Induction of Bradyzoite-Specific *Toxoplasma gondii* Antigens in Gamma Interferon-Treated Mouse Macrophages

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By using stage-specific monoclonal antibodies, an in vitro model has been developed to analyze the kinetics of expression of stage-specific antigens during the conversion process between tachyzoites and bradyzoites of *Toxoplasma gondii*. Following infection of murine macrophages with bradyzoites, the expression of bradyzoite-specific antigens declined, whereas the expression of tachyzoite-specific antigens increased during the first 72 h postinfection. Conversely, in gamma interferon-treated murine macrophages infected with tachyzoites, the inhibitory effect of gamma interferon on replication of parasites was accompanied by the induction of bradyzoite-specific antigens.

*Toxoplasma gondii* exists in two forms in the human host; tachyzoites are involved in acute infection and seem to be converted to slowly dividing bradyzoites during the onset of a specific immune response (20). So far, it is generally believed that retransformation from bradyzoites to tachyzoites occurs during reactivation of cysts in immunocompromised patients (11, 29). However, it is not entirely clear whether stage conversion or a mere selection of tachyzoites and bradyzoites is responsible for this process. In addition, it is important to consider possible alterations of the immune regulation in immunocompromised patients to analyze the conditions leading to reactivation of previously quiescent cysts. It has been shown that lymphocytes of AIDS patients have a defect in production of gamma interferon (IFN-γ), which is supposed to be a predisposing factor for these patients to develop opportunistic infections (15). In vivo studies have proven that IFN-γ seems to be the major mediator of resistance against *T. gondii* infection (14, 25, 26). Mice chronically infected by a *T. gondii* strain of low virulence developed severe encephalitis upon injection of a monoclonal antibody (MAb) to IFN-γ (24). In vitro studies have demonstrated that IFN-γ does inhibit *T. gondii* growth in several different cell types, including mouse and human macrophages, human fibroblasts, and human endothelial cells (1, 10, 16, 17, 19, 22, 23, 30). The functions of other lymphokines such as interleukin-2 or tumor necrosis factor in *T. gondii* infection have been discussed controversially (3, 7, 10, 17, 21, 22).

The aim of the present study was to establish an in vitro cell culture system by which the monitoring of the expression of stage-specific antigens during the conversion process of *T. gondii* and the influence of IFN-γ on this process might be possible. To analyze the kinetics of the expression of stage-specific antigens, it was important to define markers for tachyzoites and bradyzoites (12). As a marker for tachyzoites, MAb 6A8, which is directed against the major surface protein P30 (kindly provided by L. H. Kasper), was used (2, 8). To obtain markers for bradyzoites, we generated bradyzoite-specific MAbs by immunizing 12-week-old female BALB/c mice with 1,500 cysts of the NTE strain (5). Following three booster immunizations, splenocytes of these mice were fused with Ag 8.653 myeloma cells and resulting hybridomas were screened for reactivity with tachyzoites and bradyzoites by immunoblotting and immunofluorescence (6). Tachyzoites for all experiments were harvested from infected P388D1-macrophage cell cultures as described previously (5). Cysts were separated from brains of mice, infected intraperitoneally with 10⁵ tachyzoites 6 weeks before, by continuous density gradient centrifugation as previously described (4). For immunoblotting, lysates of 3 × 10⁶ tachyzoites per well or 200 cysts per well were separated on 11.8% acrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6, 9). Following electrophoresis, proteins were electrophoretically transferred onto 0.45-μm-pore-size nitrocellulose membranes. After blocking with 1% bovine serum albumin, the nitrocellulose membranes were incubated in turn with (i) MAbs diluted 1:100 in phosphate-buffered saline (PBS) (150 mM NaCl, 20 mM sodium phosphate, pH 7.2)-Tween and (ii) alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG). Reactive bands were visualized by using the substrates 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. For immunofluorescence, either 10⁴ tachyzoites or 10 mechanically ruptured cysts were put onto glass slides and air dried. Following fixation, the glass slides were incubated in turn with (i) one of the MAb and (ii) fluorescein-conjugated goat anti-mouse IgG, diluted 1:50 in PBS. Native staining was performed in suspension with 100 ruptured cysts. Hybridomas secreting bradyzoite-specific MAbs were further cloned by Poisson limiting dilution.

