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Differentiation between Mouse-Virulent and -Avirulent Strains of *Toxoplasma gondii* by a Monoclonal Antibody Recognizing a 27-Kilodalton Antigen

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Using a murine monoclonal antibody, we were able to differentiate between mouse-virulent and -avirulent strains of *Toxoplasma gondii*. Monoclonal antibody TB6G5 was reactive with eight clinical mouse-avirulent isolates but not with mouse-virulent laboratory strains RH and BK. The antibody-reactive antigen was identified by indirect immunofluorescence and immunoblot as a 27-kDa cytoplasmic protein expressed by tachyzoites as well as by bradyzoites.

Several different isolates of the ubiquitous protozoon parasite *Toxoplasma gondii* have been described. They are usually classified as virulent and avirulent strains on the basis of pathogenicity in the mouse model (6, 7, 13, 14, 16). The virulent *T. gondii* strains RH and BK have been maintained in mice or cell culture for decades and are widely used as an antigen source for serological tests in routine diagnosis. In contrast, strains recently isolated from patients usually are avirulent in mice and induce a chronic infection with development of cysts in the brains of these animals.

Strain-specific differences of *T. gondii* isolates have been demonstrated by immunoprecipitation and Western blot (immunoblot) with polyclonal antisera, as well as isoenzyme analysis and molecular genetic techniques (1, 3, 5, 10, 11, 17, 18). Recent data suggest that virulent strains are distinguishable from avirulent strains by some serological and genetic markers. Using a mouse monoclonal antibody (MAb), we identified a virulence-associated 23-kDa antigen which was present exclusively in virulent strains RH and BK and not in freshly isolated clinical strains of *T. gondii* (8). In a recent study, genotypes of several virulent and nonvirulent strains were compared by restriction fragment length polymorphisms. All virulent strains showed the same restriction fragment pattern and, as was suggested by the authors, probably comprise a single clonal lineage, whereas nonvirulent strains were polymorphic (15). In this report, we describe an additional MAb which can differentiate between mouse-virulent and -avirulent strains.

To generate *T. gondii*-specific MAbs, 12-week-old female BALB/c mice were immunized with 1,500 cysts of strain NTE. Fusion, hybridoma screening, and antigen preparation were done as previously described (2). The obtained hybridomas were screened for reactivity with tachyzoites and bradyzoites by immunoblotting and indirect immunofluorescence assay. For immunoblotting, lysates of 3×10^6 tachyzoites per well or 200 cysts per well were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretically transferred onto nitrocellulose membranes, and incubated first with MAb diluted 1:100 in phosphate-buffered saline (PBS)-Tween and then with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG). For immunofluorescence, 10^4 tachyzoites were put on glass slides and air dried. Following fixation with 4% paraformaldehyde, the slides were incubated first with supernatant of the MAb and then with fluorescein-conjugated goat anti-mouse IgG diluted 1:50 in PBS.

Hybridoma screening resulted in two bradyzoite-specific MAbs, which were further used to study the conversion process between the tachyzoite and bradyzoite stages (2). In addition, we identified one IgG1 MAb, designated TB6G5, that reacted with a 27-kDa protein of strain NTE bradyzoites and tachyzoites. To investigate whether the identified antigen was strain specific, various mouse-virulent and -avirulent *T. gondii* isolates maintained in P388D1 macrophage cell culture were tested for reactivity with the MAb. It could be demonstrated that MAb TB6G5 reacted specifically with mouse-avirulent strains that were isolated between 1959 and 1990 from patients with acute or reactivated toxoplasmosis (8). No reactivity was observed with the virulent reference strains RH and BK (Fig. 1). To exclude the possibility that the observed differences in strain reactivity with MAb TB6G5 were dependent on the P388D1 host cells, we also tested tachyzoites derived from mouse peritoneal cavities. Identical results for in vivo- and in vitro-derived tachyzoites were obtained (data not shown). Indirect immunofluorescence using formalin-fixed tachyzoites confirmed the specific reaction of MAb TB6G5 with mouse-avirulent clinical isolates (isolates NTE, 561, 177, GAIL, ALT, KSU, DX, and RKR). Absolutely no reaction was found with parasites of strains RH and BK.

Whether the RH and BK strains are lacking the 27-kDa protein or whether a mutation in the TB6G5-binding epitope might exist could not be investigated because a polyclonal antiserum recognizing this protein is not yet available. For the same reason, it is not clear whether the described antigen is identical with other described *T. gondii* antigens in the range of 27 kDa. For example, Charif et al. characterized 27- and 28.5-kDa secreted antigens with MAbs (4). To further identify the location of the 27-kDa protein, disrupted tachyzoites of *T. gondii* NTE were separated into cytosol and membrane fractions. A total of 5×10^6 NTE tachyzoites in 50 μl of distilled water were submitted to three cycles of freezing and thawing. After ultracentrifugation for 30 min at $40,000 \times g$, the supernatant containing the cytosolic fraction

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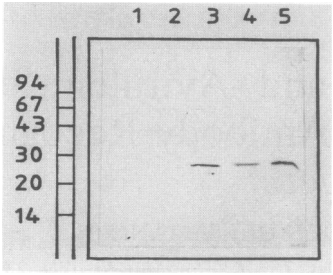

FIG. 1. Western blot analysis of reactivity of MAb TB6G5 with tachyzoites of two virulent (lanes 1 and 2) and three avirulent (lanes 3 to 5) *T. gondii* strains. Lanes: 1, RH; 2, BK; 3, NTE; 4, 561; 5, 177. Molecular masses (in kilodaltons) are indicated at the left.

and the pellet containing the membrane fraction were analyzed by immunoblotting with MAb TB6G5. The reactive 27-kDa antigen was identified exclusively in the cytosol fraction under reduced as well as unreduced conditions (Fig. 2).

