Reproducible Enzyme-Linked Immunosorbent Assay with a Magnetic Processing System for Diagnosis of Toxoplasmosis

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An enzyme-linked immunosorbent assay with polycarbonate-coated iron beads as the solid phase and with magnetic processing devices was evaluated for the quantitation of antibodies to *Toxoplasma gondii* in human serum samples. Under the parameters and other basic conditions determined in this study, the assay was highly reproducible: coefficients of variation for the absorbance values obtained with the positive serum were 2.42% in same-day tests and 3.75% in day-to-day tests. Significant correlations were observed between the present assay system and other conventional serological tests: correlation coefficients were 0.960 with the dye test and 0.929 with the latex agglutination test. Statistical analysis based on the frequency distribution of absorbance values for dye-test-positive and dye-test-negative serum samples gave feasible border lines for distinguishing between positive and doubtful samples (0.357) and between doubtful and negative samples (0.266). Under this diagnostic criterion, the results of our assay system agreed remarkably well with those obtained by the dye test and the latex agglutination test, with consistencies of 94.9 and 93.9%, respectively.

Toxoplasma gondii is a protozoan parasite producing severe congenital infections in infants and acute infections in immunosuppressed patients (17). Several authors (1, 3-5, 7, 12, 16, 23-25) have described the application of the enzyme-linked immunosorbent assay (ELISA) for the detection of *Toxoplasma* antibodies as an additional serological technique for diagnosing toxoplasmosis besides conventional tests.

The ELISA method with polycarbonate-coated iron beads as the solid phase and with a magnetic processing system was proposed by Smith and Gehle (19) and was afterward adapted for microtitration by the same authors (20). Some of its advantages over the other ELISA systems have been reported as (i) the uniformity in the surface area of the solid phase involved in the reaction, which is important for the precision of the assay, and (ii) the simplicity and economy of simultaneous processing with such a semiautomated system.

In our laboratory, immunodiagnosis by ELISA with the above-mentioned system with some modifications in the magnetic transfer devices has been studied. Previous reports dealing with Japanese encephalitis virus (10; E. Konishi and M. Yamaoka, J. Virol. Methods, in press) have demonstrated that the system is sensitive as well as reproducible and is highly suitable for antibody detection. In spite of these advantages, however, no detailed studies have been found so far concerning the adaptation of this magnetic

processing ELISA system for toxoplasmosis. This report describes not only the examination of basic conditions in our assay system but also its diagnostic evaluation for the quantitation of *Toxoplasma* antibodies in human serum samples, especially in comparison with other ELISA systems.

MATERIALS AND METHODS

Antigen. Antigen was prepared from tachyzoites of the RH strain of *T. gondii* harvested from 20- to 25-g ICR mice infected 3 days previously (15). The peritoneal exudates were centrifuged first at $50 \times g$ for 5 min to remove debris and mouse cells and again at $300 \times g$ for 5 min to collect tachyzoites. The parasites were then washed three times with phosphate-buffered saline (PBS, pH 7.4) by centrifugation and finally were suspended in PBS to a concentration of 3×10^8 parasites per ml. The suspension was then sonicated at 28 kHz five times for 1 min each in an ice bath and was clarified at $10,000 \times g$ for 1 h. The supernatant contained 1.5 mg of protein per ml as determined by the method of Lowry et al. (13) with bovine serum albumin (BSA) fraction V (Armour) as a standard, and it was stored at -20°C until used as *Toxoplasma* antigen. Just before sensitization in each test, the antigen was diluted in PBS to make a protein concentration of 15 $\mu\text{g/ml}$ and was sonicated again at 28 kHz for 1 min twice in an ice bath.

Solid phase. Iron beads coated with polycarbonate, 4.2 mm in diameter, were purchased from Toyoshima Works Ltd. Used beads were regenerated by washing with methylene chloride, immersing in 5% polycarbon-

FIG. 1. Magnetic transfer devices: a magnet plate (left) composed of twelve permanent ceramic magnets (35 by 10 by 7 mm) attached to a U-bottom microplate, and a transfer plate (right) with a hole 3 mm in diameter at the bottom of each well. Wells in the front line show placing of the iron beads with a forceps having polycarbonate-coated tips.

ate solution made in the same organic solvent, scattering on blotting paper, and allowing to dry as previously described (10).

Serum samples. Serum samples taken routinely from patients were kindly provided by N. Yamaguchi of the Central Laboratory of Kobe University and were stored at -20°C until tested. For positive and negative controls in the ELISA, serum samples were used which had antibody titers of 1:256 (positive) and $<1:4$ (negative) as determined by the methylene blue dye test described below.

