Anti-Rhodopsin Antibodies in Sera From Patients With Normal-Pressure Glaucoma

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Purpose. To explore further the potential role autoimmunity may play in the pathogenesis of normal-pressure glaucoma (NPG) in some patients, the authors examined the sera of patients with NPG for the presence of antibodies directed toward retinal antigens.

Methods. Using patient sera, immunoblotting was performed on subcellular fractions of retina, purified bovine rhodopsin, and immunoaffinity-purified recombinant human rhodopsin. A chemiluminescence–enzyme-linked immunosorbent assay (ELISA) to detect anti-rhodopsin antibodies was developed and used.

Results. A patient with NPG was found to have a high titer of immunoglobulin M antibody against a 40-kd retina-specific glycoprotein antigen subsequendy identified as rhodopsin. ELISA analysis conducted on sera from 28 patients with NPG and 26 patients with primary open-angle glaucoma (POAG) revealed highly significant differences in anti-rhodopsin antibody activity between these groups (P < 0.0002, Mann–Whitney test). For example, the majority of patients with NPG (19/28; 68%) had anti-rhodopsin antibody activity higher than the highest value obtained from among 26 age-matched patients with POAG.

Conclusions. An elevated anti-rhodopsin antibody count is related to NPG. This may indicate that there is an autoimmune component in the optic neuropathy in these patients. The specific role of these autoantibodies, if any, in the pathogenesis of the disease remains to be determined. Invest Ophthalmol Vis Sci. 1995;36:1968–1975.

Open-angle glaucoma (OAG), the second leading cause of irreversible blindness in the United States, is generally characterized by progressive optic atrophy and loss of peripheral visual sensitivity and is comprised of two major syndromes. In primary OAG (POAG), which affects approximately 0.5% of the American population and is prevalent in 1.3% of white and 4.7% of black Americans over the age of 40 years (1.6 million persons), the presence of optic nerve damage is highly correlated with chronic elevation of intraocular pressure. However, a surprisingly high percentage of patients with OAG have ophthalmologic findings identical to those in POAG except that intraocular pressure has never been demonstrated to be elevated. Several large population-based studies have documented the high prevalence of this form of glaucoma, called “normal-pressure” or “low-tension” glaucoma, and it has been estimated that on an adjusted case-ratio basis, as many as 50% of patients with OAG may indeed have normal-pressure glaucoma (NPG). Even by the conservative estimate that NPG represents 16% to 20% of OAG, NPG is a relatively common disorder.

Recently, Cartwright et al established an association between NPG and immune-related disease by identifying a 30% incidence of autoimmune disorders by epidemiologic criteria in patients with NPG. They speculate that the immune system may inflict damage to the optic nerve, or its vessels, by attacking antigens that have cross-reactivity with a primary antigenic stimulus related to the patient’s immune-related disorder (e.g., arthritis, hypothyroidism). Additional evidence
that supports the possibility that an autoimmune mechanism may underlie the optic atrophy in many patients with NPG is the recent finding of monoclonal paraproteinemias (M-proteins) in 18%, and autoantibodies to extractable nuclear antigens in 30%, of patients with NPG who were studied prospectively. M-proteins reflect the expansion of a B-cell clone secreting large amounts of a particular monoclonal antibody into the serum. No paraproteins in an age-matched cohort of patients with POAG were identified, and the incidence of antibodies to extractable nuclear antigens in the NPG group was 10 times that found in the control group of patients with POAG.

A hypothesis based on these results is that some NPG may have a humoral immune component with the autoimmunity directed toward antigens of the retina, optic nerve, or both. We have begun to examine the sera of patients with NPG for the presence of autoantibodies against retinal antigens. We have found that patients with NPG have a higher titer of anti-rhodopsin antibodies than patients with POAG. Although it is not at all clear that this correlation indicates a role for anti-rhodopsin antibodies in the pathogenesis of NPG, it does support the novel hypothesis that autoimmune mechanisms may play a role in this disease, and it points the way to new approaches to the investigation, diagnosis, and treatment of this devastating disorder.

