Detection of Circulating Antigen During Acute Infections with *Toxoplasma gondii* by Enzyme-Linked Immunosorbertent Assay

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The serodiagnosis of toxoplasmosis is usually based on demonstration of antibodies by means of different serological methods. However, for confirmation of active toxoplasmosis, examination of paired sera is still needed. In this study, a description is given of a sensitive enzyme method (enzyme-linked immunosorbent assay) with which it is possible to detect circulating antigens during the acute stage of mouse and human toxoplasmosis. Preliminary investigations of human sera suggested that circulating antigens are only found during a short period (active phase) of the toxoplasma infection and that they can be found in cases of fresh infections as well as during reinfections. The possible clinical relevance is discussed.

The diagnosis of toxoplasmosis is usually based on demonstration of antibodies. The first laboratory test for toxoplasma antibodies was the dye test, developed by Sabin and Feldman (11), and since then many other methods have been introduced. Due to the fact that a relatively high percentage of people do have antibodies without active toxoplasmosis, more than one serodiagnostic test is required to distinguish patients with active infections from patients with persisting antibodies from a past infection. The differentiation is achieved by using more than one test with various antigens and with varying degrees of sensitivity. Furthermore, in most hospitals paired sera are examined, and only a significant increase in antibody titer within 3 weeks is considered as evidence for acute toxoplasmosis.

The possibility of detection of specific immunoglobulin M (IgM) antibodies (suggesting fresh infections), introduced by Remington et al. (5), seems to be restricted to a few laboratories because of the lack of specificity of many commercially available antisera. A new direct approach is the detection of circulating antigens. Recently, Raizman and Neva (4) succeeded in detecting circulating antigens in experimental toxoplasmosis of mice and rabbits by means of counter-current electrophoresis and the agar gel diffusion test. In other parasitic infections, like schistosomiasis, circulating antigens could also be detected in experimentally infected laboratory animals (2, 3).

In this study it is shown that circulating antigens during the acute stage in mouse toxoplasmosis, as well as in human toxoplasmosis, can be detected by using the sensitive enzyme-linked immunosorbent assay (ELISA), a test that has been used successfully for the detection of serum antibodies to various parasitic antigens, including toxoplasma (1, 7, 8).

**MATERIALS AND METHODS**

**Immunization of rabbits.** Conventionally raised rabbits without antibodies against toxoplasma (dye test negative) were injected intravenously with 10⁴ tachyzoites of the RH strain of toxoplasma, obtained from mouse peritoneal fluid. This mouse-virulent strain is maintained at the Institute by constant reinfection of new animals each 3 days. The rabbits did not show any clinical sign of illness, and at day 10 they were given a booster injection of 10⁴ tachyzoites. They were bled at day 28, when the antibody titer in the dye test reached 1:32,000.

**Preparation of immunoglobulins and labeling with horseradish peroxidase.** The antitoxoplasma rabbit serum was treated with caprylic acid to obtain the immunoglobulin fraction. After sufficient dialysis against physiological saline, it was stored at -20°C, except for 10 ml, which was used for labeling with horseradish peroxidase by means of the periodate method, according to Kawaoi and Nakane (Fed. Proc. 32:840, 1973). This conjugate was stored in a refrigerator (4°C).

**Preparation of a positive antitoxoplasma mouse reference.** Twenty Swiss mice, intraperitoneally infected with 5 × 10⁴ tachyzoites, were bled at day 3 just before succumbence. The pooled sera were shown to contain circulating antigens by means of counter-current electrophoresis, carried out with antitoxoplasma hyperimmune serum, essentially according to the technique described by Raizman and Neva (4).

