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NOTES

Expression of *Toxoplasma gondii*-Specific Heat Shock Protein 70 during In Vivo Conversion of Bradyzoites to Tachyzoites

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Stage conversion between bradyzoites and tachyzoites was investigated in C57BL/6 mice chronically infected with the ME-49 strain of *Toxoplasma gondii*. In order to promote bradyzoite-tachyzoite conversion, mice were treated in vivo with neutralizing doses of anti-gamma interferon (IFN- γ) or anti-tumor necrosis factor alpha (TNF- α) antibodies. Expression of parasite-specific antigens SAG-1, SAG-2, and heat shock protein 70 (Hsp-70) was visualized in the central nervous system by immunocytochemistry and measured by photometric assay. The immunosuppressive effect of anti-IFN- γ or anti-TNF- α treatment was immediate, leading to parasite stage conversion as indicated by the increased expression of tachyzoite-specific antigens (SAG-1 and SAG-2) and by rapid parasite replication. We also observed expression of high levels of Hsp-70 during a short period of conversion of bradyzoites to tachyzoites. Our data suggest that Hsp-70 may have an important role in the process of bradyzoite-tachyzoite conversion during the reactivation of chronic toxoplasmosis.

Toxoplasma gondii is an infectious pathogen that causes toxoplasmosis. During the acute phase of infection, the tachyzoite stage of the parasite undergoes an initial period of rapid multiplication. In immunocompetent individuals, tachyzoite multiplication is inhibited by the immune response. The outcome of this immunologic response to the tachyzoite results in the development of bradyzoites, the hallmark of chronic infection. In selected immunodeficiencies, and in particular AIDS, bradyzoites may escape from the cyst and revert to tachyzoites that multiply unhampered, resulting in the extensive and often fatal tissue damage associated with toxoplasmic encephalitis (23). In vivo studies in experimental models indicate that gamma interferon (IFN- γ) is a major cytokine that mediates resistance against *T. gondii* infection (35). CD4⁺ and CD8⁺ lymphocytes are involved in the prevention of disease reactivation, probably through the production of IFN- γ (10, 11, 19, 20). In vivo and in vitro experiments also suggest a crucial role for both IFN- γ and tumor necrosis factor alpha (TNF- α) in the induction of nitric oxide-mediated microbicidal activity (1, 8, 15, 22, 29, 32).

Reactivation of a latent infection culminates in the conversion of bradyzoites to tachyzoites, an event that has been investigated in vitro (4, 5, 33). In vitro studies demonstrated that differentiation from the tachyzoite to the bradyzoite stage can be induced by external stress factors, such as increased pH of the cell culture medium, a shift of the temperature from 37 to 43°C, or treatment with sodium arsenite (34). During stage differentiation from tachyzoite to bradyzoite, a stage-specific

heat shock protein (Hsp)/BAG-1 antigen is expressed. This bradyzoite-specific protein showed similarity to the small Hsp from plants (6). In vitro exposure of tachyzoites of *T. gondii* ME-49 to pH 8.1 facilitates their conversion to bradyzoites, during which time the parasites may express a 72-kDa protein that is believed to be part of the Hsp-70 family (38).

The molecular events surrounding the conversion of the bradyzoite to the tachyzoite during reactivation of chronic infection with *T. gondii* have not been explored. In mice, relapsing toxoplasmic encephalitis is associated with an increased expression of SAG-1 and SAG-2 mRNAs in the central nervous system (CNS) (12, 13). In this study, C57BL/6 mice infected with the ME-49 strain of *T. gondii* were immunosuppressed by treatment with anti-IFN- γ or anti-TNF- α monoclonal antibody (MAb), and the effect on expression of SAG-1 and SAG-2 as well as Hsp was examined.

Female C57BL/6 mice, 4 to 5 weeks old, were infected with 10 to 20 cysts of the ME-49 strain of *T. gondii* and received weekly treatment with 3 mg of rat immunoglobulin G1 MAb specific for either IFN- γ (XMG-6), TNF- α (HT-11-22), or control β -galactosidase (GL-113), beginning at 4 weeks postinfection (11, 12). The animals treated with anti-IFN- γ antibody were killed in a CO₂ chamber and decapitated at 0, 1, 3, 5, 7, 9, 10, and 12 days after the initiation of the immunosuppressive treatment, and those treated with anti-TNF- α were killed at 12 days. The brains were removed and fixed in Bouin-Hollande fixative for 24 h and transferred to 70% ethanol before processing for paraffin sectioning (12). For immunocytochemistry (14), mouse brain sections, 4 mm thick, were obtained from paraffin blocks. To localize SAG-1, SAG-2, and 70-kDa Hsp, paraffin sections were deparaffinized and antigenic unmasking was done with a microwave oven (31). The sections were incubated for 30 min at 37°C in 2% unlabeled sheep serum to

