

## Detection of Circulating Antigens in Sera of Rabbits Infected with *Toxoplasma gondii*

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Experiments were performed to investigate the usefulness of an enzyme-linked immunosorbent assay utilizing avidin-biotin interaction as a diagnostic tool for detection of *Toxoplasma* antigen in blood. The lower limit of sensitivity of the assay by this method was ca. 4 ng/ml, and standard assays provided a linear plot of antigen concentration over a range up to 250 ng/ml. In rabbits inoculated subcutaneously with trophozoites of the RH strain, *Toxoplasma* antigen became demonstrable in the circulation 3 days after injection, before emergence of antibody in serum and development of parasitemia. Analysis of the antigen in serum from infected rabbits by high-permeation liquid gel chromatography suggested the occurrence of antigens of four different molecular weights,  $\geq 400,000$ , 220,000, 130,000, and 45,000. Of these antigens, those of molecular weights 220,000 and 130,000 showed a conspicuous elevation with time after infection.

In clinical laboratory diagnosis of acute toxoplasmosis, tests have been primarily aimed at detection of immunoglobulin M (IgM) antibody among other parameters of antibody response to *Toxoplasma* infection (3, 4, 13-15, 20). Recently, increasing attention has been directed toward detection of *Toxoplasma* antigen in the blood of infected subjects (2, 12, 18, 19), and several articles dealing with this diagnostic approach have appeared in the literature. The demonstration of *Toxoplasma* antigen in the circulation also constitutes a problem of paramount importance in connection with opportunistic infection in immunodeficient patients who cannot mount an adequate protective response (1, 7, 10, 21).

Several laboratory procedures have been reported as diagnostic means for detection of *Toxoplasma* antigen, i.e., counterimmunoelectrophoresis, agar gel immunodiffusion (12), and enzyme-linked immunosorbent assay (ELISA) (2, 18, 19). Particularly, the ELISA is considered to be a highly sensitive and useful method. A more refined method of ELISA, utilizing the avidin-biotin interaction (AB-ELISA), has recently been reported with evidence of increased sensitivity and specificity (5, 8, 16, 22).

The present study was designed to assess the usefulness of AB-ELISA as a diagnostic method for detection of *Toxoplasma* antigen by exploring the time course of changes in the serum antigen level of experimentally infected rabbits, and investigating the significance of circulating antigen in toxoplasmosis.

**Experimental infection in rabbits.** Rabbits (New Zealand White) weighing ca. 3 kg were infected with *Toxoplasma gondii* by subcutaneous injection of  $10^3$  trophozoites of the RH strain or peroral inoculation with 40 cysts of the Beverly strain per animal. Serial blood samples were drawn from each rabbit after inoculation and tested for parasitemia in mice (ddY, 10 mice per group) by injecting 1 ml of the blood intraperitoneally and determining the survival time of the mouse for 50% lethality. Sera from the rabbits inoculated were titrated for antibody to *T. gondii* by a latex agglutination test, using *Toxoplasma* antigen-coated latex particles

(Eiken Chemical Co., Ltd., Tokyo). The titration was carried out by the microtiter method.

**Biotinylated antitoxoplasmal antibody.** Immune sera were obtained from three rabbits at the height of antibody titer after two peroral inoculations of 40 cysts of the Beverly strain given ca. 4 weeks apart. Sera obtained from rabbits at 4 weeks after the final inoculation were pooled, and the pooled antiserum with an antibody titer of 160,000 in ELISA and 4,096 in the passive latex agglutination test was precipitated with ammonium sulfate and purified through a chromatographic column of DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) to obtain IgG antibody. The purified IgG antibody was dialyzed overnight against 0.1 M sodium bicarbonate at 4°C and adjusted to a concentration of 1 mg/ml. To 1 ml of this IgG preparation, 200  $\mu$ l of a biotin-*N*-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, Mo.) solution in dimethyl sulfoxide (1 mg/ml) was added, and the resulting mixture was incubated for 4 h at room temperature. The mixture was then dialyzed against phosphate-buffered saline (PBS) (pH 7.4) overnight at 4°C, dispensed in 100- $\mu$ l aliquots in ampoules, and stored at -70°C until the time of its use as a biotinylated antibody.

