BDNF Diminishes Caspase-2 but Not c-Jun Immunoreactivity of Neurons in Retinal Ganglion Cell Layer after Transient Ischemia

Toru Kurokawa, Naomi Chi Katai, Hiroto Shibuki, Sachiko Kuroiwa, Yasuo Kurimoto, Chikao Nakayama, and Nagahisa Yoshimura

PURPOSE. Retinal ischemia-reperfusion injury induces apoptosis of retinal neurons. The purpose of this study was to examine the association of c-Jun, caspase-1, -2, and -3 immunoreactivities and neuronal apoptosis in the retinal ganglion cell layer (GCL) and to study the effects of intravitreal brain-derived neurotrophic factor (BDNF) on the expression of these gene products in a rat model of retinal ischemia-reperfusion injury.

METHODS. After 60 minutes of ischemia, eyes were enucleated after 3, 6, 12, 24, and 168 hours of reperfusion. The numbers of c-Jun-, caspase-1-, caspase-2-, caspase-3, and TdT-dUTP terminal nick-end labeling (TUNEL)-positive cells in the GCL were counted. Recombinant human BDNF (5 μg) or vehicle was injected intravitreally immediately after reperfusion. At 6, 24, and 168 hours, the numbers of immunoreactive cells in BDNF- and vehicle-treated groups were compared.

RESULTS. Expression of c-Jun and caspase-2 was found in dying cells in flat-mounted retinas. The numbers of caspase-1- and caspase-3-positive cells were fewer than c-Jun- or caspase-2-positive cells. Cell death in the retinal GCL was suppressed by an intravitreal injection of BDNF. The numbers of TUNEL- and caspase-2-positive cells were lower in the BDNF-treated group at 6 hours after reperfusion (P < 0.01). The number of c-Jun-positive cells in the treated retinas was not altered by the treatment.

CONCLUSIONS. Expression of c-Jun and caspase-2 is associated with neuronal cell apoptosis in the GCL. Suppression of caspase-2 expression may explain the neuroprotective effects of BDNF. (Invest Ophthalmol Vis Sci. 1999;40: 3006–3011)

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that suppresses neuronal death from various injuries. Although little is known about how BDNF protects neurons from such injuries, it is likely that it modifies some mechanism(s) in the pathway of cell death. Retinal ganglion cell (RGC) death occurs in several ocular and systemic diseases including glaucoma, ischemic optic neuropathy, and Alzheimer’s disease. The RGC death in these diseases has been shown to be by apoptosis. In neuronal apoptosis, the caspase family proteases play an important role in the execution phase. Although BDNF is known to suppress RGC death, it is not clear how BDNF prevents apoptosis and whether BDNF suppresses the activation of caspase family proteases.

To investigate the mechanism of RGC death, experiments have been carried out on the rat axotomy and on the retinal ischemia-reperfusion models. In these models, the expression of immediate early gene products such as c-Jun is found in dying cells. We have shown previously that expression of cell cycle-related gene products such as c-Jun and cyclin D1 takes place in the dying neurons, especially those in the inner nuclear layer.

The retinal ischemia-reperfusion model is known to induce apoptosis of cells in all layers of the retina, and we used this model to study the mechanism of cell death. We chose to study the expression of cell cycle-related gene products that have been shown to be associated with cell death and to study only cells (RGCs and amacrine) in the retinal ganglion cell layer (GCL). We shall show that cell death after ischemia-reperfusion injury in the retinal GCL is associated with the expression of c-Jun and caspase family proteases and describe the neuroprotective effects of BDNF.

MATERIALS AND METHODS

Animal Ischemia Model

Adult female Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan) weighing 160 to 180 g (n = 350) were used. The experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were anesthetized by intraperitoneal pentobarbital (50 mg/kg), and retinal ischemia was induced by cannulating the anterior chamber with a 27-gauge needle connected to a bag containing normal saline. The intraocular pressure was increased to 110 mm Hg for 60 minutes by elevating the bag. The temperature of the rats was kept at 37°C by a heating pad. Sham operations were performed without increasing the intraocular pressure.

Antibodies

The following antibodies were used: rabbit anti-mouse c-Jun/ AP-1 (N) antibody raised against a peptide that corresponds to amino acids 91–105 mapping within the amino terminal domain; goat anti–caspase-1 (M-19) antibody against amino acids 3–21 mapping to the amino terminus of the 20-kDa subunit of caspase-1 of mouse origin; goat anti–caspase-2 (N-19) antibody against amino acids 3–21 mapping to the amino terminus of the precursor of caspase-2 of mouse origin.
and goat anti–caspase-3 (L-18) antibody against amino acids 157–174 mapping to the carboxyl terminus of the 20-kDa subunit of the protease precursor of human origin. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Tissue Processing and Immunohistochemical Studies

At 3, 6, 12, 24, and 168 hours after reperfusion, rats were deeply anesthetized and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The eyes were enucleated, and the retinas were separated from the eyecups in plastic petri dishes containing PBS. The retinas were then fixed for 2 hours in 4% paraformaldehyde. After rinsing in PBS, retinas were incubated for 30 minutes at room temperature with 0.2% Triton-X in PBS in plastic petri dishes.

For the detection of caspase-1 and caspase-3 immunoreactivities, the retinas were incubated with NeuroPore (Trevigen, Gaithersburg, MD) for 30 minutes (caspase-1) or 3 hours (caspase-3) at room temperature. The retinas were then rinsed with PBS and incubated with rabbit anti-mouse c-Jun antibody (1 μg/ml), goat anti–caspase-1 antibody (20 μg/ml), goat anti–caspase-2 antibody (20 μg/ml), or goat anti–caspase-3 antibody (20 μg/ml) for 48 hours at 4°C. The working concentrations of antibodies were determined after preliminary experiments with various concentrations. The retinas were then rinsed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or anti-goat IgG antibody for 75 minutes.

To count the numbers of cells in the retinal GCL, the retinas were stained with propidium iodide (PI, 20 μg/ml) for 10 minutes at room temperature, and the retinas were flat-mounted vitreous side up on glass slides. The retinas in which the primary antibodies were omitted were used as negative controls. Positive controls were generated by using retinal sections or flatmounted retinas in which these proteins were known to be expressed.3,5

TdT-dUTP Terminal Nick-End Labeling

After fixation, the retinas were subjected to three cycles of freezing and thawing and then incubated overnight with NeuroPore in plastic petri dishes. The DNA nick-end labeling was performed by a Mebstain apoptosis kit (MBL, Nagoya, Japan). The retinas were incubated with TdT and biotinylated dUTP in TdT buffer for 2 hours at 37°C, then rinsed with termination buffer containing 30 mM sodium chloride and 3 mM sodium citrate for 30 minutes at room temperature followed by rinsing with PBS. The retinas were incubated with avidin–FITC for 2 hours at 37°C and rinsed with PBS. After PI staining, the retinas were flat-mounted on glass slides. Positive controls were generated by incubating the specimens with DNase1 in water (1 μg/ml, for 1 hour at 37°C) before incubation with TdT and biotinylated dUTP. The control eyes without ischemia-reperfusion injury were used as negative controls.

Quantitative Analysis

The flatmounted retinas were photographed with a scanning laser confocal microscope (model LSM 410; Zeiss, Oberkochen, Germany) using a green filter to detect FITC and a red filter for PI; the focus was in the retinal GCL plane. The numbers of FITC-labeled c-Jun– and caspase-positive cells, TdT-dUTP terminal nick-end labeling (TUNEL)–positive cells and PI-stained cells were counted in six areas (0.2 3 0.2 mm), 1 and 2 mm away from optic disc and every 30° of the circle in each quadrant. Thus, data from 24 areas from one eye were obtained. Regions with thick nerve fiber or blood vessels were avoided, and a more central or peripheral area was chosen. Of the PI-stained cells, white blood cells, which have fragmented nuclei, and red blood cells, which have oval-shaped and spindle-shaped endothelial cells, were excluded from cell counts. The cell count was done by two examiners in a masked fashion. Data are expressed as the number of cells per square millimeter at each time point, and results are expressed as mean ± SEM.

Retrograde Labeling of RGCs

To label the RGCs, rats were anesthetized and placed in a stereotaxic frame (Narishige, Tokyo, Japan). A part of the skull and cerebral cortex was removed to expose the superior col-
liculi bilaterally. Multiple injections of neurotracer, FluoSpheres, or 1% Dil (Molecular Probe, Eugene, OR) were made in different regions of each superior colliculus with a glass micropipette attached to a 1-µl Hamilton syringe, at a depth of 0.5 or 1.0 mm. Seven days later, ischemia-reperfusion injury was induced, and the eyes were treated as described for TUNEL and immunostaining.

**Intravitreal Injection of Brain-Derived Neurotrophic Factor**

Recombinant human brain-derived neurotrophic factor (BDNF; N-terminal methionine-free, 2.0 mg/ml in 10 mM sodium phosphate and 150 mM NaCl, pH 7.0) was obtained from Regeneron Pharmaceuticals (Tarrytown, NY). Immediately after reperfusion, 2.5 µl (5.0 µg) of BDNF was injected into the vitreous cavity with a 5-µl Hamilton microsyringe attached to a 30-gauge needle. In the control group, 2.5 µl of vehicle was injected. Care was taken not to injure the lens and retinal vessels, and eyes that exhibited any complications were excluded. At 6, 24, and 168 hours after reperfusion, rats were deeply anesthetized, and the eyes taken for immunohistochemical and TUNEL studies as described.

**Statistical Analysis**

Statistical analysis was done using a two-way ANOVA followed by Fisher’s post hoc test. P < 0.05 was considered statistically significant.

**RESULTS**

**Cell Numbers in the Retinal GCL**

The number of cells in the retinal GCL decreased from 3250.2 ± 131.3 cells/mm² in sham-operated eyes (mean ± SEM, n = 5) to 2098.2 ± 45.3 at 6 hours, to 1858.5 ± 63.2 at 24 hours, and 1439.2 ± 50.8 at 168 hours after reperfusion (Fig. 1).
TUNEL Staining and Immunohistochemical Studies

TUNEL-positive cells were present at 3 hours after reperfusion (442.5 ± 47.2 cells/mm², n = 5), and the number peaked at 6 hours (545.5 ± 67.0; Fig. 1). Cells that were positive for c-Jun or caspase-1, -2, and -3 immunoreactivities were also present at 3 hours after reperfusion (Fig. 1). Among these four antibodies, anti-c-Jun and anti-caspase-2 showed consistent and strong immunostaining. Much weaker staining was obtained with anti-caspase-1 and anti-caspase-3 antibodies. The number of immunoreactive cells was highest at 6 hours for c-Jun at 202.2 ± 24.0 cells/mm² (n = 5), for caspase-1 at 51.2 ± 12.5 (n = 5), for caspase-2 at 230.2 ± 15.7 (n = 5), and for caspase-3 at 60.3 ± 9.8 (n = 5; Fig. 1). Expression of c-Jun and the caspases was also confirmed in retrogradely labeled RGCs (Fig. 2). Pertinent to this study, the expression of c-Jun and the caspases was found in cells with shrunken cell bodies and condensed chromatin (i.e., dying cells; Fig. 2).

Intravitreal Injection of BDNF

In the vehicle-treated group, the numbers of cells in the retinal GCL were 2126.8 ± 38.2 cells/mm² at 6 hours, 1970.8 ± 56.7 at 24 hours, and 1432.5 ± 78.2 at 168 hours after reperfusion (Fig. 4). After intravitreal BDNF, there were 2370.3 ± 122.5 cells/mm² (n = 5) at 6 hours (P < 0.05, compared with vehicle treatment), 2310.0 ± 51.3 at 24 hours (P < 0.01), and 2261.8 ± 16.1 at 168 hours (P < 0.01; Figs. 3 and 4). Thus, there were 10.3% more neurons in the retinal GCL at 6 hours, 14.7% more at 24 hours, and 36.7% more at 168 hours in the BDNF-treated animals.

Without BDNF treatment, the number of TUNEL-positive cells was 545.2 ± 29.7 cells/mm² (n = 5) at 6 hours, and the number was 557.4 ± 67.7 (n = 5) with BDNF. This difference was statistically significant (P < 0.01; Figs. 3 and 4). At 24 hours after reperfusion, however, the number of TUNEL-positive cells was not markedly different from the controls (Fig. 4).

The number of c-Jun-positive cells in the control group was 226.5 ± 24.3 cells/mm² (n = 5) at 6 hours after reperfusion and decreased to 68.0 ± 14.3 at 24 hours (n = 5; Figs. 3 and 4). The number of c-Jun-positive cells in the BDNF-treated group was 224.8 ± 24.9 cells/mm² (n = 5) at 6 hours and 82.0 ± 18.1 at 24 hours (Figs. 3 and 4). These values were not significantly different from those of the controls.

The number of caspase-2-positive cells in the vehicle control also showed a peak at 6 hours after reperfusion (244.6 ± 15.7 cells/mm²; n = 5) and decreased to 57.8 ± 12.6 at 24 hours (Fig. 4). In the BDNF-treated groups, there were fewer caspase-2-positive cells than in the vehicle group: 124.4 ± 35.4 cells/mm² (n = 5) at 6 hours and 35.4 ± 11.2 at 24 hours (Figs. 3 and 4). The difference between the control and BDNF-treated groups at 6 hours was statistically significant (P < 0.01). In the quantitative study of caspase-1-positive and caspase-3-positive cells, there were no statistically significant

**FIGURE 3.** Cells in the retinal GCL in a flatmounted retina stained with PI. (A) Sham operated followed by intravitreal injection of buffer. (B) Intravitreal injection of BDNF. (C through H) 6 hours after reperfusion. Green represents TUNEL staining (C and D), c-Jun immunostaining (E and F), and caspase-2 immunostaining (G and H). Colabeling of condensed PI and immunoreactivity yielded yellow color as found in (C) and (D). The number of c-Jun-positive cells is not different between the vehicle-treated control group (E) and BDNF-treated group (F). The number of TUNEL-positive or caspase-2-positive cells was decreased in the BDNF-treated group (D and H) compared with the buffer-treated group (C and G). (I and J) 168 hours after reperfusion. The number of total cells in the retinal GCL in BDNF-treated group (J) is larger than in the untreated group (I). Scale bar, 25 μm.
differences between these groups and the BDNF-treated group (graphic data not shown).

