Bradyzoite Development in *Toxoplasma gondii* and the hsp70 Stress Response

LOUIS M. WEISS, 1,2* YAN FEN MA, 2 PETER M. TAKVORIAN, 3 HERBERT B. TANOWITZ, 1,2 AND MURRAY WITTNER 2

Department of Medicine, Division of Infectious Diseases, 1 and Department of Pathology, Division of Parasitology, 2 Albert Einstein College of Medicine, Bronx, New York, and Department of Biologic Sciences, Rutgers University, New Brunswick, New Jersey 3

Received 19 September 1997/Returned for modification 3 December 1997/Accepted 15 April 1998

*Corresponding author. Mailing address: Albert Einstein College of Medicine, 1300 Morris Park Ave., Room 504 Forchheimer, Bronx, NY 10461. Phone: (718) 430-2142. Fax: (718) 430-8543. E-mail: lweiss@acomm.yu.edu.*

*Toxoplasma gondii* is a well-described ubiquitous Apicomplexan protozoan parasite of mammals and birds. It has long been recognized as an important opportunistic pathogen of immunocompromised hosts and is a major opportunistic pathogen of the AIDS epidemic (23, 43). Although overwhelming disseminated toxoplasmosis has been reported, the predilection of this parasite for the central nervous system, causing necrotizing encephalitis, constitutes its major threat to patients with human immunodeficiency virus infection (AIDS).

The development of *Toxoplasma* encephalitis is believed to be due to the transition of the resting, or bradyzoite, stage to the active and rapidly replicating tachyzoite form (11, 17). Although these stages are well defined morphologically, little is known about how interconversion from one to the other stage occurs or what signal(s) mediates this transformation. Several studies have demonstrated that bradyzoites can develop in vitro and that the development of cyst-like structures can be demonstrated by transmission electron microscopy (TEM) (16, 21, 22, 26, 35) and more recently by bradyzoite-specific monoclonal antibodies (MAbs) (4, 37, 40). Feeding experiments with cats have demonstrated that tissue culture-derived cysts are biologically identical to cysts obtained from animal tissues (15, 21). In addition, both animal- and tissue culture-derived bradyzoites are pepsin resistant.

The factors affecting the transition of bradyzoites to tachyzoites remain to be defined. In tissue culture studies, it is evident that bradyzoites spontaneously convert to tachyzoites and that tachyzoites spontaneously convert to bradyzoites. The rate of conversion appears to be strain dependent. Thus, low-virulence strains, i.e., strains that form high numbers of cysts in mice, such as ME49, have a higher spontaneous rate of cyst formation in culture than do virulent strains such as RH (36). The rate of replication of tachyzoites, which is greater than that of bradyzoites, enables tachyzoites to destroy the cell monolayer, thereby obscuring bradyzoite formation. Inhibiting the rapid growth of tachyzoites, either by drugs (pyrimethamine [5], cytokines (gamma interferon [5, 36, 40]), or frequent removal (26), gradually increases the percentage of bradyzoites in culture, consistent with their lower replication rate. However, these conditions do not induce an increase in the rate of switching of tachyzoites to bradyzoites but rather prevent destruction of the monolayer by tachyzoites and thereby permit normal bradyzoite development.

We and others have previously observed that stress conditions were associated with the induction of bradyzoite development; i.e., there were more bradyzoites under these conditions than would be expected from simple inhibition of tachyzoite replication. It was found that temperature (43°C [36]), pH (pH 6.8 or 8.2 [36, 40]), or chemical (sodium arsenite [36]) stress resulted in an increase in bradyzoite antigen expression by *T. gondii* in culture and an increase in the observed number of cyst-like structures. In murine macrophage lines derived from bone marrow, gamma interferon increased bradyzoite antigen expression, which appeared to be related to nitric oxide (NO) induction (5). Similarly, *Toxoplasma* was grown in host cells with a nonfunctional mitochondrial respiratory chain, both oligomycin (an inhibitor of mitochondrial ATP synthetase function) and antimycin A (an inhibitor of the electron transport of the respiratory chain) (5, 38) increased bradyzoite antigen expression, although not to the same extent as NO (5).
Heat shock- or stress-induced activation of a set of heat shock protein (hsp) genes, is characteristic of almost all eukaryotic and prokaryotic cells. The hssps fall into several subfamilies, namely, the low-molecular-mass hssps (16 to 35 kDa), the hsp60 family, the hsp70 family (68 to 78 kDa), and the high-molecular-mass hssps (89 to 110 kDa) (27). Heat exposure, chemical agents (sodium arsenite), mitochondrial inhibition (2,4-dinitrophenol, sodium azide, and other uncouplers of oxidative phosphorylation), transition series metals, hydrogen peroxide, and anaerobic conditions are all associated with the induction of hsps (27). Many of these agents are associated with Bradyzaote induction in vitro (5, 36, 39). In many other organisms, small hsps are developmentally regulated (13, 14).

