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Toxoplasma Antigens Recognized by Naturally Occurring Human Antibodies

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Sera of most adults have high agglutination test titers to *Toxoplasma gondii* whether or not the adults have other serological evidence of the infection. This finding has been attributed to the presence of naturally occurring antibodies to *T. gondii*. Consistent with this observation, we have recently noted that protein blots (PB) of sera of individuals not previously infected with *T. gondii* had immunoglobulin G (IgG) and IgM antibodies to antigens of the parasite. To further define the antigens recognized by these naturally occurring antibodies, we studied PB of sera of 44 adults and 9 children who had no serological evidence of the infection. Multiple antigens of *T. gondii* with molecular weights of 15,000 to greater than 205,000 were recognized by IgG and IgM natural antibodies of each of the sera. Although a relatively consistent pattern was noted on the IgM PB of the sera of the adults in the molecular weight range of 48,000 to 85,000, greater heterogeneity was noted on the IgG PB. The most common bands noted on the latter were of approximately 30,000 and 92,000 molecular weight. All of the PB obtained with the serial sera collected at yearly intervals from the children revealed bands; in some cases, new bands had appeared with time, and in others the pattern was constant. In children older than 8 years, the patterns of the PB were similar to those noted in PB of sera of the adults.

The diagnosis of toxoplasmosis is most often established on the basis of serological test results. False-positive results occur in certain of these tests with sera which are negative when tested by the highly specific Sabin-Feldman dye test (DT) (22). Fulton and Turk (11) noted that some patients developed low titers in the *Toxoplasma* agglutination test (AG) after febrile diseases other than toxoplasmosis. Subsequently, low titers of agglutinins and complement-fixing antibodies were noted in sera of individuals who were negative when tested with the DT (9, 10). Using the AG, Desmonts et al. (4) demonstrated that natural antibodies to *Toxoplasma gondii* are present in sera of almost all adults and in sera of infants older than 3 months. Reactivity of these antibodies in the AG was abolished after incubation of the sera with 2-mercaptoethanol (2ME), and it was concluded that they were of the immunoglobulin M (IgM) class (4). Additional evidence for the existence of natural antibodies comes from fluorescence or electron microscopy studies in which localized but intense staining on tachyzoites was observed when the killed organisms were incubated with sera from uninfected persons (3, 6, 7, 15, 27, 28). Studies to define the nature of these antibodies confirmed that they are mostly of the IgM class (30), are occasionally of the IgG class (8), and are rarely found in newborns and children younger than 6 months.

The origin(s) of the antigenic stimuli which account for production of these naturally occurring antibodies is unknown. Some studies have provided evidence of antigenic similarities between *T. gondii* and other organisms. However, these organisms either are not human pathogens or have only rarely been described in humans (1, 2, 20, 26).

In studies of the antigenic structure of *T. gondii*, we have consistently observed that sera which are negative in the DT and double-sandwich IgM enzyme-linked immunosorbent assay (IgM ELISA) (21) react with multiple antigens on both IgG and IgM protein blots (PBs) (I. Potasman, F. G. Araujo, G. Desmonts, and J. S. Remington, J. Infect. Dis., in press). We therefore considered it of interest to investigate the nature of the antigens recognized by these naturally occurring antibodies.

MATERIALS AND METHODS

***T. gondii* antigen.** The *T. gondii* antigen was prepared as previously described (24).

Polyacrylamide gel electrophoresis. Electrophoresis was performed on 5 to 15% gradient slab gels with the discontinuous buffer system described by Laemmli (19). Myosin, β-galactosidase, phosphorylase *b*, bovine albumin, egg albumin, and carbonic anhydrase (Sigma Chemical Co., St. Louis, Mo.) were used as molecular weight (MW) markers. Gels were run at 20 mA per gel at room temperature until the tracking dye reached the bottom of the gel.

Protein blotting. Transfer of proteins to nitrocellulose paper (0.45-μm-pore size; Schleicher & Schuell, Inc., Keene, N.H.) was performed by the method of Towbin et al. (29) with a Trans-Blot-Cell (Bio-Rad Laboratories, Richmond, Calif.). Proteins were transferred at 180 mA over 3 h.

