Standardized Quantitative Enzyme-Linked Immunoassay for Antibodies to *Toxoplasma gondii*

T. M. LIN,¹ S. P. HALBERT,^{1*} AND G. R. O'CONNOR²

Cordis Laboratories, Miami, Florida 33127¹; and Francis I. Proctor Foundation for Research in Ophthalmology, University of California, San Francisco, California 94143²

A quantitative and standardized enzyme-linked immunosorbent assay is described which uses lyophilized antigen-coated disks for the detection of human antibodies to *Toxoplasma gondii*. It does not require serial dilution of the test specimen, and the objective absorbance readings are converted into international units per milliliter traceable to the World Health Organization's reference standard preparation of toxoplasma antibodies. It was shown to be highly specific, reproducible, and sensitive. The reagents were stable, without biohazard, and ready for use. Studies of various parameters in the assay indicated that the test conditions were relatively flexible. The enzyme-linked immunosorbent assay results correlated satisfactorily with the methylene blue dye test, the indirect immunofluorescence test, and the passive hemagglutination test.

The diagnosis of human toxoplasmosis has depended heavily on serological methods for detecting antibodies to *Toxoplasma gondii*. These principally include the classical Sabin-Feldman methylene blue dye test (MBD), the indirect immunofluorescence test (IIF), and the passive hemagglutination test (PHA). The current methods of choice for clinical purposes are the MBD and IIF tests (8). However, the former uses live organisms and a scarce "accessory factor," and IIF requires a fluorescent microscope and well-standardized fluorescein-labeled antibody preparations.

Recently, enzyme-linked immunosorbent assays (ELISA) have been applied to the detection of toxoplasma antibodies, particularly in microtiter plates (1, 2, 16-18). The microtiter ELISA is generally semiquantitative and may give erratic results due to the nonuniform absorbent quality of the plastic microtiter plates (3).

In our laboratories, an ELISA for detecting hepatitis B surface antigen in human blood samples has been developed which utilizes small plastic disks for covalent binding of the solid-phase reagents (6). This procedure was incorporated into a diagnostic kit (Cordia H, Cordis Laboratories, Miami, Fla.). As reported here, this format was modified for the quantitative determination of toxoplasma antibodies in human specimens. In addition, to establish a uniform basis of reference in the reporting of results, we measured the toxoplasma antibody levels in international units per milliliter, providing values directly traceable to the World Health Organization's (WHO) first standard human anti-toxoplasma serum preparation (19). In the pres-

ent ELISA technique, serial dilution titrations and pretreatment of the test samples are not required; the total incubation time is short (1 h and 50 min); and either serum or plasma can be used. The communication describes several aspects of the general procedure and comparative results with MBD, IIF, and PHA assays.

MATERIALS AND METHODS

T. gondii organisms of the RH strain were harvested by differential centrifugation from peritoneal exudates of 3-day infected mice with heparin-containing saline (1 mg/ml). The suspensions were centrifuged and washed three times at 4°C (3,000 × g, 10 min). After being frozen at -20°C at least overnight, they were thawed, sonicated, and centrifuged at high speed (45,000 × g, 30 min). The supernatant (soluble antigen) was collected and diluted with 0.1 M NaHCO₃, pH 9.6, to an appropriate concentration. Special plastic disks containing surface isothiocyanate groups (13-mm diameter) were mixed with this antigen solution, agitated at 4°C overnight, washed, and lyophilized. These dried antigen-coated disks (containing 3 to 5 µg of protein each) were stored at 4°C in the presence of a desiccant until use. Antibodies to human immunoglobulins G and M were separately produced in goats, salted out with ammonium sulfate, and immunospecifically purified. They were then conjugated to calf alkaline phosphatase (PL Biochemicals, Milwaukee, Wis.) with glutaraldehyde (Polysciences, Inc., Warrington, Pa.) (5). The dialyzed antibody-enzyme conjugates were diluted to working concentrations in buffer at pH 8.0 (0.0025 M tris(hydroxymethyl)aminomethane [Tris], 0.2% bovine serum albumin, 0.001 M MgCl₂, 0.15 M NaCl, and 0.03 M sodium phosphate) containing 0.02% sodium azide. They were mixed proportionally for use and kept sterile at 2 to 8°C when not in use.

