

CLINICAL SCIENCE

Increased frequency of anti-retina antibodies in asymptomatic patients with chronic *t. gondii* infection

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PURPOSE: To search for anti-retina antibodies that serve as markers for eye disease in uveitis.

MATERIALS AND METHODS: Stored sera from patients with uveitis, ocular toxoplasmosis (n = 30) and non-infectious, immune-mediated uveitis (n = 50) and from asymptomatic individuals who were positive (n = 250) and negative (n = 250) for anti-*Toxoplasma* antibodies were tested. Serum anti-retina IgG was detected by an optimized ELISA using a solid-phase whole human retina extract, bovine S-antigen or interphotoreceptor retinoid-binding protein.

RESULTS: Uveitis patients showed a higher mean reactivity to whole human retina extract, interphotoreceptor retinoid-binding protein and S-antigen in comparison to the asymptomatic population. These findings were independent of the uveitis origin and allowed the determination of the lower anti-retina antibody cut-off for the three antigens. Asymptomatic anti-*Toxoplasma* serum-positive individuals showed a higher frequency of anti-human whole retina extract antibodies in comparison to asymptomatic anti-*Toxoplasma* serum-negative patients. The bovine S-antigen and interphotoreceptor retinoid-binding protein ELISAs also showed a higher mean reactivity in the uveitis groups compared to the asymptomatic group, but the observed reactivities were lower and overlapped without discrimination.

CONCLUSION: We detected higher levels of anti-retina antibodies in uveitis patients and in a small fraction of asymptomatic patients with chronic toxoplasmosis. The presence of anti-retina antibodies in sera might be a marker of eye disease in asymptomatic patients, especially when whole human retina extract is used in a solid-phase ELISA.

KEYWORDS: Retina; Uveitis; Toxoplasmosis; Autoantibodies; ELISA.

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INTRODUCTION

Individuals with uveitis may present with visual loss depending on the location of the site of inflammation.¹ The most prominent feature of uveitis is the inflammatory process, which is characterized by an intraocular immune response with several infectious and non-infectious etiologies.² Most of the physiopathological studies of uveitis have focused on the antigens that trigger the inflammatory process, which can be an autoimmune response to retina proteins³ or an infectious agent.¹

Two bilateral granulomatous uveitis conditions, sympathetic ophthalmia (SO) and Vogt-Koyanagi-Harada (VKH)

disease, share several clinical, histological and immunohistochemical features despite exhibiting diverse triggering events.⁴ In these two uveitis conditions, HLA-DR4 and T-cell responses are associated with retina antigens, which indicates the presence of an underlying T-cell-mediated autoimmunity to uveal/retinal antigens during their development.^{3,4} The only reported reliable feature that could differentiate SO from VKH disease is a history of a penetrating wound in SO and the absence of such trauma in VKH disease. The intraocular compartment lacks lymphatic drainage system and appears to function similarly to a number of alymphatic biological sites that present alterations in immune functions and antigen presentation.⁵ In an SO experimental model, a subconjunctival injection of retina S-antigen in one eye induced a bilateral sympathetic uveitis, whereas an intraocular injection in one eye did not induce disease.⁶ However, autoimmune antibodies against the outer segments of photoreceptors and Muller cells have been detected in patients with VKH disease, in some

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patients with Behçet's syndrome, and in a few patients with sympathetic ophthalmia.⁷ These results suggest that retina autoimmunity may play an important role in the pathogenesis of posterior uveitis and that anti-retina antibodies are present in this condition.

