Antibody Responses to *Toxoplasma* Antigens in Mice Infected with Strains of Different Virulence

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Swiss Webster mice were infected with either the relatively virulent C56 strain or the relatively avirulent C37 strain of *Toxoplasma gondii*, and the sequence of their antibody response to surface antigens of the parasite was studied. An immunoglobulin M (IgM) agglutinating antibody was the first serologically detectable antibody and was first detected on day 2 in mice infected with the C37 strain and on day 5 in mice infected with the C56 strain. IgG antibodies were first detected on day 8 for both strains. The major component of the IgG antibodies was IgG2; IgG3 antibodies had lower titers, and no IgG1 antibodies were detected. The IgG antibodies were active in direct parasite agglutination and in the complement-dependent cytotoxicity assay of Sabin and Feldman (Science 108: 660-663, 1948). On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, sera from mice infected with *T. gondii* detected all major radioiodinated surface proteins of toxoplasma tachyzoites. The earliest time point at which these antigens were detected differed for the two strains. Serum from mice infected with the C56 strain immunoprecipitated all cell surface antigens by day 10 of infection, whereas serum from mice infected with the C37 strain did not do so until day 15 of infection.

Despite the high titers of serum antibodies to toxoplasma observed after natural or experimental infection with *Toxoplasma gondii* in humans or animals, the role of these antibodies in resistance to infection has been the subject of considerable debate (J. S. Remington and J. L. Krahenbuhl, in A. J. Nahmias and R. O'Reilly, ed., *Immunology of Human Infections*, part II, in press). Antibodies in the presence of complement are effective in killing extracellular toxoplasmas in vitro (20). In addition, antibodies have also been shown to opsonize toxoplasma tachyzoites and thereby promote their killing by normal macrophages (1, 9). The appearance of toxoplasma antibodies of different classes in most infected humans follows the immunoglobulin M (IgM) to IgG sequence (19), which is common in infections with other organisms (7, 21). In rabbits, however, an almost concurrent appearance of IgM and IgG antibodies was observed by Strannegard (23). In mice, the main experimental laboratory animals used for studies of immunity to *T. gondii*, the sequence of development of toxoplasma antibodies and their isotypes has not been defined.

In view of the different biological functions of the various immunoglobulin isotypes (22) and

the different biochemical natures of the antigens that they recognize (17), we considered it of interest to study the time course of antibody development in mice infected with *T. gondii*. We examined the appearance of toxoplasma antibodies as detected by common immunodiagnostic assays, the dye test, the agglutination test, and immunofluorescence. Two strains of *T. gondii* of different virulence were used for infection to assess possible strain-dependent variations in the pattern of antibody development due to virulence of the parasite. Using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, we also attempted to identify the sequence in which parasite membrane antigens are recognized by IgG antibodies at various times after infection.

MATERIALS AND METHODS

Mice. Swiss Webster female mice (Simonsen Laboratories, Gilroy, Calif.) were 6 to 8 weeks old at the time of the experiments.

Infection. Mice were injected subcutaneously with 2×10^4 tachyzoites of either the C56 strain (relatively virulent, 50% lethal dose = 5×10^4) or the C37 strain (relatively avirulent, 50% lethal dose = 5×10^5) of *T. gondii*. Tachyzoites of these strains were obtained from the peritoneal cavities of infected mice. Briefly, homogenized brain tissue containing cysts was injected intraperitoneally into mice. After 5 days, tachyzoites

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were collected from the peritoneal cavities of the infected mice. Debris and host cells were removed by filtration through a polycarbonate membrane (pore size, 3 μ m; Nuclepore Corp., Pleasanton, Calif.). The numbers of organisms used for infection cause mild disease but no mortality in Swiss Webster mice. Antimicrobial agents were not used. Animals were bled just before infection and at various times thereafter, as described below. The day of infection was designated as day 0. Sera from groups of mice were pooled and stored at -20°C until used.