The human positive control was a pool of eight recalcified plasmas containing relatively high levels of *Toxoplasma* antibodies. It served as the secondary standard and was calibrated by the present ELISA method against the WHO's international standard for anti-toxoplasma serum, human, first standard, 1959 (19). The human negative control was a pool of recalcified human plasma with negligible amounts of toxoplasma antibodies (less than 20 IU/ml). Both controls were preserved with 0.1% sodium azide, made sterile by filtration, and stored in portions at 4°C until use.

For each test, one of the antigen-coated disks was incubated with 10 μ l of sample diluted in 500 μ l of specimen diluent (containing 0.01 M phosphate-buffered saline [pH 8.0], 6% bovine serum albumin, 0.05% Tween 20, and 0.1% sodium azide). After incubation at 37°C in a shaking water bath for 45 min, the sample solution was removed by aspiration, and the antigen disk (with antibody attached if present) was washed five times each with 2.5 ml of 0.002 M Tris-buffered saline, pH 7.2 to 8.0. Antibody-enzyme conjugate (0.5 ml) was then added to the disk, which was incubated as above for 45 min. The disk was again washed five times as above, transferred to an unused vial, and incubated as above for 20 min with 1 ml of the enzyme substrate, *p*-nitrophenyl phosphate (1 mg/ml in 0.028 M sodium bicarbonate buffer [pH 9.8], containing 0.01 M $MgCl_2$). The enzyme activity was stopped with 0.1 ml of 3 N NaOH and read with a spectrophotometer at a wavelength of 405 nm. To facilitate the assays, equipment used for the Cordia H procedure (Cordis Laboratories) was employed, i.e., devices for transferring and washing the disks.

The positive control and three fivefold serial dilutions of it in specimen diluent were tested simultaneously in each assay. The absorbance values and anti-toxoplasma activity (international units per milliliter) were plotted on log-log paper to establish a standard curve from which the anti-toxoplasma activity of each test sample was converted to international units per milliliter, based on its absorbance reading. Tests with the WHO standard, as well as with the secondary positive control, revealed that the straight-line relationship between absorbance readings and the anti-toxoplasma activity covered a range from 2 to 1,000 IU/ml. Because of the linearity of the relationship, the conversion from absorbance value to international units per milliliter could be made easily and rapidly with minimum error with an inexpensive hand-held calculator (e.g., Texas Instruments TI-55).

In addition to blood samples from local volunteers or blood donors, we obtained serum samples from three laboratories doing routine toxoplasma antibody immunoassays for clinical purposes and assayed them in a coded manner (after they had been tested in the laboratories supplying them) by the PHA, MBD, or IIF procedures. Comparisons of titers obtained by the present ELISA test and these other tests were analyzed by the correlation coefficient of Pearson and Lee (R) (10) after logarithmic transformation (15), with the negative results assuming the titers to be either 1, or one or more dilution level(s) below the lowest level determined in the titration system (15). The nonparametric rank correlation coefficient of Spearman (R_s) (10) was also used to avoid the bias due to this as-

sumption for negative results, especially where they covered a wide range of titers (e.g., less than 16). For the same reason, where the sample size was large enough for a meaningful conclusion, results from samples with titers of 16 or greater were also separately analyzed by R. In addition to the conventional arithmetic calculation for standard deviation and coefficient of variation, the geometric standard deviation and geometric mean were also used for comparison of reproducibility of the present ELISA with other test results, as recommended by the Center for Disease Control (15).

RESULTS

A typical standard curve established with serial fivefold dilutions of the positive control is shown in Fig. 1. It can be seen that a straight-line relationship was obtained on a log-log scale and that the coefficient of correlation of the international units per milliliter and absorbance values in the example shown was 0.998. This coefficient was generally greater than 0.995. Similar straight-line relationships were found with the WHO primary standard, as well as with several individual high-titered serum specimens. The slopes of these lines tended to be similar.

