Aqueous Humor and Serum Immunoblotting for Immunoglobulin Types G, A, M, and E in Cases of Human Ocular Toxoplasmosis

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The purpose of this study was to compare the local and systemic *Toxoplasma*-specific humoral immune responses in individuals with ocular toxoplasmosis (OT). To this end, paired aqueous humor and serum samples from 46 individuals with active OT and from 30 individuals without inflammatory eye disease (controls) were analyzed by immunoblotting for anti-*Toxoplasma* immunoglobulin G (IgG), IgA, IgM, and IgE directed against 20- to 120-kDa antigens. The presence in the aqueous humor of a unique band, or of at least three bands that were at least three times more intense in aqueous humor than in serum, was taken as evidence of local antibody production. IgG bands were detected in 98% of the aqueous humor samples, while IgA bands were detected in 76%, IgM bands were detected in 8%, and IgE bands were not detected in any. Evidence of local production of specific antibodies was found in 32 cases (70%) (IgG in 23 [50%]; IgA in 16 [35%]). In 10 instances (22%), routine laboratory tests were not indicative of OT. In 14 cases (30%), no local antibody production was detected by immunoblotting; 3 of these cases yielded evidence of local antibody production according to the Goldmann-Witmer coefficient. Local antibody production was revealed for 7 of the 30 controls (23%). Hence, the sensitivity of immunoblotting for IgG and IgA is 70%, and the specificity is 77%. We conclude that immunoblotting for local specific IgG and IgA supports the clinical diagnosis of OT in 70% of cases. In 22% of these, the diagnosis is not confirmed by other laboratory tests. Hence, immunoblotting increases the sensitivity of routine laboratory tests and should be considered for samples that register negative by such tests.

The clinical recognition of ocular toxoplasmosis is still held to be the "gold standard " among all diagnostic efforts (32). If it were infallible, it would be confirmed by laboratory tests in all instances, which unfortunately is not the case. Confirmation rates range between 50 and 80%, depending on the centers involved and the diagnostic tools employed. The errant 20 to 50% of cases do not all represent clinically false or insufficient diagnoses. In a substantial proportion of instances, discrepant clinical and laboratory data reflect our incomplete understanding of the pathophysiology of this disease.

At present, the best means of confirming the clinical diagnosis is to establish the occurrence of local specific antibody production. This is achieved either by demonstrating a relative increase in specific antibody production within the eye compared to that within the blood, at a quantitative or qualitative level, or by demonstrating relative differences in the production of different antibody classes or subclasses. In patients with ocular toxoplasmosis, the quantitative immune response, as reflected in the Goldmann-Witmer or antibody coefficient, is augmented above the accepted negative cutoff level of 3 (13, 30) in 50 to 70% of cases. In the majority of these "Goldmann-Witmer coefficient-positive" patients, no parasitic DNA is detected by PCR. And in two-thirds of the cases in which parasitic DNA is detected, no local antibody production is revealed. Hence, pathophysiologically, the liberation and proliferation

of the parasites do not coincide with the humoral immune response to the infection.

Reactivation of *Toxoplasma* parasites probably occurs during a quiet immune situation at the local site (38), and thus it precedes the humoral and cellular immune responses, which are activated as a consequence of cytokine production (5). At this early stage, the infection runs at a subclinical level. Only rarely does an individual present before the local immune response is activated to a measurable degree (1, 31, 33). In 60% of cases with no signs of local specific antibody production at the time of clinical presentation for ocular toxoplasmosis, a late-onset humoral immune response has been detected 2 to 6 weeks later (6). But this still leaves a substantial proportion of cases in which no evidence of local specific antibody production is detected at any time. It is conceivable that antibodies are bound at the infection site, but this process should not interfere with the direct detection of the parasite by DNA amplification. Alternatively, an early, highly specific humoral immune response directed against single antigenic epitopes might occur. Although this event would escape detection by a quantitative analysis, it should be revealed by a qualitative analysis, as suggested by Klaren et al. (15). In this context, we wished to ascertain whether qualitative immunoblotting could serve as a useful tool in the laboratory confirmation of ocular toxoplasmosis by yielding evidence of local specific antibody production.