Conjugate. Alkaline phosphatase (type VII-S; Sigma Chemical Co.) was conjugated to goat anti-human immunoglobulin G (Fujizoki Pharmaceutical Co., Ltd.) as described by Engvall and Perlmann (6).

Substrate. Enzyme activity was measured with 0.1% *p*-nitrophenylphosphate in 10% diethanolamine buffer (pH 9.8) containing 0.01% MgCl_2 .

Magnetic processing system. The magnetic processing system fundamentally followed the technique of Smith and Gehle (20) with some modifications in (i) the magnet plate, which was composed of 12 permanent ceramic magnets (35 by 10 by 7 mm) attached to a U-bottom microplate (Dynatech Laboratories, Inc.; Sanko Jun-yaku Co.) and (ii) the transfer plate, which was a microplate with wells, each having a hole (diameter, 3 mm) at the bottom (Fig. 1). The beads

were simultaneously transferred from one reaction mixture to another through a wash bath in the following manner. The microplate containing the beads in its wells was covered with the transfer plate, and then the magnet plate was put on the transfer plate. The beads were magnetically accepted by the transfer plate. The transfer plate with the beads was immersed in washing fluid and was then placed on blotting paper to remove the fluid from the wells through the holes at their bottoms. The beads were washed twice more. The transfer plate with the washed beads was set on the magnet plate, turned upside down, and afterward placed on the microplate containing the reagent for the next reaction. The beads dropped into the reagent upon the removal of the magnet plate.

ELISA procedure. The test was performed essentially as described by Takeda (21) and Smith and Gehle (19, 20). The parameters and other basic conditions in the assay were determined as described below. Polycarbonate-coated iron beads were sensitized by immersion for 1 h at 37°C in the *Toxoplasma* antigen (15 $\mu\text{g}/\text{ml}$). The beads were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween), and then they were each introduced into 100 μl of 100-fold diluted test serum which had been distributed into a well of the microplate. After 1 h of incubation at 28°C (the first reaction), the beads were picked up with the transfer devices, washed three times with PBS-Tween, and placed in the wells, each of which contained 100 μl of 100-fold diluted conjugate. The diluent for the test serum samples and conjugate was PBS-Tween supplemented with 1% BSA and 0.02% NaN_3 . After 1 h of incubation at 28°C (the second reaction), the beads were washed three times with PBS-Tween and another three times with distilled water and then were transferred to 100 μl of the substrate each. After 30 min of incubation at 28°C (the third reaction), the reaction was halted by adding 50 μl of 1 N NaOH to each well, and the absorbance at 410 nm (A_{410}) was read by Microelisa Minireader MR 590 (Dynatech Laboratories). The test serum samples, conjugate, and substrate were preincubated in the microplate wells at 28°C for 1 h before each reaction.

In the test, two beads were used for each test serum sample, and at most, 48 samples, including the controls, were tested per microplate. The absorbance values obtained with two beads were averaged and then adjusted with the value for the positive control as 1.0. This was done to minimize experimental error caused by differences between microplates.

Other serological tests. The methylene blue dye test was based on the method of Sabin and Feldman (18) as

TABLE 1. Effects of washing with PBS-Tween and addition of BSA to diluent on specific and nonspecific reactions in the ELISA system^a

Serum ^b	A ₄₁₀ after treatment with:						
	Distilled water	PBS-Tween	BSA at concn (%) ^c :				
			0.0	0.5	1.0	2.0	4.0
Positive	1.20	1.50	1.15	1.47	1.52	1.60	1.66
Negative	0.56	0.07	0.13	0.11	0.08	0.08	0.06

^a Experimental conditions were as described in the text.

^b Positive and negative control serum samples with dye test antibody titers of 1:256 and $<1:4$, respectively.

^c Diluent for BSA was PBS-Tween containing 0.02% NaN_3 .

FIG. 2. Effect of the pH of the coating buffer in sensitization on ELISA results. Beads were sensitized for various periods with the antigen (15 $\mu\text{g/ml}$) made in 0.1 M sodium carbonate buffer, pH 9.6 (○, △), and PBS, pH 7.4 (●, ▲), followed by the first reaction with positive control serum (○, ●) and negative control serum (△, ▲). The abscissa shows the incubation period used in sensitization; the ordinate shows A_{410} adjusted with the value for the positive control serum sensitized at pH 7.4 for 60 min as 1.0.

modified by Kobayashi et al. (9). The latex agglutination (LA) test was performed as described by Lunde and Jacobs (14) and Tsubota et al. (22) with Toxotest-MT (Eiken Chemical Co., Ltd.).

