Detection of *Toxoplasma gondii* Antigens by a Dot-Immunobinding Technique

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A sensitive assay for the detection of antigens of *Toxoplasma gondii* by spotting samples directly onto nitrocellulose paper was developed. The sensitivity ranged from 10 to 40 pg of antigen diluted in phosphate-buffered saline and 40 to 130 pg of antigen diluted in normal mouse serum, normal human serum, or human cerebrospinal fluid. *T. gondii* antigen in serum samples taken from mice infected with *T. gondii* was detectable by day 2 of infection. Antigen was also detectable in cerebrospinal fluid samples taken from four of six infants congenitally infected with *T. gondii* and in serum samples from two of these infants.

The diagnosis of toxoplasmosis by conventional serological methods which measure immunoglobulin G (IgG) or IgM antibodies may be particularly difficult in congenitally infected neonates (8) and in patients who are severely immunocompromised (17). This difficulty is compounded by the false-positive and false-negative results which occur in these particular patients. In some instances the vagaries are due to the tests themselves (5), whereas in others they are due to lack of formation of demonstrable antibody by the patient (8). It is in such situations that demonstration of *Toxoplasma gondii* antigen in serum or other body fluids may be particularly helpful in making an early diagnosis of acute toxoplasmosis.

Antigenemia due to *T. gondii* in experimentally infected animals was first reported by Raizman and Neva in 1975 (9) and has subsequently been confirmed by several other authors (4, 15, 16). Antigenemia has also been found in human serum samples tested by enzyme-linked immunosorbent assay (ELISA) (1). The techniques used are time consuming and require special equipment.

Spotting of specimens directly on nitrocellulose paper has been reported to be a very sensitive method for the detection of nucleic acid (7) as well as proteins (6, 12). The indicator system for these assays has usually been a radiolabeled DNA probe or specific antibody with results obtained by autoradiography. In addition, this dot-immunobinding (3, 6) technique has been used to screen hybridomas for the secretion of monoclonal antibodies (3). We have attempted to use this method to determine whether it might be useful in detecting the presence of *T. gondii* antigens in body fluids. Our results demonstrate the potential clinical utility of this test in the diagnosis of toxoplasmosis.

MATERIALS AND METHODS

Antigen. *T. gondii* antigen was prepared by using tachyzoites from the peritoneal exudates of mice infected 2 days earlier with *T. gondii* RH. The exudate was passed through a 3- μ m-pore-size filter (Nuclepore Corp., Pleasanton, Calif.) to remove host cells and centrifuged at $2,000 \times g$ for 15 min. The pellet was suspended in distilled water and disrupted with a Biosonik IV sonicator (Bronwill Scientific Inc., Rochester, N.Y.) set at 60 cycles for five 30-s periods. After

being observed by light microscopy for the absence of whole parasites, the solution was standardized to 10 μ g/ml, divided into 1-ml aliquots, lyophilized, and stored at -20°C until use.

Protein determination. Protein determinations were performed with the Bio-Rad protein assay reagents (Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as the standard.

Antibody. Antibodies to *T. gondii* were obtained by immunization of New Zealand white rabbits. Initial immunization was with 500 μ g of antigen (as prepared above) and 500 μ l of complete Freund adjuvant. Rabbits were injected subcutaneously at five sites (0.2 ml per site), after which the animals were boosted at 3- to 4-week intervals with antigen plus incomplete Freund adjuvant until a serum dye test titer of 1:8,000 to 1:16,000 was obtained. The rabbits were then sacrificed, and the serum was precipitated with a 45% saturated ammonium sulfate solution, resuspended in 0.01 M phosphate-buffered saline (PBS) (pH 7.2), and stored at -20°C until use.

Serology. The Sabin-Feldman dye test (DT) and double-sandwich (DS)-IgM-ELISA were performed as previously described (11, 13).

Infection of mice. Female Swiss Webster mice (Simonsen Laboratories, Gilroy, Calif.) were injected intraperitoneally with 5×10^6 *T. gondii* RH cells, and four mice were sacrificed daily for 5 days. Two uninfected mice were sacrificed daily as controls. In the experiments to determine the effect of various inoculum sizes of *T. gondii* RH on the detection of antigenemia, Swiss Webster mice were infected with 5×10^3 , 5×10^4 , 5×10^5 , or 5×10^6 *T. gondii* RH cells, and two mice from each group were bled every 2 days until death. In a separate set of experiments, 20 mice were injected with 2×10^4 , 2×10^5 , or 2×10^6 *T. gondii* cells obtained from the peritoneal fluid of mice injected 5 days earlier with brain tissue of mice chronically infected with *T. gondii* C56. One day after infection, and every third day through day 19, four infected mice and two control mice were bled from their orbital fissures. Sera were stored at -20°C until use.