Numerous parameters in the assay were varied singly to study their effects on the final absorbance values, as well as on the linearity and slopes of the standard curves. The variables included such factors as the amount of antigen used for coating the disks, incubation times, volumes of test sample, volumes of conjugates, etc. The results are summarized in Fig. 2. When various amounts of antigen were used for coating, the absorbance achieved reached a plateau at 5 μ g/disk. However, it was found that the absorbance values for the negative control and background decreased when the disk was coated with more antigen, resulting in greater slopes for

FIG. 1. Example of a typical standard curve of standardized quantitative ELISA for toxoplasma antibodies. Experimental data indicate that this straight-line relationship covers a range at least from 2 to 1,000 IU/ml.

the standard curves. When the concentration of substrate was increased, the slope dropped appreciably, although the absorbance increased slightly. Both absorbance and slope decreased

FIG. 2. Effect of some parameters on the absorbance values of the positive control and on the regression coefficient (slope) of the standard curve. The latter was established with the readings from the positive control and its three serial fivefold dilutions. These data represent averages from two or three experiments each run in duplicate. All of these standard curves closely fit with correlation coefficients ranging from 0.995 to 0.999, except for the two marked with a check. The standard procedure used in the present study employed the second condition listed in all cases. The conjugate concentration was presented as fold of that used in the standard procedure (#).

substantially as the volume of substrate (at 1 mg/ml) was increased. Increased volumes of test sample increased the absorbance values slightly but decreased the linearity and slope of the standard curve. Varying the incubation times with the test sample, or with the conjugate, had relatively little effect on these values, whereas increasing the substrate incubation time increased the absorbance, but had only a minor effect on the slope. Increasing the conjugate volume or its concentration tended to increase the absorbance and the slope, but also increased the background absorbance unacceptably. Background absorbance decreased to a steady level as the concentration of bovine serum albumin in the specimen diluent was increased to 4% and above. Incubation at 37°C gave increased absorbance and provided better standard curves than at room temperature. In general, these data indicate that many of the conditions for the assay are not critical and could be tailored to meet particular needs within most of the parameters studied.

Assays of specimens derived from a single bleeding from the same donors indicated that either serum or plasma could be used in the ELISA assay, with equivalent results. Fifteen volunteers donated blood, and the anticoagulants used were heparin, citrate, dextrose citrate, and ethylenediaminetetraacetic acid. Five of the donors showed toxoplasma antibodies 200 IU/ml or higher, and three showed values of 10 IU/ml or less. After correction for any dilution factor due to the anticoagulant used, it was found that the toxoplasma antibody levels in the serum and various plasmas of any individual determined by ELISA were equivalent, i.e., they were within the assay error (average coefficient of variation, 15%).

The reproducibility of the ELISA procedure was quite satisfactory, as shown in Table 1. The "within-run" reproducibility of the assay showed

TABLE 1. Within-run reproducibility of ELISA for *Toxoplasma* antibodies

Test specimens (no. of replicates)	ELISA observed values			
	Absorbance		IU/ml	
	Arithmetic mean (% C. V.) ^a	Geometric mean $\times/\pm$ SD ^b	Arithmetic mean (% C. V.)	Geometric mean $\times/\pm$ SD
A (9)	0.42 (9.2)	0.42 $\times/\pm$ 1.10	14 (15.5)	14 $\times/\pm$ 1.19
B (9)	2.80 (6.0)	2.80 $\times/\pm$ 1.06	420 (10.7)	418 $\times/\pm$ 1.11
C (9)	1.58 (5.2)	1.58 $\times/\pm$ 1.05	152 (9.2)	151 $\times/\pm$ 1.10
D (8)	0.14 (3.3)	0.14 $\times/\pm$ 1.03	2.1 (6.0)	2.1 $\times/\pm$ 1.06
E (8)	0.84 (5.0)	0.84 $\times/\pm$ 1.05	49 (8.9)	49 $\times/\pm$ 1.09
F (8)	2.60 (7.0)	2.59 $\times/\pm$ 1.07	367 (12.9)	356 $\times/\pm$ 1.15
G (8)	0.19 (3.0)	0.19 $\times/\pm$ 1.03	3.5 (5.3)	3.5 $\times/\pm$ 1.05
Avg % C. V. or SD	5.5	1.06	9.8	1.1

^a C. V., Coefficient of variation.

