Use of the Enzyme-Linked Immunosorbent Assay (ELISA) and Its Microadaptation for the Serodiagnosis of Toxoplasmosis

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The enzyme-linked immunosorbent assay (ELISA) has proved to be a sensitive and specific quantitative procedure for the serodiagnosis of toxoplasmosis. Using the toxoplasma model, several parameters of the test were investigated. Day-to-day reproducibility was 90% within one twofold dilution and 98% specific when tested against batteries of sera from other diseases. Both the tube method and the microtitration method were used successfully. ELISA results are equivalent to those found in the indirect immunofluorescence test, yet the ELISA procedure is simpler and more rapid to perform.

A number of serological tests have been used for the detection of antibodies to *Toxoplasma gondii*. The enzyme-linked immunosorbent assay (ELISA) appears to offer a combination of the best qualities of all. Described first by Engvall and Perlmann (4), ELISA is a modification of the radioimmunosorbent technique (RIST), in which an enzyme is substituted for the radiolabel of the antiserum. In the short time since its inception, the test has been applied to the detection of a number of metabolites (7, 12) and bacterial (3, 5) and parasitic diseases (2, 9-11, 15). Ruitenberget al. (9-11) have published numerous articles on the use of ELISA in trichinosis, and Voller and others have described its use in amebiasis (1), schistosomiasis (6), malaria (7), and Chagas' disease (4). Recently, Voller et al. (13) described a qualitative ELISA for toxoplasmosis.

Although originally described as a single tube, photometrically quantitated procedure, ELISA was readily adapted to microtitration and automation. In a semiautomated system, Ruitenberget al. (11) demonstrated that on a routine basis as many as 4,000 sera could be tested daily.

Within the limits of the adsorptive qualities of the polystyrene sorbent, any soluble antigen apparently can be incorporated into the test. Consequently, it seemed logical that a soluble antigen prepared from disrupted whole *T. gondii* organisms would contain both the cell wall antigen (the active antigen in the indirect immunofluorescence test [IIF] and the methylene blue dye test) and the cytoplasmic antigen (which is active in the passive hemagglutination test). ELISA with a soluble antigen should

give reactions characteristic of all the former procedures.

We describe here an ELISA procedure for toxoplasmosis in which an antigen derived from solubilized whole organisms is used. Both the tube and microtitration procedures have been successfully used.

MATERIALS AND METHODS

Antigen. Tachyzoites of the RH strain of *T. gondii* were harvested from mice infected 3 days previously. The peritoneal fluid was withdrawn and mixed with at least 10 volumes of 0.5% formalinized phosphate-buffered saline (PBS), pH 7.2. After remaining for 1 h at room temperature, the cells were collected by centrifugation and washed three times with PBS. After the final wash, the packed cells were resuspended to a 1% concentration (vol/vol) in distilled water and disrupted in a Ribi cell fractionator at 20,000 lb/in² at 7°C. After the mixture had settled overnight, gross particles were removed by centrifugation at 2,000 × g for 30 min. The supernatant was collected and extracted with an equal volume of trifluorotrichloroethane (Genitron 113, Allied Chemical Corp., Morristown, N.J.). The resulting clear antigen was stored as 1-ml aliquants in the vapor phase of a liquid nitrogen storage box.

Conjugate. Horseradish peroxidase conjugated to goat anti-human immunoglobulin G-Fab was prepared by the method of Kawaoi and Nakane (Fed. Proc. 32:840, 1973) modified by Ruitenberget al. (9). The enzyme preparation used was horseradish peroxidase type VI (Sigma Chemical Co., St. Louis, Mo.), with an RZ activity of 2.68. A 10-mg amount of horseradish peroxidase was dissolved in 2 ml of 0.3 M sodium bicarbonate, pH 8.1. Two-tenths milliliter of 1% (vol/vol) 1-fluoro-2,4-dinitrobenzene (Eastman Kodak Co., Rochester, N.Y.) in absolute ethanol was added, and the preparation was mixed for 1 h at room temperature. A 2-ml amount of aqueous 0.08 M

