysts produced by this strain were equally infectious by the oral route. However, the four other exotic strains studied here showed partial or complete defects in this process that may explain their underlying differences in oral transmissibility. These defects can be grouped into two processes, underdevelopment and ineffective differentiation, as summarized here and further defined below. The exotic strains CAST, GPHT, and MAS were less efficient at forming cysts in vivo and produced smaller cysts with fewer parasites. Additionally, the two exotic strains

MAS and FOU showed more-severe defects that were refractory to the induction of bradyzoite transformation in vitro in response to alkaline pH-induced stress. Collectively, these traits are likely to result in less-efficient oral transmission between intermediate hosts.

The previously characterized exotic strain CAST has decreased oral transmission in the mouse model compared with the clonal lineages (39). A similar phenotype was observed here for the unrelated exotic strain GPHT. These two exotic strains underwent normal differentiation in vitro following alkaline pH stress designed to induce bradyzoite development. They efficiently expressed bradyzoite antigens (i.e., BAG1 and DBL staining) and demonstrated ultrastructural features of cysts. However, these strains formed small cysts in vivo and failed to establish high tissue burdens, unlike the clonal lineages. Exotic strains MAS and FOU also produced small cysts that were low in number. Because the dose of bradyzoites necessary to cause oral infection in the mouse model is relatively high, i.e., $\sim 1,000$ bradyzoites (15), the inefficient oral transmission of these strains may result from a low tissue density of bradyzoites. Differentiation is also likely to be influenced by passage history, and it is possible that repeated passage of these strains in animals (as bradyzoites) might resuscitate their defects in transmission. However, this process is unlikely to rescue strains MAS and FOU, which are unable to cause oral infection in rodents, thus precluding repeated passage as tissue cysts in vivo.

The exotic strains MAS and FOU also had more-profound defects in differentiation. Following in vitro culture under alkaline pH-induced stress, MAS and FOU were severely impaired in bradyzoite development. Both strains stained weakly with DBL, indicating that they fail to develop a normal cyst wall. The antigen recognized by mAb 73.18 to CST1 has been described as the major glycoprotein in the cell wall, and the reactivity of this mAb correlates with the results of DBL staining (44). The very weak staining of MAS with both DBL and antibodies to CST1 suggests that it is defective in the expression of this antigen. On the other hand, FOU reacted abundantly to mAb against CST1 despite staining only weakly with DBL, suggesting that this cyst wall antigen lacks normal glycosylation. Previous reports have indicated that the cell wall stains with DBL (recognizing *N*-acetylgalactosamine) and with succinylated wheat germ agglutinin (recognizing *N*-acetylglucosamine) (7). We have not probed the cyst wall formed by FOU and MAS with succinylated wheat germ agglutinin; however, these two strains might be useful for further studies to define the assembly and composition of the cell wall. Despite these defects, the cyst wall in these two strains appeared normal with ultrastructural examination and consisted of a convoluted cell wall and internal matrix. However, parasites within the cyst-like vacuoles formed by these strains failed to demonstrate ultrastructural features consistent with bradyzoites, but instead resembled tachyzoites. Strains MAS and FOU also failed to develop acid-pepsin resistance, consistent with a failure to undergo differentiation. This was further evident from their failure to shut off tachyzoite gene expression, as shown by qPCR. Collectively, these features indicate that MAS and FOU fail to differentiate in vitro in response to alkaline pH-induced stress. This defect would appear to result from an inability to sense or respond to the stress response, which has

been linked to differentiation (21, 41). In this regard, strains MAS and FOU resemble knockout mutants lacking the P-type (H⁺) ATPase PMA1, which fail to respond to stress conditions in vitro (24). However, since $\Delta pma1$ parasites still form cysts in vivo that are orally infectious (24), the defect in MAS and FOU must be more complex than a simple failure to respond to stress-inducing signals for differentiation. Further analysis of these exotic strains will be useful for exploring the pathways of differentiation in response to environmental signals.

Our findings reveal that the defects in oral transmission of some exotic strains relate to defects in differentiation in response to stress and an inability to form large and abundant cysts in vivo, in the mouse model. Variation in cyst size and abundance may also occur in other intermediate hosts, which would be expected to influence transmission by the asexual route in the wild. Large cysts likely enhance the transmission of *T. gondii* between intermediate hosts, since bradyzoites are not highly infectious by this route. Notably, variations in size or numbers may be less likely to influence oral transmission to cats, since the threshold for such infections is much lower (14). Consistent with this, strain MAS can be successfully transmitted through a cat, with a short prepatent period characteristic of bradyzoite infection, despite not being efficient in transmission between intermediate hosts (unpublished result). Previous studies using chemically derived mutants that were selected for an inability to differentiate suggest a hierarchy of control of gene expression during differentiation (36). These mutants had global defects in the expression of bradyzoite-specific genes, although variations in specific genes were observed between separate mutants. Because so many genes were affected in these mutants, it is difficult to identify the precise role of any one gene in altering traits such as transmission. In contrast, the naturally occurring strains examined here appear to have more subtle defects in differentiation, as they could still express early bradyzoite markers (BAG1, LDH2, and cyst wall antigens). Further molecular comparison of the strains described here may reveal more specific defects that affect differentiation and oral transmission, which is likely to be informative about the evolutionary forces that have shaped transmission dynamics in *T. gondii*.

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