Autoreactive Antibodies and Loss of Retinal Ganglion Cells in Rats Induced by Immunization with Ocular Antigens

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PURPOSE. In an experimental autoimmune animal model, retinal ganglion cell (RGC) loss was induced through immunization with glaucoma-related antigens. The target of this study was to investigate the pathomechanism behind this decline and the serum antibody reactivity against ocular and neuronal tissues after immunization with glaucoma- and non–glaucoma-associated antigens.

METHODS. Rats immunized with optic nerve antigen homogenate (ONA) or keratin (KER) were compared to control rats (CO). Intraocular pressure (IOP) was measured, and the fundi were examined regularly. Four weeks afterward, cells were counted in retinal flat mounts. Retina, optic nerve, and brain sections from healthy animals and optic nerve sections from immunized animals were incubated with serum collected at different time points. The occurrence of autoreactive antibodies was examined. Signs of antibody deposits, microglia activation, and demyelination were sought in optic nerves of immunized animals. Brain sections were examined for abnormalities.

RESULTS. No IOP or fundus changes were observed. Animals immunized with ONA showed a significant cell loss compared with the CO group. Elevated autoreactive antibodies against retina, optic nerve, and brain were observed. Animals immunized with KER, despite their immunologic response against KER, demonstrated neither RGC loss, nor increased development of autoreactive antibodies. Optic nerve from animals immunized with ONA demonstrated antibody accumulation, glia activation, and demyelination. No such observations were made in the KER or CO groups. Brain sections were without pathologic findings.

CONCLUSIONS. Systemic autoimmunity against ocular and neuronal epitopes, mediated by accordant autoreactive antibodies, is involved in the inflammatory processes that cause RGC degeneration in this experimental animal model. (Invest Ophthalmol Vis Sci. 2011;52:8835–8848) DOI:10.1167/iovs.10-6889

Glaucoma is one of the most common causes of irreversible blindness worldwide. By 2020, approximately 80 million people affected by glaucoma are expected to be bilaterally blind.1 However, the pathogenesis of the disease is not fully understood. Elevated intraocular pressure (IOP) is still considered to be one of the most important risk factors, but cannot explain all cases of glaucoma.2

Today, many research groups support the theory that immunity plays an important role in glaucoma pathogenesis.3,4 Previous studies have shown that glaucoma patients develop antibody alterations against specific retina and optic nerve proteins.5 Despite these results, it is still unclear whether the changes in antibody patterns have a causal connection with glaucoma development or are epiphenomena of the disease. In vitro experiments have already shown that RGC survival can be impaired by the presence of exogenously applied antibodies—for example, those against heat shock proteins.6 In an attempt to further investigate in vivo the role of antibodies in glaucoma, we used an animal model in which retinal cytotoxicity can be provoked through antigen immunization. As this model uses no methods of direct damage of the retina or the optic nerve, such as chronic ocular hypertension,7 acute retinal ischemia,8 or optic nerve crush,9 it is suitable to analyze the role of the immune system during RGC death. A significant decline of RGCs in the retina of rats after immunization with specific ocular antigens (heat shock proteins) was recently presented by other studies.10,11 Wax et al.11 observed infiltration of activated T-cells on retinal flat mounts from immunized animals. Joachim et al.10,12 showed that immunization with heat shock proteins leads to RGC loss and causes alterations in the antibody profile of animals, including up- and downregulation of specific retinal proteins,13 similar to those observed in patients with glaucoma.

The main objective of this study was to analyze the development and the time course of antibody autoreactivity against ocular tissues (retina and optic nerve), as well as against central nervous system (CNS) tissues (brain) in animals, after immunization with glaucoma- or non–glaucoma-associated antigens. In addition, histologic examinations of brain and optic nerve sections were conducted to search for antibody accumulation and signs of a possible inflammatory process, such as demyelination, cellular infiltrates, and glia activation, allowing a deeper understanding of the pathophysiologic mechanism behind RGC loss.

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insight into the pathomechanism of the observed neuronal decline in retina in this model.

METHODS

Animals

Twenty adult male Lewis rats were obtained from Charles River (Sulzfeld, Germany). All animals were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the project was approved by the Animal Care Committee of Rhineland-Palatinate, Germany. The animals were kept in a 12:12 hour light–dark cycle environment under standardized conditions. Food and water were provided ad libitum to the rats as usual. Clinical examinations of all animals, including ophthalmic examinations, were performed daily.

Immunization

All animals were immunized with a single intraperitoneal injection containing the antigen together with incomplete Freund’s adjuvant and pertussis toxin (both Sigma-Aldrich, Munich, Germany), as described previously.14,15 Four weeks later, all animals were euthanized.

