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Characterization of Bradyzoite-Specific Antigens of *Toxoplasma gondii*

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Monoclonal antibodies that react specifically with bradyzoite antigens of *Toxoplasma gondii* were selected by differential immunofluorescence among hybridomas produced against these organisms. These antigens were further characterized by immunofluorescence on living bradyzoites and Western immunoblotting. Four pellicular antigens (36, 34, 21, and 18 kDa) were identified; three of these are exposed on the surface of the organism and accessible to either antibodies, trypsin cleavage, or both of these surface-probing procedures. These antigens were found on recent human isolates of *T. gondii*, as well as on laboratory strain bradyzoites obtained from either brain cysts or in vitro-grown parasites.

The life cycle of *Toxoplasma gondii* comprises several distinct invasive stages in both the definitive and intermediate hosts. These stages (sporozoites, merozoites, and bradyzoites) share common structures, especially the organelles involved in cell invasion, but also manifest important differences, which are most apparent when parasite differentiation or host parasite interaction is analyzed (8, 9, 12, 14). Such is especially the case for the two different stages known in the intermediate host, namely, tachyzoites and bradyzoites. Indeed, bradyzoites which are encysted in various organs are considered the parasite response to the host immune system, whereas tachyzoites can multiply only in naive recipients. The growing interest in sequelae of immunodeficiency has focused attention on toxoplasmic recrudescence. In immunocompetent individuals, tachyzoite multiplication is progressively inhibited by the growing immune response and results in a switch to bradyzoite development. In selected immunodeficiencies, most particularly, the acquired immunodeficiency syndrome, bradyzoites are presumed to revert to tachyzoites which multiply unhampered, forming brain abscesses that are eventually fatal. Understanding the mechanism by which tachyzoite-bradyzoite interconversions occur may therefore provide clues to the management of toxoplasmic infections while simultaneously addressing an interesting issue in cell differentiation.

A prerequisite for study of such an interconversion is identification of molecules or structures specific to each stage to raise probes to trace the early events of the process. Whereas an increasing number of tachyzoite molecules have been and are being described (6, 2), very little is known about bradyzoites: one 20-kDa antigen has been recently described (14). Therefore, we developed a series of monoclonal antibodies (MAbs) specific to bradyzoites. These MAbs are described in this report.

MATERIALS AND METHODS

Parasites. Two strains of *T. gondii* were used in this study: RH (15) and 76K (11). Bradyzoites of the 76K strain were used to immunize mice for hybridoma production; the 76K strain was passed every 2 months by oral injection of cysts collected from the brains of infected animals. RH strain tachyzoites were used for differential screening of bradyzoite-specific antigens. RH strain tachyzoites were routinely maintained by serial passage in the peritoneal cavities of Swiss mice. For all of the experiments described here, RH tachyzoites were produced by simultaneous injection of TG 180 sarcoma cells and tachyzoites intraperitoneally. Parasites were collected 3 days later (3). Strain 76K tachyzoites were also produced in monolayers of HeLa cells which were maintained in Dulbecco's minimal essential medium supplemented with 5% fetal calf serum. Parasites were purified from infected monolayers by glass wool filtration (5).

MAb production. MAbs were obtained by fusion of SP2/O myeloma cells with splenocytes of BALB/c mice immunized with *T. gondii*. Immunization was done as follows: a 3-month-old BALB/c mouse was immunized intraperitoneally with 5,000 cysts of the 76K strain that had been purified through Percoll (1), frozen-thawed twice in phosphate-buffered saline (PBS), and supplemented with Freund's complete adjuvant. Intraperitoneal injection was repeated twice at a 3-week interval with the same material in incomplete Freund's adjuvant. The mouse was boosted intravenously 1 month later with the same material in PBS. Fusion was performed 3 days later (4). Hybridomas were screened by differential immunofluorescence assay (see below). Positive hybridomas were cloned by limited dilution. Mass production of MAbs was performed by injection of hybridoma cells into pristane-primed BALB/c mice and collection of ascitic fluids.