We have recently identified and cloned the Bradyzaote gene BAG5, identified by Bradyzaote-specific monoclonal antibody 74.1.8 (29), and determined that the product is related to the small hsps (29). This antigen was also identified by another group and designated BAG1/hs30 (6). It is highly represented in the Bradyzaote-specific dBEST database (http://daphne.humgen.upenn.edu:1024/toxodb/ver_2/toxodb.html), representing at least 3% of all clones identified. In addition to the small hsps, members of the hsp70 family have been associated with differentiation in several organisms, and we therefore examined whether hsp70 induction was associated with Bradyzaote forma- tion in T. gondii. We found that hsp70 expression was asso- ciated with Bradyzaote differentiation and subsequently cloned a T. gondii hsp70 homolog.

MATERIALS AND METHODS

T. gondii isolation and culture. Cysts were obtained from BALB/c mice at 5 days postinfection with T. gondii ME49 and purified by isopycnic centrifugation (40). After rupture of the cysts with trypsin, 1,000 to 2,000 Bradyzaotes were added to a flask of 50% confluent human fibroblast cultures (ATCC CRL 1475 [CCD-25SK]), and subsequently 1,000 to 5,000 tissue culture-derived T. gondii organisms, up to passage 20, were used to inoculate a new fibroblast monolayer. Dubbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco-BRL, Gaithersburg, Md.), 10 mM HEPES (pH 6.8, 7.1, or 8.1) and 1% penicillin-streptomycin complex were replaced weekly. Bradyzaote monolayers were subcultured weekly with 0.25% trypsin-0.03% EDTA at a subcultivation ratio of 1:4 and used between passages 6 and 30. T. gondii RH was maintained by twice-weekly passage in human fibroblasts

Bradyzaote in vitro assay. We previously demonstrated by immunofluorescence, TEM, and Western blotting the in vitro development of Bradyzaotes and development of cysts together with tachyzoites in continuous cell cultures of human fibroblasts (40). Four thousand parasites were used to inoculate a new fibroblast monolayer in a two-chamber culture slide (Permanox; Nalge-Nunc, Napersville, III.), or 10,000 parasites were used in a T25 flask. At the time of infection, pH-adjusted medium containing the drugs of interest was added. At 3 days postinfection, the slides were washed in phosphate-buffered saline, fixed for 30 min with 2% buffered formalin, permeabilized with 0.2% Triton X-100 for 20 min, and blocked with 1% bovine serum albumin overnight. They were then incubated with the primary antibody(ies) at the appropriate dilution for 90 min at 37°C, washed, incubated with the secondary antibody (rhodamine [RHOD]- conjugated anti-mouse immunoglobulin G [IgG] at 1:75 or RHOD-conjugated anti-rabbit IgG or fluorescein isothiocyanate-conjugated anti-rabbit IgG at 1:50) or fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G [IgG] at 1:75 or RHOD-conjugated anti-rabbit IgG or fluorescein isothiocyanate-conjugated anti-rabbit IgG at 1:50) and subsequently incubated with nitroblue tetrazolium-5-bromo-4-chloro-3-(3-dimethylaminophenyl)-2(5H)-tetrazolium chloride (NBT/BCIP; Promega) at 1:250 and visualized with DABCO (1,4-diazobicyclo[2,2,2]octane)-phosphate-buffered saline, and examined with a Nikon Diaphot inverted fluorescence microscope. For dual-antibody staining on the same slide, RHOD-conjugated anti-mouse IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG were utilized together. The total number of Bradyzaote-bearing tachyzoites and the total number of tachyzoite-bearing Bradyzaotes were determined, as was the total number of Toxoplasma vacuoles for each experiment.

