

# Cataractogenic Lens Injury Prevents Traumatic Ganglion Cell Death and Promotes Axonal Regeneration Both In Vivo and in Culture

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**PURPOSE.** To examine and quantify neuroprotective and neurite-promoting activity on retinal ganglion cells (RGCs) after injury of the lens.

**METHODS.** In adult albino rats, penetrating lens injury was performed by intraocular injection. To test for injury-induced neuroprotective effects in vivo, fluorescence-prelabeled RGCs were axotomized by subsequent crush of the optic nerve (ON) with concomitant lens injury to cause cataract. The numbers of surviving RGCs were determined in retinal wholemounts and compared between the different experimental and control groups. To examine axonal regeneration in vivo, the ON was cut and replaced with an autologous piece of sciatic nerve (SN). Retinal ganglion cells with axons that had regenerated within the SN under lens injury or control conditions were retrogradely labeled with a fluorescent dye and counted on retinal wholemounts. Neurite regeneration was also studied in adult retinal explants obtained either after lens injury or without injury. The numbers of axons were determined after 1 and 2 days in culture. Putative neurotrophins (NTs) were studied within immunohistochemistry and Western blot analysis.

**RESULTS.** Cataractogenic lens injury performed at the same time as ON crush resulted in highly significant rescue of  $746 \pm 126$  RGCs/mm<sup>2</sup> (mean  $\pm$  SD; approximately 39% of total RGCs) 14 days after injury compared with controls without injury or with injection of buffer into the vitreous body ( $30 \pm 18$  RGCs/mm<sup>2</sup>). When lens injury was performed with a delay of 3 days after ON crush, 49% of RGCs survived, whereas delay of 5 days still rescued 45% of all RGCs. In the grafting paradigm virtually all surviving RGCs after lens injury appeared to have regenerated an axon within the SN graft ( $763 \pm 114$  RGCs/mm<sup>2</sup> versus  $79 \pm 17$  RGCs/mm<sup>2</sup> in controls). This rate of regeneration corresponds to approximately 40% of all RGCs. In the regeneration paradigm in vitro preceding lens injury and ON crush 5 days previous resulted in a maximum of regeneration of  $273 \pm 39$  fibers/explant after 1 day and  $574 \pm 38$  fibers/explant after 2 days in vitro. In comparison, in control retinal pieces without lens injury  $28 \pm 13$  fibers/explant grew out at 1 day, and  $97 \pm 37$  fibers/explant grew out at 2 days in culture. Immunohistochemical and Western blot analysis of potential NTs in the injured lens revealed no expression of ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), NT-4, nerve growth factor (NGF), and basic fibroblast growth factor (bFGF).

**CONCLUSIONS.** The findings indicate that the lens contains high neuroprotective and neuritogenic activity, which is not caused by NT. Compared with the data available in the literature, this neuroprotection is quantitatively among the highest ever reported within the adult rat visual system. (*Invest Ophthalmol Vis Sci.* 2000;41:3943-3954)

It has been established that adult retinal ganglion cells (RGCs) of rodents serve as a suitable model to study injury-induced degeneration and regeneration in vivo<sup>1-3</sup> and in vitro.<sup>4-6</sup> The observed vulnerability of neurons to axotomy and their responsiveness to various target-derived neurotro-

phic factors (NTs) prompted the hypothesis that their fate may be effected by one of the various NTs. Indeed, external application of brain-derived neurotrophic factor (BDNF) and NT-4/5 to the explanted retina supports the survival of RGCs and promotes axonal regeneration.<sup>5,7,8</sup> In analogy, several trophic factors such as nerve growth factor (NGF),<sup>9</sup> basic fibroblast growth factor (bFGF),<sup>10</sup> BDNF,<sup>11,12</sup> NT-4,<sup>8</sup> and ciliary neurotrophic factor (CNTF)<sup>11</sup> prolong survival of RGCs when injected into the vitreous body after optic nerve (ON) sectioning and support regeneration in vitro.<sup>5,13,14</sup> By comparing various NTs in dissociated RGCs, it has been shown that of a number of factors, only CNTF shows neuritogenic effects.<sup>6</sup>

Other substances reported to influence survival of RGCs after intraocular injection were giant cell line-derived neurotrophic factor (GDNF),<sup>15</sup> inhibitors of cyp32-like proteases,<sup>16</sup> inhibitors of nitric oxide synthase,<sup>17</sup> and inhibitors of micro-

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glial cells.<sup>13</sup> Some of these NTs are produced by Schwann's cells<sup>11</sup> and may account for survival and regeneration in the peripheral nerve-ON grafting paradigm.<sup>1</sup> In addition to targeting of RGCs, bFGF has been intraocularly injected to rescue dystrophic photoreceptors in the rat strain of the Royal College of Surgeons (RCS).<sup>18</sup> Finally, several NTs were injected to rescue photoreceptors that undergo degeneration on light illumination.<sup>19</sup>

Neuroprotective manipulations may have been effective because of complex influences on the retina that involve non-neuronal cells in the retina or even nonretinal compartments. Injections of solvents or merely a heavy penetrating injury to the anterior portion of the eye strengthened the resistance of RGC to ON cut.<sup>12</sup> Similarly, experimentally induced intraocular inflammation supported survival of axotomized RGCs, therefore ascribing to immigrating macrophages a supportive role during regeneration.<sup>20</sup> Deactivation of resident intraretinal microglial cells<sup>13</sup> or increasing macrophage recruitment in the ON enhances the rate of RGC survival, whereas NT-4's effects on RGCs may be mediated by astrocytes.<sup>7</sup> Conceivably, several of the effective substances that are contained in injected media or are represented by the various neurotrophic molecules may prolong the survival of axotomized neurons by modulating the glial responses. Alternatively, they may induce intraocular responses with consecutive local transcription of effective factors other than those originally injected. This interpretation is strengthened by various reports that the rescue effects are limited to a proportion of RGCs, whereas there are almost no reports on additive effects. A mere mechanical injury to the eye induces, for example, transcription of FGF-2 and CNTF.<sup>21</sup>

The activation of intrinsic antiapoptotic genes is an alternative concept to the approach of external substitution with neurotrophic or neuroprotective agents. Members of the *bcl-2* family<sup>22</sup> have been reported to rescue such neurons in culture with dependence on neurotrophic factors.<sup>23</sup> In transgenic mice overexpressing *bcl-2*, a greater proportion of neonatal RGCs survive and extend axons in culture, although neuritegenesis was assumed to be mediated by a different mechanism than antiapoptosis.<sup>24</sup> RGCs also survived better in adult stages of *bcl-2* transgenic mice.<sup>25–27</sup> *Bcl-2* seems to regulate apoptosis within the lens epithelium, and lens-specific overexpression of *bcl-2* in transgenic mice inhibits lens cell death.<sup>28</sup> Also, NTs such as BDNF and NT-3 show a patterned expression within the lens epithelium.<sup>29</sup> These molecules are assumed to regulate differentiation and maintenance of ocular tissues including lens epithelia.

The experiments reported here were undertaken to answer the following questions in relation to penetrating injury of the lens: Is injury to the lens and subsequent opacity associated with survival of RGCs? If so, can the activity be attributed to identified NTs or other cell survival promoting factors? Is lens-derived activity due to neuroprotective and/or to neurotogenic activity? To achieve these goals, we performed three major series of experiments. First, we prelabeled RGCs from the superior colliculus (SC) and then axotomized them by crushing the ON and analyzed whether simultaneous or delayed lens injury resulted in greater survival of RGCs. In the second set of experiments, axonal regeneration was studied in a grafting paradigm *in vivo*. For this, the cut ON was replaced with an autologous piece of SN, and RGCs regenerating after lens injury were retrogradely labeled from the graft. Third, in a series of cell culture experiments, crush of the ON and lens

injury were performed to analyze whether the retinal stripes obtained from these eyes showed greater axonal regeneration in culture than control stripes. Finally, immunohistochemical and Western blot analyses were performed to detect candidate molecules involved to prevent neuronal death.