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TABLE 1. Free tachyzoites, cyst numbers, cyst diameters, and cyst PAS staining in brains of C57BL/6 mice chronically infected with *T. gondii* and treated with various MABs^a

MAB treatment and day after initiation	Free-tachyzoite score ^b	No. of cysts per section	Diameter of cysts (μ m)	% PAS-positive cysts
Anti- β -galactosidase (control)	1+	3.5 \pm 2.1 ^c	45.4 \pm 14.4 ^d	96
Anti-IFN- γ day 0	1+	6.8 \pm 3.8 ^c	39.8 \pm 14.2 ^d	100
Anti-IFN- γ day 1	—	10.1 \pm 1.3	46.7 \pm 23.5	100
Anti-IFN- γ day 3	—	8.6 \pm 4.4	43.6 \pm 12.0	81
Anti-IFN- γ day 5	2+	11.8 \pm 5.8	46.8 \pm 19.7	80
Anti-IFN- γ day 7	3+	30.0 \pm 20.5	29.1 \pm 17.1	60
Anti-IFN- γ day 9	4+	49.5 \pm 0.7	25.6 \pm 14.2	27
Anti-IFN- γ day 10	3+	21.6 \pm 7.4	31.1 \pm 12.5	45
Anti-IFN- γ day 12	5+	128.5 \pm 49.4 ^e	25.6 \pm 14.0	50
Anti-TNF- α day 12	4+	31.6 \pm 28.3	23.3 \pm 12.7	80

^a The results are the means and standard deviations of assays performed on three mice/group.

^b 1+, one lesion containing few tachyzoites in three to five histopathologic sections; 2+, one or more lesions containing tachyzoites in two sections; 3+, one or more parasite-containing lesions per section; 4+, one or more parasite-containing lesions per section and at least one extensive lesion containing abundant tachyzoites; 5+, more than one extensive lesion containing abundant tachyzoites. —, not detected.

^c Significantly different from values obtained for mice treated with IFN- γ and sacrificed on day 1 and days 5 to 12 and mice treated with TNF- α and sacrificed on day 12 ($P \leq 0.0295$).

^d Significantly different from values obtained for mice treated with IFN- γ and sacrificed on days 7 to 12 and mice treated with TNF- α and sacrificed on day 12 ($P \leq 0.0255$).

^e Significantly different from values obtained for mice with all other conditions of immunosuppression ($P \leq 0.0290$). For the methodological details, see the text.

reduce nonspecific binding and then incubated in polyclonal rabbit primary antibody against SAG-1 or SAG-2 antigen or Hsp-70 (1:25) at 4°C overnight. The polyclonal antibody to Hsp was raised against the 3/4 C-terminal region of Hsp-70 from *Leishmania (Viannia) braziliensis*, the most polymorphic portion of this molecule (2). Secondary biotinylated antibodies were sheep anti-rabbit antibodies. The sensitivity was improved with the avidin-biotin technique (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, Calif.). The reaction was visualized by incubating the section with 3,3'-diaminobenzidine tetrahydrochloride (Sigma) for 5 min. The slides were studied with an Olympus light microscope and photographed with Kodak film (100 ASA). Control slides were incubated in the unlabeled rabbit serum. The measurement of the staining intensity was done with the UTHSCSA Image Tool program from the University of Texas Health Science Center, San Antonio.

Morbidity was assessed by histochemical enumeration of cyst numbers and determination of sizes as well as distribution within the CNS with brain sections from chronically infected mice. Periodic acid-Schiff stain (PAS) was used as a specific stain to identify the cyst membrane that contains the bradyzoite stage of *T. gondii*. Polyclonal antibodies against SAG-1 or SAG-2 were used as specific markers for tachyzoites (16–18, 24). The expression of parasite Hsps during bradyzoite-tachyzoite conversion was evaluated because these proteins may be involved in the stage transformation of parasites (38). The parameters measured to determine *T. gondii* stage conversion included (i) the frequency of free tachyzoites; (ii) the average number of cysts within the brain; (iii) cyst diameters; and (iv) the intensity of SAG-1, SAG-2, Hsp-70, or PAS staining during bradyzoite-tachyzoite conversion in brains of chronically infected animals, analyzed before and after the treatment with anti-IFN- γ or anti-TNF- α MAB (Table 1 and Fig. 1 and 2).

