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INFLAMMATORY DISORDERS

Anti-retinal autoantibodies in experimental ocular and systemic toxoplasmosis

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Abstract
Background Patients with ocular toxoplasmosis (OT) develop autoreactivity to several retinal antigens, including retinal S-antigen. By establishing an experimental rabbit model of systemic and of primary and secondary ocular toxoplasmosis, we wished to investigate the onset and development of humoral response to retinal S-antigen.
Methods Of twelve infection-naïve rabbits, six were left untreated, and the other six were infected subcutaneously with 5,000 tachyzoites of the highly virulent, non-cyst-forming BK-strain of *Toxoplasma gondii*. Three months later, the left eye of each animal was infected transvitreally with 5,000 tachyzoites of the same strain. The right eye of each rabbit served as an uninfected control. Blood and aqueous humor were collected prior to infection, and up to 90 days thereafter. Using the ELISA technique, all samples were analyzed in parallel for total IgG, and antibodies against toxoplasmic, bovine retinal S-antigen and peptide 35 from human S-antigen.
Results In infection-naïve rabbits *Toxoplasma*-specific antibodies were detected 10 to15 days after systemic and ocular infection. Serum antibodies against retinal S-antigen and peptide 35 were not detected in response to systemic *Toxoplasma* infection. After ocular challenge, aqueous-humour levels of antibodies against retinal S-antigen and peptide 35 in the infected eye began to rise 10 to 15 days later in infection-naïve, but not in infection-immunized animals. During the early post-infection period, the concentrations of anti-retinal antibodies in the infected eye correlated with the severity of inflammatory tissue destruction, but returned to baseline later even though the inflammatory response persisted. In the uninfected partner eye, concentrations of anti-retinal and toxoplasmic antibodies did not correlate with each other.
Conclusion Our data afford no evidence of similarities between toxoplasmic and retinal antigens, nor of infection-induced humoral autoimmunity. They indicate rather that retinal autoantigens are liberated in the context of inflammatory tissue destruction due to ocular toxoplasmosis.

Keywords *Toxoplasma gondii* · Toxoplasmosis · Ocular toxoplasmosis · Retinochoroiditis · Chorioretinitis · Autoimmunity · ELISA · Retinal S-antigen · Animal experiments · Animal model · Aqueous humor

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Introduction

Patients with ocular toxoplasmosis (OT) are known to develop autoreactivity to retinal antigens, such as the S-type. However, the contribution of this antibody production to the OT pathology is not clear [1–7]. That autoimmune processes do indeed play a pathophysiological role in OT is evidenced by the greater prevalence of anti-*Toxoplasma gondii* antibodies in patients with non-toxoplasmic endogenous uveitis than in healthy individuals [8]. On the other hand, autoimmunity against retinal antigens can develop also in patients with retinal detachment and diabetic retinopathy, as well as in those with autoimmune diseases in which the eye is not involved [9]. Cells that have been derived from patients with Fuchs' anterior uveitis without evident retinal disease present a positive cellular response to the retinal S-antigen compared to healthy controls [10].

According to current opinion, OT may be capable of inducing autoimmunity in the context of an infection-associated immunopathological process [11]. The autoimmune disease could be precipitated by at least one of three basic influences, namely by genetic [12], environmental [13–16] or immunoregulatory factors [17–19].

The immunopathological response could evolve at either a cellular [1, 2, 20] or an humoral antigen-driven level [3, 4, 6]. In a murine model of OT, the destruction of retinal tissue is effected mainly by an infiltration of mononuclear cells [21]. Whether autoimmune phenomena are involved also in the development of recurrences is still unclear [22]. In experimental toxoplasmic encephalitis, chronic disease is associated with a maturation and prolonged activity of dendritic cells [23, 24], which could thus well be involved in its recurrences.

An infectious agent may predispose an individual to, or actually precipitate autoimmune reactions by unmasking sequestered autoantigens against cells that have been induced to express class-II HLA antigens in response to the underlying infection. On the other hand, the parasite may be comprised of proteins that share an antigenic similarity with human retinal antigens, thereby rendering it capable of inducing an autoimmune reaction against ocular proteins, as is the case in herpetic keratitis [25–27]. The disruption of the ocular vascular barrier that follows the infectious and inflammatory destruction of ocular tissue may propagate the accumulation and binding of anti-retinal autoantibodies, and exacerbate the destruction of tissue [7]. Interestingly, naturally-occurring anti-retinal antibodies have been detected in healthy individuals who have no history of ocular disease [7, 28, 29]. Autoimmune reactivity in OT could represent an unspecific epiphenomenon of retinal damage, in conjunction with a secondary, parasite-induced release of antigens.

By drawing on an experimental rabbit model of systemic and primary and secondary ocular toxoplasmosis [30], we wished to investigate the onset and development of the humoral response to the retinal S-antigen, using an established autoantibody ELISA technique [31–33]. A comparison between primary ocular and systemic toxoplasmosis will reveal whether any cross-reactivity exists between toxoplasmal and retinal antigens, and that between primary and secondary OT will disclose whether a correlation exists between the levels of anti-retinal antibodies and the degree of ocular tissue destruction.