## MATERIALS AND METHODS

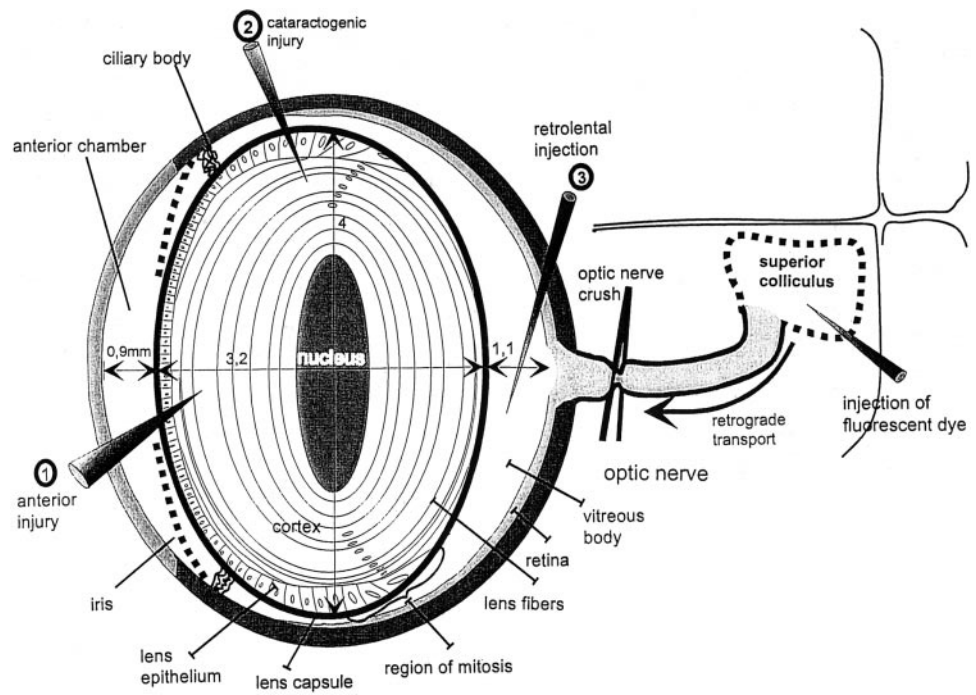
### Surgical Procedures

All experiments were performed with 94 adult rats of the Sprague-Dawley strain of either sex, weighing 180 to 220 g. The care and maintenance of the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For anesthesia, the animals received an intraperitoneal injection of a mixture of 12.5 mg ketamine sulfate (50 mg/ml; Parke-Davis, Morris Plains, NJ) and 0.1 ml xylazine (2%, Bayer, West Haven, CT) per 100 g body weight. To perform open crush on RGC axons, the left (ONs) were surgically exposed intraorbitally, and, after longitudinal incision of the meninges, the ONs were mechanically crushed (10 seconds) by means of a jeweller's forceps under a microsurgery microscope (OPMI 19; Carl Zeiss, Oberkochen, Germany). This surgery avoids damage of blood supply to the retina, because the central retinal artery and vein enter the retina within the nasal inferior meninges, which remain untouched by the crush. Because differences in the extent of injury may affect survival rates, all experiments were performed with animals in which nerves were injured 0.5 mm from the lobe.

### Effects of Lens Injury *In Vivo*

To search for lens-derived neuroprotection of RGCs, these neurons were identified by retrograde tracing. To do this, 48 rats received the fluorescent dye D291, *N*-4-[4-didecylaminostyryl]-*N*-methylpyridinium iodide (4Di-10ASP; Molecular Probes, Eugene, OR) into the SC. Figure 1 shows the anatomic relationships within the eye and the projection of ganglion cell axons through the ON to the SC. Eight days later, the ON contralateral to the injected SC was surgically exposed in its intraorbital aspect and mechanically crushed at 0.5 mm beyond the ON head for 10 seconds, as described earlier. The rats were then divided into six groups. The first group ( $n = 6$ ) received ON crush, intraocular penetration and injury of the lens (perforation routes 1 and 2, Fig. 1) containing the lens proliferative zone of the epithelium, and injection of 10  $\mu$ l phosphate-buffered saline (PBS, pH 7.4) according to standard protocols.<sup>13</sup> The second group ( $n = 6$ ) received ON crush, retrolenticular intraocular penetration (perforation route 3, Fig. 1), and injection of 10  $\mu$ l PBS without lens damage. A third group of control animals ( $n = 6$ ) received ON-crush, but no eye injection or lens injury. A fourth group ( $n = 18$ ) received ON-crush, with lens injury plus PBS (perforation route 2, Fig. 1) by 3, 5, or 7 days (six animals per subgroup) after ON-crush. The fifth group of control rats ( $n = 6$ ) received only retrograde labeling of RGCs but no further manipulation at the ON or in the eye. This group was used to determine the numbers of retrogradely filled RGCs, and the rats were killed 8 days after the staining procedure. A group of six rats underwent labeling only and were killed at 22 days after staining—that is, after the total study period of the other experimental groups—to assure that the long-term staining did not affect cell survival by itself. Finally, a group of five rats were treated the same as the animals

**FIGURE 1.** Experimental design of the present work. The graph shows the anatomic relations within the adult rat eye and indicates the various methods of perforation to approach and interfere with intraocular compartments. In addition, it schematically shows the projection of the retina to the SC and the procedure of labeling and ON crush. The intraocular distances and sizes of the different compartments are given in millimeters; the ON and its connection are not drawn to size.



of the fourth group, but without PBS injection to test the effect of the injection route alone.

In all groups, the corresponding retinas were ophthalmologically screened to exclude ischemia, dissected, flatmounted, fixed in 4% paraformaldehyde, embedded (Moviol; Merck, Darmstadt, Germany), and examined with a fluorescence microscope to detect labeled RGCs and phagocytotic microglial cells under the different experimental conditions.

Cell densities were determined with the aid of a fluorescence microscope (Axiovert; Carl Zeiss) equipped with an ocular grid of  $200 \times 275 \mu\text{m}$  (magnification,  $\times 400$ ). The results from the counts, which covered the total retinal surface, were averaged for every retina and were calculated to cell density values per area unit. To estimate the total number of RGCs per retina, the average was multiplied with the retinal surface, which was determined to be  $40 \text{ mm}^2$  at this age. From the mean densities of all retinas per group, mean values and SDs were calculated. Data of cell survival under the different treatments were compared with an analysis of frequency distribution. The results of the different conditions of injections were compared with the control condition by using Student's *t*-test. Data are presented as means  $\pm$  SD.

The rodent lens has an oval shape that fills most of the posterior eye cavity, thus providing limited space for intravitreal injections of larger amounts of material (Fig. 1). The approximate sizes of the intraocular cavities and compartments are indicated for an adult rat of approximately 200 g body weight in Figure 1. The perforation routes 1 and 2 of Figure 1 are commonly reported to be associated with intraocular injury. The only perforation route that avoids no direct injury to lens is route 3 in Figure 1, which passes through the posterior pole of the eye cup to approach the vitreous body transsclerally and transretinally. It appeared from the injections performed in the present study that the use of a pulled glass capillary of 20 to  $30 \mu\text{m}$  diameter is the most suitable method to obtain consistent results with all the perforation routes. All

perforation routes were tested in pilot experiments with injection of  $10 \mu\text{l}$  of PBS. However, the only method that consistently resulted in reproducible cataract was route 2.

