Protection of Retinal Ganglion Cells and the Optic Nerve During Short-term Hyperglycemia in Experimental Glaucoma

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Objective: To evaluate the neuroprotective effect of short-term hyperglycemia on the retinal ganglion cell body and axon in a rat model of experimental glaucoma.

Methods: Using a well-described limbal laser technique, unilateral ocular hypertension was induced in 2 groups (26 per group) of Sprague-Dawley rats. One group remained normoglycemic; the other was rendered hyperglycemic by means of an intraperitoneal injection of streptozocin. After 2 weeks of elevated intraocular pressure, axonal and retinal damage profiles were compared using several histological techniques. Immunohistochemical changes in the retina and optic nerve were also assessed.

Results: We found convincing evidence of delayed axonal degeneration and retinal ganglion cell death in hyperglycemic rats. Axon loss was reduced by about 50% 2 weeks after induction of ocular hypertension. Survival of retinal ganglion cell perikarya increased to a similar extent in hyperglycemic rats.

Conclusions: The optic nerve and retinal ganglion cells are partially protected by short-term hyperglycemia in this rat model of experimental glaucoma. Energy substrate availability may therefore play a role in glaucomatous optic neuropathy.

Clinical Relevance: Our findings, to some extent, support the claims of the Ocular Hypertension Treatment Study, in which diabetes appeared to protect against the conversion to glaucoma. Targeted manipulation of neuronal energy metabolism may delay optic nerve degeneration and may represent a novel neuroprotective strategy for neurodegenerative diseases of the visual system such as glaucoma.

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GLAUCOMA REFERS TO A family of ocular conditions with multifactorial causes united by a clinically characteristic optic neuropathy. The pathogenesis of glaucoma remains unclear, but there is considerable evidence that inadequate blood supply to the optic nerve (ON) head may be involved, in at least some types of glaucoma.1-3 Given that vasculopathy is a hallmark of diabetes and that compromised retinal and ON blood flow is a feature of glaucoma, it seems reasonable to suggest that diabetes might exacerbate glaucoma; however, despite decades of searching, there remains, at best, conflicting evidence regarding a clinical or an epidemiological association between these 2 diseases. In large-scale clinical trials, no association was found in the Framingham,4 Barbados,5 or Baltimore Eye6 studies. In the Beaver Dam Study, glaucoma was weakly associated with older-onset diabetes only.7 A weak association was also reported in the Los Angeles Latino Eye Study,8 whereas the strongest reported association was documented in the Blue Mountains Eye Study.9 This lack of convincing epidemiological association seems odd, particularly when considering the fact that studies using intraocular pressure (IOP) in their definition of glaucoma were biased toward finding an association based on corneal biomechanical changes in persons with diabetes.10 There is increasing evidence that hyperglycemia causes cross-linking and is associated with increased central corneal thickness, which results in augmented stiffness of the cornea.11,12 This means that IOP measurements taken by methods that depend on these corneal variables, such as Goldmann applanation tonometry, overestimate the actual IOP; hence, there is an inherent measurement bias. Data from the Ocular Hypertension Treatment Study13 are even more curious. In that study, the presence of diabetes protected against the conversion of ocular hypertension (OHT) to glaucoma. In fact, this

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finding was one of the strongest associations reported, and it has subsequently been incorporated into risk calculators for clinical use. It is possible that the finding is spurious; however, Quigley has recently outlined reasons why diabetes may be protective.

Glucose is an essential energy substrate for central nervous system tissues, including the retina and ON, but it has become dogma within clinical medicine that elevated serum glucose levels (hyperglycemia) are deleterious to neurons, including those in the retina, under conditions of energy deprivation. Contrary to this viewpoint, we have previously demonstrated that providing glucose to retinal neurons in vivo during acute and sustained periods of ischemia affords a robust neuroprotective effect, arguably one of the strongest and most durable demonstrations of retinal preservation. Recent studies further showed that hypoglycemia exacerbated ischemic retinal injury, that retinal neuronal cells in vitro preferentially metabolized glucose compared with lactate, and that inhibition of lactate transport did not exacerbate ischemic retinal injury in vivo. Together, these findings indicate that glucose and not lactate is responsible for the protective effect.