MATERIALS AND METHODS

Patients. This study drew on data from a series of 104 consecutive patients presenting with active posterior uveitis during a 10-year period (October 1993 to April 2003) at the Department of Ophthalmology, University of Bern, Bern,

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Switzerland. All individuals unambiguously met our criteria for the clinical diagnosis of ocular toxoplasmosis, namely, the manifestation of a lesion involving primarily the retina, with secondary spread to the choroid and cellular vitreous and infiltration of the anterior segment. In most cases, a preexisting scar was evident. In all patients, samples of aqueous humor and serum were routinely collected for laboratory testing. This procedure was undertaken with the approval of the local Ethics Committee and with the patients' informed consent. Patients from whom at least 50 μl each of aqueous humor and serum was available for immunoblotting after the completion of routine analyses qualified for inclusion in our study group, which thus consisted of 46 individuals.

Patients first presented at a median time of 8 days (range, 2 to 60 days; standard deviation, ±12.6 days) following the appearance of symptoms. On this occasion, the retinal lesions were photographed, and samples of aqueous humor and serum were taken. To assess the specificity of our findings, we included paired aqueous humor and serum samples from 30 control patients who manifested no evidence of inflammatory eye disease.

Laboratory tests. Aqueous humor samples were centrifuged at $13,000 \times g$. The sediments thus obtained were used for amplification of parasitic DNA by PCR, and the supernatants were used for analysis of antibody levels. Levels of total immunoglobulin G (IgG), anti-*Toxoplasma* (specific) IgG, specific IgA, and specific IgM in samples of aqueous humor (diluted 1:20) and serum (diluted 1:100) were determined as described elsewhere (6), by using commercial products as standards (Platelia Toxo IgG, IgA, or IgM [Bio-Rad, Marnes la Coquette, France]). In analogy to the calculation of intrathecal antibody levels, the Goldmann-Witmer coefficient (*C*) affords a quantitative estimate of local specific antibody production. It was calculated for antibodies of the IgG type by using the following formula: C = (anti-*Toxoplasma* IgG in aqueous humor/anti-*Toxoplasma* IgG in serum)/(total IgG in serum/total IgG in aqueous humor). Residual amounts of aqueous humor and serum were stored at $-20°C$ prior to the immunoblot analysis.

Immunoblotting. Immunoblotting was performed according to an established protocol by using commercially available anti-*Toxoplasma* immunoblotting test membranes (LD Bio Diagnostics, Lyon, France). The membranes were first equilibrated and blocked with 3% goat serum and 3% dry milk powder (dissolved in phosphate-buffered saline [PBS] containing 0.05% Tween) and then washed in PBS containing 0.5% Tween for 10 min. They were incubated with the samples (aqueous humor diluted 1:40 and serum diluted 1:100, both in PBS containing 0.05% Tween [pH 7.4]) for 14 h (overnight) at ambient temperature under shaking conditions and were then washed three times, for 10 min each time, with PBS containing 0.5% Tween. Alkaline phosphatase-labeled antibodies (anti-human IgG, IgA, IgM, and IgE [Sigma Chemicals, Buchs, Switzerland]) were diluted 1:200 in PBS containing 0.05% Tween. After three 10-min washes in PBS containing 0.5% Tween, the bound secondary antibody was visualized by using nitroblue tetrazolium as a substrate for the conjugated alkaline phosphatase. The reaction was terminated by the addition of water, which was removed after 2 min, when the background began to stain. The membranes were air dried, mounted on strips of paper, and photographed.

Interpretation of immunoblotting results. According to the separation technique employed, only antibodies directed against antigens in the 20- to 120-kDa size range were detected. The band patterns revealed for each antibody class within paired aqueous humor and serum samples were evaluated and compared qualitatively. Bands that were present in the aqueous humor but absent from the serum were identified. The presence within the aqueous humor of a unique band for a particular antibody type, or of at least three bands for a particular antibody type that stained at least three times more intensely than in the serum (by optical inspection), was taken as evidence of local specific antibody production.

The results obtained for ocular toxoplasmosis and control patients were compared by using the chi-square test.