Immunochemical detection of antigens. The serum of each patient was tested with a strip (1.4 by 14 cm) cut from a nitrocellulose blot (10 by 14 cm) containing the separated *T. gondii* antigens. The strips were incubated overnight with the appropriate serum diluted 1:50 in phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 20 (solution A). After incubation, the strips were washed five times for 5 min each time with phosphate-buffered saline–0.05% Tween 20. Thereafter, strips were cut in half longitudinally and overlaid with horseradish peroxidase-conjugated rabbit anti-human IgG or with IgM antibodies (Cooper Biomedical, Inc., West Chester, Pa.). Each conjugate was diluted 1:1,000 or 1:8,000 in solution A and allowed to react with the strips for 1 h. The strips were again washed as described above and immersed in the substrate

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TABLE 1. Serological test results in 15 adults without serological evidence of infection with *T. gondii*[a]

Patient (sex)[b]	AG titer without 2ME	IgM IFA tips[c]
1 (F)	640	+1
2 (F)	≥2,560	+2
3 (M)	640	0
4 (M)	1,280	+3
5 (M)	≥2,560	+1
6 (M)	640	+1
7 (M)	1,280	+1
8 (M)	≥2,560	+2
9 (M)	≥2,560	+1
10 (F)	≥2,560	+2
11 (F)	1,280	+1
12 (F)	≥2,560	+2
13 (M)	160	0.5
14 (M)	1,280	+1
15 (F)	1,280	+2

[a] The adults were all negative when tested in the AG (with 2ME), DT, and double-sandwich IgM ELISA.
[b] F, Female; M, male.
[c] IgM IFA tips, IgM immunofluorescent antibody confined to the tips of tachyzoites. See Materials and Methods for explanation of scoring system.

solution (0.1 mg of diaminobenzidine [Litton Bionetics, Charleston, S.C.] per ml plus 0.25% H_2O_2 in phosphate-buffered saline) for 15 min. To test for possible binding of the conjugate directly to the antigen, controls were run in which both IgG and IgM conjugates diluted 1:500 in solution A were allowed to react overnight with the blots and were then developed with diaminobenzidine. No bands were seen in these blots (data not shown).

Sera. Sera were from 15 normal healthy adults, 29 adults for whom *Toxoplasma* serology was requested because toxoplasmosis was suspected, a child who was apparently infected with *T. gondii* soon after birth, and 9 children with various malignancies. The last group was chosen because follow-up sera were available and because we considered it of interest to follow the evolution of the PB patterns. The follow-up sera were tested in parallel with the earlier sera from each patient by using nitrocellulose strips cut from the same blot.

Serological tests. To exclude prior exposure to *T. gondii* and for the purpose of this study, all sera were tested in the AG with and without 2ME (10, 11), DT, IgM ELISA (Potasman et al., in press), and IgM indirect fluorescent-antibody test (IFA) (31). Sera were considered negative for specific antibodies if the DT titer was negative in the undiluted (1:2) serum. A serum negative in the AG was nonreactive at dilutions of 1:20 or higher. All sera had negative IgM ELISA titers. Some sera showed polar (tip) staining in the IgM IFA. This staining was recorded and graded on a scale of 0 to 4+. The IFA slides were read independently by two observers, and the final score represents the mean of the two readings. The sera of the 15 healthy adults were screened for rheumatoid factor to exclude the possibility of interference in the IgM IFA (16).

RESULTS

Healthy adults. Each of the 15 healthy adults (9 men, 6 women) was negative in the DT, in the IgM ELISA, in the AG (with 2ME), and for rheumatoid factor. Sera of 14 of them gave tip reactions in the IgM IFA (Table 1). We were unable to demonstrate a significant correlation between the AG (without 2ME) and tip reactions.

The PBs of 7 of the 15 individuals are shown in Fig. 1. Each of the PBs contained some bands; the intensity of the staining and the MWs of the bands varied from patient to patient. The IgG PBs appeared to have a higher patient-to-patient variability than did the IgM PBs. Bands in the PBs spanned MWs from approximately 15,000 to greater than 205,000.

Analysis of the IgG PBs of the 15 individuals revealed the most common bands to be located at approximate MWs of 22,000 in 6, 27,000 in 5, 30,000 in 10, 35,000 in 7, 48,000 to 50,000 in 5, 68,000 in 8, 92,000 in 10, and 120,000 to 125,000 in 6 patients. There was no band which appeared consistently in the PBs of the 15 individuals. Analysis of the IgM strips revealed a more homogenous pattern; the MWs of the most common bands were 10,000 to 14,000 in 6, 30,000 in 10, doublet at 48,000 to 50,000 in 12, 56,000 to 60,000 in 9, 66,000 in 9, 80,000 to 85,000 in 15, 105,000 in 15, and 115,000 in 13 patients. When the differences in intensity of bands were marked (patients 3 and 4, corresponding to strips 3 and 4 in Fig. 1), the paucity or abundance of bands on the IgM strips from these patients correlated with the intensity of the tip reactions and to some degree with the results of the AG (without 2ME).