To date, little research has investigated the effect of diabetes in models of experimental glaucoma. Herein, we provide evidence that retinal ganglion cell (RGC) axons and their perikarya are partially protected by hyperglycemia.

**METHODS**

**OUTCOMES**

Primary outcomes of the study were RGC loss and axonal injury. Complementary outcomes included measurement of microglial and macroglial activation, RGC stress, and neurofilament breakdown.

**STUDY PLAN**

In preliminary experiments, we demonstrated that 2 weeks of elevated IOP caused measurable loss of RGCs and their axons, together with activation of retinal and ON glia. Furthermore, we found that 2 weeks of hyperglycemia in normotensive rats caused no significant loss of RGCs (data not shown). Hence, the 2-week point was chosen for the present study. This time frame is short enough for diabetes per se not to cause any confounding RGC death but long enough to allow measurable retinal and axonal damage resulting from chronic OHT.

Rats were randomly divided into 2 groups (26 rats per group). One group retained physiologic serum glucose levels (normoglycemic [NG-OHT]) and the other was rendered hyperglycemic (HG-OHT). Ocular hypertension was induced in the right eye of each animal by laser photocoagulation of the trabecular meshwork using a slightly modified protocol of the method described by Levkovitch-Verbin et al. After brief anesthesia with isoflurane, IOPs were measured bilaterally at baseline and on days 1, 3, 5, 7, 8, 10, and 14 using a rebound tonometer (TonoLab; Icare Finland, Espoo, Finland). To characterize IOP profiles for each rat peak IOP, IOP exposure (positive IOP integral) and the IOP integral were calculated using the following formulas:

Positive IOP Integral = \sum_{i=1}^{k} (t_i - t_{i-1}) \times \frac{1}{2} \times ([IOP_{R} - IOP_{L}]_{i} - IOP_{L})

IOP Integral = \sum_{i=1}^{k} (t_i - t_{i-1}) \times \frac{1}{2} \times ([IOP_{R} + IOP_{L}]_{i-1} - IOP_{L}),

where \( t_i \) indicates the time point \( i \) (in days), \( IOP_{R} \), IOP in the right eye; and \( IOP_{L} \), IOP in the left eye.

Serum glucose levels were measured in all rats at day 14 before the animals were humanely killed by means of cardiac perforation with physiologic saline solution under terminal anesthesia.

**TISSUE PROCESSING AND HISTOLOGY**

Each eye with the ON and chiasm attached was carefully dissected. A short piece of proximal ON, 1.5 mm behind the globe, was removed for resin processing. The globe, middle ON, and chiasm were fixed in 10% buffered formalin for 24 hours and processed for routine paraffin-embedded sections. Globes were embedded sagittally, whereas ON and chiasmata were embedded longitudinally. In all cases, 5-µm serial sections were cut. Toluidine blue staining of resin sections of the proximal ON was performed as previously reported.

**IMMUNOHISTOCHEMISTRY**

Immunohistochemistry was performed as previously described. In brief, tissue sections were deparaffinized and treated with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was achieved by microwaving the sections in 10 mM citrate buffer (pH 6.0) for 10 minutes at 95°C to 100°C. Tissue sections were then blocked in phosphate-buffered saline containing 3% normal horse serum, incubated overnight at room temperature in primary antibody (Table 1), followed by consecutive incubations with biotinylated secondary antibody and streptavidin-peroxidase conjugate. Color development was achieved with 3',3'-diaminobenzidine. Sections were typically counterstained with hematoxylin, then dehydrated and mounted. Specificity of antibody labeling was confirmed by incubating adjacent sections with mouse IgG1 isotype control (50878; BD Pharmingen, North Ryde, Australia) or normal rabbit serum.

