Circulating Anti-galectin-1 Antibodies Are Associated with the Severity of Ocular Disease in Autoimmune and Infectious Uveitis

Marta D. Romero,[1,2,3,4] *Juan C. Muiño,*[3,4,5] *Germán A. Bianco,*[1] *Mercedes Ferrero,*[2,3] *Claudio P. Juarez,*[3] *José D. Luna,*[3] *and Gabriel A. Rabinovich*[1]

Purpose. Galectin (Gal)-1, an endogenous lectin found at sites of immune privilege, plays a critical role in the regulation of the immune response. Therapeutic administration of Gal-1 or its genetic delivery suppresses chronic inflammation in experimental models of autoimmunity. The purpose of this work was to investigate the occurrence of circulating anti-Gal-1 antibodies in patients with autoimmune and infectious uveitis as potential determinant factors of disease progression.

Methods. IgG, IgE, and IgA anti-Gal-1 antibodies were assessed by ELISA and Western blot in sera from patients with autoimmune ($n = 47$) and infectious ($n = 15$) uveitis compared with healthy control subjects ($n = 30$). The frequency of anti-Gal-1 antibodies was examined in patients experiencing poor clinical outcome ($n = 21$) or good evolution ($n = 9$). Anti-Gal-1 antibodies were eluted by incubating patient sera with nitrocellulose filters adsorbed with rGal-1. The ability of these antibodies to recognize retinal tissue was assessed by ELISA, Western blot, and immunohistochemistry.

Results. IgE, IgG, and IgA anti-Gal-1 antibodies were increased in sera from patients with autoimmune uveitis ($P < 0.001$ vs. controls) and toxoplasmic retinochoroiditis ($P < 0.001$). The level of anti-Gal-1 IgE and IgG antibodies was associated with progressive disease and poor outcome in autoimmune and infectious uveitis. Furthermore, these antibodies strongly immunoreacted with retinal lysates and recognized retinal structures mainly photoreceptors in retinal sections.

Conclusions. Anti-retinal Gal-1 antibodies are present in sera from patients with uveitis and can be associated with the progression of ocular disease, suggesting their potential use in follow-up observations of these patients. (*Invest Ophthalmol Vis Sci.* 2006;47:1550–1556) DOI:10.1167/iovs.05-1234

From the [1]División Immunogenética, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina; the [2]Laboratorio of Inmunopatología LIIDO, Córdoba, Argentina; the [3]Fundación Ver, Córdoba, Argentina; and the [5]División Medicina III, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Argentina.

[4]Contributed equally to the work and therefore should be considered equivalent authors.

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Corresponding author: Gabriel A. Rabinovich, División Inmunogenética, Hospital de Clínicas "José de San Martín," Facultad de Medicina, Universidad de Buenos Aires, Av. Córdoba 2351, 3er Piso (C1120)Ciudad de Buenos Aires, Argentina; gabyrabi@ciudad.com.ar.

Intraocular inflammatory diseases are a major cause of severe impairment, accounting for 10% of blind registrations in the working population.[1,2] These uveitic syndromes may occur as autoimmune or infectious processes, which are typically characterized by inflammation and subsequent destruction of the neural retina.[1–3] How the inflammatory process is initiated is still not clear, but current knowledge indicates that subversion of immune privilege mechanisms by autoimmune insults and pathogens may play a critical role.[3] In this context, it has been demonstrated that apoptotic cell death promotes immune privilege within the ocular microenvironment and contributes to amelioration and clinical resolution of autoimmune uveitis (AU).[3,4] Furthermore, it has been speculated that specific blockade of immunosuppressive factors in the ocular microenvironment contributes to the initiation and perpetuation of Th1-mediated inflammation.[3]

Galectin (Gal)-1, a member of a highly conserved protein family,[5,6] is expressed at sites of immune privilege such as the eyes and testis[7–10] and has the potential to regulate immune tolerance and inflammation.[11–19] How Gal-1 exerts its anti-inflammatory response is poorly understood, primarily because of its pleiotropic nature where it has been shown to suppress T-cell activation,[20] T-cell adhesion to extracellular matrix,[21] and Th1 cytokine secretion.[11,16–18] Foremost, however, has been the ability of Gal-1 to promote T-cell apoptosis through binding to specific cell surface glycoconjugates.[13–18] In this context, Ishida et al.[8] have recently reported the ability of retinal pigment epithelium to suppress T-cell activation in vitro through secretion of Gal-1, suggesting the potential role of this protein in the establishment of immune privilege.[8]

We have recently shown that Gal-1 plays a critical role in the maintenance and re-establishment of immune tolerance in experimental models of chronic inflammation and cancer.[11,12]

Taken together, these observations led us to speculate that specific blockade of immunosuppressive Gal-1 may contribute to the breakdown of immune privilege and to the perpetuation of the inflammatory response. In the present study, we determined the occurrence of IgG, IgA, and IgE anti-Gal-1 antibodies in sera from patients with AU or IU and posed the question of whether these autoantibodies recognize retinal structures and play a role in the immunopathogenesis of human uveitis.