**Human sera.** A total of 1,116 sera, which were sent in for routine toxoplasma antibody examinations, were tested by means of the conventional immunofluorescent antibody test and the ELISA for specific IgM antibodies as well as free circulating antigens (see below).
ELISA. (i) Detection of circulating antigen. Based on the principle described previously (7, 8), a sandwich method was carried out as follows. Polystyrene microtiter plates (Microtiter, Cooke Engineering Co., Alexandria, Va.) were coated with rabbit antitoxoplasma immunoglobulin. In previous studies it was found the immunoglobulin fraction, with total protein content of 3.4 g/liter, could be diluted 500 times with phosphate-buffered physiological saline (0.01 M, pH 7.2). The cups were incubated with 100 μl of the diluted immunoglobulin solution at 4°C for 48 h. After proper flushing with tap water containing 0.05% Tween 20 for 1 min, samples of undiluted fresh patient sera (100 μl/cup) were added and incubated at 37°C for 4 h under gentle rotation. Then, after proper flushing, 100 μl of the enzyme-labeled antitoxoplasma globulins (the conjugate) optimally diluted in phosphate-buffered saline containing 4% bovine serum albumin and 0.05% Tween 20 was added to each cup. The incubation time was 1 h at 37°C. After flushing, the specific substrate was added, i.e., 5-aminosalicylic acid (80 mg/100 ml of distilled water, pH 6.0) plus H₂O₂ (0.05%) in a 9:1 ratio. A change of color from yellow to brown indicated that the peroxidase was bound to the wall, i.e., that circulating antigens existed in the patient serum. Tests were read after 1 h. As controls for the test, we used: (i) conjugate control, i.e., immunoglobulin-coated wells, without patient serum incubation, treated with the conjugate and the substrate; (ii) substrate control, i.e., immunoglobulin-coated wells, without patient serum incubation and without conjugate incubation but treated with the substrate; (iii) positive control serum (mouse origin); (iv) no serum (vi) positive human serum (i.e., normal human serum to which soluble toxoplasma antigen was added); (v) normal human donor serum as a negative control (i.e., the activator serum used in the dye test). Controls i, ii, iv, and vi all yielded a negative reaction.

(ii) Detection of specific IgM antibodies. For the detection of specific IgM antibodies, an anti-human IgM antiserum (specific for heavy chains) was purchased from the Pasteur Institute, Paris. The assay was principally carried out as described elsewhere for antitoxoplasma immunoglobulin G (IgG) antibodies (8). The results are expressed as either the presence or absence of specific IgM antibodies.

IF test. The conventional indirect immunofluorescent (IF) test was carried out in the widely accepted way, with the difference that, as antigen, cryostat sections of brain tissue from mice infected 2 days previously in the cerebellum cavity with the RH strain of toxoplasma (11) were used. The results are expressed as end point titers of twofold dilutions.

RESULTS

Mouse sera. In the sera of infected mice, circulating antigens could be detected from 1 day after infection onwards (Table 1). This correlates very well with the onset of parasitemia observed previously (10). The pooled sera from day 3 after infection still reacted positively in a 1:100 serum dilution and were further used as positive control sera.

Human sera. A total of 1,116 sera of patients suspected of having toxoplasmosis and sent to the Institute for conventional antibody titer measurement was also examined for circulating antigens; 64 of them reacted positively (5.7%).

We compared this phenomenon with two other serological data, the end point titer in the IF test and the presence or absence of specific IgM antibodies (ELISA). High antibody titer (IF ≥ 1:1,024) and/or the presence of specific IgM antibodies suggest toxoplasmosis in the active stage.

Because clinical data were not available from all patients, the patients were divided into two groups, those with histories and those without. In Table 2 are presented the results of the patients with circulating antigens in their sera and who showed clinical symptoms that could be caused by toxoplasma, for example, enlarged lymph nodes, icterus, hepato- and splenomegaly, fever, and chorioretinitis. In Table 3 are presented the results of the patients with circulating toxoplasma antigens from whom we did not

| TABLE 1. Correlation of the presence of circulating toxoplasma antigens (detected with ELISA) and parasitemia in mice infected with T. gondii |
| Determination | Presence at (days postinfection): |
| | 0* | 1 | 2 | 3 |
| Circulating antigens | - | + | + | + |
| Parasitemia | - | + | + | + |
* Infection with 0.5 × 10⁷ tachyzoites intraperitoneally on day 0.

