Demonstration of Intraocular Synthesis of Immunoglobulin G Toxoplasma Antibodies for Specific Diagnosis of Toxoplasmic Chorioretinitis by Enzyme Immunoassay

HANNU J. TURUNEN,1* PAULI O. LEINIKKI,1 AND K. MATTI SAARI2

Institute for Biomedical Sciences1 and Department of Clinical Sciences,2 University of Tampere, Tampere, Finland

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Toxoplasmosis, caused by the protozoan parasite Toxoplasma gondii, is a very common infection in humans. In adolescence and adulthood, most infections are subclinical or run a very mild clinical course (8). A primary infection during pregnancy may lead to a congenital infection of the fetus with or without clinical manifestations (3). A localized infection in the eye with a relapsing course is known to be the most common single manifestation of congenital toxoplasmosis (4). Its outbreak may be delayed for months or even years after birth (1). Recurrences of primary lesions are usually not reported until after the first 10 to 30 years of life (13).

The diagnosis of toxoplasmic chorioretinitis is based mainly on the typical clinical picture and a positive serological test for toxoplasmosis (11, 13). However, other inflammatory processes, such as tuberculosis, candidiasis, and syphilis, and even some noninflammatory conditions may mimic this entity (12), thus emphasizing the need for an accurate differential diagnosis.

Conventional serology is of little value because significant changes of toxoplasma antibody levels in serum are only rarely seen (12). The demonstration of elevated levels of toxoplasma antibodies in the aqueous humor by the dye test has been used for the accurate diagnosis of toxoplasmic chorioretinitis (2, 9).

In a preliminary report (10), we have described the suitability and usefulness of the enzyme immunoassay (EIA) for the determination of intraocular toxoplasma antibodies. In this paper we report detailed results from a larger material of clinically diagnosed toxoplasmic chorioretinitis, other types of uveitis, and controls by using a modified application of EIA.

MATERIALS AND METHODS

Cataract patients. Thirty-two cataract patients without clinical history of uveitis were studied as controls as described earlier (10). An anterior chamber tap was made during normal cataract extraction through a partial limbal incision, with special care to avoid blood contamination. The aqueous humor was collected with a 27-gauge needle on a tuberculin syringe. The samples of aqueous humor and corresponding serum samples were preserved at −20°C.

Patients with posterior uveitis. Thirteen patients with acute posterior uveitis and one patient with generalized uveitis were studied. They were further divided into two groups based on the clinical picture of the disease. The first group consisted of nine patients with typical fundal lesions of recurrent active toxoplasmic chorioretinitis. This group consisted of six males and three females, ranging in age between 13 and 48 years with a mean of 25 years.

The other group consisted of four patients with other forms of posterior uveitis and one patient with general...
ized uveitis. This group consisted of two males and three females, ranging in age between 16 and 62 years with a mean of 44 years.

All patients were studied from 10 days to 10 weeks after the onset of ocular symptoms. The anterior chamber tap was made in an operating room by means of an oblique corneal puncture near the inferior limbus made with a 27-gauge needle on a tuberculin syringe. The amount of aqueous humor obtained was approximately 200 μl. Serum samples were obtained from all patients at the same time as the samples of aqueous humor; second serum samples from the patients with toxoplasmic chorioretinitis were collected about 1 year later. Samples of aqueous humor and of serum were stored at −20°C.

Enzyme immunoassays for toxoplasma and mumps antibodies. A modification of the EIA for the determination of immunoglobulin G (IgG), IgM, and IgA anti-toxoplasma antibodies as described earlier (H. Turunen, K. A. Vuorio, and P. O. Leinikki, Scand. J. Infect. Dis., in press) was used. This modification was found to be suitable for the determination of mumps virus antibodies also.

Antigens. Toxoplasma trophozoites for the preparation of the antigen were obtained from the peritoneal exudates of mice infected 3 to 4 days earlier with the RH strain of T. gondii. Contaminating mouse cells were removed by differential centrifugation at 300 rpm for 10 min followed by three washes with 0.9% NaCl solution. The pellet was resuspended in distilled water and sonicated. The lysate was centrifuged at 10,000 × g for 30 min, and the supernatant was dispensed in 0.1-ml portions and stored at −70°C.