Materials and Methods

Antibodies and rGal-1 Preparation

Peroxidase-conjugated goat anti-human IgG; peroxidase-conjugated goat anti-human IgA; alkaline-phosphatase-conjugated goat anti-human IgE; goat anti-human IgG, IgA, and IgE and peroxidase-conjugated anti-goat IgG were purchased from Sigma-Aldrich (St. Louis, MO). Anti-human Gal-1 polyclonal antibody was obtained as previously described.[12] This antibody was highly specific for Gal-1, because it did not recognize other members of the galectin family, such as Gal-3.[22] Human rGal-1 was produced and purified as described.[23]

Preparation of Bovine Retinal Cell Lysates

Retina was obtained from fresh bovine eyes, and extracts were prepared by homogenizing retina in cold PBS containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 10 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich) and kept for 30 minutes on ice. The solution was centrifuged at 4°C for 20 minutes at 10,000*g*, and the supernatants were collected and stored at −20°C.

Patients

We studied 62 patients with uveitis, 32 males and 30 females (mean age, 36.9 ± 14.9 years; range, 5–72). The diagnosis of uveitis was established according to clinical criteria as described.[2,24] These patients with uveitis were treated for 3 months with methylprednisolone in decreased immunosuppressive doses from 120 mg/d to 10 mg on alternating days. In cases of poor response to treatment, patients were included in a tacrolimus protocol in a dose of 5 mg/d for at least 1 year of treatment. Patients were classified according to their clinical evolution in subgroups of severe relapsing uveitis with poor evolution and uveitis with good evolution.

According to the etiology of uveitis, patients were classified as previously described[24] into the following groups: patients with AU ($n = 47$; 27 males and 20 females) and patients with IU, ($n = 15$; 5 males and 10 females). Sera from age-matched healthy individuals ($n = 30$) were used as the control. Patients with progressive disease were clinically characterized by poor response to tacrolimus treatment, with continuous crisis of uveitis and severe impairment of visual function, whereas patients with good evolution responded successfully to treatment with improvement of vision or only slight vision impairment. Thirty patients treated with similar drug protocols with progressive ($n = 21$) and good ($n = 9$) evolution were selected for longitudinal analysis of anti-Gal-1 antibodies, which were determined at different times (approximately every 6 months) during the course of the disease.

The studies were approved by the Institutional Review Board of the Hospital de Clínicas José de San Martín and The Ethics Committee of Fundación Ver and complied with the tenets of the Declaration of Helsinki.

Immunoadsorption

To examine the potential cross-reactivity between Gal-1 and retinal tissue, immunoadsorption assays were performed as described by Giordanengo et al.[25] Briefly, rGal-1 (5 μg/mL) was adsorbed to nitrocellulose filters. After adequate washing and blocking, filters were incubated overnight at 4°C with 1:40 dilution of sera from 13 patients with uveitis or 7 healthy control subjects. Bound antibodies were then eluted with 0.02 M glycine pH 2.8 and neutralized with 1 M Tris-HCl (pH 8.6). Each eluted sample was tested against rGal-1 (5 μg/mL) and bovine retinal tissue (50 μg/mL) by ELISA and Western blot.

Enzyme-Linked Immunosorbent Assay

Serum levels of anti-Gal-1 IgE, IgG, and IgA antibodies were determined by ELISA in sera from patients with uveitis or healthy control subjects. Anti-Gal-1 antibodies recognizing rGal-1 or bovine retina were also determined by ELISA in eluted material obtained from uveitis or control sera. Briefly, microtiter plates (Nunc, Rochester, NY) were coated with 5 μg/mL human rGal-1 in PBS, blocked, and incubated with a 1:100 dilution of human sera. Bound IgG and IgA were detected by incubation with peroxidase-conjugated goat anti-human IgG (1:1000) or peroxidase-conjugated goat-anti human IgA (1:1000), followed by substrate (orthophenilendiamine). Bound IgE was detected with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-human IgE followed by substrate (dinitrophenylphosphate). Optical densities were measured at 490 or 405 nm respectively in an ELISA reader (Bio-Rad, Richmond, CA). A serially diluted polyclonal rabbit anti-human Gal-1 antibody revealed with a peroxidase-conjugated anti-rabbit IgG was used as a positive control for immunoreactivity. A result was considered positive if optical density was two or more standard deviations above the mean of normal control sera.

