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Toxoplasma gondii: Antigenic differences between endozoites and cystozoites defined by monoclonal antibodies *

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Abstract. The antigenic differences between endozoites and cystozoites of *Toxoplasma gondii* were analyzed by indirect immunofluorescent assay and Western blotting using monoclonal antibodies. An antigenic determinant of an antigen of 20 kDa mol. wt. that appeared to be localized in the cytoplasm was observed to be specific to cystozoites alone. The 20-kDa antigen was diffused throughout the parasite and was observed in cystozoites produced both in vivo and in vitro.

Toxoplasma gondii, an obligate intracellular protozoan, invades many kinds of mammalian and avian somatic cells, proliferating in the host cells by endodyogeny. In the intermediate host the parasite exhibits two forms, the endozoite (tachyzoite) and cystozoite (bradyzoite), whose distinct biological characteristics are expressed in the rapid multiplication rate of the former and the slow multiplication rate of the latter. The relative number of both forms in the host differentiates between *T. gondii* strains with low and high virulence.

Previous studies on the effects of pepsin-HCl have indicated certain differences between the two forms that could subsequently play an important role in the infection process (Jacobs et al. 1960). Lunde and Jacobs (1983) have reported such differences to be antigenically expressed, as shown by immunofluorescence tests using polyclonal antibodies.

Our studies using monoclonal antibodies (mAbs) have thus far confirmed such observations. In this paper, we describe a cystozoite-specific antigenic epitope common to the different strains of *T. gondii* tested. This epitope was detected in cysts produced both in vivo and in vitro (cell culture), indicating antigenic similarity between the two cystic forms.

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Materials and methods

Mice. BALB/c adult mice bred in our laboratory were used in this experiment.

Parasites. The high-virulence RH strain, a low-virulence, cyst-forming population isolated from the RH strain and designated as RH-cyst III (Yano and Nakabayashi 1986), and the low-virulence S-273 and Beverley strains of *T. gondii* were used throughout this study. Endozoites of the S-273, Beverley, and RH-cyst III strains were obtained from the peritoneal cavity of mice 7 days after IP injection with infected mice brain. The virulent RH strain was maintained by serial passage in mice at 3-day intervals, and the endozoites were collected from the peritoneal cavity.

In vitro cysts. To obtain cysts produced in vitro, endozoites were injected into mouse embryo cells (MECs) cultured in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum, 20 m*M* L-glutamine, and antibiotics, and maintained by changing the medium at 3-day intervals. At 7 days postinfection, when the inoculated parasites multiplied and the host cells ruptured, the parasites released into the cell-culture medium were harvested, washed three times by centrifugation at 3000 rpm for 10 min with chilled saline solution, and used for various experiments. Subsequent collections of parasites were done in synchrony with the changing of the medium and MECs were added every 3–4 days. Such MEC-cultured *T. gondii* were maintained for approximately 6 months, after which they were passaged in mice.

In vivo cysts. Cysts produced in vivo were obtained from the brains of mice chronically infected with the Beverley strain; the brains were homogenized in a Potter-Elvehjem glass homogenizer and diluted in phosphate-buffered saline (PBS). The cysts in the brain homogenate were isolated by percoll density gradient centrifugation (Cornelissen et al. 1981). The isolated cysts were suspended in 50 m*M* acetate buffer (pH 2.9) containing 0.15 *M* NaCl at 4° C for 1 h to remove the mouse immuno-

globulin on the surface of the cysts. Cysts were subsequently washed with PBS by centrifugation at 3000 rpm for 10 min at least three times.

Mouse polyclonal anti-T. gondii antibody. Hyperimmune polyclonal antibody was obtained from mice infected with 1×10^4 Beverly strain parasites. The mice were boostered with 1×10^4 RH strain parasites on day 30 postinfection; those surviving after 14 days were considered hyperimmune.

Mouse monoclonal anti-T. gondii antibodies. Spleen cells from mice chronically infected with S-273 strain parasites were hybridized with SP 2/0-Ag 14 BALB/c myeloma cells by fusion in the presence of polyethylene glycol 1500. Standard procedures were followed for cell fusion and cloning (Mishell and Shiigi 1980). Hybridoma clones were screened for anti-*T. gondii* antibody production by the indirect immunofluorescence assay (IFA).