**DISCUSSION**

These results showed that c-Jun and the caspase family proteases, especially caspase-2, were expressed in cells in the retinal GCL that had morphologic and histochemical signs of dying cells (Figs. 2 and 3). Although the observations made with the retrograde labeled retinas suggest that the dying cells were mainly RGCs, we cannot rule out amacrine cells as being part of this group. In any case, these observations suggest an association between the cell death and c-Jun and/or caspase-2 expression. This relationship agrees with the role played by c-Jun in cell death in the central and peripheral nervous systems and in the RGC death in the axotomy model.2

Among the caspase family of proteases, expression of caspase-2 was detected in the highest number of cells, with much fewer cells expressing caspase-1 and -3. There is good evidence that caspase-2 induces apoptosis,7,8 and more recently, an independent processing of caspase-2 and upregulation of caspase-3 activity have been shown in trophic factor–deprived PC12 cell death. This type of cell death required caspase-2 activation, and the upregulation of caspase-3–like activity was neither necessary nor sufficient to induce cell death.9 Our data are in keeping with these findings.

Expression of c-Jun and caspase-2 was already present at 3 hours after reperfusion (Fig. 1), and their peak expression was seen at 6 hours. Thus, most of the c-Jun- and caspase-2-related...
cell death occurred within 24 hours after reperfusion, which also corresponds to the time course of TUNEL-positive cells (Fig. 1). However, there were a number of TUNEL-positive cells that did not express c-Jun or caspase immunoreactivities. One possible explanation for this discrepancy is that the window for the expression of c-Jun and caspases is shorter than the window to detect dying cells by TUNEL staining. Another possible explanation is that cell death may occur independent of c-Jun expression, caspase expression, or both.

The protective effects of BDNF were evident as early as 6 hours after reperfusion (Fig. 4). Intravitreal injection of BDNF decreased the number of TUNEL-positive cells in the GCL, but the number of c-Jun–positive cells remained unchanged. This suggests that BDNF does not influence the expression of c-Jun, which agrees with De Felipe and Hunt,10 who suggested that BDNF does not regulate c-Jun expression in damaged neurons. However, BDNF did suppress the expression of caspase-2, both directly and indirectly. Similar regulation of caspase-2 expression by nerve growth factor occurred in sympathetic neurons and PC12 cells.8

The relationship between c-Jun- and caspase-2–dependent pathways is not known: They may be distinct pathways or they may be linked. In this experiment, the number of caspase-2–positive cells was significantly decreased by BDNF but not the c-Jun–positive cells. These findings suggest two hypotheses: One is that c-Jun and caspase-2 are downstream of the same signal in both the c-Jun and caspase-2 steps and the other is that c-Jun and caspase-2 have distinct pathways and BDNF only blocks expression of caspase-2. From our experiments, it is not possible to select which of these hypotheses is correct because we do not know whether a single cell expresses c-Jun and caspase-2 sequentially. Expression of c-Jun and caspase-2 was detected in cells with a shrunken cell body and condensed chromatin (i.e., morphological signs of cell death; Figs. 2 and 3). Thus, even if BDNF works between c-Jun and caspase-2, it is unlikely that the process of apoptosis can be reversed. We hypothesize that c-Jun–dependent and caspase-2–dependent cell death occurs via distinct pathways and that the cascade associated with caspase-2 is blocked by BDNF but c-Jun–dependent pathways are not.

In this study, retrograde labeling of the RGCs was not done routinely; instead, we used confocal microscopy and counted the number of cells stained with PI in the retinal GCL. Because amacrine cells are also found in the retinal GCL, their presence will alter the overall numbers of cells. Thus, the cell counts presented represent both RGCs and amacrine cells. Even when the RGCs were labeled retrogradely, it was not easy to distinguish cells that lost the dye due to the destruction of cell body from displaced amacrine cells (Fig. 2). Because our analysis consisted of the cell numbers in the retinal GCL, and because our conclusions depended on changes in the cell numbers, the presence of amacrine cells should not alter our conclusions.

In summary, we have shown that the expression of c-Jun and caspase-2 is associated with cell death in the retinal GCL in a rat ischemia-reperfusion injury model. BDNF had a neuroprotective effect on cell death, possibly by suppressing caspase-2 expression, but had no influence on c-Jun expression. Although the pathway linked to caspase-2 is suppressed by BDNF, there may exist other pathways through which BDNF works.

References
Antioxidant Pattern in Uveal Melanocytes and Melanoma Cell Cultures

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PURPOSE. To investigate the antioxidant status of cultured uveal melanocytes from patients with uveal melanoma and uveal melanoma cells to characterize some of the biochemical properties of these cells in respect to the normal cutaneous melanocytes.

METHODS. The fatty acid pattern of membrane phospholipids, intracellular vitamin E level, and superoxide dismutase (SOD) and catalase activities were studied in uveal melanocytes (n = 10) and uveal melanoma cell (n = 10) cultures, by gas chromatography mass spectrometry or by spectrophotometer.

RESULTS. Among the uveal melanocyte cultures, two groups were differentiated, according to catalase activity: group A with catalase values comparable to those of cutaneous ones and higher SOD activity and group B with catalase values 2 SD lower (P < 0.001) and lower SOD activity. Vitamin E concentration was not significantly different between melanoma cells and melanocytes, whereas a significantly higher percentage of polyunsaturated fatty acids was found in melanoma cells and the B group of melanocytes (P = 0.022). In uveal melanoma cells SOD activity was significantly lower than that detected in uveal melanocytes (P < 0.005).

CONCLUSIONS. These results show a different pattern of antioxidants in uveal melanocytes with respect to cutaneous ones, possibly related to the anatomic distribution. However, as in cutaneous melanocytes, two subgroups were identified on the basis of the antioxidant pattern that could be the expression of a constitutional increased susceptibility to oxidative stress in some subjects. Moreover, an imbalance of the antioxidants was observed in melanoma cells, possibly related to the disease status and progression. (Invest Ophthalmol Vis Sci. 1999;40:3012–3016)

METHODS

Xanthine, xanthine oxidase, and nitro blue of tetrazolium (NBT) were from Sigma (St. Louis, MO). Ham’s F-10 medium, fetal calf serum (FCS), and antibiotics were provided by Gibco (Paisley, Scotland, UK). Butylated hydroxytoluene, tricosanoic acid (C23:0), N,O-bis(trimethylsilyl)trifluoro-acetamide and trimethyl chorsilane, sodium methoxide, and other reagents and solvents were from Merck AG (Darmstadt, Germany) and were of the highest purity grade.

Cell Cultures

Normal uveal melanocytes from uveal melanoma patients (UM, n = 10) were collected from eyes, enucleated for large choroidal and ciliary body melanomas, at the site diametrically opposed to the tumor, and uveal melanoma cells (UMC, n = 10) were taken from the same eyes. Uveal melanocytes and uveal melanoma cells were isolated after mechanical and enzymatic dissection and cultured as previously described for cutaneous melanocytes.5-4 Cells were cultured in Ham’s F-10 medium, with 5% FCS and 5000 U/ml penicillin and 5000 ng/ml streptomycin, and in melanocyte cultures, bovine pitu-
itary gland extract (50 µg/ml) was added. Subconfluent cultures at the fourth or fifth passage were studied. The batch of FCS was the same for all the experimental periods and was analyzed for fatty acid pattern and vitamin E level.

Antioxidant Enzyme Assays

Cells (4 × 10⁶) were collected and sonicated in phosphate-buffered saline (pH 7.4; Gibco; 1 ml) and centrifuged at 10,000g for 10 minutes at 4°C. Enzymatic activities were evaluated by a spectrophotometer (Beckman DU 70) on cell supernatants. Catalase activity was determined by the disappearance of hydrogen peroxide. One unit of catalase was defined as the amount that degrades 1 µmol of H₂O₂, and 1 unit of SOD was defined as the amount of enzyme that produces 50% inhibition of NBT reduction by superoxide produced by xanthine-xanthine oxidase system. One unit of catalase was defined as the amount that degrades 1 µmol of H₂O₂, and 1 unit of SOD was defined as the amount of enzyme that produces 50% inhibition of NBT reduction. The activities of cell supernatants were compared with the known purified enzymes and then calculated on each supernatant, and experiments were repeated twice. The results are reported as the mean of different determinations and expressed as unit/10⁶ cells. For each group of cultures data are reported as mean ± SD.

Vitamin E Analysis

Cells (4 × 10⁶) were extracted three times in hexane-ethanol (3:1) with 1% sodium dodecyl sulfate in the presence of 25 µg of γ and δ tocopherols as internal standards. Tocopherols were derivatized with N,N-bis-(trimethylsilyl)-trifluroaceticamide with 1% trimethyl chlorosilane as catalyst and were analyzed by gas chromatography mass spectrometry on SPB1 column (30 m × 0.20 µm ID, 0.25 mm; Supelchem) by a selected ion(s) monitoring technique. Analyses were repeated twice in each extract, and a SD less than 1% was found. Results are expressed as nanograms per 10⁶ cells.

Polyunsaturated Fatty Acid Analysis

Cell pellets were extracted twice in chloroform:methanol (1:1) in the presence of butylated hydroxy toluene (50 µg) as antioxidant and 25 µg tricosanoic acid ethyl ester as internal standard. The fatty acids of phospholipid fraction were transmethylated with sodium methoxide in methanol and analyzed by gas chromatography mass spectrometry on capillary column (FFAP, 60 m × 0.32 µm × 0.25 mm; Hewlett-Packard). The results were obtained after time integration of the chromatogram and final processing of the peak areas. The identity of each fatty acid was determined by comparing the mass spectrum of the peaks with those obtained using reference standards.

Statistical Analysis

Student’s t-test was used to determine the statistical significance. Statistical significance was accepted as P < 0.05. The correlation existing was studied by linear regression analysis and r values were calculated by the Pearson test.

Morphologic Analysis

Cell cultures of normal uveal melanocytes and uveal melanoma cells were seeded into 2% gelatin-coated tissue Transwell and cultured in complete melanocyte medium. Seventy-two hours afterward cells were washed three times in PBS and incubated for 60 minutes at 4°C in fixative buffer (5% milk, 0.01% Tween 20, 0.5 M NaCl, and 2% glutaraldehyde). After fixative treatment, samples were postfixed in 1% osmium tetroxide in Veronal acetate buffer (pH 7.4) for 2 hours at 4°C, stained with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812. Thin sections were examined both unstained or poststained with uranyl acetate and lead hydroxide.

RESULTS

The clinical and histologic features of the lesions of the studied cases are reported in Table 1. The cultures obtained were studied at subconfluence, at the fourth or fifth passage grown in medium containing the same batch of fetal calf serum, so that the amounts of vitamin E, external fatty acids, and essential elements were the same in each culture. The levels of antioxidants were expressed as units per 10⁶ cells or nanograms per 10⁶ cells, to minimize differences among results due to cell size and shape and to compare the values with those previously obtained in normal cutaneous melanocytes.

Electron microscopic examinations revealed pure cultures of melanocytes and melanoma cells defined on the basis of the presence of characteristic markers such as melanosomes and melanin granules, respectively. No significant differences were observed among the cultures of melanocytes, whereas spindle and epithelioid cells were observed in melanoma cultures.

Antioxidant Pattern

In uveal melanocytes mean catalase activity was 1.06 ± 0.81 U/10⁶ cells, a value not significantly different from that reported in cutaneous melanocytes (CM) (Table 2), but the elevated SD suggested a wide range of variability in this population. A similar situation was reported in a previous work of ours, in cultures of cutaneous melanocytes from the apparently normal skin of subjects with melanoma. Two populations with different morphologic and biologic proprieties were differentiated according to catalase activity. Therefore, using the same scheme, two groups were identified among those with uveal melanocytes (UM): In the first one (UM-A, of 10), catalase activity was 1.79 ± 0.42

**Table 1. Clinical and Histological Data**

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* According to COMS criteria.
U/10^6 cells, a value similar to that observed in normal cutaneous melanocytes. On the contrary, in the second one (UM-B, 5 of 10), catalase activity was 2 SD lower (0.33 \pm 0.065 U/10^6 cells, P \leq 0.001; Table 2). Mean SOD activity in those with UM was 1.12 \pm 0.38 U/10^6 cells, significantly higher than that previously observed in normal cutaneous melanocytes; however, the mean in the UM-A group was 1.42 \pm 0.22 U/10^6 cells, whereas in UM-B it was significantly lower at 0.81 \pm 0.21 U/10^6 cells (P < 0.01, Table 2).

Levels of vitamin E were 1.15 \pm 0.15 ng/10^6 cells in UM-A and 1.04 \pm 0.73 ng/10^6 cells in UM-B. Both values were significantly lower than those previously observed in normal cutaneous melanocytes (Table 2).

In UM, SOD activity was 0.34 \pm 0.11 U/10^6 cells, significantly lower than that observed in the UM-A and UM-B groups, respectively (P < 0.005). In the same cells, catalase activity was 0.42 \pm 0.18 U/10^6 cells, a value significantly lower than that observed in UM-A but not dissimilar from that found in UM-B (Table 2).

In UMC mean vitamin E concentration was 3.09 \pm 2.68 ng/10^6 cells, higher but not statistically different from that observed in both UM-A and UM-B even if with a wider range of variability (Table 2). A relationship was found between lipophilic and enzymatic antioxidants, with a significant direct correlation between SOD/catalase ratio and vitamin E level (R = 0.73, P = 0.015; Fig. 1).

<table>
<thead>
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<th>Table 2. Antioxidants and PUFA Percentage in Cutaneous Melanocytes, Uveal Melanocytes, and Uveal Melanoma Cells</th>
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Each result represents the mean of 2 experiments in duplicate.

\* P < 0.05 versus UM-A.
\† P < 0.001.