Materials and methods

Protocol of rabbit infection with virulent BK strain of *Toxoplasma gondii* tachyzoites

All animal experiments were approved by the local and institutional Animal Ethics Committee, and were performed in accordance with ARVO's Guidelines for Animal Research under the surveillance of the local Public Veterinary Health authorities. Experimental ocular toxoplasmosis was induced either as a primary disease in infection-naïve rabbits or as a secondary one in infection-immunized animals by the intraocular injection of tachyzoites of the highly virulent BK strain of *Toxoplasma gondii*, as previously described [30]. Twelve 4-month-old seronegative Dutch belt rabbits of both sexes, weighing 3.5 to 5 kg, were used in this study. They were obtained from a local breeder and housed in an animal-keeping unit at the University of Bern. Six of the rabbits were left untreated until the time of ocular infection. Each of the other six rabbits was infected subcutaneously with 5,000 tachyzoites of the virulent, non-cyst-forming BK-strain of *Toxoplasma gondii*. Between 8 and 28 days later, they were injected intramuscularly with clindamycin (20 mg/kg of body weight/day) to prevent a lethal course of events. Nevertheless, two of the six rabbits died. A 3-month period was then allowed for the infection to quieten down. At the end of this period, the left and the right eyes of the rabbits in each group were fundoscopically examined after pupillary dilatation to exclude the presence of any ocular pathology. The left eye of the four infection-immunized and of the six infection-naïve rabbits was then infected transvitreally with 5,000 tachyzoites of the BK-strain of *Toxoplasma gondii* under indirect ophthalmoscopic control. The right eye of each rabbit served as an uninfected control. Between 8 and 28 days later, all animals were intramuscularly injected with clindamycin (to prevent a lethal course of events). The rabbits were examined regularly. Samples of blood from the ear vein and of aqueous humor from both eyes were collected under conditions of general anaesthesia, both prior to systemic or ocular infection (day "0"), and on days 5, 10, 15, 21, 28, 35, 42, 60, 72 and 90. All samples were

processed immediately as described below. They were then stored at −20°C until required for the antibody analyses, which were run in parallel.

Methods

Collection and preparation of samples 150- to 250-µl aliquots of aqueous humor were collected by puncturing the anterior chamber with a 30-gauge needle, which was connected to a tuberculin syringe. Each sample was centrifuged immediately after its collection. The sediment was used for the amplification of DNA (data not presented) and the supernatant for the antibody analysis. Samples of blood were withdrawn from the ear vein. They were clotted and centrifuged to yield the serum, which was also used for the antibody analysis.

Total rabbit IgG This was quantified using the ELISA technique. A triplicate set of controls with a known antibody concentration was run in parallel with the test samples on each test plate to internally equilibrate the system. The test samples were run in duplicate, and the average measurement was used for subsequent calculations. As outlined in a previous study report [34], we attempted to quantify not only IgG but also IgM and IgA, but none of these were sufficiently specific to permit their unique determination without binding to rabbit IgG, so running the corresponding analyses was judged not useful.

Anti-Toxoplasma rabbit IgG This was also quantified using the ELISA technique. For this purpose, tachyzoites of *Toxoplasma gondii* that had been killed by freezing and thawing were employed, as previously described [34]. To detect the local production of antibodies, the cut-off level for the Goldmann–Witmer coefficient (antibody ratio C) was arbitrarily set at C≥3.

The level of antibodies against the retinal S-antigen and peptide 35 were similarly quantified using the enzyme-linked immunosorbent assay (ELISA) technique [32, 33]. Since peptide 35 is a major epitope in the S-Ag molecule,

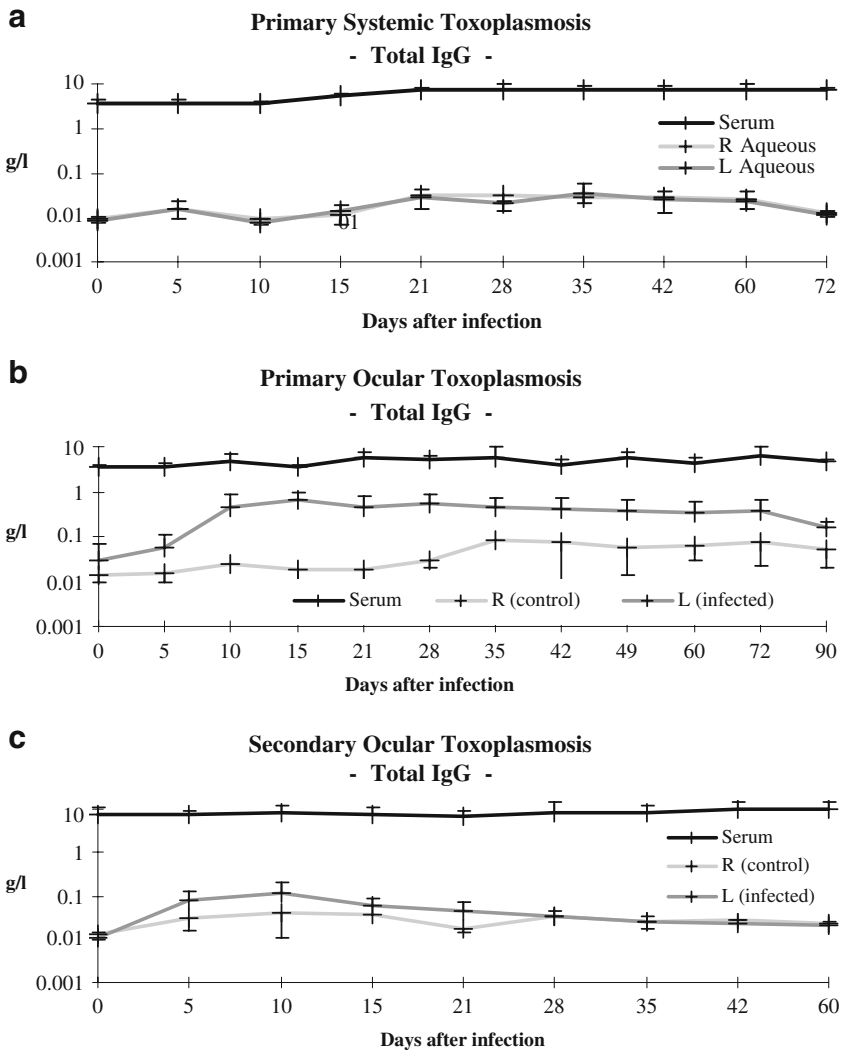

Fig. 1 Concentrations of total IgG in the serum and aqueous humour of rabbits that had been infected with 5000 tachyzoites of the BK strain of *Toxoplasma gondii*. **a** Primary systemic toxoplasmosis. **b** Primary OT. **c** Secondary OT

and since there is a sequence homology between peptide 35 from rabbit, rat and human, we checked the antibody response to both, the entire bovine S-Ag molecule and to peptide 35 in toxoplasmic rabbits.