Human sera and cerebrospinal fluid. Six infants with congenital toxoplasmosis suspected on the basis of clinical and serological findings in the infants and a history in the mothers compatible with the acute acquired infection during pregnancy were chosen (10). All infants had both serum and

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cerebrospinal fluid available for testing. Sera were also obtained from 40 patients with biopsy-proven toxoplasmic lymphadenopathy; sera were obtained from 3 weeks to 5 months after the onset of clinically evident lymphadenopathy. All were positive in the DT and DS-IgM-ELISA. Twenty DT-negative human sera from individuals with no clinical symptoms or signs of lymphadenopathy were used as controls.

Immunoblotting technique. Lyophilized preparations of *T. gondii* antigen were reconstituted with 1 ml of PBS, mouse serum (a pool from five seronegative control mice), human serum (a pool from five seronegative normal human volunteers), or human cerebrospinal fluid (a pool from five individuals with neurological disorders of noninfectious etiology). These control samples were serially diluted 1:3 with PBS and used as positive controls. Negative control samples consisted of the same pools without the addition of antigen. Positive and negative control samples were run with each test. All tests were run in parallel on separate days.

Nitrocellulose paper (Bio-Rad) was cut into strips and overlaid with a template consisting of 10 equally spaced holes. A glass petri dish was used to minimize nonspecific adherence of protein to the container. Samples (4 μ l) of the positive and negative controls and of serum or cerebrospinal fluid to be tested were placed on the nitrocellulose paper and allowed to air dry for 1 h. The paper was then blocked with a 3% gelatin solution in 0.02 M Tris-0.5 M NaCl (TBS) (pH 7.5) and incubated for 1 h at room temperature on an agitator. After the 3% gelatin solution was pipetted off, 20 ml of a solution of rabbit anti-*T. gondii* antibodies in 1% gelatin solution was added and incubated at room temperature on an agitator. After being rinsed with distilled water, the plates were washed for three 10-min periods with TBS containing 0.025% Tween 20. A solution of goat antirabbit antibody bound to horseradish peroxidase (Bio-Rad) diluted in 1% gelatin-TBS was then incubated at room temperature and washed with distilled water and TBS-0.025% Tween 20 as described above.

Twofold dilutions of rabbit anti-*T. gondii* antibody (1:125 to 1:16,000) were incubated for 2, 4, 8, or 16 h. Goat antirabbit antibody bound to horseradish peroxidase was tested in twofold dilutions (1:1,000 to 1:8,000) and incubated for 1, 2, or 4 h. For preparation of the substrate, 60 mg of horseradish peroxidase color development reagent (Bio-Rad) containing 4-chloro-1-naphthol was mixed with 20 ml of ice-cold methanol and then added to 100 ml of TBS with 60 μ l of 30% hydrogen peroxide. The plates were incubated with the substrate for 10 min, rinsed twice with distilled water, and blotted dry. The results were compared visually with the positive and negative control dots run on the same strip. The development of color with equal or greater intensity than that of the positive control sample was read as a positive specimen.

RESULTS

Determination of optimal conditions for the antigen assay. Optimal concentrations of rabbit anti-*T. gondii* and goat antirabbit conjugate were determined by block titration against serial dilutions of the appropriate positive and negative controls. For detection of antigen in mouse serum, human serum, or human cerebrospinal fluid, concentrations of 1:4,000 of rabbit anti-*T. gondii* antibody and 1:8,000 of goat antirabbit conjugate were found to be optimal. Use of the rabbit anti-*T. gondii* antibody at higher concentrations often resulted in a ring-shaped pattern which made detection of antigen difficult, and a similar pattern would sometimes

occur with negative controls. Use of the goat antirabbit conjugate at higher concentrations gave a darker background without increasing the sensitivity. After optimal concentrations of antibody were determined, the length of incubation was varied to determine the optimal period for incubation. Incubation with rabbit anti-*T. gondii* antibodies for 2 h decreased the sensitivity two- to fourfold compared with incubation for 16 h. For specimen testing, therefore, we used an incubation period of 16 h with rabbit anti-*T. gondii* antibody. Since results after incubation with goat antirabbit conjugate for 1, 2, or 4 h showed no significant differences, we chose an incubation time of 1 h for this step.