Fatty Acids of Cell Membranes

To study the peroxidizable components of cell membranes, the fatty acid pattern of membrane phospholipids was analyzed. In all the cultures, among saturated fatty acids, palmitic (C16:0) and stearic acids (C18:0) were the most representative; oleic acid (C18:1 n9) was the main monounsaturated and linolenic acid (C18:2 n6), C20:3 n6, arachidonic acid (C20:4 n6) and C22:6 n3 the polyunsaturated fatty acids (PUFAs). Arachidonic acid was the most representative long-chain PUFA present in phospholipid fraction. In the UM-A group, PUFA percentage, compared with total fatty acids analyzed, was 7.9\% \pm 4.37\%, lower than those of cutaneous melanocytes, whereas in the UM-B group the PUFA percentage was 21.49\% \pm 7.02\% (P < 0.001). A significant direct correlation was found between the PUFA percentage and the SOD/catalase ratio (r = 0.69), indicating that the increased concentration of peroxidizable compounds was associated with an imbalance in the enzymatic antioxidant activities (Fig. 2). In UMC the PUFA percentage was 18.16\% \pm 5.02\% (Table 2), a value not statistically different from that observed in the UM-B group.

Discussion

Our study demonstrates that cultured uveal melanocytes from patients with melanoma, compared with cultured normal cu-
taneous melanocytes,3,4 have a higher level of enzymatic antioxidant activities. In particular, SOD activity appears to be significantly higher, possibly related to the high O₂ tension found in the choroidal district. SOD, in fact, is important in clinical situations such as in reperfusion after ischemia, where huge amounts of superoxide anion (O₂⁻) are generated and need to be dismutated by this enzymatic activity.

More interestingly, as previously described in cutaneous melanocytes from melanoma patients,3,4 two subgroups of uveal melanocytes were identified on the basis of catalase activity: one with values comparable to those observed in cutaneous melanocytes (UM-A) and the other with significantly lower values (UM-B). Consequently, in the B group, the SOD/catalase ratio, which is correlated with the susceptibility of the cells to a peroxidative stress,5 was significantly increased compared with that of group A. The SOD/catalase ratio imbalance represents an alteration of the scavenger system. Antioxidants, in fact, interact in a complex fashion, so that changes in the...
concentration or activity in one component can affect the whole system. SOD dismutates superoxide anion radicals, generating hydrogen peroxide and oxygen. Catalase and glutathione peroxidase (GSH-Px) are the main enzymes involved in removing H$_2$O$_2$. If the production of H$_2$O$_2$ overwhelms the activities of these latter enzymes, in the presence of transitional metals (Fe$^{2+}$, Cu$^{+}$), H$_2$O$_2$ becomes a substrate of the Fenton reaction, giving rise to extremely toxic and mutagenic hydroxyl radicals (HO$^·$). Moreover, the imbalance of the SOD/catalase ratio was associated with an increased PUFA percentage in the cell membranes of UM-B, suggesting that these cells are more susceptible to the deleterious effects of prooxidants. In vitro, cutaneous melanocytes with alteration of the SOD/catalase ratio, in fact, undergo a significant proliferation after treatment with a low concentration of cumene hydroperoxide. Moreover, cell cultures deficient in catalase activity showed an increased DNA alteration after exposure to peroxidizing agents. Therefore, the imbalance of the intracellular antioxidants has been considered as a possible additional risk factor for the development of melanoma.

In uveal melanoma cells, instead, a significant decrease of SOD activity, compared with that of uveal melanocytes, was detected, whereas catalase activity, even if lower, was not significantly modified. An increased level of the polyunsaturated component of cell membranes and vitamin E concentration was observed. The higher vitamin E level is likely to be a compensatory mechanism adopted by the cells, at least in vitro, to reduce the intracellular oxidative events. In fact, in UMC a significant direct correlation was observed between the SOD/catalase ratio and the vitamin E level. These results are in agreement with previous data that demonstrated the correlation among the differentiation status, antioxidant systems, and percentage of PUFA in cultured cells: the higher the proliferation rate, the less differentiated the cells; the lower the total antioxidant protection system, the higher the PUFA percentage. However, no correlation was found between alteration of the antioxidant pattern and the melanoma cell type.

The source of ROS for the development of cutaneous melanoma could be UV exposure, but uveal melanocytes, especially those embedded in the ciliary body, are reached by low amounts of UV radiation; therefore, different free radical sources should be considered. Uveal melanocytes are tightly connected with the vascular bed and the oxidative insults might be related to hemodynamic changes. Alterations in blood flow can induce free radical release, and free radical-mediated injury has been involved in the pathophysiological alterations observed during ischemia and reperfusion.

Episodes of choroidal ischemia-reperfusion may take place many times during a lifetime without causing clinical manifestations, because of the reservoir in choroidal blood flow. In conclusion these data demonstrate that an alteration of the antioxidant pattern can be detected in uveal melanoma cells, as well as in cutaneous ones, possibly related to the disease status and progression. Moreover, data obtained from apparently normal uveal melanocytes suggest that in some subjects a constitutional imbalance of the antioxidant system, detectable by a decrease of catalase activity, can exist. This could be the basis for increased susceptibility to free radical-mediated damage and possibly to the development of uveal melanoma. As for skin melanoma, we can suggest that an individual predisposition together with environmental and general risk factors could play an important role in tumor onset and that the occurrence of the uveal melanoma might be the expression of acute repeated damages.

**References**


Retinal Expression of \( \gamma \)-Crystallins in the Mouse

Stephen E. Jones, Catherine Jomary, John Grist, Jayneeta Makwana, \(^1\) and Michael J. Neal

**Purpose.** High levels of expression of a form of \( \gamma \)-crystallin mRNA in mouse retina have been identified. Because the six murine \( \gamma \)-crystallins have generally been regarded as specific to the lens, the expression of these crystallins at the mRNA and protein levels in the retina were evaluated in more detail.

**Methods.** Expression of \( \gamma E/F \)-crystallin mRNA was examined by northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) analysis applied to murine retinal and lens total RNAs. For \( \gamma A-D \)-crystallin mRNAs, a multiplex RT-PCR was used on total cDNAs. The detection of total \( \gamma \)-crystallin protein in the retina was performed using an antibody to bovine lens \( \gamma \)-crystallins, applied to protein extracts in immunoblot analysis and to cryostat sections of ocular tissues in immunofluorescence studies.

**Results.** By RT–PCR, we confirmed expression of both \( \gamma E \) and \( \gamma F \)-crystallin as well as all four (\( \gamma A-\gamma D \)) remaining crystallins at the mRNA level in the mouse retina. \( \gamma \)-Crystallin proteins were also detectable in murine retina by immunoblot analysis, although at a lower level than in the lens. By immunocytochemistry, \( \gamma \)-crystallins were localized particularly to the inner retina, outer plexiform layer, and the photoreceptors during postnatal development.

**Conclusions.** Our findings of \( \gamma \)-crystallin mRNA and protein expression in the retina indicate that none of the major crystallin classes is uniquely expressed in the lens. The expression of \( \gamma \)-crystallins in the developing murine retina suggests a role analogous to the anti-stress properties established for the small heat-shock protein \( \beta \)-crystallin, perhaps in response to varying exposure to light.


The \( \alpha \), \( \beta \), and \( \gamma \)-crystallins comprise the three main families of major soluble proteins ubiquitously expressed in vertebrate lenses. Together with the taxon-specific members, crystallins appear to have evolved from preexisting proteins that were recruited to new structural roles in the lens.\(^1\) Thus, the \( \alpha \)-crystallins, \( \alpha A \) and \( \alpha B \), resemble heat-shock proteins with chaperone functions; the \( \beta/\gamma \) superfamily shows similarities to bacterial spore coat protein and to *Physarum* species stress-induced proteins; and the taxon-specific crystallins are closely related or identical to certain metabolic enzymes such as lactate dehydrogenase and aldose reductase. Although extraocular expression has been established for \( \alpha \)- and \( \beta \)-crystallin families, the expression of \( \gamma \)-crystallins (of which there are 6, \( \gamma A-\gamma F \) in the mouse) has generally been considered lens specific, based particularly on transgenic mouse studies using a \( \gamma \)-crystallin promoter to drive reporter gene expression.\(^2\) To date, there have been three reports providing evidence of extralenticular \( \gamma \)-crystallin expression. These are of the mRNAs in the developing *Xenopus laevis* tadpole, occurring at very low levels throughout much of the embryo,\(^3,4\) and, most recently, evidence for a seventh member of the \( \gamma \)-crystallin family, \( \gamma S \)-crystallin, expressed in murine lens, retina, and cornea.\(^5\) Such evidence has provided initial indications that none of the crystallins classes can be characterized as lens specific.

During the course of an analysis of retinal gene expression patterns occurring in wild-type and retinal degeneration (rd) mice, using the technique of mRNA differential display, we identified a gene showing high levels of expression in retinal RNAs. The cloned product corresponded to a partial \( \gamma E \)-crystallin cDNA and prompted us to examine further the expression of the \( \gamma \)-crystallin family in the mouse retina. Our evidence indicates that none of the \( \gamma \)-crystallins is uniquely expressed in the lens and suggests that nonrefractive functions for these proteins may be retained in the retina.

**Materials and Methods**

**Tissues**

C57BL/6(J) or nondegenerative rd/+ strain mice were reared in a cyclic 12-hour light/12-hour dark environment and were cared for and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were the source of tissues for RNA and protein extraction and for immunohistochemical analyses. Postmortem eyes, enucleated during the light phase, were either dissected to retrieve the lenses and the retinas (the latter free of any lens tissue), for subsequent storage in liquid nitrogen until RNA extraction or at \(-70^\circ C\) for protein extraction, or fixed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer overnight, followed by embedding, freezing, and cryosectioning as previously described.\(^6\)

**RNA Extraction and Northern Blot Analysis**

Total RNA was extracted from pooled frozen tissues (a minimum of 6 retinas or lenses per time) using either the RNeasy kit (Qiagen GmbH, Hilden, Germany) or a guanidinium thiocyanate/phenol–based protocol (Clontech, Palo Alto, CA), according to the manufacturers’ instructions. For northern blot analysis, 4-µg samples were denatured, electrophoresed in 1.3% agarose gels, and blotted onto nylon membranes (Amersham International, Slough, UK). Cloned inserts from plasmids, or polymerase chain reaction (PCR) products, were labeled with \( \alpha \)-[\( ^{32} \)P]dCTP using the Rediprime kit (Amersham International) and hybridized to the blots in the presence of 50% formamide at 42°C overnight, followed by stringent washing and autoradiography.
Reverse Transcription–PCR and Restriction Enzyme Digestion

Single-strand cDNA was obtained by reverse transcription (RT) of retina or lens RNA using approximately 1-µg RNA samples, incubated in 20-µl reactions with 1.25 U AMV reverse transcriptase (GIBCO–BRL, Paisley, UK) and random hexamer primers (Pharmacia Biotech, Uppsala, Sweden) for 60 to 90 minutes at 42°C, followed by heat inactivation of the enzyme. PCR amplifications were performed using primers according to the strategy of Goring et al.

For detection of γE/F-crystallin expression, primers GECRY.1: 5'-AGGCCATGGGGGAGATCAGCCTTCTATG-3' and GCRYR: 5'-AAGGCTCTGCCAGGGTGAGCAGCAG-3' were used, in 30-µl reactions incorporating 0.75 U Taq polymerase (GIBCO–BRL) and 2 µl cDNA in a thermal cycler (Hybaid, Teddington, UK) with the following parameters: 94°C for 5 min, then 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 30 sec, and a final step of 72°C for 5 min. Aliquots of 10 µl were cut with BglII (New England Biolabs, Beverly, MA) for a minimum of 2 hours and the products resolved on ethidium bromide-stained 1.0% agarose (Pharmacia Biotech, Uppsala, Sweden)/3.0% NuSieve GTG (FMC, Flowgen, Lichfield, UK) gels. For the detection of γA-, γB-, γC-, and γD-crystallins, primers were as described by Goring et al. and amplification conditions were as above with the exception that the anneal temperature was reduced to 50°C. Molecular size markers were obtained from GIBCO–BRL.

Verification of the amplified products was performed by probing of a Southern blot of the γE/F-crystallin reaction products with labeled insert of the sequenced γE-crystallin clone, which confirmed cross-hybridization, and by HindIII restriction digestion of the γA-γD-crystallin multiplex reaction products, which led to the expected reduction in size of all 4 bands and generation of a single additional small fragment, due to cleavage at a conserved site (data not shown). The primer pairs in all cases were set across an intron, but additional amplifications in the absence of reverse transcriptase were performed to confirm that the resultant products were not due to genomic DNA contamination.

Western Blot Analysis

Equal amounts of total protein (~5 µg) from extracts of retina, lens, heart, and liver tissue were subjected to electrophoresis under denaturing conditions and transferred to Immobilon-P (Millipore, Bedford, MA) membranes according to established protocols. Immunodetection was with a rabbit anti-bovine lens γ-crystallin antibody as the primary antibody (used at 5 µg/ml), and peroxidase-conjugated goat anti-rabbit IgG at 1:1000 (Sigma, St Louis, MO) as the secondary antibody, followed by development in the presence of 3,3'-diaminobenzidine and hydrogen peroxide. The analysis of retina and lens samples was repeated a minimum of 4 times.

Immunocytochemistry

Enucleated eyes were fixed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer overnight, incubated in 30% sucrose, and embedded and frozen as described previously. Cryostat sections (10-µm-thick) were processed for immunocytochemistry using the classic immunofluorescence technique. The primary antiserum was as described above for immunoblot analysis, used at a concentration of 2.5 µg/ml, and the secondary antibody a fluorescein isothiocyanate–coupled anti-rabbit IgG used at 1:200 (Sigma, St Louis, MO). Sections from 3 different animals were examined for each time, and typical results presented.