**AB-ELISA.** The purified anti-*Toxoplasma* IgG antibody was diluted with 0.1 M bicarbonate buffer (pH 9.6) to an optimal concentration of 10  $\mu$ g/ml (for the lot used in the present study, as previously determined by checkerboard titration), and 100  $\mu$ l was pipetted into wells of a polystyrene microtiter plate (Costar, Cambridge, Mass.) and incubated at 4°C overnight. The wells were then washed five times with PBS containing 0.05% Tween 20 (PBS-Tween). Serial two-fold dilutions of serum sample and those of antigen purified as described below from a suspension of toxoplasmas, as a control, were prepared in PBS-Tween containing 10% normal rabbit serum, and 100  $\mu$ l of each dilution was transferred to the microtiter plate. The plate was incubated at 37°C for 1 h, and the wells were rinsed five times with PBS-Tween. Subsequently, 100  $\mu$ l of biotinylated IgG antibody (a 1:1,000 dilution of a 1-mg/ml solution in PBS-normal rabbit serum-Tween, prepared immediately before use) was added to each well, followed by incubation for 30 min at 37°C. The plate was washed again five times with PBS-Tween, and 100  $\mu$ l of a 1:500 dilution of avidin conjugate horseradish peroxidase

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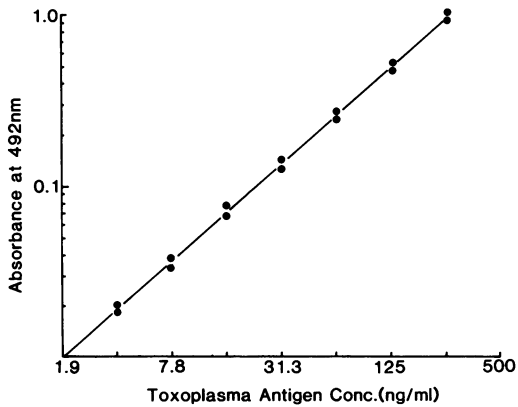


FIG. 1. Standard curve of *Toxoplasma* antigen as determined by AB-ELISA. The antigen concentration was determined by the Folin phenol reagent on sonicated (10 kc for 20 min) trophozoites and is expressed as the bovine serum albumin amount in nanograms per milliliter.

(E. Y. Laboratories, Inc., San Mateo, Calif.) in PBS-normal rabbit serum-Tween was added to each well and incubated for 15 min at 37°C. After the washing was repeated with five changes of PBS-Tween, a substrate solution for horseradish peroxidase (made up by dissolving 60 mg of *o*-phenylene-

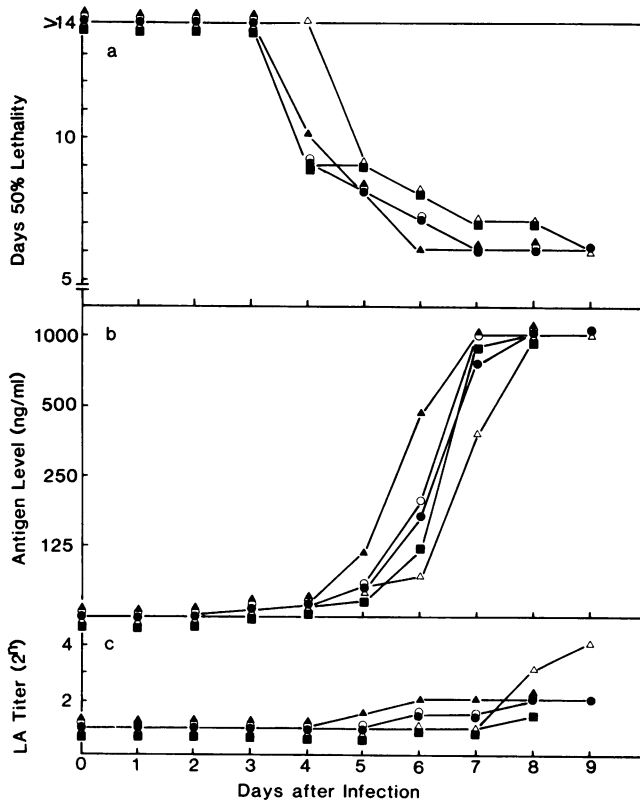


FIG. 2. Serum antigen and antibody levels and parasitemic condition in rabbits after subcutaneous inoculation of *T. gondii* (RH strain). The severity of parasitemia (a) is expressed as days of infection for 50% lethality in groups of mice inoculated intraperitoneally with 1 ml of infected rabbit blood. The serum antigen concentration (b) was determined by AB-ELISA, and the serum antibody titer (c) was determined by latex agglutination test. Symbols indicate data obtained from individual rabbits.