RESULTS

The quantitative findings for our patients (including determination of the levels of total and specific IgG, specific IgA, and specific IgM in paired aqueous humor and serum samples) are summarized in Table 1. Anti-*Toxoplasma* (specific) IgG was detected in concentrations that were compatible with chronic infection in all of the serum samples but in only 50% of the aqueous humor samples. A Goldmann-Witmer coefficient above 3 was registered in 39% of cases (18 of 46). Specific IgM was not detected in any of the serum or aqueous humor

TABLE 1. Quantitative anti-*Toxoplasma* antibody information for 46 patients[a]

Antibody and Sample	Unit	Min.	Max.	Avg	SD	Median
Total IgG						
Serum	g/liter	6.5	16.7	10.7	2.3	10.7
Aqueous humor	mg/liter	2.0	469	19.3	70.1	2.0
Anti-*Toxoplasma* IgG						
Serum[b]	IU	8	2,000	253	431	134
Aqueous humor[c]	IU	0	2,400	122	416	1.5
Goldmann-Witmer coefficient	Index	0	732	28.3	110.3	0.2
Anti-*Toxoplasma* IgA						
Serum	Index	0	1.6	0.06	0.25	0
Aqueous humor	Index	0	40	2.1	8.4	0

[a] Min., minimum; Max., maximum.
[b] Anti-*Toxoplasma* IgG was detected at a minimal concentration of 8 IU in all 46 serum samples.
[c] In 23 of 46 aqueous humor samples, no specific anti-*Toxoplasma* IgG was detected by using a commercial ELISA system (Platelia Toxo; Bio-Rad, Marnes la Coquette, France).

samples. Specific IgA was detected in 7% (3 of 46) of the serum samples and in 29% (13 of 46) of the aqueous humor samples; in 18% (8 of 46) of the aqueous humor samples, the IgA index was ≥0.5. Of the 32 samples that were analyzed for the presence of parasitic DNA, 6 (19%) registered positive, and only 2 of the 6 had a Goldmann-Witmer coefficient of ≥3.

Qualitative immunoblot analysis was undertaken for each of the 46 pairs of serum and aqueous humor samples. The first 24 patients were evaluated for the presence of specific IgG, IgA, IgM, and IgE. But since no specific IgE bands were detected, and specific IgM bands were revealed in only two aliquots of aqueous humor, paired samples from the remaining 22 patients and from the controls underwent immunoblotting only for antibody types IgG and IgA. For the ocular toxoplasmosis patients, specific IgG bands were detected in 98% (45 of 46), and specific IgA bands were detected in 76% (35 of 46), of the aqueous humor samples. For the control patients, specific IgG bands were detected in 77% (23 of 30 [*P* = 0.01]), and specific IgA bands were detected in 53% (16 of 30 [*P* = 0.07]), of the aqueous humor samples (Table 2). Specific bands that satisfied the criteria for local specific antibody production occurred in 70% (32 of 46) of the ocular toxoplasmosis samples. In 16 of the 32 cases (35%), the antibodies were exclusively of the IgG type, in 9 cases (20%) they were exclusively of the IgA type, and in 7 cases (15%) they were of both types, IgG and IgA. Of the 32 cases that revealed evidence of local specific antibody production, 29 (91%) manifested unique bands within the aqueous humor; the other 3 cases exhibited at least three bands that stained at least three times more intensely in the aqueous humor than in serum. Among the control patients, specific bands that satisfied the criteria for local specific antibody production occurred in 20% (6 of 30 [*P* = 0.00007]) of cases. In one of the six cases (3%), the antibodies were exclusively of the IgG type, in four cases (13%) they were exclusively of the IgA type, and in one case (3%) they were of both types, IgG and IgA. Immunoblotting for the local production of specific antibodies of the IgG and IgA types thus yields an overall sensitivity of 70% and a specificity of 77%. Immunoblotting for the local production of specific IgG alone yields a sensitivity of 50% and a specificity of 93%. Immunoblotting for the local

TABLE 2. Qualitative immunoblot analysis for specific anti-*Toxoplasma* antibody bands within aqueous humor samples

Anti-*Toxoplasma* antibody type	No. (%) of aqueous humor samples in which specific antibody bands were detected by immunoblotting		No. (%) of paired samples meeting the criteria for local specific antibody production[a]	
	Noninflammatory eye disease ($n = 30$)	Ocular toxoplasmosis ($n = 46$)	Noninflammatory eye disease ($n = 30$)	Ocular toxoplasmosis ($n = 46$)
IgG	23 (77)	45 (98)	2 (7)	23 (50)
IgA	16 (53)	35 (76)	6 (20)	16[b] (35)
IgM		2/24[d] (8)		1[c] (4)
IgE		0/24 (0)		0
Total	29 (97)	46 (100)	7 (23)	32 (70)

[a] See Materials and Methods.
[b] In nine of these cases, no unique specific IgG bands were detected.
[c] Associated with a comparable band pattern for IgG (Fig. 2).
[d] Immunoblot analysis performed for the first 24 paired samples (see Results).

production of specific IgA alone yields a sensitivity of 35% and a specificity of 83%. Immunoblotting evidence of local specific antibody production was not obviously related to the assumed mode of disease transmission, i.e., congenital (Fig. 1) or acquired (Fig. 2).

