Antigen(s) Responsible for Immunoglobulin G Responses Specific for the Acute Stage of *Toxoplasma* Infection in Humans

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An agglutination test for immunoglobulin G (IgG) *Toxoplasma* antibodies with acetone-fixed tachyzoites (AC antigens; AC agglutination test) was positive only with sera from patients during the acute stage of their infection. In contrast, when the test was performed with Formalin-fixed tachyzoites (HS antigens; HS agglutination test), positive results were obtained during both the acute and chronic (latent) stages of the infection. Studies were performed to define the antigen(s) of *T. gondii* which are detected by IgG antibodies present only during the acute stage of the infection. Sera of mice immunized with AC antigens recognized predominantly 10 antigens of tachyzoites by immunoblot analysis. Sera from individuals with the acute but not chronic infection reacted strongly with these same 10 antigens in immunoblots. Sera of mice immunized with HS antigens recognized more *Toxoplasma* antigens on immunoblots than did mouse AC antibodies. Absorption of the latter with HS antigens removed detectable reactivity of AC antibodies in immunoblots and in the AC and HS agglutination tests, suggesting that AC antigens are a selected portion of HS antigens. AC antigens were specific for tachyzoites in that AC antibodies reacted with the cell membranes of tachyzoites but not those of bradyzoites, whereas HS antibodies reacted with cell membranes of both forms of the organism in an indirect fluorescent-antibody test. Tachyzoite-specific antigen(s) appear to be useful in differentiating between the acute and chronic stages of *Toxoplasma* infection through their detection by IgG antibodies.

The high prevalence of *Toxoplasma* antibodies in human populations and the fact that titers may remain elevated for years following the acute infection have complicated the interpretation of serologic test results obtained in individuals suspected of having acute toxoplasmosis. Until recently, no serological test for immunoglobulin G (IgG) antibody, when used alone, could differentiate between recently acquired infection and chronic (latent) infection with a single serum sample. In 1986, Thulliez et al. (13) reported a new antigen preparation which could accomplish this. These authors used Formalin-fixed tachyzoites (HS antigens), as used in the conventional agglutination test (2), and acetone-treated tachyzoites (AC antigens). Their results suggest that tachyzoite antigens recognized by IgG antibodies formed during the acute infection have specificities different from those formed later in the infection. We therefore considered it of interest to attempt to define the unique antigens present on the acetone-treated parasites to identify those antigens most important for the early diagnosis of this infection. We report that tachyzoite-specific antigen(s) are important in distinguishing the stage of infection with *Toxoplasma gondii*.

MATERIALS AND METHODS

Mice. Outbred female Swiss-Webster mice (Simonsen Laboratories, Gilroy, Calif.) were 6 to 8 weeks old when used in the experiments.

Human sera. Sera were from more than 20 adult patients who had acute lymphadenopathic toxoplasmosis. Diagnosis of acute lymphadenopathic toxoplasmosis was by both serology and histopathology (16).

***Toxoplasma* antigens.** Tachyzoites of the RH strain were harvested from peritoneal fluids of mice infected 2 days

earlier and purified as described previously (17). For the agglutination test, tachyzoites were fixed with either Formalin or acetone as previously described (13). In brief, tachyzoites were washed by centrifugation and suspended in phosphate-buffered saline (PBS; pH 7.2). For acetone fixation, tachyzoites were treated with 30% acetone in PBS at 4°C for 72 h. For Formalin fixation, tachyzoites were suspended in 20% Formalin in PBS at 4°C overnight.

Gel electrophoresis and immunoblotting. Electrophoresis was performed in 5 to 15% polyacrylamide slab gels with the discontinuous sodium dodecyl sulfate buffer system described by Laemmli (6). Fresh (not fixed in acetone or Formalin) tachyzoites were solubilized in the sample sodium dodecyl sulfate buffer as described earlier (6). Molecular weight standards were myosin, β -galactosidase, phosphorylase b, bovine serum albumin, egg albumin, and carbonic anhydrase (Sigma Chemical Co., St. Louis, Mo.). Proteins separated by electrophoresis were transferred to nitrocellulose paper as described by Towbin et al. (14). Blots were first soaked in 5% nonfat dried milk in PBS for 1 h at room temperature to saturate unused protein-binding sites. The blots were then rinsed in PBS containing 0.05% Tween 20 (PBS-Tween) and incubated overnight with test serum at a 1/100 dilution in 5% dried milk in PBS-Tween. The nitrocellulose sheets were then washed with PBS-Tween and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-human IgG or anti-mouse IgG antibodies (Tago Inc., Burlingame, Calif.) at dilutions previously determined to be optimum. After another wash, blots were soaked in a solution of 0.1 mg of diaminobenzidine per ml and 0.1% H₂O₂ in PBS. Color development was stopped by washing with PBS-Tween.