**EVALUATION OF IMMUNOHISTOCHEMISTRY**

Immunolabeling for each antigen was performed in a single batch, and all analyses were conducted in a masked fashion. Left retinas and left ONs served as control specimens.
Sections were evaluated by 3 independent observers (including A.E. and G.C.). To minimize sampling errors, all analyses were performed on sections taken at the level of the ON head. Retinal ganglion cells immunostained for NeuN, Brn-3, and heat shock protein 27 (HSP27) were counted across the entire retina. Two sections per animal were analyzed for NeuN and the counts were averaged. Loss of RGCs was calculated by subtracting the NeuN count of the glaucomatous right eye from the normotensive left eye. Immunoreactivities for macroglia (labeled with glial fibrillary acidic protein) and microglia (labeled with Iba1 and ED1) were scored using a 4-point grading system, as described previously,21 after appraisal of the entire retina.

Optic Nerve

For each antigen, immunohistochemistry was performed using 1 section of the middle ON and 1 section of the distal ON (adjacent to the optic decussation). One photomicrograph from each location was taken using the 20×/0.11032 microscope objective. Evaluations were performed using a software package platform (ImageJ 1.42q; http://rsb.info.nih.gov/ij/). For counterstained sections, color deconvolution was applied to extract the 3',3'-diaminobenzidine staining. After thresholding, the area of staining was measured.

EVALUATION OF SEMITHIN TOLUIDINE BLUE STAINED ON CROSS SECTIONS

Axon numbers were estimated using a semiautomated, quantitative method with targeted sampling, similar to that used by others20 (eMethods section and eFigure; http://www.archophthalmol.com).

STATISTICAL ANALYSIS

Our hypothesis was that short-term hyperglycemia would protect RGC axons and their somata. This was a unified hypothesis, and no correction for multiple hypothesis testing was made. Other analyses of the ON and retina were considered as complementary outcomes. An unpaired t test was used if parametric assumptions were met; a Mann-Whitney test was used otherwise. Regarding the complementary outcomes, we were not interested in the notion of finding at least 1 statistically significant result; hence, adjustments for multiple statistical tests would have unnecessarily inflated the type II error rate and were not performed. No post hoc comparisons were made, and P values were interpreted in the context of the experiment, relevant biological factors, and scientific knowledge. Statistical analyses were performed using commercially available software (GraphPad Prism 5.0b; GraphPad Software Inc, La Jolla, California).

RESULTS

Streptozocin caused a sustained elevation of the serum glucose level. Peak pressure and IOP exposure were comparable in both experimental groups (Table 2) and were similar to values reported by other groups using the same model.29

OPTIC NERVE

Estimated axon counts of ON cross sections, the primary outcome, revealed protection of the ON by hyperglycemia (Figure 1). In NG-OHT and HG-OHT rats, the mean (SEM) counts were 66 500 (4000) and 77 600 (2800), re-
respectively, which corresponded to a 21% axonal loss in NG-OHT rats and a 8% loss in HG-OHT rats, given that the estimated axon count in control nerves was 84 300 (1600).

Quantitative analyses of distal and middle ON sections immunolabeled for markers of axonal cytoskeletal damage, microglial activation, and astroglial and oligodendroglial stress also indicated white matter protection by hyperglycemia. Representative immunohistochemical images are shown in Figure 2, whereas the results are documented in Table 3. In detail, Smi32, an antibody that labels nonphosphorylated neurofilament heavy, is a sensitive marker of axonal neurofilament abnormalities.25,30 Compared with control ONs, increased Smi32 abnormalities were evident in the treated ONs of NG-OHT rats. The Smi32 abnormalities tended to be less prevalent in the HG-OHT group, although the difference did not reach statistical significance (Table 3; \( P = .39 \) and \( P = .40 \) for distal and middle ONs, respectively).

We have previously demonstrated that microglial number and phagocytic activity, as detected immunohistochemically using antibodies against Iba1 and ED1, correlate well with axonal injury during experimental glaucoma.25 In the present study, both markers were significantly less abundant in the distal and middle ONs of HG-OHT rats compared with NG-OHT rats (Table 3; \( P = .04 \) and \( P = .02 \), respectively, for ED1; \( P = .007 \) and \( P = .01 \), respectively, for Iba1), a result that is consistent with greater axonal preservation in the HG-OHT group.

