Epitope Mapping of Anti-Rhodopsin Antibodies from Patients with Normal Pressure Glaucoma

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Purpose. The presence of anti-rhodopsin antibodies in patients with normal pressure glaucoma (NPG) has been previously demonstrated with western blot analysis and enzyme-linked immunosorbent assay. To learn more about the characteristics, origin, and possible significance of these antibodies, the epitopic specificity of the anti-rhodopsin antibodies was examined in four NPG patients.

Methods. Antibodies in patient sera were assayed by western blot analysis against purified bovine rhodopsin. Peptides derived from particular segments of the rhodopsin sequence were tested for activity in competing for rhodopsin-antibody binding.

Results. Of a series of nine peptides that constitute most of the hydrophilic regions of rhodopsin, only one, consisting of the C-terminal 25 amino acids, prevented binding of the patient antibodies to rhodopsin. Higher resolution mapping using a set of dodecamers of overlapping sequences from the C-terminal region demonstrated that...
antibody binding is completely dependent on the last two amino acids. Removing the C-terminal alanine alone, or amidating the C terminus carboxyl group, also eliminated antibody binding.

**Conclusions.** Because four of four patient antibodies examined exhibited the identical epitopic specificity, it is likely that a common mechanism underlies their generation. This may indicate that molecular mimicry has occurred, because several pathogens contain similar C-terminal sequences. Although they may serve as diagnostic markers, and provide evidence that there is an autoimmune component in some patients with glaucoma, the role, if any, that these antibodies play in the pathogenesis of the disease remains unknown. *(Invest Ophthalmol Vis Sci. 1999;40:1275–1280)*

Glaucoma is no longer viewed simply as elevated intraocular pressure that damages the optic nerve. Studies have indicated that in a significant percentage of patients with glaucoma, intraocular pressure has never been demonstrated to be elevated. The prevalence of this form of the disease, often called “normal pressure” or “low tension” glaucoma, is conservatively estimated to be approximately 20% to 30% of patients with glaucoma.1,2 Apoptotic cell death has been proposed to mediate the glaucomatous process in both high and normal pressure forms of experimental and human glaucoma.3–6 Although neurodegenerative processes such as ischemia, excitotoxicity, neurotrophin insufficiency, and peroxynitrite damage have all been hypothesized to play a role in causing apoptotic glaucomatous ganglion cell loss, none of these mechanisms have been definitively linked to either the high or normal pressure form of the disease.

We have proposed that one form of normal pressure glaucoma (NPG) may represent an autoimmune neuropathy. Evidence that supports this includes immunoglobulin deposition in the ganglion cell layer observed in the postmortem examination of eyes from a patient with NPG,6 an epidemiologic association of immune-related disease in 30% of patients with NPG,7 and the presence of aberrant or elevated serum autoantibodies including monoclonal paraproteins; antibodies to DNA, RNA, and nuclear proteins; and antibodies to heat-shock proteins in patients with NPG.8,9 In addition, autoantibodies to small heat-shock proteins are elevated in patients with glaucoma, and these antibodies induce apoptosis in native human retina and immortalized retinal cells in culture.10

**Methods**

**Patients**

All subjects were treated in accordance with the tenets of the Declaration of Helsinki. Blood samples were taken after detailed consent was obtained from patients with NPG. Serum was prepared, heat inactivated, and stored at −70°C until use. The inclusion and exclusion criteria for these patients were described previously.11,12 Briefly, NPG consisted of the presence of open iridocorneal angles, no evidence of intraocular pressure greater than 23 mm Hg, glaucomatous changes in visual fields and optic nerve cupping, and the absence of alternative causes of optic neuropathy. Visual field loss of patients was evaluated with the Humphrey Field Analyzer, 30-2 program (Humphrey Instruments, San Leandro, CA). Our criteria for visual field abnormalities included a corrected pattern standard deviation (SD) with a P < 0.05 or a glaucoma hemifield test outside normal limits obtained with at least two reliable and reproducible visual field examinations.

**Peptide Synthesis**

Solid-phase peptide synthesis was performed using phenylacetyl amonomethyl resin using an Applied Protein Technology peptide synthesizer (model Pss 80; Cambridge, MA). tert-Bu-tyloxycarbonyl amino acids were coupled using diisopropyl-carbodiimide/yi-hydroxybenzotriazole, and the completed peptide was cleaved from the resin using liquid hydrogen fluoride.13 Peptides were purified by preparative high-performance liquid chromatography and were demonstrated to have the expected composition by amino acid analysis.

