Detection of Antigens in Urine during Acute Toxoplasmosis

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Received 27 September 1988/Accepted 24 January 1989

***Toxoplasma* antigens were detected in sera and urine of mice acutely infected with *Toxoplasma gondii*. The concentrations of antigens in the urine samples measured by enzyme-linked immunosorbent assay were similar to those detected in the sera of the corresponding mice. The major antigens were not dialyzable and were largely destroyed by treatment with trichloroacetic acid and heat (100°C for 1 h). *Toxoplasma* antigens were demonstrable on Western blots (immunoblots) of the urine samples.**

The alarming incidence of toxoplasmic encephalitis in patients with acquired immunodeficiency syndrome and the difficulty encountered in establishing the diagnosis without brain biopsy has served as an impetus for development of new, noninvasive methods for diagnosis of this entity (7). The earlier demonstration of *Toxoplasma* antigens in serum samples from acutely infected animals and humans (1, 3, 4, 6, 8, 10, 11) suggested to us that if antigens also appear in the urine, their detection might prove to be of diagnostic value. The experiments reported here were performed to determine whether *Toxoplasma* antigens can be detected in urine from a murine model of acute toxoplasmosis.

Female Swiss Webster mice weighing 18 to 20 g (Simonson Laboratories, Gilroy, Calif.) were placed in metabolic cages, two mice per cage, 1 day prior to infection. A total of five experiments were performed. The first two were pilot experiments for determining the dose of *Toxoplasma gondii* to be used and to ascertain the optimal times for collection and the optimal storage conditions for urine and serum. Eight mice were used for each experiment.

Urine was collected for 24 h and used as normal urine. Thereafter, the mice were infected intraperitoneally with 10⁵ trophozoites of the RH strain of *T. gondii* obtained from the peritoneal fluid of mice infected 2 days previously (12). Urine was collected on days 4 through 7 after infection (all infected mice died by day 8). Urine samples collected on a given day from the 8 mice were pooled and centrifuged for 10 min at 1,300 × g, and the pellet was discarded. The supernatant was used undialyzed or was dialyzed overnight against 0.01 M phosphate-buffered saline, pH 7.2 (PBS), by using dialysis membrane (Spectrum Medical Industries Inc., Los Angeles, Calif.) with a 12,000- to 14,000-molecular-weight cutoff. The undialyzed and dialyzed specimens were stored at –20°C. Preliminary experiments revealed that results with urine placed at 4°C and studied within 10 days did not differ from those obtained with urine samples stored at –20°C for up to 30 days.

Serum was collected on days 4, 5, and 6 of infection from mice infected at the same times and with the same inocula as those from which urine was collected. Sera obtained from uninfected mice were used as controls. The sera were stored at –20°C and run in parallel with the urine samples.

Urine samples and sera were tested for the presence of *Toxoplasma* antigen in a modification (J. S. Remington, Y.

Suzuki, and P. Stepick-Biek et al., manuscript in preparation) of an enzyme-linked immunosorbent assay (ELISA) previously described (3). Urine and serum samples were tested at a dilution of 1:1,000 in 0.05 M carbonate buffer (pH 9.6); when tested undiluted or at dilutions of 1:10 or 1:100, the readings were off scale (MicroELISA reader; Dynatech Laboratories, Inc., Chantilly, Va.)

Heat denaturation at 100°C for 60 min was performed on dialyzed normal urine and urine collected on day 6 following infection. Trichloroacetic acid (TCA; Sigma Chemical Co., St. Louis, Mo.) was mixed with dialyzed normal urine or dialyzed urine collected on day 6 to effect a final TCA concentration of 10%. The samples were incubated at room temperature for 2 h, after which they were centrifuged at 11,600 × g for 5 min. The supernatant was then dialyzed overnight against PBS.

Polyacrylamide gel electrophoresis and protein blots were performed as previously described (9). Samples were run on 5 to 15% polyacrylamide gradient slab gels with the use of the discontinuous sodium dodecyl sulfate (SDS) buffer system (5). Phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Bio-Rad Laboratories, Richmond, Calif.) were used as molecular weight standards. Gels were run at 20 mA per gel at room temperature until the tracking dye was approximately 5 mm from the bottom of the gel. The separated proteins were then transferred to nitrocellulose paper (0.45-μm-pore-size; Schleicher & Schuell, Inc. Keene, N.H.) for 1.5 h at 200 mA with a Bio-Rad Trans-blot apparatus. After transfer, the nitrocellulose paper was blocked with 5% nonfat dry milk in PBS.