Western Blot Analysis

SDS-PAGE was performed in an electrophoresis apparatus (Mini Protean-II; Bio-Rad) as described.[12] To assess the presence of anti-Gal-1 antibodies in patient sera, we resolved rGal-1 (5 μg) and bovine retinal lysates (30 μg) on a 15% polyacrylamide gel. After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes and probed with appropriate dilutions of human sera or eluted antibodies from patients with uveitis or healthy control subjects. Blots were then incubated with goat anti-human IgG, IgA, and IgE (1:100) followed by peroxidase-conjugated anti-goat IgG (1:500) and developed using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ), and the films were analyzed (Image Analysis software; Scion, Frederick, MD).

Immunohistochemistry

Semithin sections (0.5 μm) of human eyes obtained for autopsies were blocked with H_2O_2 followed by incubation with an adequate dilution of rabbit anti-human Igs for 20 minutes at room temperature and then incubated for 24 hours at 4°C with anti-Gal-1 antibodies eluted from sera corresponding to patients with uveitis or healthy control subjects. Incubation with optimal dilutions of goat anti-human IgG and IgE was performed for 2 hours at room temperature. The reaction was completed by incubation with peroxidase-conjugated anti-goat IgG. All reactions were revealed using bisdiaminobenzidine-H_2O_2 and visualized by microscope (Olympus, San Diego, CA).

Statistical Analysis

Statistical analysis was performed by ANOVA for continuous variables and χ^2 statistics for categorical variables. All tests for significance were two-sided (Microstat-Ecosoft Inc., Indianapolis, IN). $P < 0.05$ was considered statistically significant.

Results

Prevalence of Circulating Anti-Gal-1 Antibodies in Sera from Patients with AU or IU

A quantitative determination of anti-Gal-1 antibodies was performed by indirect ELISA in sera from patients with uveitis ($n = 62$) and healthy control subjects ($n = 30$). As shown in Figure 1A, Gal-1 immunoreactivity was detected in 80.6% of patients with uveitis compared with 16.6% of healthy control subjects ($P < 0.001$). To determine whether anti-Gal-1 antibodies occur in uveitis of different etiology, we classified patients according to their clinical features as having AU or IU. In addition, the last group was subdivided into toxoplasmic retinochoroiditis (TR), tuberculosis uveitis (TU), and bacterial endophthalmitis (BE). The prevalence of anti-Gal-1 antibodies in each group is shown in Figure 1B.

Frequency of Different Anti-Gal-1 Antibody Isotypes in Patients with AU or IU

The next issue we attempted to elucidate was related to specific anti-Gal-1 antibody isotypes present in sera from patients with uveitis. Therefore, anti-Gal-1 IgG, IgA, and IgE isotypes were investigated by ELISA in sera from patients with uveitis (Fig. 1C). The highest percentage of immunoreactive sera in the whole uveitic population corresponded to the IgE isotype in 30 of 62 cases (48.4%), followed by IgG in 27 of 62 cases (43.5%), whereas the lower frequency was found to be associated with the IgA isotype in 21 of 62 cases (33.9%). In addition, sera from control subjects were positive in 2 of 30 cases (3.3%)