Seronegative adults. PBs (not shown) of the 29 patients with suspect acute toxoplasmosis, who were negative by conventional serology, revealed patterns similar to those obtained in the 15 individuals described above.

Children. Each of the serum samples of the nine children was negative in the DT, AG (with 2ME), and IgM ELISA. The AG (without 2ME) titers ranged from 1:20 to 1:2,560 or higher (Table 2). Of the children from whom more than one serum sample was available, three of six had a rise in titer in the AG (without 2ME) and four of six had a rise in the score of the tip reaction in the IgM IFA.

The patterns noted on the PBs of patients C to I are shown in Fig. 2 and 3. The PBs of patients A and B are not shown, because the bands were too faint to reproduce. Bands were present on the IgG and IgM blots of each of the children and tended to be fainter in the younger group and to increase in intensity with age. For example, in Fig. 2, strips 1 to 3

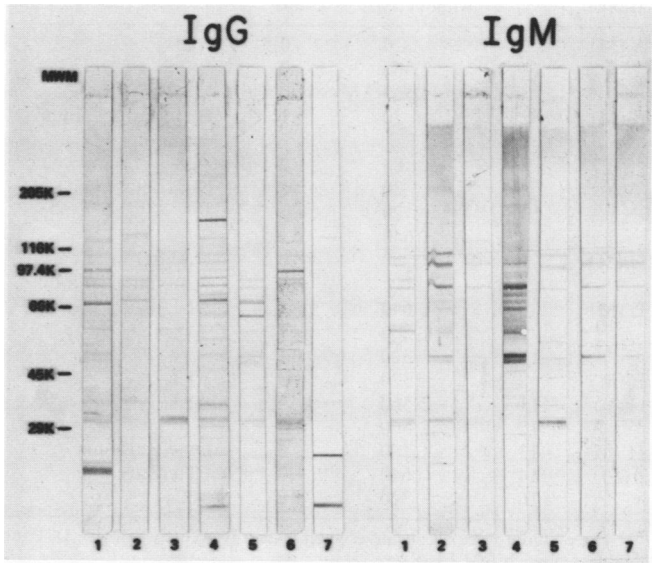

FIG. 1. PBs of sera of seven healthy adults (strips 1 through 7 correspond to patients 1 to 7 in Table 1). MW markers (MWM) are on the left. See Table 1 for serological test results. K, 10^3.

(patient C) revealed at least three very faint bands on the IgG strip (1) obtained with the first serum sample. Addition of a new band (below the 29,000 MW marker) and intensification of existing bands (at approximately 85,000 MW) were noted with subsequent serum samples over a period of almost 2 years. Intensification of bands was also noted in the IgM blots of the same child; at 4 years of age for the child, a strongly staining band was found at the approximate MW of 30,000. In contrast, in Fig. 2, strips 4 to 6 (patient E), a major change in IgG or IgM PB pattern with time did not appear to occur.

The IgG blots of the nine children showed considerable variability in the number and distribution of bands. The following bands were most common: a doublet at approximate MWs of 85,000 and 80,000 was found in 13 of 18 IgG strips, a 50,000-MW band was found in 11 of 18, and a wide, but faint band of 30,000 MW was found in 5 of 18. In contrast, the patterns on the IgM strips were remarkably similar, especially in children older than 8 years. This pattern (Fig. 3) included a dominant band with an approximate MW of 88,000 (noted in all nine children), a band at 105,000 in eight of nine, and a doublet at 50,000 to 52,000 in strips of all four children older than 8 years. In addition, weakly staining bands on the IgM PB were found at 70,000 MW in six of the strips and at 30,000 MW in at least four of the strips. The patterns found on the IgM strips of the older children were remarkably similar to the ones found on the strips developed with sera obtained from adults.

Acutely infected child. This patient (Fig. 4) was included in this study even though he was infected with *T. gondii* soon after birth. The first serum sample from his mother was obtained on the day of delivery (strip 6, Fig. 4). At 3 months later, the patient became positive in the DT and IgM ELISA. Serum samples used for developing strips 1 through 5 were drawn at weeks 16, 23, 30, 35, and 155 after birth. The corresponding DT titers were (reciprocals) 8,000, 2,048, 2,048, 1,024, and 512. The respective IgM ELISA titers were 8.9, 6.6, 2.4, 0.8, and 0.0. Bands in his IgM strips corresponding to those detected by naturally occurring antibodies appeared by approximately 3 years of age (strip 5 in the IgM blot). There was a remarkable similarity of the band pattern in the MW range of 48,000 to 85,000 on this strip with the pattern in the corresponding area on strips 6 to 8 obtained with the serum of his mother, the negative control, and a case of acute toxoplasmosis (strip 8 in the IgM blot).