Animals were divided in four groups (n = 5 each). Two groups (ONA I and ONA II) were immunized with homogenate of bovine optic nerve. The animals in group ONA I received 8 mg homogenate plus 500 μL incomplete Freund’s adjuvant and 120 μL (3 μg) pertussis toxin. The animals in group ONA II were immunized with half this dose, consisting of 4 mg optic nerve homogenate, 250 μL incomplete Freund’s adjuvant, and 60 μL (1.5 μg) pertussis toxin. A third group (KER) received KER (MP Biomedicals, Solon, OH), an antigen currently not associated with glaucoma. The immunization contained 1 mg KER diluted in 500 μL incomplete Freund’s adjuvant together with 120 μL (3 μg) pertussis toxin. KERs are a family of fibrous structural proteins that form hard but unmineralized structures, such as hair and nails. Such proteins are contained mainly in the intracytoplasmic cytoskeleton of epithelial tissue and are essential in the maintenance of its mechanical stability and integrity.16 In the eye, only the corneal epithelium contains KERs in high quantities. KER 3 and 12 are expressed in all corneal epithelial cell layers. An exception is the limbus area, where only suprabasal cells are KER positive, whereas the basally located corneal stem cells are negative.17 Mutations in the genes of these KERs are responsible for Meesmann’s corneal dystrophy.18 The lack of KER 12 in genetically engineered mice results in a mechanically fragile, easily detachable corneal epithelium.19 For immunization in animals, KERs are used to obtain anti-KER sera or to specify and isolate anti-KER antibodies.20,21 Therefore, they have been shown to be effective immunogens. The CO group received 1 mL sodium chloride, together with 500 μL incomplete Freund’s adjuvant and 120 μL (3 μg) pertussis toxin.

Measurement of IOP

Rats were subjected to IOP measurement before immunization, as well as 2 and 4 weeks afterward. Measurements were conducted with a

| Table 1. IOP in the Experimental and Control Groups |
|----------------------------------|--------|--------|--------|
|         | Baseline | 2 Weeks | 4 Weeks |
| CO      | 12.8 ± 0.3 | 12.6 ± 0.3 | 12.4 ± 0.2 |
| ONA I   | 12.9 ± 0.2 | 12.2 ± 0.2 | 12.3 ± 0.1 |
| ONA II  | 12.7 ± 0.2 | 12.5 ± 0.2 | 12.6 ± 0.2 |
| KER     | 12.9 ± 0.2 | 12.7 ± 0.3 | 12.5 ± 0.2 |

IOP of all groups before immunization and 2 and 4 weeks afterwards. Data are expressed as the mean ± SEM.

Figure 1. (A) Intraocular pressure of all study groups before as well as 2 and 4 weeks after immunization. (B) Fundus photographs from a rat before and 2 weeks after immunization with optic nerve homogenate (ONA I).
hand-held tonometer (TonoPen; Medtronic, Basweiler, Germany), as described previously. During each examination, 10 measurements per eye were performed, and mean counts were calculated (Table 1; Fig. 1A).

**Funduscopy**

Fundus were examined directly after IOP was measured. During this procedure, the animals were anesthetized with gaseous isoflurane. The fundi were inspected and photographed through a binocular surgical microscope (Carl Zeiss, Jena, Germany). Images obtained at the three time points were compared at the end of the study (Fig. 1B).

**Retinal Flat Mounts and Cell Counts**

Eyes were enucleated and fixed in 4% paraformaldehyde solution (VWR, West Chester, PA), and retinal flat mounts were prepared. Cells were stained with cresyl blue according to standard protocols. After de- and rehydration by increasing and decreasing concentrations (70%–100%) of ethanol, respectively, the flat mounts were placed in distilled water and stained with 2% cresyl blue (Merck, Darmstadt, Germany). After staining, they were differentiated, dehydrated in ethanol, incubated in xylene, and fixed in quick-hardening mounting medium (Eukitt; Sigma-Aldrich, Munich, Germany). To quantify the different neurons in the superficial

![Retina flat mount after cresyl blue stain. Sixteen areas were photographed per flat mount at four eccentricities from the optic nerve: (1) central, (2) mid-central, (3) mid-peripheral, and (4) peripheral.](image)

![Micrographs of retinal flat mounts from the different study groups. The density of the neuronal cells stained with cresyl blue is reduced in the ONA group compared with the CO and the KER group. Between the KER and the CO groups, no difference was noted.](image)

![Percentages of surviving neuronal cells in the ONA I, ONA II, and KER groups in relation to the neuronal cells of the CO group.](image)

![Percentage of RGCs preserved in the ONA I, the ONA II, and the KER groups, this time in correlation with the different areas of the flat mounts: (1) central, (2) mid-central, (3) mid-peripheral, and (4) peripheral.](image)
retinal ganglion cell layer, micrographs were taken as in former studies in 16 predefined areas, four in each quadrant with 40× magnification28-30 (Figs. 2A, 2B). As shown in Figure 2A, these areas were located in the central, mid-central, mid-peripheral, and peripheral areas of the flat mount in relation to the optic nerve head (1–4). An epifluorescence microscope (Axio Imager M1; Carl Zeiss) equipped with a digital camera (AxioCam MRc; Carl Zeiss) was used for photography and analysis of retinal flat mounts. All cells in the superficial retinal ganglion cell layer were manually counted by an experienced examiner masked to the protocol, who was not involved in previous parts of the study. The cells were subdivided into three cell types by morphologic criteria, such as shape, location, structure, and size of the Nissl substance using the cell counter plug-in Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). Neurons rich in Nissl substance with a prominent nucleolus have a diameter above 8 μm, and their shape is characterized by irregular outlines. Glial cells (GCs), diameter <8 μm, are usually round with regular outlines and acquire a darker stain. Endothelial cells, easy to see due to their more longitudinal shape, were excluded from further analysis. The classification of retinal cells according to morphologic criteria after staining with cresyl violet is an established method already used in previous studies in different species.25-31-35

**Collection of Blood Samples**

Blood samples from all animals were obtained via tail vein puncture before and 2 weeks after immunization.34-35 At the end of the study, blood was collected via heart puncture. All blood samples were transferred into reaction tubes (Eppendorf, Hamburg, Germany) immediately after collection. After 20 minutes of clotting, they were centrifuged at 12,000 rpm for 20 minutes at 4°C (Biofuge Heraeus, Hanau, Germany). Serum samples were obtained and stored at −80°C for later analyses.