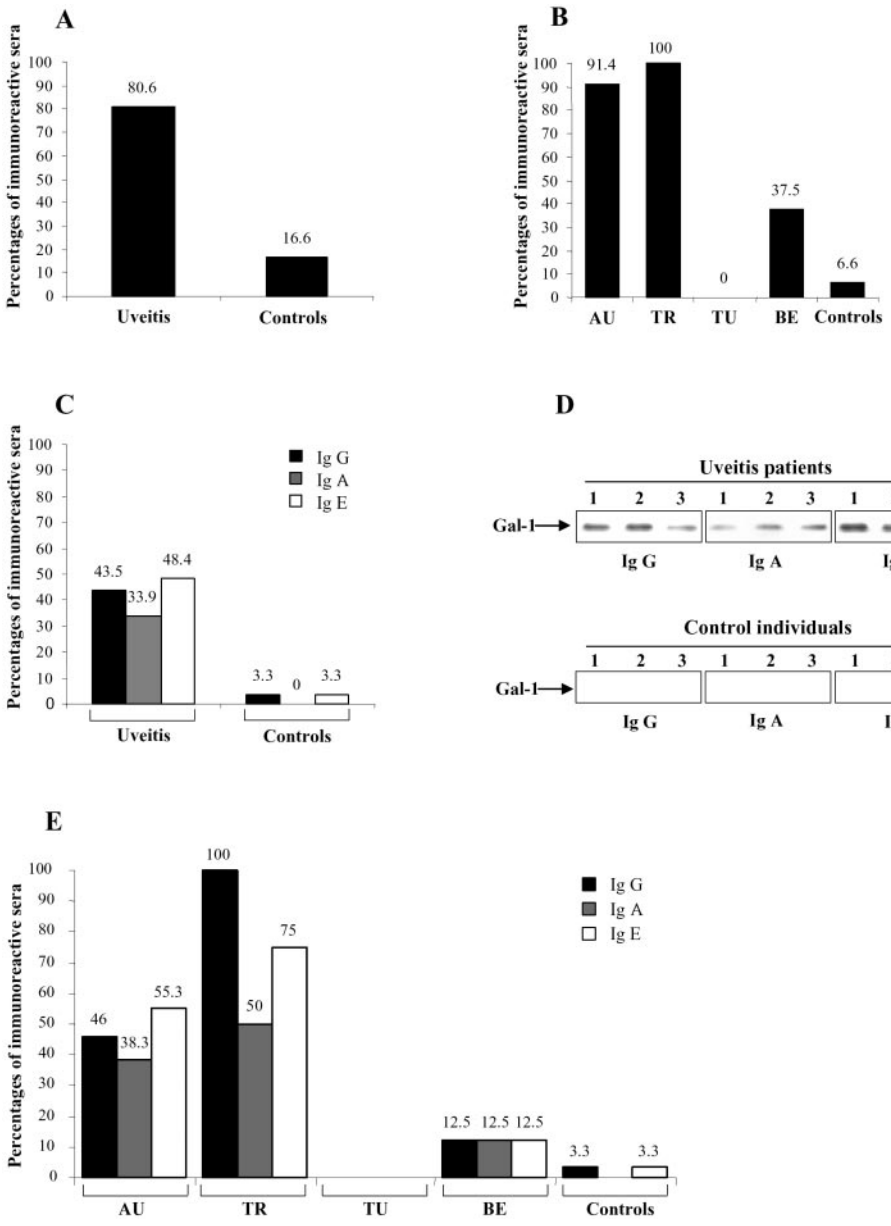

FIGURE 1. Anti-Gal-1 antibodies in sera from patients with AU or IU. (**A**) Percentages of immunoreactive sera from patients with uveitis ($n = 62$) compared with control sera ($n = 30$; $P < 0.0.001$). Reactivity was considered positive if optical density (OD) was two or more standard deviations above the mean of normal values. (**B**) Percentages of immunoreactive sera from patients with uveitis discriminating between AU ($n = 47$), TR ($n = 4$), TU ($n = 3$), BE ($n = 8$), and control subjects ($n = 30$; $P < 0.001$ AU and TR versus control). (**C**) Percentages of sera showing IgG, IgA, and IgE reactivity against rGal-1 in patients with uveitis ($P < 0.001$ versus control for all isotypes tested). (**D**) IgG-, IgA-, and IgE-immunoreactive profiles by Western blot of representative sera from patients with uveitis and control subjects. (**E**) Percentages of sera exhibiting positive IgG, IgA, and IgE immunoreactivity in different subgroups of patients with uveitis and control subjects ($P < 0.001$ AU and TR versus the control for all isotypes tested; $P < 0.05$ BE versus the control for all isotypes tested).

for IgE and IgG ($P < 0.001$; IgE-, IgG-, and IgA-positive uveitis sera versus the control). These findings were confirmed by Western blot analysis of sera from different patients with AU compared with sera from control individuals (Fig. 1D).

We then determined the percentages of different isotypes in patients with uveitis by discriminating the ocular disease into AU or IU. As shown in Figure 1E, all anti-Gal-1 isotypes were significantly increased in patients with AU and TR ($P < 0.001$ AU and TR versus control group; $P < 0.05$ BE versus control group for all isotypes tested). Furthermore, distinct levels of IgG, IgA, and IgE anti-Gal-1 antibodies were present in the different subgroups of AU and IU (Fig. 2).