The isotypes of the MAbs were identified by immunodiffusion of hybridoma supernatants against isotype-specific antibodies.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed by the method of Laemmli (10) by using 12 or 15% (wt/vol) acrylamide separating gels. Molecular weight standards (Pharmacia LMW kit) were used for calibration.

Western blotting. Tachyzoites or cyst proteins were separated by SDS-PAGE under nonreducing conditions (2.5×10^6 zoites or 250 cysts in a 1-cm-wide slot) and electrophoretically transferred to nitrocellulose (19) with a semidry apparatus. The nitrocellulose sheet was then saturated for 30 min in 5% nonfat dry milk in TNT buffer (140 mM

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NaCl, 0.5% [vol/vol] Tween 20 in 15 mM Tris-HCl [pH 8]). Strips were cut and incubated with the different MAb solutions (mouse ascites fluid diluted 1:200 in TNT) for 1 h at 37°C. After washing, the sheet was incubated in alkaline phosphatase-conjugated anti-mouse immunoglobulin (Ig) antibodies and revealed with the appropriate substrate (Promega).

Controls were performed by SDS-PAGE and Western blotting of mouse brain proteins (50 μg/1-cm-wide slot) or the material obtained by mock purification of uninfected brains and adjusted to the dilution used for the cyst pellet.

Immunofluorescence assay (IFA). For hybridoma screening, purified RH tachyzoites were washed three times with PBS and dried on standard IFA slides which were stored at −20°C. Bradyzoites were released from purified 76K cysts by grinding with a Dounce homogenizer and dried on IFA slides. IFA was performed at 37°C after 10 min of fixation in cold acetone.

For surface IFA, 76K bradyzoites were released as described above and processed as live parasites without fixation. They were incubated with normal goat serum diluted 1:10 in PBS and then in MAbs (1:100 dilution of mouse ascites fluid), washed, and incubated with fluorescein-conjugated goat anti-mouse IgG. All steps were performed at 4°C.

Effect of enzymatic release of bradyzoites on MAb reactivity. Cysts were purified as described above, suspended in PBS, distributed among three tubes, and pelleted. One pellet was suspended in 0.5% trypsin (Difco 1:250) in Dulbecco PBS, and a second was suspended in 0.5% pepsin in the same buffer. Both tubes were incubated at 37°C for 5 min and centrifuged, and the zoites washed in PBS and numbered. The third pellet was suspended in PBS, and the zoites were freed by mechanical grinding (Dounce homogenizer). Equal numbers of bradyzoites from the three assays were solubilized in SDS-PAGE sample buffer, electrophoresed, and transferred to nitrocellulose. The nitrocellulose was then probed with MAbs.

RESULTS

Many of the hybridomas obtained by the procedure described above reacted with both RH tachyzoites and 76K bradyzoites either in IFA or on immunoblot and usually in both assays; these hybridomas were not investigated further. Only hybridomas which reacted exclusively with 76K bradyzoites were retained; they produced four different major blotting patterns (Fig. 1); one strongly reacting hybridoma was selected for each pattern, cloned, and characterized further.