**Quantification of Antibody Reactivity against Ocular Tissues**

To evaluate the antibody reactivity of serum against retina and optic nerve, tissues of healthy Lewis rats embedded in paraffin were used. Retinal and optic nerve cross sections were cut in 1-μm slices on a microtome (Reichert-Jung, Depew, NY), mounted on glass slides (Superfrost Plus; Menzel, Braunschweig, Germany), and subsequently stained.36-37 The tissues were initially pretreated for 10 minutes with 0.3% hydrogen peroxide (Roth, Karlsruhe, Germany) in phosphate-buffered saline solution (PBS; Invitrogen, Carlsbad, CA) to decrease endogenous peroxidase activity. To increase their binding sensitivity with the primary antibody, they were immersed in preheated target retrieval solution (Dako, Carpinteria, CA) and incubated for 45 minutes. Afterward, they were incubated with 1% bovine serum albumin in 0.5% Triton X-100 (both Sigma-Aldrich) to prevent nonspecific binding. Subsequently, they were incubated with serum samples (dilution 1:200 for retina and 1:750 for optic nerve sections). Serum samples from all groups (CO, ONA I, ONA II, and KER) collected at three time points during the study (0, 2, and 4 weeks after immunization) were used. A monoclonal secondary anti-IgG antibody (H+L, 1:500; Pierce Biotechnology, Rockford, IL) was subsequently applied. Color was developed through application of 3,3-diaminobenzidine tetrahydrochloride (DCHS, Hamburg, Germany) used as a co-substrate. Finally, all slides were counterstained with hematoxylin (Merck) and mounted. Micrographs of stained sections were taken with the same epifluorescence microscope with digital camera (Figs. 3, 4) as that used for cell counts. All cross sections were examined and evaluated microscopically by three independent examiners. The scores ranged from 0, no staining, up to 3, intense staining.37-38

To evaluate endogenous changes in tissues of immunized animals favoring antibody binding, as well as a possible already present antibody autoactivity in their serum, the same immunohistochemistry process was followed on longitudinal optic nerve sections from immunized animals of all groups of the study (Fig. 5).

**Detection of Antibody Reactivity against Brain and Dermal Tissues**

To test whether autoreactive antibodies can be detected against neuronal tissues besides the eye, brains were obtained from healthy rats and embedded in paraffin. Brain cross sections of 3-μm thickness were prepared and pretreated according to a modified protocol of the one described earlier. After de- and rehydration, the tissues were incubated in a water bath (99°C) with target retrieval solution (Dako) for 1 hour, followed by incubation in 1% bovine serum albumin with 0.1% Triton X-100 and 1% goat serum in PBS solution (1 hour). Afterward, the slides were incubated with the different serum samples obtained from the study animals as previously described for the ocular tissues. Sera from all time points (before the immunization, as well as 2 and 4 weeks afterward) were used. Serum incubation continued overnight, followed by incubation with goat anti-rat IgG immune fluorescence FITC-labeled antibody as a secondary antibody. Finally, the sections were mounted with DAPI.

Several slides of dermal tissue from the ear obtained from healthy rats underwent the same procedure.

All slides were examined directly afterward through a fluorescence microscope (Eclipse TE 2000; Nikon; Düsseldorf, Germany) equipped with a CCD camera (Fig. 6). Two independent examiners, masked to the protocol, scored all brain sections, as described above. Scores ranged from 0, no staining, to 3, intense staining.37,38

**Histopathology of Optic Nerve**

Optic nerve tissues were obtained from all groups. They were cut at a distance of 2 mm from optic chiasm, fixed in 4% paraformaldehyde, and embedded in paraffin. Longitudinal sections (5 μm) were stained with hematoxylin-eosin (H&E) and Luxol fast blue (LFB), with or without Nissl (LFB/Nissl), to detect possible pathologic changes, such as inflammation, aberration, or demyelination. Demyelination was graded by two examiners masked to the protocol and using a scoring system from 0 to 3 (0, no demyelination; 1, rare foci of demyelination; 2, small areas of demyelination; and 3, large areas of demyelination).39

**IgG Antibody Accumulation and Microglia in Optic Nerves**

Optic nerve sections were prepared for detection of possible IgG antibody deposits and microglia activation.50 Sections were transferred onto slides, deparaffinized, and rehydrated. The slides were initially pretreated with target retrieval solution for 45 minutes (Dako), fol-

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**FIGURE 3.** (A) Retina cross sections after incubation with rat sera. Sera collected before immunization triggered no IgG antibody reactivity. Two weeks after immunization, some serum samples showed weak signs of antibody reactivity. Four weeks after immunization, serum from ONA immunized rats produced a much stronger staining, whereas no staining was detectable in the CO and the KER groups. (B) Example of retina cross sections incubated with ONA and CO serum. The basic retinal layers are labeled: RGC, retinal ganglion cells; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PHR, photoreceptors. The autoreactive antibodies bound particularly to the retinal ganglion cell layer and the nerve fiber layer. On the other side, no antibody binding can be detected in the different retinal layers after incubation with serum from the CO group. (C) IgG antibody reactivity against retina before the immunization, as well as 2 and 4 weeks afterward. Scale bars, 50 μm.