IgG and IgE anti-Gal-1 Antibodies and the Progression and Severity of Ocular Inflammation in Patients with Uveitis

To determine whether anti-Gal-1 antibodies could be associated with the evolution of ocular disease in patients with AU or IU, we investigated the possible relationship between the levels of IgG, IgE, and IgA anti-Gal-1 antibodies and clinical manifestations in patients with progressive ($n = 21$) and good ($n = 9$) evolution treated under similar drug protocols. Levels of specific antibodies were determined during the course of the disease. Longitudinal analysis is shown in two representative age-matched polar cases with either progressive (Fig. 3A) or good evolution (Fig. 3B) in samples collected approximately every 6 months. The patient with progressive disease was clinically characterized by poor response to tacrolimus treatment, continuous uveitis crises, and the persistent presence of anti-Gal-1 IgE antibodies. This patient was blind in one eye, and the second eye displayed continuous and persistent clinical manifestations of uveitis (Fig. 3A). In contrast, the second patient responded to treatment with improvement of vision, and this clinical outcome was associated with a decrease in the levels of anti-Gal-1 IgE antibodies (Fig. 3B). Thus, the levels of IgE anti-Gal-1 antibodies positively correlate with the severity of inflammatory disease and poor clinical evolution during the course of the ocular inflammatory disease.

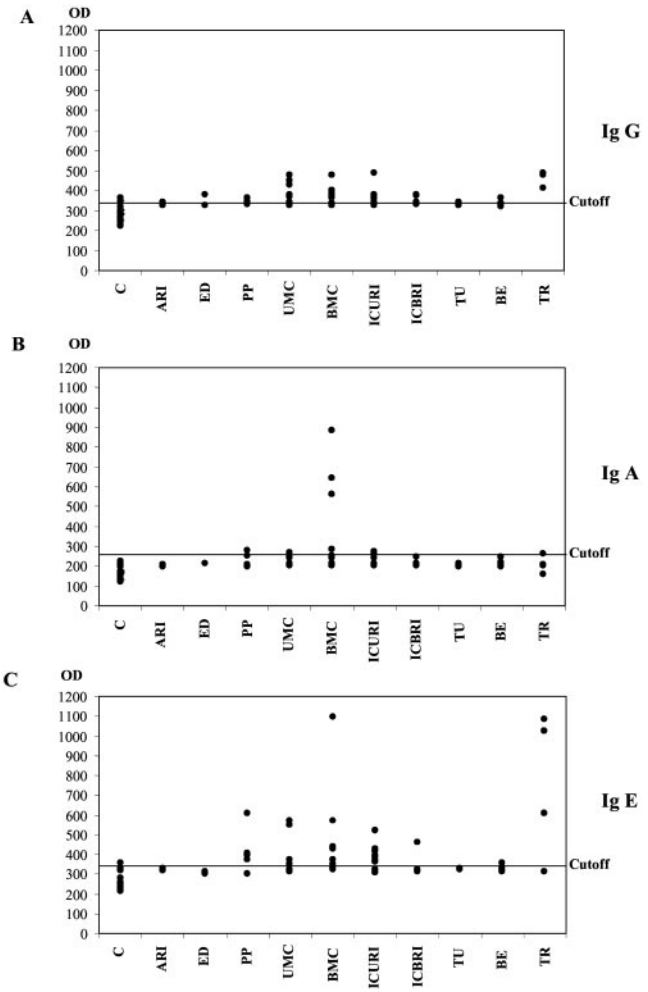

FIGURE 2. Anti-Gal-1 IgG, IgA, and IgE isotypes in subgroups of patients with AU or IU. The AU group was subdivided into acute recurrent iridocyclitis (ARI), Eales' diseases (ED), pars planitis (PP), unilateral multifocal choroiditis (UMC), bilateral multifocal choroiditis (BMC), idiopathic chronic unilateral recurrent iridocyclitis (ICURI), and idiopathic chronic bilateral recurrent iridocyclitis (ICBRI). Cases of IU were subdivided into TU, BE, and TR. Results were considered positive if optical density (OD) was two or more standard deviations above the mean in the control group (cutoff).

Circulating Anti-Gal-1 Antibodies in Bovine and Human Retina

To gain insight into the functional significance of anti-Gal-1 antibodies present in sera from patients with uveitis, we explored whether these antibodies may specifically recognize retinal structures. For this purpose, nitrocellulose filters were coated with human rGal-1 and exposed to sera from selected patients with uveitis (n = 13) and control subjects (n = 7). Bound antibodies were specifically eluted as described in the Materials and Methods section. Anti-Gal-1 IgG antibodies were detected by ELISA in nine of the eluted fractions (Fig. 4A), six of which strongly reacted with bovine retinal lysates (Fig. 4B). In contrast, IgE anti-Gal-1 antibodies were detected in nine of the eluted fractions (Fig. 4A), all of which immunoreacted with bovine retinal lysates (Fig. 4B). Finally, IgA anti-Gal-1 antibodies were detected in four of the eluted fractions (Fig. 4A), but only one fraction reacted against bovine retina (Fig. 4B). No signs of immunoreactivity against rGal-1 or bovine retina were detected using fractions eluted from control sera (data not shown).