To study axonal regeneration in vivo, the exposed ON ( $n = 7$ ) was cut and replaced with an autologous piece ( $\sim 2 \text{ cm}$  length) of sciatic nerve as reported earlier.<sup>1</sup> Two groups of animals were formed. In the first group ( $n = 4$ ) cataractogenic lens injury was performed. The animals of the control group ( $n = 3$ ) received grafting but no lens injury. In both groups rats were allowed to survive 14 days. In a second control group of three rats, 1 mg bovine serum albumen (BSA; Sigma, Munich, Germany) was injected into the vitreous body without lens injury. The purpose of this control was to monitor whether proteins within the vitreous body induce secondary effects that may result in better regeneration. At this stage the graft was surgically exposed again, and those RGCs whose axons had regenerated into the graft were retrogradely labeled, as described earlier.<sup>13</sup> The densities of RGCs were counted 5 days later in wholemounts by using the same conditions as were used for the survival studies.

### Pretreatment of Retina and Explantation

Lens-derived factors may promote axonal regeneration in culture, in addition to supporting ganglion cell survival and regeneration in vivo. The procedure of culturing was identical with that described previously.<sup>4,5</sup> In nine rats, the lens capsule was damaged by an injection using a microcapillary penetrating through the sclera (perforation route 2, Fig. 1). Control animals were rats that received no lens injury and rats ( $n = 3$ ) that received an intravitreal injection of 1 mg BSA through perforation route 3—that is, without lens injury. The retinas of the different groups were used to produce explants for axonal regeneration in vitro. The ganglion cell origin of the growing axons has been studied with various methods showing that only RGCs are able to extend axons under the conditions imposed in this study.<sup>4,5</sup> The numbers of regrowing axons



were determined after 24 and 48 hours *in vitro* by means of an inverted phase-contrast microscope (Axiovert; Carl Zeiss) with a  $\times 20$  lens.

As a measure of axonal growth, all axons were counted that encountered an imaginary line of between 50 and 100  $\mu\text{m}$  from the explant's margins. With increasing numbers of axons per explant, fasciculation occurred, in particular in the lens-damaged group. The fasciculation sometimes prevented the clear distinction of individual axons within bundles, even at larger microscope magnifications. The results from the single counts were averaged for each experimental group. To extrapolate over the total retina, the averaged number was multiplied by the number of explants per retina, usually eight. The data of regeneration under the different treatments were evaluated with Student's *t*-test. All data of cell densities or cells per retina are presented as mean  $\pm$  SD.

### Immunohistochemistry

Eyes obtained from damaged lens samples ( $n = 3$ ) and those from control ( $n = 3$ ) rats were examined for the appearance of molecules that are known to exert neuroprotective effects. The eyes were frozen in liquid nitrogen and cryosectioned for immunohistochemistry. The following monoclonal antibodies and antisera were used: antibodies to CNTF (dilution 1:100; Promocell, Heidelberg, Germany), NT-4 (dilution 1:200; Chemicon, Temecula, CA), NGF (dilution 1:100; Sigma), and bFGF (dilution 1:100; Promocell). To examine immigrating immunocompetent cells as result of the lens injury, CD45 antigen staining was performed on all rat samples (leukocyte common antigen [LCA] clone CD45; Serotec, Oxford, UK; dilution 1:200). In addition to the antibodies against NTs or leukocytes, glial markers were used to examine whether lens injury is associated with glial cell activation. Sections from the same samples were therefore processed to stain either astrocytes and Müller cells (glial fibrillary acidic protein, [GFAP] dilution 1:200–1:500; Boehringer-Mannheim, Mannheim, Germany) or intraretinal microglial cells (OX-42 antibody that recognizes the complement factor 3 receptor, dilution 1:500; Dako, Zug, Switzerland). Immunohistochemistry protocols were performed according to instructions of the suppliers. The secondary antibodies were coupled with fluorescein-isothiocyanate, and evaluation was performed with the aid of a fluorescence microscope equipped with photographic camera and computer-assisted image analysis.

### Biochemistry and Western Blot Analysis

The anterior halves of five lens capsules carrying the epithelium were dissected at the level of the equator, removed from the lens cortex with jeweller's forceps, and collected in PBS. Histologic examination of wholemount samples with hematoxylin stain revealed that with this method most of the lens epithelium remained attached to the capsule. Epithelium-containing capsules were homogenized with an ultrasound homogenizer (10 minutes; 20 W) in 200  $\mu\text{l}$  PBS. In addition to epithelium, five whole lenses were dissected into small pieces followed by ultrasound homogenization (5–10 minutes). After centrifugation at 7,500 rpm for 5 minutes, the supernatant was collected, and protein contents were determined photometrically with protein dye binding of Coomassie brilliant blue. Separation of proteins was performed with 14% sodium-dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) ac-

cording to standardized protocols (Slab gels; Bio-Rad, Hercules, CA). After SDS-PAGE, the gels were fixed and stained with the silver method, or used for Western blot analysis.

To immunologically identify neurotrophic factors, we separated five total lens homogenates including epithelium with SDS-PAGE in an electrophoresis chamber (Bio-Rad) using 1-mm-thick acrylamide gels. After SDS-PAGE the proteins were transferred to nitrocellulose membranes (0.45- $\mu\text{m}$  thick). The blots were blocked with 5% dried milk in Tris-buffered saline-Tween 20 (TBS-T) and processed for immunostaining with primary antibodies to the NTs (CNTF, NT4, bFGF, and NGF, dilution 1:2000 each). The monoclonal antibody to human BDNF (gift from Ilse Bartke, Roche Diagnostics, Panzberg, Germany) was diluted 1:2000 to 1:5000. Antigen antibody complexes were detected by enhanced chemiluminescence (ECL; Amersham, Amersham, UK).

## RESULTS

All rats that underwent surgery or repeated treatments for the different purposes of this study survived the procedures and did not exhibit pathologic behavior. Ophthalmoscopic examination of the eyes in the different groups did not reveal detectable changes (microphthalmia, infections, or ischemic lesions), unless such a change was experimentally induced, such as a lens injury that resulted in swelling, opacity, and cataractogenesis.