sodium periodate was added and mixed for 30 min at room temperature. Then, 2 ml of 0.16 M ethylene glycol was added and mixed for 1 h. The resulting solution was centrifuged at $900 \times g$ for 10 min to remove the small amount of precipitated impurities and was subsequently dialyzed at 4°C against four 1-liter changes of 0.01 M carbonate buffer, pH 9.5. The dialysate was mixed with 10 mg of anti-immunoglobulin for 3 h at room temperature and then was dialyzed against 0.15 M PBS, pH 7.2, at 4°C. The conjugated material was separated from the unconjugated protein and enzyme by filtration on a Sephadex G-200 column equilibrated with 0.15 M PBS containing 0.02% NaN_3 . The peaks that showed optical density activity at both 280 nm (for the protein) and 403 nm (for the peroxidase) were collected and concentrated to at least 1 mg of protein per ml. The conjugate was stored in the cold (4°C).

Test sera. Test sera consisted of five pools of human sera of known levels of reactivity in the IIF procedure for toxoplasmosis. Additional sera used for testing sensitivity and specificity were selected from sera submitted to the Center for Disease Control that had titers in the conventional serological tests for the diseases suspected.

Immunosorbent. Disposable polystyrene tubes (11 by 55 mm, Pharmacia Laboratories, Inc., Piscataway, N.J.) were filled with 1 ml of antigen adjusted to 5 μg of protein per ml in 0.1 M carbonate buffer, pH 9.6, containing 0.02% NaN_3 . The tubes were incubated in a 37°C water bath for 3 h and stored in the cold (4°C) until used.

Substrate. The substrate was prepared by dissolving 80 mg of 5-aminosalicylic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in 100 ml of hot distilled water (80°C). Immediately before the solution was used, the pH was brought to 6.0 with 1 N NaOH. To 9 parts of 5-aminosalicylic acid solution, 1 part of 0.05% (vol/vol) H_2O_2 was added.

ELISA tube test. The method of Ruitenberg et al. (11) was followed for the tube test, except for a few minor modifications. The day before assay, the immunosorbent tubes were emptied by suction, refilled with 2 ml of 1% (wt/vol) bovine serum albumin in 0.1 M sodium carbonate buffer (pH 9.6) containing 0.02% NaN_3 , and incubated at 4°C overnight. To begin the test, the tubes were washed with distilled water three times for 5 min each. Then, 1 ml of each dilution of test serum in PBS was added to each tube, and the tubes were incubated at 37°C for 30 min. The tubes were washed three times as before and then incubated with 1 ml of 1% (wt/vol) aqueous bovine serum albumin solution at 37°C for 30 min. The tubes were washed again and then refilled with 1 ml of conjugate diluted to the proper dilution in PBS with 1% bovine serum albumin. The tubes were incubated at 37°C for 30 min and then washed as before. One milliliter of substrate was added and allowed to react at room temperature for 1 h. The reaction was terminated by adding 0.1 ml of 1 N NaOH. The brown-colored product was measured in a Coleman Jr. spectrophotometer at 450 nm.

ELISA microtitration procedure. The procedure followed for the microtitration test was that of Ruitenberg et al. (10). All reagents used were the same

as those for the tube tests. Disposable polystyrene microtitration trays with 96 flat-bottom wells (Flow Laboratories, Inc., Rockville, Md.) were coated with antigen by adding 100 μl of the diluted antigen solution to each well and incubating the trays in a water bath at 37°C for 3 h. Trays containing the antigen solution were then stored at 4°C until used. Before the assay, the trays were washed three times by flooding them with distilled water and then draining, inverting, and vigorously shaking them. Test sera were diluted with PBS, and 100 μl of each dilution was transferred to a well of a tray. The trays were incubated in a 37°C water bath for 30 min. They were then washed as before, and 100 μl of conjugate, diluted in PBS with 1% bovine serum albumin, was added. The trays were again incubated at 37°C for 30 min and then were washed. Finally, 100 μl of substrate was added, and the trays were allowed to remain at room temperature for 1 h while the reaction occurred. The reaction was then terminated by adding 25 μl of 1 N NaOH. The brown reaction product was evaluated visually. The last serum dilution showing darker color than the lowest dilution of the negative serum was regarded as the end point.