In some cases, peptides were obtained from commercial sources (Research Genetics, Huntsville, AL).

**Western Blot Analysis**

Patient sera were routinely tested for reactivity toward bovine rhodopsin because we had previously demonstrated that NPG patients have serum antibodies that recognize bovine rhodop- sin.11 There is 96% amino acid identity (99% identity or conservative substitution) between bovine and human rhodopsins. Bovine rhodopsin was prepared by chromatography on Con A Sepharose in dodecyl maltoside, based on the procedure of Litman.14 Some experiments used rhodopsin generously provided by Steven Fiesler (St. Louis University, St. Louis, MO).

Among the most interesting and perplexing associations of glaucoma and autoimmunity are our previous findings that autoantibodies to rhodopsin are elevated in patients with normal pressure compared with those with high pressure glaucoma or age-matched control patients.1,2 Glaucoma is traditionally considered to be a disease of ganglion cell layer loss, with little or no subjective symptoms or signs that suggest photoreceptor involvement. Therefore, although we considered the presence of elevated serum antibodies to rhodopsin a biochemical parameter indicating that the mechanism(s) of optic atrophy in NPG and high pressure glaucoma may be distinctly different, we were unable to conclude that elevated rhodopsin antibodies were of pathogenic importance. To better understand the origin and potential significance of anti-rhodopsin antibodies in patients with NPG, we performed epitope mapping to define in detail the immunogenic specificity of these antibodies.
Each gel lane contained 1 μg or 2 μg rhodopsin (determined using the BCA assay; Sigma Chemicals, St. Louis, MO).

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 Bio-Rad MiniTrans Blot Apparatus (Bio-Rad, 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 at least 15 minutes to block nonspecific protein adsorption to the membrane. Strips corresponding to individual gel lanes were cut out and used for incubation with patient sera in the presence or absence of competing peptides. For competition, peptides (50 μg/ml) were incubated with diluted antisera (usually 1:2000 final) for 1 hour before application to strips. Antibody containing solutions, including sodium azide (0.1% wt/vol) as a preservative, were added to the strips for overnight incubation. After several washes in TTBS, the membranes were incubated in TTBS/2.5% milk containing goat anti-human μ chain coupled to horseradish peroxidase (2000/1; Sigma) 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).

**RESULTS**

Antibodies that bind to macromolecules are actually directed to small structural features, or epitopes, that usually consist of short delimited regions of the primary sequence. Such antibodies will bind to small peptides containing the epitopic sequence. Therefore, to determine the epitopic specificity of antibodies directed against rhodopsin found in NPG patient sera, we incubated the sera with peptides derived from the rhodopsin sequence (Fig. 1) and determined whether the peptides prevented interaction with full-length rhodopsin immobilized on a western blot.

This strategy is illustrated in Figure 2A, using two mouse monoclonal antibodies directed against different parts of the rhodopsin sequence. Antibodies K16-107 and B6-30 are directed against the C- and N-terminal sequences of rhodopsin, respectively. Incubation of either antibody with its cognate peptide completely prevented binding to rhodopsin on the western blot. This is not a nonspecific inhibitory effect of either peptide, because they were completely ineffective at preventing the binding of the disparate antibody.

Nine peptides (p1-p9, Fig. 1) spanning the hydrophilic segments of the rhodopsin sequence were synthesized and tested for competition against sera from patient MW, a patient with anti-rhodopsin antibodies that provide a robust signal on western blot analysis. As shown in Figure 2B, only p9, consisting of the C-terminal 25 amino acids, prevented binding. This is strong evidence that the antibody present in this serum is nonspecific and directed toward the C-terminal region of rhodopsin.

Because individual epitopes are much smaller than 25 amino acids, the next experiment was aimed to determine which subset of residues in the C-terminal 25 were critical for antibody binding. A second set of peptides (sp1-sp8, Fig. 1) was synthesized. Each peptide was 12 amino acids in length as determined by sequences taken from the C-terminal region of rhodopsin, the first beginning at residue 323 (peptide sp1, amino acids 323-344) and the start of the following peptides serially incremented by 2 amino acids until the actual C terminus was included (sp8, amino acids 337-348).