Sucrose gradient-purified mumps virus antigen was obtained from Orion Diagnostica, Helsinki, Finland. The stock antigen was preserved at −70°C.

EIA method. For the determination of toxoplasma and mumps antibody levels in serum samples and corresponding samples of aqueous humor, microtiter plates (Linbro/Titertek; Flow Laboratories, Irvine, Scotland) were sensitized by overnight incubation at 4°C with 150 μl (per well) of the proper antigen diluted in phosphate-buffered saline pH 7.2. In optimal dilutions as described earlier. The plates were then washed three times with phosphate-buffered saline containing 0.5% (vol/vol) Tween 20. The samples of serum and of aqueous humor were diluted in phosphate-buffered saline containing 5% (vol/vol) horse serum, 0.05% (vol/vol) Tween 20, and NaCl to give a final concentration of 0.5 M. The serum samples were tested in three dilutions: 1:100, 1:300, and 1:1,000. The samples of aqueous humor were tested in one dilution, 1:10. All specimens were run in duplicate by incubating them in 100-μl volumes per well for 90 min at 37°C. After the washing procedure, heavy-chain-specific anti-human immunoglobulins of rabbit origin (DAKO, Copenhagen, Denmark), 100 μl per well, were incubated for 45 min at 37°C. The dilutions, made in the same diluent buffer as the samples, were 1:4,000 for anti-y, 1:2,000 for anti-μ, and 1:1,000 for anti-α. After the washing procedure, alkaline phosphatase-conjugated anti-rabbit immunoglobulins (Orion Diagnostica) diluted 1:100 in the same diluent buffer as above were added, 100 μl per well, and were incubated for 2 h at 37°C. The amount of bound enzyme was visualized by adding 100 μl (per well) of p-nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo.), 1 mg/ml, diluted in diethanolamine-magnesium chloride buffer, pH 10.0. The substrate was allowed to react for 30 min at 37°C followed by the addition of 100 μl (per well) of 0.3 M NaOH to stop the reaction. The resulting yellow color was measured in a Titertek Multiskan spectrophotometer (EliLab, Helsinki, Finland) at a wavelength of 405 nm.

Determination of serum antibody levels. The quantitation of antibody activities in the serum samples was based on the use of positive and negative references. The antibody activities were determined at one optimal dilution in each immunoglobulin class, namely, 1:300 for IgG-class antibodies and 1:100 for IgM- and IgA-class antibodies. Antibody levels are expressed as arbitrary EIA units (EIU) which calculated by the formula antibody level(sample) = [(ODsample − ODnegative control/ODpositive control − ODnegative control)] × 100, where OD is the optical density at 405 nm. The World Health Organization anti-toxoplasma reference serum (1,000 IU/ml) was tested and found to correspond to 170 EIU of IgG toxoplasma antibodies.

Determination of the ratio between antibody levels in the serum and the corresponding sample of aqueous humor. The ratio between antibody levels in the serum and in the aqueous humor was calculated by first drawing a dose-response curve between the OD and the serum dilution based on the three dilutions used and then measuring the logarithmic distance between the OD for the aqueous humor and the corresponding serum curve. The logarithmic distance was transformed to the arithmetic ratio, illustrating the relative difference in antibody activities in the two samples. This method is a modification of the "effective dose" method for comparing antibody activities in different samples as described by Leinikki et al. (5).

Confirmatory test for the specificity. To exclude the possible influence of increased capillary permeability and polyclonal antibody stimulation, parallel determinations with mumps virus as the antigen were carried out as described earlier (10) in all cases with an abnormal antibody ratio between the serum and the aqueous humor. Mumps virus was selected because of the high prevalence of natural mumps antibodies in the population and because the sensitivities of the tests for mumps antibodies and toxoplasma antibodies were equal.