To examine the effects of various modulators on extracellular parasites, T. gondii was isolated from human fibroblasts by rupture with a 27-gauge needle followed by filtration through a 3.0-μm-pore-size Nuclepore filter. These purified organisms were then incubated for 3 h in pH 8.1 or 7.1 medium prior to their being used to infect slides. After treatment, slides were maintained in pH 7.1 medium.

hsp antibody. C92F3A-5, a MAb specific to the inducible form of hsp70 (SPA810; StressGen, Victoria, British Columbia, Canada), was used at 1:100 to 1:200 for immunofluorescence and at 1:1,000 for Western blotting. C92F3A-5 does not react with cognate hsp70 (hsc70).

28-kDa antigen BAG1/hsp30 [BAG5] (41) was used at a 1:50 dilution, poly-
been submitted to GenBank (accession no. AF045559).

To Northern analysis, total RNA was prepared from T. gondii ME49 by using Triazol reagent (Gibco-BRL), electrophoresed on a 1.2% formaldehyde–morpholine propane sulfonic acid (MOPS)–agarose gel, transferred to a nitrocellulose membrane, UV cross-linked by using 0.300 J/cm² in a UV Translink (ISS) (3). The membrane was then washed twice in 2× SSC–0.1% SDS at 25°C and twice for 15 min each in 2× SSC–0.1% SDS at 60°C, followed by autoradiography (3).

To determine the hsp70 copy number, genomic DNA (2 μg) from T. gondii ME49 was digested with 20 U each of EcoRI, PstI, BamHI, EcoRV, HindIII, SacI, and XbaI, separated by electrophoresis on a 1% agarose gel, and transferred to a nylon membrane (Boehringer Mannheim), and probed with random-primed (Boehringer Mannheim) 32P-labeled C1-1 insert (3). The membrane was then washed twice in 2× SSC–0.1% SDS at 37°C, separated by electrophoresis on a 1% agarose gel, and transferred to a nylon membrane (Boehringer Mannheim), and the membrane was UV cross-linked by using 0.300 J/cm² in a UV Translink (ISS) (3). The membrane was then prehybridized in DIG Easy Hyb (Boehringer Mannheim) at 42°C for 2 h, hybridized with the digoxigenin random primer-labeled (Boehringer Mannheim) TgHSPB3 insert (680 bp) in DIG Easy Hyb (Boehringer Mannheim) at 42°C for 2 h, washed, and then developed with NBT–BCIP as described in “Cloning of the hsp70 gene of T. gondii” above.

**RESULTS**

In order to separate the effects of stress conditions known to induce bradyzoite formation directly on the parasite from the effects of these conditions on both host cells and parasites, we evaluated whether a relatively brief exposure (i.e., 1 h) of extracellular T. gondii tachyzoites to various conditions would induce bradyzoite formation. When extracellular T. gondii organisms were incubated in pH 8.1 medium for 1 h before they were used to infect a fibroblast monolayer at pH 7.1, there was a significant increase in bradyzoite antigen expression at 48 h compared to when extracellular T. gondii organisms were incubated at pH 7.1 prior to infection. After pH 7.1 exposure, there were 297 ± 16 cysts/flask, which was a 2-fold increase in the percentage of vacuoles expressing bradyzoite antigens. SNP, an NO donor, is a strong inducer of bradyzoite development (5). After 3 days in culture with 100 μM SNP, 80% of all vacuoles expressed bradyzoite antigens, and there was a threefold increase in the total number as well as the percentage of organisms expressing bradyzoite antigens. Pretreatment of human fibroblasts with pH 8.1 medium or SNP followed by removal of these agents prior to infection with T. gondii at pH 7.1 did not affect bradyzoite formation. After a 1-h exposure of extracellular organisms to 100 μM SNP followed by culture at pH 7.1 without SNP, we observed a 1.5- to 2-fold increase in the total number as well as percentage of organisms expressing bradyzoite antigens (40% without SNP exposure and 80% after SNP exposure). Thus, short-term exposure of extracellular tachyzoites to inducing conditions should facilitate the study of early genes in bradyzoite development which may act as triggers for stage conversion.