When these peptides were incubated with sera from patient MW, only sp8 provided competition (sp1 and sp 8 shown in Fig. 3). These data indicate that the C-terminal two amino acids (proline-alanine, PA) are critical components of the epitope recognized by this antibody. Furthermore, all vertebrate opsins end with the sequence PA.

Because the last two amino acids were necessary for antibody interaction, we wanted to determine whether in fact the C-terminal amino acid was critical. Peptide “-1,” which is identical to peptide sp8 except that it lacks the C-terminal alanine, was synthesized and tested. This peptide did not compete for binding (Fig. 3B), indicating a requirement for the C-terminal alanine as a component of the epitope.

Because the data indicated that the C-terminal residue of rhodopsin was an important component of the epitope recognized by this antibody, we tested the possibility that the carboxyl group defining the C terminus may participate in the rhodopsin-antibody interaction. The C-terminally amidated anologue of sp8 was synthesized. This substitutes the uncharged amide for the negatively charged carboxyl. This peptide was unable to prevent antibody binding to rhodopsin (Fig. 3B), pointing to a critical role for the negatively charged C-terminal carboxyl group in antibody binding.

**FIGURE 1.** Schematic diagram of peptides derived from the sequence of bovine rhodopsin used as competitors for antibody binding in this study.
FIGURE 2. Specific competition of antibody binding to rhodopsin on western blot analysis by appropriate peptides. (A) Control antibodies of known specificity. Preincubation of monoclonal antibodies (mAb) directed against the N-terminal (B6-30) or C-terminal (K16-107) region of rhodopsin without (—) or with peptides derived from the N-terminal (p1) or C-terminal (p9) region prevented antibody-rhodopsin interaction only for the antibody directed toward the relevant portion of the sequence. Arrow indicates position of the rhodopsin monomer band. (B) C-terminal peptide competes for patient antibody-rhodopsin interaction. Serum from patient MW was preincubated with peptides of sequence derived from the hydrophilic portions of bovine rhodopsin. Only p9, containing the C-terminal 25 amino acids, prevented antibody-rhodopsin interaction.

We repeated all the experiments above using sera from three additional patients. In our experience, human sera usually exhibit a high background staining, visible on the blots shown in Figure 4. Nonetheless, the results of the peptide competition experiments were unambiguous and reproducible. All three patients had anti-rhodopsin antibodies of identical epitopic specificity as patient MW (Fig. 4).

Of the initial set of 9 peptides that span the whole structure, only p9, containing the C terminus, effectively competed for rhodopsin binding with each of the three sera (p1 and p9 illustrated in Fig. 4). Of the C-terminal 12 amino acid peptides, only sp8, the one containing the C terminus, was an effective competitor of each of the three sera (sp1, sp7, and sp8 illustrated in Fig. 4). These data indicate that, like the antibody from patient MW, each of these three patient antibodies was monospecific and directed toward the C terminus of rhodopsin.

When peptide competition was performed using the peptide that lacked the C-terminal alanine, or the peptide that had the amidated C terminus, no competition was seen against any of the three patient antibodies (Fig. 4). Therefore, the anti-rhodopsin antibodies present in the sera of these three additional NPG patients have an identical epitopic specificity to antibody from patient MW.

FIGURE 3. (A) The last two amino acids of rhodopsin are critically required for antibody binding. Eight small peptides derived from the C terminus (see Fig. 1 and text) were tested for their ability to compete for patient antibody binding to rhodopsin. Only sp8, which contains the last two amino acids, was an effective competitor (sp1, sp9, and the no peptide control, —, are shown). (B) The carboxyl terminus is required for patient antibody binding to rhodopsin. Removing the last amino acid (—1) or amidating the C-terminal carboxylic acid (am) renders sp8 an ineffective competitor (no peptide control, —). Lower and upper arrows indicate positions of rhodopsin monomer and dimer bands.