RESULTS

To increase objectivity and reproducibility, we set 50% transmittance (T) on the colorimeter as the maximum reading to be considered positive. We titered all reagents to the 50% end point to determine optimal dilution or serum end points.

Our conjugate, prepared as an anti-human immunoglobulin G-Fab, is highly specific and reactive. Table 1 illustrates a typical conjugate titration. In the lower dilutions, excessive background readings caused the negative serum to appear to be positive. At the optimum dilution in this titration there was essentially no reactivity in the negative serum (61% T at 1:10), but the positive serum was reactive to its known IIF titer of 1:2,560 and gave a sharp end point.

Consistency in results of serological procedures for the diagnosis of toxoplasmosis is extremely important. Since the most widely used procedure is the IIF test, which gives the same results as the methylene blue dye test, we considered it important to compare the IIF and ELISA procedures. Table 2 illustrates the comparative results of these two tests. Because the ELISA test is relatively easy to perform, we tested it in twofold dilutions; the IIF test was tested in fourfold dilutions. This resulted in an apparent skewing of the data, but the data still exhibit a close straight-line relationship. The only obvious discrepancy is the one serum that is 1:16 by IIF and 1:1,024 by ELISA.

To determine reproducibility, we tested one positive serum (IIF 1:1,024) and one negative

TABLE 1. *Conjugate titration for ELISA in the toxoplasmosis system*

Conjugate dilution	Test serum ^a	% T (at 450 nm) at serum dilution of:						
		10	40	160	640	2,560	5,120	10,240
400	Pos	11	7	5	5	13	28	31
	Neg	19	20	23	30	X ^b	X	X
600	Pos	5	9	5	9	25	42	46
	Neg	35	39	35	41	X	X	X
800	Pos	9	29	10	19	38	48	69
	Neg	43	62	50	50	X	X	X
1,000	Pos	10	35	15	21	50	68	76
	Neg	61	66	66	55	X	X	X

^a Pos, Positive control (IIF 1:1,024); Neg, negative control (IIF < 1:4).^b X, Not done.TABLE 2. *Comparison of IIF and ELISA tests for toxoplasmosis*

IIF	ELISA								
	<16	16	32	64	128	256	512	1,024	≥2,048
<16	22 ^a								
16			1		3			1	
64				2		2			
256				2	8	9	1	2	
1,024				1	1	1	3	2	2
≥4,096								2	

^a Number of sera.

serum (IIF < 1:16) on 10 different days. Table 3 shows that in 8 of the 10 tests, titers of 1:512 or 1:1,024 were obtained for the positive serum. In one instance the titer was 1:256, and in only one instance was there a major variation, in which the titer was 1:4,096. The high titer was obtained in a test that normally would have been considered invalid because the negative control was strongly positive.

Perhaps a better measure of reproducibility is seen in the results of the negative control serum. Since all dilutions of the serum are negative, only the colorimeter readings of the initial dilution were compared. In 8 of 10 tests, T varied only from 42 to 68%. In the one instance that was low, 28% T, the positive serum was similarly low. When the negative serum gave 81% T, there appeared to be no adverse effect on the positive serum. (Although the data do not show it, the titer of the positive serum was 1:1,024 on that day.)