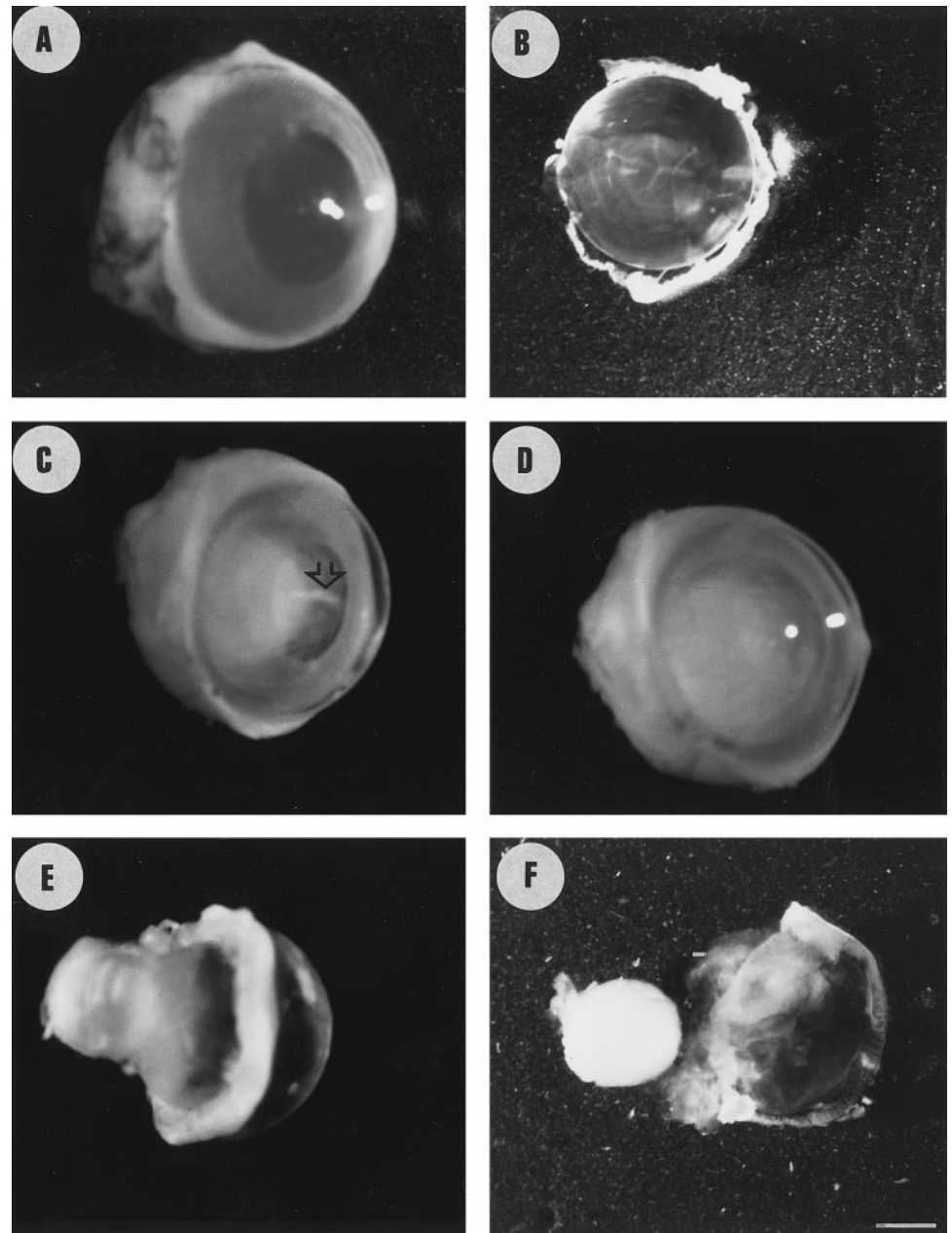
### Injury-Induced Cataractogenesis

On fundoscopic examination at the day of injection using a cornea applanation coverslip and a surgical microscope, perforation of the lens could be visualized easily and documented. Re-examination of the same animals was performed at the day of retinal explantation to prepare cultures or on the day when retinal flatmounts were prepared to count RGCs. In all cases, the degree of lens injury-induced swelling, nuclear opacity, and capsular rupture and the progression of cataract were documented and related to the method of eye penetration.

It appeared that perforation route 1 through the cornea and the pupil to damage the anterior lens capsule did not induce cataract during the 14 days of analysis. Nor did perforation route 3 induce any cataract (Fig. 2A), and the extracted lenses were found to be translucent and free of opacity (Fig. 2B). Such transparency was independent of whether buffer was injected into the vitreous. In contrast, most lenses that received injury through perforation route 2 showed a fast reduction or loss of transparency (within 1 day), swelling of lens cortex, and a progressive cataractous opacity in the lens nucleus (Figs. 2C, 2D). Cataractogenesis continued during the 14 days of observation and reached the typical shapes and morphologies shown in Figures 2C and 2D and in Figures 2E and 2F after lens extraction. The anterior part of the cortex of such lenses could be described as crystalline and transparent, although they also showed some signs of swelling and moderate opacity (Figs. 2E, 2F). In control animals ( $n = 3$ ), perforation route 2 was used to damage only the ciliary body without affecting the lens capsule.

### Lens Injury-Induced Rescue of Axotomized RGCs

Deposition of the fluorescent dye 4 Di-10ASP into the SC (Fig. 1) resulted in dye uptake by retinofugal terminals and retro-



**FIGURE 2.** Morphologic appearance of the lenses after different treatments. (A) Anterior-lateral view of the transparent rat eye after retrolenticular intravitreal injection of PBS without lens injury. (B) The same transparent lens after dissection and extraction viewed from its posterior pole. (C, D) Lateral-anterior view of two eyes with retrolenticular injection of PBS and simultaneous lens injury through perforation route 2. In both cases the lens nucleus was opaque. (C, arrow) Penetration channel of the needle. (E) The same lens as in (C) after dissection of the posterior eye segment. The lens nucleus was cataractous and protruded through the lateral posterior capsule, whereas the anterior lateral lens cortex was still transparent. (F) The same lens as in (D) shown after dissection. The cataractous nucleus was separated from the noncataractous but swollen cortex. Scale bar, 1 mm.

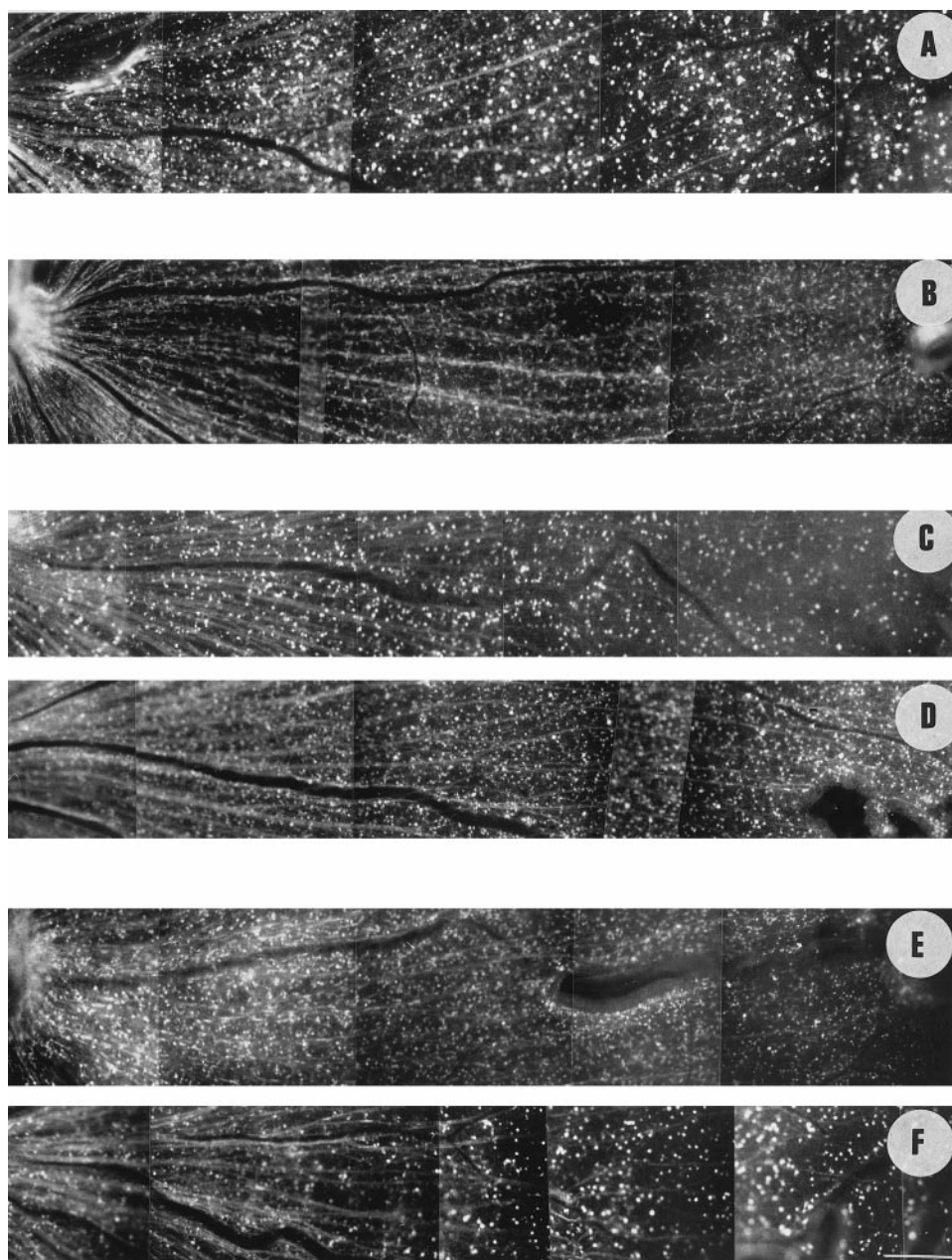
grade transport. The images of retinal wholemounts prepared 8 days later (Figs. 3A, 4A) showed a uniform distribution of ganglion cells all over the retinal quadrants and over the whole retinal eccentricity (Fig. 3A). Considering typification of RGCs, the major types and sizes of cells were consistently represented. Determination of RGC density revealed that  $1,935 \pm 78$  RGCs/mm<sup>2</sup> or  $77,400 \pm 3,120$  RGCs/retina (Fig. 5) were labeled, thus confirming that most RGCs had taken up and transported the dye. In control retinas intended to exclude potential toxic effects of the dye itself and analyzed at 22 days after dye injection without further treatment, no decrease of this cell density or leakage of the dye was observed. This result indicated no toxicity or damage of the fluorescent cells by the substance.