RESULTS

During an analysis of altered patterns of gene expression associated with murine retinal degeneration, we isolated a differential display PCR product, which, when used as a northern blot analysis probe, detected highly abundant transcripts of approximately 0.8-kb size in control mouse retinal RNA. Figure 1A shows the corresponding detection of this mRNA species in postnatal day (P)20 mouse retina using the cloned version of the PCR product. Sequence analysis of this clone indicated that the insert corresponded to approximately 0.3 kb cDNA of murine γE-crystallin, one of the family of γ-crystallin genes highly expressed in vertebrate lenses but not previously reported in the retina. In the mouse, γA-, γB-, γC-, γD-, and γE-crystallin genes form a linked tandem array on chromosome 1, with γF-crystallin 200 to 400 kbp upstream of the cluster (see Ref. 7 and references therein). Sequence data have shown that the γF gene is almost identical to that of γE, and use of a γE probe would not permit discrimination between these two genes by northern blot analysis. To establish whether both γE- and γF-crystallins are expressed in the retina, we adopted a strategy of using RT–PCR based on that of Goring et al. This approach exploits the fact that by selecting appropriately sited primers the resulting PCR products can be cut by the restriction enzyme BglII within the γE but not within the γF sequence, due to a nucleotide difference at position 272 in the γE cDNA. In the selected samples tested (C57BL/6 retina, at P15, P17, and P20), we found that expression of γE and γF transcripts was detected (Fig. 1B, lanes 4–6); and while the PCR was not strictly quantitative, the levels of the two appeared similar. The pattern obtained with the retinal samples resembled that seen with the lens control (Fig. 1B, lane 7).

To examine further whether the remaining four γ-crystallin genes were expressed in the retina, we performed a multiplex PCR on murine retinal and lens total cDNAs (Fig. 1C). All four (γA–γD) crystallins showed detectable expression in both tissues, although the level of γC-crystallin expression appeared more variable as well as lower than that of the others, the latter observation in agreement with the findings of Goring et al. on total ocular RNA. In all cases of PCR amplification, the primers were designed to span an intron, thus any genomic DNA contamination should not yield products of the sizes obtained. Additional confirmation that the RT–PCR was detecting crystallin expression was provided by the absence of products when reverse transcriptase was omitted (Fig. 1D).

Evidence for expression of a gene at the mRNA level does not necessarily imply the presence of translation products, however. We therefore used an antibody raised against bovine total lens γ-crystallin proteins in immunochromatographic assessments of expression at the protein level in the retina. Total retinal and lens extracts from mice were subjected to western blot analysis, and reactive bands of ~20 kDa were detected in both tissues, although at a much higher concentration in the lens (Fig. 2: lanes 1, 2, and 5). Close inspection (and of blots from repeat experiments) revealed that multiple bands were present in each sample, most likely corresponding to the different γ-crystallin forms, although discrimination among these was
not possible. No bands were detected in extracts of mouse heart or liver (Fig. 2, lanes 3 and 4).

Typical patterns of localization of immunoreactive γ-crysalins by fluorescence staining in the mouse retina at selected postnatal ages (P10, P16, and P18) are shown in Figures 3A

**Figure 1.** Analysis of γ-crystallin mRNA expression in murine ocular tissues. (A) Northern blot analysis of C57BL/6 mouse retinal total RNA (4 μg) at P20 days using γE-crystallin cDNA probe. (B) RT–PCR analysis of γE and γF-crystallin expression. Total cDNA was subjected to PCR using primers GECRY.1 and GCRYR. Products were digested with BglII and separated on an ethidium bromide-stained agarose gel. Uncut product of 351 bp corresponds to γF transcript; cut products of 276 and 75 bp to γE transcript. Lane 1, molecular size markers (200- and 300-bp bands indicated); lane 2, uncut product, P20 retina; lane 3, uncut product, P20 lens; lanes 4 through 7, cut products: lane 4, P15 retina; lane 5, P17 retina; lane 6, P20 retina; and lane 7, P20 lens. (C) Multiplex RT–PCR analysis of γA–γD-crystallin mRNA expression. Lane 1, molecular size markers as in (B); lane 2, P15 retina; lane 3, P17 retina; lane 4, P20 retina; and lane 5, P20 lens. (D) RT–PCR control reactions using P20 retinal RNA in the presence (lanes 2, 5) or absence (lanes 3, 6) of reverse transcriptase, followed by amplification using the γE/F (lanes 2, 3) and γA–γD (lanes 5, 6) primers. Molecular size markers, lanes 1, 4; 300 bp markers indicated by arrowheads.

**Figure 2.** Western blot analysis of γ-crystallin expression in murine tissues (~5 μg protein/lane). Lane 1, P20 retina; lane 2, P20 lens; lane 3, P18 heart; lane 4, P18 liver; and lane 5, P20 lens (duplicate of lane 2).

**Figure 3.** Immunofluorescence detection of γ-crystallin expression in sections of murine retinal tissue at different postnatal ages. (A) P10; (B) P16; (C) P18. (D) control section (P20) lacking primary antibody. PR, photoreceptors; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar, 100 μm. Results similar to those shown in (D) were obtained with additional control sections at ages P10, P16, and P18.
through 3C. We found that despite some variability in fluorescence intensity during postnatal development, the main sites of γ-crystallin protein localization were the ganglion cell layer, the outer plexiform layer, and photoreceptors, particularly in the outer segment regions. The immunostaining also extended from the inner edge of the inner nuclear layer through the inner plexiform layer and nerve fiber layer to the ganglion cell layer. At the times examined, there was no detectable immunoreactivity at the retinal pigment epithelium. Control sections lacking primary antibody (including those of retinas from P10, P16, and P18, not shown) displayed minimal background fluorescence, mainly associated with the retinal vasculature (Fig. 3D).

**DISCUSSION**

In their analysis of the expression of the γA-γF-crystallin genes in mouse eye development, Goring et al. 7 assayed total ocular mRNA on the assumption that γ-crystallin expression was entirely lens specific. Our present study indicates that at least in certain cases the retinal component of ocular γ-crystallin expression can be significant and that all 6 genes are expressed in the retina and the lens. In the lens, the spatial distribution of the different crystallin proteins has been interpreted as contributing, perhaps via differing biophysical properties such as water exclusion, to the final optical characteristics of this structure. The potential function of γ-crystallins in the neural retina is less clear. Although it is possible that the molecular packing properties of the crystallins contribute to retinal translucency, it seems more plausible that nonrefractive properties may be preserved from the evolutionary precursor proteins. From parallels with the heat-shock and stress-induced genes, γ-crystallins may, in conjunction with β B- and possibly also certain β-crystallins, constitute a family of defensive proteins, which are induced during critical periods of stress on the retina. The most obvious stressor is incident light, particularly after the opening of the eyes at about P13 to P14 in the mouse. Exposure to intense light is a well-characterized inducer of photoreceptor apoptosis and is associated with the induction of specific genes including that for the glycoprotein clusterin. 10 It will be of interest to examine the response of crystallin gene expression to light damage, and in relation to another well-established modulator of retinal gene expression, the diurnal cycle of light exposure. It remains possible that retinal γ-crystallins have no specific function and represent an adventitious form of expression, rudiments perhaps of early interactions between the developing lens placode and the optic vesicle. Whichever is the case, it is clear that the lens can no longer be regarded as the unique repository of any of the major crystallin families.

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**References**

Refractive Associations with Cataract: the Blue Mountains Eye Study

Ridia Lim, Paul Mitchell, and Robert G. Cumming

PURPOSE. To assess the relationship between myopia and age-related cataract in a defined older population.

METHODS. A cross-sectional study of 3654 people aged 49 to 97 years was conducted in the Blue Mountains near Sydney, Australia, from 1992 through 1994. General medical, eye, and refractive history and information about confounders were collected by questionnaire. Participants had a detailed determination of refraction, and the spherical equivalent refraction of each eye was calculated. The Wisconsin Cataract Grading System was used in masked grading of slit lamp and retroillumination lens photographs, to assess presence and severity of nuclear, cortical, and posterior subcapsular (PSC) cataract. Data from both eyes were analyzed by the generalized estimating equation method, adjusting for cataract risk factors.

RESULTS. Included in the analysis were 7308 eyes. A history of wearing distance glasses, excluding eyes with current hyperopic refraction, was used as a proxy for myopia. Subjects who had worn distance glasses were more likely to have nuclear cataract (odds ratio [OR] 1.3; confidence interval [CI] 1.0–2.1). After stratification by age at first wearing distance glasses, this relationship remained only for people who first wore distance glasses after age 40 years (OR 1.3; CI 1.0–1.8), which suggested a myopic refractive shift from developing nuclear opacity and was supported by the weak association found between current myopic refraction and nuclear cataract (OR 1.3; CI 1.0–1.6). Eyes with onset of myopia before age 20 years had the greatest PSC cataract risk (OR 3.9; CI 2.0–7.9). This was supported by the finding of an association between current myopic refraction and PSC cataract (OR 2.5; CI 1.6–4.1). PSC cataract was inversely associated with hyperopia (OR 0.6; CI 0.4–0.9). Refraction-related increasing odds were found between PSC cataract and myopia: low myopia (OR 2.1; CI 1.4–3.5), moderate myopia (OR 3.1; CI 1.6–5.7), and high myopia (OR 5.5; CI 2.8–10.9). High myopia was associated with PSC, cortical, and late nuclear cataract.

CONCLUSIONS. Early-onset myopia (before age 20 years) may be a strong and independent risk factor for PSC cataract. The findings suggest the possibility of a dose response between levels of myopia and PSC cataract. Nuclear cataract was associated with presumed acquired myopia, whereas high myopia was associated with all three types of cataract. (Invest Ophthalmol Vis Sci. 1999;40:3021–3026)

Moderate to high myopia has a known association with age-related cataract. However, for lower levels of myopia this relationship has been disputed. Although many studies have suggested that low myopia may be an important risk factor for cataract, there have been few recent reports of examination of this association. In particular, no population-based studies have explored the potential relationship between low levels of myopia and the principal types of age-related cataract.

Perkins performed a retrospective study of patients who had undergone cataract surgery and reported an association between low myopia and cataract. However, this study was limited by the availability of past refraction records in only 17% of subjects. In two hospital-based case–control studies from Oxfordshire (United Kingdom), Harding reported an association between childhood myopia and cataract, but did not subdivide the age-related cataract types. He estimated that 7% of cataract was directly attributable to myopia. Two other studies that reported an association also did not subdivide cataract types.

Recent studies have examined the relationship between a history of wearing glasses in youth and individual types of age-related cataract. Although no association was found with either nuclear, cortical, or PSC cataract, the Lens Opacities Case–Control Study reported an association between a history of wearing glasses before age 20 years (interpreted as a proxy for myopia) and mixed cataract (OR 1.44). The Beaver Dam Eye Study examined individual cataract types in the worse eye and compared people who had worn distance glasses before age 21 years with those who had begun wearing distance glasses after age 40 years. Use of distance glasses before age 21 years had a statistically significant association with PSC cataract (OR 1.20) in women, but not in men (OR 1.06), after controlling for age. After controlling for other confounders, this association in women was stronger (OR 1.43), although not statistically significant (95% CI 0.98–2.08). A history of wearing distance glasses before age 21 years in men was associated with a significantly lower risk of nuclear cataract (OR 0.77), and wearing glasses was also interpreted as protecting against ultraviolet radiation.

We designed in the present population-based cross-sectional study to explore whether refractive error, particularly increasing levels of myopia, could precede and be an independent risk factor for age-related cataract, after taking into account the effects of other known cataract risk factors. Further, we attempted to examine temporal aspects of the relationship between myopia and development of cataract.

METHODS

The Blue Mountains Eye Study is a population-based study of the prevalence and causes of age-related vision loss, conducted in two urban postal code areas of the Blue Mountains region near Sydney, Australia. The study population and methods have been described previously. After a private census, all perma-
urrent residents aged 49 years or older, were invited to participate. Of 4433 age-eligible residents, 3654 people (82.4%) aged 49 to 97, participated from 1992 through 1994, including 2072 women and 1582 men (mean age, 66 years). There were 68 people who died and 210 who moved from the area (6.3%) before they could be examined. The remaining 501 people refused examination, including 353 (8.0%) who permitted a brief interview and 148 (3.3%) who refused any participation. Residents who attended for the examination were more likely to wear glasses currently and to have hypertension and were less likely to have ever seen an ophthalmologist than were nonattenders.12 Nonparticipants were also slightly older but had a similar gender distribution and prevalence of doctor-diagnosed eye disease (cataract, glaucoma, and age-related macular degeneration) to participants. Ethical approval for the study was obtained from the Western Sydney Area Human Ethics Committee and written, informed consent was obtained from all subjects. The research was conducted according to the recommendations of the Declaration of Helsinki.

A standardized questionnaire was administered by trained interviewers that included eye and general medical histories, use of medications, and demography. Several questions regarding myopia were included, such as: “Do you wear glasses (that includes bifocals or multifocals) to see clearly in the distance, or have you in the past?” and “How old were you when you first needed to wear glasses to see clearly in the distance?” Objetive refraction was performed using an autorefractor (model 530; Humphrey, San Leandro, CA) and was followed by subjective refraction, according to the Beaver Dam Eye Study modification of the Early Treatment Diabetic Retinopathy Study (ETDRS) protocol and a logMAR chart.11,13 The spherical equivalent refraction (SER) defined as the sum of the best-corrected spherical refraction plus half the cylindrical refraction, was used to categorize current refractive status.

The questionnaire also asked about known and potential risk factors for cataract, including a history of diabetes, hypertension, smoking, and use of inhaled or oral steroids. Hypertension was defined as a history of treated hypertension and/or systolic blood pressure higher than 160 mm Hg or diastolic pressure higher than 90 mm Hg. Sun-related skin damage to the hands, forearms, and face was assessed by a single senior interviewer that included eye and general medical histories, use of medications, and demography. Several questions regarding myopia were included, such as: “Do you wear glasses (that includes bifocals or multifocals) to see clearly in the distance, or have you in the past?” and “How old were you when you first needed to wear glasses to see clearly in the distance?” Objetive refraction was performed using an autorefractor (model 530; Humphrey, San Leandro, CA) and was followed by subjective refraction, according to the Beaver Dam Eye Study modification of the Early Treatment Diabetic Retinopathy Study (ETDRS) protocol and a logMAR chart.11,13 The spherical equivalent refraction (SER) defined as the sum of the best-corrected spherical refraction plus half the cylindrical refraction, was used to categorize current refractive status.