diamine in 150 ml of 0.2 M phosphate-0.1 M citrate buffer [pH 4.8] and adding 100  $\mu$ l of 5% H<sub>2</sub>O<sub>2</sub>) was pipetted into the wells, and the plate was incubated for 20 min at room temperature in a dark place. Then 100  $\mu$ l of 2 N HCl was added to each well to stop the reaction, and the absorbance of each reaction mixture was determined at 492 nm with a photometric microtiter plate reader (Corona MTP 12; Corona Electric Co., Ltd., Katsuta, Ibaraki). A parallel series of mixtures prepared and processed in the same way, but containing the same quantities of PBS-normal rabbit serum-Tween in place of the sample, served as the reagent blank.

***Toxoplasma* antigen.** *Toxoplasmas* collected from peritoneal exudates from infected mice were washed three times with cold PBS and passed through a CF11 column to eliminate mouse peritoneal constituents (17). The purified *Toxoplasmas* were sonicated for 20 min at 10 kc and centrifuged at 10,000  $\times g$  for 30 min. The amount of *Toxoplasma* antigen was determined with the Folin phenol reagent (9) and expressed as the equivalent of bovine serum albumin.

**High-permeation liquid gel chromatography.** A 0.3-ml sample of infected rabbit sera was chromatographed on TSK Gel G3000 SW in two coupled columns (0.7 by 60 cm) (Tokyo Soda Manufacturing Co., Ltd., Tokyo) at a constant flow rate of 1 ml/min. The elution pattern of circulating toxoplasmal antigen was compared with that of serum IgM (970,000), IgA (160,000), albumin (66,000), C3 (195,000), and C3c (140,000), which served as marker proteins for estimation of the molecular weight and which were quantitated by laser nephelometry. Secretory IgA (390,000) and egg albumin (45,000) were also employed as marker proteins.

**Sensitivity of AB-ELISA for antigen.** The limit of sensitivity of the AB-ELISA for *Toxoplasma* antigen, as assessed with sonicated antigen preparations, was ca. 4 ng/ml with a linear plot for calibration up to a concentration of 250 ng/ml (Fig. 1).

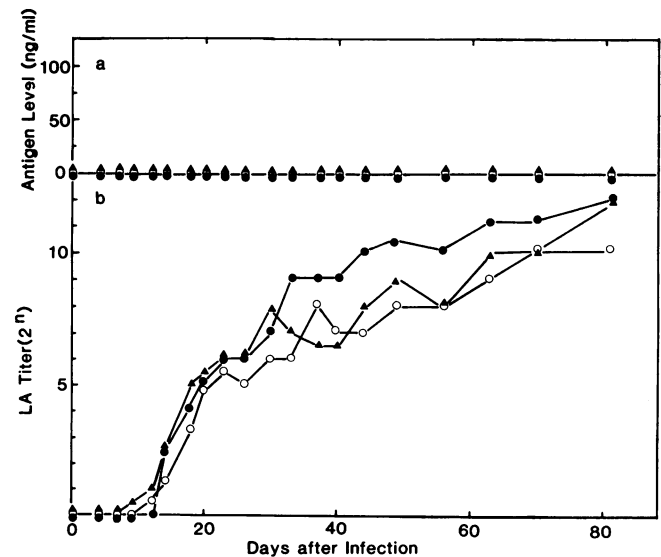


FIG. 3. Serum antigen and antibody levels in rabbits after peroral inoculation of *T. gondii* (Beverly strain). (a) Serum antigen concentration determined by AB-ELISA. (b) Serum antibody titer determined by latex agglutination test. The agglutination test was carried out by the microtiter method. Symbols indicate data obtained from individual rabbits.