The antibodies produced by these four clones produced similar patterns on standard IFA: the entire periphery of the bradyzoite was labelled (data not shown) in an IFA pattern identical to that obtained with tachyzoites and surface-specific MAbs (2). On immunoblots of bradyzoite lysates (Fig. 1), the antigens recognized by these MAbs had the following molecular masses (estimated under nonreducing conditions by comparison with the Pharmacia LMW standard): MAb T8 4A12, 36 kDa (lane b); MAb T8 2C2, 34 kDa (lane c); MAb T8 4G10, 21 kDa (lane e); MAb T8 3B1, 18 kDa (lane a). The antigens recognized by these MAbs will be referred to as Pb36, Pb34, Pb21, and Pb18, respectively. Only two of the MAbs also reacted with reduced samples: T8 4G10 and T8 3B1 (Pb21 and Pb18); little difference in molecular mass was found when the migrations of Pb21 and Pb18 were compared on reduced and unreduced gels (data not shown). The four MAbs obtained corresponded to major antigens recognized by polyclonal mouse anti-bradyzoite serum (Fig. 1, lane d). On some blots, depending on the amount of protein loaded, both Pb36 and Pb34 migrated as doublets or broad bands (Fig. 1), leading to molecular mass overlap between the two antigens. None of the antibodies reacted with uninfected mouse brain proteins (data not shown). The respective isotypes of the MAbs were as follows: T8 4A12, IgG1; T8 2C2, IgG1; T8 4G10, IgG1; T8 3B1, IgM.

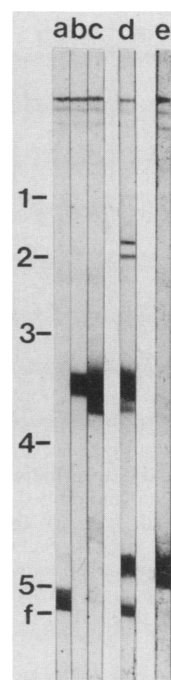

FIG. 1. Immunoblot of *T. gondii* cyst lysates probed with MAbs T8 3B1 (a), T8 4A12 (b), T8 2C2 (c), and T8 4G10 (e) and mouse serum taken prior to fusion (d). On this 12% acrylamide run, the 18-kDa antigen migrated with the front. The mobilities of standard proteins (Pharmacia LMW kit) were as follows: 1, 94 kDa; 2, 67 kDa; 3, 43 kDa; 4, 30 kDa; 5, 20 kDa; F, dye front.

Surface localization of immunofluorescence was detected for Pb36, Pb34, and P21 when the assay was performed on living zoites. No conclusive result was obtained with the Pb18-specific MAb.

Trypsin treatment of zoites before SDS-PAGE and immunoblotting (Fig. 2) eradicated reactivity with the Pb34 antigen (lane b) and almost of all of the reactivity with the Pb21 antigen (lane h), whereas reactivity with Pb36 and Pb18 underwent little variation. Pepsin had no effect on the antigens under the conditions used (which led to cyst rupture and bradyzoite freeing).

The reactivity of MAbs directed against the tachyzoite major surface antigens (2) was assayed on bradyzoites by fluorescence and immunoblotting (data not shown). Two MAbs reacted with bradyzoites and gave the same pattern as with tachyzoites (MAb T4 1F12, protein P43; MAb T4 2E12, protein P23). Three other MAbs specific for P30 (T4 1E5), P35 (T4 3F12), and P22 (T4 3G11) did not react with bradyzoites.

To confirm that the nonreactivity of the four bradyzoite-specific MAbs with RH tachyzoites was due to stage specificity and not to strain differences between 76K and RH, we