Crush of the ON contralateral to the site of the dye injection after 8 days ( $n = 6$ ) and microscopic examination of the retina 14 days after the crush without any injection provided

the retinal images shown in Figure 3B. Identical images were obtained when PBS was injected into the retrolenticular space ( $n = 6$ ; perforation route 3, Fig. 1). In both groups the treatment resulted in strong reduction of RGC numbers and in coverage of the whole retinal surface with microglial cells that become fluorescent after phagocytosing the dying RGCs (Figs. 3B, 4B). They are easily distinguishable on the basis of their small sizes and their shapes (Fig. 4B). The rationale of determining microglial cells, was also that these cells are only visible after phagocytosis. Thus, their numbers can be used as an additional indicator of the degree of degeneration of prelabeled RGCs. The numbers of RGCs and microglial cells are shown in Figure 5.

In the experimental series used to analyze and quantify effects of lens injury, the lens was perforated according to perforation route 2 (Fig. 1), because pilot experiments using the noncataractogenic perforation routes 1 and 3 did not show





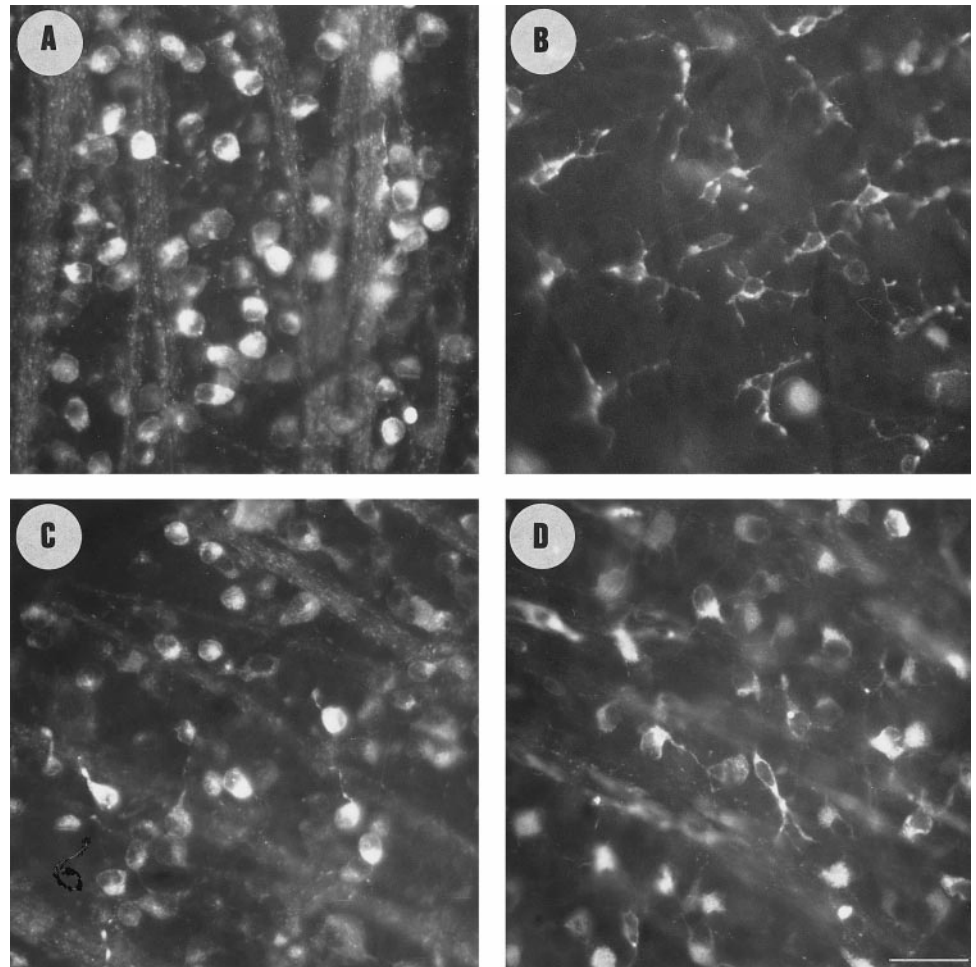
**FIGURE 3.** Photomontages of retrogradely labeled and treated retinas. In all montages the ON head is located on the *left*, the retinal periphery on the *right*. These magnifications do not allow for distinction between RGCs and microglial cells (see Fig. 4). (A) Normal retina: RGCs were retrogradely filled with 4Di-10ASP from the contralateral SC. (B) Retina was labeled from the SC, and the ON was crushed 8 days later. Note the uniform distribution of microglial cells along the degenerated optic fiber layer. (C, D) Two retinas that were labeled from the SC were crushed after 8 days and received lens injury on the day of ON crush. Note the numerous surviving RGCs in comparison to (B). (E, F) Two retinas that were treated the same as those in (C) and (D) but received a lens injury 5 days after the ON crush. All retinas from (B) through (F) were observed at the same time (14 days) after crush. Note the high numbers of surviving RGCs. Scale bar, 100  $\mu$ m.

a marked effect on survival of RGCs. Also the pilot experiments using perforation route 2 to damage the ciliary body and pars plana did not reveal higher incidence of cell survival in the retina (data not shown). It appeared that ON crush and concomitant or delayed cataractogenic lens injury resulted in massive protection of retrogradely labeled RGCs (Figs. 3C, 3D). Morphologically intact RGCs (Fig. 4C) were uniformly distributed over the whole retinal surface and over its total eccentricity (Figs. 3C, 3D). Quantitatively,  $746 \pm 126$  RGCs/mm<sup>2</sup> or  $29,840 \pm 5,040$  RGCs/retina were present (that is, approximately 39% of the total population;  $n = 6$ , Fig. 5). In the same retinas, labeled microglial cells were reduced to  $288 \pm 74$ /mm<sup>2</sup> or  $11,520 \pm 2,960$  RGCs/retina (Fig. 5). The number of rescued RGCs could even be increased, when the cataractogenic lens injury was performed with a delay of 3 or 5 days after ON crush. In these cases 49% (3 days' delay) and 45% (5 days' delay) of all RGCs survived (Fig. 5). The 7 days' delayed

lens injury rescued fewer RGCs (Fig. 5). In the cases with delayed injuries (5 and 7 days), the peripheral retina contained as many viable cells (Fig. 3F) as the control retina (Fig. 3A). These cells are morphologically shown in Figures 3E and 3F and enlarged in Figure 4D. The difference between the procedures with concomitant injuries was the significantly higher density of ganglion and microglial cells ( $467 \pm 294$  RGCs/mm<sup>2</sup>; Fig. 5), which indicated that some phagocytosis was performed at days 5 and 7 after crush.