RESULTS

Basic assay conditions. Experiments were performed to determine the conditions under which the ELISA would be best suited for quantitating antibodies to *T. gondii*. First, we examined the conditions for reducing the nonspecific (background) reaction with Tween 20 and BSA (Table 1). Comparison of the washing fluids indicates that PBS-Tween reduced the absorbance value obtained with the negative control serum much more than distilled water did; on the other hand, the positive control serum yielded a higher absorbance value with PBS-Tween washing. In addition, adding BSA to the diluent helped reduce the absorbance value in the negative serum and increase it in the positive serum. Therefore, the conditions of PBS-Tween in the washing process and 1% BSA in the diluent were introduced into our assay system.

Second, the parameters in the present ELISA procedure were examined with the positive and negative control serum samples by changing one or two parameters and making the others, which were described above, constant. As for the pH of the coating buffer in sensitization, pH 7.4 was compared with the commonly used alkaline pH 9.6 (Fig. 2). The time courses for the positive control serum indicate that both pHs were al-

most equal in sensitizing efficiency when the incubation period was 30 min or more. Since higher absorbance values were obtained with the negative serum at pH 9.6 than at pH 7.4, we adopted pH 7.4 for sensitization. The antigen concentration was examined by sensitizing iron beads with various concentrations of antigen (Fig. 3). From the dose-dependent absorbance curve for the positive and negative serum samples, a concentration of 15 $\mu\text{g/ml}$ was considered to be optimal.

Figure 4 shows the relationship between absorbance value and incubation period for the first and the second reactions. In the first reaction, the absorbance value increased with the incubation period, reached a maximum after 1 h, and remained there for another 1 h, whereas in the second reaction, no such plateau was observed, and the values increased continuously. For the sake of reactivity and economy of time each 1 h was selected. The other parameters had the same tendencies as previously reported (10; Konishi and Yamaoka, in press). For serum and conjugate concentrations, it was found that each 1:100 dilution gave an appreciable difference in absorbance value between positive and negative serum samples. In the incubation period of the third reaction, 30 min was regarded as sufficient for our system.

Reproducibility. Replicate tests on single serum samples were performed to evaluate the reproducibility of the ELISA system. Six repli-

FIG. 3. Effect of antigen concentration for sensitization on ELISA results. Beads were sensitized with various concentrations of antigen in PBS for 1 h followed by the first reaction with positive (○) and negative (●) control serum samples. The abscissa shows the antigen concentration; the ordinate shows A_{410} adjusted with the value for the positive control serum sensitized at 15 $\mu\text{g/ml}$ as 1.0.

FIG. 4. Effects of various incubation periods in the first reaction (a) and the second reaction (b) upon ELISA results, with positive (○) and negative (●) control serum samples. The ordinate shows A_{410} adjusted with the value for 1 h of incubation as 1.0.

cates of each of the positive and negative serum samples were assayed in one day, and eight replicates were examined on different days. The coefficients of variation (CVs) were 2.42% in same-day tests and 3.75% in day-to-day tests with results obtained with the positive control serum (Table 2). The fact that the negative control serum gave greater CVs than the positive control serum was attributed to the small means in the negative serum.

Diagnostic criterion. A total of 113 positive and 98 negative serum samples as determined by the dye test were assayed by the present ELISA to get the frequency distribution of absorbance values (Fig. 5). There was a distinct separation between the dye-test-positive group and the dye-test-negative group, with no serum found in the range of 0.35 to 0.45. These distributions were then statistically analyzed by the method of Heck et al. (8) (Table 3). For positive samples, the confidence limit calculated from the mean and standard deviation by a one-tailed *t*-test at the 0.01 probability level was 0.2661. In the

TABLE 2. Reproducibility of the ELISA system

Test ^a	Serum	A_{410}	CV (%)
Same-day	Positive	1.57 ± 0.038	2.42
	Negative	0.06 ± 0.007	11.67
Day-to-day	Positive	1.52 ± 0.057	3.75
	Negative	0.05 ± 0.007	14.00

^a Single serum samples were tested repeatedly six times in the same-day test and eight times in the day-to-day test.

same manner, 0.3567 was obtained as the confidence limit at the 0.0005 probability level for negative samples. Supposing that the serum samples showing absorbance values of >0.357 are positive, those of <0.266 are negative, and those between these two values are doubtful, the probability of classifying negative serum erroneously as positive is $<0.05\%$, and the probability of classifying positive serum erroneously as negative is $<1\%$.

Comparison with other serological tests. The absorbance values obtained with 98 human serum samples were compared with the results of the dye test (Fig. 6) and the LA test (Fig. 7). High and significant correlations were observed between the ELISA and each of these two tests: correlation coefficients were 0.960 with the dye test and 0.929 with the LA test.