For 17 of the 32 ocular toxoplasmosis patients with immunoblot evidence of local specific antibody production, this finding was not supported by the Goldmann-Witmer coefficient, which was <3. In 7 of these 17 cases, the local specific antibodies produced were exclusively of the IgG type, in 7 cases they were exclusively of the IgA type, and in the other 3 instances they were of both types, IgG and IgA. Immunoblot evidence of local specific antibody production was supported by amplification of parasite DNA in 3 of the 32 immunoblot-positive cases and by a specific IgA index of >0.5 in 2 cases. For 15 of the 18 patients whose Goldmann-Witmer coefficient was ≥3, indicative of local specific antibody production, this finding was supported by immunoblotting, while for the other 3 it was not. For 15 of the 46 ocular toxoplasmosis patients (33%), evidence of local specific antibody production was afforded by both immunoblotting and the Goldmann-Witmer coefficient. In 9 of these 15 cases, the antibodies were exclusively of the IgG type, in 2 instances they were exclusively of the IgA type, and in 4 cases they were of both types, IgG and IgA (Table 3).

DISCUSSION

Our results demonstrate that immunoblotting can serve as a useful tool in the laboratory confirmation of ocular toxoplasmosis by yielding evidence of local specific antibody production. If the analysis includes an estimation of specific IgA as
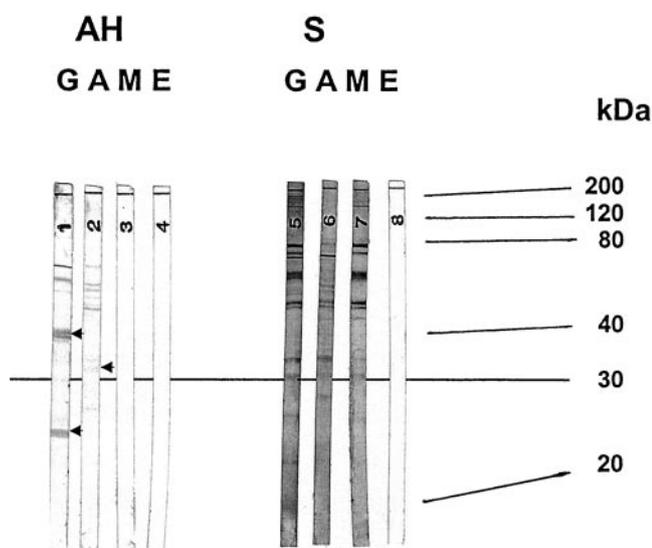

FIG. 1. Immunoblot analysis for a patient with ocular toxoplasmosis of clinically assumed congenital origin. The presence within the aqueous humor (AH) of a unique band, or of at least three bands for a particular antibody type (i.e., IgG, IgA, IgM, or IgE) that stained at least three times more intensely than in the serum (S), was taken as evidence of local specific antibody production. In this example, three unique bands, binding to antigens with molecular sizes of 26, 32, and 43 kDa, are indicated by arrowheads. G, IgG; A, IgA; M, IgM; E, IgE.
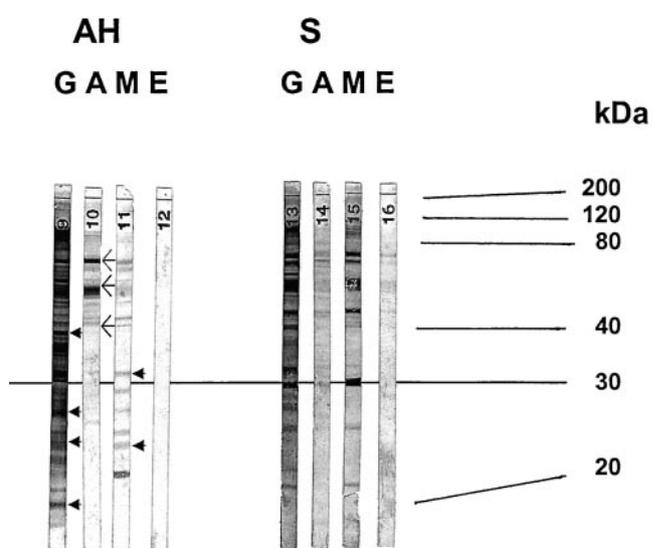