**Serum antigen levels in rabbits after inoculation with *T. gondii* (RH strain).** In rabbits inoculated subcutaneously with *T. gondii* (RH strain), *Toxoplasma* antigen emerged in the circulation 3 days after infection and the serum antigen level rose sharply thereafter (Fig. 2b). All of these animals died 8 to 9 days after infection. Antitoxoplasma antibody was detected, but only with a titer of as low as 1:16 in only one rabbit surviving for 9 days (Fig. 2c). As no parasites could be demonstrated microscopically in rabbit blood after infection, the rabbits were tested for parasitemia by determining the days after infection for 50% lethality in mice. Groups of 10 mice each were inoculated intraperitoneally with 1 ml of infected rabbit blood obtained at various intervals after infection. Times for 50% lethality longer than 14 days were considered as no parasitemia. All rabbits developed parasitemia, four of them after 4 days of infection and the fifth after 5 days (Fig. 2a).

**Serum antigen levels in rabbits after inoculation with *T. gondii* (Beverly strain).** Rabbits perorally inoculated with the Beverly strain showed elevation of antitoxoplasma antibody in serum (Fig. 3b), but all remained seronegative for antigen throughout the observation period of 120 days postinoculation (Fig. 3a). All rabbits survived over the entire period of observation in this group.

**Analysis of antigen in the serum of rabbits infected with *T. gondii* (RH strain).** Sera obtained from rabbits on days 0, 7,

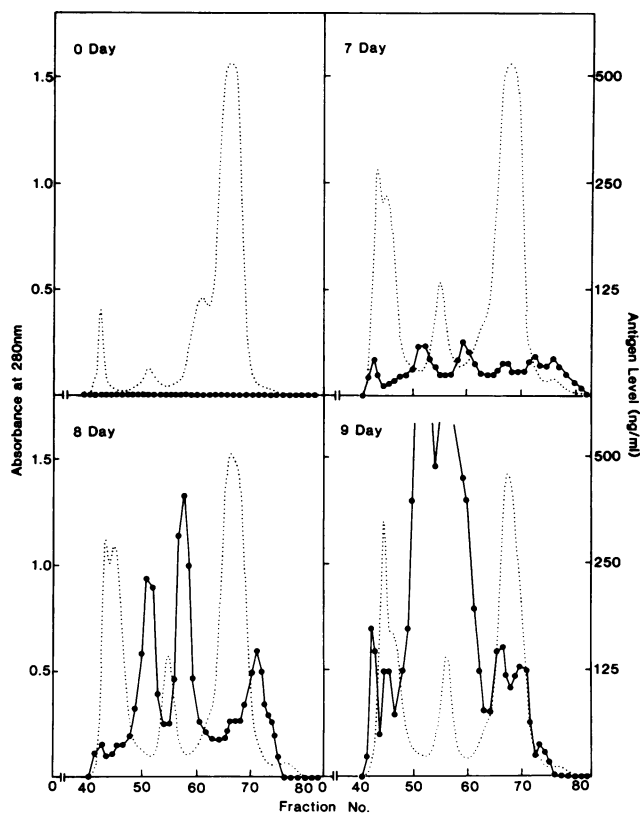


FIG. 4. High-permeation liquid gel chromatographic analysis of *Toxoplasma* antigen in the blood of infected rabbits. Rabbit sera were chromatographed on two coupled columns of TSK Gel G3000 SW (0.7 by 60 cm each) at a constant flow rate of 1 ml/min, and 0.5-ml fractions were collected. Symbols indicate absorbance at 280 nm (.....) and antigen levels (●).