Given the association of the induction of bradyzoite development in vitro with temperature, pH, mitochondrial inhibitors, sodium arsenite, and many of the other stressors associated with hsp induction, we sought evidence that such induction is an early event in bradyzoite development. Therefore, we examined extracellular T. gondii after a 1-h exposure to pH 8.1 versus pH 7.1 for the expression of inducible hsp70. This protocol allowed examination of the parasitic hsp60 without significant contamination by host cell proteins. For detection by Western blotting, we utilized MAB C92F3A-5 (StressGen), which is specific to the inducible form of hsp70. As can be seen from Fig. 1, an hsp70 is induced at 1 h in ME49 exposed to pH 8.1 medium as compared to that incubated with pH 7.1 medium, demonstrating that a preexisting hsp is induced by the pretreatment protocol that also induced bradyzoite formation (equal numbers of organisms were loaded in each lane).

In order to demonstrate that the T. gondii hsp was also induced in intracellular parasites, we took advantage of the observation that the antigen recognized by C92F3A-5 in T. gondii ME49 was 72 kDa in size and the fact that in human fibroblasts this antibody recognizes a 72-kDa protein. We therefore examined by Western blotting intracellular T. gondii ME49 that had been subjected to pH 8.1 or 7.1 treatment prior to infection. At day 3 postinfection the infected fibroblast monolayer was harvested, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis followed by Western blotting with MAb C92F3A-5. As can be seen in Fig. 1, there is a 72-kDa band specific to T. gondii above the human 70-kDa hsp band in T. gondii-infected cultures at pH 7.1 and 8.1, and this band is not seen in human fibroblasts without infection. By densitometry this antigen was found to be increased fourfold in pH 8.1-treated T. gondii compared to the amount seen in pH 7.1-treated cells. A recombinant human hsp70 was included as

![Figure 1](http://iai.asm.org/DownloadedFrom)
a positive control. A similar increase in hsp70 was demonstrated when T. gondii was purified from the human fibroblast monolayer prior to electrophoresis (data not shown).

By using degenerate primers to conserved regions of hsp70, an amplicon of ≈680 bp (Fig. 2) corresponding to the predicted size based on homology with other hsp70 genes was demonstrated in T. gondii. Sequencing of this amplicon (Fig. 3, positions 582 to 1257) and analysis by using the National Center for Biotechnology Information Blastx program (http://www.ncbi.nlm.nih.gov/BLAST/) (12) was performed. This gene fragment had high-level homology (Blast score of >500) to the following genes, all of which were from Apicomplexan parasites: Eimeria acervulina hsp (accession no. Z26134), Eimeria maxima hsp70 (cytosolic) (Z40684), Plasmodium cynomolgi hsp70 (cytoplasmic) (M90978), Plasmodium falciparum hsp70 (cytoplasmic) (M19753), and Cryptosporidium parvum hsp70 (U71161 and U11781). This suggests that the amplicon is an inducible hsp70 from T. gondii. By RT-PCR this presumptive hsp70 appeared to be increased by exposure of T. gondii to pH 8.1 (Fig. 2). Standardizing the amount of hsp70 amplicon on the gel to the amount of β-tubulin mRNA amplicon detected by RT-PCR gives a three- to fourfold induction of hsp70 in extracellular T. gondii tachyzoites exposed to pH 8.1 medium for 1 h. When strain RH tachyzoites were used, no increase in hsp70 was seen by RT-PCR, and no increase in the bradyzoite antigen BAG1/hsp30 (BAG5), which is reactive with MAb 74.1.8, could be demonstrated by immunofluorescence or Western blotting following pH 8.1 or SNP treatment (data not shown).