SDS-polyacrylamide gel electrophoresis. One-dimensional gel electrophoresis of toxoplasma antigens was performed as previously described (E. Handman, J. W. Goding, and J. S. Remington, J. Immunol., in press). In brief, tachyzoites of the RH strain were harvested in phosphate-buffered saline (PBS), pH 7.4, from the peritoneal cavities of mice infected for 2 days. Debris and host cells were removed as described above, and the parasites were washed three times in PBS, pH 7.4. Viability was greater than 95%, as assessed by staining with acridine orange and ethidium bromide (16). Tachyzoites (5×10^7 in 200 μ l of PBS, pH 7.4) were radioiodinated by the lactoperoxidase technique (13) with 1 mCi of ^{125}I (sodium iodide; Amersham Corp., Arlington Heights, Ill.) and 10- μ l pulses of H_2O_2 (0.3, 1, 3, and 9 mM) (Eastman Kodak, Rochester, N.Y.) at 2-min intervals at 20°C . Data obtained in separate experiments in our laboratory have demonstrated that the radiolabeled proteins detected by electrophoresis are integral membrane proteins (E. Handman et al., in press). The cells were then washed twice and lysed in 0.5% Nonidet P-40 (Particle Data, Inc., Elmhurst, Ill.) in PBS. The detergent extract was incubated for 1 h at 4°C with sera collected from mice at various times during infection. All sera were inactivated for 30 min at 56°C . Immune complexes were precipitated on protein A bearing *Staphylococcus aureus* strain Cowan 1 (New England Enzyme Center, Boston, Mass.), as described by Kessler (10). The washed pellets of bacteria were boiled in SDS, and one-dimensional polyacrylamide gel electrophoresis was performed as described by Laemmli (11), using 13% acrylamide. Molecular weight standards (bovine serum albumin, ovalbumin, lactate dehydrogenase, immunoglobulin light chains, and lysozyme) were run on each slab gel, and the resulting Coomassie brilliant blue-stained bands were used to determine the approximate molecular weights of bands on the autoradiograms. In other experiments, radiolabeled proteins were electrophoresed in two dimensions by the technique of O'Farrell et al. (15). For separation in the first dimension, samples were run in a non-equilibrium pH gradient in thin glass tubes (8). Separation in the second dimension was done on an SDS-polyacrylamide slab gel, as described above. For identification of molecular weight standards, gels were stained in 0.1% Coomassie brilliant blue R-250 in 50% trichloroacetic acid, destained in 7% acetic acid, dried, and autoradiographed with Kodak X-Omat R film and Du Pont Cronex Lightning-Plus intensifying screens (12). The exposure time was 24 h.

Serological tests. All sera were tested in parallel at the same time in each of the assays.

The Sabin-Feldman dye test was performed with a micromodification of the technique described by Sabin

and Feldman (20). This micromodification is routinely used as a diagnostic test for human and animal sera in our laboratory. The serum to be tested is diluted two- or fourfold in PBS in round-bottom microtiter wells (Dynatech Laboratories, Alexandria, Va.). A 50- μ l volume of a suspension of 2×10^6 of living tachyzoites per ml in PBS, pH 7.2, containing 40% normal human serum (as the source of complement) is added to each well. The microtiter trays are shaken for 45 s on a Microshaker II (Dynatech Laboratories) and incubated for 1 h at 37°C . Then, 50 μ l of 1% methylene blue in borate buffer, pH 10.8, is added to each well. The trays are shaken again and allowed to stand for 10 min at room temperature before reading the test.

Preparation of antigen for the agglutination test and performance of the test were as previously described (4). In brief, sera were diluted two- or fourfold in round-bottom microtiter trays in PBS, pH 7.2, or in PBS containing 2-mercaptoethanol (2ME; Eastman Kodak) at a final concentration of 0.1 M. To each well was added 50 μ l of a specially prepared suspension of Formalin-fixed tachyzoites (4×10^8 /ml). The trays were shaken for 5 min on a Microshaker II and allowed to settle overnight at room temperature. Normal mouse serum served as the negative control, and a pool of sera from chronically infected mice served as the positive control. When human serum is assayed at this concentration of 2ME, complete ablation of IgM antibody activity occurs.

The indirect fluorescent-antibody (IFA) test was performed as described by Walton et al. (24). To eliminate a high background of fluorescence due to the presence of toxoplasma IgM antibodies on the surface of parasites harvested from the peritoneal cavities of mice infected for 2 days, mice were treated subcutaneously with 200 mg of cyclophosphamide (Cytoxan; Mead Johnson & Co., Evansville, Ind.) per ml 2 days before intraperitoneal infection with the RH strain of *T. gondii*. Tachyzoites were collected from the peritoneal cavities of these mice infected for 2 days, washed in PBS, and fixed in 1% Formalin. Slide preparations of these parasites were stored at -20°C until used.

Preparation of specific antisera. Specific antisera to heavy chains of mouse immunoglobulins were produced by immunization of rabbits with Fc fragments of myeloma proteins MOPC-21 (G1 κ) and HOPC-1 (G2a λ) and with intact molecules of Y-5605 (G3 λ). Anti-IgM antibodies were produced against the myeloma protein HPC-76 (μ κ).

