Plate Hemolysin Test for the Rapid Screening of Toxoplasma Antibodies

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A plate hemolysin test was developed to screen serum specimens for the presence of toxoplasma antibodies. When we tested 130 sera by both this test and the standard toxoplasma dye test, we found the plate hemolysin test to be a rapid, sensitive, and economical method for detecting toxoplasma antibodies. In all but one instance it paralleled the dye test. A comparison of the results of testing six sera by the hemolysin, hemagglutination, and dye-test techniques suggested that the hemolytic antibodies were more closely related to hemagglutinating antibodies than to dye-test antibodies. We could not store sheep erythrocytes sensitized with toxoplasma lysate for more than 3 days without altering the sensitivity of the test. Concanavalin A proved to be an effective coupling agent for binding toxoplasma antigens to red-cell membranes, a quality attributed to its affinity for specific polysaccharide-combining sites.

Although many serologic tests are available for the detection of toxoplasma antibodies, few are practical as rapid, mass-screening procedures. Such tests, if available, would be of great value in uveitis clinics where facilities for the prompt serologic confirmation of a presumptive diagnosis of toxoplasmic chorioretinitis are often lacking. The risk of acquiring primary fetal toxoplasmic infection is greatest in the absence of maternal antibodies (1). If a rapid screening test were available, women attending obstetric clinics could be tested routinely, and those lacking antibodies could be identified. Recent studies (7) have shown that about 40% of the fetuses carried by mothers who become infected during pregnancy acquire the disease.

A rapid screening test need only give a positive or negative result quickly, economically, and with a sensitivity comparable to the dye test. Since no correlation has been established between the severity of toxoplasmic eye disease and antibody titer, it is only important for the ophthalmologist to determine whether the serum of the patient in question tests positively or negatively for toxoplasmosis (5).

Previous work performed at the Proctor Foundation with the Jerne plaque technique, and with a modification of this technique for the detection of hemolytic antibodies (4), prompted an effort to develop a plate hemolysin test for

the detection of toxoplasma antibodies. Initial attempts to sensitize sheep erythrocytes with a water-soluble extract of toxoplasma organisms by means of standard coupling agents, such as carbodiimide or chromium chloride, were unsuccessful, possibly due to the low protein and relatively high carbohydrate content of the antigen. On the basis of a report by Leon and Young (8) that concanavalin A (Con A) could bind glycoproteins or polysaccharides to sheep erythrocytes for use in a hemagglutination test, we decided to use Con A as a coupling agent in a hemolytic assay system.

MATERIALS AND METHODS

Toxoplasma antigen. From the pooled peritoneal exudates of white Swiss mice (weighing 20 g each) that had been infected with the RH strain of *Toxoplasma gondii* 3 days previously, we harvested and purified toxoplasma organisms according to the method of Nozik and O'Connor (10). The final suspension (approximately 10^8 organisms in 2.0 ml of Hanks solution) was frozen quickly and then stored for 24 to 48 h at -20 C. After thawing the suspension, we lysed the parasites mechanically in a 5-ml cylindrical glass tissue grinder (Misco Corp., Berkeley, Calif.) by forcing the tightly fitting plunger up and down 20 times. The tissue grinder was kept in an ice bath (4 C) during this procedure. The suspension was finally centrifuged at 16,000 × g for 20 min at 4 C. We then separated the supernatant "lysate" from the residual sediment by decantation, and stored the opalescent fluid at -15 C.

Test sera. We obtained fresh and frozen sera from

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the Uveitis Survey Clinic of the University of California, San Francisco. All of the sera were drawn from uveitis patients, some of whose lesions were strongly suggestive of toxoplasmic retinochoroiditis on morphologic grounds alone. Approximately 1 ml of each serum was first heat-inactivated at 56 C for 30 min and then absorbed with 0.1 ml of packed washed sheep erythrocytes for 30 min at room temperature.