FIG. 2. Immunoblot analysis for a patient with ocular toxoplasmosis of clinically assumed acquired origin. The presence within the aqueous humor (AH) of a unique band (solid arrowheads), or of at least three bands for a particular antibody type (i.e., IgG, IgA, IgM, or IgE) that stained at least three times more intensely than in the serum (S) (arrows), was taken as evidence of local specific antibody production. Note the similarity of the band profiles for IgG and IgM. G, IgG; A, IgA; M, IgM; E, IgE.

TABLE 3. Comparison of qualitative (immunoblotting) and quantitative (ELISA) findings for anti-*Toxoplasma* antibodies of the IgG and IgA types in paired aqueous humor and serum samples of patients with ocular toxoplasmosis

Comparison of immunoblot results with data obtained using other laboratory tests	No. (%) of paired aqueous humor and serum samples in which unique bands or bands of heightened intensity were detected ($n = 46$)
No laboratory confirmation of the clinical diagnosis[a]	10 (22)
No unique bands or bands of heightened intensity detected by immunoblotting	14 (30)
No unique bands or bands of heightened intensity detected in samples with a Goldmann-Witmer coefficient of ≥ 3	3 (7)
Unique bands or bands of heightened intensity detected in samples with a Goldmann-Witmer coefficient of <3	17 (37)
No other laboratory confirmation of the clinical diagnosis	12 (26)
Unique bands or bands of heightened intensity detected in samples with a Goldmann-Witmer coefficient of ≥ 3	15 (33)
Definitive laboratory diagnosis of OT[b]	36 (78)

[a] Includes determination of levels of total and specific IgG, specific IgA, and specific IgM in the aqueous humor and serum, amplification of parasite DNA in the aqueous humor by PCR, and immunoblotting for anti-*Toxoplasma* IgG and IgA.
[b] OT, ocular toxoplasmosis. In one PCR-positive sample, no specific antibodies were detected in the aqueous humor despite the presence of a clinically typical active lesion.

well as specific IgG antibodies (34), it attains a satisfactory level of sensitivity, which is not further improved by including an evaluation of specific IgM and specific IgE.

For immunoblot results to yield clinically relevant information about local antibody production in the context of ocular toxoplasmosis, a rigorous band selection strategy must be implemented. In contrast to other authors (12, 15), we did not consider either the pattern or the staining intensity of bands alone to suffice as evidence of local specific antibody production.

The detection of local antibody production according to the staining intensity of bands may be influenced by many factors which are incompletely understood, such as the dynamics of uveovascular barrier stability, the persistence of local antibody production, and the effect of nonspecific triggers on the local specific humoral immune response. Moreover, a quantitative assessment of band staining intensity would have necessitated a further dilution of serum and aqueous humor samples to obtain comparable levels of specific IgG. By so doing, aqueous humor samples in which the concentration of specific IgG lay below the level of detection by ELISA (50%) would have been excluded from the analysis. We therefore chose to evaluate our immunoblots qualitatively and considered that specific antibodies were being produced locally if bands were present in the aqueous humor but absent from the serum or if at least three bands stained at least three times more intensely in the aqueous humor than in the serum. The sensitivity of the method might have been enhanced by adopting less stringent exclusion criteria, but probably at the expense of specificity.

No common recognition patterns exist for the detection of local specific antibody production by immunoblotting. In one report, antibody binding to antigens with molecular sizes below 16 kDa and above 116 kDa was reported (3), whereas in another, only antibodies recognizing a 28-kDa antigen were revealed (15). In two further reports (29, 34), no detailed information was furnished. We considered that the major soluble and surface antigens of *Toxoplasma gondii* would most likely fall within the 20- to 120-kDa size range, and antibodies directed against these were monitored.

Comparison of the sensitivities of a quantitative ELISA (on the basis of the Goldmann-Witmer coefficient for IgG and the IgA index) and qualitative immunoblotting for detection of local specific antibody production revealed discordant findings in 37% of instances for both IgG and IgA. This difference is most likely understandable in terms of local antibody turnover, which is poorly understood (23).