Rapid parasite replication was observed between days 5 and 7 after treatment with anti-IFN- γ MAB, as indicated by the presence of free tachyzoites and PAS-negative pseudocysts (20%), which represent newly formed cysts (Table 1). Morphologic analysis indicated that the maximum parasite burden occurred between days 7 and 9. Increased numbers of free tachyzoites and pseudocysts (Table 1) were observed in samples of brain tissue, suggesting cyst rupture, bradyzoite-tachy-

zoite conversion, tachyzoite replication, and active infection of host cells. Of note was an increase in the percentage and absolute number of PAS-positive cysts after day 9 of treatment with anti-IFN- γ MAB, indicating that the transformation of tachyzoites to bradyzoites persisted in spite of immunosuppressive treatment. Although the relative numbers of pseudocysts decreased, there was an increase in the absolute numbers of pseudocysts and free tachyzoites. These observations demonstrate that development of new cysts as well as maximal tachyzoite replication occurred after day 9 of treatment with anti-IFN- γ .

In contrast to a previous study (39), the results of the immunocytochemistry analysis demonstrated the expression of stage-specific tachyzoite antigens on parasites within the cyst membrane (14, 31). Some cysts from infected mice treated with MAB had few parasites expressing SAG-1 and SAG-2 antigens. The discrepancy between our observations and the previous report may be explained in part by our use of *T. gondii* ME-49. During chronic infection in highly susceptible C57BL/6 mice (7, 36), this strain can maintain a continuous and more dynamic cyst turnover (9, 26). Nevertheless, our data also show that in vivo neutralization of either IFN- γ or TNF- α in mice chronically infected (for 4 weeks) with *T. gondii* ME-49 resulted in a dramatic enhancement and homogeneous expression of SAG-1 and SAG-2 inside the brain cysts. This suggests that the majority of the parasites inside cysts begin to express tachyzoite-specific antigens after cytokine neutralization. A photometric assay was used to measure the expression of SAG-1 and SAG-2 in brain cysts. As shown in Fig. 1A, 1 day after initiation of anti-IFN- γ treatment, the expression of SAG-1 was enhanced. The highest expression of SAG-1 by parasites in the CNSs of animals was recorded at 7 to 9 days after treatment with anti-IFN- γ MAB (Fig. 1A and 2B). A decrease in the intensity of SAG-1 expression was observed after day 10 of treatment with anti-IFN- γ . Similar kinetics were observed when the expression of SAG-2 by *T. gondii* during treatment with anti-IFN- γ MAB was evaluated (Fig. 1B). An increase in SAG-1 and SAG-2 expression was also observed in parasites in the CNSs of mice chronically infected with *T. gondii* and treated with anti-TNF- α MAB (Fig. 1A and B).

In order to study the expression of *T. gondii* Hsp-70 during stage conversion from bradyzoites to tachyzoites, we used rab-