Two MAb of the IgG1 subclass that were specific for bradyzoites were identified and designated MAb 7E5 and MAb 4F8. By using immunoblotting under reduced conditions, MAb 7E5 reacted specifically with a 30-kDa antigen of bradyzoites but not with tachyzoites of *T. gondii* (Fig. 1). No strain specificity was observed, because reactivity could be obtained with bradyzoites of strains NTE, ALT, and 561 (5). In immunofluorescence with fixed bradyzoites (5 min; 4% paraformaldehyde), MAb 7E5 led to a diffuse staining pattern, with the exception of a small area in the apical pole. By using native bradyzoites, no reactivity could be obtained. MAb 4F8 reacted with fixed (5 min, 4% paraformaldehyde, or 5 min, methanol) as well as with native bradyzoites of different strains, indicating that it probably recognized a surface antigen (Fig. 2). No reactivity with either bradyzoi-
tes or tachyzoites could be obtained with MAb 4F8 by immunoblotting under reduced as well as nonreduced conditions. To analyze whether the antigens recognized by the MABs are sensitive to trypsin or pepsin, 300 cysts of T. gondii NTE were incubated for 2 h at 37°C either in a buffer containing 0.25% trypsin in PBS or in a buffer containing 1,000 IU of pepsin in 0.9% NaCl, pH 1.5. The samples were then applied to SDS-PAGE followed by immunoblotting or put on glass slides and fixed. The 30-kDa antigen recognized by MAb 7E5 was pepsin resistant but trypsin sensitive, whereas the 4F8 reactive antigen was pepsin and trypsin resistant (data not shown). The difference in trypsin sensitivity of our MABs indicated that they reacted not only with different epitopes but with different antigens. To date, only a few bradyzoite-specific MABs have been described (18, 27, 28). Exchange of MABs with Tomavo and coworkers (27) has proven that our MAb 7E5 was different from their bradyzoite-specific MABs (4a). Weiss et al. have described a MAb of the IgG2b isotype being reactive with a 28-kDa antigen, similar to the size range of our MAb 7E5 (28). However, our MAb is of the isotype IgG1 and reacted with a 30-kDa bradyzoite-specific antigen.

Surprisingly, when tachyzoites of strain NTE were used, MAb 4F8 as well as MAb 7E5 reacted with around 1% of these T. gondii cells (Fig. 3). An unrelated control MAb of the same isotype was not able to react with any T. gondii cells within a tachyzoite population of the NTE strain. To further analyze this subpopulation, a double immunofluorescence using the combination MAb 4F8-MAb 7E5 and the combination MAb 4F8-MAb 6A8 was performed by incubating the glass slides in turn with (i) MAb 4F8, (ii) fluorescein isothiocyanate-conjugated goat anti-mouse IgG, (iii) MAb 7E5 or MAb 6A8, and (iv) tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG. Both bradyzoite-specific MABs reacted with the same subpopulation within tachyzoites, and 80% of this subpopulation also exhibited reactivity with the tachyzoite-specific MAb 6A8. No cross-reactivity of the TRITC-conjugated IgG to MAb 4F8 existed, indicating that all binding sites were saturated with the fluorescein isothiocyanate-conjugated IgG. The detection of a subpopulation within tachyzoites that was reactive with bradyzoite-specific MABs is in agreement with
a previous finding demonstrating the existence of a heterogeneous *T. gondii* population (27). It seems possible that *T. gondii* cells coexpressing the tachyzoite-specific P30 antigen as well as bradyzoite antigens represent an intermediate stage between tachyzoites and bradyzoites.

For the subsequent in vitro infection experiments, three different murine cell types were used. Mouse peritoneal macrophages (MPMs) were obtained by performing a peritoneal lavage on CBA/J mice and cultured in CLICK-RPMI (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS), 300 mg of L-glutamine per liter, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), and antibiotics. L929 murine fibroblasts were maintained in minimal essential medium (Biochrom) supplemented with 6% FCS, L-glutamine, HEPES, and antibiotics. The medium for murine P388D1 permanent macrophages consisted of RPMI 1640 (Biochrom), supplemented with 5% FCS, L-glutamine, and antibiotics. P388D1 macrophages, MPMs, and L929 murine fibroblasts were infected with bradyzoites of strain NTE, and the kinetics of the expression of stage-specific antigens during the conversion process from bradyzoites to tachyzoites was monitored by double immunofluorescence with stage-specific Mabs. Probes were fixed for 5 min in methanol and incubated in turn with (i) Mab 4F8, Mab 7E5, or Mab 6A8; (ii) fluorescein isothiocyanate-conjugated goat anti-mouse IgG; (iii) polyclonal mouse antitoxoplasma serum; and (iv) TRITC-conjugated goat anti-mouse IgG.

Freshly isolated bradyzoites were entirely reactive with Mab 7E5 and 4F8, but completely nonreactive with the tachyzoite-specific Mab 6A8. Following infection, the fraction of *T. gondii* cells being reactive with the bradyzoite-specific Mab 4F8 declined from 100% on day 1 postinfection (p.i.) to 15% on day 3 p.i. The results with Mab 7E5 were similar to the results with Mab 4F8. Along with the decline of the fraction of *T. gondii* cells showing reactivity with bradyzoite-specific Mabs, the fraction of those *T. gondii* cells that were strongly reactive with the tachyzoite-specific Mab increased from day 1 p.i. to day 3 p.i. (Fig. 4). The tachyzoite-specific antigen P30 seemed to be expressed very early during infection: already 24 h after infection with bradyzoites, 22% of the *T. gondii* cells reacted strongly with the tachyzoite-specific Mab. From day 2 to day 3 massive replication had occurred and the whole population was positive for P30, suggesting that at this time, the conversion from bradyzoites to tachyzoites might have been completed.