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and Luxol fast blue with periodic acid Schiff staining (LFB/PAS) staining, as well as for immunohistochemistry. An autostaining device (Autostainer Plus; Dako) was used for immunostaining of horizontal sections of the brains as well as transverse sections of the spinal cord with monoclonal antibodies to CD3 (polyclonal, dilution 1:150; Dako), which is a marker for T cells; CD20 (clone L26, dilution 1:500; Dako), which is a B-cell marker; and CD68 (clone PG-M1, dilution 1:600; Dako), which is a marker for monocytes and macrophages. Processing by the autostaining program consisted of 5 minutes in peroxidase block (S2025; Dako), a 30-minute incubation in primary antibody, followed by a 30-minute incubation in secondary reagent (Histofine; Dako) and 10 minutes in diaminobenzidine substrate. After completion of the staining procedure, the stained slides were removed from the device and counterstained for 5 minutes in hemalum. All slides (Fig. 9) were examined microscopically (Vanox-T; Olympus, Tokyo, Japan).

**Statistical Analysis**

All data collected from IOP measurements, retinal cell counts, IgG antibody autoreactivity, demyelination, and microglia activation scoring were transferred to the software (Statistica, ver. 8.0; StatSoft, Tulsa, OK) for statistical analysis. Results of all animal groups were compared using two-way ANOVA analysis and are presented in mean counts. The Tukey test for multiple comparisons was applied, and differences reaching \( P < 0.05 \) were considered significant.

**RESULTS**

**Intraocular Pressure**

IOP was found to be stable in all groups throughout the study (Table 1, Fig. 1A). The IOP within each study group showed no significant alterations between the different points in time (\( P > 0.05 \)), and no significant difference was found between study groups (\( P > 0.05 \)).

**Funduscopy**

No animal developed pathologic fundus changes during the study (Fig. 1B). Fundus examinations revealed neither abnormalities in blood vessels nor neovascularization or retinal bleedings. The optic discs and blood vessels were clearly visible at all points in time, and no differences were noted between study groups. Corneas were also inspected microscopically, and no signs of opacity or other pathologic findings were observed.

**Evaluation of Retinal Flat Mounts**

To evaluate the possible cell loss in rats after immunization, the CO group was used as a point of reference and comparison. As the CO group demonstrated the highest cell counts in the superficial retinal ganglion cell layer (Table 2A), the other groups kept only a percentage of these numbers (Fig. 2C). In the two ONA groups, only 69.7% (ONA I) and 70.1% (ONA II) of the RGCs survived after immunization. The mean counts for the ONA groups were significantly lower than those of the CO group (\( P = 0.000008 \) for both ONA groups). On the other hand, in the animals that received KER, a higher percentage (95.1%) of RGCs was preserved. The mean counts of the KER

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**FIGURE 4.** (A) Optic nerve cross sections after IgG antibody detection. Before and 2 weeks after immunization, there was no or very little detectable staining. Four weeks after immunization of autoreactive antibodies was observed regarding ONA samples. The CO and KER groups revealed no such signs. (B) Micrograph of optic nerve cross sections incubated with serum from the ONA and the CO group. Staining can be seen on the whole mass of the section around the nuclei of the Schwann cells. On the other hand, no staining is detected on the neuronal tissue after incubation with serum from the CO group. (C) IgG antibody reactivity against optic nerve related to the time points before and after immunization. Scale bar, 50 \( \mu \)m.

**FIGURE 5.** Left: Longitudinal optic nerve sections from healthy rats incubated with serum samples from the different groups of the study. Right: longitudinal optic nerve sections from study rats (ONA, KER, and CO groups) incubated with their own serum. The incubation of healthy tissues with serum from the CO or KER groups led to no antibody binding. When optic nerve tissues from these animals are incubated with probes of their own serum, a very moderate staining is observed. On the other hand, intense antibody reactivity is seen in incubation with serum samples from the different groups of the study.

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Histologic Processing of Brain

The brains for all rats were immersion-fixed in 4% buffered formalin (Vector Laboratories, Burlingame, CA), 1% BSA, and 0.1% Triton X-100 dissolved in PBS. For detection of IgG depositions, tissues were incubated overnight with an FITC-labeled anti-rat IgG antibody (1:500; GenWay Biotech, San Diego, CA) and then mounted with antifade medium (Vectashield; Vector Laboratories).