At the clinic visit, a detailed eye examination was performed. Photographs of the lens of each eye were taken after pupil dilatation with 1% tropicamide and 10% phenylephrine drops. The protocol for lens photography and grading closely followed the Wisconsin Cataract Grading System14,15 developed for the Beaver Dam Eye Study. Slit lamp photographs were taken to assess the severity of nuclear cataract (camera model SL-7E; Topcon Optical, Tokyo, Japan). Retrolumination photographs of the anterior and posterior lens were taken to assess the presence and severity of cortical and PSC cataracts (cataract camera model CT-R; Neitz, Tokyo, Japan).

The severity of nuclear cataract on a 5-point scale was assessed by comparing subject photographs with a set of four standard photographs. The presence and severity of cortical cataracts were graded by placing over the Neitz photographs a circular grid divided into eight equal wedges and a central circle. Graders estimated the area percentage for each of these nine segments involved by cataract. The percentages were summed to give an estimate of the total lens area involved by cataract. PSC cataract was graded similarly. Photographs taken of pupils less than 4 mm in diameter were excluded from cortical cataract analyses. All photographs were graded by one of two masked graders. The k values for intergrader reproducibility were 0.79 for nuclear (260 eyes), 0.78 for cortical (379 eyes), and 0.57 for PSC cataract (383 eyes). The quadratic weighted k statistic (intraclass correlation coefficient) was used, because this measure correlates two graders on the same scale in reproducing the actual grade.15 The values for nuclear and cortical cataract represent good reproducibility, whereas the value for PSC cataract is fair.

Data for cortical and PSC cataracts were missing from approximately 3% of subjects because photographs were ungradable or were not taken. Because of intermittent camera malfunction (underexposure of some photographs), 1045 (29%) subjects did not have photographs suitable for nuclear cataract grading. These subjects did not differ in any important way from subjects withgradable photographs.15

We analyzed data from both eyes, using the generalized estimating equation method described in Zeger et al.16 and Liang and Zeger.17 Although usually bilateral, both refractive error and cataract are eye specific. The generalized estimating equation method allows use of data from both eyes while accounting for the correlation between the two eyes in a single subject. It affords greater precision of estimation and is less sensitive to missing data for some eyes.18 Cataract was a dichotomized variable in all analyses. Cortical cataract was considered present if 5% or more of the lens was involved, whereas PSC cataract was considered present if any PSC opacity was graded. Nuclear cataract was considered present if graded level 3 or higher, in keeping with the definition of early cataract used previously in the Beaver Dam Eye Study19 and in our prevalence report.15

In the multivariate analyses, we controlled for age, sex, hypertension, diabetes (history), smoking (current, past, or never), use of oral or inhaled steroids (ever or never), and level of sun-related skin damage (mild, moderate, or severe). Age was a continuous variable, whereas all other variables were categorical. Statistical software (Statistical Analysis System, ver. 6.12; SAS Institute, Cary, NC) was used for statistical analysis, including generalized estimating equation analyses. Odds ratios (OR) and 95% confidence intervals (CI) are presented.

**RESULTS**

We excluded 325 (4.5%) aphakic, pseudophakic, or enucleated eyes from the analyses. Eyes with missing or ungradable photographs accounted for other missing cataract data. Using data from both eyes and the definitions outlined, 2182 (47.4%) eyes had nuclear, 1211 (18.0%) eyes had cortical, and 265 had PSC (3.9%) cataract. Of the mixed types of cataract, 491 (10.9%) eyes had mixed cortical and nuclear cataract, 124 (2.7%) had mixed nuclear and PSC cataract, and 70 (1.0%) had mixed cortical and PSC cataract. All three cataract types coexisted in only 39 eyes (0.9%). A much smaller number of eyes had pure cataract types: Pure nuclear cataract was found in 1554 eyes (34.4%), pure cortical in 272 eyes (6.0%), and pure PSC in 52 eyes (1.2%).

Current refraction data were available on 7243 (99.1%) eyes. Emmetropia was defined as a spherical equivalent refrac-
### Table 1. Adjusted Odds Ratios for Associations between Current Refraction and Types of Age-Related Cataract

<table>
<thead>
<tr>
<th>Type of Cataract</th>
<th>Current Refraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hyperopia</td>
</tr>
<tr>
<td>Eyes with PSC cataract</td>
<td></td>
</tr>
<tr>
<td>Model adjusted for age and sex</td>
<td>0.7</td>
</tr>
<tr>
<td>Multivariate model†</td>
<td>0.6</td>
</tr>
<tr>
<td>Number of cases</td>
<td>99</td>
</tr>
<tr>
<td>Total number of eyes (%)</td>
<td>3104</td>
</tr>
<tr>
<td>Eyes with nuclear cataract</td>
<td></td>
</tr>
<tr>
<td>Model adjusted for age and sex</td>
<td>1.5</td>
</tr>
<tr>
<td>Multivariate model*</td>
<td>1.5</td>
</tr>
<tr>
<td>Number of cases</td>
<td>1225</td>
</tr>
<tr>
<td>Total number of eyes (%)</td>
<td>2131</td>
</tr>
<tr>
<td>Eyes with cortical cataract</td>
<td></td>
</tr>
<tr>
<td>Model adjusted for age and sex</td>
<td>1.2</td>
</tr>
<tr>
<td>Multivariate model†</td>
<td>1.4</td>
</tr>
<tr>
<td>Number of cases</td>
<td>674</td>
</tr>
<tr>
<td>Total number of eyes (%)</td>
<td>3091</td>
</tr>
</tbody>
</table>

Low myopia, −1 to more than −3.5 D; medium myopia, −3.5 to more than −6 D; high myopia, −6 D or less. Data in parentheses beside ORs are 95% CIs.

* Adjusted for age, sex, smoking, hypertension, diabetes, use of oral or inhaled steroids, and examiner-assessed sun-related skin damage.

† Also adjusted for nuclear cataract grade.

1. Data were available on use of distance glasses in the past 99.9% of subjects (7302 eyes) and age at first wearing distance glasses for 99.1% (6832 eyes). Of these, 32.1% had never worn distance glasses, 47.4% had begun wearing distance glasses after age 40 years, 9.9% between ages 21 and 39 years inclusive, and 10.6% before age 20 years. In using a history of wearing distance glasses measure as a proxy for myopia, we excluded from analyses eyes with a current hyperopic refractive error. We considered that this would exclude most subjects who wore glasses for hyperopia at an early age. The age at which subjects reported first wearing distance glasses was divided into three groups: Age less than 20 years defined an early-onset myopia group; age 20 to 39 years, a later onset myopia group; and age 40 years or older, the group with latest onset. Subjects in the referent group had never worn distance glasses.

2. We found an association between any current myopia and presence of nuclear cataract (OR 1.3; CI 1.0–1.6) after adjusting for other cataract risk factors. This weak association was present for all three levels of myopia but was only significant for moderate myopia, as shown in Table 1. Any history of wearing distance glasses was significantly associated with presence of nuclear cataract. This was present before (OR 1.4; CI 1.1–1.7; Table 2) as well as after, excluding eyes with current hyperopic refraction (OR 1.3; CI 1.0–2.1; Table 3). However, after stratification by the age at which distance glasses were first worn, a statistically significant association was found only for persons who began wearing distance glasses after age 40 years (OR 1.3; CI 1.0–1.8; Tables 2, 3). We performed further analyses, defining late nuclear cataract as level 4 or higher. Using this definition, current myopia had a stronger association with cataract. Any myopia was associated with late nuclear cataract (OR 2.3; CI 1.7–3.2) and a statistically significant relationship was found with lower degrees of myopia (−1 to more than −6 D; OR 2.3; CI 1.7–3.2) as well as with high myopia (less than −6 D; OR 2.5; CI 1.1–4.5) after multivariate adjustment.

3. Any current myopia was associated with presence of PSC cataract (OR 2.5; CI 1.6–4.1), before and after adjusting for the level of nuclear cataract. In the PSC cataract model, we controlled for the severity of nuclear cataract because of the known myopic shift in refraction caused by development of nuclear opacity. We found a weak negative association between PSC cataract and hyperopic compared with emmetropic eyes (OR 0.6; CI 0.4–0.9). A refraction-related trend was found for increasing risk of PSC cataract with increasing myopic refractive error. The magnitude of this association was OR 2.1 (CI 1.2–3.8) for low myopia, OR 3.0 (CI 1.3–6.9) for moderate myopia, and OR 4.9 (CI 2.1–11.4) for high myopia. The association between a history of wearing distance glasses and PSC cataract was not statistically significant (OR 1.3; CI 0.8–2.1) in the multivariate analysis. However, after excluding eyes with current hyperopic refraction, this relationship was statistically significant (OR 2.6; CI 1.5–4.4; Table 3). The model was also adjusted for the severity of nuclear opacity.
The strongest association was found in people with early-onset myopia (OR 3.9; CI 2.0–7.9), with a weaker association found with later onset myopia (OR 3.1; CI 1.3–7.3). A weak association was present between cortical cataract and hyperopia, after adjusting for other risk factors (OR 1.4; CI 1.1–1.7). High myopia was also associated with cortical cataract (OR 2.9; CI 1.4–6.0). No associations were found between history of wearing distance glasses and presence of cortical cataract.

The Beaver Dam Eye Study compared people who wore distance glasses before age 20 years with those who began wearing distance glasses from age 40 years. When we made a comparison between these two groups, we found that people who wore distance glasses before age 20 years were more likely to have PSC cataract than those who began wearing distance glasses from age 40 years (Table 4). Odds for the association with PSC cataract were greater for women (OR 3.1; CI 1.8–5.3) than men (OR 2.2; CI 1.0–4.8). Women who wore distance glasses from age 40 years were more likely to have nuclear cataract. There was thus an association between older age at wearing distance glasses and nuclear cataract in women.

Data in parentheses beside ORs are 95% CIs.

* Adjusted for age, sex, smoking, hypertension, diabetes, use of oral or inhaled steroids, and examiner-assessed sun-related skin damage.
† Also adjusted for nuclear cataract grade.

<table>
<thead>
<tr>
<th>Type of Cataract</th>
<th>Distance Glasses Worn</th>
<th>Age at Which Distance Glasses Were First Worn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyes with PSC cataract</td>
<td>Never</td>
<td>Ever</td>
</tr>
<tr>
<td>Model adjusted for age and sex</td>
<td>1.0 (referent)</td>
<td>1.3 (0.9–1.9)</td>
</tr>
<tr>
<td>Multivariate model†</td>
<td>1.0 (referent)</td>
<td>1.3 (0.8–2.1)</td>
</tr>
<tr>
<td>Number of cases</td>
<td>58</td>
<td>206</td>
</tr>
<tr>
<td>Total number of eyes (%)</td>
<td>2070 (2.8)</td>
<td>4670 (4.4)</td>
</tr>
<tr>
<td>Eyes with nuclear cataract</td>
<td>Model adjusted for age and sex</td>
<td>1.0 (referent)</td>
</tr>
<tr>
<td>Multivariate model*</td>
<td>1.0 (referent)</td>
<td>1.4 (1.1–1.7)</td>
</tr>
<tr>
<td>Number of cases</td>
<td>500</td>
<td>1680</td>
</tr>
<tr>
<td>Total number of eyes (%)</td>
<td>1400 (35.7)</td>
<td>3206 (52.4)</td>
</tr>
<tr>
<td>Eyes with cortical cataract</td>
<td>Model adjusted for age and sex</td>
<td>1.0 (referent)</td>
</tr>
<tr>
<td>Multivariate model†</td>
<td>1.0 (referent)</td>
<td>0.9 (0.7–1.1)</td>
</tr>
<tr>
<td>Number of cases</td>
<td>305</td>
<td>904</td>
</tr>
<tr>
<td>Total number of eyes (%)</td>
<td>2059 (14.8)</td>
<td>4648 (19.4)</td>
</tr>
</tbody>
</table>

Data in parentheses beside ORs are 95% CIs. Subjects with current hyperopic refraction were excluded.

* Adjusted for age, sex, smoking, hypertension, diabetes, use of oral or inhaled steroids, and examiner-assessed sun-related skin damage.
† Also adjusted for nuclear cataract grade.
Further, in using this history as a proxy for myopia, we excluded people with a current refraction of more than +1.0 D, because hyperopic subjects could also have worn distance glasses from a young age. Although the findings were similar when these subjects were included in analyses (Table 2), excluding people with hyperopia strengthened the odds for PSC cataract, particularly for the early-onset group (Table 3). These findings suggest that early-onset myopia may be a risk factor for development of PSC cataract in later life.

Could the association found between myopia and PSC cataract have been confounded by difficulty in grading PSC in the presence of advanced nuclear cataract? It is not possible to exclude this as a possibility, but it seems unlikely to have had a major effect on our findings because of the relatively small number of subjects with the highest nuclear grade.

Low myopia has not been previously implicated in the development of cortical cataract, and we also found no association. Our data, however, indicate that high myopia is associated with all three types of age-related cataract, particularly PSC. This finding is in keeping with the long-accepted notion that high myopia is an important risk factor for cataract.

We had the opportunity to compare our results with Beaver Dam findings. The two studies used similar techniques, and our grading protocols were the same. Overall, the patterns of association found are similar. Both studies found that wearing distance glasses before age 20 years was associated with PSC cataract and that wearing myopic distance correction beginning in older age was associated with nuclear cataract.

Mechanisms for the association between myopia and cataract are not known. It has been suggested that there may be a biochemical basis for cataractogenesis in myopic patients with retinal lipid peroxidation playing a role. Our results showed a trend for an increasing association between PSC cataract and myopic refraction that extended from hyperopia to high myopia. Could the relationship with PSC cataract be based on axial length? Increasing axial length may tend to deprive the posterior lens of nutrition. To our knowledge, no study to date has studied axial length and cataract prevalence in a population. Further work in this area is warranted. The true refractive status of our study population remains unknown. Although we have measures of current refraction,
greater clarity would have been provided by having axial length and keratometry measurements to differentiate axial from index myopia.