***Toxoplasma* serology.** IgG antibodies were measured by the Sabin-Feldman dye test (10), and IgM antibodies were

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TABLE 1. *Toxoplasma* antibody titers in four representative patients in the acute and chronic stages of the infection

Patient no. (sex-age [yr]) ^a	Date ^b	Stage of infection	Anti- <i>Toxoplasma</i> antibody titer by:			
			IgM ELISA ^c	Dye test	Agglutination test	
					AC	HS
1 M-27	01/20/75	Acute	12.4	1,600	1,600	3,200
	03/27/78	Chronic	3.9	819	<40	400
2 F-43	09/23/76	Acute	3.6	2,400	400	1,600
	10/07/80	Chronic	0.6	410	<40	200
3 F-24	04/14/76	Acute	4.8	3,200	400	1,600
	02/24/78	Chronic	0.6	26	<40	400
4 M-34	05/26/81	Acute	10.6	4,000	800	1,600
	02/01/83	Chronic	4.3	179	<40	800

^a M, Male; F, female.^b Expressed as month/day/year.^c ELISA, Enzyme-linked immunosorbent assay.

measured by the double-sandwich IgM enzyme-linked immunosorbent assay as described previously (8). Titers in the double-sandwich IgM enzyme-linked immunosorbent assay were calculated by a slight modification of the formula described by Siegel and Remington (12).

The agglutination tests with HS (HS test) or AC (AC test) antigens were performed as described previously (13). In brief, HS antigen was suspended in PBS at a concentration of 3×10^7 tachyzoites per ml. Fifty microliters of the suspension was added to 50 μ l of double dilutions of sera in microdilution plates. Sera were diluted in PBS containing 0.2 M 2-mercaptoethanol. The trays were incubated at room temperature overnight, and the agglutination patterns were then read. AC antigen was suspended in alkaline buffer (pH 8.7) containing 1% bovine serum albumin. The concentration of tachyzoites for AC antigen was the same as that for HS antigen. Sera were diluted in the same buffer containing 0.2 M 2-mercaptoethanol. *Toxoplasma* antibody titers in the dye and agglutination tests are expressed in international units per milliliter for both human and mouse sera. For each test, the relationship between titers expressed as the reciprocal of the dilutions and international units was determined by using the World Health Organization standard serum, which contains 2,000 IU per vial (13).

IFA test. The indirect fluorescent-antibody (IFA) test was performed with either tachyzoites or bradyzoites as described for tachyzoites by Walton et al. (15). Cysts of *T. gondii* were purified by Percoll density gradient centrifugation (1) from brains of mice chronically infected with the ME49 strain. Free bradyzoites were obtained by treatment of cysts with pepsin (4). The bradyzoites were fixed in 2% Formalin in PBS and attached to the surface of glass slides by drying the suspension on the slides. Separate slides were coated with Formalin-fixed tachyzoites. The slides were incubated at 37°C for 1 h with dilutions of the test sera. After being washed, slides were incubated with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Organon Teknika, Malvern, Pa.) for 1 h at 37°C.

Preparation of antisera against AC and HS antigens. Mice were immunized by the intraperitoneal route with AC or HS antigens. Each antigen preparation contained 1.5×10^7 cells. The mice were injected twice at 4 weeks apart. They were sacrificed, and pooled sera from three mice were obtained 1 week after the second immunization.

Absorption of AC antibodies with HS antigens. A total of 300 μ l of a 1:10 dilution of the AC antisera in PBS was

incubated with 4.5×10^7 cells of HS antigens at 37°C for 1 h. After incubation, HS antigens were removed by centrifugation at $1,300 \times g$ for 10 min. Repeat absorptions were performed to remove reactive antibodies.