Buffer. Phosphate-buffered saline solution (PBS)—0.15 M NaCl, 0.01 M phosphate (Na_2HPO_4: KH_2PO_4 = 9:1), pH 7.3—was used throughout the experiment.

Sheep erythrocytes. Sheep erythrocytes were obtained under sterile conditions and stored in Alsever's solution at 4 C for from 5 to 7 days. Before using them, we washed them twice with normal saline solution and then three times with PBS.

Preparations of sensitized erythrocytes. Sheep erythrocytes were sensitized with toxoplasma antigen by an adaptation of a technique of Leon and Young (8), as follows. A 20% suspension of sheep erythrocytes was incubated for 30 min at 37 C with an equal volume of Con A (Pharmacia Fine Chemicals, Piscataway, N.J.) in a concentration of 100 μg of PBS per ml. After washing the cells three times to remove the unbound Con A, we reconstituted the coated cells in PBS to the original volume and added 1 ml of the cell suspension to 5 ml of toxoplasma lysate, diluted 1:2 in PBS. After incubating the sensitized erythrocytes at 37 C for 30 min, we washed them three times in PBS and finally resuspended them in 2 ml of PBS containing 2% dye-test-negative, heat-inactivated fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N.Y.).

Con-A-coated erythrocytes without toxoplasma lysate served as controls.

Preparation of test plates for the hemolytic assay. We prepared the test plates by a modification (4) of the procedure described by Hübner and Gengozian (6). According to this procedure, we added 0.1 ml of the sensitized erythrocytes, or 0.1 ml of the Con-A-treated cells without antigen, to 1 ml of warm (48 C) 0.7% agarose (Marine Colloids, Inc., Biomedical Systems, Rockland, Me.) made up in double-strength Eagle minimal essential medium (Microbiological Associates, Inc., Bethesda, Md.).

The suspension was mixed thoroughly and then poured quickly onto 60-mm plastic Petri dishes containing 2.0 ml of solidified 0.7% agarose. The agarose containing the sensitized erythrocytes solidified within 5 min at room temperature.

Detection of toxoplasma hemolytic antibodies. One drop (approximately 5 μliters) of undiluted serum was placed on the surface of the prepared test plate with a Pasteur pipette. A drop of the same serum was placed on a similar plate containing Con-A-treated erythrocytes without antigen. After incubating the plates for 1 h at 37 C, we added 1 ml of a 1:8 dilution of dye-test-negative guinea-pig complement (Grand Island Biological Co., Grand Island, N.Y.). After standing for 15 min at room temperature, the plates were incubated at 37 C. We examined them after an hour's incubation, and again after 4 h, for evidence of lysis. Any hemolysis, complete or incomplete, that appeared at the site of the test drop and did not appear at the corresponding site on the control plate was considered positive for toxoplasma antibodies.

Toxoplasma dye test. Toxoplasma antibodies in the test sera were assayed by a modification of the Sabin-Feldman dye test (12).

RESULTS

The results of testing 50 frozen and 80 fresh sera by both the hemolysin test and the dye test are given in Table 1; 61 sera were positive by both tests, 68 were negative by both tests, and one frozen serum was negative repeatedly by the hemolysin test but positive at a titer of 1:4 by the dye test. None were hemolysin-test positive and dye-test negative.

The distribution of the dye test titers in the 61 positive sera is given in Fig. 1; 43% of the positive sera had titers of less than 1:16, and 75% had titers of 1:16 or less.

It was readily apparent that positive sera with high dye-test antibody titers produced more complete hemolysis faster and in a wider zone than sera with titers of 1:8 or less. Occasionally, a highly positive serum showed a ring of hemolysis at the periphery of the test spot that progressed to complete lysis of the cells within that spot over a period of 1 or 2 h. No attempt was made to quantitate the extent of the hemolysis, but serial twofold dilutions of 18 dye-test-positive sera were examined by the hemolysin test so as to compare the titers of the toxoplasma antibodies elicited by the two assays (Table 2). The two tests yielded titers that agreed well when the dye test titers were 1:8 or higher, but below that level there was considerable disparity. Fortuitously, none of the dye tests was positive at 1:32.