To assess the macroglial stress response, we quantified immunolabeling for 2 members of the small HSP family, HSP27 and αB-crystallin, which are differentially expressed in astrocytes and oligodendrocytes, respectively, after axonal injury.31 Marked upregulation of both small HSPs were detected in the treated ONs of NG-OHT rats. These changes were significantly attenuated in the distal and middle ON of HG-OHT animals (Table 3; \( P = .01 \) and \( P < .001 \), respectively, for HSP27; \( P < .001 \) and \( P = .02 \), respectively, for αB-crystallin).

The combined results demonstrated decreased axonal loss, attenuated microglial proliferation and phagocytic activity, and a less elevated macroglial stress response in the glaucomatous ONs of HG-OHT rats.

**RETINA**

The primary retinal outcome of the study was survival of RGCs. This was assessed by counting NeuN-positive cells in the ganglion cell layer (Table 4 and eTable). NeuN has been proved to be a reliable marker for quantification of RGC survival in models of RGC degeneration.32,33 In the treated retinas of NG-OHT rats, the mean (SEM) loss was 64.3 (10.9) vs 31.1 (9.3) RGCs in HG-OHT rats (\( P = .02 \)). In contrast to the primary outcome, the secondary retinal outcomes did not point toward greater protection in the HG-OHT group. Representative immunohistochemical images are shown in Figure 3, and the results are provided in Table 4. In detail, grading of sections immunolabeled with markers of microglial and macroglial activation revealed similar magnitude glial injury responses (\( P = .34 \) and \( P = .46 \), respectively). Similarly, quantification of the number of HSP27-positive RGCs in the retinas of NG-OHT and HG-OHT rats demonstrated a small number of RGCs in all laser-treated eyes but no difference between the 2 experimental groups (\( P = .75 \)). Heat shock protein...
27 is not constitutively expressed by RGCs; however, within an ongoing pathological setting, such as experimental glaucoma or chronic ischemia, it is persistently upregulated in some RGCs, indicating cellular stress.

Hyperglycemia exacerbates ischemic brain injury. The situation in the retina, however, which exhibits unique features of glucose metabolism, is different. Previous studies have shown that providing glucose to retinal neurons in vivo during acute and prolonged periods of ischemia affords a robust neuroprotective effect. Epidemiological data investigating the association between diabetes mellitus and glaucoma is controversial and published results are inconsistent. The aim of this study was to evaluate the short-term effect of hyperglycemia on axonal damage and RGC survival in a rat model of experimental glaucoma, dissecting the potentially beneficial effect of additional energy supply (ie, elevated serum glucose levels) from the noxious chronic changes that occur in diabetic retinopathy.

As for our primary outcome, we found convincing evidence that hyperglycemia attenuated axonal and somal RGC degeneration after experimentally induced OHT. The secondary outcomes of the study, which provided information on the status of the axonal cytoskeleton and the response of microglial and macroglial cells in the ON and retina, yielded mixed results.

**OPTIC NERVE**

Immunolabeling with Smi32 highlights any axon fibers in the process of degeneration and provides information about ongoing, rather than cumulative, injury. Although there was a trend for decreased Smi32 labeling in the HG-OHT rats, statistical significance was not attained. The short period during which axons show abnormalities in Smi32 immunolabeling might explain this result. Alternatively, it may simply be the case that axonal degeneration was proceeding at a similar rate in NG-OHT and HG-OHT animals by 14 days after elevation of IOP. In contrast to Smi32, analysis of microglial markers in the glaucomatous ONs supported the finding of increased axonal survival in HG-