This finding was in agreement with a previous study indicating that only a few generations were necessary to result in the loss of bradyzoite-specific markers following infection of cell cultures with bradyzoites (13). The kinetics suggest that during a short period of time within the conversion process, parasites seem to express tachyzoite- and bradyzoite-specific antigens simultaneously. No significant difference for reactivity with the stage-specific Mabs was obtained for the different cell types.

Since it was shown that IFN-γ seems to be the main mediator of resistance against *T. gondii* infection, it was also of interest to investigate the possible influence of IFN-γ on expression of bradyzoite-specific antigens. Thus, 2.5 × 10^5 MPMs, 2 × 10^6 P388D1 murine permanent macrophages, or 2 × 10^6 L929 murine fibroblasts were seeded on glass slides and were stimulated with 100 IU of IFN-γ per ml after 24 h of incubation at 37°C with 5% CO₂. Infection with 10³ and 10⁴ tachyzoites of strain NTE was carried out at three different time intervals: (i) 24 h after IFN-γ stimulation, (ii) 3 h after IFN-γ stimulation, and (iii) 3 h before IFN-γ stimulation. Every 2 days medium was exchanged and 100 IU of IFN-γ per ml was added. As a control, unstimulated MPMs were also infected with 10⁴ or 10⁵ tachyzoites. The probes were fixed for 5 min in methanol from day 1 to day 6 and analyzed by double immunofluorescence using in turn, (i) Mab 4F8, (ii) fluorescein isothiocyanate-conjugated goat anti-mouse IgG, (iii) polyclonal mouse anti-toxoplasma serum, and (iv) TRITC-conjugated goat anti-mouse IgG. All experiments were performed at least twice. No difference in *T. gondii* replication between IFN-γ-stimulated and -nonstimulated P388D1 macrophages and L929 fibroblasts seemed to exist because tachyzoites divided rapidly and led to host cell lysis within 5 days. The portion of 4F8-reactive parasites did not increase from day 1 to day 5 p.i. and was less than 1%. The failure of IFN-γ to inhibit *T. gondii* growth in murine fibroblasts is in agreement with results of a previous study (24). The reason that stimulated P388D1 murine macrophages did not show any inhibitory effect on *T. gondii* replication was unclear. In contrast, in IFN-γ-stimulated MPMs, replication of *T. gondii* was significantly

![FIG. 4. Representative experiment showing the expression of stage-specific antigens in P388D1 murine macrophages infected with bradyzoites of *T. gondii* NTE with bradyzoite-specific Mabs (4F8 and 7E5) and a tachyzoite-specific Mab (p30). Reactivity with Mabs was classified into strongly reactive (+ +) and weakly reactive (+).](image-url)
inhibited to the extent that no host cells were lysed during the first 6 days p.i. In addition, the number of parasites expressing the bradyzoite-specific MAb 4F8-reactive antigen increased from 1% on day 1 p.i. to 32% on day 6 p.i. when MPMs were stimulated 3 h after infection with NTE tachyzoites (Fig. 5). It was not possible to investigate the kinetics of conversion from tachyzoites to bradyzoites for longer than 6 days because the entire number of parasites decreased during the experiment probably because of the microbicidal effect of the activated MPMs. These results were similar and independent of the time of IFN-γ-stimulation in relation to infection with T. gondii. When MPMs were stimulated with IFN-γ solely in the first 24 h of infection, slow replication of parasites from day 2 to day 4, followed by extensive replication and host cell lysis 6 days p.i., was observed. Under these conditions, the fraction of MAB 4F8-reactive parasites increased up to 50% from day 1 to day 4. When rapid replication occurred on day 5 and day 6 p.i., that fraction declined to less than 5%.

Increase of the relative number of parasites expressing bradyzoite-specific antigens could be due to a real induction of these antigens or to a selection process favoring the survival of those parasites already expressing these antigens. To differentiate between these possibilities, we determined the absolute number of MAB 4F8-reactive parasites on the cover slides, and we found a 10-fold increase during the first 4 days after infection. Since replication of parasites stopped in IFN-γ-treated MPMs, the increase in absolute numbers of MAB 4F8-reactive parasites suggested that a real induction of bradyzoite-specific antigens had occurred. So far, it is not clear whether parasites expressing bradyzoite-specific antigens are also more resistant to the antitoxoplasmaclidal activity of macrophages. The inhibitory effect of IFN-γ on T. gondii growth in MPMs is well established (1, 10). Our study suggested that IFN-γ also had an indirect effect on induction of bradyzoite-specific antigens in vitro.

This in vitro system has been shown to be a useful tool for studying the influence of IFN-γ on T. gondii infection and expression of stage-specific antigens. Experiments are now under way to study the role of other immunomodulators or chemotherapeutic agents on induction of stage-specific T. gondii antigens. Moreover, it remains to be elucidated whether parasites expressing bradyzoite-specific antigens are capable of forming cysts.

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