### Regeneration of Retinal Ganglion Cell Axons

To assess whether lens injury promoted survival of ganglion cells with regenerative properties (proof of viability), axonal regrowth was studied *in vivo* (grafting example) and *in culture* by using a chemically defined medium containing neither serum nor neurotrophic substitutes.<sup>4,5</sup>



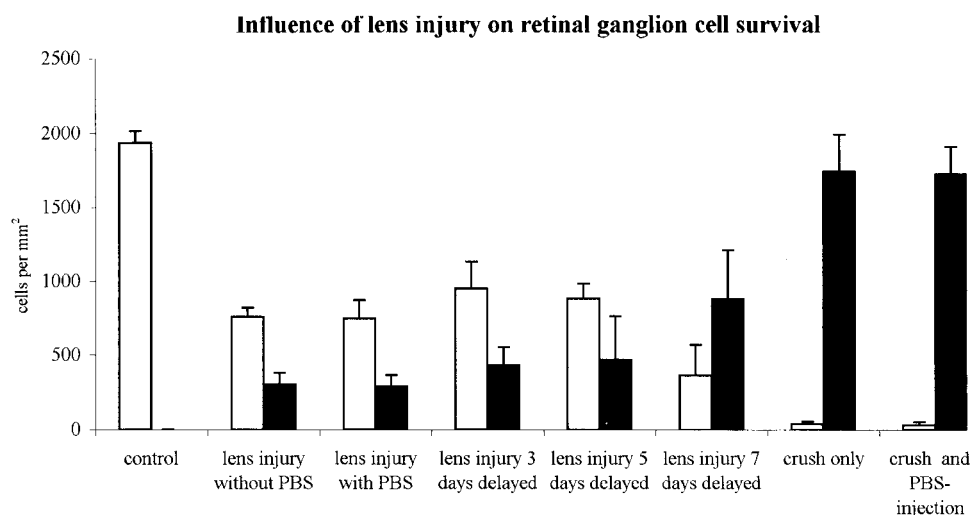
**FIGURE 4.** Higher microscopic magnifications of selected retinal regions in the photomontages in Figure 3 show how RGCs could be discerned from microglia. (A) RGCs, but no microglial cells, in the normal retina of Figure 3A. (B) Microglial cells that became fluorescent while phagocytosing degenerating RGCs in the axotomized retina shown in Figure 3B. (C) Surviving RGCs rescued after crush of the ON and simultaneous lens injury of Figure 3C. (D) Surviving RGCs were easily distinguishable from microglial cells in the retina, which received delayed lens injury, in Figure 3C. Note the comparable numbers of RGCs in (C) and (D). Scale bar, 25  $\mu\text{m}$ .

In the grafting paradigm *in vivo*, lens injury resulted in cataract that was visible in all animals of this group ( $n = 4$ ). Deposition of the fluorescent dye 4Di-10ASP into the graft 14 days after grafting and examination of the flatmounted retinas 5 days later revealed that fluorescent, and thus regenerating, cells were distributed across the retina (Figs. 6A through 6D). All regions of the retina contributed to regeneration, whereas a slight central-to-peripheral decrease in cell density was observed (Figs. 6A, 6C). Quantitatively, an average of  $763 \pm 114$  RGCs/ $\text{mm}^2$  ( $n = 4$ ) were retrogradely labeled across the retinal surface (Fig. 6E). Extrapolated over the total retinal surface, this number corresponds to  $30,520 \pm 4,560$  RGCs/retina, which is approximately 40% of the total population. This number is 10 times higher, and thus significantly higher ( $P < 0.001$ ), than that in the control group ( $79 \pm 17$  RGCs/ $\text{mm}^2$  or  $3,160 \pm 680$  RGCs/retina ( $n = 3$ ; Fig. 6E). The comparison of this number of regenerating RGCs with the numbers of surviving RGCs under conditions of cataractogenic lens injury (compare with Fig. 5) shows that it is likely that all surviving neurons were able to regenerate their axons into the SN graft.

In the explantation paradigm, most retinal stripes obtained from 18 retinas remained attached to the substrate and were examined for axonal growth at 1 and 2 days in culture. In the first hours in culture, axons grew out and increased in length and number. Measurements of axon numbers were relatively easy and reproducible during the first day *in vitro*. However, a preceding lens injury increased dramatically both

the number of axons and the degree of their fasciculation (Figs. 7A, 7B), rendering it difficult to count single axons easily. A quantitative analysis of the fibers grown under different experimental conditions is shown in Figure 7C. In control retinal explants from crushed ONs without lens injury, an average of  $28 \pm 19$  axons/explant or  $224 \pm 152$  axons/retina ( $n = 72$  explants from nine retinas) was determined after 1 day, and  $97 \pm 34$  axons/explant or  $776 \pm 272$  axons/retina after 2 days in culture (Fig. 7C). Cataractogenic injury of the lens at the day of ON crush further increased the numbers of axons both after 1 day ( $273 \pm 39$  axons/explant or  $2184 \pm 312$  axons/retina) and 2 days ( $574 \pm 38$  axons/explant or  $4592 \pm 304$  axons/retina;  $n = 72$  explants from nine retinas) in culture (Fig. 7C). Compared with the massive regeneration *in vivo*, significantly fewer axons regenerate in culture.

To limit the probability that secondary immunologic responses to intravitreal proteins may influence the regeneration of axons, the following control experiments were performed. In three rats that received grafting of the SN and injection of BSA without lens injury,  $131 \pm 30$  RGCs/ $\text{mm}^2$  had regenerated, which is not significantly higher than in noninjected control animals ( $121 \pm 21$ , Fig. 6). In three rats that received BSA injection, no lens injury, and explantation  $79 \pm 30$  axons/explant grew *in vitro*. This was comparable with PBS-injected control retinas (Fig. 5). Both control experiments indicate that proteins within the vitreous body did not induce immunologic responses with effects relevant to increased regeneration.



**FIGURE 5.** Quantitative numbers of ganglion and microglial cells as shown in Figures 3 and 4. Controls were retrogradely labeled  $1935 \pm 78$  RGCs/mm<sup>2</sup> representing the total population at the beginning of the experiment. Crush of the ON and injection of PBS reduced these number to  $30 \pm 18$  RGCs/mm<sup>2</sup> with parallel increase of phagocytotically stained microglial cells by a factor of approximately 25. Crush of ON and simultaneous lens injury causing rupture of capsule and cataract resulted in a 25-fold increase of RGCs ( $746 \pm 126$  cells/mm<sup>2</sup>) and, as expected, reduction of labeled microglial cells. Lens injury without PBS injection had indistinguishable effects on survival ( $760 \pm 60$  cells/mm<sup>2</sup>), indicating the pronounced role of causing cataract through perforation route 2. Crush of the ON with lens injury delayed by 3 or 5 days resulted in even stronger rescue of RGCs ( $997 \pm 185$  cells/mm<sup>2</sup> at 3 days' delay and  $879 \pm 102$  cells/mm<sup>2</sup> at 5 days' delay). An expected decrease of survival ( $363 \pm 205$  cells/mm<sup>2</sup>) was observed when injury occurred with 7 days' delay. These values are statistically significant compared with control retinas ( $P < 0.01$ ) and with simultaneous lens injury ( $P < 0.05$ ).

When processed for immunohistochemistry with the CD45 antibody no leukocyte immigration could be seen in the retina or other intraocular compartments as a result of lens injury. However, Müller cells responded to the lens injury with increased GFAP content, and intraretinal microglial cells within different layers responded with expression of the C-3 receptor as shown with the OX-42 antibody (data not shown).

#### Potential Neuroprotective Substances within the Lens

To analyze whether some known neurotrophic factors are produced and released from the injured lens, immunohistochemical analysis was performed on cryostat sections, and Western blot analysis in SDS gels. It appeared that immunohistochemistry was negative when antibodies were used against CNTF, NT 4/5, bFGF, NGF, and BDNF. Western blot analysis confirmed these data (Fig. 8) and supported the view that lens injury did not release immunodetectable amounts of either of the factors investigated.