Indirect immunofluorescence assay. Washed parasites were either fixed in 1% paraformaldehyde-PBS (pH 7.2) at 4° C for 15 min or suspended in PBS under living conditions at 4° C. The assay previously described by Sethi et al. (1984) was followed. Briefly, the fixed parasites (or parasite-enriched cell suspension) were incubated with undiluted hybridoma cell-culture supernatant or ascites from hybridoma-injected mouse diluted 1:100 in PBS at 37° C or 4° C, respectively, for 30 min. Fluorescein isothiocyanate-conjugated anti-mouse IgG (TAGO, Inc., Code no. 4350) diluted 1:100 in PBS was reacted under conditions similar to those used for the second antibody. The fluorescence pattern was visualized using a fluorescence microscope.

Solubilization of antigens. Parasite-enriched cell pellets obtained from MEC cultures were suspended in lysis buffer composed of 50 m*M* TRIS-HCl (pH 8.0) containing 1% Triton X-100, 2 m*M* *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (PMSF), 5 m*M* ethylenediamine tetraacetic acid-disodium salt (EDTA), 10 μg/ml aprotinin and pepstatin at a concentration of 100 mg wet wt. parasites/ml lysis buffer. The suspension was ultrasonicated on ice with a 30-s pulse at least three times and centrifuged at 15000 rpm for 20 min at 4° C. The supernatant was divided into aliquots and kept at −20° C until use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), solubilized antigens were mixed with the same volume of sample buffer [4.6% SDS, 10% 2-mercaptoethanol, 12.5 m*M* TRIS-HCl (pH 6.8)]. After boiling for 3 min, the samples were electrophoresed on 12.5% acrylamide slab gels according to the method previously described by Laemmli (1970).

Western blotting assay. After SDS-PAGE, gels were applied on nylon membrane sheets (Durapore, Millipore Ltd.) and the proteins in the gels were transferred electrophoretically (Towbin et al. 1979). After transfer, the membrane sheets were immersed for 1 h in 5% skim milk containing PBS to block the remaining protein-binding sites and subsequently incubated for 1 h at room temperature with either noninfected mouse serum or anti-*T. gondii* antibodies diluted 1:500 in 5% skim milk containing PBS. The sheets were washed several times in 0.05% Tween-20 containing PBS and reacted with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Ltd., Code no. 170-6516) diluted 1:1000 in 5% skim milk-PBS for 1 h at room temperature. Reaction was revealed by immersion in 0.1 *M* TRIS-HCl buffer (pH 7.4) containing 0.2 mg/ml diaminobenzidine and 0.1% H_2O_2, and the reaction was stopped by adding 0.1 *M* H_2SO_4.

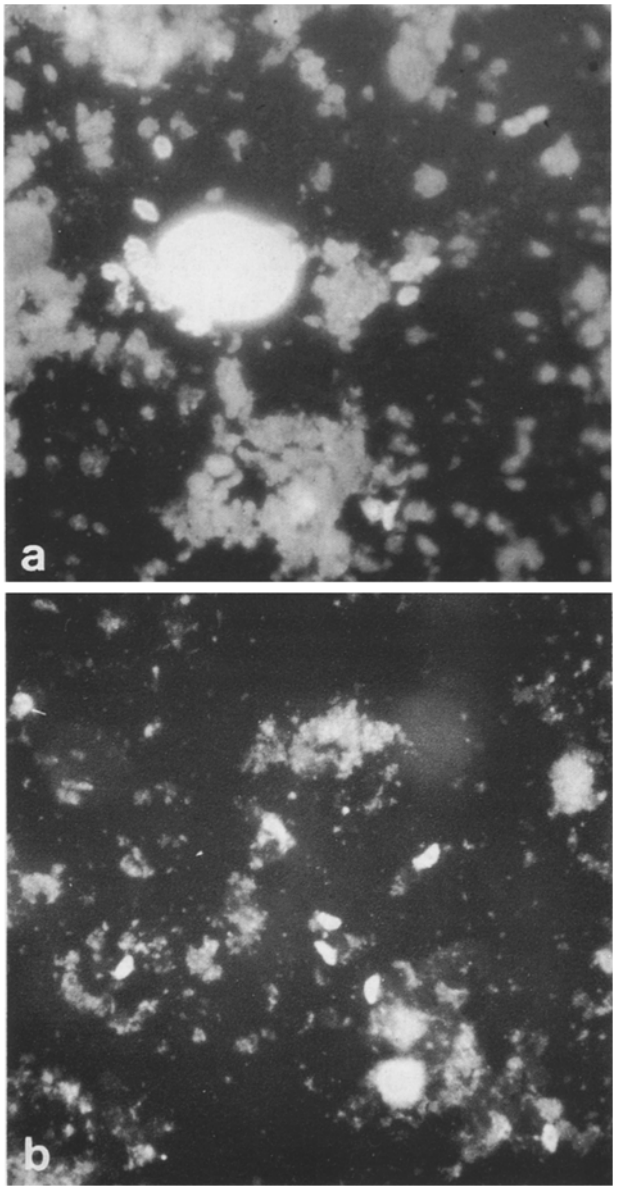

Fig. 1 a, b. Fluorescence pattern of Beverley strain parasites reacted with mAb. **a** Parasites from 6-month MEC cultures reacted against mAb BE6CC. Note that only parasites in cyst-like bodies and a few extracellular ones show a positive reaction (counterstained with Evan's blue). **b** Parasites in the cysts harvested from mouse brain reacted against mAb BE6CC. Note that cystozoites obtained from the broken cyst show a positive reaction