To confirm these findings, we analyzed IgG immunoreactivity of eluted fractions by Western blot. Figures 4C and 4D show the immunoreactive profile of eluted anti-Gal-1 antibodies from two representative sera with high immunoreactivity against rGal-1 (Fig. 4C; lanes 2 and 3) and bovine retinal lysates (Fig. 4D; lanes 2 and 3). Controls using the rabbit anti-Gal-1 polyclonal antibody are shown in Figures 4C and 4D (lane 1).

To determine whether the circulating anti-Gal-1 antibodies can recognize specific retinal structures, sections of human retinal tissue were exposed to eluted antibodies. As shown in Figures 4E and 4F, anti-Gal-1 antibodies eluted from sera from patients with AU demonstrated widespread immunostaining along all retinal layers, particularly at the level of photoreceptors (Figs. 4E, 4F for IgG and IgE, respectively). Eluted anti-Gal-1 antibodies preadsorbed with rGal-1 were used as the control for specificity and did not show any immunoreactivity (Figs. 4E, 4F, insets). As expected, eluted material from control individuals did not show significant immunoreactivity against retinal sections (Figs. 4G, 4H for IgG and IgE, respectively).

DISCUSSION

Uveitis is a generic term that encompasses a variety of intraocular inflammatory responses of infectious or autoimmune origin.[1–3] Despite considerable progress in elucidating the immunopathogenesis of these ocular disorders, there is still scarce information about reliable immunologic markers of disease evolution.[26] In this context, no significant differences in the levels of anti-retinal IRBP antibodies were detected between sera from patients with uveitis and healthy control subjects,[26] although evidence has been demonstrated regarding the presence of IgG and IgE autoantibodies to retinal S antigen in patients with uveitis.[24]

In the present study, we report the occurrence of anti-Gal-1 antibodies in sera from patients with AU or IU compared with healthy control subjects. Of note, the frequency and levels of anti-Gal-1 IgE antibodies correlated with poor evolution of

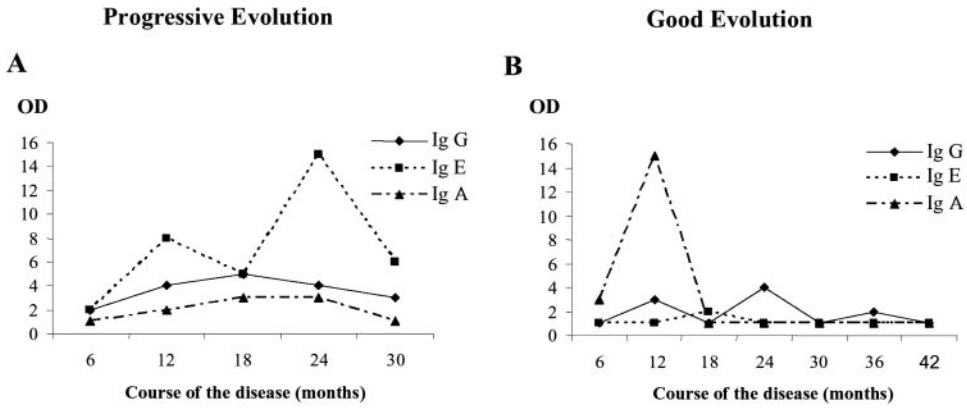

FIGURE 3. Anti-Gal-1 antibodies are associated with progression and poor outcome in patients with uveitis. Longitudinal analysis of anti-Gal-1 antibodies (ELISA) was performed in two polar representative sera from age-matched patients with progressive disease (**A**) or good evolution (**B**). Both patients were treated in the same protocols. A total of 21 sera samples from patients with uveitis who had progressive disease and 9 from patients with good evolution were analyzed and had profiles comparable to those shown.