#### DISCUSSION

We used the axotomized RGCs as a model to analyze lens-derived substances with neuroprotective activity *in vivo* and neurite-promoting activity *in vitro*. The principal new findings of the present study were: (1) It was the lens injury and not the simple perforation of the anterior eye that caused release of neuroprotective agents. (2) Moreover, only cataractogenic lens injury with capsule rupture, epithelium damage, and swelling resulted in extensive survival of axotomized RGC *in vivo*. (3)

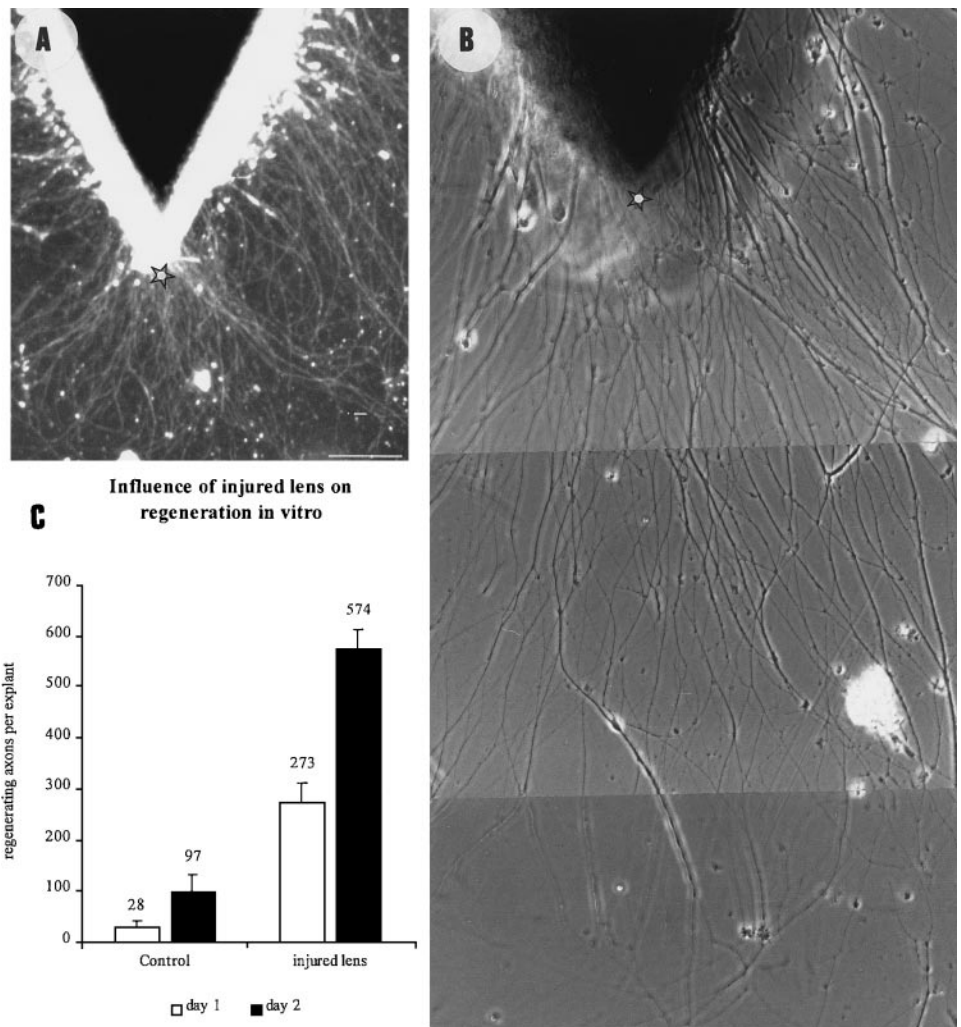
All surviving RGCs are capable of regenerating axons *in vivo* when the ON is replaced with a piece of SN. (4) Explantation of retinal pieces from eyes with posttraumatic cataract resulted in a marked increase of the numbers of regenerating axons in culture.

#### Survival and Regeneration of Axotomized RGCs In Vivo

Perforation of the ocular tissue through the cornea, or through the pars plana-ciliary body, or through the posterior sclera without lens injury did not increase the numbers of surviving RGC, thus indicating that opening of the lens capsule is essential to release the intralens trophic agents. The findings do not completely exclude the appearance and the possible release of additional factors from such nonlens tissues as iris, cornea, ciliary body, vitreous body, uvea, and retina. The different routes of penetration and the combinations of lesions are best explained by the selective release of lens factors when injury was followed by onset of swelling and cataractogenesis. Immunologic responses cannot be completely excluded, although the injection of BSA into the vitreous body did not cause regeneration-relevant responses. In addition, the failure to immunohistochemically detect CD45 antigen on leukocytes in the retina is a further line of evidence against a massive immigration of these cells in this experimental paradigm.

The greater survival of RGCs after targeted lens injury and cataractogenesis is consistent with previous observations that show that penetrating lesions causes release of neurotrophic activities. Moreover, it has been reported<sup>12</sup> that eye injury through an anterior approach has a more beneficial effect than





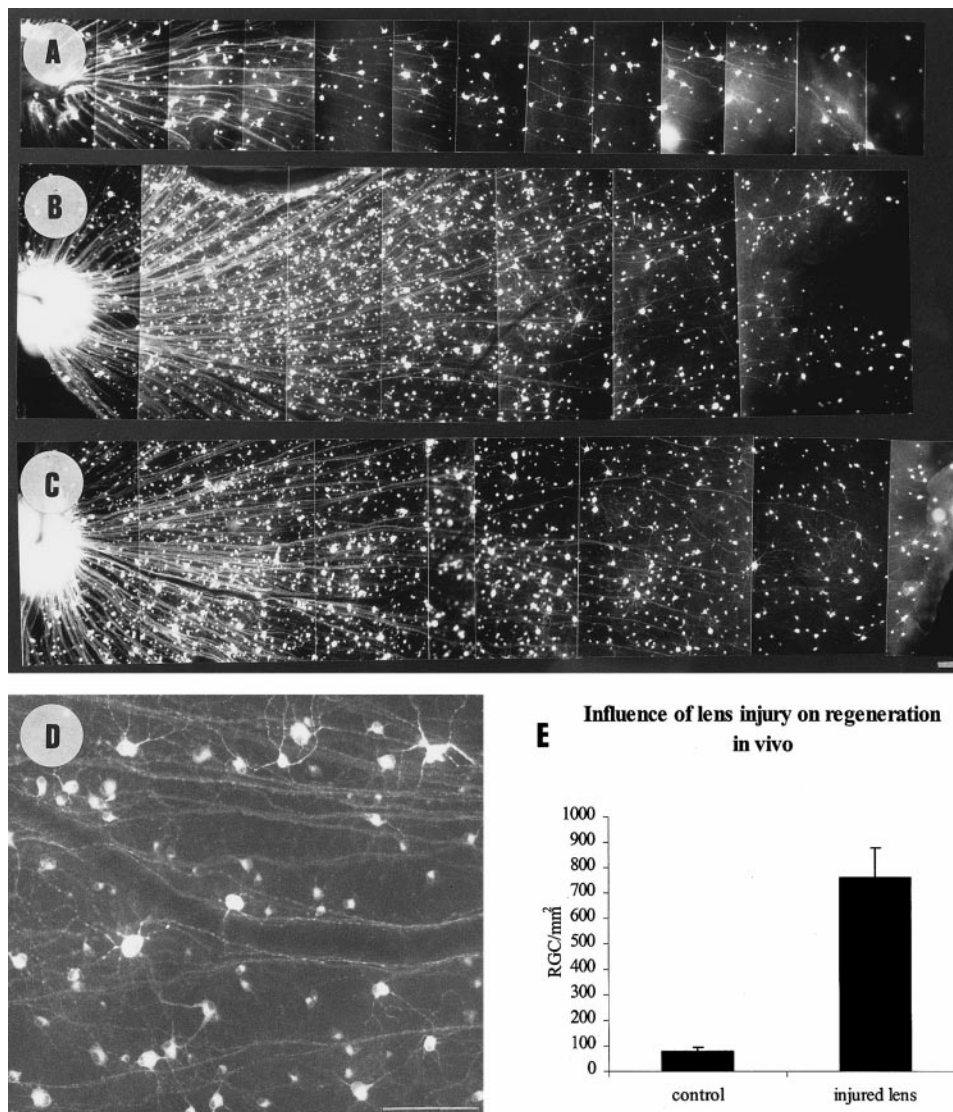
**FIGURE 6.** Regenerating RGCs in the grafting paradigm after cataractogenic lens injury. (A) Montage from whole-mounted control grafted retina ranging from the ON head (*left edge*) to the periphery (*right edge*). (B, C) Montages from retinas after grafting and simultaneous lens injury. *White* area on the *left* shows the ON head with the axon bundles spreading toward the individual RGCs. In all photographs, each fluorescent spot represents one RGC. (D) Larger magnification showing individual RGCs of the different types and sizes. (E) Average numbers of RGCs regenerating under control and cataractogenic conditions. All data were obtained at 18 days after grafting. Scale bar, 50  $\mu$ m.