Toxoplasma gondii, which is a successful obligate intracellular protozoan parasite, is widely distributed throughout the world and infects more than two billion people worldwide.⁸ *Toxoplasma* infection is usually asymptomatic in humans, and persistent infection with the cyst form of this parasite is controlled by the host immune system. However, in fetuses and immunosuppressed patients (such as AIDS patients or organ transplant recipients), the parasite becomes activated and causes life-threatening disease.⁹ A specific retina involvement may be present in up to 20% of all *Toxoplasma* infected individuals, regardless of their immune status.¹⁰ Toxoplasmosis is the most frequent cause of posterior uveitis in the USA and Brazil, and it is associated with visual impairment and blindness. The diagnosis is usually based on characteristic funduscopy findings and the clinical presentation; the disease is usually progressive and recurrent, and it can cause severe morbidity. These outcomes occur despite the availability of an effective treatment based on pyrimethamine, which is an anti-parasitic drug that is associated with anti-inflammatory drugs such as corticosteroids.¹¹ In the eye, the primary target tissue for ocular toxoplasmosis is the neural retina, which displays a surrounding, intense granulomatous reaction with numerous intracellular parasite cysts.¹² Free tachyzoites and cysts are also observed within retina pigment epithelium (RPE) cells.¹³ Ocular reactions also involve necrosis of the retina and RPE, subretinal and choroidal neovascularization, and focal inflammation.¹⁴ The inflammatory processes that are associated with retina infection by *T. gondii* may damage Bruch's membrane, which results in a disruption of the choroidoretinal interface.⁴

Toxoplasma uveitis can present the same autoimmune response as SO and VKH: a disruption of the parasite cysts that exposes the retina antigens. When central vision is threatened, ocular toxoplasmosis is treated with specific antibiotics together with corticosteroids, which suggests that the host immune response plays an active role in the disease process. In ocular toxoplasmosis, the involvement of the eye and inadequate autoimmune responses of memory cells to retina antigens in the blood have been reported previously,^{10,14} but few studies have reported a high frequency of retina autoantibodies titers in this condition.^{15,16} Furthermore, another group detected robust levels of cellular responses against retina antigens in the blood of ocular toxoplasmosis patients¹⁰. Alternatively, cross-reactive antigens could be encoded and produced by the infectious agent, which could induce autoimmunity via a cross-reactive immune response of antigen mimicry, which has also been reported for American trypanosomiasis.¹⁷ Autoimmunity that is induced by mechanical antigen exposure or by cross-reactive antibody production cannot be distinguished in established usual experimental models with active or chronic infection because both processes can occur concomitantly.

To search for anti-retina antibodies that could serve as markers for eye disease, we investigated the serum levels of anti-retina antibodies in asymptomatic anti-*Toxoplasma* seropositive and seronegative uveitis patients who were diagnosed with or without ocular toxoplasmosis. Whole

human retina extracts, S-antigens and IRBP were analyzed using an optimized ELISA.

MATERIALS AND METHODS

Serum samples

Five hundred serum samples were recovered from storage at the Protozoology Laboratory of IMTSP; these samples were previously used in epidemiological studies of the seroprevalence of toxoplasmosis in São Paulo State. The samples had been collected from healthy individuals with no symptoms at the time of blood collection, and according to defined patterns for routine ELISA and IFA anti-*T. gondii* IgG serology, 250 samples were positive (Tg IgG+ group), and 250 samples were negative (Tg IgG- group). We prospectively collected 80 serum samples from patients who were diagnosed with uveitis and undergoing treatment at the Uveitis Service in the Department of Ophthalmology at the Hospital das Clínicas, University of São Paulo, School of Medicine. Thirty uveitis patients were diagnosed with ocular toxoplasmosis based on a funduscopy and laboratory tests (Tg Uveitis), and 50 uveitis patients were diagnosed with non-infectious, immune-mediated uveitis (non-Tg Uveitis). The samples comprised blinded sera from patients with Vogt-Koyanagi-Harada disease (28), Behçet's disease (19), vitiligo (2) and ankylosing spondylitis (1). All of the patients were informed about the nature of the study and consented to participate. This study was approved by the institutional ethics committee (CAPesq HCFMUSP) under protocol no. 961/04.