^b SD, Standard deviation.

an average coefficient of variation of 5.5% and a geometric standard deviation of 1.06 in the absorbance values. These values were 9.8% and 1.11, respectively, after conversion to international units per milliliter. Tests of day-to-day interlaboratory reproducibility showed approximately equivalent results.

The specificity of the ELISA values for toxoplasma antibodies was confirmed by absorption experiments. In the examples shown in Fig. 3, six human serum specimens (5 positive and 1 negative) were preincubated with *T. gondii* antigen, *Entamoeba histolytica* antigen, *Trichinella spiralis* antigen, or no antigen. The latter two parasitic antigens were crude extracts incorporated into diagnostic kits using immunodiffusion (Cordis Laboratories) and were known to possess relatively high specific antigenic activity, i.e., sufficient for gel diffusion techniques. The *T. spiralis* was derived from infected mouse tissue. The preincubated specimens were then assayed as above to determine whether neutralization of the antibody in the sample had occurred by the particular antigen in question. It is clear from the results that the toxoplasma antigen was the only preparation capable of significantly reducing the ELISA values of the positive specimens, indicating the immunological specificity of the present ELISA for toxoplasma antibodies. Reciprocally, when the disks were coated with *E. histolytica* antigens and used in similar neutralization experiments with human sera containing anti-amoeba antibodies,

only *E. histolytica* antigen (not toxoplasma antigen) was shown to reduce significantly the anti-amoeba antibody activity (unpublished data).

Comparisons between the present ELISA and three other immunoassays (MBD, IIF, and PHA) are summarized in Fig. 4-7. High and significant correlation coefficients were observed in all instances. These data also indicated that specimens showing up to 30 IU/ml by ELISA were mostly negative by the other three assays. In addition, almost all specimens positive by the other assays showed greater than 10 IU/ml, and most of them were above 30 IU/ml. Four of the 206 specimens shown in Fig. 4 were nonreactive or reactive undiluted in the MBD test, but showed high ELISA values (>50 IU/ml). Absorption experiments as described above revealed that only the *Toxoplasma* antigen (but not normal mouse plasma, or amoeba or *Trichinella* antigens) was able to significantly reduce the high ELISA values in these specimens.

The Center for Disease Control (Atlanta, Ga.) distributes reference human serum controls for the assay of toxoplasma antibodies. One lot of these reference sera was assayed by ELISA, with good agreement in the results as shown in Table 2. These values are in accord with the distribution of values shown in Fig. 7.

DISCUSSION

The ELISA procedure described here has several advantages over other commonly used as-

FIG. 3. Demonstration of the immunological specificity of ELISA reactions for toxoplasma antibodies by absorption studies. Six human serum specimens (5 positive and 1 negative) were preincubated at 37°C for 30 min with soluble antigens derived from: (i) *T. gondii*, (ii) *E. histolytica*, (iii) *T. spiralis*, or (iv) none. After this preincubation, each specimen was assayed by the ELISA procedure. It can be seen that the soluble toxoplasma antigens dramatically blocked the ELISA reactions in each of the positive serum specimens, whereas the other antigens had no significant effect.

FIG. 4. Scatter diagram and regression lines of ELISA compared to the MBD results on 206 serum specimens (both assays were done in the laboratory of G.R.O.). R_s , the rank correlation coefficient of Spearman. R_1 and R_2 , the correlation coefficient of Pearson and Lee for all 260 specimens and those with MBD titer of 16 or greater, respectively. The regression lines are for R_1 and R_2 .