Briefly, microtiter 96-well plates (Nunc-immuno plate, Roskilde, Denmark) were coated with retinal S-antigen (2 μg/ml) and peptide 35 (2 μg/ml; 35). Serum and aqueous humor samples of each rabbit were added to the wells at dilution 1/50. Serial dilutions between I/20 to 1/1000, of rat sera with high antibody titers to S-Ag followed by incubation with horseradish peroxidase-conjugated goat anti-rat IgG (H + L), and of randomly selected test rabbit sera and aqueous humor samples, were included in each assay to validate the sensitivity and the variation of the ELISA. The dilution 1/50 was chosen since no background was detected at this dilution. Since we had to titrate numerous samples of blood and aqueous humors, and to avoid variations between the plates, we incubated the plates coated with dilutions of samples overnight and processed for antibody titration all the plates at the same time the day

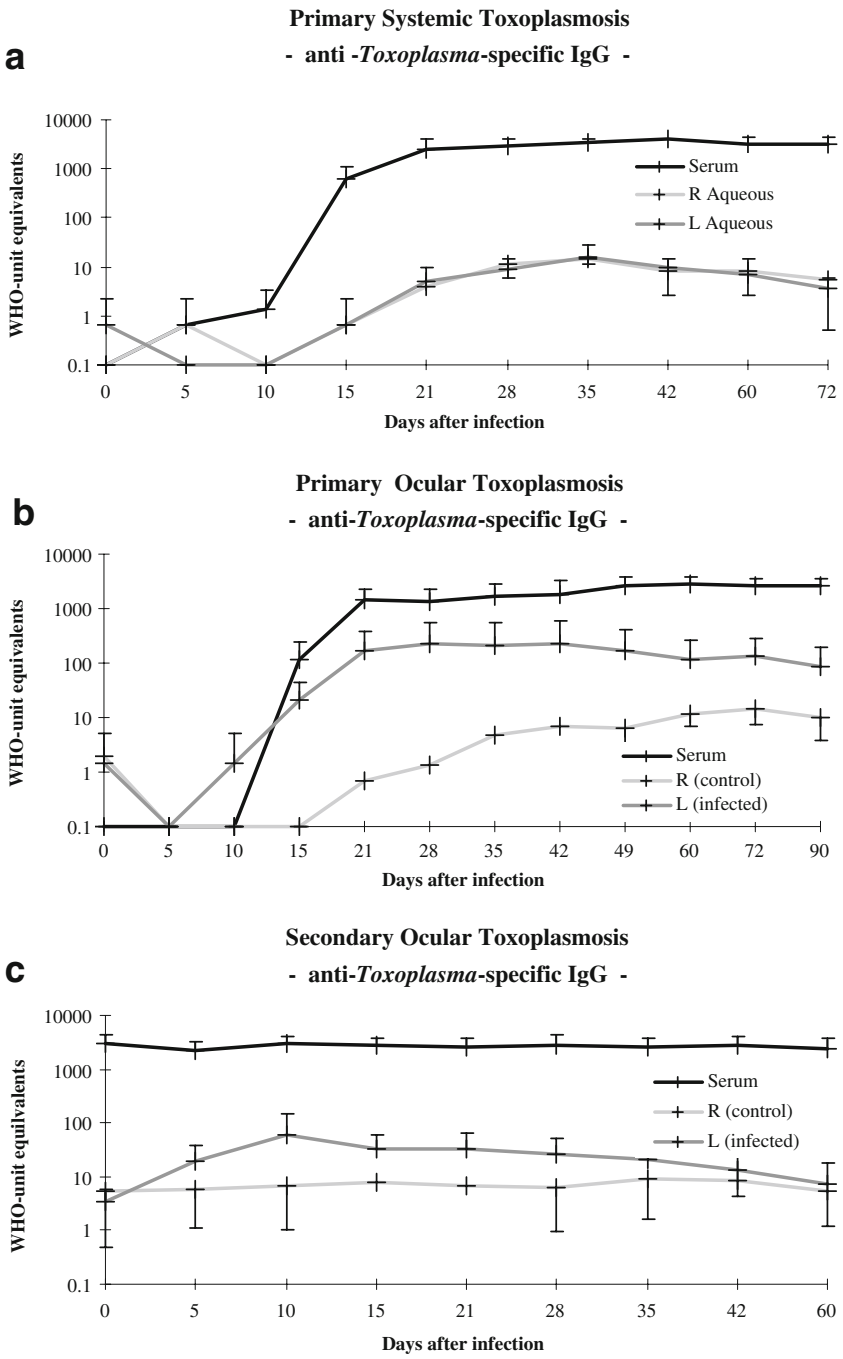

Fig. 2 Concentrations of anti-*Toxoplasma*-specific IgG in the serum and aqueous humour of rabbits that had been infected with 5000 tachyzoites of the BK strain of *Toxoplasma gondii*. **a** Primary systemic toxoplasmosis. **b** Primary OT. **c** Secondary OT

after. Results were not different for samples which had been incubated for 2 hours or overnight. After incubation overnight at 4°C to allow antibody to bind antigen, the samples were removed and the wells were washed. Bound antibody was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L; 1/1000 diluted) (Zymed Laboratories, San Francisco, CA, USA) and application of *O*-phenylenediamine substrate in 0.1 M citrate buffer (Sigma, L'Isle D'Abeau 219 Chesnes, France). Absorbance was read at 490 nm, and mean optical density (OD) ± SD was calculated for each group. Data are represented as median, minimal and maximal values, together with the standard deviation. For the statistical analysis (Student's *t*-test), the data were assumed to be normally distributed. Since the number of samples was limited, the level for statistical significance was set at $p<0.01$.