RESULTS

Cataract patients. The toxoplasma antibody levels in serum samples and the ratios between antibody levels in serum and aqueous humor are shown in Table 1. Out of 32 cataract patients, 11 (32.4%) had IgG toxoplasma antibodies in the serum. The IgG antibody levels ranged between 7 and 180 EIU with a mean of 49 EIU. All were negative for IgM-class antibodies. Low levels of IgA toxoplasma antibodies were detected in two patients; only one patient had a clearly elevated IgA antibody level of 50 EIU. This patient also had very high levels of IgG toxoplasma antibodies.

The exact difference between IgG toxoplasma antibody levels in the serum and the aqueous humor could be calculated in one cataract pa-
TABLE 1. Toxoplasma antibody levels in cataract patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Antibody level in serum (EIU)</th>
<th>Ratio of IgG antibody levels (serum/aqueous humor)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
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<td>23</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
<td>7</td>
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</tr>
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<td>5</td>
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</tr>
<tr>
<td>9</td>
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<td>0</td>
</tr>
<tr>
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</tr>
<tr>
<td>11</td>
<td>39</td>
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The ratios between IgG toxoplasma antibody levels in serum and in aqueous humor showed a clearly different pattern when compared with the cataract controls (Table 2). The ratios in the first eight patients varied between 6 and 56 with a mean ratio of 24, whereas in the controls the ratio was 100 or more. In the ninth patient, the ratio was over 100. In this case, the sample had been tapped only 10 days after the onset of ocular symptoms; in all other patients, the interval between the tap and the onset of the clinical disease was longer than 15 days.

For all patients showing a lowered ratio between IgG toxoplasma antibodies in serum and in aqueous humor, parallel determinations of the IgG antibody ratios with the mumps virus antigen were carried out. The results are shown in Table 2. The mumps antibody ratios were over 100 in every case. The samples of aqueous humor were also studied for the presence of IgM or IgA anti-toxoplasma antibodies. No IgM or IgA activity could be detected.

New serum samples were collected from these patients about 1 year later to detect possible significant changes in serum toxoplasma antibody levels. Only minor variations, about ±10 EIU, were observed; these were completely within the normal limits of variation.

Patients with other types of uveitis. Four patients with an active chorioretinal inflammatory process and one patient with generalized uveitis were studied. The ophthalmological findings for these patients were not compatible with toxoplasmosis.

All patients had IgG toxoplasma antibodies in serum ranging from 15 to 180 EIU with the mean of 67 EIU (Table 3). All were negative for IgM-class antibodies. IgA-class toxoplasma antibodies were found in three patients, with low levels in two patients and a high level in one patient, who also had a high level of IgG toxoplasma antibodies.

Four of these patients showed high ratios between IgG antibody levels in serum and aqueous humor comparable to the ratios observed in the cataract controls. The patient with generalized uveitis had a clearly lowered ratio of 2. However, the ratio was also low for mumps.

TABLE 2. Antibody levels in patients with clinically diagnosed toxoplastic chorioretinitis

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Toxoplasma antibody levels in serum (EIU)</th>
<th>Ratio of IgG antibody levels (serum/aqueous humor)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
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</tr>
<tr>
<td>9</td>
<td>36</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> NT, Not tested.
virus antibodies, with a value of 3 indicating nonspecific reactivity. Also, IgA toxoplasma antibodies were detected in the aqueous humor from this patient in a ratio of 5 between serum and aqueous humor, suggesting a leakage of extraocular antibodies into the eye.

**DISCUSSION**

The majority of toxoplasmic chorioretinitis cases are late sequelae of congenitally acquired infection; an acquired form is a rarity (7, 11). The diagnosis of toxoplasmic chorioretinitis has been based mainly on the typical clinical picture (11, 13). However, other infections and even noninflammatory conditions may resemble the ophthalmological findings of ocular toxoplasmosis (12), thus generating a need for additional diagnostic procedures.

By using the dye test, detailed studies about the synthesis of intraocular toxoplasma antibodies have been carried out (2, 9). The dye test has been widely replaced by immunoassays in the serological diagnosis of toxoplasmosis because of the inherent technical problems of the dye test.