FIG. 5. Comparison between the ELISA and the MBD tests on 20 human serum samples for *Toxoplasma* antibodies. MBD, run at the University of California, San Francisco; the ELISAs were run independently and coded at the Cordis Laboratories. R_s , the rank correlation coefficient of Spearman. R_1 , the correlation coefficients of Pearson and Lee for all 20 specimens. The regression line is for R_1 .

says for detecting and measuring antibodies to *T. gondii*. The MBD, IIF, PHA, and most of the micro-ELISA tests require serial dilutions of the test samples for establishing the level of antibodies, whereas our ELISA yields such quantitative values on the basis of a determination

with a single dilution of test sample. Moreover, the present assay was standardized against the WHO reference preparation, as the antibody concentrations were reported in international units per milliliter. Such a single basis of reference should minimize technical variations in different laboratories and permit more uniform measurements of toxoplasma antibodies in any laboratory using this procedure. This problem with the other assays is underscored by the description submitted with the WHO international standard for anti-toxoplasma serum. This single serum preparation was assayed in a number of qualified laboratories, and the titers found ranged from 400 to 10,000 in the MBD test, from 25 to 500 in the complement fixation test, and between 250 and 6,000 in the IIF test.

The ELISA described here was satisfactory for serum or plasma, and sodium azide as a preservative did not interfere with the results. No pretreatment of the test specimens is required, the assay is relatively rapid, and the reagents, which are ready for use, revealed considerable stability without any biohazard. Preliminary data indicated that all reagents could be kept refrigerated at 2 to 8°C for at least 2.0 years without noticeable effect on the test results. The antigen on the solid phase could even be kept at room temperature (23°C) for at least 10 months without loss of activity.

FIG. 6. Comparison between the ELISA and the PHA tests on human serum samples for *Toxoplasma* antibodies. PHA was run by the Bio-sciences Laboratories, and the ELISA were run independently and coded at the Cordis Laboratories. R_s , the rank correlation coefficient of Spearman. R_1 and R_2 , the correlation coefficients of Pearson and Lee for all 95 samples and those with titers of 16 or greater, respectively, assuming the PHA titer of 1 for those less than 16.

FIG. 7. Comparison between the ELISA and the IIF tests on 22 human serum samples for *Toxoplasma* antibodies. IIF was run by the Parasitic Serology Laboratories, Center for Disease Control, Atlanta; and the ELISA were run independently and coded at the Cordis Laboratories. R_s , the rank correlation coefficient of Spearman. R_1 , the correlation coefficient of Pearson and Lee, assuming the IIF titers of 4 for the negative results. The regression line is for R_1 .

A rather good correlation was found between the ELISA values (international units per milliliter) and titers of toxoplasma antibodies found in several panels of test specimens assayed by

MBD and IIF. In a separate, ongoing clinical research study with 34 specimens with MBD titers between 1,024 and 8,192, there was no indication of a plateau of the ELISA test results

TABLE 2. *ELISA assay results with Center for Disease Control toxoplasmosis control human sera*^a

Serum	Center for Disease Control-stated titer, IIF	ELISA observed (IU/ml) ^b	
		Arithmetic mean $\pm$ SD ^c	Geometric mean $\times/\div$ SD
Negative	<32	2.1 $\pm$ 0.3	2.1 $\times/\div$ 1.17
Low titer	32-128	62 $\pm$ 11	62 $\times/\div$ 1.19
High titer	512-2,048	203 $\pm$ 30	202 $\times/\div$ 1.17

^a Catalog no. KK0501, lot 76-0339K.^b Measured in six experiments at different times.^c SD, standard deviation.

with these high-titered samples (unpublished data). Failure to achieve even higher correlations may be due to two factors. The MBD and IIF procedures allow a plus-or-minus fourfold error in the endpoint titers (12). Thus, a sample with a "true titer" of 1:64 may reveal titers of 1:16 or 1:256 in replicate assays. The reproducibility of the ELISA is considerably better, showing a geometric standard deviation of 1.11 in international units per milliliter, which is smaller than most of those reported for immunoglobulin quantitation (13, 14). The latter, in turn, has been found to provide appreciably better reproducibility than those serological tests using serial titrations, which allow accuracy to within one twofold dilution (14). In addition, the ELISA determination yields results which are presumably a summation of all the antibodies directed against the several toxoplasma antigens (at least seven) known to induce antibody responses in humans (4, 7, 9, 11; unpublished data, G.R.O.), since the preparations used to coat the disks were total extracts of the organisms prepared by very mild sonication procedures. In contrast, the MBD and the IIF tests undoubtedly measure a more limited set of antibody responses, presumably those situated on the surface membrane of the toxoplasma organisms (9).