The anti-IgG1 antiserum was purified before conjugation to fluorescein isothiocyanate by passage over a series of immunoabsorbents containing the following myeloma proteins bound to cyanogen bromide-activated Sepharose (Pharmacia Fine Chemicals Inc., Piscataway, N.J.): GPC-8 (G2a κ), MPC-11 (G2b κ), Y-5605 (G3 λ), HPC-76 (μ κ), and normal mouse F(ab) fragments. The anti-IgG2 antiserum was absorbed against MOPC-21 (G1 κ), Y-5605 (G3 λ), HPC-76 (μ κ), and normal mouse F(ab). The anti-IgG3 antiserum was absorbed against MOPC-21 (G1 κ), HOPC-1 (G2a λ), MPC-11 (G2b κ), and HPC-76 (μ κ).

Fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, Mo.)—conjugated rabbit anti-mouse IgM and rabbit anti-mouse IgG1, IgG2, and IgG3 were prepared as described by Goding (6). The sera were

found to be specific by double diffusion in agar and by immunofluorescence. Using monoclonal antibodies of the IgG2 and IgG3 subclasses, which are directed against membrane and cytoplasmic antigens of toxoplasmas (E. Handman and J. S. Remington, Immunology, in press), and the chain-specific fluorescent antibodies, we found the staining restricted to the correct subclass, with no cross-reactions.

RESULTS

The various antibodies produced in mice infected subcutaneously with either the C37 or C56 strain of *T. gondii* are shown in Tables 1 and 2. The first detectable toxoplasma antibody was an IgM which was reactive in the IFA and agglutination tests. In the agglutination test, antibody was detected as early as day 2 of infection in mice infected with the C37 strain and by day 5 in mice infected with the C56 strain. When this test was performed in the presence and absence of 2ME, a titer decrease was noted in

the early time points examined, suggesting the presence of IgG antibodies.

Antibodies of the IgG2 and IgG3 subclasses were detected in the IFA test on day 8 of infection. The dye test, which measures primarily IgG antibodies, became positive on the same day. IgG1 antibodies were not detected in any of the mice.

In mice infected with the C56 strain, relatively high titers of IgG antibodies appeared on day 8 and were mostly of the IgG2 subclass. IgG2 antibody titers remained remarkably stable during the 50 days of observation when compared with the titers of IgG3 antibodies or with the titers in the agglutination and dye tests, which rose gradually and plateaued on day 15 or 20 of infection.

In mice infected with the C37 strain, IgG antibodies were first detected in the IFA test on day 8, and their titers increased more gradually than those in mice infected with the C56 strain.

TABLE 1. *Toxoplasma* antibody titers in Swiss Webster mice during infection with the C56 strain of *T. gondii*

Day of infection	Dye test	Antibody titer					
		Agglutination test		IFA test			
		+2ME	-2ME	IgM	IgG1	IgG2	IgG3
0	— ^a	—	—	—	—	—	—
1	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—
5	—	16	—	16	—	—	—
8	128	16	256	256	—	4,096	—
10	128	16	256	4,096	—	4,096	16
15	1,024	256	16,000	4,096	—	1,024	64
20	1,024	256	4,096	1,024	—	1,024	400
28	6,400	400	6,400	1,600	—	6,400	400
43	25,600	25,600	25,600	6,400	—	6,400	400
50	12,800	25,600	25,600	1,600	—	6,400	400

^a —, Negative when diluted 1:4 or undiluted.

TABLE 2. *Toxoplasma* antibody titers in Swiss Webster mice during infection with the C37 strain of *T. gondii*

Day of infection	Dye test	Antibody titer					
		Agglutination test		IFA test			
		+2ME	-2ME	IgM	IgG1	IgG2	IgG3
0	— ^a	—	—	—	—	—	—
2	—	8	16	2	—	—	—
4	—	4	8	16	—	—	—
6	—	16	32	16	—	—	—
8	4	8	64	256	—	4	—
10	32	64	128	256	—	64	100
14	512	256	4,096	2,560	—	10,240	6,400
16	512	400	10,000	1,600	—	1,600	400
20	2,048	1,600	25,600	1,600	—	6,400	400
35	25,600	400,000	100,000	6,400	—	25,600	400
57	12,800	100,000	100,000	6,400	—	25,600	400

^a —, Negative when diluted 1:4 or undiluted.

The IgM titers also increased more gradually in these mice.

In an attempt to identify the antigens recognized by toxoplasma IgG antibodies at various times after infection, radiolabeled toxoplasma membrane antigens were immunoprecipitated with sera from infected mice and electrophoresed on SDS-polyacrylamide gels. Figure 1A shows the relatively simple pattern of radioiodinated proteins on the membrane of toxoplasma tachyzoites. Four major bands and some additional minor spots were identified on two-dimensional gels (Fig. 1A). Each of the four major radioiodinated proteins seen when the whole extract was run on a two-dimensional gel (Fig. 1A) was recognized by antibodies from mice chronically infected (on day 50 of infection) with the C37 (Fig. 1B) or C56 (Fig. 1C) strain of *T. gondii*. Only a few of the minor spots were precipitated by mouse antibodies. Toxoplasma membrane proteins (p) have been designated according to their approximate molecular weights: p43,000, p35,000, p27,000, and p14,000 (E. Handman et al., in press).