that performed through a posterior approach. Although we cannot conclude from their description whether they used perforation route 1 or 2, these investigators may have used a combination of perforation routes 1 and 2. To localize the source of lens-derived trophic activities within a certain intraocular compartment, we used a fine glass capillary instead of a 26-gauge needle and injured the lens without affecting the iris or the ciliary body. In addition, we compared three perforation routes that allowed an exact localization of the lens injury, ranging from noncataractogenic retrolenticular injection or anterior capsule rupture to induction of cataractogenic changes of varying degrees. It clearly appeared that induced lens swelling and cataractogenesis enhanced RGC survival, but not intravitreal injection or puncture of lens capsule without traumatic cataract.

That delayed lens injury preferentially affected survival of the RGC in the peripheral half of the retina can be explained with the central-to-peripheral gradient in the progression of death after ON crush. Indeed, more peripheral neurons were rescued with delayed lens injury. In the same context, more microglial cells compared with those found after simultaneous injury may indicate onset of phagocytosis before the fifth day. The most likely interpretation is that the event of lens swelling initiates the release of neuroprotective factors. Most likely, because RGCs enter the apoptosis at days 4 to 6 after ON

transection,<sup>2</sup> delayed lens injury can retard this apoptotic cascade even when performed 5 days later than nerve lesion. The results do not exclude, however, that other intraocular structures such as the iris or ciliary body may contribute secondarily to the production of neurotrophic agents in response to the phacolytic events. This is of particular relevance, because NT-4 and BDNF mRNAs were detected in the iris.<sup>12</sup> BDNF and NT-3 mRNAs are thought to play a regulatory role in the developing mouse eye including the lens epithelium.<sup>29</sup> However, the Western blot analysis performed in the present work did not reveal detectable protein levels of BDNF in the lens homogenates. This is explicable by either a downregulation of protein in the adult lens or by low-level protein production not detectable by the monoclonal antibodies. Cellular candidates for release of such factors may be Schwann cells in the iris,<sup>30</sup> iris muscle cells,<sup>31</sup> or glial cells that are activated after lens injury, or even immigrating immune cells. Regardless of which cells produce these additional neurotrophic or protective factors, it appears from the present work that the lens injury and not the ocular injection itself initiates their production, and thus causes the marked survival effects on RGCs.

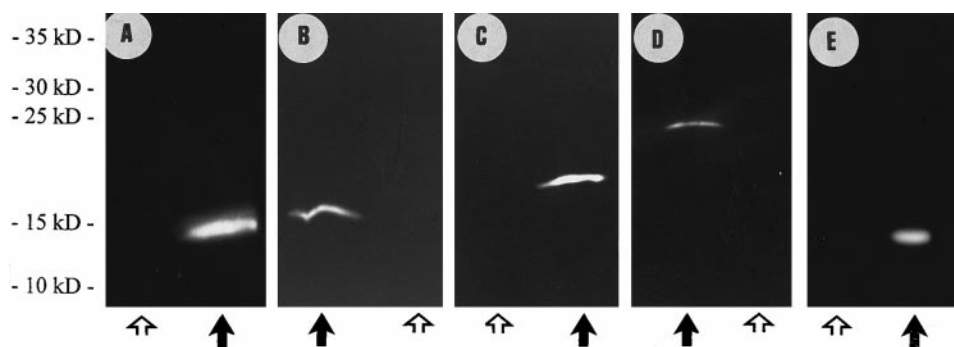
In a number of ganglion cell rescue studies, it has been reported that intraocular injection of either of several molecules results in prolonged survival of ganglion cells. These studies include injection of NGF,<sup>9</sup> bFGF,<sup>10</sup> BDNF, CNTF,<sup>11,32</sup>



**FIGURE 7.** Photomicrographs showing a retinal explant obtained from a retina 5 days after ON crush and lens injury causing lens opacity, as shown in Figure 2D. (A) Low-power phase contrast shows at 24 hours in vitro a massive regrowth of axons predominantly at the explant's tip (*arrow* toward the bottom), which corresponds to the position of the ON head. (B) Higher magnification of the same explant showing the density and length of axons. This particular explant showed more than 1400 fibers. (C) Quantitative analysis of axons counted after 1 and 2 days in culture. The numbers of axons taken from lens-injured eyes was increased by approximately a factor of 10 compared with control retinas.

and inhibitors of caspases.<sup>16,17</sup> In addition, intraocular injection of CNTF results in better axonal regeneration within a peripheral nerve graft.<sup>14</sup> However, the exact methodologies of injection and in particular whether injection was accompanied with lens injury is not described in any of these studies. The only description of performing such injury in connection with survival was in a study by Mansour-Robaey et al.,<sup>12</sup> whereas further experiments applying intravitreal injections were per-

formed without lens injury and using transscleral perforation route 3.<sup>11,13</sup> Injections of neurotrophic factors to rescue retinal photoreceptors in dystrophy models were performed with bFGF and CNTF.<sup>18,19</sup> Although these investigators also reported higher survival with injection of PBS, there is no clear determination of whether this effect was due to lens injury or not. The present immunohistochemical and Western blot studies did not show abundant production of any of the aforemen-



**FIGURE 8.** Western blot analysis showing the search for some NTs in lens homogenates. (A) Comparison between 10 ng NGF (*open arrow*) and 250 µg homogenate (*filled arrow*). Comparison of (B) NT-4, (C) bFGF, (D) CNTF, and (E) BDNF (10 µg each; *open arrows*) with 250 µg lens homogenate (*filled arrows*) in each lane. None of the NTs was detectable.



tioned factors in the normal or the injured lens. However, these factors may be produced at lower concentrations not detectable with the methods used in the present study. Although transfer of peripheral nerve pieces into the vitreous body resulted in regeneration of ganglion cell axons within the severed ON,<sup>33</sup> this study does not report whether the lens was injured.

The lens is an evolutionary highly conserved tissue that subserves refractive functions and therefore contains very high amounts of soluble structural proteins called crystallins.<sup>34,35</sup> The complete encapsulation of the tissue keeps it protected from vascularization, whereas the subcapsular epithelial layer serves as a stem cell population to recruit equatorial addition of new fibers (Fig. 1). A remarkable feature of this process of cell proliferation and transformation of cells into fibers occurs by incomplete autophagy and subsequent elongation of the crystallin-containing fibers.

The transformation occurs throughout life with the crystallins being the structural proteins in vertebrates with the longest turnover half-life. In addition to functioning as source of transparent lens fibers, the lens epithelial cells contain high amounts of factors that are essential to the protection of the tissue from light-induced damage—for instance solar UVA and UVB irradiation.<sup>36–38</sup> Such cell survival factors may account for the neuroprotection of ganglion cells as well.<sup>22–25</sup> The importance of lens factors in rescuing retinal cells has also been demonstrated by showing that lensectomy and vitrectomy but not vitrectomy alone decrease the rate of photoreceptor loss in transgenic pigs.<sup>39</sup> It seems therefore reasonable to be aware of accidental lens injury and avoid it, if the scope of a study is targeted to intraocular injection of neuroprotective or neurotrophic agents to rescue retinal cells in the various retinodegenerative diseases.

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