Results

Despite prophylactic treatment with clindamycin, only eight out of 12 rabbits (four of the six with primary systemic toxoplasmosis and four of the six with primary ocular

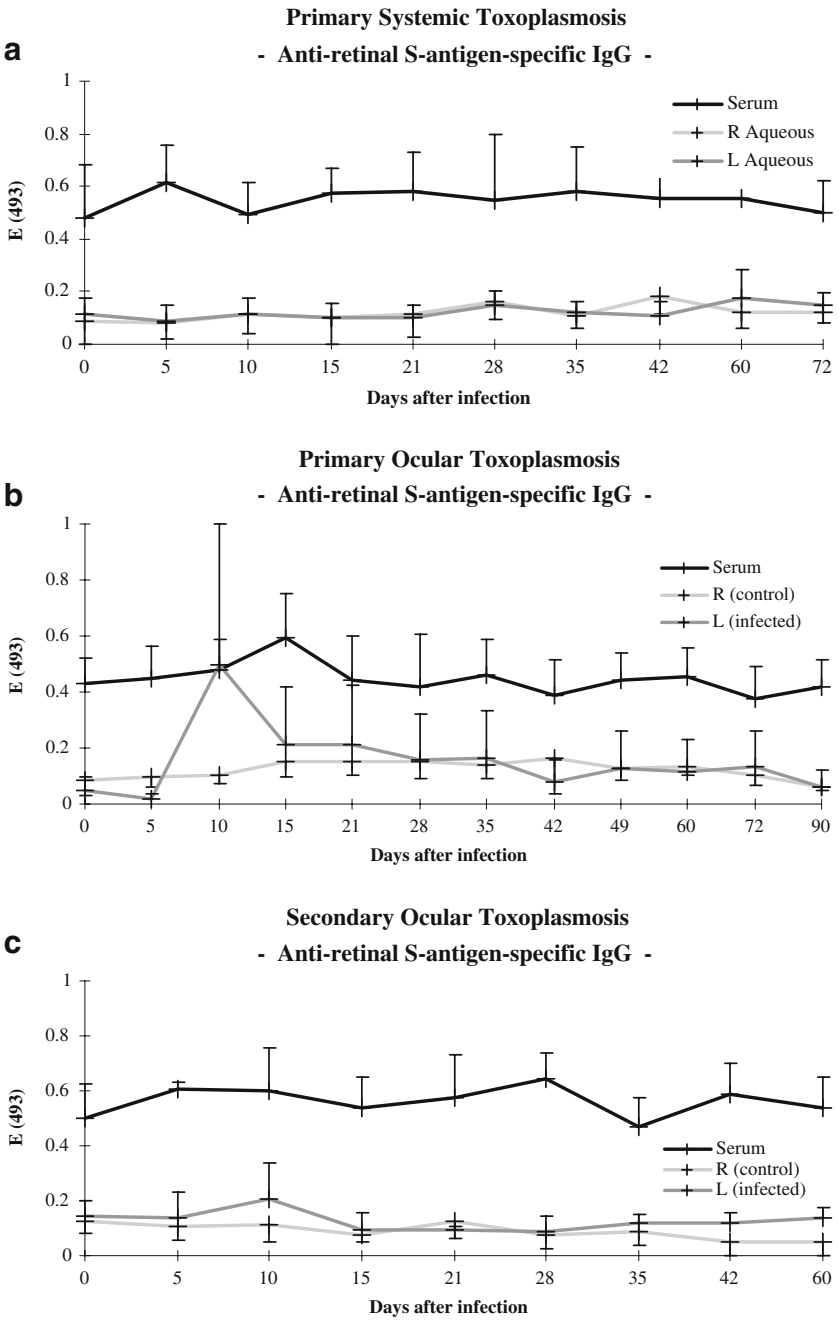

Fig. 3 Concentrations of anti-retinal S-antigen-specific IgG in the serum and aqueous humour of rabbits that had been infected with 5000 tachyzoiotes of the BK strain of *Toxoplasma gondii*. **a** Primary systemic toxoplasmosis. **b** Primary OT. **c** Secondary OT

toxoplasmosis (OT) survived the infection with *Toxoplasma* until day 90. Hence, the group of animals with secondary OT consisted of four rabbits.

In Figs. 1, 2, 3 and 4, temporal changes in the concentrations of total IgG (Fig. 1), of *Toxoplasma*-specific IgG (Fig. 2), of retinal S-antigen-specific IgG (Fig. 3) and of retinal p35-antigen-specific IgG (Fig. 4) are depicted for the rabbits with primary systemic toxoplasmosis (Figs. 1a, 2a, 3a and 4a), with primary OT (Figs. 1b, 2b, 3b and 4b), and with secondary OT (Figs. 1c, 2c, 3c and 4c). These sets of data were used to calculate the antibody coefficients for *Toxoplama*-specific IgG (Fig. 5), for retinal S-antigen-specific IgG (Fig. 6) and for retinal p35-antigen-specific IgG (Fig. 7) in each disease category: primary systemic toxoplasmosis (Figs. 5a, 6a and 7a), primary OT (Figs. 5b, 6b and 7b) and secondary OT (Figs. 5c, 6c and 7c).

The systemic injection of rabbits with *Toxoplasma* elicited a twofold increase of levels of total IgG in the serum and aqueous humor (Fig. 1a).

This rise indicates that a humoral immune response had been stimulated. Ocular infection with *Toxoplasma* elicited an increase in the local level of total IgG (Fig. 1b and c), which may reflect the stimulation of a local humoral immune response and/or a breakdown of the blood ocular