**METHODS**

**Patient Criteria**

All patients with glaucoma (both POAG and NPG) were evaluated initially by MBW in the ophthalmology outpatient clinic at Barnes Hospital between January 1991 and August 1994. Normal-pressure glaucoma is defined by the clinical constellation of open angles, normal intraocular pressure (on at least three separate visits), optic nerve damage, and subsequent visual field loss that is progressive in nature and ultimately may impair central vision. Inclusion criteria for patients with normal-pressure glaucoma are no evidence of intraocular pressure greater than 23 mm Hg, progressive changes in either visual fields or optic nerve cupping, and absence of alternative causes of optic neuropathy (i.e., meningeal disease, infections such as syphilis). Inclusion criteria for patients with primary open angle glaucoma are evidence of intraocular pressure greater than 23 mm Hg, progressive visual field loss or optic nerve cupping, and absence of alternative causes of optic neuropathy (i.e., meningeal disease, infections such as syphilis).

The enzyme-linked immunosorbent assay (ELISA) (see below) was performed on sera from 28 patients with NPG (21 women, 7 men; 26 white, 2 black) with an age of 69.7 ± 1.4 years (mean ± SEM) and 26 patients with POAG (15 women, 11 men; 24 white, 2 black) with an average age of 73.3 ± 1.7 years. Ages of the two groups were not statistically different (P > 0.05, t-test). The degree of glaucomatous visual field loss of NPG and patients with POAG was ascertained from the mean decibel loss on automated perimetry using the Humphrey (San Leandro, CA) instrument, the 30-2 program, and a size III stimulus. For patients with NPG, 48/56 eyes (86%) available for data analysis had a mean loss of 11.6 ± 1.0 dB (mean ± SEM). For patients with POAG, 45/52 eyes (86%) available for data analysis had a mean loss of 17.0 ± 1.2 decibels (mean ± SEM). The difference in visual field loss between the two groups was statistically significant (P < 0.001, t-test).

The tenets of the Declaration of Helsinki were followed in all experiments involving humans. Informed consent was obtained from all participants, and blood was drawn in concordance with a protocol approved by the Institutional Review Board.

**Western Blot Analysis**

Bovine retinas were dissected from eyes obtained at a local abattoir or purchased frozen (Pel-Freez, Rogers, AR), human eyes were donated by the MidAmerica Eye and Tissue Bank (St. Louis, MO), and rats were adult Sprague-Dawley (Harlan, Indianapolis, IN). All experimental and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinas (and other tissues) were homogenized in ice-cold lysis buffer containing 2 mM HEPEs, 2 mM ethylenediaminetetraacetic acid, pH 7.4, and protease inhibitors (50 μM phenylmethyl sulfonyl fluoride and 1 μg/ml each of aprotinin, antipain, bacitracin, bestatin, chymostatin, leupeptin, pepstatin A). After centrifugation at 1000g for 10 minutes, the pellet (consisting of nuclei and unbroken cells) was discarded and the membrane fraction was pelleted by centrifugation at 35,000g for 20 minutes. The supernatant, or soluble fraction, was saved, and the membrane pellet was washed twice more in lysis buffer. Fractions were stored at −80°C until use. The protein concentrations in the membrane and soluble fractions were determined using the BCA method (Pierce, Rockford, IL).

Proteins were separated by electrophoresis in 12% sodium dodecyl sulfate polyacrylamide gels (SDS–PAGE) and electrophoretically transferred to polyvinyl membranes (Millipore, Marlboro, MA) in a BioRad MiniTrans Blot Apparatus (BioRad, Hercules, CA). After transfer, membranes were incubated in TTBS (50 mM Tris HCl, 154 mM NaCl, 0.1% Tween-20, pH 7.5) containing 2.5% nonfat dry milk for 15 minutes, then overnight in the same buffer containing a dilution of patient serum and sodium azide (0.1%
wt/vol). After several washes in TTBS, the membranes were incubated in TTBS–2.5% milk containing goat anti-human γ, μ, κ, or λ chain coupled to horseradish peroxidase (2000/1, Fisher) for 2 hours. After several washes in TTBS, immunoreactive bands were visualized by enhanced chemiluminescence using commercial reagents (products from Amersham [Arlington Heights, IL] and NEN [Boston, MA] gave comparable results).