RESULTS

Sera of patients with acute lymphadenopathic toxoplasmosis. Table 1 shows representative serological test results from more than 20 patients from whom serum was obtained both during the acute stage of their toxoplasmic lymphadenopathy and later, during their chronic (latent) infection. The high IgG (dye test) and IgM test titers are characteristic of the acute phase of toxoplasmic lymphadenopathy. Whereas the HS test was positive for both the acute and chronic stages of the infection, the AC test was positive only for the acute stage of the infection. These results suggest that sera obtained during different stages of the infection contain IgG antibodies that recognize different antigens of *T. gondii*. AC antigens appear to be recognized by acute-phase-specific antibodies.

***Toxoplasma* antigens recognized by antibodies formed during acute and chronic stages of the infection.** Immunoblots were used to define and compare *Toxoplasma* antigens recognized by the acute- and chronic-phase sera of three of four patients shown in Table 1. A sodium dodecyl sulfate lysate of tachyzoites was used as the antigen for these studies. Acute-phase sera recognized more antigens than did chronic-phase sera (Fig. 1), and certain bands were present in only the former or were more strongly stained in those blots.

Antigens recognized by anti-AC and anti-HS antibodies. To identify antigens unique to AC and HS antigens, mice were

FIG. 1. Immunoblot analysis of *T. gondii* antigens recognized by IgG antibodies in sera of patients obtained during the acute (A) and chronic (C) stages of infection. Numbers represent molecular weight standards. Arrows show antigens with molecular weights comparable to those recognized by AC antibodies (see Fig. 2). 1, 2, and 3, Patient numbers in Table 1.

FIG. 2. Immunoblot analysis of *T. gondii* antigens recognized by mouse AC (anti-AC) and HS (anti-HS) antisera. NMS, Normal mouse serum. Numbers represent molecular weight standards.

immunized with either AC or HS antigens to prepare antisera for testing by immunoblot. Antisera from mice immunized with either antigen preparation had high antibody titers in both AC and HS tests. Pooled sera of mice immunized with AC antigens had titers of 1:3,200 in both AC and HS tests. Antisera from mice immunized with HS antigens had an AC titer of 1:1,600 and an HS titer of 1:12,800. These results indicate that AC and HS antigens share some of the same antigens on their surfaces.

Toxoplasma antigens recognized by the antisera prepared against AC and HS antigens were analyzed by immunoblotting with the sodium dodecyl sulfate *Toxoplasma* lysate antigen preparation. The AC antisera recognized fewer numbers of antigens than did the HS antisera (Fig. 2). Approximate molecular weights of the most prominently stained bands recognized by the AC antibodies were 116,000, 78,000, 64,000, 61,000, 54,000, 52,000, 42,000, 30,000, 24,000, and 6,000. Antigens with molecular weights comparable to these ten values were more intensely stained when reacted with the acute-phase human sera than when reacted with the chronic-phase sera (arrows in Fig. 1), but not all antigens were recognized by all of the acute sera.

All bands in the blots obtained with the AC antibodies were present in blots obtained with the HS antibodies. Additional antigens which were present in the blots treated with HS antibodies were not present in the blots treated with AC antibodies (Fig. 2).

Absorption of AC antibodies with HS antigens. Because the results described above suggest that AC antigens are a part of HS antigens, we performed absorption studies to determine whether HS antigens could remove the mouse AC antibodies. AC antibodies reacted with only cell membranes

FIG. 3. Reaction of AC and HS antibodies with tachyzoites and bradyzoites in an IFA test (Materials and Methods). Formalin-fixed tachyzoites (A and C) and bradyzoites (B and D) were incubated with AC (A and B) and HS (C and D) antibodies, respectively. No fluorescence was noted when normal mouse serum was used.

of HS antigens (Fig. 3A). Changes in the AC and HS agglutination test titers when the AC antibody preparation was used following each of several absorptions with HS antigens are shown in Table 2. Both the AC and HS test titers decreased and ultimately were undetectable.

Following the final absorption, immunoblots were performed. All but one (molecular weight, 116,000) of the bands recognized by the AC antibodies disappeared after the absorption (Fig. 4). The intensity of the band after absorption was much weaker than that before absorption.

Reaction of AC and HS antibodies with tachyzoites and bradyzoites. It is known that tachyzoites and bradyzoites of *T. gondii* each have specific antigens (5, 7). We examined the possibility that AC antigens are tachyzoite specific. Reactivities of AC and HS antibodies with tachyzoites and bradyzoites were examined by the IFA test with Formalin-fixed organisms. HS antibodies recognized the cell membranes of both the tachyzoites and bradyzoites (Fig. 3C and D). In contrast, AC antibodies reacted with cell membranes of tachyzoites only (Fig. 3A and B). Normal mouse sera did not give a positive reaction with either form. A bright spot was noted in the center of the bradyzoites when they were incubated with either AC or HS antibodies.