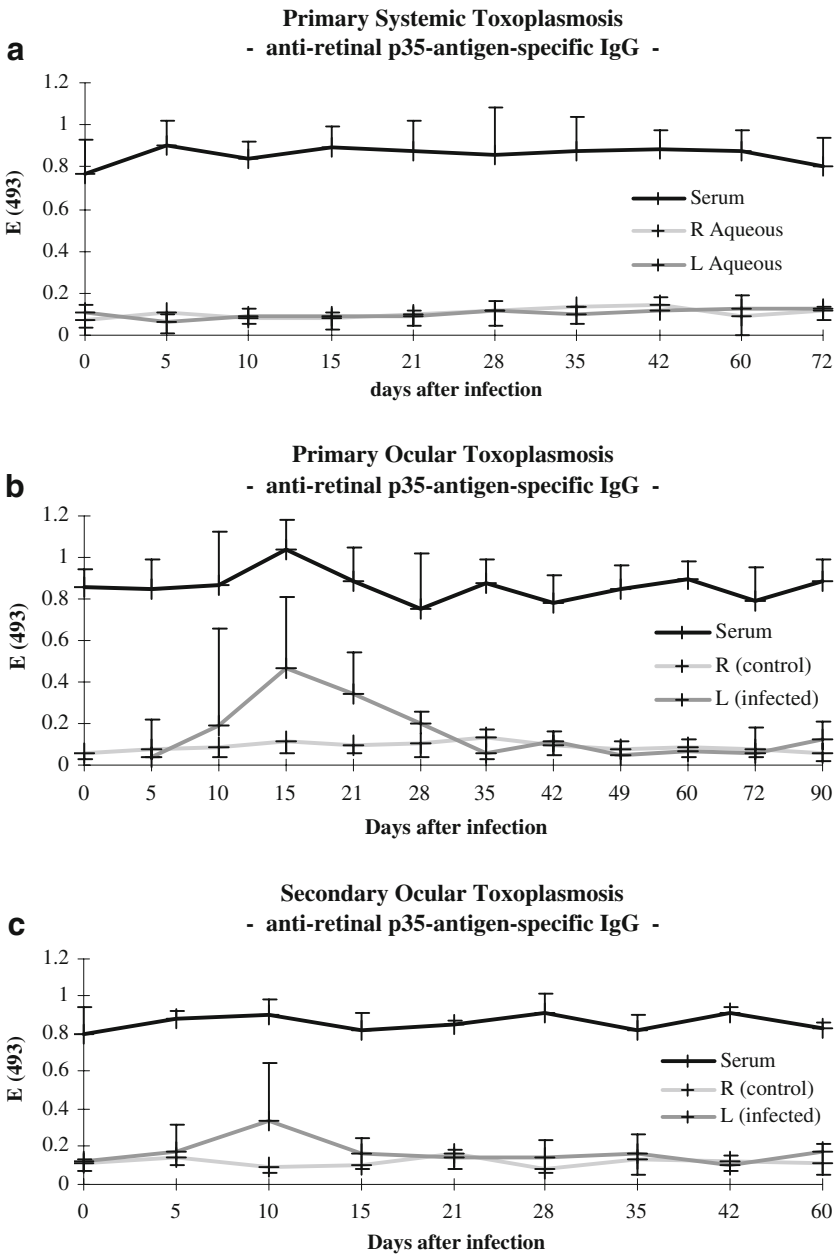

Fig. 4 Concentrations of anti-retinal p35-antigen-specific IgG in the serum and aqueous humour of rabbits that had been infected with 5000 tachyzoiotes of the BK strain of *Toxoplasma gondii*. **a** Primary systemic toxoplasmosis. **b** Primary OT. **c** Secondary OT

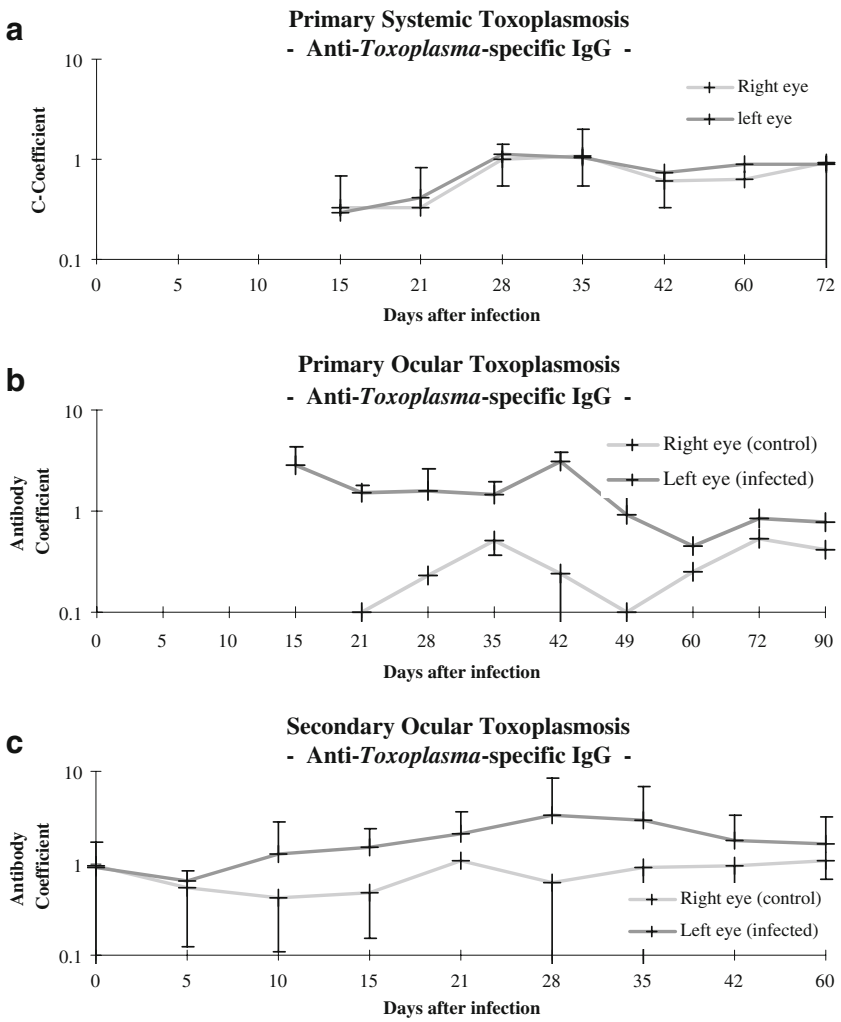

Fig. 5 Antibody (Goldmann–Witmer) coefficients for *Toxoplasma*-specific IgG in rabbits that had been infected with tachyzoites of the BK-strain of *Toxoplasma gondii*. **a** Primary systemic toxoplasmosis. **b** Primary OT. **c** Secondary OT

barrier. The time-lapse that was required for the production of antibodies was 10 to 15 days (Fig. 2b and c), which is consistent with previously published data [33]. In infection-immunized animals, intravitreal reinfection with *Toxoplasma* did not induce any change in the serum antibody levels, which is in accordance with the situation in humans. However, in the infected eye, the local level of *Toxoplasma*-specific IgG increased (Fig. 2c).