As a microglia marker, an antibody against the ionized calcium binding adaptor molecule 1 (rabbit anti-Iba 1, 1:500; Wako Pure Chemical Industries, Osaka, Japan) was used. Tissues were incubated with this antibody overnight, followed by secondary antibody incubation with a Cy3-conjugated goat anti-rabbit IgG antibody (1:500; Linaris, Wertheim-Bettingen, Germany) for 3 hours. Images were taken via a fluorescence microscope (Fig. 8B). For microglia analysis, the number of nuclei with Iba1-positive perikarya was counted in a 214 × 173-\( \mu \)m region of interest (ROI, 37.02 mm\(^2\)) after background subtraction by ImageJ software. Activated and ramified microglia were distinguished based on morphologic criteria such as major branches with their ramified processes or an amoeboid shape (activated).30,40 The ROI quadrate was always placed axially in the longitudinal sections approximately 1 mm distal to the cutting edge to the optic chiasm, representing the middle part of the optic nerve.

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group were not significantly different from those of the CO group ($P = 0.28$). Moreover, when the counts of the ONA I and II groups were compared with those of the KER group, significant differences were also observed ($P = 0.000008$ for both ONA groups). Notable is the fact that despite the different antigen doses they received, the ONA I and II groups showed no statistically significant difference from each other ($P = 0.28$).

Examination of four areas of the retinal flat mounts revealed a predominant neuronal cell loss in the peripheral sections of the retina (Table 2C, Fig. 2D). Regarding the number of glia measured in the surface of the retinal ganglion cell layer, only a slight elevation of the cell count was observed in animals immunized with ocular antigens and it was not statistically significant.

**Figure 6.** (A) Brain cross sections of the cerebellum after IgG antibody detection through immunofluorescence staining. Before the immunization, only slight staining was observed on brain tissue. Four weeks after immunization, there was considerable immunofluorescence staining on brain sections incubated with serum from ONA animals. The staining was most intense around the Purkinje cells and their dendrites. On the other hand, no staining was detected on brain sections incubated with KER serum. (B) IgG antibody reactivity (score) against brain tissues in relationship to the time points of the study. Scale bar, 10 μm.
Autoreactive Antibodies against Ocular Tissues from Naïve and Immunized Animals of the Study

Incubation of cross sections with serum obtained from all animals before immunization showed no detectable autoreactive IgG antibodies against ocular tissues (Table 3). Mean scores for retina and optic nerve sections in all groups were approximately 0. Hence, no significant differences were observed between the study groups.

After 2 weeks, some signs of autoreactivity against the retina were observed after incubation with ONA sera (Table 3; Fig. 3). In both ONA groups, scores were significantly higher than the mean score of the CO group (P = 0.02 for both ONA groups). The KER group showed no significant signs of autoreactivity, with a mean score not significantly different from that of the CO group (P = 0.8). The comparison between ONA groups and the KER group revealed no significant difference (P = 0.1 for both ONA groups). Similar observations were made for the optic nerve cross sections (Table 3, Fig. 4). In comparison to the CO group, elevated antibody reactivity was found in the ONA I group (P = 0.001). The ONA II and KER groups showed levels of autoreactive antibodies similar to that of the CO group (ONA II: P = 0.4; KER: P = 0.99). Of the ONA groups, only ONA I showed increased reactivity in comparison to the KER group (ONA I: P = 0.0008; ONA II: P = 0.45).

Four weeks after immunization, the antibody reactivity against retina (Table 3, Fig. 3) increased further in the animals immunized with ONA than in the CO group (ONA I: P = 0.0003; ONA II: P = 0.0008). The KER group continued to

### Table 2. Neuronal Cell Data

#### A. Count of Neuronal Cells on Retinal Flat Mounts

<table>
<thead>
<tr>
<th></th>
<th>Total Flat Mount</th>
<th>Central</th>
<th>Mid-central</th>
<th>Mid-peripheral</th>
<th>Peripheral</th>
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<tr>
<td>CO</td>
<td>297 ± 37</td>
<td>313 ± 35</td>
<td>301 ± 35</td>
<td>301 ± 36</td>
<td>273 ± 32</td>
</tr>
<tr>
<td>ONA I</td>
<td>207 ± 51</td>
<td>247 ± 44</td>
<td>225 ± 38</td>
<td>183 ± 40</td>
<td>172 ± 42</td>
</tr>
<tr>
<td>ONA II</td>
<td>220 ± 42</td>
<td>239 ± 36</td>
<td>241 ± 32</td>
<td>215 ± 36</td>
<td>184 ± 40</td>
</tr>
<tr>
<td>KER</td>
<td>284 ± 28</td>
<td>295 ± 17</td>
<td>296 ± 22</td>
<td>281 ± 25</td>
<td>263 ± 36</td>
</tr>
</tbody>
</table>

#### B. P Values of Comparisons between the Animal Groups Regarding the Neuronal Counts in the Whole Retinal Flat Mounts

<table>
<thead>
<tr>
<th></th>
<th>CO</th>
<th>ONA I</th>
<th>ONA II</th>
<th>KER</th>
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<tr>
<td>CO</td>
<td>—</td>
<td>0.000008</td>
<td>0.000008</td>
<td>0.28</td>
</tr>
<tr>
<td>ONA I</td>
<td>0.000008</td>
<td>—</td>
<td>0.28</td>
<td>0.000008</td>
</tr>
<tr>
<td>ONA II</td>
<td>0.000008</td>
<td>0.28</td>
<td>—</td>
<td>0.000008</td>
</tr>
<tr>
<td>KER</td>
<td>0.28</td>
<td>0.000008</td>
<td>0.000008</td>
<td>—</td>
</tr>
</tbody>
</table>