DISCUSSION

The AC test, which measures IgG antibodies (13), was positive with sera of patients with toxoplasmic lymphadenitis only during the acute stage of their infection. These results, obtained in serial sera collected from the same patients during the acute stage of their infection and for several years thereafter, are consistent with our previous results obtained from larger numbers of patients (13). Thus, in the acute stage of the infection in humans, the IgG antibody response appears to be directed against antigens which are different from those which stimulate the IgG antibody response during the chronic stage of infection. AC antigens can detect these acute-phase-specific antibodies. Whether this fact is due to the absence of the AC antigen among those antigens which stimulate *Toxoplasma* antibody production for years after acute infection or to a lack of host recognition of the antigen(s) is unclear.

The results of the immunoblots obtained with the mouse AC antisera suggest that at least 10 antigens are important in the antibody response against *T. gondii* in the acute stage of the infection. These antigens reacted much more strongly with acute-phase sera than with sera obtained during the chronic stage of the infection. Some additional antigens (molecular weights of 48,000 and 37,000) which were not recognized by the AC antibodies also seem to be important in the acute-phase-specific antibody response. Potasman et al. (9) reported that the 35,000-molecular-weight (35K) and 4K antigens were most intensely stained in immunoblots of acute-phase sera. These two antigens would be comparable to the 37K and 6K antigens in the present study. Erlich et al.

FIG. 4. Immunoblot analysis of *T. gondii* antigens recognized by mouse AC antibody before and after absorption with HS antigens. Numbers represent molecular weight standards.

(3) and Sharma et al. (11) also reported that the antigen with an apparent molecular weight of 6,000 is important for the IgM antibody response in acute *Toxoplasma* infection.

Although more bands were noted in immunoblots with the HS antibodies than in those with the AC antibodies, each of the bands in the blots treated with AC antibodies was present in the blots treated with HS antibodies. The observation that AC antibodies were completely removed when absorbed with HS antigens suggests that AC antigens are a selected part of HS antigens. This hypothesis is also supported by results of experiments in which HS antibodies were absorbed with AC antigens. The AC test titer of HS antibodies became undetectable, whereas the HS test titer remained positive (data not shown).

From the results obtained in the IFA test with AC antibodies and the HS antigen preparation, the antigens recognized by AC antibodies are cell surface antigens. This is further supported by results of separate studies (data not shown) of immunoblots in which the antigen was a membrane fraction of *T. gondii*. In blots with AC antibodies, the membrane fraction revealed the same 10 bands as did blots of whole organisms, although the band intensities of the 116K and 76K antigens were much weaker with the membrane fraction (unpublished data). Sharma et al. (11) also reported that the membrane fraction contains the greatest number of antigens which react with both human IgM and IgG *Toxoplasma* antibodies.

Our results reveal that AC antigens are tachyzoite specific. In the IFA test, AC antibodies reacted with the cell surfaces of tachyzoites but not those of bradyzoites, whereas HS antibodies reacted with the surfaces of both tachyzoites and bradyzoites. The presence of antigenic differences between tachyzoites and bradyzoites has also been reported by Lunde and Jacobs (7) and Kasper et al. (5). The latter investigators observed that two major membrane antigens of tachyzoites (molecular weights of 30,000 and

TABLE 2. Absorption of mouse AC antibodies with HS antigens

No. of absorptions	Agglutination test titer	
	AC	HS
0	1,600	800
1	400	400
2	100	100
3	50	50
4	<50	<50

22,000) are absent in bradyzoites. These antigens might be comparable to the 30K and 24K antigens observed in the blots treated with AC antibodies. Since the AC antigen preparation consisted of acetone-treated tachyzoites, acetone may have removed certain antigens from the cell membrane or altered the antigens such that they no longer cross-reacted with antigens of bradyzoites. Tachyzoites proliferate only during the early stages of the infection; thereafter, the organism persists as tissue cysts. Thus, stimulation of antibody formation by antigens which are tachyzoite specific (AC antigens) should occur solely during the acute stage of the infection and likely explains why the AC test can diagnose this stage of the infection.

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