Retina antigens

Whole human retina extracts (WHREs) were prepared from discarded, fresh human eyes that were obtained after corneal removal for transplantation from our hospital corneal transplant program. The retina was carefully removed and placed in a 1.5-ml conical tube containing 0.5 ml of sample buffer comprising 0.125 M Tris/HCl, pH 6.8, 1% sodium dodecyl sulfate, 4 M urea, 1 mM phenylmethanesulfonyl fluoride, 5 mM EDTA and 1 mg/ml bromophenol blue. These were stored at -17°C until use. The extract was prepared as follows: the contents of the tube were placed in a mortar, frozen with liquid nitrogen, and ground to a powder with a porcelain pestle. The frozen powder was returned to the tube, heated to 65°C for 15 minutes and centrifuged at 10,000 × g for 2 minutes. The protein content of the supernatant fraction was measured using the Bradford assay, adjusted to 250 µg/ml and used for microplate adsorption. SDS-PAGE was performed and revealed typical protein bands without degradation (data not shown). Bovine S-antigen was purified according to the reported method¹⁸, whereas bovine interphotoreceptor retinoid-binding proteins (IRBPs) were isolated as elsewhere reported¹⁹ and stored at -70°C until use.

ELISA assays

The population samples were randomly assorted in each plate. All of the selected samples were tested using an improved ELISA. Briefly, WHRE, IRBP and S-antigen were dissolved in 8 M urea at 2 µg protein/ml, and 100 µl/well were adsorbed overnight at 4°C. The plates were washed and blocked with phosphate-buffered saline containing 0.3% non-fat dry milk and 0.05% Tween-20 (PBST); this buffer was used for all of the washes and serum or

conjugate dilutions. After washing and blocking in PBST, 1100 serum dilutions in PBST containing 0.125 M urea were allowed to react for 2 h at 37°C. After additional washes, the wells were incubated with specific anti-human Fc IgG conjugates for 1 h at 37°C. The wells were washed, and the bound conjugates were visualized with o-phenylenediamine (OPD) and hydrogen peroxide. The reaction was stopped with 4 N HCL, and the optical densities at 492 nm were determined using a microplate reader. The samples were considered reactive according to an arbitrary cut-off that was based on the results of whole group of uveitis patients. The positive cut-off level was defined using the lower 99% confidence interval of uveitis samples; data below this level were considered negative for each specific reaction.

Statistical analysis

Mean antibody levels were compared between the populations using the Student's t-test or a one-way ANOVA following a variance assessment using Bartlett's test. The ANOVA was followed by a post-hoc comparison using Bonferroni's multiple comparisons test. The results were also confirmed using the Mann-Whitney or the Kruskal-Wallis test with Dunnett's post-tests. The frequency of positivity and the odds ratio were analyzed using chi-square tests. The calculated frequencies and means were considered significantly different when the probability of equality was less than 5% ($p < 0.05$). All statistical analyses were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

RESULTS

Optimized ELISA for the detection of human anti-retina IgG

We standardized a selective and efficient ELISA for the detection of anti-retina antibodies using three different retina antigens, as described in the Methods section. In this test, there was a low concentration of protein antigens in the solid phase, and the serum was diluted using a chaotrope-containing dilution buffer that diminished non-specific binding. We also used a selective anti-human Fc conjugate, which provided greater assay efficiency. Eighty uveitis patient samples were tested in the ELISA, including 30 serum samples from patients with *Toxoplasma* uveitis, and the results determined the cut-off of the positive reaction in each test using the lower 99% confidence interval of all uveitis samples. Five hundred samples from the adult asymptomatic population were tested; 250 of these samples were positive for anti-*T. gondii* IgG (contact with the infectious agent), and 250 samples were from asymptomatic patients (no contact with the infectious agent). The results obtained for each individual assay are presented below.

WHRE ELISA

The distribution of the quantitative results for the WHRE in each group is presented in Figure 1. The samples from uveitis patients showed higher levels of anti-WHRE IgG in comparison to the associated asymptomatic population ($p < 0.001$). No differences in reactivity were found with respect to ocular toxoplasmosis or other uveitis etiologies, which allowed the determination of a cut-off of 0.12 A_{492nm} . In the asymptomatic groups, the mean level of anti-retina antibodies in the samples from patients with *T. gondii* IgG