ACKNOWLEDGMENT

This work was supported in part by grant EY-01204 from the National Institutes of Health, Bethesda, Md.

LITERATURE CITED

- Camargo, M. E., A. W. Ferreira, J. R. Mineo, C. K. Takiguti, and O. S. Nakahara. 1978. Immunoglobulin G and immunoglobulin M enzyme-linked immunosorbent assays and defined toxoplasmosis serological patterns. *Infect. Immun.* 21:55-58.
- Capron, A., D. Bout, and J. C. Dugimont. 1975. Apport des méthodes immunoenzymologiques utilisant des antigènes purifiés au diagnostic spécifique et automatisé des infections parasitaires. *Ann. Soc. Belge Med. Trop.* 55:443-454.
- Chessum, B. S., and J. R. Denmark. 1978. Inconstant ELISA. *Lancet* i:161.
- Chordi, A., K. W. Walls, and I. G. Kagan. 1964. Analysis of *Toxoplasma gondii* antigens by agar diffusion methods. *J. Immunol.* 93:1034-1044.
- Engvall, E., and P. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay for immunoglobulin G. *Immunochemistry* 8:871-874.
- Halbert, S. P., and M. Anken. 1977. Detection of hepatitis B surface antigen (HB_sAg) with use of alkaline phosphatase-labelled antibody to HB_sAg. *J. Infect. Dis.* 136:S318-S323.
- O'Connor, G. R. 1957. Precipitating antibody to toxoplasma. *Am. J. Ophthalmol.* 44:75-83.
- Palmer, D. F., J. J. Cavallaro, K. Walls, A. Sulzer, and M. Wilson. 1976. A procedural guide to the performance of the serology of toxoplasmosis. Center for Disease Control, Atlanta, Ga.
- Remington, J. S., and J. L. Krahenbuhl. 1976. Immunology of toxoplasma infection, p. 236-267. *In* S. Cohen and E. Sadun (ed.), *Immunology of Parasitic Infections*. Blackwell Scientific Publications, Philadelphia, Pa.
- Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods, 6th ed. Iowa State University Press, Ames.
- Strannegard, O. 1962. Studies of toxoplasma precipitinogen and their corresponding antibodies by means of diffusion-in-gel methods. *Br. J. Exp. Pathol.* 43:600-613.
- Sulzer, A. J., and E. C. Hall. 1967. Indirect fluorescent antibody test for parasitic diseases. IV. Statistical study of variation in the indirect fluorescent antibody (IFA) test for toxoplasmosis. *Am. J. Epidemiol.* 86:401-407.
- Taylor, R. N., K. M. Fulford, and A. Y. Huong. 1978. Comparison of kinetic and end-point diffusion methods for quantitating human serum immunoglobulins. *J. Clin. Microbiol.* 8:23-27.
- Taylor, R. N., K. M. Fulford, and V. A. Przybyszewski. 1977. Proficiency testing summary analysis: diagnostic immunology 1977 I: rubella, carcinoembryonic antigen, immunoglobulin quantitation, C-reactive protein, salmonella (typhoid O) agglutination. Center for Disease Control, Atlanta, Ga.
- Taylor, R. N., A. Y. Huong, and K. M. Fulford. 1975. Measurement of variation in serologic tests. Center for Disease Control, Atlanta, Ga.
- Voller, A., D. E. Bidwell, A. Bartlett, D. G. Fleck, M. Perkins, and B. Oladehin. 1976. A microplate enzyme-immunoassay for toxoplasma antibody. *J. Clin. Pathol.* 29:150-153.
- Walls, K. W. 1978. Serodiagnosis of toxoplasmosis. *Lab. Manage.* 16:26-31.
- Walls, K. W., S. L. Bullock, and D. K. English. 1977. Use of the enzyme-linked immunosorbent assay (ELISA) and its microadaptation for the serodiagnosis of toxoplasmosis. *J. Clin. Microbiol.* 5:273-277.
- World Health Organization. 1977. Biological substances. International standards, reference preparations, and reference reagents, p. 20-21. World Health Organization, Geneva.