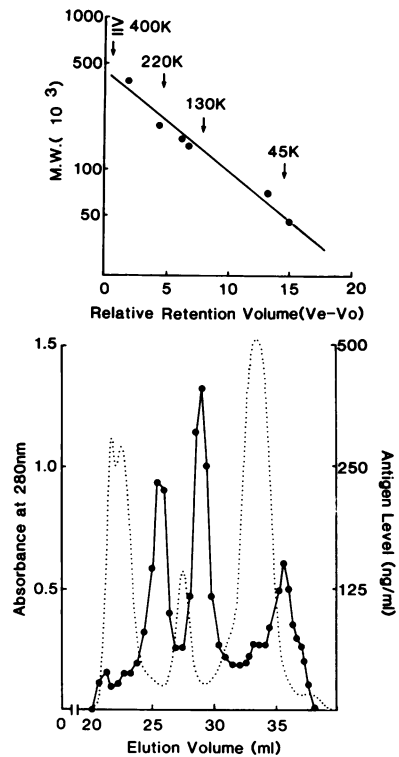


FIG. 5. Molecular weight estimation of antigen peaks. Secretory IgA (400,000), C3 (195,000), serum IgA (160,000), C3c (140,000), serum albumin (66,000), and egg albumin (45,000) served as standard proteins for estimation of molecular weights of *Toxoplasma* antigens. Symbols in the bottom panel indicate absorbance at 280 nm (.....) and antigen levels (●).

8, and 9 after infection with the RH strain were fractionated by high-permeation liquid gel chromatography and assayed for *Toxoplasma* antigen by the AB-ELISA technique. The typical elution patterns with levels of antigen are shown in Fig. 4. Four principal peaks (designated peaks 1 to 4) of *Toxoplasma* antigen became evident in the serum 7 days after inoculation, with prominent elevation of peaks 2 and 3 on days 8 and 9 in contrast to the rest of the peaks.

Comparison of the elution pattern with that of highly purified preparations of reference proteins gave molecular sizes for these antigen peaks of  $\approx 400,000$ , 220,000, 130,000, and 45,000, respectively (Fig. 5).

The diagnostic usefulness of countercurrent electrophoresis and agar gel diffusion for detection of *Toxoplasma* antigen in the blood of infected subjects was investigated by Raizman and Neva (12). ELISA was shown to be a highly sensitive procedure by van Knapen and Panggabean (19) and by Araujo and Remington (2). Nevertheless, these methods appear to be not sensitive enough as a diagnostic tool to detect the protozoan parasite antigen in the circulation. It has been shown that avidin has a remarkably high degree of affinity for biotin, some  $10^6$  times as great as that of antibody for antigen (16). The AB-ELISA system, therefore, provides a noticeable enhancement of sensitivity of the assay and has been reported to be capable of detecting IgE at a concentration as low as 100 pg/ml in serum (16). In the detection of *Toxoplasma* antigen in blood, our assay system demonstrated an improved lower limit of assay sensitivity of 4 ng/ml, compared with 30 to 50 ng/ml with the conventional ELISA procedure (2). The demonstration of the antigen in

the sera of infected rabbits was accomplished by AB-ELISA without requiring any procedure for serum concentration in this study, whereas concentration of serum by affinity chromatography was necessary for detection of the antigen in sera of experimentally infected rabbits, according to Raizman and Neva (12).

In the rabbits inoculated per os with cysts of the Beverly strain, our data showed elevation of serum antibody to *Toxoplasma* but did not reveal any demonstrable antigen in the serum throughout the postinoculation course of 120 days. This is attributed to an exceedingly low circulating antigen level, i.e., less than 4 ng/ml, that escaped detection by this procedure.

High-permeation liquid gel chromatographic analysis of *Toxoplasma* antigen in sera of rabbits infected by the RH strain yielded data that suggest the occurrence of at least four antigens of different molecular weights ( $\geq 400,000$ , 220,000, 130,000, and 45,000). From these molecular weights and the relatively low serum antibody titers seen in the rabbits at this stage of infection, it would be reasonable to infer that these antigen molecules occur in free form rather than as immune complexes. It was reported by Handman et al. (6) and Ogata et al. (11) that *Toxoplasma* antigen preparations obtained by the use of a monoclonal antibody showed a molecular weight of 43,000 or less, as estimated on polyacrylamide disc gel electrophoresis run in the presence of sodium dodecyl sulfate. Most of the antigens recovered from the blood of infected rabbits had apparently greater molecular weights in this study. The disparity may be explained by the fact that the antigenic components probably undergo resolution into their ultimate subunits in the presence of a surfactant, e.g., sodium dodecyl sulfate, whereas no such dissociation of antigen ordinarily takes place in vivo.

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