Glycosidase Treatment
Retinal membranes were dissolved using 1% SDS in 50 mM potassium phosphate, pH 7.0. After centrifugation in a microfuge, the supernatant was diluted 10-fold in 50 mM potassium phosphate, pH 7.0, containing the nonionic detergent Nonidet P40 (Pierce), 1%. Peptide N-glycosidase F (Boehringer–Mannheim, Indianapolis, IN) was added, and the reaction proceeded for 18 hours at 37°C. Aliquots of the mixture were analyzed by Western blot analysis as above.

Chemiluminescence ELISA for Anti-rhodopsin Antibodies
Bovine rhodopsin (50 ng) in 50 μl carbonate buffer (50 mM sodium carbonate, pH 8.8) was added to each well of 96-well black plastic microtiter plates (Packard Instruments, Meriden, CT), and the buffer was permitted to evaporate overnight. Each well was then filled with wash buffer (50 mM sodium phosphate–0.5 M NaCl, pH 7.2) containing 3% normal goat serum and 0.1% sodium azide and incubated at 37°C for at least 6 hours. Sister plates prepared without rhodopsin were treated identically. Patient sera diluted 1:5000 in phosphate-buffered saline (PBS, 50 mM sodium phosphate, 150 mM NaCl, pH 7.4) plus 1% normal goat serum and azide were added to wells of rhodopsin-coated plates and control plates and incubated overnight at 4°C. The primary antibody was removed by washing twice with PBS and once with distilled water, then secondary antibody (goat anti-human IgM coupled to HRP, 1:2000) was added. After a 1-hour incubation at room temperature, the secondary was washed out. The chemiluminescence reagents were added, and the plate was counted immediately in a TopCount plate reader (Packard Instruments, Meriden, CT). The difference in counts between the rhodopsin and the control plate reflects specific anti-rhodopsin antibody binding. For individual experiments, sera were assayed in duplicate wells, and the data presented are means of two independent experiments.

RESULTS
Sera from several patients with M-proteins and NPG were used to probe Western blots of retinal proteins. Various immunoreactive bands were observed, several of which seemed to be present in many of the patients (not shown). The serum from one of these patients (MW, a 68-year-old white woman), provided a robust signal on Western blots and was selected for further biochemical studies aimed at antigen identification. The selection of this patient's sera was based not only on the strength of the signal provided on the Western blot but also on the fact that this autoantibody was an immunoglobulin M (IgM, see below). In some cases, IgM M-proteins play a pathogenic role in producing polyneuropathy, and the antigenic targets of IgM M-proteins are more readily identified than the targets of IgA or IgG M-proteins.11

A 40-kd band was labeled in a membrane, but not supernatant, fraction prepared from bovine retina (Fig. 1A) using serum from this patient. This band was labeled using a secondary antibody selective for human IgM (anti-μ heavy chain) or human λ light chains, but not labeled with secondary antibody against human IgG (anti-γ heavy chain) or human κ light chains (data not shown).

This band was not labeled using any of several control human sera (Fig. 1A). Using 20 μg of retinal membrane protein, the 40-kd band was observed at patient serum dilutions up to and including 1:300,000. This serum labeled a similar band in human (Fig. 1B), rat (Fig. 2), and frog (Fig. 4A), but not chick embryo (not shown) retinal tissue.

The 40-kd antigen was specifically present in the
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**FIGURE 2.** The 40-kd antigen is retina specific. Membrane fractions (40 μg protein) prepared from rat retina (R), cerebellum (Cb), cerebrum (Cr), heart (H), kidney (K), liver (Lv), lung (Lg), and spleen (S) were subjected to Western blot analysis as described in the legend to Figure 1, using serum from patient MW at 1:2000. The 40-kd antigen (arrow) is greatly enriched in retina.