The hsp70 amplicon was used to identify corresponding cDNA clones from a λZAP library. Two of the clones, C1-1 and C3, containing inserts of about 2,500 bp were sequenced in both directions. The resultant 2,382-bp sequence (Fig. 3) had a repetitive GGMP motif toward the carboxy-terminal portion of the protein, which is seen in other Apicomplexan hsp70 genes (10). Analysis of this sequence with the Blastx program (http://www.ncbi.nlm.nih.gov/BLAST/) (12) was performed and identified this protein as belonging to the inducible hsp70 group. It had the highest homology (Blast scores of over 500) to other Apicomplexan hsp70 genes as described above for the hsp70 gene fragment. This T. gondii hsp70 gene had 89.2% similarity to the E. acervulina hsp70 gene (accession no. Z26134) and only 73.2% similarity to the human hsp70 gene. Northern analysis of T. gondii mRNA demonstrated a single band of about 2.3 kb (data not shown). Southern blotting of T. gondii genomic DNA cut with EcoRI, PsiI, BamHI, EcoRV, HindIII, SacI, or XhoI and probed with clone C1-1 (T. gondii hsp70) demonstrated a pattern consistent with this T. gondii hsp70 gene being a single-copy gene (data not shown).

Utilizing immunofluorescence with MAb C92F3A-5 on human fibroblasts infected with T. gondii ME49 that had been treated at pH 8.1, we were able to demonstrate that hsp70 staining was localized most strongly to T. gondii expressing bradyzoite-specific antigens (Fig. 4A and B). Little to no staining was observed in tachyzoites (Fig. 4A and D). Similarly, by immunoelectron microscopy, we were able to demonstrate reactivity of MAb C92F3A-5 with bradyzoites isolated from mouse brain and from tissue culture (Fig. 5A and B). Thus, we believe that hsp70 expression is associated with the development of bradyzoites.

Quercetin, a bioflavonoid, has been reported to inhibit the synthesis of many hsps, including hsp90, hsp70, and hsp27, while having no effect on the synthesis of other cellular proteins (9). Quercetin at 100 µM inhibited the pH 8.1-associated induction of bradyzoite antigens (Table 1) (1.8- and 1.1-fold increases in the percentages of bradyzoite antigen-positive vacuoles for control and quercetin treatments, respectively). In addition, the total number of bradyzoite antigen-positive vacuoles was lower in the presence of quercetin at either pH 7.1 or 8.1 (control, 86 ± 9 cysts/slide at pH 7.1 and 142 ± 9 cysts/slide at pH 8.1; quercetin [100 µM], 56 ± 2 cysts/slide at pH 7.1 and 59 ± 4 cysts/slide at pH 8.1). Quercetin was also found to decrease the growth of T. gondii (at 100 µM there was a 30 to 40% decrease in the total number of vacuoles compared to that with the control (214 ± 10 versus 147 ± 5). Thus, at pH 8.1, in the absence of quercetin 60% of organisms expressed bradyzoite antigens and in the presence of quercetin only 39% expressed bradyzoite antigens. Quercetin also decreased the expression of hsp70 in pH 8.1-treated T. gondii ME49 as ascertained by immunoblotting with MAb C92F3A-5 (Fig. 6). The induction of bradyzoites due to SNP was also inhibited by quercetin (Table 1). To our knowledge, quercetin is the first compound that has been reported to decrease bradyzoite formation.

Nonsteroidal antiinflammatory drugs have been reported to induce hsps through effects on heat shock transcription factors (20). Treatment of cells with indomethacin has been associated with a decrease in the temperature required to obtain a heat shock response, i.e., a synergistic interaction resulting in a decrease in the heat shock threshold and protection of cells against cytotoxic conditions (20). Thus, we examined the effects of indomethacin on bradyzoite antigen expression (Table 1). In the presence of 100 µg of indomethacin per ml, a 1.6-fold increase in bradyzoite antigen-positive organisms was seen at pH 7.1 and a 3.0-fold increase was seen at pH 8.1, compared to values for controls (no indomethacin) at pH 7.1. At pH 8.1 without indomethacin, there was a 1.6-fold increase in bradyzoite antigen-positive organisms compared to that at pH 7.1. Immunoblotting with MAb 92F3A-5 demonstrated an increase in hsp70 in indomethacin-treated T. gondii (Fig. 6). Indomethacin thus appeared to facilitate the development of bradyzoites and may have been synergistic with pH shock.

**DISCUSSION**

The data presented indicate that hsps play a role in bradyzoite differentiation. For example, quercetin, a bioflavonoid which inhibits the synthesis of hsp90, hsp70, and hsp27, suppressed the induction of bradyzoite development in vitro. Indomethacin, which increases hsp expression, was associated with an increase in bradyzoite formation. An inducible hsp70 homolog was demonstrated in bradyzoites by Western blotting, immunoelectron microscopy, and immunofluorescence with...
Mab C92F3A-5. This MAb was reported to react with inducible hsp70s.