Infection with *Toxoplasma* did not elicit any change in the serum levels of antibodies against the retinal S-antigen and peptide 35. Looking at individual response, there is a delayed increase of serum levels of anti-S-Ag. Presence of low concentrations of specific IgG to S-antigen and peptide 35 is constitutively detected in the serum at day 0 in all groups of rabbits, but not in the aqueous humor when the blood–ocular barrier is intact. The local levels of antibodies against the retinal antigens increased after 10 to 15 days in an interindividually highly variable fashion in the infected eye of the infection-naïve, but not (or to a lesser extent), again in an interindividually highly variable fashion, in that of infection-immunized rabbits (Figs. 3 and 4). A local synthesis of anti-retinal antibodies has to be anticipated, since an elevation of specific IgG to S-antigen and peptide 35 in aqueous humor was found in the infected eye of the infection-naïve and infection-immunized rabbits, whereas no such antibodies were detected in the uninfected eye.

The antibody coefficient C for *Toxoplasma* in the infected eye reached levels above 2 after 15 to 42 days in rabbits with primary OT, and after 28 to 48 days in those with secondary OT. These levels were never reached in any of the animals with systemic toxoplasmosis (Fig. 5). An antibody coefficient of 3 or more was never consistently achieved in any of the animals, which is in accordance with previously published data [34].

In animals with systemic toxoplasmosis, the antibody coefficients in the infected eye for the retinal S-antigen and for peptide 35 rose non-significantly from day 42 after the systemic infection. In animals with primary or secondary OT, the antibody coefficients in the infected eye tended to drop during the early post-infection phase and then to

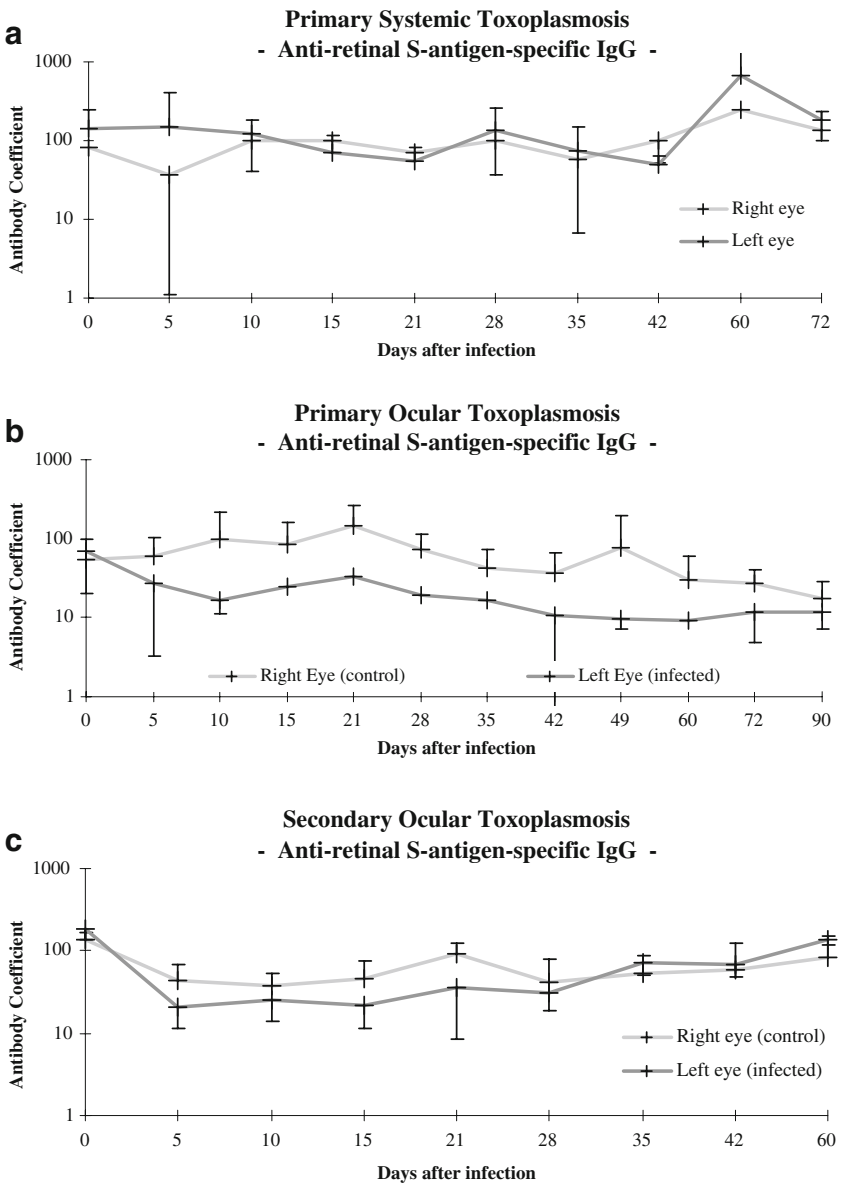

Fig. 6 Antibody (Goldmann–Witmer) coefficients for retinal S-antigen-specific IgG in rabbits that had been infected with tachyzoites of the BK-strain of *Toxoplasma gondii*. **a** Primary systemic toxoplasmosis. **b** Primary OT. **c** Secondary OT

recover (Figs. 6 and 7), but were always lower than in the non-infected eye. This may either reflect a wash-out of these antibodies in consequence of the blood–ocular barrier breakdown or a binding of antibodies to (liberated as well as tissue-bound) local retinal antigens. Interestingly, the antibody coefficient for anti-retinal antibodies was more than 10-fold higher than those for the *Toxoplasma*-specific antibodies. However, owing to the high standard deviations, the differences were not consistently significant ($p<0.01$) The rabbits used in this study were not congenic, which may explain the inconstistancy of the results between individual rabbits (Fig. 8a and b show this exemplarily for the group of primary ocular toxoplasmosis). These results suggest either that the humoral immune response against retinal antigens is a more localized phenomenon than that against toxoplasmal antigens, or that the response to *Toxoplasma* infection is not a strictly compartmentalized event, even after ocular challenge.