Results

Parasites in MEC culture

To eliminate the effects of host immune reactions specific against cystic forms of *T. gondii* in the analysis of its antigens using monoclonal antibodies (mAbs), we used parasites cultured in MEC

Table 1. Immunofluorescence patterns and molecular weight analysis of *Toxoplasma* parasite antigens detected with monoclonal and polyclonal antibodies

Antibodies	Immunofluorescence patterns					Mol. wt. SDS-PAGE	
	RH fixed	RH-III fixed	Bev fixed	S-273		RH	Bev
				fixed	living		
None	–	–	–	–	–	–	–
Noninfected serum	–	–	–	–	–	–	–
T. gondii chronic serum	rim	rim	rim	rim	rim	>20 bands	
mAb BE6CC	–	rim	rim	rim	–	–	20 kDa

Fixed, 1% paraformaldehyde-PBS (pH 7.2); living, reacted at 4° C for 30 min

in vitro. Endozoites of low-virulence Beverley, S-273, and RH-cyst III isolates, which infected MECs, multiplied slowly in the host cells as cyst-like bodies or clumps of parasites enclosed by a thin membrane, rupturing the host cells about 7 days postinfection. The endozoites of the virulent RH strain multiplied rapidly within MECs, rupturing host cells and reinvading new ones within 3–4 days without producing cyst-like bodies. Such parasites maintained in cell cultures for long periods did not show any change in their strain-specific virulence, as tested by mouse inoculation.

Immunofluorescence patterns

The hybridoma cells were selected and cloned according to their ability to produce specific antibodies against parasites of the S-273 or RH strain. One hybridoma clone was observed to produce mAbs that recognized an epitope in the cystozoites of S-273, Beverley, and RH-cyst III strains produced in MEC cultures (Fig. 1a). The same mAb also detected the same epitope in in vivo-produced cystozoites obtained from *T. gondii*-infected mouse brain (Fig. 1b); however, it could not detect a similar epitope in the endozoite forms, including those of the virulent RH strain. Immunofluorescence tests showed a "rim" pattern characterized by diffused fluorescence throughout the parasite. Neither qualitative nor quantitative differences in the reactivities between cystozoites from the different strains were detected by IFA. Both parasites that had been released from the broken cysts and those that were still within the cyst-like bodies exhibited the same fluorescence pattern. The localization of this specific antigenic epitope appears to be intracellular, because nonfixed cytozoites reacted with the mAb at 4° C for 30 min failed to show any specific fluorescence (Table 1). Incubation under such conditions eliminates the possibility of shedding of the antigen-antibody complex. Increasing the duration of cell culture resulted in an increase in the number of nonreactive extracellular parasites. However, no such change in reactivity was observed in the parasites inside the cyst-like bodies.

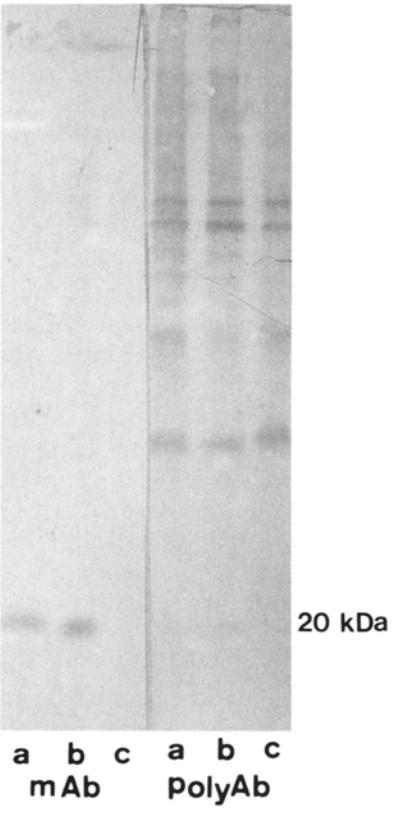

Fig. 2. Western blot analysis of solubilized antigens of Beverley (*a*), S-273 (*b*), and RH (*c*) strains reacted with BE6CC (*mAb*) and mouse immune serum (*PolyAb*). mAb BE6CC recognized a 20-kDa antigen in low-virulence strains Beverley and S-273 but not in the highly virulent RH strain