Finally, RT-PCR with degenerate primers to conserved regions of hsp70 demonstrated the presence of an hsp70 homolog in *T. gondii* on exposure to conditions which induce bradyzoite formation. This hsp70 amplicon was subsequently utilized to clone a *T. gondii* hsp70 gene. The identified *T. gondii* hsp70 gene is a single copy gene. In other Apicomplexa, such...
as *P. cynomolgi*, the hsp70 gene is also present as a single copy in the genome. An hsp70 gene has been mapped to chromosome V of *T. gondii* by using an hsp70 gene of *Trypanosoma brucei* as a probe (34). Using a polyclonal antiserum to *P. falciparum* hsp70, Lyons and Johnson (24) were able to demonstrate an increase in hsp70 in *T. gondii* RH during in vivo infection. This protein was not observed in vitro by using standard culture techniques (pH 7.1). This suggests that the stress

FIG. 4. Immunofluorescence of human fibroblasts infected with *T. gondii* following a 1-h extracellular pH 8.1 exposure. (A and B) Immunofluorescence with MAb C92F3A-5, specific for inducible hsp70 (A), and rabbit polyclonal antibody to MAG1 (30). Colocalization of hsp70 and MAG1 in *T. gondii* ME49 is demonstrated. Both hsp70 and MAG1 also localized with BAG1/hsp30 (BAG5) (MAb 74.1.8 [21]) (data not shown). The arrows point to a tachyzoite (T) vacuole in which no staining is seen with C92F3A-5 or MAG1. (C) Photomicrograph (phase-contrast; magnification, ×60) of *T. gondii* RH in human fibroblasts. (D) Same field as panel C, with fluorescence filters demonstrating the absence of MAb C92F3A-5 staining of RH tachyzoites (a small amount of fluorescent particulate material is present, but no staining is present in tachyzoite vacuoles).

as *P. cynomolgi*, the hsp70 gene is also present as a single copy in the genome. An hsp70 gene has been mapped to chromosome V of *T. gondii* by using an hsp70 gene of *Trypanosoma brucei* as a probe (34). Using a polyclonal antiserum to *P. falciparum* hsp70, Lyons and Johnson (24) were able to demonstrate an increase in hsp70 in *T. gondii* RH during in vivo infection. This protein was not observed in vitro by using standard culture techniques (pH 7.1). This suggests that the stress

FIG. 5. Immunoelectron microscopy with C92F3A-5. (A) In vitro bradyzoites, demonstrating localization of C92F3A-5 to essentially the cytoplasm of the bradyzoites (arrows). Some staining between organisms in the vacuole is also demonstrated. By electron microscopy this vacuole demonstrated bradyzoite characteristics (posterior nucleus, amylopectin granules, and a thickened cyst wall). Bar, 1 μm. (B) In vivo bradyzoites in a cyst isolated from a murine brain, demonstrating localization of C92F3A-5 to the cytoplasm of the bradyzoites. Less staining is present in bradyzoites from these mature in vivo cysts than is seen in the developing day 3 bradyzoites in vitro. This is consistent with the expression of hsps in development, where expression occurs during the process of differentiation and declines after differentiation is complete. The magnification is the same as in panel A. (C) In vitro bradyzoites, demonstrating a lack of gold staining with negative control antiserum (normal mouse serum). Bar, 1 μm.
A duplicate SDS-polyacrylamide gel confirmed equal loading of each lane. Equal numbers of parasites were loaded in each lane. Coomassie blue staining of host hsp70, due to residual host cell debris, are present in the different lanes. lane 4 compared to lane 2) at day 3 of culture. Note that different amounts of (hsp70) in compared to lane 2) and a threefold increase in C92F3A-5 reactive protein expression in

By densitometry, there was shown to be a three- to fourfold decrease in antigen induction of tachyzoites to bradyzoites. We hypothesize that the yield information on the regulation of the process of transformation, study of the factors regulating this stress response may

experienced by the parasite during the infection (i.e., the immune response of the host) induced hsp70 gene expression in the parasite (24). The three- to fourfold change in hsp70 levels in T. gondii seen with stress as well as differentiation in our current study are consistent with the magnitude of the hsp70 response demonstrated for other Apicomplexa, such as Theileria annulata (33).