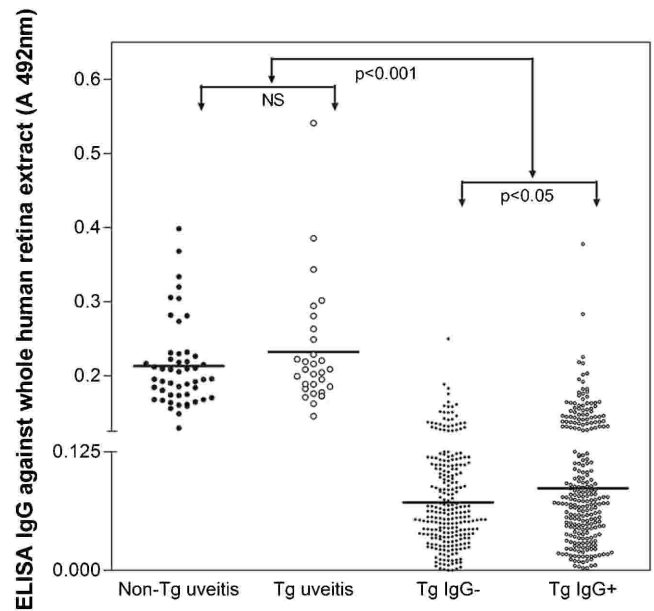


Figure 1 - Antibody reactivity against whole human retina extracts (WHRE) as determined using chaotrope in the serum dilution. Non-Tg uveitis: samples from patients with uveitis other than toxoplasmosis (n = 50; see Methods). Tg uveitis: *Toxoplasma* uveitis samples (n = 30). Tg IgG -: samples from individuals who were seronegative for *T. gondii* (n = 250). Tg IgG+: samples from individuals who were seropositive for *T. gondii*. Bars represent mean values. The y-axis was interrupted at cut-off using the mean minus 2 SD of samples from all uveitis patients.

was higher than that in patients without contact with this agent, as detected by ANOVA ($p < 0.05$). Using the cut-off that was determined for the uveitis patients, the qualitative data revealed a greater number of samples with values above the cut-off (67/250) in the group with *T. gondii* IgG than in that (39/250) without *T. gondii* contact ($p < 0.005$); the OR for the association of anti-retina antibodies and anti-*T. gondii* IgG in the serum was 1.98 (C.I.95% 1.25 to 3.16).

S-antigen ELISA

The presence of antibodies against bovine S-antigen in our samples was also tested by ELISA, and the results are shown in Figure 2. The samples from the uveitis patients showed higher mean values of anti retina antibodies in comparison to those determined for the asymptomatic population. However, some high outliers were detected using this assay ($p < 0.001$). The cut-off value was 0.089 A_{492nm} , and the difference between the groups was not significant, which likely reflects the low reactivity to this antigen. Both asymptomatic populations presented similar mean levels of anti-retina antibodies and no differences with respect to the presence of chronic *T. gondii* infection. Using the cut-off obtained from the uveitis patients, the frequency of samples that were positive for S-antigen among the anti-*T. gondii*-IgG-positive patients (116/250) was found to be similar to that in the samples (99/250) from the group without *T. gondii* contact.

IRBP ELISA

Antibodies against bovine IRBP antigen were also tested using the low-protein ELISA, and the results are shown in Figure 3. This assay showed the lowest level of reactivity,