Detection of antigen in PBS. Between 10 and 40 pg of *T. gondii* sonic extract diluted in PBS was consistently demonstrable (Fig. 1). A relatively concentrated preparation of the rabbit anti-*T. gondii* (1:100) and goat antirabbit (1:1,000) antibodies could be used; nonspecific cross-reactivity, as was sometimes observed with high concentrations of antibody in tests of serum or cerebrospinal fluid (see below), was not observed. This method could also detect whole *T. gondii* organisms diluted in PBS equivalent to 400 pg of protein (data not shown).

Detection of antigen in mouse serum. To determine whether this technique could be used to detect *T. gondii* antigen in mouse serum, antigen was suspended in normal mouse serum; 130 pg of *T. gondii* antigen could be consistently demonstrated in mouse serum by this assay. Additional experiments were performed in which mice were infected with *T. gondii* RH or C56; 50% of mice infected with 5×10^6 *T. gondii* RH cells intraperitoneally had demonstrable antigen in their serum on day 2, whereas 100% of mice tested on days 3, 4, and 5 had detectable antigen (Fig. 2). All mice infected with 5×10^4 or 5×10^5 *T. gondii* RH cells had

FIG. 1. Dot-immunobinding assay of *T. gondii* antigen in (A) PBS; (B) normal mouse serum; (C) normal human serum; (D) human cerebrospinal fluid. Details of performing the assay are given in the text.

FIG. 2. Percentage of mice with detectable antigenemia after infection with *T. gondii*. Symbols: ▨, RH strain; □, C56 strain.

detectable antigenemia by day 4, and all mice infected with 5×10^3 *T. gondii* RH cells had detectable antigenemia by day 6 after infection. None of the control mice were positive. None of the mice infected intraperitoneally with *T. gondii* C56 and followed until death or day 19 of infection had antigenemia detectable by this method (Fig. 2).

Detection of antigen in human serum. We next determined whether antigen could be detected in human serum. Lyophilized *T. gondii* antigen was suspended in normal human serum. *T. gondii* antigen was detectable, and the sensitivity ranged from 40 to 130 pg by this method. Of the sera from the adults with biopsy-proven toxoplasmic lymphadenopathy, 8 of 40 (20%) were positive, whereas only 1 of 20 (5%) sera from DT-negative controls was positive. The one positive DT-negative control was only faintly visible (corresponding to a positive control of 13 pg or less). Two of the six sera tested from the cases of congenital toxoplasmosis were positive (Table 1).

Detection of antigen in human cerebrospinal fluid. When *T. gondii* was added to normal human cerebrospinal fluid, a minimum of 130 pg of antigen was detected. Antigen was detected in the cerebrospinal fluid of four of the six congenitally infected infants (Table 1).

DISCUSSION

The diagnosis of toxoplasmosis is most commonly made by serological means. This is usually done by finding the presence of *T. gondii*-specific IgM antibodies or a fourfold or

greater rise in IgG antibody titer in the serum. There are some circumstances, however, in which these criteria may not be met and in which a rapid diagnosis of *T. gondii* infection cannot be made by measuring antibody levels alone. Of infants congenitally infected with *T. gondii*, ca. 20% will not have detectable IgM antibody against this organism when tested by the DS-IgM-ELISA and at least 75% will not have demonstrable IgM antibody against *T. gondii* when measured by the IgM-immunofluorescent antibody test (8). In patients with the acquired immunodeficiency syndrome, IgM antibody may also be absent even with biopsy-proven infection with *T. gondii* (17; B. J. Luft, R. G. Brooks, F. Conley, R. E. McCabe, and J. S. Remington, J. Am. Med. Assoc., in press). It would therefore be clinically useful to develop a test which directly detects the presence of the whole parasite or its antigens in body fluids to document the presence of recent or ongoing infection.