Both SNP and pH 8.1 treatment have been previously reported to induce bradyzoite differentiation (5, 36, 40) in vitro. We have demonstrated that differentiation in this parasite can be triggered by a brief (1-h) exposure of extracellular T. gondii to these agents. This suggested that much of the effect of these inducing agents is directly on the parasite rather than due to effects of these agents on the host cells. In our experience this effect is most easily demonstrated with T. gondii strains such as ME49 or PLK (a clonal derivative of ME49).

It has become clear that hsps are not limited to stress responses but are developmentally regulated as well. The heat shock response of fungi such as Saccharomyces cerevisiae and Neurospora crassa has been extensively studied, and the pattern of hsps induced varies with fungal development (2, 13, 19). For example, expression of hsp34 and hsp38 is induced during fungal development (19). The transition from vegetative growth to differentiation resulted in production of mRNAs encoding hsps (2). Our group (29) as well as Bohne et al. (6) have identified a bradyzoite-specific antigen in T. gondii, BAG1/hsp30 (BAG5), that has homology to small hsps. Given recent data on the presence of plant-like structures in T. gondii (18, 38), it is interesting that BAG1/hsp30 (BAG5) displays its highest homology at the carboxy terminus to a number of small hsps of plants with molecular sizes of 17 to 22 kDa. In many plants, differentiation events, such as seed formation, are associated with the induction of small hsps (13, 27). In invertebrates, mammals, and birds the small hsps have been associated with differentiation. In Drosophila, for example, hsp27, hsp26, hsp23, and hsp22 are expressed in a tissue-specific manner during development and appear to have key functions in development (1).

Members of the hsp70 family have also been associated with differentiation in many different organisms. In Blastoectidilla emersonii, hsp70 expression is induced during sporulation, and hsp70 has been associated with hyphal branching and secretion in response to steroids in Achlya ambisexualis (13). For Histoplasma capsulatum, mitochondrial ATPase activity and hsp70 induction have been correlated with the transition from mycelium to yeast phase (31). For protozoa such as Leishmania chagasi (42) and Trypanosoma cruzi (32), hsp70 has been associated with the capacity to survive oxidant stress and may also play a role in the differentiation of promastigotes to amastigotes. For P. cynomolgi, hsp70 expression has been associated with the sexual stages (10). In addition to affecting gene expression, the heat shock responses have been associated with changes in cellular metabolism in Xenopus, including interruption of oxidative phosphorylation leading to anaerobic glycolysis (13, 28). In this regard, it is interesting that bradyzoites may depend on such glycolytic pathways more than the tachyzoite stage for energy metabolism, due to the absence of a functional tricarboxylic acid cycle (8) as well as the presence of developmentally regulated lactate dehydrogenase genes (44).

During the process of differentiation, multiple genes are expressed and structural remodeling of T. gondii occurs. The process of differentiation probably depends on the ratio of regulatory factors (growth) to DNA templates (division) over time (33). Heat shock proteins are clearly induced during these events. They may function as chaperones, like hsp70, or have as-yet-unknown functions, like the small hsps. Knockout mutations of hsps in T. gondii may shed light on the interactions of hsps and their involvement in the process of stage transitions (tachyzoite to bradyzoite and bradyzoite to tachyzoite). In addition, study of the factors regulating this stress response may yield information on the regulation of the process of transformation of tachyzoites to bradyzoites. We hypothesize that the process of bradyzoite differentiation in T. gondii is a stress response triggered by environmental conditions related to the inflammatory process in the host and that the heat shock response is related to the metabolic adaptations in this parasite during differentiation.

ACKNOWLEDGMENTS

We acknowledge Denise LaPlace for her technical assistance. This work was supported by Public Health Service grant AI39454 from the National Institutes of Health.