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**Table 3. Effect of Hyperglycemia on Chronic OHT–Induced Neurofilament Degeneration and Glial Activation in Distal and Middle ON**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Neurofilament Degeneration, Smi32</th>
<th>Microglial Activation</th>
<th>Macroglial Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED1</td>
<td>Iba1</td>
<td>αB-crystallin</td>
</tr>
<tr>
<td>Distal ON</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n=51)</td>
<td>Absent</td>
<td>Absent</td>
<td>35.2 (2.4)</td>
</tr>
<tr>
<td>NG-OHT (n=26)</td>
<td>21.0 (7.1)</td>
<td>7.9 (2.1)</td>
<td>77.7 (6.6)</td>
</tr>
<tr>
<td>HG-OHT (n=26)</td>
<td>10.6 (3.3)</td>
<td>3.1 (1.1)</td>
<td>54.9 (4.6)</td>
</tr>
<tr>
<td>P valueb</td>
<td>.39c</td>
<td>.04c</td>
<td>.007c</td>
</tr>
<tr>
<td>Middle ON</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NG-OHT (n=23)</td>
<td>16.0 (5.9)</td>
<td>5.4 (2.0)</td>
<td>76.4 (7.2)</td>
</tr>
<tr>
<td>HG-OHT (n=25)</td>
<td>9.9 (4.5)</td>
<td>1.1 (0.3)</td>
<td>53.9 (5.0)</td>
</tr>
<tr>
<td>P valueb</td>
<td>.40c</td>
<td>.02c</td>
<td>.01b</td>
</tr>
</tbody>
</table>

Abbreviations: HG-OHT, hyperglycemic group; NG-OHT, normoglycemic group; OHT, ocular hypertension; ON, optic nerve.

Mean (SEM)a numbers given represent the area of immunostaining expressed in pixels (× 103). The total area of a microphotograph is 1920 × 103 pixels. Controls are contralateral ONs.

b Calculated as a comparison between the NG-OHT and HG-OHT ONs.
c Calculated using the Mann-Whitney test.
d Calculated using the unpaired t test.

d | Mean (SEM)a |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RGC Absolute Loss, NeuN</td>
<td>RGC Stress, HSP27</td>
</tr>
<tr>
<td>Controls (n=44)</td>
<td>Nil</td>
</tr>
<tr>
<td>NG-OHT (n=24)</td>
<td>64.3 (10.9)</td>
</tr>
<tr>
<td>HG-OHT (n=20)</td>
<td>31.1 (9.3)</td>
</tr>
<tr>
<td>P valueb</td>
<td>.02</td>
</tr>
</tbody>
</table>

Abbreviations: HG-OHT, hyperglycemic group; NG-OHT, normoglycemic group; OHT, ocular hypertension; RGC, retinal ganglion cells.

a The NeuN-measured loss and heat shock protein 27–positive RGCs were counted along the entire retina at the level of the optic nerve head. Glial cell–marker immunostaining was scored on a 4-point grading system ranging from 0 to 3. The mean (SEM) NeuN count for control eyes was 346 (6) neurons. Loss of RGCs was calculated by subtracting the NeuN count of the glaucomatous right eye from that of the normotensive left eye.

b Calculated as the comparison between NG-OHT vs HG-OHT groups using the Mann-Whitney test. All treatment groups are significantly different from the controls (P < .01).
Heat shock protein 27 and abundant in HG-OHT nerves, denoting reduced axonal damage.36,37 That HSP27 and they are also markedly upregulated in many neurodegenerative diseases.36,37 That HSP27 and multiple functions acting as molecular chaperones, apoptosis inhibitors, antioxidants, and cytoskeleton stabilizers. They are also markedly upregulated in many neurodegenerative diseases.36,37 That HSP27 and αB-crystallin are differentially expressed in astrocytes and oligodendrocytes during ON degeneration has been previously demonstrated.35 The more limited induction of these proteins in HG-OHT rats points toward a reduced pathological profile in these ONs compared with those of NG-OHT rats.