Molecular weight determination

SDS-PAGE of solubilized antigens from the different *T. gondii* strains detected more than 20 protein

bands, and approximately the same bands were recognized by polyclonal antibody from chronically infected mouse serum. Figure 2 shows that the mAb recognized one specific protein band with a mol. wt. of approximately 20000 daltons (20 kDa) in the Beverley and S-273 strains but not in the RH strain. Polyclonal hyperimmune mouse serum reacted with the same 20-kDa protein band in the Beverley and S-273 strains but could not detect it in the RH strain.

Discussion

The ease with which low-virulence *T. gondii* produces cysts markedly distinguishes it from the virulent strains that form only cysts under suitable conditions (Shimada et al. 1974). This characteristic ensures a longer survival time in susceptible animals after infection. Whereas endozoites are responsible for the major damage and destruction of host cells due to their rapid growth, cystozoites appear to be more effective in transmission and are probably responsible for certain recurrent symptoms in chronic toxoplasmosis (Frenkel 1955). Such differences between the two forms are very important and, as such, necessitate more studies on their antigenic specificities and roles in infection.

In this study, low-virulence strains of *T. gondii* (Beverley and S-273), were observed to form cysts in tissues of infected mice and cyst-like bodies in MEC cultures. This was also observed with the RH-cyst III strain, a *T. gondii* population with low pathogenicity isolated from the brain of a mouse immunized with the RH strain (Yano and Nakabayashi 1986; Omata et al. 1987). The highly virulent RH strain multiplied as endozoites, inducing the rupture of infected host cells; cyst formation was seldom observed either in vivo or in vitro. Cyst formation by virulent strains under normal conditions, as opposed to that under suitable conditions previously reported by Shimada et al. (1974), probably occurs at a very low proportion compared with the total parasite population in the mouse system.

Comparison of the antigenicity between the different strains of *T. gondii* showed at least one antigenic determinant with a mol. wt. of 20 kDa that was detected in all low-virulence strains by immunoblotting. The results of IFA indicated an antigenic epitope specific to cystozoites produced both in vivo and in vitro.

Despite the ease with which in vitro cysts can be obtained, there is always the question regarding their antigenic similarity or dissimilarity with those produced in vivo. Difficulties in obtaining large numbers of in vivo-produced cysts make it hard to present a complete antigenic profile for the purpose of comparison. Although more studies are needed, our experiments showed the similarity of the 20-kDa epitope in both in vivo and in vitro cysts. Such observations of antigenic similarity tend to support the contention that cyst-like bodies could be a process of tissue cyst development (Matsubayashi and Akao 1963).

It is also interesting to note that increasing the duration of *T. gondii* cell culture led to an increase in the number of extracellular parasites not reacting with the mAb. However, since all of those within cyst-like bodies remained reactive, such an observation could be explained either by a gradual transformation from the in vitro cystozoites back to the endozoite form, accompanied by a switching off of the production of cystozoite-specific antigenic epitopes, or by an increase in a specific subpopulation of *T. gondii* that does not produce the epitope. Lunde and Jacobs (1985) have reported the transformation of in vivo cystozoites in cell culture, resulting in the loss of cystozoite-specific antigenic determinants. In the present study, it is unclear as to whether the parasites not reacting with mAb have the characteristics of endozoites. We are now extending our study in this direction to clarify this phenomenon.

As suggested by our results, the cytoplasmic localization of the antigen indicates that it is not the parasitic component that, together with host cell substances, has been proposed to form the cyst wall (Matsubayashi and Akao 1966). Nevertheless, such stage specificity could be indirectly related to cyst formation or to specific characteristics that make cystozoites different to and, in the case of transmission, more effective than endozoites.

Our inability to detect the 20-kDa antigen in the RH strain with mAbs or polyclonal hyperimmune serum could simply be a reflection of the inability of this strain to form cyst-like bodies in vitro. The RH-cyst III strain, a low-virulence population of *T. gondii* obtained from immune mice infected with the RH strain that produces cyst-like bodies in vitro (Yano and Nakabayashi 1986; Omata et al. 1987), produces the antigen in the same way as the Beverley and S-273 strains. This suggests that the 20-kDa antigen is specifically synthesized by cyst-forming parasites. Studies to check the presence of other epitopes in the 20-kDa antigen are under way.

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