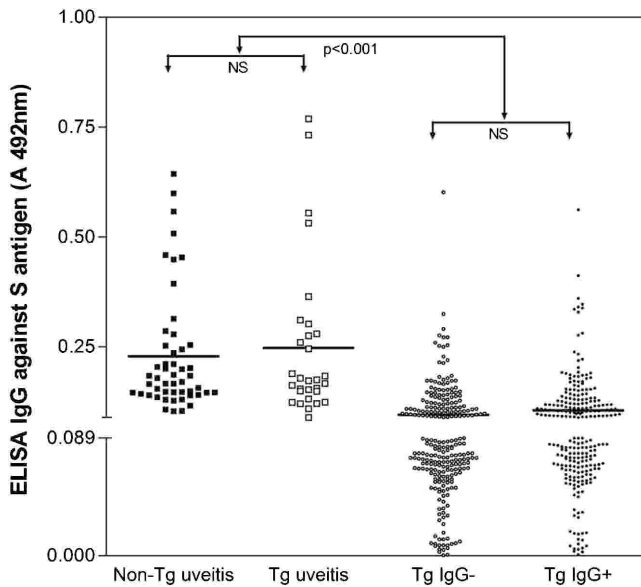


Figure 2 - Antibodies against bovine retina S-antigen in selected patients. Non-Tg uveitis: samples from patients with uveitis other than toxoplasmosis (n = 50; see Methods). Tg uveitis: samples from patients with *Toxoplasma* uveitis (n = 30). Tg IgG-: samples from patients who were seronegative for *T. gondii* (n = 250). Tg IgG+: samples from patients who were seropositive for *T. gondii*. Bars represent mean values. The y-axis was interrupted at cut-off using the mean minus 2 SD of samples from all uveitis patients.

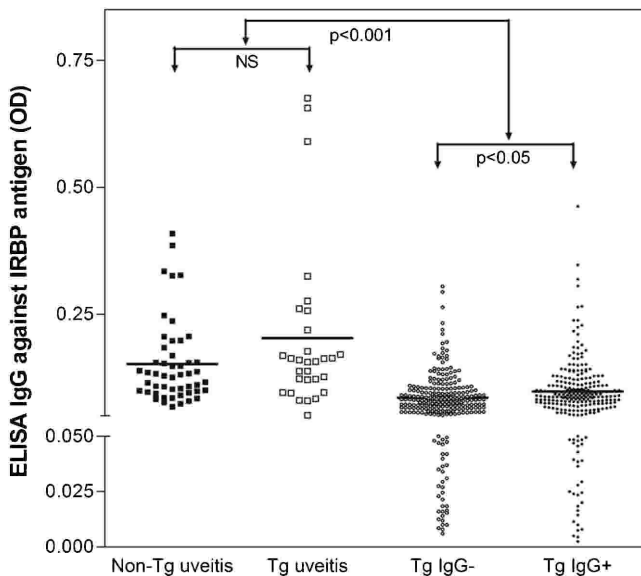


Figure 3 - Antibodies against bovine interphotoreceptor retinoid-binding protein (IRBP) antigen in selected patients. Non-Tg uveitis: samples from patients with uveitis other than toxoplasmosis (n = 50; see Methods). Tg uveitis: samples from *Toxoplasma* uveitis patients (n = 30). Tg IgG-: samples from patients who were seronegative for *T. gondii* (n = 250). Tg IgG+: samples from patients who were seropositive for *T. gondii*. Bars represent mean values. The y-axis was interrupted at cut-off using the mean minus 2 SD of samples from all uveitis patients.

but it also demonstrated that the samples from uveitis patients had higher mean levels of anti-IRBP IgG in comparison to those from the asymptomatic population ($p < 0.001$). *Toxoplasma* uveitis samples presented the highest

mean levels of anti-retina antibodies in comparison to the other groups, but these levels were highly variable and did not differ statistically from those determined in non-infectious uveitis patients. A higher mean level of anti-IRBP IgG was found in asymptomatic chronic toxoplasmosis samples compared to asymptomatic *Toxoplasma*-free patient samples ($p < 0.05$). The cut-off determination failed to resolve the asymptomatic populations according to the anti-*T. gondii* IgG serology due to a low cut-off of 0.05 A_{492nm} in this assay. Therefore, a high frequency of positive samples was observed: 216/250 positive samples among the asymptomatic patients without *T. gondii* contact and 220/250 positive sample among the anti-*T. gondii* IgG-positive patients.