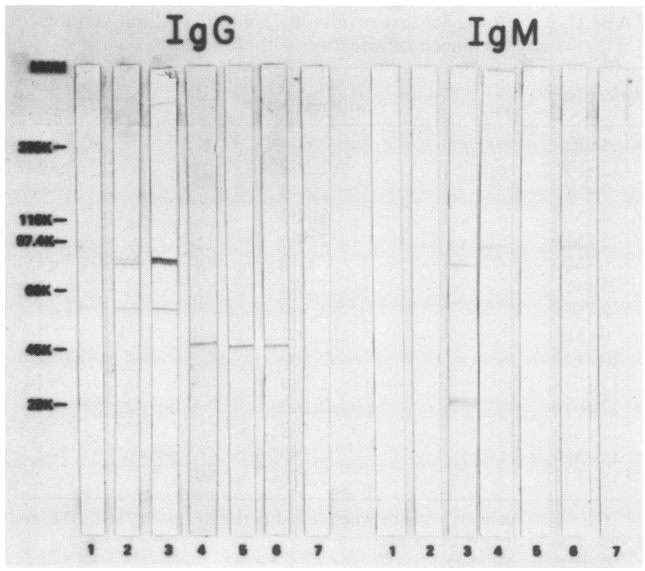

FIG. 2. PBs obtained with sera (see Table 2 for details) of three children. Strips 1 to 3 were developed with sequential sera of patient C; strips 4 to 6 were developed with sequential sera of patient E; strip 7 is from patient D. MW markers (MWM) are on the left. K, 10^3.

TABLE 2. Serological test results in nine children without serological evidence of infection with *T. gondii*[a]

Patient (sex)[b]	Age (yr)	AG titer without 2ME	IgM IFA tips[c]	Accompanying condition
A (M)	1	320	0.5	ALL[d]
	2	2,560	0	
	3	2,560	0.5	
B (M)	2	20	+1	Endodermal sinus tumor
	3	80	+2	
C (M)	2	20	0.5	Neuroblastoma
	3	20	+1	
	4	640	1.5	
D (M)	5	80	0.5	Undifferentiated pulmonary sarcoma
E (M)	8	640	+1	ALL
	9	1,280	+1	
	10	320	+1	
F (M)	8	320	+3	ALL
G (F)	11	≥2,560	+2	ALL
	12	≥2,560	+3	
H (M)	13	1,280	+3	Osteosarcoma
I (F)	14	640	+2	Osteosarcoma
	16	320	+2	

[a] The children were all negative when tested by AG (with 2ME), DT, and double-sandwich IgM ELISA.
[b] M, Male; F, female.
[c] IgM IFA tips, IgM immunofluorescent antibody confined to the tips of tachyzoites. See Materials and Methods for explanation of scoring system.
[d] ALL, Acute lymphoblastic leukemia.

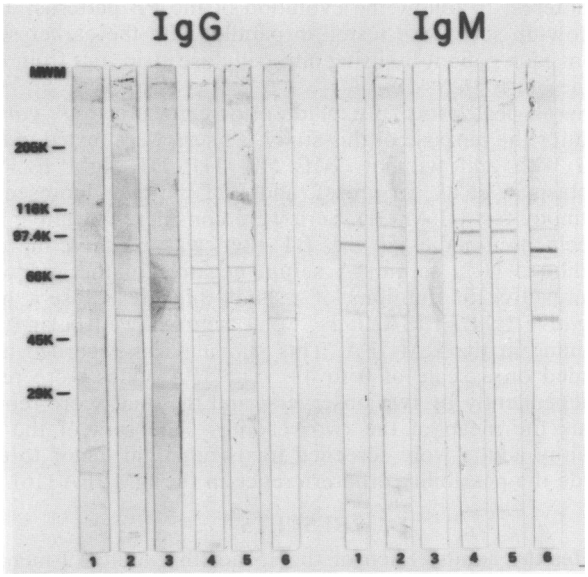

FIG. 3. PBs obtained with sera (see Table 2 for details) of four children. Strips 1 and 2 were developed with sequential sera of patient I; strip 3 is from patient H; strips 4 and 5 were developed with sequential sera of patient G; strip 6 is from patient F. MW markers (MWM) are on the left. K, 10^3.