#### C. Percentage of Surviving Neuronal Cells in the Study Groups in Relation to CO Group Counts

<table>
<thead>
<tr>
<th></th>
<th>Total Flat Mount</th>
<th>Central</th>
<th>Mid-central</th>
<th>Mid-peripheral</th>
<th>Peripheral</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONA I</td>
<td>69.7 ± 17.1</td>
<td>79.1 ± 14.1</td>
<td>80.2 ± 10.6</td>
<td>71.6 ± 11.8</td>
<td>67.5 ± 14.8</td>
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<td>ONA II</td>
<td>74.1 ± 14.2</td>
<td>76.3 ± 11.3</td>
<td>98.3 ± 7.1</td>
<td>93.5 ± 8.4</td>
<td>96.6 ± 13.1</td>
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<tr>
<td>KER</td>
<td>95.6 ± 9.6</td>
<td>84.6 ± 5.3</td>
<td>98.3 ± 7.1</td>
<td>93.5 ± 8.4</td>
<td>96.6 ± 13.1</td>
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</tbody>
</table>

(A) Multiple comparisons between all animal groups were performed by two-way ANOVA, followed by Tukey’s post hoc test. Values are expressed as mean counts ± SD. Results are presented for all examined retinal areas together, as well as for the four retinal areas separately. (B) P values from Tukey’s post hoc test after two-way ANOVA of all comparisons between the study groups of RGC counts in the whole retinal flat mounts. Only P < 0.05 was regarded as statistically significant and is shown in bold. (C) Percentages of RGCs that were preserved in the ONA I, the ONA II, and the KER groups (% ± SD), in the whole retina flat mounts and in the different areas of the flat mounts.

### Table 3. Scores of Autoreactive IgG Antibodies

#### Retina Cross Sections

<table>
<thead>
<tr>
<th></th>
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<th>ONA II</th>
<th>KER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.4 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.2</td>
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<tr>
<td>2 Weeks</td>
<td>0.1 ± 0.1</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>0.1 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>2.1 ± 0.4</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>

#### Optic Nerve Cross Sections

<table>
<thead>
<tr>
<th></th>
<th>CO</th>
<th>ONA I</th>
<th>ONA II</th>
<th>KER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2 Weeks</td>
<td>0.1 ± 0.1</td>
<td>1.6 ± 0.4</td>
<td>0.5 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>0.1 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>1.7 ± 0.5</td>
<td>0.3 ± 0.3</td>
</tr>
</tbody>
</table>

#### Brain Cross Sections

<table>
<thead>
<tr>
<th></th>
<th>CO</th>
<th>ONA I</th>
<th>ONA II</th>
<th>KER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>2 Weeks</td>
<td>0.2 ± 0.1</td>
<td>1.6 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>0.3 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>1.7 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM.
demonstrate low levels of autoreactive antibodies not significantly different from that of the CO group \((P = 0.6)\). At this time point, the antibody reactivity was significantly higher in the ONA groups than in the KER group \((ONA I: P = 0.002; ONA II: P = 0.007)\). The examination using optic nerve cross sections revealed similar results (Table 3, Fig. 4). Both ONA groups had significantly higher antibody reactivity than the CO group \((ONA I: P = 0.0003; ONA II: P = 0.005)\). Again, the KER group showed no significant difference from the CO group \((P = 0.9)\). The comparison between the ONA groups and the KER group revealed significant differences \((ONA I: P = 0.0005; ONA II: P = 0.018)\).

Regarding the antibody autoreactivity against ocular tissues from immunized animals of the study, a very moderate staining was observed in optic nerves of animals of the CO and KER groups after incubation with their own serum (obtained after 4 weeks). A much more intense staining was seen when optic nerves from the ONA groups were incubated with serum from these animals (Fig. 5).

**Autoreactive Antibodies against Brain Tissue**

Almost no immunofluorescence staining was detected on all brain cross sections incubated with serum taken before immunization (Table 3; Fig. 6). Therefore no difference between groups was observed.

Two weeks after immunization, antibody reactivity was detected in sera from some animals immunized with ONA. The difference between the ONA I group and the CO group was statistically significant \((P = 0.0001)\). The ONA II and the KER groups showed no significant difference from the CO group \((ONA II: P = 0.29; KER: P = 0.99)\). Of the ONA groups, only ONA I had significantly higher antibody reactivity than the KER group \((ONA I: P = 0.0003; ONA II: P = 0.12)\).

Four weeks after immunization, the antibody reactivity against brain tissues was increased in ONA groups. The other groups demonstrated almost no signs of antibody reactivity. The differences between both ONA groups and the CO group was statistically significant \((ONA I: P = 0.0003; ONA II: P = 0.0001)\). There was no significant group difference between KER and CO \((P = 0.99)\), but significant differences were noted between the KER group and the two ONA groups \((ONA I: P = 0.00016; ONA II: P = 0.00013)\).