DISCUSSION

Independent of *T. gondii* infection, we discovered higher levels of antibodies that were reactive to retina antigens in the sera from uveitis patients in comparison to stored samples from asymptomatic individuals. This result was observed for each of the three antigens used in the solid-phase ELISA, but the best discrimination was obtained using our human whole human retina extract. The extract from the human retinas comprised several proteins, and the method used to extract the proteins yielded more antigens than did techniques employing non-denaturing saline or detergent solutions as described elsewhere.²⁰ We had previously performed a WHRE ELISA that demonstrated a higher reactivity and thresholds in the reaction, which impeded the qualitative analysis; the addition of urea to the dilution buffer eliminated the non-specific binding and provided a clear qualitative discrimination of uveitis patients (data not shown). The urea-containing sample buffer used for SDS-PAGE allowed the extraction of hydrophobic domains of the membrane proteins that are highly prevalent in the retina, which is composed of several membrane-associated proteins.²¹ This method of extract preparation results in a more sensitive antibody detection in comparison to the use of the less reactive purified retina antigens, such as S-antigen and IRBP, which are usually of a bovine origin. These findings have been described previously in a study that found that the purified antigens provided less significant results than did the whole retina in an intact retina immunofluorescence assay.¹⁶ In the present study, the use of IRBP as an ELISA antigen demonstrated the least efficiency for anti-retina antibody detection despite the demonstration of definitive results for the mean sample reactivity in uveitis patients compared to the asymptomatic population. This test also revealed a higher mean reactivity for samples from *Toxoplasma*-infected asymptomatic individuals than for those from the non-infected asymptomatic population; however, the individual discrimination was poor due to the large numbers of reactive samples. The S-antigen ELISA demonstrated similar results with clear differences in the mean values of antibody levels and a good discrimination of the presence of uveitis; however, the qualitative results were poor. These data demonstrate that purified antigens may be less efficient in comparison to the whole extract for determination of the type of retina involvement, as described previously.²²

Several isolated antigens have been proposed for as targets of autoimmune eye disease and some of them were associated to the specific disease.²³ Our results demonstrating a higher resolution for the whole retina extract suggest

that exposure to a variety of *T. gondii* antibodies may affect the whole retina through a large spectrum of antigens instead of a specific antigen, which is observed with other diseases, such as viral²⁴ or degenerative diseases.²⁵ In qualitative studies of autoantibodies against the retina using ELISA, the main challenge is the determination of threshold values. In the current study, an unusual approach was used. The samples from uveitis patients served as positive controls for all of the tested antigens, and inflammation was assumed to result in a humoral response. Based on these criteria, our WHRE ELISA revealed a few responders in the asymptomatic population, which permitted its use as a screening tool. A similar result was not obtained using specific antigens, such as S-antigen or IRBP, which demonstrated higher proportions of positive samples in the asymptomatic population.

The mechanism responsible for the induction of anti-retina antibodies is controversial because the eye is a specialized compartment with respect to the immune response. The eye is considered a sanctuary compartment in which antigens evade immune detection and clonal selection until the protective eye barriers are impaired.²⁶ No lymphatic drainage occurs in the intraocular compartment, which appears to function similarly to immunologically privileged biological sites, and therefore, an alteration of immune function and antigen presentation are observed.²⁷ This phenomenon is sustained in sympathetic ophthalmia, in which the traumatic exposure of intraocular components to the immune response may lead to intraocular inflammation in the non-affected healthy eye.⁴ Other examples of autoimmune eye disease included tissue-specific diseases, such as VKH,²⁸ or systemic autoimmune diseases, such as Behcet's disease and systemic lupus erythematosus.³⁰

During the first episode of intraocular inflammation caused by *T. gondii*, retina antigens are exposed to the immune response, which leads to the selection of antigen-specific reactive memory cells both for humoral or cell mediated immunity. Cyst reactivation or re-infection of the eye could establish secondary anamnestic and granulomatous responses, which are typical of the adaptive immune response. This process has been reported using cell proliferation assays to assess cellular immunity to eye self-antigens, and the results demonstrated a weak cellular response to uncontrolled disease.³⁰

The sera of uveitis patients demonstrated higher levels of anti-retina autoantibodies, regardless of the etiology. Our screening analysis also revealed higher levels of those antibodies in samples from the asymptomatic populations that demonstrated a high titer of anti-retina specific antibodies, and these results could imply the presence of a retinal disease. This association highlights the importance of the use of serological surveys to detect anti-retina antibodies in patients as a screening tool for ophthalmologic consultations, the selection of patients for follow-up, and early diagnosis and treatment, especially in patients with serologies that are positive for *Toxoplasma*. Our data strongly suggest that the immune damage caused by *Toxoplasma* invasion of the retina results in uveitis both by a direct mechanism and by retina antigen exposure, as determined by the humoral immune host response against retina antigens.

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