Although a very faintly labeled 40-kd band was found in membranes prepared from rat cerebrum and cerebellum, no robust labeling was found in heart, kidney, liver, lung, or spleen (Fig. 2; the faint 55-kd bands present in the heart, kidney, and liver lanes were not visible when the sera was diluted 1:10,000). No labeling of membrane proteins from human cortical white or gray matter was found (not shown). These results suggested that the 40-kd antigen is expressed specifically in retina and that this patient does not have additional high-titer serum autoantibodies directed at organs other than retina that can be detected by Western blot analysis.

To characterize further the antigen and the epitope(s) bound by the autoantibody, bovine retinal membranes were incubated with peptide N-glycosidase F before Western blot analysis. This caused a decrease in the apparent molecular weight of the antigen to 35 kd (Fig. 3). These results indicate that the 40-kd antigen is a glycoprotein containing a large N-linked carbohydrate and the epitope recognized by the antibody resides on the protein, not the carbohydrate, portion of this molecule.

When a detergent extract of retinal membranes was fractionated on a chromatofocusing column and the fractions were analyzed by SDS–PAGE and Western blot analysis, it was apparent that the 40-kd antigen comigrated with a major 40-kd protein detectable by silver staining (not shown). This suggested that the 40-kd antigen may be an abundant retinal protein. Two abundant retinal proteins have apparent molecular weights of 40-kd on SDS–PAGE, rhodopsin, and transducin. Because rhodopsin is a glycoprotein whereas transducin is not, rhodopsin appeared to be the best candidate antigen. Bovine rhodopsin, purified by subcellular fractionation and lectin-affinity chromatography, was recognized by the serum (Fig. 4A). An anti-rhodopsin antibody provided an identical pattern of labeling as the patient serum on a Western blot containing pure rhodopsin, bovine retinal membranes, and outer segments prepared from frog retina (Fig. 4A).

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**FIGURE 3.** The 40-kd retinal antigen is a glycoprotein. Bovine retinal membranes (13 μg/lane) were solubilized and incubated overnight at 37°C in the presence (+) or absence (−) of peptide N-glycosidase F (2 U/ml; Boehringer), then subjected to Western blot analysis as described above. Serum from patient MW was used at 1:2000. After incubating in the presence of the enzyme, the immunoreactive band migrated at an apparent molecular weight of 35 kd.

**FIGURE 4.** Serum from patient MW recognizes rhodopsin. (A) Bovine retinal membranes (brm, 6 μg), pure bovine rhodopsin (rho, 0.5 μg), and frog rod outer segments (fros, 20 μg) were subjected to Western blot analysis and probed with serum from patient MW (1:2000) or anti-bovine rhodopsin antisera raised in rabbit (1:1000). (B) Recombinant human rhodopsin (R-rho, 2 μg) and bovine retinal membranes (brm, 15 μg) were both recognized by serum from patient MW (1:2000).
glycoprotein that potentially contaminates preparations of rhodopsin biochemically purified from retina, we used recombinant human rhodopsin expressed in COS cells and purified by immunoaffinity chromatography on a monoclonal antibody column. No other retinal proteins are present in this starting material. The patient serum robustly labeled the recombinant rhodopsin (Fig. 4B). To the best of our knowledge, this is the first description of a human anti-rhodopsin antibody.

To determine whether these results were generalizable to other patients with NPG, sera from 28 patients with NPG and 26 patients with POAG were examined for anti-rhodopsin antibodies using a specific ELISA. By several criteria, patients with NPG have significantly higher levels of anti-rhodopsin antibody than patients with POAG: 19/28 patients with NPG have values higher than the highest value obtained from among the patients with POAG (P < 0.0002; Mann-Whitney test), and 13/28 (46%) have values higher than twice the highest values obtained from the patients with POAG; the mean levels of anti-rhodopsin antibody activity are significantly different (POAG, 543 ± 59; NPG, 2883 ± 527 cpm; P < 0.01, t-test). The individual values are shown in Figure 5. Interestingly, but perhaps expectedly, the highest activity was provided by patient MW (Fig. 5).