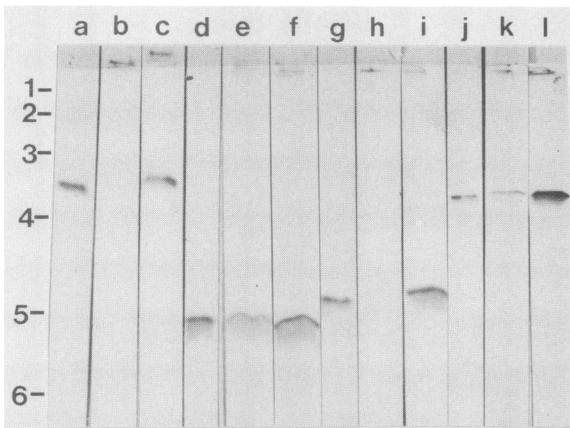

FIG. 2. Immunoblot of bradyzoites liberated from cysts by mechanical rupture (a, d, g, and j), trypsin treatment (b, e, h, and k), or pepsin treatment (c, f, i, and l) and probed with MAbs T8 2C2 (Pb34; a, b, and c), T8 3B1 (Pb18; d, e, and f), T8 4G10 (Pb20; g, h, and i), and T8 4A12 (Pb36; j, k, and l). Samples equivalent to equal numbers of bradyzoites were loaded into each well. The mobilities of standard proteins (Pharmacia LMW kit) were as follows: 1, 94 kDa; 2, 67 kDa; 3, 43 kDa; 4, 30 kDa; 5, 20 kDa; 6, 14.4 kDa.

investigated the reactivity of the MAbs with culture-grown 76K organisms. Immunoblotting showed faint reactivity of MAb T8 4A12 with parasites that were supposed to behave as tachyzoites and did indeed react with the five MAbs specific for tachyzoite surface proteins. IFA explained these findings by showing that two populations of zoites were found simultaneously in culture-grown 76K; i.e., a small number of parasites reacted with MAb T8 4A12 (and also with the other bradyzoite-specific antibodies), whereas most of the parasites were completely unreactive with bradyzoite-specific MAbs, as were RH tachyzoites.

The four bradyzoite antigens described here were also found on bradyzoites of a recent human isolate, which showed that they were not restricted to strain 76K (data not shown).

DISCUSSION

Although several previous reports (8, 12) have shown antigenic differences between bradyzoites and tachyzoites of *T. gondii*, very little is known about bradyzoite-specific molecules. Omata et al. (14) have produced a MAb that reacts with a 20-kDa protein located in the cytoplasm of bradyzoites. Kasper has obtained MAbs that react specifically with bradyzoites in an enzyme-linked immunosorbent assay, but the corresponding antigens could not be characterized (8).

Three of the bradyzoite proteins identified here behave as surface antigens, although not always meeting all of the criteria for surface localization. Pb36 was IFA^+, Pb34 was IFA^+ and trypsin sensitive, and Pb21 was IFA^+ and trypsin sensitive. Since tachyzoite antigens P43 and P23 were also found on bradyzoites, bradyzoites are likely to express at least five surface antigens. Pb18, which has peripheral distribution but does not fulfill any of the criteria for surface accessibility, is likely to be located deeper in the pellicle of bradyzoites.

Attempts at surface iodination or biotinylation of bradyzoites and study of phosphatidylinositol-specific phospholipase C sensitivity have been unsuccessful. This was due mainly to the low amounts of bradyzoites available and difficulties in purifying them completely from brain contamination. Further investigation will be necessary to determine whether bradyzoite-specific surface proteins possess a glycosylphosphatidylinositol anchor, as described for tachyzoite surface molecules (13, 18).

The results obtained with culture-grown 76K strain parasites confirmed the bradyzoite specificity of the MAbs raised during this study; they also confirmed the findings of Shimada et al. (17), Jones et al. (7), and Omata et al. (14), who described the simultaneous growth of tachyzoites and bradyzoites like organisms in vitro.

The possible relationship between one of the antigens described here and the 20-kDa antigen described by Omata et al. (5) is unclear; since Omata et al described the antigen as internal, it should not be identical to the 21-kDa antigen we have identified.

The different sensitivities of surface antigens to trypsin and pepsin under conditions that led to cyst rupture can be related to the results of Sharma and Dubey (16), who showed better preservation of bradyzoite viability in pepsin solutions. However, alteration of some of these molecules by trypsin does not seem to impair the infectivity of bradyzoites dramatically, since we used this procedure before successful infection of cell cultures.

These bradyzoite-specific probes will facilitate analysis of interconversion between acute and chronic stages of toxoplasmosis. The mechanism of interconversion can now be studied in vitro by using modulation of surface antigen expression as a marker of parasite differentiation.

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