**Optic Nerve Demyelination**

Analysis of the LFB-stained longitudinal optic nerve sections revealed moderate demyelination in both ONA groups 4 weeks after immunization (Fig. 7A). Demyelination was characterized by disruption or alterations in the organization of the neuron myelin sheaths, whereas the optic nerve sections from animals of the CO and the KER group showed no or few such signs. Moreover, several cellular infiltrates were seen in longitudinal optic nerve sections of the ONA groups. After scoring and statistic analysis, the differences between both ONA groups and the CO \((ONA I: P = 0.0005; ONA II: P = 0.013)\) and KER \((ONA I: P = 0.0005; ONA II: P = 0.013)\) groups were found to be significant. No significant difference \((P = 1.0)\) was found between the CO and KER groups (Fig. 7B).

**IgG Deposition and Activation of Microglia in Optic Nerves**

Several IgG accumulations were noted in almost all optic nerves of ONA animals. Beside focal diffuse depositions, stronger staining patterns were observed on single axons (Fig. 8A). Optic nerves of CO and KER animals revealed only staining at the blood vessels and some unspecified background fluorescence staining, but no axon-guiding IgG deposits. Ramified microglia decreased in the immunized groups (Fig. 8B), whereas a significant increase in activated microglia was seen. In optic nerve tissues from the CO and the KER groups no such increase was noted. After scoring and statistical analysis, the differences between both ONA groups \((ONA I: P = 0.00017; ONA II: 0.00018)\) as well as between the ONA groups and the KER group \((ONA I: P = 0.00017; ONA II: P = 0.00076)\) were statistically significant. Between the CO and the KER groups, no difference was noted \(P = 0.64\); Fig. 8C).

**Brain Sections**

Brains from all animals were also examined 4 weeks after immunization. Contrary to the optic nerve cross sections, in

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**Figure 7.** (A) Longitudinal optic nerve section from the CO and the KER groups stained with LFB/Nissl revealed normal myelin patterns with no infiltrates. In sections from ONA immunized animals, rare foci (arrowheads) of moderate demyelination with disruption of the lamellar structure are shown. Noticeable are darker stained nuclei of cellular infiltrates beside the demyelination (arrows). (B) Both ONA groups showed a significantly increased demyelination of the optic nerve in comparison to the CO and the KER groups. Scale bar, 50 μm.
these cases, neither demyelinated lesions nor mononuclear cell infiltrates consisting of macrophages and/or lymphocytes were detectable in the rat brains by routine histology (H&E and LFB/PAS staining; Fig. 9) and immunohistochemistry with monoclonal antibodies to CD3, CD20, and CD68 (not shown).

**DISCUSSION**

In previous studies, complex antibody profiles and altered levels of antibodies against specific ocular antigens were detected in serum samples of glaucoma patients. However, it is still unclear whether these antibodies are involved in the initial pathogenesis of glaucoma or they develop during the course of the disease as an accessory phenomenon. To further investigate the role of antibodies, we used an animal model of experimental autoimmune RGC loss.

A decrease in RGCs in rats after immunization with heat shock proteins was recently shown. Wax et al. reported a decrease in RGC density 1 and 4 months after immunization. Joachim et al. observed similar results 5 weeks after immunization. In our study, we showed that immunization with an optic nerve homogenate, a complex mixture of neuronal antigens, triggered neuronal cell loss. In contrast, the immunization with KER, a non–glaucoma-associated antigen, left these cells predominantly unaffected. These results suggest that the immune response triggered through the immunization could be target-specific and lead to RGC death. Increased IgG antibody reactivity against ear cross sections (structures rich in KER) was detected in sera of animals immunized with KER (data not shown). In the eye, however, KERs can be found in high quantities only in corneal tissue. As the retina contains no KER, the RGCs remain unaffected by an immunologic response, which would include the development of antibodies against such molecules. This fact could be the reason that the KER injection did not affect neuronal cells in these animals.

We also noted that the immunization with different doses of the same antigen had a similar effect on the RGCs. Different doses of immunization have been tested in studies of experimental autoimmune uveitis. Results were not uniform, but depended on the antigen used for immunization. Broekhuysen et al. were able to induce a mild posterior retinitis in Lewis rats with opsins, which was not influenced by the amount of the injected antigen. On the other hand, the same study revealed a stronger dose-dependent uveitis caused by immunization with...
interphotoreceptor retinoid-binding protein or retinal S-antigen. We hypothesize that the doses of optic nerve homogenate used in our study were above a crucial level that did not allow significant distinction of the intensity of RGC decline. Future studies, including a greater range of immunization doses, should be conducted to investigate in depth the possible correlation between the antigen quantity and cell loss.

Furthermore, we found that the animals immunized with ONA developed antibodies that were autoreactive against ocular tissues, more specifically against retina and optic nerve. These results agree with findings of Joachim et al., who identified complex alterations in antibody patterns in rats after immunization with heat shock proteins. We assume that, in our study, immunization with the homogenate of optic nerve led to a complex systemic immune response, which included the increase of antibody reactivity in serum against several retinal epitopes, possibly including the interphotoreceptor retinoid-binding protein or retinal S-antigen. The animals in our model immunized with optic nerve antigens also developed autoreactive antibodies against neuronal tissues in naïve rats, as well as against brain tissues, after their immunization with a neuronal homogenate, which was actually expected to include several neuronal antigens common in the nervous system, among them glaucoma-associated antigens such as tau protein.