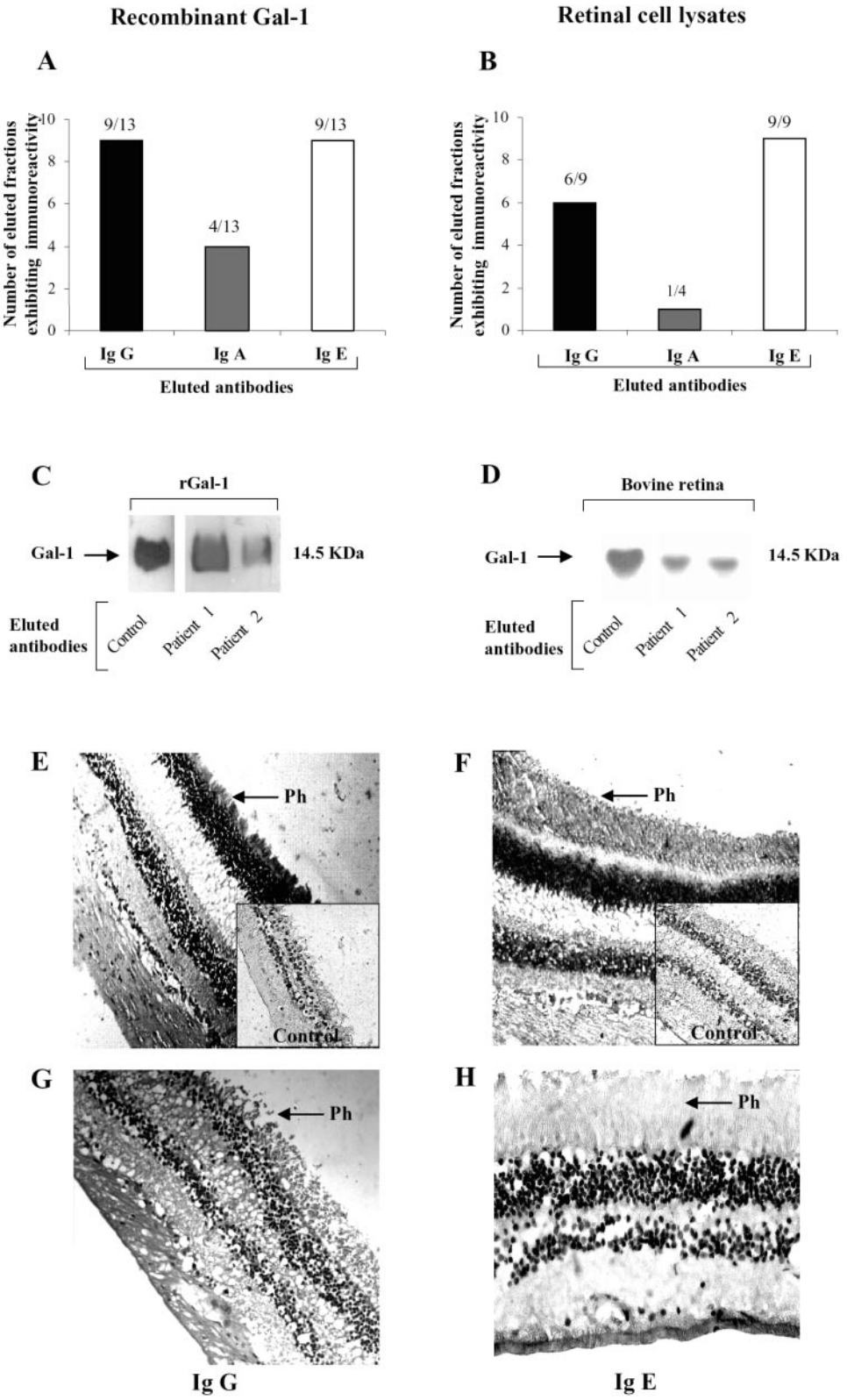

FIGURE 4. Immunoreactivity of anti-Gal-1 antibodies against retinal tissue. Eluted anti-Gal-1 antibodies from patients' sera were assayed against rGal-1 (**A**, **C**) and retinal lysates (**B**, **D**) by ELISA (**A**, **B**) and Western blot (**C**, **D**). IgG, IgA, and IgE isotypes were determined by ELISA, and IgG immunoreactivity was confirmed by Western blot. (**E–H**) Sections of human retina were exposed to anti-Gal-1 antibodies eluted from sera of patients with uveitis (**E**, **F**) or healthy control subjects (**G**, **H**). Bound antibodies were detected using peroxidase-labeled goat anti-human IgG (**E**, **G**) and IgE (**F**, **H**). *Arrow*: photoreceptors layer (Ph). Eluted antibodies preadsorbed with rGal-1 were used as the immunoreactivity control (*insets*).

ocular inflammatory disease in patients with uveitis. IgE and IgG anti-Gal-1 antibodies specifically immunoreacted with Gal-1 in retinal cell lysates and recognized retinal structures, mainly photoreceptors, in sections of human retinal tissue.