Although prepared plates stored at 4 C for more than 2 days yielded inaccurate results, with many false negatives, sensitized erythrocytes stored at 4 C in PBS with 2% FCS could be used for 3 days without altering the test's sensitivity.

In an attempt to define the nature of the hemolytic antibody, we were fortunate enough

TABLE 1. *Comparison of the sensitivities of the hemolysin plate test and the Sabin-Feldman dye test for toxoplasma antibodies*

Hemolysin plate test	Sabin-Feldman dye test	No. of sera
+	+	61
−	−	68
−	+	1
+	−	0

DISTRIBUTION OF DYE TEST TITERS AMONG 61 SERA
THAT WERE POSITIVE BY THE HEMOLYSIN TEST

DYE TEST TITERS

FIG. 1. *Dye test titers of patients found to be positive by the hemolysin test.*

to obtain from the Uveitis Clinic six frozen sera that had all been tested by the immune adherence hemagglutination test, the hemagglutination test, and the dye test. In all six sera, only the dye test was positive at titers of from 1:4 to 1:16. We tested these sera by both the hemolysin test and the dye test, and again only the dye test was positive.

DISCUSSION

Hemagglutinating, precipitating, complement-fixing, and dye-test antibodies have all been detected in the sera of patients infected with *T. gondii*. Hemolytic antibodies to toxoplasma have not previously been reported, however, probably due to inadequate means of sensitizing test erythrocytes.

The results presented in Table 1 indicated that sheep erythrocytes could be sensitized with water-soluble toxoplasma antigens when Con A was used as the coupling agent, and that such sensitized cells could be utilized in a practical hemolytic assay. The test appeared to be very sensitive, paralleling the dye test in all but one of 130 sera tested. The titers of 18 sera subjected to the two tests in parallel (Table 2) showed a high degree of correspondence except at very low antibody titers. Thus, in general, both the quantitative and the qualitative goals that had been set for the hemolysin test have been met.

The finding of seven sera in which both the hemagglutination and the hemolytic antibody tests were negative, when the dye test was weakly positive, suggests that the hemolytic and hemagglutinating antibodies may be closely related. They are both probably different from dye-test antibodies, however. Various investigators have attributed this difference to heterogeneity of the antigens responsible for antibody stimulation, since the dye test depends upon membrane-bound antigens, and both the hemagglutination and hemolysin tests use water-soluble extracts of whole organisms (9). When hemolysin and hemagglutinin titers were compared by one of us (J.M.H.) in rabbits injected intravitreally with bovine gamma globulin or ovalbumin, the titers of most rabbits' sera corresponded closely (4). Additional sera must be tested for toxoplasma antibodies by both tests to confirm their similarity.

Recently developed, passive hemolysin tests in agarose appear to constitute an easy, economical, and rapid method for testing large numbers of sera (4). In the present study, from 50 to 60 sera were tested on a single 60-mm Petri dish. Since the individual serum drops were visible whether the test was positive or negative

TABLE 2. *Comparison of titers of toxoplasma antibody detected in 18 sera by the hemolysin test and dye test*

Dye test titers	Hemolytic assay titers										
	Neg.	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:1024
Neg.											
1:1				1							
1:2											
1:4		1				3					
1:8					2	1					
1:16					1	2	1				
1:32											
1:64						1	1	3			
1:128											
1:256											
1:512											
1:1024											1

(Fig. 2 and 3), extensive labeling of the specimens was unnecessary. Although it is conceded that other tests for the rapid screening of patients' sera for antibodies to toxoplasma are available (those utilizing lyophilized sensitized erythrocytes for the passive hemagglutination reaction being most useful for this purpose [11]), we feel that the ease of identifying positive sera by a single glance at a hemolysin plate containing 60 or more specimens offers a special advantage for mass screening.