Discussion

Using an experimental rabbit model of systemic and ocular toxoplasmosis [33], and an established autoantibody ELISA technique [32, 33], we demonstrated that the concentrations of anti-retinal antibodies increased in the infected eyes during the early post-infection period. The data correlated with the degree of infectious tissue destruction at the clinical level (data not presented). In the uninfected partner eyes, the concentrations of anti-retinal antibodies did not

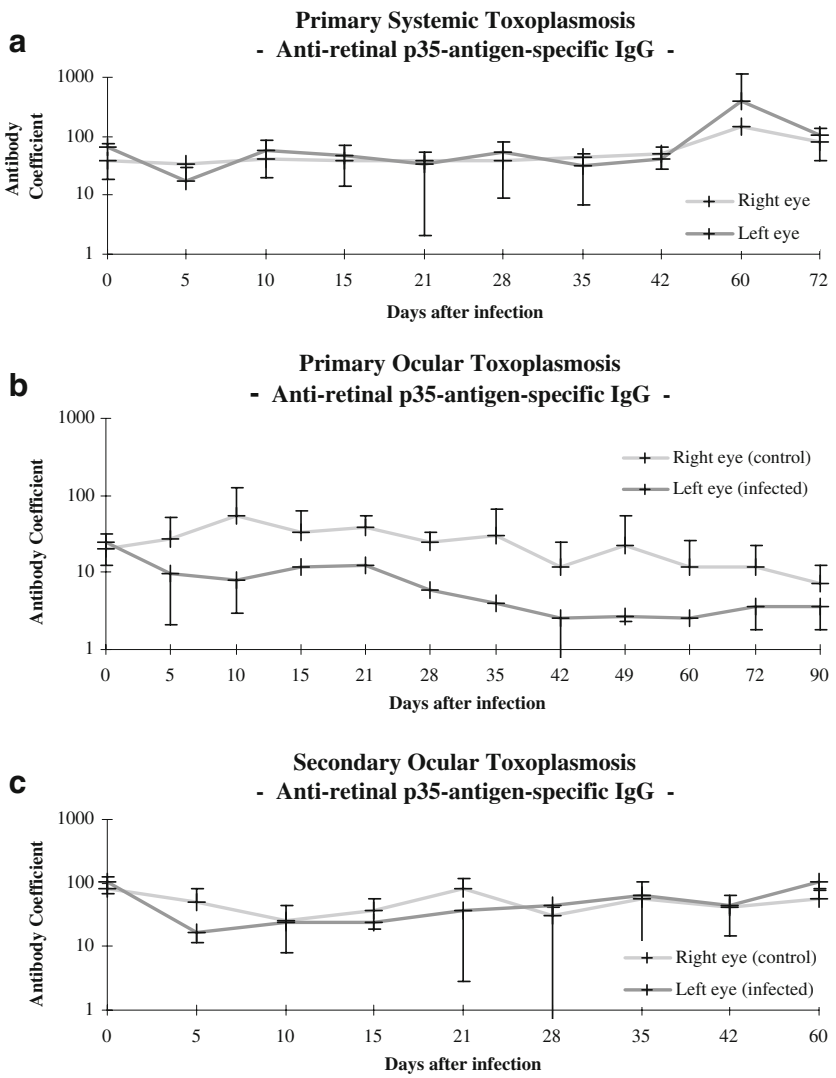

Fig. 7 Antibody (Goldmann–Witmer) coefficients for retinal p35-antigen-specific IgG in rabbits that had been infected with tachyzoites of the BK-strain of *Toxoplasma gondii*. **a** Primary systemic toxoplasmosis. **b** Primary OT. **c** Secondary OT

rise, and no tissue destruction was observed. The fact that the partner eye did not respond with antiretinal antibody production strongly argues against an infection-induced autoimmune response to retinal antigens. Hence, this would be expected to evolve bilaterally. Instead, the antibodies may have been induced locally in response to the retinal lesioning. The concentrations of anti-retinal antibodies failed to increase after the systemic infection of rabbits with *Toxoplasma*. This finding indicates that the causes for retinal and toxoplasmic antigen synthesis are distinct. In rabbits with either primary or secondary OT, the levels of the anti-retinal antibodies were restored to baseline levels during the later post-infection period, even though the inflammatory infiltration of the anterior chamber, of the vitreous and of the intraocular tissues persisted. Moreover, the clinically observed degree of tissue destruction did not parallel the levels of anti-*Toxoplasma* antibodies. Hence, our data offer no support for the existence of an infection-induced humoral autoimmune response that is based upon a cross-reactivity between toxoplasmic and retinal antigens [6]. They suggest rather that retinal autoantigens are liberated in the context of inflammatory tissue destruction [20]. On the other hand, in mice that have been infected with *Toxoplasma gondii* in utero, retinal vasculitis develops, as well as the almost uniform and highly-selective destruction of the photoreceptor layer. These share features in common with experimental autoimmune retinitis [36, 37].