Besides such quantitative agreements, the results obtained by the ELISA agreed qualitatively with the results obtained by the dye test and the LA test under the above-mentioned diagnostic criterion, with 0.266 and 0.357 as the border lines (Table 4). In relative ratio, the consistency

FIG. 5. Frequency distribution of ELISA A_{410} values for 113 dye-test-positive and 98 dye-test-negative human serum samples.

TABLE 3. Statistical analysis of 211 positive and negative human serum samples

Serum	No. of samples	A_{410}	Confidence limit ^a	
			99%	99.95%
Positive	113	1.1662 ± 0.3806	0.2661	
Negative	98	0.1112 ± 0.0721		0.3567

^a Determined by one-tailed *t*-test.

was 94.9% with the dye test and 93.9% with the LA test.

DISCUSSION

Various ELISA methods have been reported for the detection of *Toxoplasma* antibodies in human serum samples (1, 3–5, 7, 12, 16, 23–25). As to the choice of solid phases, these studies are divided into two broad groups. One group (3–5, 7, 16, 23–25) used test tubes or depression microplate wells as the solid phase (henceforth designated as the plate method), and the other (1, 12) used disks or beads (the bead method; in one study [1], polystyrene beads, not iron beads were adopted). The essential difference between the methods is considered to be that in the former the reagents are changed on immobile solid phase and in the latter the mobile solid phase is transferred into the prepared reagent. The bead method offers several advantages. For example, although the temperature differences across a plate are to a great extent responsible for such inconsistencies as the edge effect which have been reported in the plate method (2, 5, 11), this thermal difficulty can be easily eliminated in the bead method by prewarming the reagents distributed in microplate wells and by a sensitization process in which many solid-phase units are totally immersed in the incubated antigen solution. Moreover, Smith and Gehle (20) have pointed out that the ELISA system with iron beads as the solid phase is superior to the bead method with other materials in that simultaneous processing is easily done by magnetic transfer devices. Synchronized incubation, as well as the uniform surface area of the solid phase (19), leads to high precision in the assay. In our experiment, the CVs obtained by same-day and day-to-day tests (Table 2) were smaller than any of the CVs described in the above references with the plate and bead methods, showing the higher reproducibility of the present iron bead method.

In the preliminary examinations of the basic assay conditions, PBS-Tween washing and the addition of 1% BSA to the diluent contributed not only to the reduction of the nonspecific reaction but also to the enhancement of the specific reaction (Table 1). This phenomenon seems to be related to the pronounced separa-

FIG. 6. Comparison between the ELISA and the dye test for *Toxoplasma* antibodies in 98 human serum samples. A total of 56 samples were negative by both the ELISA and the dye test.

FIG. 7. Comparison between the ELISA and the LA test for *Toxoplasma* antibodies in 98 human serum samples. A total of 56 samples were negative by both the ELISA and the LA test.

TABLE 4. Qualitative agreements between results of ELISA and of dye and LA tests for 98 serum samples

Antibody ^a	ELISA antibody ^b			Agreement with ELISA (%)
	Positive	Doubtful	Negative	
Dye test				94.9
Positive	33	0	0	
Doubtful	1	4	2	
Negative	0	2	56	
LA test				93.9
Positive	32	2	1	
Doubtful	2	4	1	
Negative	0	0	56	

^a Dye test titers were classified as positive, $\geq 1:16$; doubtful, 1:4; and negative, $< 1:4$. LA test titers were classified as positive, $\geq 1:32$; doubtful, 1:16; and negative, $< 1:16$.

^b Absorbance values were classified as positive, > 0.357 ; doubtful, 0.266 to 0.357; and negative, < 0.266 .

tion of dye-test-positive from dye-test-negative groups in the frequency distribution (Fig. 5). The optimal antigen concentration for sensitization was 15 $\mu\text{g/ml}$ (Fig. 3). Because the volume necessary for immersion of 96 beads in a test tube was 4 ml, approximately 0.6 μg was calculated as the antigen amount corresponding to one bead. Peritoneal exudates taken from two mice served well enough for the examination of about 1,000 samples. In the relationship between absorbance value and each of the incubation periods in the first and second reactions (Fig. 4), the time courses have almost the same tendency as those reported by Engvall and Perlman (6).

The present ELISA system correlated significantly with other serological tests (Fig. 6 and 7), and the high qualitative agreements (Table 4) were obtained with the diagnostic criterion given by statistical analysis of the frequency distribution (Table 3). This consistency indicates that our ELISA system was sufficiently evaluated for the quantitation of *Toxoplasma* antibodies in human serum samples. In view of its capacity to examine many samples (48 samples per plate) within a limited time (about 4 h in all), this assay system may be applied not only to clinical diagnosis but also to epidemiological surveillance for humans and domestic animals. This method is now being tested for immunoglobulin M quantitation, which is of greater importance for the diagnosis of acute acquired toxoplasmosis (16, 17).

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