Many of the sera were tested repeatedly with different batches of lysate, and the results were completely reproducible when the antigen was used in a dilution of 1:2. In dilutions greater than 1:4, there was a loss of sensitivity for sera manifesting dye titers of 1:8 or less. Attempts to reuse the lysate to sensitize a second suspension of Con-A-coated cells were unsuccessful, indicating that most of the antigen had been bound during the first exposure to Con A. It is recommended that reference sera with known low dye-test titers be included with the test sera to ensure the test's accuracy.

It is unfortunate that sensitized erythrocytes cannot be stored for more than 3 days without affecting the reproducibility of the test. Although only 3 h are necessary to prepare the plates, the potential advantage of being able to store sensitized erythrocytes for later use is
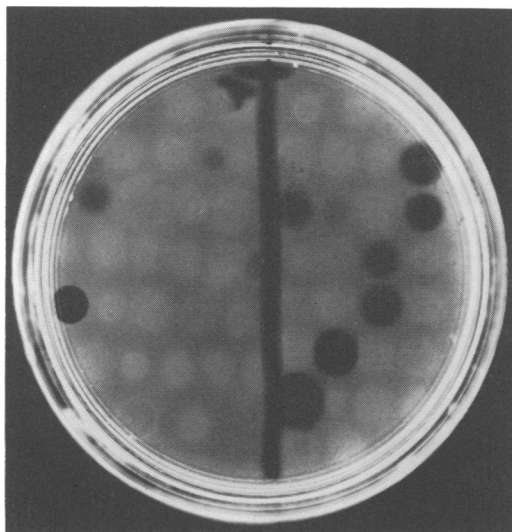

FIG. 3. *Hemolysin control plate prepared with Con-A-coated erythrocytes without toxoplasma antigen. The same 58 sera were tested as in Fig. 2. No nonspecific hemolysis occurred with any of the serum specimens tested.*

obvious. This aspect of the hemolysin test is currently under study.

In an attempt to shorten the test procedure, 80 sera were subjected to the hemolysin test without prior absorption with sheep erythrocytes. In 23% of these sera, nonspecific lysis on the control plates reduced the test's accuracy. Absorption for 30 min at room temperature was found to eliminate all nonspecific lysis in the 130 samples tested.

The demonstration that antigens derived from various microbial agents have the necessary combining sites for Con A (2, 3, 13) suggests that the plate hemolysin test prepared with Con A may have much wider use than as a serologic test for toxoplasmosis alone.

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FIG. 2. *Hemolysin test plate prepared with toxoplasma sensitized sheep erythrocytes. Fifty-eight sera were tested, each represented by one spot. Dark spots represent zones of hemolysis indicating a positive result. Light areas show that no hemolysis occurred; those sera are negative for toxoplasma antibodies in undiluted form.*
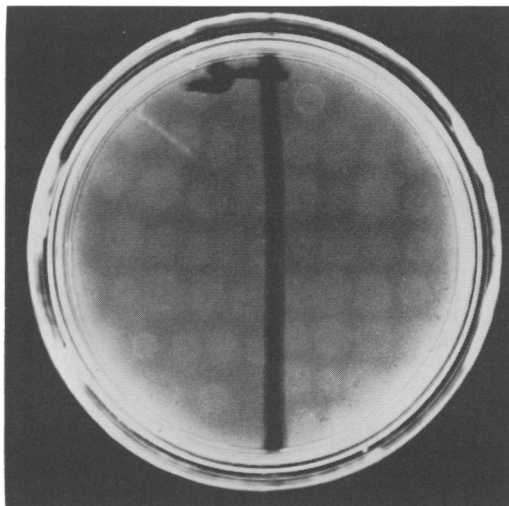
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