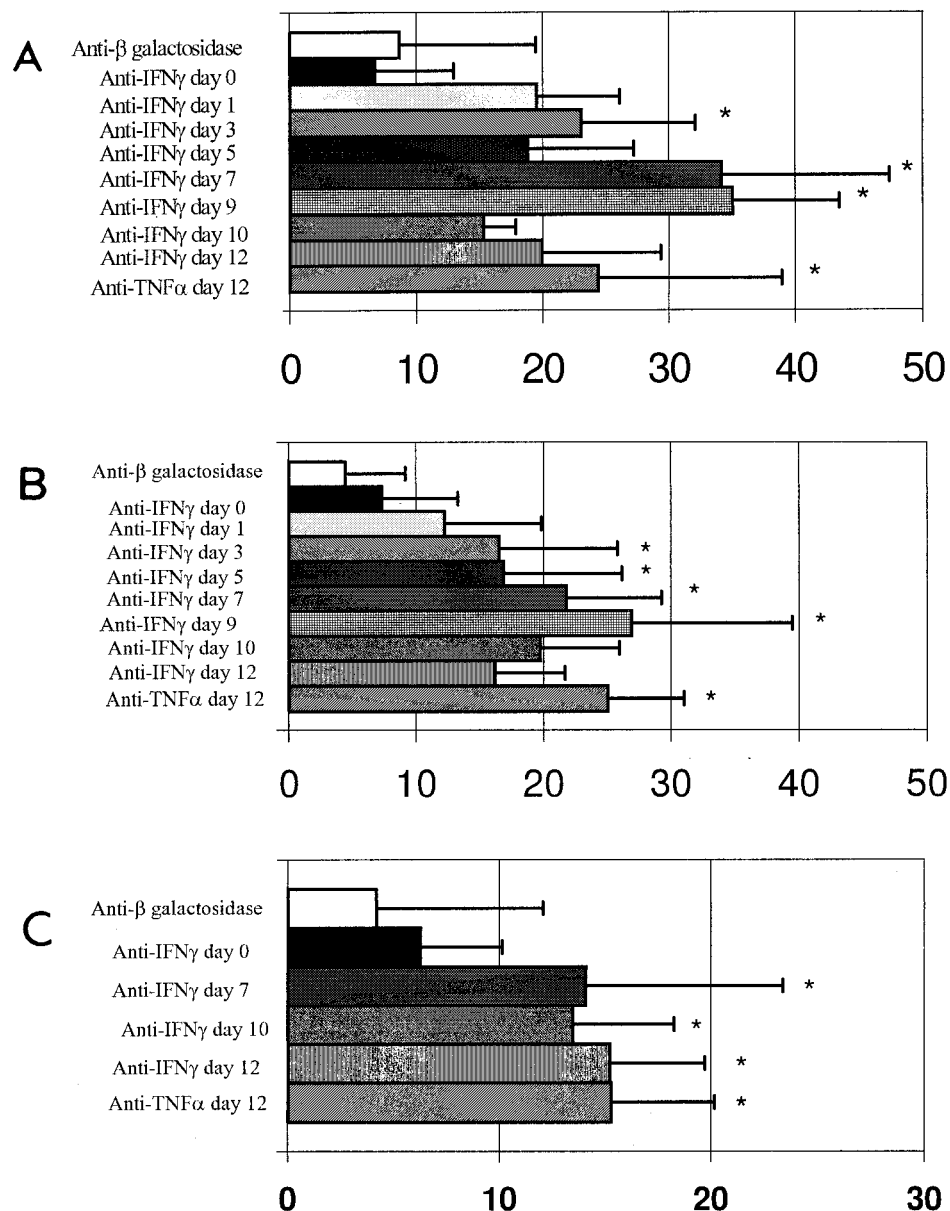


FIG. 1. Detection of SAG-1 (A), SAG-2 (B), and Hsp-70 (C) antigens by photometric assay in brain cysts from mice chronically infected with *T. gondii*. The data were obtained from several cysts analyzed from groups of three mice. Intensities of expression under the indicated treatment conditions are shown in absorbance units. Asterisks indicate values significantly different from those obtained with controls ($P < 0.05$).

bit polyclonal antibodies raised against the last three quarters of the C-terminal region of Hsp-70 from *L. (V.) braziliensis* (2). Neutralization of endogenous IFN- γ or TNF- α in chronically infected C57BL/6 mice resulted in homogeneous expression of Hsp-70 in some brain cysts (Fig. 1C and 2E). Interestingly, some of the cysts from immunosuppressed animals did not express this protein. By photometric assay, increased expression of Hsp-70 was observed in animals receiving anti-IFN- γ MAb after 7 days of treatment. These mice had an increase in number of free tachyzoites in relation to cyst numbers (Table 1). Fully differentiated free tachyzoites in the brain tissue did not express Hsp-70 (Fig. 2F). Thus, our data indicate that maximal expression of Hsp-70 occurs in encysted parasites during a short period of parasite stage conversion. We were unable to determine whether expression of Hsp-70 occurs dur-

ing bradyzoite-tachyzoite conversion or tachyzoite-bradyzoite conversion. The fact that the maximal intensity of Hsp expression was observed at late stages of reactivation may indicate that Hsp-70 is primarily expressed during tachyzoite-bradyzoite conversion.

The Hsps have been shown to be highly conserved among a wide variety of organisms. Although Hsp functions are not completely understood, these proteins are essential for survival of the cell (3, 30) and are involved in a variety of biological functions within the cell, including preservation and recovery of various protein complexes and degradation of denatured proteins. Environmental stresses, such as heat shock, starvation, and alkaline pH, can induce cell differentiation, a process associated with induction of Hsp expression (21, 28). In nature, transfer of parasites from one environment to another or par-