The precise pathogenic role of retinal autoimmune responses in eye disease is unknown [38]. The humoral autoimmune response may be no more than an epiphenomenon which develops after damage to the retina by either physical agencies, micro-organisms, or immunological impairment [39, 40]. And even if autoimmunity does not itself initiate ocular inflammation, it might perpetuate and sustain the inflammatory state, and thereby exacerbate the tissue damage [9]. In our study, the quantitative disparity

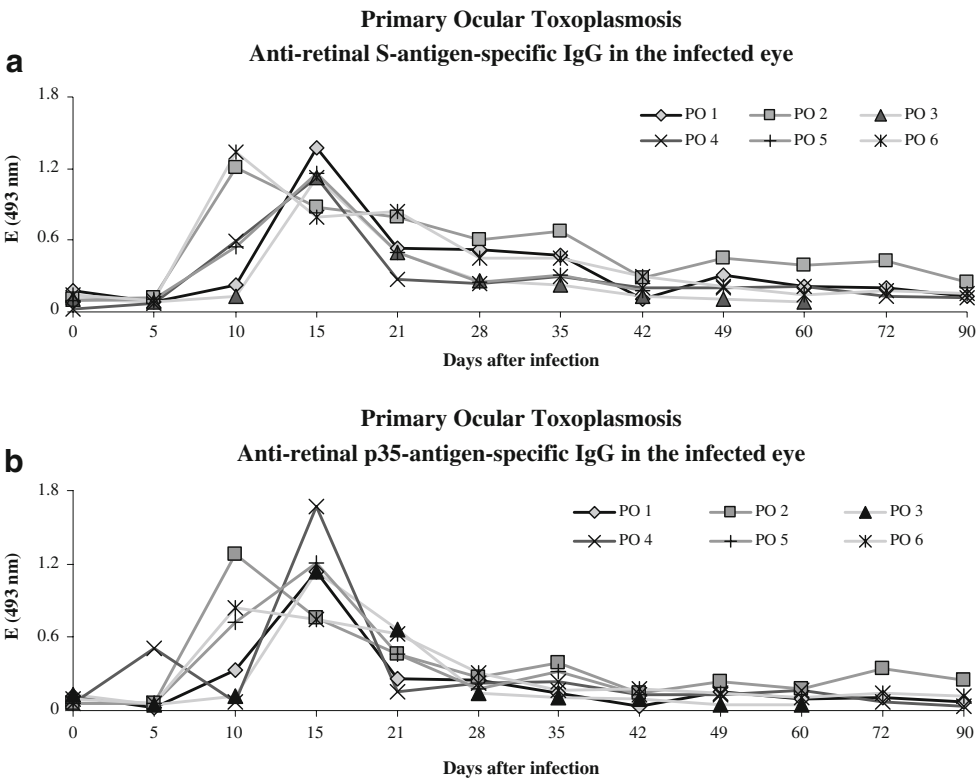

Fig. 8 Concentrations of anti-retinal S-antigen-specific (**a**) and anti-p35.specific (**b**) IgG in the aqueous humour and serum of single rabbits (PO1–PO6) that had primarily been infected in the left eye with 5,000 tachyzoites of the BK strain of *Toxoplasma gondii*

and the temporal asynchrony in the evolution of anti-retinal and anti-*Toxoplasma* antibodies suggest that humoral autoimmunity might well develop as an epiphenomenon in the context of inflammatory tissue destruction [19]. However, the levels of autoantibodies that are produced in consequence of the infectious tissue destruction may not be sufficient to yield evidence of permanent parasite-induced autoreactivity [6, 38].

Our data do not permit the drawing of any conclusions relating to autoimmunity at the cellular level and the interrelation between cellular and humoral immune response [41]. Interestingly, it has been reported that in the eyes of horses with equine recurrent uveitis, both IgG antibodies and autoreactive T-cells specific for retinal antigens were detected [42]. The role of infectious agents at the origin of the autoimmune response to retinal autoantigens has remained less clear [42]. In addition, the analysis of certain serum markers during equine recurrent uveitis has shown that serum is a valuable tool for analysing the onset and possible recurrences of the disease [43].

T-cells that have been derived from the peripheral blood of patients with mild OT react more strongly to retinal autoantigens than do those that have been derived from individuals with a more aggressive form of the disease [6, 38]. Exogenous, non-self antigens can stimulate type-2 T-helper cells, and thereby modify the cytokine environment sufficiently to alter the cytokine phenotype of inflammatory, autoreactive T-cell clones [41, 44]. This response may afford the host some protection against experimental autoimmune encephalitis and possibly other T-cell-mediated autoimmune diseases. Hence, infection with *Toxoplasma* could even protect the host against the development of overt autoimmune diseases at immuno-privileged sites such as the eye and the brain [44]. Outside the retina, anti-retinal antigens could help in the development of peripheral tolerance, as well as in protecting the host against autoimmunity [45]. The result may be the consequence of conflicting cellular immune responses [46]. In OT, as in more overt autoimmune diseases, the cellular immune response is mediated by type-1 T-helper cells. Consequently, the cytokine profiles may be similar in each case, but tailored to suit the pathogenic agency [46, 47]. Such tailoring might explain why the autoimmune response is lower in OT than in autoimmune diseases [48].

Our own study suffered from two major drawbacks. Firstly, some interesting information may have been lost due to the smallness of the animal cohort, which precluded a rigorous statistical evaluation of the data. Secondly, the generally poor specificity of anti-rabbit antibodies precluded a quantification of specific IgM, IgA and IgE antibodies. A hypersensitivity reaction to *Toxoplasma* antigens or an autoimmune response against retinal autoantigens could conceivably contribute to the clinical disease [2, 49, 50], although IgE antibodies are not usually detected [51]. In OT, the degree of anti-retinal reactivity correlates not only with the concentrations of antibodies against the retinal S-antigen, but also with those of antibodies against the interphotor-

eceptor binding protein [52] and other retinal antigens [39, 40]. Interestingly, the antibody coefficients for retinal autoantigens were consistently more than 10-fold higher than those for toxoplasmic antigens. This finding reconfirms the assumption that the local production of an antibody parallels the strictly local availability of the corresponding antigen. Moreover, the antibody coefficients tended to be lower in the infected than in the uninfected eye. This finding may be accounted for by a breakdown of the uveovascular barrier and the ensuing dispersal of the antibodies.

In conclusion, our data indicate that at the humoral level, infection-induced immunopathological rather than autoimmune phenomena contribute to the inflammatory response in OT, and that the latter do not influence the clinical evolution of the disease.

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