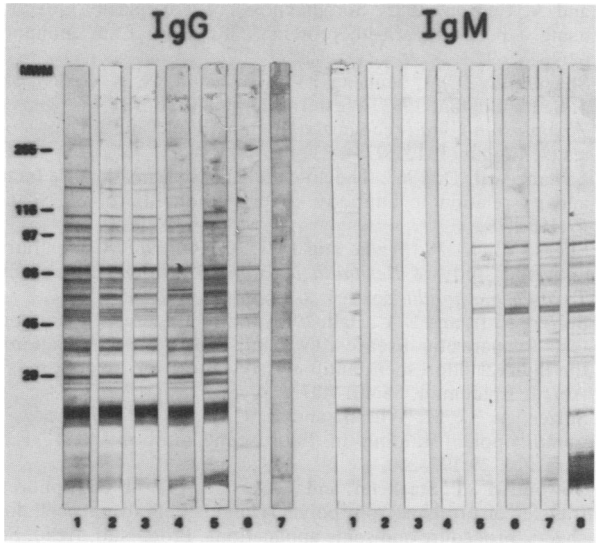

FIG. 4. PBs obtained with sera of a child who acquired acute *T. gondii* infection after birth. See Results for details. Strips 1 through 5 were developed with sequential sera of this child drawn at 16, 23, 30, 35, and 155 weeks of age; strip 6 was developed with serum from the child's mother; strip 7 was developed with the negative pool; strip 8 (IgM only) is of a case of acute toxoplasmosis in an adult. Note the similarities in the middle portions of strips 5 to 8 on the IgM blot. MWM, MW markers.

DISCUSSION

The results described above demonstrate that sera of individuals who have not been infected with *T. gondii* contain antibodies which react with a variety of antigens of the parasite in PBs. Antibodies of both the IgG and IgM class and mainly directed against antigenic moieties with MWs of 15,000 to 205,000 were found in the PB of each of the 44 adults and 9 children we examined.

The patterns of the IgG PB varied considerably among individuals, whereas a more consistent pattern was noted on the IgM PB. There was considerable variation in intensity of individual bands in PBs obtained with serum of each individual, and the intensity of reactions to the same antigen differed with sera of different individuals, even though the PBs were processed with strips from the same piece of nitrocellulose paper. The most common bands on the IgM PB were located at approximate MWs of 30,000, 48,000 to 50,000 (usually a doublet), 105,000, and 115,000.

The finding that sera of uninfected individuals react with the 30,000-MW band seems especially important in view of the recent reports of the use of a monoclonal antibody against the 30,000-MW antigen for diagnosis of toxoplasmosis (5, 23). The 30,000-MW antigen is a cell membrane constituent first described by Handman et al. (13). This antigen, recognized by monoclonal antibody 1E11 (13, 14), has been shown to be identical to the antigen referred to as P30 by Kasper et al. (18), Dubremetz et al. (5), and Santoro et al. (23).

Also of interest was the frequent finding of bands in the MW region of 105,000 and 115,000. We have recently demonstrated (Potasman et al., in press) that after acute infection, the intensity of staining of these and other bands increases remarkably. In that study, we also noted that individuals whose sera did not reveal these bands on their PBs before infection acquired them soon after they became infected. Our data suggest that these antibodies persist throughout life. Insofar as the half-life of IgM antibodies is brief, it has to be assumed that a persistent antigenic stimulus exists to account for the continued presence of the naturally occurring IgM antibodies.

A common pattern seen on the IgM PBs of some adults was remarkably similar to the patterns seen with sera from some of the older children, indicating that by adolescence these antibodies have already been acquired. Furthermore, a similar pattern was observed in the PB obtained with serum from a 3-year-old child who had been acutely infected with *T. gondii* 3 years earlier. The IgM PBs of the sequential sera obtained from this child revealed that while he lost the strongly staining band located below the 29,000-MW marker, he acquired a set of bands in the MW area of 48,000 to 85,000; this set is remarkably similar to that displayed on the PB of his uninfected mother and to the set seen on the PB of the negative serum pool.

The ideas that normal human serum might contain nonspecific antibodies against *T. gondii* and that these antibodies create what was referred to as a *Toxoplasma*-hostile environment were entertained by various investigators over the past three decades (4, 11, 12, 17, 25). Further characterization of the antigens which react with naturally occurring antibodies should facilitate our understanding of the immunology and pathogenesis of *T. gondii* infection.

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