REFERENCES


---

**TABLE 1. Bradyzoite antigen expression in vitro**

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>BAG1/hsp30 (BAG5) immunostaining* of day 3 cultures at pH 7.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1.0</td>
</tr>
<tr>
<td>Indomethacin**</td>
<td>1.6</td>
</tr>
<tr>
<td>Quercetin***</td>
<td>0.9</td>
</tr>
<tr>
<td>SNP</td>
<td>3.0</td>
</tr>
<tr>
<td>Quercetin and SNP</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Data are expressed as fold stimulation with respect to the control (no drug, pH 7.1) percentage of bradyzoite-positive vacuoles. The standard errors of the means for calculated fold stimulations were less than 7%. A fold stimulation of 1.5 is significant (P < 0.05).

** pH of medium containing HEPES buffer.

---

**FIG. 6. Effects of quercetin and indomethacin on hsp70 expression in T. gondii.** Lane 1, recombinant human inducible hsp70 (StressGen); lane 2, T. gondii ME49, pH 8.1, day 3 of culture; lane 3, T. gondii ME49, pH 8.1 and 100 μM quercetin, day 3 of culture; lane 4, T. gondii ME49, pH 8.1 and 100 μM of indomethacin per ml, day 3 of culture. T. gondii ME49 was purified from day 3 cultures as described in Materials and Methods. The recombinant human hsp70 reactivity with MAb C29F3A-5 is located at 70 kDa (arrow), as demonstrated in lane 1. A 72-kDa reactive band (T. gondii hsp70) is evident in lanes 2, 3, and 4. By densitometry, there was shown to be a three- to fourfold decrease in antigen expression in T. gondii exposed to 100 μM quercetin at pH 8.1 (i.e., lane 3 compared to lane 2) and a threefold increase in C29F3A-5 reactive protein (hsp70) in T. gondii exposed to 100 μg of indomethacin per ml at pH 8.1 (i.e., lane 4 compared to lane 2) at day 3 of culture. Note that different amounts of host hsp70, due to residual host cell debris, are present in the different lanes. Equal numbers of parasites were loaded in each lane. Coomassie blue staining of a duplicate SDS-polyacrylamide gel confirmed equal loading of each lane.

---

**TABLE 1. Bradyzoite antigen expression in vitro**

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>BAG1/hsp30 (BAG5) immunostaining* of day 3 cultures at pH 7.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1.0</td>
</tr>
<tr>
<td>Indomethacin**</td>
<td>1.6</td>
</tr>
<tr>
<td>Quercetin***</td>
<td>0.9</td>
</tr>
<tr>
<td>SNP</td>
<td>3.0</td>
</tr>
<tr>
<td>Quercetin and SNP</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Data are expressed as fold stimulation with respect to the control (no drug, pH 7.1) percentage of bradyzoite-positive vacuoles. The standard errors of the means for calculated fold stimulations were less than 7%. A fold stimulation of 1.5 is significant (P < 0.05).

** pH of medium containing HEPES buffer.

---

**FIG. 6. Effects of quercetin and indomethacin on hsp70 expression in T. gondii.** Lane 1, recombinant human inducible hsp70 (StressGen); lane 2, T. gondii ME49, pH 8.1, day 3 of culture; lane 3, T. gondii ME49, pH 8.1 and 100 μM quercetin, day 3 of culture; lane 4, T. gondii ME49, pH 8.1 and 100 μg of indomethacin per ml, day 3 of culture. T. gondii ME49 was purified from day 3 cultures as described in Materials and Methods. The recombinant human hsp70 reactivity with MAb C29F3A-5 is located at 70 kDa (arrow), as demonstrated in lane 1. A 72-kDa reactive band (T. gondii hsp70) is evident in lanes 2, 3, and 4. By densitometry, there was shown to be a three- to fourfold decrease in antigen expression in T. gondii exposed to 100 μM quercetin at pH 8.1 (i.e., lane 3 compared to lane 2) and a threefold increase in C29F3A-5 reactive protein (hsp70) in T. gondii exposed to 100 μg of indomethacin per ml at pH 8.1 (i.e., lane 4 compared to lane 2) at day 3 of culture. Note that different amounts of host hsp70, due to residual host cell debris, are present in the different lanes. Equal numbers of parasites were loaded in each lane. Coomassie blue staining of a duplicate SDS-polyacrylamide gel confirmed equal loading of each lane.
(ed.), Heat shock and development. Springer-Verlag, Berlin, Germany.