DISCUSSION

Our findings reveal that the serum autoantibodies in patients with NPG are directed toward the C terminus of the rhodopsin molecule. Because the competition by the C-terminal peptide was complete, it is highly unlikely that antibodies directed toward epitopes other than the C terminus contribute to the signal observed on the western blot analysis. Our results do not preclude the possibility that antibodies against conformationally dependent epitopes may also be present, but because our assay utilizes denatured rhodopsin they would not be detected. The peptide competition experiments demonstrate the critical importance of the presence of the terminal amino acid alanine and the terminal carboxylate group as a prerequisite for antibody recognition because the synthetic dodecamer sp8, minus either the terminal amino acid alanine, or with an amidated carboxyl-terminus, did not compete for rhodopsin binding.

The C terminus of rhodopsin is not the only, or even the most immunogenic, portion of the rhodopsin molecule, based
Additional patient sera contain anti-rhodopsin antibodies directed against the C terminus itself. In each of three patients, only the peptide containing the C-terminal carboxylic acid (sp8) effectively competes for antibody–rhodopsin binding. Other peptides tested were ineffective (illustrated for p1, sp1, and no peptide control, —). Additional patient sera contain anti-rhodopsin antibodies directed against the C-terminal region. In each of three patients, only the peptide containing the C-terminal carboxylic acid (sp8) effectively competes for antibody–rhodopsin binding. Removing the last amino acid (–1) or amidating the carboxylic acid (amide) prevents competition.

**Figure 4.** (A) Additional patient sera contain anti-rhodopsin antibodies directed against the C-terminal region. In each of three patients, only peptides containing the C terminus of rhodopsin (p9, sp8) effectively compete for antibody–rhodopsin binding. Other peptides tested were ineffective (illustrated for p1, sp1, and no peptide control, —). (B) Additional patient sera contain anti-rhodopsin antibodies directed against the C terminus itself. In each of three patients, only the peptide containing the C-terminal carboxylic acid (sp8) effectively competes for antibody–rhodopsin binding. Removing the last amino acid (–1) or amidating the carboxylic acid (amide) prevents competition.

For both sera and monoclonal antibodies to rhodopsin, the majority of antigenic binding sites were localized to the amino terminal half of the protein. This may indicate that in our patients the eliciting immunogen is unlikely to have been intact endogenous rhodopsin.

The rhodopsin antibodies present in our patients have remained detectable at approximately the same titers from the time of their earliest detection to the present, with a follow-up period of at least 5 years for the patients reported here. A potential explanation for the immunoreactivity observed to rhodopsin in patients with NPG may be molecular mimicry. A premise of molecular mimicry is that immune responses to infectious agents may generalize to native cellular proteins with similar epitope homology, resulting in serum antibodies that recognize these proteins. The hypothesis whereby infectious agents initiate aberrant autoimmune tissue-specific responses due to molecular mimicry between the infectious agent and native tissue proteins is well recognized and has been implicated in the development of numerous organ-specific autoimmune diseases. If mimicry were operative in some patients with NPG, antibodies made in response to exogenous rhodopsin in patients with NPG may be molecular mimicry.

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**References**

Mice Deficient in Inducible Nitric Oxide Synthase Are Susceptible to Experimental Autoimmune Uveoretinitis

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Purpose. Nitric oxide (NO) is an important mediator of inflammatory tissue damage. The present study addresses the question whether inducible nitric oxide synthase (iNOS), and consequently the ability to upregulate NO, is required to effect the pathogenesis of experimental autoimmune uveoretinitis (EAU) in mice.

Methods. Mice with a homologous disruption of the iNOS gene (iNOS KO) were evaluated for their ability to develop EAU and associated cellular responses after immunization with the interphotoreceptor retinoid-binding protein. EAU was determined by histopathology 21 days after uveitogenic immunization, and antigen-specific cellular responses were assessed by delayed type hypersensitivity and lymphocyte proliferation.

Results. iNOS knockout (iNOS KO) mice developed EAU with scores similar to wild-type mice and exhibited good cellular responses to the immunizing antigen.

Conclusions. A functional iNOS gene is not necessary for EAU pathogenesis. Therefore, upregulation of NO is not required to mediate autoimmune tissue damage in the eye.

Experimental autoimmune uveoretinitis (EAU) is a T-cell-mediated autoimmune disease model that targets the neural retina. EAU can be induced in mice, rats, and primates by immunization with retinal antigens or their fragments, or by the adoptive transfer of retinal antigen-specific Th1-like...