A mechanical cause for the association between myopia and cataract was suggested by Weale. He argued that the lens matrix in myopia may be subject to greater pressure from the lens capsule than in other refractive states because of the reduced need for myopic subjects to accommodate. Weale further speculated that overcorrection of myopia in the late 20s may decrease the excess risk of cataract.²

Our findings may have important implications. Low myopia and cataract are both common problems worldwide, and our study also provides some evidence that the age-specific prevalence of myopia may be increasing.²² Much research targets modifiable cataract risk factors to relieve the future public health burden of cataract. To date, however, there is no proven therapy that reduces development or progression of myopia.

In summary, this cross-sectional study has examined several refractive parameters in attempting to build a picture of the temporal relationship between myopia and age-related cataract. A statistically significant relationship was found between high myopia, which could be based on axial length. Further work is indicated to examine possible mechanisms for the relationship.

References

Effects of Abnormal Light-rearing Conditions on Retinal Physiology in Larvae Zebrafish

Shannon Saszik1,2 and Joseph Bilotta

PURPOSE. Anatomic studies have found that zebrafish retinal neurons develop in a sequential fashion. In addition, exposure to abnormal light-rearing conditions produces deficits in visual behavior of larvae zebrafish, even though there appears to be little effect of the light-rearing conditions on the gross morphology of the retina. The purpose of this study was to assess the effects of abnormal light-rearing conditions on larval zebrafish retinal physiology.

METHODS. Larvae zebrafish (Danio rerio) were exposed to constant light (LL), constant dark (DD), or normal cyclic light (LD) from fertilization to 6 days postfertilization (dpf). After 6 days, the animals were placed into normal cyclic light and tested at 6 to 8, 13 to 15, and 21 to 24 dpf. Electrotetroinogram (ERG) responses to visual stimuli, consisting of various wavelengths and irradiances, were recorded. Comparisons were made across the three age groups and the three light-rearing conditions.

RESULTS. Deficits from the light-rearing conditions were seen immediately after exposure (6–8 dpf). The LL-condition subjects showed the greatest deficit in the UV and short-wavelength areas and the DD-condition subjects showed a slight deficit across the entire spectrum. At 13 to 15 dpf, the LL and DD groups showed an increase in sensitivity and by 21 to 24 dpf, the groups no longer differed from controls.
CONCLUSIONS. Abnormal lighting environments can adversely influence the physiological development of the larvae zebrafish retina. The pattern of damage that was seen in zebrafish is similar to that found in other vertebrates, including higher vertebrates. However, unlike higher vertebrates, the zebrafish appears to be capable of regeneration. This suggests that the zebrafish would be a viable model for light environment effects and neural regeneration. (Invest Ophthalmol Vis Sci. 1999;40:3026–3031)

In both lower and higher vertebrates there are many examples of the effects of abnormal light environments on the retina. For example, Harwerth and Sperling found a decrease in the sensitivity of M-cones and L-cones when adult primates were exposed to middle- and long-wavelength light, although the damage did not appear to be permanent. However, they also found that the S-cones showed a permanent decrease in sensitivity after exposure to short-wavelength light. Interestingly, when monkeys were reared in constant dark for up to 2 months, no anatomic differences were found at the retinal level or the dorsal lateral geniculate nucleus. The effects of different light-rearing environments on developing zebrafish have been assessed using behavioral and anatomic methods. Studies using pigmented zebrafish found no differences in retinal anatomy, including outer segment size and retinal layer lamination, among subjects raised in constant light, constant dark, and normal cyclic light. However, the anatomic work does not agree with behavioral data from zebrafish raised under similar lighting conditions. Larvae zebrafish exposed to constant light from fertilization to 6 days postfertilization (dpf) had a visual acuity below that of constant dark and normal subjects when measured using the optomotor response; however, zebrafish raised from fertilization to 6 dpf in constant dark had only a slight deficit in acuity compared to normal subjects.

The purpose of the present study was to examine light-rearing effects on visual physiology, as measured with the electroretinogram (ERG). This study used light-rearing conditions similar to those used in the previously mentioned anatomic studies. It was hypothesized that animals reared in constant light would show deficits in sensitivity in the UV and short-wavelength areas of the spectrum. Zebrafish S-cones are similar qualitatively in structure to the S-cones in primates, and primate S-cones appear to be the most susceptible to light damage. Also, studies with other fish species have shown that the U-cones are very susceptible to environmental factors. Finally, it is anticipated that animals reared in constant dark should show some visual deficits, but these should not be as severe as those for animals reared in constant light.

METHODS
Participants
Larvae zebrafish (*Danio rerio*), bred in-house, were maintained until 6 dpf in one of three light-rearing conditions: constant light (LL), constant dark (DD), or a 14-hour lights on–10-hour lights off cycle (LD). Light levels in LL and LD conditions were approximately 500 lux (F40/D fluorescent lights; Sylvania, Danvers, MA). These conditions correspond to those used in past work that showed differences in visual behavior. All procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Apparatus
A two-channel optical system was used to present the visual stimuli (see Ref. 8). Monochromatic light was presented to the subjects through one channel, which had a 150 W xenon arc lamp as its light source (model LH 150; Spectral Energy, Westwood, NJ). The second channel had a 250 W tungsten-halogen bulb (model 6334; Oriel, Stratford, CT) light source that provided a 5 μW/cm² broadband background. The two channels were combined optically and focused onto one end of a liquid light guide (model 77556; Oriel); the other end was placed in front of the subject’s eye. Stimulus wavelength and irradiance were controlled using interference and neutral density filters.

Testing Procedures
Testing began on day 6 when the fish were removed from the light-rearing environment. Detailed procedures have been described elsewhere. Briefly, the subject was anesthetized with tricaine methanesulfonate; the dose (0.01%, 0.02%, or 0.04%) varied according to age (6–8, 13–15, and 21–24 dpf, respectively). The subject was placed on a piece of cotton in a Petri dish; the cotton was moistened with an anesthetic solution. The recording and reference electrodes, glass pipettes with a 10-μm tip diameter, were filled with teleost saline solution and housed 36-gauge chlorided silver wires. The recording electrode was placed on the subject’s eye, and the reference electrode was placed on the body. For the 21 to 24 dpf group, the recording electrode was inserted into the vitreal chamber. This was done to make the procedures of this age group more comparable to previously published adult data. Electrical signals were differentially amplified (AC amplifier with a bandpass of 0.1–100 Hz) and recorded by the laboratory computer at a rate of 250 Hz. The animal adapted to the broadband background for 5 minutes before trials began. The irradiance at any given wavelength began below threshold and was increased until the desired response was obtained. Each trial consisted of three 200-ms stimulus presentations. The order of wavelength presentation was staggered in 40-nm steps; the remaining wavelengths were then filled in, such that 20-nm steps existed in the final set of data.

RESULTS
ERG waveforms were digitally filtered for 60-Hz noise and averaged across the three stimulus presentations. Spectral
Sensitivity functions were calculated for both the a- and b-wave ERG components when they were apparent. Spectral sensitivities of the 6 to 8 dpf subjects from the LD, DD, and LL conditions were calculated using the a-wave component from responses to 320- to 440-nm stimuli; after 440 nm, the a-wave was no longer evident in the subjects’ waveforms. The a-wave was measured from baseline (response before stimulus onset) to the first negative peak. For the b-wave component, spectral sensitivity was calculated from 320 to 640 nm for all age groups and all conditions. The b-wave was measured from baseline or the first negative peak to the first positive peak. There were no apparent differences in the subjects’ ERG waveforms across all rearing conditions, including normal subjects (LD). The young subjects’ ERG waveforms had strong a- and b-waves in response to UV stimuli, as opposed to being dominated by the b- and d-waves as found in adult responses across wavelengths. To obtain sensitivity to each stimulus wavelength, the reciprocal of the log stimulus irradiance (quanta s⁻¹ cm⁻²), which produced a criterion response, was calculated from the log irradiance-response function.

To make comparisons across the lighting conditions, the data were first normalized to values at 640 nm. This is because, based on previous research, it was hypothesized that the deficits due to light-rearing conditions would be found in the UV and short-wavelength areas of the spectrum. Thus, normalizing the data to the peak in the UV area, which is where larvae zebrafish are normally most sensitive, would be inappropriate because that is where the deficits were expected. The decision to normalize the curves at the long wavelengths was supported empirically by attempting to normalize the functions at other parts of the spectrum. For example, normalizing at the UV wavelengths (which was the most sensitive portion of all the functions) gave the impression that the 6 to 8 dpf subjects in the three rearing conditions were identical in sensitivity to UV wavelengths and that the LL group was more sensitive to middle and long wavelength stimuli when compared to normal subjects. This interpretation is inconsistent with normal zebrafish ERG development. Therefore, the data were normalized to values at 640 nm. The data were renormalized to the peak sensitivity of the LD-condition subjects for each age group. Normalizing the data to the peak sensitivity of the LD-condition subjects was done so the sensitivity of the DD- and LL-condition functions could be compared, relative to the normal LD-condition function. After calculating the relative spectral sensitivities for the three conditions and the three age groups, a quantitative assessment of the cone contributions to the spectral sensitivity function was performed. A multiple mechanism model was used to derive the cone inputs (λmax = 362, 415, 480, and 570 nm; U-, S-, M-, and L-cones, respectively) to the spectral sensitivity data (see Ref. 8 for details).

Figure 1 shows the spectral sensitivity functions of the 6 to 8 dpf subjects from the LD (squares), DD (circles), and LL (triangles) conditions. The lines represent the best-fit models and the error bars indicate ±1 SEM. As expected, compared to the LD-condition subjects, the LL-condition subjects showed the greatest deficit in sensitivity, especially to UV and short-wavelength stimuli. There were differences between the LL- and the LD-condition subjects in the middle- and long-wavelength areas, but they were relatively small. The subjects in the DD condition also showed a deficit in sensitivity when compared to the LD-condition subjects, but it was not as large as the deficit of the LL-condition subjects. Unlike the LL-condition function, there was a uniform sensitivity deficit of a half log unit in DD-condition subjects’ relative sensitivity compared to the sensitivity of the LD-condition subjects. Figure 2A shows the spectral sensitivity of the 13 to 15 dpf subjects from the LD (squares), DD (circles), and LL (triangles) conditions. In general, all three spectral sensitivities are similar in shape. At this age, the difference in the spectral sensitivities that was apparent in the 6 to 8 dpf subjects has disappeared. Interestingly, there are no sensitivity differences for the three groups in the UV area. The LL- and the DD-condition subjects appear to show...
only a slight deficit in sensitivity to the short- and middle-wavelength areas of the spectrum when compared to the LD-condition subjects. Figure 2B shows the spectral sensitivity functions of the 21 to 24 dpf subjects in the LD (squares), DD (circles), and LL (triangles) conditions. The damage seen initially in the 6 to 8 dpf subjects raised in the LL and DD conditions gradually disappeared and by 24 dpf, the sensitivity of the LL- and DD-condition subjects returned to normal.

To assess whether photoreceptor function was responsible for the differences seen in the LD, LL, and DD conditions, a-wave spectral sensitivity functions were calculated. Figure 3A shows the spectral sensitivity functions of the a-wave component of the ERG response from 6 to 8 dpf subjects in the LD (squares), DD (circles), and LL (triangles) conditions. The data are shown in absolute sensitivity values (quanta s⁻¹ cm⁻²). The curves from the three groups are very similar in shape and absolute sensitivity. There do not appear to be any differences in the a-wave spectral sensitivity functions of the 6 to 8 dpf subjects exposed to the LL and DD conditions when compared to the LD condition, suggesting no problems with photoreceptor function.

Because the functions across the three conditions were similar, the data were averaged and modeled. In Figure 3B, the points represent the data and the line represents the model. Because the spectrum range was limited to primarily UV wavelengths, only the U-cone spectra were used in the model. There appears to be a good fit between the model and the data, suggesting that this spectral sensitivity function reflects U-cone activity in the retina. Because the function is derived from the a-wave component of the ERG response, it suggests normal U-cone function.

**DISCUSSION**

The main objective of the present study was to examine the effects of abnormal light-rearing conditions on early retinal development. On the basis of work done in primates,¹ it was anticipated that after exposure to constant light, there would...
be deficits in the UV and short-wavelength areas of the spectrum. Also, subjects exposed to constant dark conditions were expected to show some deficits, but these deficits would not be as severe as those observed in the constant light condition and would not be limited to the UV and short-wavelength areas.

The immature system of the larvae zebrafish appears to be affected by abnormal light-rearing environments. The abnormal conditions (LL and DD) used in this study altered the b-wave spectral sensitivity of subjects compared to those raised in normal cyclic light. These physiological deficits are similar to the behavioral deficits that have been reported, suggesting problems with the U- and S-cones and/or their connections. In the UV and short-wavelength areas of the spectrum, both the LL and DD subjects were found to have lower sensitivity when compared to normal subjects of the same age. However, subjects raised in constant light showed the greatest deficit in sensitivity, and the constant dark subjects’ sensitivity fell between that of the LL and DD subjects.

These findings are consistent with those reported in primates, where subjects raised in constant light displayed a large deficit in sensitivity to short-wavelength stimuli. As expected, the results show that the U- and S-cones’ contribution to the b-wave response are more susceptible to light damage than the other cone types. Interestingly, there was no difference in a-wave spectral sensitivity at the UV wavelengths. This suggests there is no problem with the U-cones, and the functional deficits found must be the result of either direct damage to the bipolar cells or damage to the synaptic connections between the U-cones and bipolar cells. At the middle- and long-wavelength areas of the spectrum, the deficits seen in the b-wave responses of subjects across the two conditions were similar. Subjects from both abnormal light-rearing conditions showed deficits in sensitivity, although the deficits in the middle- and long-wavelength areas of the spectrum were not as severe as those in the UV area of the spectrum. These findings also support studies of other fish species that have shown the U-cones to be labile and susceptible to environmental factors. For example, trout lose their U-cones with age and the presence of U-cones may be altered by exposure to the hormone thyroxine.