| TABLE 2. Correlation of the presence of circulating toxoplasma antigens (detected with ELISA) with IF and ELISA IgM results of 35 sera from patients with clinical symptoms suggestive of toxoplasmosis |
| Antitoxoplasma IgG (H + L)* (IF) | Antitoxoplasma IgM (ELISA) |
| Titer | No. of sera | + | - |
| ≥1:1,024 | 4 | 4 | 0 |
| ≤1:512 | 31 | 19 | 12 |
* H, Heavy; L, light chain.

| TABLE 3. Correlation of the presence of circulating toxoplasma antigen (detected with ELISA) with IF and ELISA-IgM results of 29 sera from patients without detailed clinical data |
| Antitoxoplasma IgG (H + L)* (IF) | Antitoxoplasma IgM (ELISA) |
| Titer | No. of sera | + | - |
| ≥1:1,024 | 8 | 7 | 1 |
| ≤1:512 | 21 | 11 | 10 |
* H, Heavy; L, light chain.
obtain clinical data. In one of the patients in the latter group we were not able to detect antibodies in either the serum or in the liquor cerebrospinalis, although circulating antigens were found. We could detect the parasite itself in the liquor sediment by means of the direct fluorescent-staining technique (10).

**DISCUSSION**

Usually the clinical diagnosis of toxoplasmosis is supported by serodiagnosis. A rise in antibody titer within a few weeks is believed to be the only real evidence for an acute toxoplasmosis besides the detection of the parasite itself. The detection of specific IgM antibodies against toxoplasma is not yet widely used; first, because of a lack of specificity of biological reagents, and second, because IgM antibodies are not formed in all cases of acute toxoplasmosis and the production does not stop, as it usually does within 6 months, in all cases.

Therefore, it would be of great clinical value to develop a serological test that indicates directly that the parasite is present and active. Circulating toxoplasma antigen can easily be detected under experimental conditions with relatively insensitive precipitation tests (4). With very sensitive immunoenzyme methods like ELISA, it is possible to detect low amounts of antibodies as well as antigens (1, 7-9).

Circulating toxoplasma antigens are not often found in patients, not even in those suspected of having toxoplasmosis. In the latter group we found a percentage of 5.7, suggesting that the antigens do not circulate for a long period. Even when they are partly neutralized by antibodies, antigenic material remains in circulation, since both antigens and antibodies have been detected in sera with high antibody titers. As far as we know, there is no description of immune complexes in toxoplasmosis, and we were not able to detect the circulating antigens as part of a complex with human globulins by using peroxidase-labeled anti-human IgG conjugate. The fact that both high and low, or negligible, antibody titers were found in combination with circulating antigen leads to the hypothetical conclusion that two categories of patients with circulating antigen exist: (i) those with high antibody titer, IF ≥ 1:1,024 (possibly in combination with other positive tests such as specific IgM antibody, the complement fixation test, etc.), suggesting a primary and fresh infection; and (ii) those with low antibody titer, IF test ≤ 1:512, in combination with a negative complement fixation test and no IgM antibodies, suggesting exacerbation of an old (latent) infection, or reinfection. However, in combination with a positive IgM test, it could very well be a primary infection at the onset of infection, when no IgG antibodies have as yet been formed.

The obvious advantage of testing both antibody level and circulating antigen is the possibility of diagnosing an active toxoplasmosis on the basis of only one serum sample instead of using paired sera at 3-week intervals. Even in ocular toxoplasmosis, in which generally low antibody titers are found, we were able to detect circulating antigen in three cases of uveitis. Furthermore, testing for the presence of circulating toxoplasma antigen could be of importance for donor blood used for (transplantation) patients undergoing immunosuppressive treatments. Although nothing is known about the nature of the circulating toxoplasma antigens thus far, these results encourage further studies.

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**LITERATURE CITED**