Through the use of a dot-immunobinding assay we demonstrated antigen in both experimentally infected mice and in serum and cerebrospinal fluid of humans with toxoplasmosis; 50% of mice infected with *T. gondii* RH had detectable antigen by day 2 of infection, and 100% had detectable antigen by day 3. These results are similar to those recently reported by Turunen, who used a DS-ELISA method (15). Of interest is the fact that we could not demonstrate circulating antigen in mice infected with *T. gondii* C56. Several possibilities may account for this observation. The fact that antigenemia was detectable even when small inocula of *T. gondii* RH (corresponding to similar inocula used with the C56 strain) were used suggests that the difference in detectable antigenemia was not a function of a different number of organisms. It is also unlikely that the antibody used in the present test recognizes antigens of only the RH strain, since previous results from our laboratory show that antibodies from animals infected with RH, C56, or C37 strain recognize similar antigens (unpublished data). If circulating antigens exist in infection with the C56 strain, these antigens may differ from those which circulate after infection with the RH strain. Characterization of the circulating antigens in the cotton rat model of *T. gondii* infection has shown them to be intracellular in origin (4). The intracellular antigens of these two strains of *T. gondii* have not been directly compared, although the outer membrane antigens of these two strains have been found to be similar (2). It is also conceivable, although unlikely, that the polyclonal anti-*T. gondii* antibodies made by immunization of rabbits with the

TABLE 1. Presence of *T. gondii* antigen in infants with congenital toxoplasmosis

Patient no. (birth date [mo/day/yr])	<i>T. gondii</i> antigen in:								Signs and symptoms
	Serum				Cerebrospinal fluid				
	Date of specimen (mo/day/yr)	DT	DS-IgM- ELISA	Antigen	Date of specimen (mo/day/yr)	DT	DS-IgM- ELISA	Antigen	
1 (12/7/81)	12/11/81	1:2,048	—	+	1/26/82	—	—	+	Hydrocephalus
2 (1/5/81)	1/28/81	1:4,096	—	+	1/30/81	1:8,192	—	+	Hydrocephalus
3 (11/21/83)	12/3/83	1:8,192	—	—	12/3/83	1:4,096	+	+	Hydrocephalus, cerebral calcifications, chorioretinitis
4 (2/7/83)	3/9/83	1:2,048	—	—	2/7/83	1:512	+	+	Hydrocephalus, cerebral calcifications
5 (12/22/79)	5/16/80	1:32,768	1:256	—	5/16/80	1:128	+	—	Seizures, chorioretinitis
6 (11/20/83)	12/14/83	1:8,192	—	—	12/14/83	1:512	—	—	Hydrocephalus, chorioretinitis

T. gondii RH did not recognize these antigens. More likely is the possibility that, because of the less virulent nature and slower replication of the C56 strain, the level of free circulating antigen remains below the level of detection or is absent. Immune complexes have also been demonstrated in clinical infections with *T. gondii* strains and may have played a role in removing low levels of circulating antigen (14).

Antigenemia has been previously demonstrated in 63% of patients with toxoplasmic lymphadenopathy by an ELISA technique (1). We were able to demonstrate antigenemia in 20% of 40 cases of toxoplasmic lymphadenopathy by using the dot-immunobinding technique. The lower incidence of positive tests for antigen in our study may reflect differences in the populations studied, the time after infection that individual sera were obtained, or the prolonged storage of many of our samples. In addition, two of six congenitally infected infants had detectable antigenemia.

Four of six congenitally infected infants (including the two with antigenemia) also had *T. gondii* antigen detectable in their cerebrospinal fluids. These results are similar to those reported by Araujo and Remington (1), in which four of six infants congenitally infected with *T. gondii* had cerebrospinal fluid which was positive for antigen by an ELISA technique.

There are several advantages to the dot-immunobinding technique over other antigen detection systems such as radioimmunoassay or ELISA. The first is the increased sensitivity of this method, which can regularly detect picogram amounts of antigen (1, 15, 16). When suspended in PBS, 10 pg of *T. gondii* antigen could be detected. We have found, as have others (15), that addition of antigen to body fluid or tissues decreases the sensitivity of a test for antigen by a factor of at least 3. This may be due to the binding of antigen to other serum proteins, to the formation of immune complexes, or to degradation of antigen by proteases present in clinical samples being tested.

The second advantage of the dot-immunobinding technique is the small volumes of fluid which can be tested for antigen. We routinely used 4 μ l of sample, although even smaller volumes may be used with good results (3). Larger volumes may also be used by respotting the nitrocellulose after allowing the original sample to dry thoroughly (6). In addition to these advantages, the test is easy to perform and results are read by visual observation, which eliminates the need for a gamma counter or an ELISA plate reader. When used in conjunction with other serological tests for toxoplasmosis and clinical picture, this test may be useful for the diagnosis of toxoplasmosis.

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