The fact that the two abnormal lighting conditions had differential effects on spectral sensitivity suggests that there may be two separate phenomena being observed for the LL and DD conditions. One possible explanation for this phenomenon in the LL group may be the result of damage to the system, whereas deficits found in the DD group may be due to a developmental delay. In the constant light condition, the system may develop normally and then begin to degenerate because of the overexposure to light. However, in the constant dark condition, the deficits may not be due to damage from the environment, but rather because of the lack of light stimulation, the visual system may fail to develop normally. Thus, subjects exposed to constant light conditions may have resulting damage to synaptic connections, but constant dark subjects are simply at an earlier developmental level. The possibility of a delay in visual development for the DD subjects is supported by the findings that many of the DD larvae, when removed from the DD condition at 6 dpf, were still not hatched. Zebrafish normally hatch at 3 dpf.

Unlike those seen in higher vertebrates, the deficits seen in the b-wave spectral sensitivity of zebrafish subjects were not permanent. Once the subjects were returned to the normal environment, the differences found in the 6 to 8 dpf subjects were no longer evident at 13 to 15 and 21 to 24 dpf. By 21 dpf, all subjects appeared to have regained normal spectral sensitivity functions. There were no apparent differences across the three conditions. This is significant because it suggests that the zebrafish may be a good model for studying neural regeneration.

Further work in this area should investigate the specific relationship between the light environment and visual function. The light environment used in the present study consisted of “daylight” fluorescent lights. Thus, the spectral emission of this source most likely contained energy spikes at certain wavelengths. This lighting condition was chosen because it was similar to the lighting used in past work that showed differences in zebrafish visual behavior and because fluorescent lighting is often used in phototherapy.

SUMMARY AND CONCLUSIONS

Like primates, the zebrafish is affected by constant light-rearing. The pattern seen in primates appears to be replicated in the zebrafish, with a severe deficit in the UV and short-wavelength areas and only a slight deficit in the middle- and long-wavelength areas. Interestingly, the effects found with constant dark rearing were different. These subjects showed a slight deficit across their entire visible spectrum. The difference between the zebrafish and primates is that the deficits were not permanent in the zebrafish.

In conclusion, this study has established the viability of the zebrafish as a model for studying the effects of abnormal light-rearing conditions on retinal development. The unique features of early zebrafish retinal development offer avenues of research that have not previously been found in other species. For example, the high degree of retinal immaturity, in addition to its rapid development and its transparent shell, allow researchers to assess the effects of an abnormal environment in a short period without interrupting development.

Acknowledgments

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References

Binocular Depth-from-Motion in Infantile and Late-Onset Esotropia Patients with Poor Stereopsis

Manami Maeda,1 Mibo Sato,1 Tomobisa Ohmura,1 Yoji Miyazaki,1 Ai-Hou Wang,2 and Shinobu Awaya1

PURPOSE. There are at least two possible ways to detect motion-in-depth binocular without monocular cues: the binocular disparities at different times and a mechanism that detects interocular velocity differences. The perception of interocular velocity differences (Binocular depth-from-motion [BDFM]) depends on the relative velocity of the images on the retina of the left and right eyes, and this information can be experienced by normal and some strabismic patients. The purpose of this study was to determine the characteristics of esotropic patients who have BDFM but have poor stereopsis.

METHODS. Forty-one infantile and 28 late-onset esotropia patients with poor stereopsis were studied. Dynamic stereopsis and BDFM were tested with computer-generated random dot stereograms and kinematograms. The correlations between BDFM and other binocular functional tests were determined.

RESULTS. A total of 31 (44.9%) patients, 15 (36.5%) of the infantile and 16 (57.1%) of the late-onset esotropia group, passed the BDFM test. None of these patients passed the random dot stereo test under static or dynamic conditions. Fusion of the Worth four dot test at near 0.3 m was correlated with the presence of BDFM. Three of the 15 infantile and 10 of the 16 late-onset esotropic patients with positive BDFM showed gross stereopsis as measured by the Titmus Fly. The angle of strabismus was significantly smaller in the patients with positive BDFM for the infantile and the late-onset esotropia groups.

CONCLUSIONS. BDFM was present in about half of the esotropic patients who do not have fine stereopsis. Ocular alignment within 10 to 15 prism diopters is an important factor in obtaining BDFM. Strabismus surgery still provides some binocular benefit for infantile esotropic patients who were bypassed for early surgery. Separate mechanisms may underlie static stereopsis and BDFM. 

Static stereopsis, which arises from the disparity of the retinal images on the two retinas, gives rise to the finest depth perception. To obtain a high level of stereopsis, good ocular alignment is essential; therefore, obtaining or regaining good ocular alignment is one of the important goals for strabismus treatment.

In strabismus clinics, conventional static stereopsis is routinely examined, but dynamic stereopsis, which requires expensive and space-occupying equipment, has not been routinely used. Fusion and stereopsis are considered to be static depth perception processes, which comes from the retinal disparity of the binocular images. However, in a dynamic world, the retinal images are always in motion, and there are both monocular and binocular depth cues from the motion. Monocularly, objects moving in depth result in changing retinal image size and motion parallax. There are at least two possible ways to detect motion-in-depth binocular without monocular cues: the binocular disparities at different times and a mechanism that detects inter-ocular velocity differences. The perception of interocular velocity differences (binocular depth-from-motion [BDFM]) depends on the relative velocity of the images on the retina of the left and right eyes, and this information can be experienced by normal and some strabismic patients1 (Fig. 1). For objects moving in the real world, interocular velocity differences occur at the same time as changes in binocular disparity. Kitaoji and Toyama2 reported that motion stereopsis can be preserved in patients with small angle strabismus who do not have static stereopsis. Thus, testing for the presence of binocular motion-in-depth in a
A clinical setting and learning the prerequisites for BDFM perception can provide important information to assess the visual capabilities of strabismic patients.

One of the authors (AHW) has developed a computer program that generates dynamic random-dot stereograms. In this program, disparity and motion cues can be included or omitted independently. We have tested patients with either early- or late-onset esotropia with this program to see whether BDFM was present despite the absence of static stereopsis.

**METHODS**

This study was performed in the Department of Ophthalmology of Nagoya University between December 1996 and December 1997.

**Controls**

We examined 12 normal subjects (age, 26-30 years, 6 men and 6 women) without any ocular abnormality other than refractive errors (visual acuity; better than 20/20) to determine the normal responses and to select the optimal testing conditions.

**Esotropia Patients**

Informed consent was obtained from the subjects or their guardians, and all procedures were conducted in accordance with the principles embodied in the Declaration of Helsinki.

Forty-one patients with infantile esotropia and 28 with late-onset esotropia were examined. The patients in both groups showed worse than 3000 seconds arc stereopsis as determined by the large fly (+) in the Titmus Stereo Test. Patients with infantile esotropia were defined as those whose esotropia was diagnosed before 6 months of age by an ophthalmologist or was confirmed by photographs; whose deviation was not abolished by a hyperopic correction; who had no central nervous system disorders or developmental delay; and who were born after 37 weeks of gestation. Late-onset esotropia was defined as an accommodative or partially accommodative esotropia with hyperopia, which became manifest after 18 months of age. Patients with paralytic esotropia, esotropia from organic disorders, premature birth (gestation before 37 weeks), and developmentally delayed children were excluded. The mean age at the time of examination was 8.8 years (age range, 4–19 years) for the infantile esotropic group and 13.1 years (age range, 5–31 years) for the late-onset esotropic group.

**Visual Stimulator**

The computer program was run on a FMV-5100D4 computer (FUJITSU, Tokyo, Japan) and displayed on a 17-inch CRT in a dimly lit room. Anaglyphic random-dot stereograms and kinematograms were made up of red and green random-dots. The size of the screen was $31.0 \times 22.0$ cm with $320 \times 200$ pixels. When viewed at a 55.5-cm distance, each pixel subtended 360 second-arc. Four rectangles of $60 \times 50$ pixels ($6^\circ \times 5^\circ$ arc) were displayed on the monitor, and one was programmed to provide a depth cue. Throughout the testing session, the background was made up of a stable random-dot pattern with the same density as the 4 rectangles.

The pair of rectangles seen by right and left eyes, t1R and t1L, was called a stereogram pair if they had the same random-dot pattern and included a disparity cue. If the rectangles were different and therefore had no motion cue, they were called a temporal correlogram. The rectangles seen by the same eye, t1R and t2R, were called kinematograms if they had the same random-dot texture and had cues for apparent motion. If the rectangles were different and therefore had no motion cues, they were called a temporal correlogram. In the kinematogram pair, if the rectangles moved in opposite directions, the fused target invoked a depth sensation (i.e., a movement in depth) in normal subjects.

Test 1 was designed to test binocular motion-in-depth elicited by interocular disparity cues and/or BDFM elicited by movement cues. For this, t1L, t1R, t2L, and t2R were made up of the same random-dot pattern. Test 2 was designed to test only stereopsis, and t1R = t1L, but t1R and t1L were different from t2R and t2L. When the program is stopped, only one rectangle with a disparity of 360 second-arc is seen in depth by normal subjects, but no depth is seen by stereo blind subjects.
Test 3 tested only BDFM without disparity cues, and t1R was different from t1L; however, t1R and t1L had the same pattern as t2R and t2L, respectively. The other three rectangles were seen by both eyes and were designed to move in the same directions. When the program is paused, four indistinguishable rectangles are seen by normal subjects, and no rectangles can be seen in depth by stereo blind subjects (Fig. 2).

Each patient sat facing the screen at 55.5-cm distance with a green filter on the right eye and red filter in front of the left eye. The computer randomly determined which one of the four rectangles would provide a depth cue. The subjects were asked to select the figure that appeared to move back and forth in the “Z” direction. For control, we occluded one eye or paused the program (Test 3) during the course of the tests to be certain that answers were based on disparity or motion cues. The correct answer was given to the patients immediately after each test. The test was repeated 10 times in a forced-choice manner, and a passing score was set at eight correct answers. After the subject passed Test 1, Tests 2 and 3 were conducted. If a subject passed both Tests 1 and 2, he/she was designated as having stereopsis from disparity. If the subject passed both Tests 1 and 3, but not Test 2, he or she was defined as having BDFM-positive without stereopsis.

Testing Methods

Controls. All three tests were first performed on the normal subjects. Test 1 and Test 2 were performed on control subjects. Test 3 was performed with various range of movement and with different velocities of the random-dot pattern to determine the optimal stimulus conditions. The range and speed that allowed the control subjects to detect the depth most easily were selected as the testing condition for the esotropic patients. To determine the minimum visual acuity necessary to pass the BDFM test, we blurred the vision in one or both eyes with Einschleich occlusion parietal filters (Ryser Optik, Basel, Germany) and conducted Tests 1, 2, and 3 on 5 normal patients. In addition, to verify that this test can be passed by horizontal disparity but not by flickering or odd sensation, we tested with 3 normal subjects by rotating the monitor 90°.

Esotropic Subjects. We performed complete ophthalmic examinations including visual acuity, Titmus Stereo tests, TNO stereo test, Bagolini striated lenses test, and Worth four-dot test at near (0.3 m) and distance (5 m) on all the subjects. The angle of strabismus was measured by simultaneous prism cover test at near and far.
Data Analysis

The differences between the patients having BDFM (BDFM+) and those lacking BDFM (BDFM−) were analyzed statistically with either the chi-square test or Mann–Whitney test, and $P < 0.05$ was accepted as statistically significant.

RESULTS

Normal Subjects

The results obtained from the normal subjects showed that the optimal repetition rate and range of movement of the two targets was 3.5 Hz and 1 pixel, respectively. All normal subjects perceived a depth sensation on all tests. When the visual acuity was artificially reduced to 16/20 or better, no control subjects failed Test 3. However, they also reported that they felt depth sensation more strongly on Tests 1 and 2 than Test 3. No one passed any tests when the monitor was rotated 90°.

Patients with Esotropia

The relationship between BDFM and the sensory tests is shown in Table 1. None of the patients passed Test 2 regardless of the time of onset of the ocular deviation (i.e., none had stereopsis). Fifteen (36.5%) patients in the infantile group and 16 (57.1%) in the late-onset group (total = 31, 44.9%) passed Tests 1 and 3 but not 2 and were classified as having positive BDFM without stereopsis. Of the other 38 patients, 26 in the infantile group and 12 in the late-onset group did not pass any test. There was no statistical difference between early-onset esotropia and late-onset esotropia in the incidence of positive BDFM.

The distribution of the angle of deviation at near for the infantile esotropia and the late-onset esotropia groups is shown in Figures 3A and 3B, respectively. The patients with positive BDFM (black squares) had significantly smaller angles of deviation (infantile onset, 4.33 SD, 5.68 prism diopters [pd]; late-onset, 8.00 SD, 6.65 pd) than the patients lacking BDFM (infantile-onset, 12.2 SD, 2.46 pd; late-onset, 18.4 SD, 9.00 pd; $P = 0.0016$; Mann–Whitney test). There was no statistical difference between the age at the time of examination and the presence of BDFM in both groups (infantile-onset, $P = 0.205$; late-onset, $P = 0.0885$ Mann–Whitney test). Of the 41 infantile esotropia patients, 36 had undergone strabismus surgery at a mean age of 166 months (range, 24–356 months). There was no statistical difference between the presence of BDFM and whether the patient had undergone surgery. In addition, there was no statistical difference be-

### Table 1. Comparative Data between BDFM and Conventional Binocular Tests (Chi-Square Test)

<table>
<thead>
<tr>
<th>BDFM(+) n = 31</th>
<th>BDFM(−) n = 38</th>
<th>Chi-Square Test</th>
</tr>
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<tbody>
<tr>
<td>Titmus stereo tests</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fly(+)</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Fly(−)</td>
<td>18</td>
<td>35</td>
</tr>
<tr>
<td>Worth four-dot test at 0.3 m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusion(+)</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>Fusion(−)</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td>Bagolini striated lenses test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUP(+)</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>SUP(−)</td>
<td>17</td>
<td>17</td>
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SUP, suppression. NS, not significant ($P > 0.05$).

Figure 3. (A) Present angle of strabismus and BDFM (infantile esotropia). (B) Present angle of strabismus and BDFM (late-onset esotropia).
tween a positive BDFM and the age at the time of surgery in both groups (infantile-onset, \( P = 0.796 \); late-onset, \( P = 0.517 \) Mann–Whitney test).