In a previous report (10), we were able to show that intraocular antibody synthesis can indeed be demonstrated by using EIA. In this study, we have applied a modified four-layer EIA to get more detailed information about humoral immune responses in ocular toxoplasmosis. This modification has proved to be very sensitive and suitable for the demonstration of immune responses in different immunoglobulin classes during acute systemic toxoplasmosis (Turunen et al., in press).

To study the ratio between antibody levels in serum and aqueous humor in the noninflammatory state, we used cataract patients without any clinical history of uveitis. In seropositive patients, the mean IgG antibody level of 49 EIU was very close to the level of 53 EIU observed in Finnish blood donors (Turunen et al., in press). In all cases, the ratio between IgG toxoplasma antibody levels in serum and aqueous humor was at least 100; in one case, the exact ratio could be calculated and was found to be 250. Interestingly, this figure is very close to the ratio observed by using comparable methods values for serum and cerebrospinal fluid in healthy individuals (6).

In eight of the nine patients with clinically diagnosed active toxoplasmic chorioretinitis, the ratio between IgG toxoplasma antibody levels in serum and aqueous humor was lowered, ranging between 6 and 56 with the mean value of 24, reflecting increased IgG antibody levels in the aqueous humor. To prove that this was not a result of increased capillary permeability or polyclonal antibody stimulation, these ratios were also determined with unrelated mumps virus antigen. The ratios were constantly over 100, confirming that the elevated levels of IgG toxoplasma antibodies in the aqueous humor resulted from an active intraocular antibody synthesis. Only one patient in this group showed a ratio comparable to that obtained for control cataract patients. This patient had been tapped for the aqueous humor only 10 days after the onset of ocular symptoms. There is evidence from some earlier studies that the synthesis of intraocular antibodies is transient, being associated with acute recurrences, and the highest levels of local antibodies are demonstrable at the late stages of the ocular attack (2). This might explain the lack of the local antibody response in this particular patient.

No IgM or IgA toxoplasma antibody activity could be detected in the aqueous humor of these patients, indicating that the local antibodies in the aqueous humor were exclusively IgG. The lack of IgM-class antibodies supports the concept that the ocular lesions in the present series of patients resulted from recurrent antigenic stimuli.

IgG toxoplasma antibody levels in serum from these patients varied between 30 and 85 EIU with the mean level of 59 EIU. This figure, indicating a quite low antibody level, is in good agreement with the generally accepted view that patients with toxoplasmic chorioretinitis usually have low antibody levels in serum (12). No IgM antibody response could be detected; this excludes the possibility of acute or recent systemic toxoplasmosis. The changes in toxoplasma antibody levels observed between serum samples collected in the acute phase of the ocular disease and about 1 year later were within normal limits of variation and could not offer any additional aid in diagnosis. This also is consistent with the results of earlier studies (9).
Patients with other types of uveitis had IgG toxoplasma antibody ratios similar to those of the control cataract patients. Only one patient had a low ratio, but this patient also had a lowered ratio for mumps virus antibodies. In this case the most obvious explanation is increased capillary permeability, leading to the leakage of serum antibodies into the eye. This case demonstrates very clearly the necessity and usefulness of the control antigen in the test.

In this study, we were able to prove that IgG-class anti-toxoplasma antibodies are produced locally within the eye in cases of recurrent active toxoplasmic chorioretinitis. The clinical picture in all those cases in which intraocular antibody synthesis was demonstrated was quite typical (11). However, it must be recognized that many inflammatory and noninflammatory conditions may mimic the clinical findings of ocular toxoplasmosis, thus causing difficulties in the differential diagnosis, especially in countries and areas where such clinical entities are common. This emphasizes the usefulness of the demonstration of local antibody production for an accurate etiological diagnosis.

It is probable that the best results by the determination of the antibody ratios are obtained at the late phases of an acute relapse of ocular toxoplasmosis. In the early phases the demonstration of toxoplasma antigen in the aqueous humor, if possible, could be of paramount importance. We have developed a sensitive EIA for the detection of toxoplasma antigen, and studies of the suitability of this method for the diagnosis of ocular toxoplasmosis are in progress.

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LITERATURE CITED