Determining the specificity of tests for toxoplasmosis is complicated by the high prevalence of antibody in the normal population. Table 4 shows the results of batteries of sera submitted for the serodiagnosis of various diseases. With the single exception of the 1:256 result in an amebiasis serum, all sera positive in ELISA either were confirmed by being also positive by IIF or had titers too low to be of clinical importance. Of some concern is the one rheumatoid specimen that was positive by both IIF and

TABLE 3. *Reproducibility of ELISA titers for toxoplasmosis on 10 successive days*

Serum tested	Titer				
	256	512	1,024	2,048	4,096
Positive (times tested)	1	3	5		1 ^a
Negative (% T at 1:16 dilution)	64, 54, 68, 64, 81, 28, ^a 50, 42, 48, 45				

^a Tested on same day.TABLE 4. *Specificity of ELISA test for toxoplasmosis*

Sera submitted for:	- IIF + ELISA ^a	No. of sera	
		+ IIF + ELISA	- IIF - ELISA
Amebiasis	256	2	7
Echinococcosis	32, 32	3	6
Histoplasmosis	64	3	11
Cytomegalovirus	16, 16	1	8
Rheumatoid		1	12

^a Reciprocal of ELISA titer for each serum negative by IIF but positive by ELISA.

ELISA. Although only 1 of 13 sera was positive, rheumatoid serum has been recognized as a complicating factor in the IIF procedure, so this finding justifies a more thorough study of these sera by ELISA.

DISCUSSION

These data clearly delineate the sensitivity,

specificity, and reproducibility of ELISA. As evaluated here with toxoplasmosis as the test system, this procedure clearly shows great promise as a system for serodiagnosis of a great variety of diseases and conditions. We have found it to be as sensitive and specific as the IIF procedure and highly reproducible. Although Voller et al. (13) found discrepancies between results from ELISA and the methylene blue dye test and between ELISA and the indirect hemagglutination test, we found excellent agreement between ELISA and IIF.

With the microtitration techniques in particular, reagents are inexpensive and stable. The conjugate, as prepared, was diluted 1:1,000 for use, and it has been used for 6 months without detectable deterioration. The conjugate used in these studies was prepared according to published techniques for ELISA, except that NaN_3 was included in the buffer for the Sephadex column. Since these data were obtained, new conjugates have been prepared according to the method of Nakane and Kawaoi (8), and the NaN_3 was omitted since it is an enzyme inhibitor. This change provided superior conjugates that give more dependable reactivity, but the sensitivity and specificity must still be evaluated. The high working dilution and long shelf life of the conjugates make them very economical reagents for large-scale testing. The stability of horseradish peroxidase permits prepared conjugates to be easily shipped and stored.

The high degree of reproducibility is very encouraging. Plus or minus one twofold dilution is a demanding criterion for a serological procedure and is not attained by most with any confidence; yet only one test fell markedly outside this range. The negative serum only varied a few percent T and was never considered positive in any of the tests. This level of reproducibility assures a procedure that is reliable and that lends itself to modification and refinement.

Specificity was well within expected limits. Clinical information with which to evaluate the toxoplasma reactivity of the one negative-positive serum submitted for amebiasis serology was not available. Whether the IIF result is a false negative or the ELISA result is a false positive is undecided. Of more importance, however, is the positive rheumatoid serum. One of the major problems with the IIF test for toxoplasmosis is the reaction with sera from autoimmune diseases. We hoped that the partial purification of the antigen might eliminate this cross-reaction. One of the 13 rheumatoid sera reacted in both IIF and ELISA. One questions whether the reaction is a specific toxo-

plasma reaction in both tests or simply a non-specific reaction in each.

Although ELISA has been used for a variety of diseases and conditions, it still needs to be standardized. We have attempted to delineate some of the parameters of the technique itself, using toxoplasmosis as the test model. Nevertheless, optimum conditions for virtually every step of the procedure still need to be defined. All investigators who have reported so far have used the original method of Engvall and Perlmann (4) or Ruitenberget al. (10), or minor modifications of them. Little has been done to evaluate and standardize the methodology. Now that we have established the limits of specificity and reproducibility with a somewhat purified toxoplasma antigen, we can use this quantitative procedure to define more clearly the optimum conditions for ELISA.

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