The quantification by ELISA of local specific IgG antibody production has a clinically acceptable specificity of 89%, but the sensitivity of this method (63%) is not sufficiently high to furnish a meaningful basis for negative prediction in the context of ocular toxoplasmosis (34). Comparable though variable levels of specificity (89%) and sensitivity (ranging from 53% for a sample size of 19 [34] through 77% for a sample size of 13 [15] to 100% for a sample size of 4 [29] or 3 [3]) have been achieved by immunoblotting. If quantitative ELISA and qualitative immunoblot analyses for IgG are combined, the sensitivity may be increased to 68%, and if detection of parasite DNA by PCR is also included, a sensitivity level of 83% may be attained (34). Sensitivity can be further improved by broadening the spectrum of specific antibody types analyzed (30). By quantitative ELISA, specific antibodies of the IgA, IgM, and IgE types have been demonstrated to occur in ocular toxoplasmosis patients at the following frequencies: IgA, from 26% (6) through 52% (30) to 63% (17) in the aqueous humor; IgM, from <1% (6, 30) to 11% (17) in the aqueous humor and to 50% in the serum (21, 25); IgE, from 0% (18) to 14% (7) in the vitreal fluid and to 66% in the serum (21, 27). The levels of IgE within the aqueous humor have not been determined, which is surprising given the purported role of this class of immunoglobulin in ocular toxoplasmosis-related hypersensitivity (24, 37) and autoimmunity (11, 14, 22, 35, 36). The broad range of sensitivities reported for the different types of specific antibodies probably reflects the usually small number of patients included and the case selection criteria employed, e.g., the mode

of disease transmission (acquired or congenital), age (25), and, eventually, genetic origin.

Given the variability of these findings, it is generally deemed useful to estimate only levels of IgG, which is quantitatively the most abundant immunoglobulin type in the aqueous humor, during routine testing of samples for ocular toxoplasmosis. Indeed, quantitative or qualitative determination of specific IgM may not be helpful, due to its natural occurrence in the sera of patients otherwise registering seronegative for toxoplasmosis (16, 28) and its persistence in chronically infected individuals (8). However, there are indications that the determination of specific IgA within either the aqueous humor (6, 15, 30) or tears (20) may yield valuable information, but of unknown sensitivity or specificity (9, 19). The results of our study support this contention. Immunoblotting revealed the presence of IgA bands in 76% of aqueous humor samples (Table 2) and yielded evidence of local specific IgA antibody production in 35%. In 22% of the patients, this evidence of local specific IgA antibody production was not supported either by immunoblot data for IgG or by quantitative ELISA (the Goldmann-Witmer coefficient for IgG and the IgA index). These results are not suggestive of an inhibition or blocking of IgA-specific binding by IgG.

By combining the immunoblot results for IgG and IgA, we improved the sensitivity of detection of immunoblotting from 50% (for IgG alone) to 70%, but at the price of specificity. Immunoblotting for IgM and IgE failed to yield any useful information, which accords with previously published data relating to adult patients (2, 4, 18, 35), although in young children these classes of immunoglobulins may be of greater importance (27). Hence, we do not recommend the evaluation of IgM and IgE on a routine basis. It is, of course, conceivable that the low incidence of local IgM bands (8%) and the complete absence of IgE bands may be accounted for by an inhibition or blocking of IgM- or IgE-specific binding by IgG. In the case of IgM this seems unlikely, since no IgM was detected by an immunocapture assay in the corresponding serum samples. But blockage by IgG of IgE-specific binding could have occurred. Indeed, local production of IgE would be expected to contribute to inflammatory eye diseases (10), including ocular toxoplasmosis (26).

In conclusion, our data furnish sufficient evidence for the importance of immunoblotting in the laboratory confirmation of ocular toxoplasmosis to justify its inclusion in the panel of routine tests to which aqueous humor samples are subjected. Our findings also reveal a previously unreported discrepancy between quantitative and qualitative estimates of the local immune response to *Toxoplasma*. Possible explanations for this difference may include an early, highly specific humoral immune response directed against single antigenic epitopes (and thus not measurable on a quantitative basis) and a local capture of antibodies during the early stages of infectious activity. However, our understanding of the pathophysiology of ocular toxoplasmosis is still deplorably poor, and until this situation is improved, we will not be in a position to completely reconcile the clinical picture with immunological findings.

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