For polyacrylamide gel electrophoresis, pelleted trophozoites of the RH strain were placed in 2% SDS sample buffer containing 50 mM dithiothreitol plus protease inhibitors (10 mM EDTA, 1 mM leupeptin, 2 mM iodoacetamide, and 1 mM phenylmethylsulfonyl fluoride [Sigma]) to give a final concentration of 1 mg of protein per ml. Protein concentration was determined by using the Bio-Rad protein assay kit. The sample was then boiled for 3 min, left at room temperature for 30 min, and centrifuged for 10 min at 210 × g, and the pellet was discarded. The supernatant was stored at –80°C in aliquots after the addition of 10 μl of 0.1% bromophenol blue per ml. A 10-μg sample of protein per lane was loaded onto the gels. Urine samples for polyacrylamide gel electrophoresis were diluted 1:2 with 4% SDS sample buffer. Sera were diluted 1:100 and 1:1,000 with 2% SDS sample buffer. The samples were then treated as for the

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FIG. 1. (A) Detection by ELISA of *Toxoplasma* antigens in pooled dialyzed and undialyzed urine from 8 mice infected intraperitoneally with 10^5 *T. gondii*. (B) Detection by ELISA of *Toxoplasma* antigens in pooled dialyzed urine and pooled serum from mice infected intraperitoneally with 10^5 *T. gondii*. O.D., Optical density.

trophozoites and 50 μ l of diluted urine per lane was loaded onto the gels.

Control rabbit serum was from noninfected, nonimmunized animals. The antiserum used for developing the blots was from the same pool of rabbit anti-*Toxoplasma* immunoglobulin G used for the antigenemia ELISA. All sera were diluted 1:100 in 5% nonfat dry milk in PBS-0.05% Tween 20 (PBS-T). The conjugate was goat anti-rabbit horseradish peroxidase-labeled immunoglobulin G (Caltag Laboratories, San Francisco, Calif.) diluted 1:1,000 in 3% bovine serum albumin in PBS-T. The substrate was 3,3'-diaminobenzidine tetrahydrochloride (Organon Teknika, Durham, N.C.) and was used at a final concentration of 0.1 mg/ml. Controls were run to test for direct binding of the conjugate to *Toxoplasma* antigens and to samples of normal mouse urine and serum. After being blocked, the nitrocellulose paper was incubated with the conjugate and then developed with the substrate. No bands were seen in these blots (data not shown).

Toxoplasma antigens were demonstrable by ELISA in the urine of mice as early as 5 days postinfection (Fig. 1A). The concentration of antigens rose most sharply between day 5 and day 6 of infection. Results of two additional experiments were almost identical. To ensure that the findings in the urine were not nonspecific because the mice had an acute infection, urine from mice infected with *Trypanosoma cruzi* was also studied. Eight mice were infected intraperitoneally with 10^5 trypomastigotes of the Tuluhuen strain (2). All mice died by day 11 postinfection. Urine from infected mice was collected daily from day 6 through day 10 following infection and was treated and studied as for urine from mice infected with *T. gondii*. Acute lethal infection with *Trypanosoma cruzi* did not result in a false-positive ELISA in urine samples from the mice.

Toxoplasma antigens in sera of mice were demonstrated by ELISA at 4 days after infection, a day earlier than in the urine (Fig. 1B). By day 6, the values for detectable antigen in urine and serum were similar (Fig. 1B).

Dialysis of urine collected on days 4 through 7 of infection did not reduce antigen detection in the ELISA (Fig. 1A). Treatment at 100°C for 1 h resulted in a 91% loss (reduction in optical density) in activity, and TCA treatment resulted in a 95% loss in activity in the supernatant (data not shown). Normal urine, run as a control, showed no alteration in the optical density in the ELISA for either the heat or TCA treatment.

Patterns of protein blots of urine collected on days 6 and 7 of infection differed from those obtained with normal urine.

Bands (mainly with molecular weights between 20,000 and 40,000) were noted in patterns obtained with the day 7 urine that were either not present or were much less intensely stained in the patterns of normal urine (Fig. 2). Two other bands were noted with apparent molecular weights of 75,000 and 6,000.

These results reveal that *Toxoplasma* antigens are demonstrable in urine of mice during the acute stage of *Toxoplasma* infection. *Toxoplasma* antigens were also detected in sera from the same mice. Interestingly, the concentration of antigens in the urine samples, as measured in the ELISA system, was similar to the concentrations in the sera of the corresponding mice. The remarkable decrease in detectable antigens in the urine samples following treatment with TCA suggests that the antigens are mainly protein in nature. When

FIG. 2. Protein blots developed with normal rabbit immunoglobulin G (lane 1) and polyclonal rabbit anti-*Toxoplasma* immunoglobulin G (lanes 2 through 6). Lanes 1 and 4 were with urine collected 7 days after infection, lane 2 was with normal urine, and lane 3 was with urine collected 6 days after infection. Lane 5 was with normal mouse serum, and lane 6 was with serum collected 6 days after infection. Arrows indicate bands present in patterns of urine from infected mice that were either not present or were at a much lesser intensity in patterns of urine from normal mice. Numbers on left represent molecular weight standards.

the antigens were studied by protein blots, the number of bands that appeared to be *Toxoplasma* specific was greater in the urine blots than in the serum blots.

Detection of antigen in urine may be useful for diagnosis of the acute infection in humans and especially for those in whom the infection may be widely disseminated, such as congenitally infected infants and immunocompromised patients. In preliminary studies in patients with acute toxoplasmic encephalitis and acquired immunodeficiency syndrome, we have detected *Toxoplasma* antigens in urine samples from 5 of 20 patients tested. Of the 20 samples, 18 were also tested for antigenemia; 3 serum samples were positive and were from three of the five patients whose urine was also positive for *Toxoplasma* antigen.

This work was supported by Public Health Service grant AI04717 from the National Institutes of Health and grant 000719 from the American Foundation for AIDS Research.

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