**RETINA**

A recent study21 demonstrated that hyperglycemia is neuroprotective against chronic retinal ischemia caused by permanent ligation of the common carotid arteries. In addition to enhancing RGC survival, retinas from hyperglycemic rats had fewer RGCs that were metabolically stressed (ie, HSP27-positive), a reduced microglial response, and a less elevated reactive gliosis in astrocytes and Müller cells compared with retinas from normoglycemic rats. This was not the case in the present study. Although a greater number of RGCs were evident in the retinas of HG-OHT rats, the glial responses were effectively of identical magnitude in NG-OHT and HG-OHT retinas, and there were a similar number of HSP27-positive RGCs in both groups. Glial markers are very sensitive to homeostatic imbalance and respond rapidly to pathological events. A possible explanation for the discrepancy between RGC counts and glial stress in the retina could be that hyperglycemia merely delays RGC death. The ON findings with respect to Smi32 would support this hypothesis. Overall, hyperglycemia afforded less retinal protection in this model of glaucoma than in the other ischemic models. If we assume that hyperglycemia is acting to increase energy availability, the results imply that ischemialike mechanisms constitute a modest component of the injury in this glaucoma model and point toward a mechanical insult featuring transport disruption as a more significant primary event.

The present results are not in agreement with the conclusions drawn by Kanamori et al38 who found that hyperglycemia had an additive effect on the rate of retinal apoptosis induced by chronic elevation of the IOP in a rat model of glaucoma. However, there are explanations for the perceived inconsistencies. First, Kanamori et al38 elicited hyperglycemia for twice the length of time before induction of OHT than in our study, which may have affected the health of RGCs. Second, and most important, that study showed no evidence that diabetes actually increased RGC death. Not only was the number of eyes examined not stated, but the authors did not delineate whether TUNEL (terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate–biotin nick-end labeling) staining was associated with neurons or glia. Furthermore, axons and RGCs were not quantified. It is possible that streptozocin-induced diabetes prolonged the labeling period for TUNEL positivity, without actually causing greater cell loss.

We did not address possible molecular mechanisms responsible for the attenuation of axonal degeneration, but one such key mechanism could relate to the increased tissue availability of glucose as a metabolic substrate in experimental hyperglycemia. Baltan et al39 showed that the metabolic reserve was diminished in ONs of aged DBA/2J mice. Terada et al40 demonstrated delayed Wallerian degeneration in the transected sciatic nerve of diabetic rats.
CONCLUSIONS

We found a neuroprotective effect of hyperglycemia on the ON and RGCs in this model of experimental glaucoma. In light of this experiment, the results from the Ocular Hypertension Treatment Study,1,4,16 which suggested delayed glaucoma progression in patients with diabetes, seem plausible. Quigley16 has proposed several mechanisms by which underlying diabetes may protect the retina from glaucoma-related insults. These include the intracellular invasion of the trophic factors from leaky vasculature, localized episodes of hypoperfusion leading to localized ischemic preconditioning, or increased glycation of structural tissue elements such as collagen, culminating in increased connective tissue rigidity and prevention of pressure-related structural damage. Finally, we do not stipulate changes in clinical practice patterns in patients with glaucoma and diabetes mellitus. However, a bioenergetic approach to delay neurodegeneration is worth further pursuit.

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Author Contributions: Dr Ebneter had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Role of the Sponsor: The supporting institutions had no role in the design and conduct of the study; in the collection, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript.

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Online-Only Material: The eMethods section, eFigure, and eTable are available at http://www.archophthalmol.com.

Additional Contributions: Mark Daymon provided expert technical assistance.

REFERENCES


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**Ophthalmic Images**

Giant Conjunctival Mucosa-Associated Lymphoid Tissue Lymphoma

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Mika Noda, MD, PhD
Kan Ishijima, MD
Susumu Ishida, MD, PhD

Slitlamp examination of a 70-year-old man who had blurred vision and eyelid swelling for 2 years in both eyes (A [left eye] and B [right eye]). Giant salmon-pink conjunctival tumors obstructed the pupil and affected the patient’s vision. Histologically, the conjunctival tumors consisted of a monomorphous population of atypical B lymphocytes (C), positive for CD20 immunohistochemical stain (D) (original magnification x1100). DNA analysis obtained from tumor tissue by Southern blot demonstrated immunoglobulin heavy-chain gene rearrangement in the JH region (E, arrows). kb indicates kilobase; OD, right eye; and OS, left eye.