On the other hand, animals of the KER group showed no alteration of their antibody autoreactivity against ocular or neuronal tissues throughout the study. This applies also to the control group of our study which showed no autoimmune reactivity.

According to these results, both groups with no signs of a specific autoimmune reactivity, which targets neuronal epitopes in eye and brain, retained their retinal neuronal cells through the study. Since only animals with high levels of antibody autoreactivity against such tissues suffered additionally from RGC loss, it allows us to propose that these two findings are connected.

Results of other studies demonstrate that antibodies are indeed able to induce neuronal cell death through a variety of pathways. Matus et al. showed that antibosomal P-protein autoantibodies cause apoptotic cell death in brain cellular cultures by increasing the calcium influx into neurons significantly. A decline in neuronal cultures of the rat cortex was also induced by GluR3 autoantibodies via complement and membrane attack complex activation. In vitro experiments by Lu et al. revealed that autoantibodies against the intermediate filament α-internexin can mediate neuronal damage by inhibiting axonal elongation.

Therefore, we suggest that in our model, elevated antibody autoreactivity against neuronal antigens in eye and brain tissues impaired the survival of RGCs through similar mechanisms. Since evidence of the presence of autoreactive antibodies against retina, optic nerve, and brain tissue in serum of immunized animals was found in vitro, a possible in vivo binding of superficial or intracellular (after endocytosis) antigens could trigger mechanisms that lead to the observed RGC death.

In vitro experiments in a study by Tezel and Wax have already shown that the survival of retinal cells can be impaired by the presence of exogenously applied antibodies—for example, against heat shock proteins. Moreover, this study offered evidence of later presence of these antibodies in cytoplasmic and nuclear structures. Of course, such a pathogenic mechanism would have as an essential prerequisite the capability of the developed autoantibodies to pass through the blood–retinal barrier. Examination of tissues from immunized animals by electron microscopy may reveal whether antibodies are indeed able to enter RGCs and on which epitopes they may bind. In our model, antibody accumulation could be detected on optic nerve tissue from animals immunized with ocular antigens. No detection was possible in animals of the CO or the KER group, allowing us once more to connect these findings with the retinal decline observed in the ONA groups.

Even if these antibodies do not directly trigger cell death, autoreactive antibodies could indirectly lead to RGC degeneration through anatomic alterations or through the activation of cellular mechanisms, whose role in the pathogenesis of glau-
coma has been discussed.61 In our model, pathologic alterations in the anatomy of the optic nerve with a significant reduction of the myelin sheaths of optical neurons were observed in animals immunized with ocular antigens. Furthermore, cellular infiltration of the neuronal tissue was detected in the same animals, whereas animals from the CO and KER groups demonstrated no such signs. Demyelination and activated cytotoxic T cells are known to be pathogenic factors in animal models of experimental autoimmune diseases, such as experimental autoimmune uveitis62 and experimental autoimmune encephalitis.63 On the other hand, no such observations were made in the brain tissues examined in the study, indicating an inflammatory process targeting specifically the optic nerve. Beside the possible contribution of T cells, the involvement of microglia has to be discussed also.64,65 Qualitative changes were observed regarding the glia cells detected in our model, as the normal ramified glia cells were in large scale replaced from activated cells in animals of the ONA groups, in comparison to the CO and the KER groups.

According to all these results, only the animals immunized with ONA demonstrated fully developed pathology, with abnormalities in anatomic (demyelination), cellular (cellular infiltration, glia activation, and molecular (antibody reactivity) levels. This pathology probably led to the reduction of RGCs, which was also observed only in animals immunized with ONA. How these anatomic, cellular, and molecular mechanisms combine to lead to neuronal death in the retina in our model remains for further investigation. For example, an activation of T cells via autoreactive antibodies could result in the release of smaller molecules, which can easily pass the blood-retina barrier and induce RGC apoptosis66 (e.g., soluble Fas ligand, whose potential role in RGC death has already been proposed in a model of autoimmune glaucoma), 11 or ligands associated with the tumor necrosis factor death receptor family.67 On the other hand, microglia are the resident immuno-competent and phagocytic cells in the CNS, and their activation is closely linked to neurodegenerative processes induced by various stimuli.68 In this regard, microglia could be stimulated, as described above for T cells or by antibody cross-linking of retinal and optic nerve proteins.69–70

Further studies should be performed to investigate the role of these autoreactive antibodies in cell death in more detail. Can a transfer of serum antibodies from immunized to naïve animals cause cell death in these animals?

In summary, it is known that cellular and chemical autoimmune are critical for RGC survival.8 Regulatory errors, such as over- or undertactivity of these complex mechanisms, could be fatal for RGCs. Based on the results of this study, we assume that specific autoantibodies against neuronal tissues in eye or brain could be involved in the RGC apoptosis in this experimental autoimmune animal model.

References