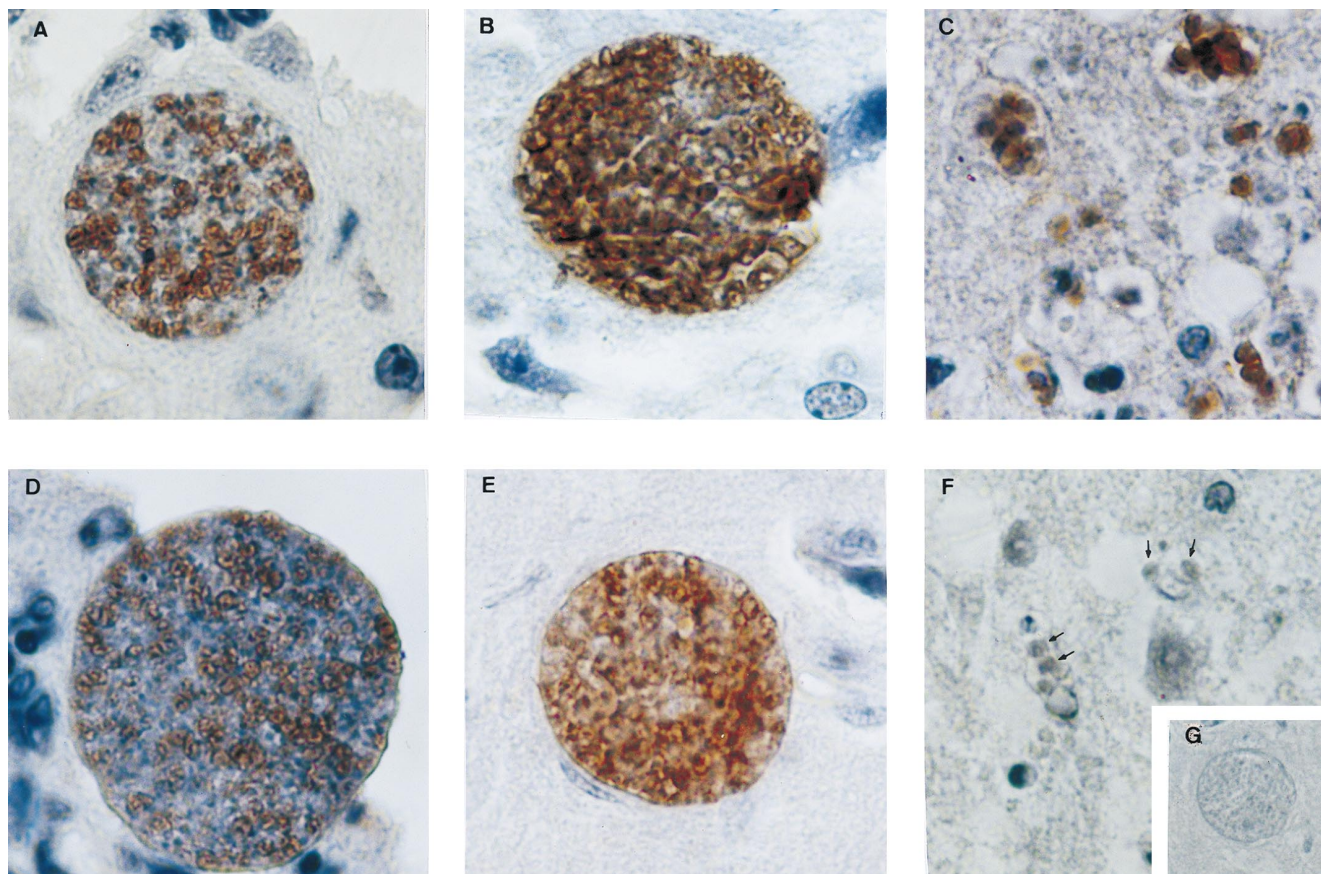


FIG. 2. Illustration of SAG-1 (top panels) and Hsp-70 (bottom panels) immunoperoxidase staining in parasites inside cysts (A, B, D, and E) and free tachyzoites (C and F) of *T. gondii* present in the CNSs of animals treated with either control MAb (A and D) or anti-IFN- γ (B, C, E, and F) and shown at a magnification of $\times 244$. Background staining with an unrelated control polyclonal antibody is shown in the inset (G) at a magnification of $\times 101$. For the methodological details, see the text.

asite stage conversion is frequently associated with expression of Hsp (3, 27, 37). Hsps are also important immunologic targets in response to pathogens (25). In vitro, environmental stresses, such as alkaline pH, can drive the transformation of tachyzoites to bradyzoites. Associated with this transformation is the expression of a *T. gondii*-specific antigen that has some homology to the small Hsp from plants (6). In our study, neutralization of endogenous IFN- γ or TNF- α or depletion of T-cell subsets (data not shown) enhanced expression of Hsp-70 inside brain cysts from immunosuppressed mice. Interestingly, Hsp-70 was not intensively expressed by bradyzoites from immunocompetent mice or by free tachyzoites in brain lesions from immunosuppressed animals. These findings suggest that Hsp-70 may have an important role in *T. gondii* adaptation during this differentiation event.

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