Whether serological differences between *T. gondii* strains are due to differences in protein expression or due to posttranslational modifications, such as different glycosylation, has barely been investigated yet. Ware and Kasper suggested that differences in antigenicity between strain RH and avirulent isolates might be due to losses of carbohydrate chains in RH tachyzoites stimulated by continuous passages in mice (17). To analyze possible glycosylation of the 27-kDa antigen, the cytosolic fraction of 5×10^6 tachyzoites was denatured by boiling at 100°C for 2 min in 0.5% SDS–5% 2-mercaptoethanol. The sample was diluted fivefold in a buffer containing final concentrations of 20 mM sodium phosphate (pH 7.5), 50 mM EDTA, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. One unit of N-Glycosidase-F (Boehringer, Mannheim, Germany) was added, and the mixture was incubated for 12 h at 25°C. Samples were analyzed by SDS-PAGE and Western blotting. No shift in molecular mass was found for the 27-kDa protein, suggesting that this protein is not N-glycosylated. In a control digestion, 1 μg of transferrin was added to 5×10^6 tachyzoites and treated as described above. After staining with Ponceau S, a decrease in the molecular weight of transferrin was seen, indicating that no N-Glycosidase-F-inhibiting factors were present in the tachyzoite lysates.

To investigate whether the human antibody response to *T. gondii* generates TB6G5-cross-reactive antibodies, we tested sera of patients in a competition assay. Ten serum samples from patients with acute and chronic toxoplasmosis were pooled, respectively. Acute sera were characterized as having a Sabin Feldman dye test result of >1:1,000, having a complement fixation test result of >1:10, and being IgM–enzyme-linked immunosorbent assay (IgM-ELISA) positive and IgA-ELISA positive, with rising titers in follow-up sera. Sera of chronically infected patients had a Sabin Feldman dye test result of <1:1,000 and a complement fixation test result of <1:10 and were IgM-ELISA negative and IgA-ELISA negative, with stable titers in follow-up sera. Negative sera were negative in the serological tests mentioned above. Western blot strips with NTE tachyzoites were incubated with 1 ml of human sera (diluted 1:100) in PBS–0.5% Tween for 3 h. After being washed, the strips were treated with MAb TB6G5 diluted 1:100 in PBS–0.5% Tween 20. These sera did not inhibit the binding of MAb TB6G5 in Western blot analysis, suggesting that the TB6G5-reactive epitope seems unlikely to belong to the dominant epitope repertoire of the human antibody response to *T. gondii*. This result was not surprising, since the immunodominant antigens of *T. gondii* are localized to the surface (12), whereas the 27-kDa antigen has been localized to the cytoplasm of the parasite.

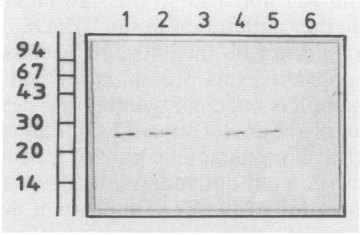

FIG. 2. Western blot analysis of the cytosol and membrane fractions of *T. gondii* tachyzoites under reduced (lanes 1 to 3) and unreduced (lanes 4 to 6) conditions. Lanes 1 and 4, total tachyzoite lysates; lanes 2 and 5, cytosol fractions; lanes 3 and 6, membrane fractions. Molecular masses (in kilodaltons) are indicated at the left.

The expression of the 27-kDa antigen on individual *T. gondii* strains seemed to be entirely complementary to the expression of the 23-kDa antigen recently described by us (8). MAb 5B10 detected the 23-kDa antigen in virulent strains RH and BK but not in avirulent strains (8). Taken together with these earlier results, this preliminary study has revealed, besides MAb 5B10, an additional marker upon which a serological classification of *T. gondii* isolates into mouse-virulent and -nonvirulent strains might be possible. These findings are consistent with the suggestion of Sibley and Boothroyd (15) that virulent strains of *T. gondii* originated from a single lineage, which is clearly different from nonvirulent isolates. Although the major antigens seemed to be present in both virulent and avirulent strains (10, 11, 17), minor differences in antigenic structure might have an impact for serological diagnosis of toxoplasmosis. In most diagnostic laboratories, the virulent strains RH and BK are used as an antigen source, whereas most *T. gondii* strains isolated from patients belong to the nonvirulent group. Whether some clinical isolates differ in immunodominant epitopes from strains RH and BK has not been investigated yet, but such possible differences might explain false-negative results in serological tests when strain RH or BK is used as the sole antigen source (9). It therefore should be investigated whether a nonvirulent *T. gondii* strain or a mixture of different isolates as an antigen source would be more suitable in serological tests.

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