Eight of the highest activity NPG sera were examined for their reactivity against 1 μg of purified rhodopsin on Western blots. At a dilution of 1:2000, four of these clearly labeled the 40-kd band. The Western blots from four of the eight—three positive and one negative—are shown in Figure 6.

**DISCUSSION**

Normal-pressure glaucoma, a common disease with devastating peripheral and central visual loss, is a progressive optic atrophy with no known pathophysiology or well-accepted, efficacious therapy. Our suspicion that autoimmunity may play a role in the pathogenesis of NPG has led us to pursue the methods by which several subgroups of neuropathies, thought to be immune-mediated, have been studied. One method of defining such syndromes has been to study their association with serum antibodies directed against neural glycolipids or proteins. Patients with NPG in whom anti-retina antibodies can be identified may serve as index patients to facilitate identification of retinal autoantigen(s) using Western blot analysis or other techniques. We identified a high titer of antibodies against a retina-specific 40-kd antigen in a patient with NPG. On subsequent analysis, the antigen was identified as rhodopsin.

The presence of anti-rhodopsin antibodies in this patient with NPG is unlikely to signify a generalized immune reaction to damaged photoreceptors. First, antibodies to other, highly immunogenic components

**FIGURE 5.** Sera from patients with NPG show high anti-rhodopsin antibody activity. Sera from 28 patients with NPG and 26 patients with POAG was examined in a chemiluminescence-based enzyme-linked immunosorbent assay for the presence of anti-rhodopsin immunoreactivity. Each bar represents an individual patient. NPG = normal-pressure glaucoma; POAG = primary open-angle glaucoma.

**FIGURE 6.** Sera from some, but not all, patients exhibiting high anti-rhodopsin antibody activity in the enzyme-linked immunosorobent assay recognize rhodopsin on Western blots. Rhodopsin (1 μg) was recognized in Western blots by sera from 3 of 4 patients shown. All four show high activity (>2500 cpm) in the enzyme-linked immunosorbent assay.
of photoreceptors, such as retinal S-antigen, are not present in this serum. Moreover, the anti-rhodopsin antibodies are of a single heavy-chain and single light-chain type. This suggests that a specific clonal expansion of antibody-producing cells, not a generalized immune reaction, has occurred. Despite the presence of anti-rhodopsin antibodies, there is no evidence of diffuse inflammatory disease, such as uveoretinitis. (Experimental autoimmune uveoretinitis can be elicited in animals by immunizing with rhodopsin and adjuvant and is accompanied by the presence of anti-rhodopsin antibodies.)

Last, there is no electrophysiological evidence of photoreceptor disease: Patient MW had normal electroretinogram results (data not shown).

It is interesting that the high-titer, anti-rhodopsin antibody detected in the serum of patient MW by Western blotting is an IgM-κ, whereas the paraprotein present in high concentration in her serum and detected by immunofixation is an IgM-κ. Western blot analysis using chemiluminescence for visualization of bands is much more sensitive than the immunofixation technique used in the clinical laboratory for detection of paraproteins. These results suggest there is a biclonal gammopathy in this patient. We have not identified the antigen the IgM-κ is directed against. We see no evidence for any IgM-κ anti-retinal protein antibodies in our Western blot analysis. However, the IgM-κ antibody may be directed against a retinal antigen, but this interaction may not be detectable by Western blot analysis. For example, the harsh conditions necessary for sample preparation (e.g., denaturation in 2% SDS, reduction with 20 mM dithiothreitol) may destroy sensitive features of the epitope; the antibody–antigen interaction may be a low-affinity one; the antigen may be present in low amounts and, hence, may be below the level of sensitivity; and the antigen may not be a protein (e.g., it may be a glycolipid). Among the other patients with NPG with highest activity of anti-rhodopsin antibodies on ELISA, only a fraction recognized rhodopsin in Western blot analysis. This may have resulted from the harsh treatment of the protein necessary to prepare it for electrophoresis.