Some alternative nonexclusive hypotheses could be postulated to explain the increased frequency of anti-Gal-1 antibodies in inflammatory ocular disorders. First, the occurrence of these antibodies might be a direct consequence of the release of tissue-associated Gal-1 during the inflammatory response accompanying the ocular disease. However, these anti-Gal-1 antibodies could also appear in response to autoimmune or infectious insults and may contribute to the breakdown of immune tolerance and privilege by blocking functionally active sites of retina-associated Gal-1.

Because of its ability to inhibit T-cell effector functions, it has been speculated that endogenous Gal-1 expression in the eye[7–9] could function as a novel mechanism of immune privilege. In the mammalian retina, Gal-1 is preferentially expressed by the retinal pigment epithelium, photoreceptors, and the outer limiting membrane.[7] Ishida et al.[8] demonstrated that cells from retinal pigment epithelium suppress T-cell activation, at least in part, through Gal-1-mediated mechanisms. Accordingly, we hypothesize that Gal-1 may also be secreted to the aqueous humor as an immunosuppressive cytokine,[4] where it may endow T cells with the capacity to regulate the inflammatory response. This hypothesis, which warrants further investigation, is consistent with our recent findings demonstrating that Gal-1 suppresses experimental autoimmune retinal disease in a murine model by promoting a shift toward a T-regulatory response (Rabinovich et al., manuscript submitted).

To counteract inflammation, the eye is confined to an immunosuppressive microenvironment that uses multiple mechanisms to suppress the activation and cytotoxic activity of T cells.[3] Some of these mechanisms involve soluble factors found in aqueous humor, which suppress the generation of pathogenic effector T cells and the production of proinflammatory cytokines.[3] In this context, the presence of anti-Gal-1 antibodies recognizing retinal structures may play a potential role in blocking the immunoregulatory activity of this protein and promoting an increased survival of pathogenic T cells.

In addition to the potential impact of these antibodies in the modulation of immune tolerance, anti-retinal Gal-1 antibodies may also have implications in the modulation of retinal architecture. In this regard, Uehara et al.[7] reported retinal detachment and vacuolation of the outer plexiform layer when an anti-Gal-1 antibody was injected intravitreously in rat eyes, suggesting that endogenous Gal-1 may also be involved in the adhesion of photoreceptors and outer plexiform layers by interacting with specific glycoconjugates.[7] Accordingly, in the present study, sera from patients with AU showed high levels of specific IgE and IgG anti Gal-1 antibodies that were found to be clinically associated with severe impairment and loss of vision. It is noteworthy that IgE was the most frequently detected isotype in sera from patients with poor clinical outcome and that patients with good evolution did not show IgE immunoreactivity. The occurrence of high levels of IgE autoantibodies in autoimmune disorders has been reported in clinical settings and experimental models, including uveitis, thyroiditis, and chemically induced autoimmunity.[24,27–29] However, the role of IgE in the development and/or perpetuation of autoimmune response remains to be elucidated.

The occurrence of anti-Gal-1 antibodies has been described in inflammatory and neurologic processes,[30] including acute and chronic stages of *Trypanosoma cruzi* infection.[25] In this regard, we found in the present study that a greater proportion of anti-Gal-1 antibodies observed in patients with IU corresponds to infections caused by the protozoan *Toxoplasma gondii*. This observation is worthy of discussion in terms of previous findings describing the presence of a galactose-binding protein (with significant amino acid sequence homology to Gal-1) in tachyzoites of a virulent *T. gondii* strain.[31] In this sense, it should be emphasized that TR is different from other types of IU in that it is strikingly similar to AU. We and other investigators recently highlighted the importance of autoantibodies against retinal antigens in determining disease severity in toxoplasmosis uveitis.[24,32] In this regard, we have demonstrated in an experimental model of arthritis induced by *T. gondii* the presence of autoantibodies against retinal S antigen, iris antigens, type-II collagen, and proteoglycans, which positively correlated with clinical manifestations of arthritis and iridocyclitis.[33] In addition, autoantibodies against other members of the galectin family (Gal-3 and Gal-9) have been detected in physiological and pathologic settings, including colon carcinoma, Crohn's disease and systemic lupus erythematosus.[34–37]

In conclusion, our results highlight the clinical importance of specific anti-retinal Gal-1 antibodies in sera from patients with AU or IU, indicating their potential prognostic use in follow-up observation of inflammatory ocular diseases.

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