IgG antibodies from mice infected with the C56 strain detected the four radiolabeled membrane proteins of toxoplasmas on day 8 of infection (Fig. 2, lane C), whereas antibodies from mice infected with the C37 strain immunoprecipitated these antigens only on day 10 of infection (Fig. 2, lane E). The relative intensities of the antigen bands varied; p43,000 and p27,000 were the most prominent when serum from mice infected for 10 days with the C37 strain was used for immunoprecipitation (Fig. 2, lane E),

whereas p43,000 was the prominent band precipitated by serum from mice infected for 8 days with the C56 strain (Fig. 2, lane C).

The time of appearance of IgG antibodies as detected by immunoprecipitation and electrophoresis correlated remarkably well with the time of their detection by the serological assays.

DISCUSSION

Our studies on the time course of antibody response in mice infected with the C37 and C56 strains of *T. gondii* revealed that the appearance of IgM antibodies preceded that of IgG antibodies. (IgM antibodies appear before IgG antibodies in humans [18] but were found to appear almost concurrently in rabbits [23].) In mice, we found that these IgM antibodies were not transient but persisted for at least 50 days, by which time the infection was in its chronic stage. During the chronic stage, there is a permanent residual parasite load and possibly continuous recruitment of new B-cell clones.

IgG antibodies were detected at the same time by the IFA test and dye test. The agglutination test, performed with and without 2ME, appeared to detect IgG antibodies much earlier than did the IFA test in mice infected with the C37 strain. The agglutination test is exquisitely sensitive for detection of IgM antibodies, and it is possible that it detected IgM antibodies that were less susceptible to reduction with 2ME and had not been reduced. Nonspecific (2ME-sensitive) agglutination has been observed in humans devoid of IgG antibodies detectable by either the IFA or dye test (3).

FIG. 1. Two-dimensional polyacrylamide gel electrophoresis patterns of ^{125}I -labeled toxoplasma antigens bound by antibodies from mice chronically infected with the C37 and C56 strains of *T. gondii*. (A) Proteins in whole lysate; (B) proteins precipitated by mouse anti-strain C37 antibodies; (C) proteins precipitated by mouse anti-strain C56 antibodies. Numbers represent the following molecular weight standards: bovine serum albumin (68,000), ovalbumin (43,000), lactate dehydrogenase (30,000), and immunoglobulin light chains (22,000).

FIG. 2. One-dimensional polyacrylamide gel electrophoresis patterns of ^{125}I -labeled toxoplasma antigens bound by antibodies from mice infected with the C56 strain of *T. gondii* at (A) day 20, (B) day 15, and (C) day 8 of infection or with the C37 strain of *T. gondii* at (D) day 8, (E) day 10, and (F) day 15 of infection. (G) Proteins that had bound to staphylococci (no antiserum). Numbers represent the following molecular weight standards: bovine serum albumin (68,000), ovalbumin (43,000), immunoglobulin light chains (22,000), and lysozyme (14,000).

Antibodies of the IgG1 subclass were not detected in mice infected with either strain of *T. gondii*. This is of interest in view of the inability of this IgG subclass to fix complement and to bind to macrophages (22). IgG1 may be a blocking antibody (5) and may have a possible parasite-protective effect on the larval cestode *Me-socestoides corti* (14). One of the notable features of the analysis of IgG subclasses associated with toxoplasma infection was the presence of the greatest antibody activity in the IgG2 subclass, which is known to be particularly active in classical complement fixation and in binding to phagocytic cells (22). Another interesting feature of this analysis was the presence of antibodies of the rare IgG3 subclass. Recruitment of IgG3-producing clones by bacterial carbohydrate antigens has been documented by Perlmutter et al. (17). The biological function of IgG3 is not clearly understood, but it may be relevant in antibacterial immunity.

We demonstrated that IgG antibodies to all four radiolabeled membrane proteins detectable on toxoplasma tachyzoites are produced simultaneously in the infected mice. However, the relative intensity of the protein bands detected by autoradiography of one-dimensional SDS-polyacrylamide gels varied. This variability may be due to a quantitative difference in the amount of antibody produced against each antigen at any one time, as well as to a different affinity and avidity of each antibody. The time of ap-

pearance of toxoplasma antibodies detectable by serological assays correlated remarkably well with the time of antigen detection by SDS-polyacrylamide gel electrophoresis.

It is of interest that antisera to the C56, RH, and C37 strains recognize the same membrane antigens on tachyzoites of the RH strain, thus supporting the concept that *T. gondii* is one species with strains of various degrees of virulence (2; E. Handman et al., in press).

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