The significant difference in anti-rhodopsin antibody activity in sera from patients with NPG versus patients with POAG is consistent with the finding that clinical differences exist in the manifestation of visual field loss and optic nerve atrophy in NPG compared with POAG. For example, the visual field scotomas in NPG are closer to fixation, have steeper slopes, and appear to represent more localized retinal damage than those of POAG. Although it is not clear what the implications of these observations are regarding the pathogenesis of glaucoma in either syndrome, the possibility that a novel mechanism of optic atrophy exists in patients with NPG is consistent with these reported differences. The difference in anti-rhodopsin antibody activity between these two groups is, therefore, the first significant retinal biochemical parameter that differentiates NPG and POAG.

Could antibodies to a photoreceptor antigen be involved in atrophy of the nerve fiber layer? The answer is unclear, but it seems unlikely that these antibodies are of direct pathogenic significance. However, considering the higher levels of anti-rhodopsin antibodies in the sera of patients with NPG, some speculation concerning a possible pathogenic role for these antibodies is warranted. It may be relevant that patients with NPG have significantly greater peripapillary atrophy of the retinal pigment epithelium than patients with POAG or patients with ocular hypertension.

This focal defect in the blood–retina barrier may provide circulating elements of the immune system access to retinal antigens. Perhaps a combination of localized anatomic abnormalities and immunologic predisposition (e.g., elevated circulating complement) in some patients results in functional damage, hence, glaucomatous optic neuropathy. We have no direct evidence to support this hypothesis. However, clinical studies have identified an estimated twofold to sixfold increase in peripapillary nerve fiber layer hemorrhages in patients with NPG (versus POAG), which may signify a focal autoimmune vasculitis that results in nerve fiber layer infarction and, ultimately, glaucomatous optic neuropathy.

These possibilities aside, it seems more likely that the anti-rhodopsin antibodies are not directly pathogenic. Nonetheless, the presence of these antibodies signifies a possible difference in humoral immunity between patients with NPG and patients with POAG. The data presented here, and in previous studies, buttress the hypothesis that there is an immunologic component to the disorder in some patients with NPG that may underlie their visual loss.

The identification of neuropathy-related antibodies is important from clinical and research points of view. Testing for serum antibodies in the clinical evaluation of neuropathy syndromes is now widely practiced. Antibodies and their patterns of cross-reactivity provide diagnostic markers for some neuropathies that are probably immune mediated and treatable. In other cases, such as Guillain–Barré-like syndromes, antibody measurement may give diagnostic and prognostic information. In multifocal motor neuropathy, antibody titers are a helpful guide to necessary and sufficient doses of immunosuppressive agents used for treatment. Finally, determination of antibody specificity and cross-reactivity also yields clues to the neural targets of the immune processes in neuropathies and motor neuron disorders. By analogy to these immune-mediated neuropathies, the identification of el-
evated anti-rhodopsin antibodies in patients with NPG may be similarly useful.

The hypothesis that autoimmune mechanisms contribute to the pathogenesis of NPG has implications for the investigation, diagnosis, and treatment of this disorder. Additional studies aimed at finding autoantibodies, toxic cytokines, or other components of the humoral immune system that may be responsible for ganglion cell dysfunction and death should be performed. It may be useful to monitor patients with NPG for anti-rhodopsin and other autoantibodies as a potentially useful means of classification, as is done for the autoantibodies of Guillain–Barré-like syndromes. Furthermore, therapeutic strategies designed and used for the treatment of autoimmune disorders should be examined for efficacy in NPG.

Key Words
autoimmune response, immunoglobulin, immunopathology, low-tension glaucoma, rhodopsin

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References