There was a high correlation between a positive Titmus Fly test and a positive BDFM (\( P = 0.0009 \), chi-square test). None of the patients passed Plate I of the TNO stereo test. Fusion of the Worth four-dot test at 0.3 m and positive BDFM were highly correlated (\( P < 0.0001 \), chi-square test). There was a significantly higher number of patients with BDFM who were able to fuse the Worth four-dot test at 0.3 m. None of the patients fused the Worth four-dot test at 5 meters. There was no correlation between suppression under Bagolini striated lenses test and BDFM and also no correlation between the visual acuity and the presence of BDFM.

**DISCUSSION**

In this study, we tested whether binocular detection of motion-in-depth, which is evoked by the simultaneous nasal and temporal shift of the retinal images in the two eyes, was present in patients with infantile and late-onset esotropia. We found that 18 of the 31 subjects who did not have stereopsis as determined by the Titmus fly test, showed binocular detection of motion-in-depth sensation. Removing the disparity cues from the visual stimulus did not interfere with their depth perception but removing motion cues from the visual stimulus completely blocked their depth judgment. This latter fact was verified by the absence of disparity sensors as tested by static stereopsis test. When one eye was occluded, none of the subjects could pass the BDFM test. We also rotated the monitor screen 90° to give a vertical directional movement of the targets and no depth sensation was evoked. These findings indicate that the binocular depth perception came solely from inter-ocular velocity differences in the horizontal direction.

In contrast, control subjects answered that they felt stronger depth sensation with Tests 1 and 2 than Test 3, which means they rely on interocular disparity cue rather than interocular velocity cue. This finding is supported by the study of Halpen, who examined the quality of motion-in-depth from interocularly uncorrelated motion-defined forms and concluded that the perceived magnitude of depth is less than that seen with interocularly correlated targets. Cumming and Parker reported that motion-in-depth is primarily detected by means of temporal changes in binocular disparity and that interocular velocity differences play a minor role, if any, in normal subjects. Our finding suggests that the subjects had grown up without the experience of interocular disparity perception, interocular velocity differences could play some role for motion-in-depth sensation.

The angle of the strabismus measured by simultaneous prism cover test, fusion of the Worth four-dot test at near, and the existence of gross stereopsis were factors that were correlated with the presence of positive BDFM. The possible reason for the strong correlation between the Worth four-dot test at near and BDFM is related to the size of the stimulus and the technique used in the computer program for binocular separation. The subtense of the Worth four-dot test at 5 m is 0.5° and that at 0.3 m is 6°, which is the same as the horizontal size of each rectangle of the BDFM test. Therefore, patients with peripheral fusion are probably good candidates for positive BDFM. The Bagolini striated test, on the other hand, was not correlated with BDFM. If the BDFM had been tested under different conditions of binocular separation, the results might have been different.

Kitaoji and Toyama reported earlier that motion stereopsis can be preserved in strabismic patients and that the existing strabismus angle was an important factor related to motion perception. In our computer-generated patterns, the movement of the images was not as smooth as that shown by a galvanometer because of the nature of computer graphic generation. When the image movement is too large, or too slow, or too fast, the sensation of continuous movement is not elicited. Because of the faster movement of the images, 3.5 Hz, compared with the 1 Hz used by Kitaoji and Toyama, it was not possible to verify whether the approaching and receding phases were correctly identified. However, the subjects with BDFM clearly stated that they perceived a back and forth movement. Some of the esotropic patients pointed out that the range of movement varies on each individual.

This study verified that the patients without good binocularity under static conditions, such as infantile esotropia patients, can obtain or regain a different kind of binocularity under dynamic conditions. Interestingly, infantile-onset esotropia patients in this study were all aligned after age two, and the age at surgery did not interfere with the success rate of the BDFM test. This finding is supported by the fact that the plasticity of the motion pathway remains “soft-wired” longer than the critical period for fine stereopsis in humans. It has been established that the peripheral retina is more sensitive to motion and that motion is transmitted to the middle temporal area (MT), where almost all neurons are directionally selective. Direction and orientation selectivity of neurons in visual are in the MT of the macaque. The detection and analysis of motion may also be required in conjunction with the depth perception. Bradley et al. reported the existence of an important link between disparity and transparent motion detection in MT and suggested that binocular disparity in MT may facilitate velocity processing. Recently, MT is reported to be important for the perception of structure-from-motion. Thus, BDFM that comes from binocular detection of velocity could be helpful in the perception of motion-in-depth and structure-from-motion with strabismic patients who lack disparity perception.

**CONCLUSIONS**

BDFM was present in more than half of the esotropic patients who do not have fine stereopsis. Ocular alignment within 10 to 15 prism diopters is an important factor in obtaining BDFM. Strabismus surgery still provides some binocular benefit for infantile esotropia patients who were bypassed for early surgery. Separate mechanisms may underlie static stereopsis and BDFM.

**Acknowledgment**

The authors thank Professor Yozo Miyake for his continuous encouragement during the course of this study.

**References**

Identification of Local Th2 and Th0 Lymphocytes in Vernal Conjunctivitis by Cytokine Flow Cytometry

Andrea Leonardi,1,2 Giuseppe De Franchis,2 Francesca Zancanaro,2 Giovanna Crivellari,2 Massimo De Paoli,2 Mario Plebani,2 and Antonio G. Secchi1

PURPOSE. Th2 lymphocytes may play a key role in the development of allergic diseases such as vernal keratoconjunctivitis (VKC). Cytokine flow cytometry of tear samples was used to identify the phenotypical and functional properties of lymphocytes at the actual site of the allergic reaction.

METHODS. Tear and blood samples were obtained from patients affected by active VKC (n = 12) and from normal control subjects (n = 10). Tears were obtained after gentle scraping of the tarsal and bulbar conjunctiva. Tear and blood samples were placed in a solution of brefeldin-A, phorbol myristate acetate (PMA), ionomycin, and RPMI for 4 hours and then processed for flow cytometry. Lymphocytes were marked with the monoclonal antibodies, anti-IFN-γ and anti-interleukin (IL)-4. Levels of IL-4, IL-2, IFN-γ, IL-2R, total IgE, eosinophil cationic protein (ECP), eosinophil protein X/neurotoxin (EPX), and myeloperoxidase (MPO) were also evaluated in serum.

RESULTS. Expression of IL-4 was observed in 9.2% ± 9.5% of lymphocytes in tears of patients with VKC. Of the 12 patients with VKC, 8 (67%) had tear lymphocytes positive for IL-4 (Th2). Two patients (17%) had a double population of lymphocytes: One was positive for Th2, and the other was positive for both IL-4 and IFN-γ (Th0). One patient (8%) was positive for IFN-γ (Th1) only, and one patient was negative for both ILs. No differences in the percentage of Th2 lymphocytes were found between tarsal and limbal patients. The percentage of Th2 lymphocytes was significantly correlated with the severity of the disease. No positive lymphocytes were found in tears of control subjects. Eosinophils, serum IgE, ECP, and EPX were all significantly higher in VKC than in control subjects.

CONCLUSIONS. In ocular allergic diseases, local lymphocytes expressed the Th2 phenotype and, to a lesser degree, the Th0 phenotype. Although results of systemic allergic markers can be inconclusive in patients with VKC, flow cytometry demonstrated a local lymphocyte phenotype that can account for the clinical and histologic abnormalities of VKC. (Invest Ophthalmol Vis Sci. 1999;40:3036–3040)

Verbal keratoconjunctivitis (VKC) is a severe, bilateral, recurrent inflammatory eye disease occurring mostly in children and young adults and histologically characterized by increased numbers of eosinophils, mast cells, and mononuclear cells, the latter mainly consisting of CD4+ Th-helper (Th) lymphocytes.1 CD4+ Th cells can be classified into distinct types by their cytokine profile.2 Type 1 (Th1) cells, which produce interleukin (IL)-2, interferon gamma (IFN-γ) and tumor necrosis factor-beta (TNF-β), are known to promote delayed-type hypersensitivity. Type 2 (Th2) cells, which produce IL-3, IL-4, IL-5, IL-10, and IL-13, are thought to aid in humoral responses such as IgE isotype switching and mast cell and eosinophil growth and differentiation. In the absence of clear polarizing signals, CD4+ T cell subsets with a more heterogeneous profile of cytokine production than Th1 or Th2 are designated Th0. These Th0 subsets mediate intermediate effects, depending on the cytokines produced and the nature of responding cells. To date, the few studies of cytokine production by conjunctival Th cells have used immunoassays or mRNA analyses, neither of which provided consistent information about the production of different cytokines from individual cells. A prevalence of Th2 type clones has been shown in lymphocyte culture derived from the conjunctival biopsy specimens of a small group of patients with VKC.3,4

In the present study, the expression of IFN-γ and IL-4 cytokines in fresh lymphocytes obtained from conjunctival scrapings and peripheral blood of patients with VKC was investigated using flow cytometry. Cytokine flow cytometry of conjunctival lymphocytes may closely reflect the actual cytokine production and thus the functional properties of these cells at the site of the inflammation, minimizing artifacts due to long-term culture. In addition to flow cytometry, serum cyto-
kines and other systemic markers of allergic inflammation were considered and correlated with the clinical condition.

**MATERIALS AND METHODS**

**Patients and Healthy Subjects**

Tear and blood samples were obtained from patients affected by active VKC (n = 12; mean age, 12.6 ± 6 years), and normal healthy control subjects (n = 10; mean age, 9.7 ± 3.3 years). A group of 20 adult healthy subjects (mean age, 38.3 ± 10 years) was included as an additional control, from whom only blood samples were obtained and analyzed. Of the 12 patients with VKC (2 girls and 10 boys), 6 had the tarsal form of the disease and 6 the limbal form. All the patients were instructed to discontinue therapy for at least 5 days before the visit. For each patient, a clinical score (range, 0–4: 0, absent; 4, severe) was assigned to the four major symptoms (itching, tearing, photophobia, and foreign body sensation) and to the six major signs (conjunctival erythema and chemosis, discharge, papillae, limbal infiltrates, and corneal epithelial disease). The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained from all subjects for participation in the study.

Tears were collected after gentle scraping of the tarsal and bulbar conjunctiva. Two hundred to 350 μl of tear fluid was collected using a capillary tube and placed in vials (Eppendorf, Mannheim, Mannheim, Germany) and cell counting in the Tear cytology on precolored slides (Testsimplet; Boehringer was performed according to Prussin. Peripheral blood and CD4/CD29) and T4/2H4-FITC/Pe (anti-CD4/CD45RA; both (anti-CD56), and anti-HLA-DR-Pe (all purchased from Becton by active VKC (n = 12; mean age, 12.6 ± 6 years), and normal protein (PerCP)–conjugated monoclonal antibodies (mAbs) to human lymphocyte cell-surface antigens were used: Leu-4-FITC (anti-CD5), Leu-3a-Pe/PerCP (anti-CD4), Leu-2a-Pe (anti-CD8), Leu-11c-Pe (anti CD-16), Leu-12-FITC (anti-CD19), Leu-19-Pe (anti-CD56), and anti-HLA-DR-Pe (all purchased from Becton Dickinson, Mountain View, CA) and T4/4B4-FITC/Pe (anti-CD4/CD29) and T4/2H4-FITC/Pe (anti-CD4/CD45RA; both from Coulter, Miami, FL). Data analysis was performed on a flow cytometer (FACScal Immunocytometry System; Becton Dickinson) equipped with an argon laser emitting at 488 nm. Correlate analysis of forward scatter and right-angle scatter was used to establish a lymphocyte gate.

Intracellular cytokine staining (cytokine flow cytometry) was performed according to Prussin. Peripheral blood and tear samples were processed in the same manner. Each blood and tear sample of patients with VKC and normal subjects was incubated for 4 hours in CO₂ at 37°C. Blood samples from the additional control group were similarly processed but incubated for 4 and 8 hours. Isotype-matched control subjects were prepared with 450 μl RPMI and 50 μl BFA. Ten micrometers of mAb anti-CD4 conjugated with PerCP was added to all samples and incubated for an additional 20 minutes. After washing, samples were fixed (100 μl of component A, Fix and Perm; Caltag, South San Francisco, CA) and incubated for 15 minutes at room temperature. After two washings, 100 μl of fixative (component B; Fix and Perm; Caltag), 20 μl of mAb anti-Hu-IFN-γ-FITC (IgG₂a), and 20 μl of anti-Hu-IL-4-Pe (IgG₁) were added. Isotype-matched control subjects were prepared with IgG₁-Pe and IgG₂a-FITC at the same concentration as the anti-cytokine mAb. After a 30-minute incubation in the dark, samples were washed and then fixed with 500 μl of 1% paraformaldehyde.

Flow Cytometric Analysis

Samples of heparinized peripheral blood were processed according to the standard procedure of double or triple direct immunofluorescence. The following: fluorescein-isothiocyanate (FITC)–, phycoerythrin (Pe)- and peridinin-chlorophyll-protein (PerCP)–conjugated monoclonal antibodies (mAbs) to human lymphocyte cell-surface antigens were used: Leu-4-FITC (anti-CD5), Leu-3a-Pe/PerCP (anti-CD4), Leu-2a-Pe (anti-CD8), Leu-11c-Pe (anti CD-16), Leu-12-FITC (anti-CD19), Leu-19-Pe (anti-CD56), and anti-HLA-DR-Pe (all purchased from Becton Dickinson, Mountain View, CA) and T4/4B4-FITC/Pe (anti-CD4/CD29) and T4/2H4-FITC/Pe (anti-CD4/CD45RA; both from Coulter, Miami, FL). Data analysis was performed on a flow cytometer (FACScal Immunocytometry System; Becton Dickinson) equipped with an argon laser emitting at 488 nm. Correlate analysis of forward scatter and right-angle scatter was used to establish a lymphocyte gate.

Intracellular cytokine staining (cytokine flow cytometry) was performed according to Prussin. Peripheral blood and tear samples were processed in the same manner. Each blood and tear sample of patients with VKC and normal subjects was divided into two aliquots. The activated aliquot was processed after stimulation with ionomycin and phorbol myristate acetate (PMA) in the presence of brefeldin-A (BFA), and the nonactivated aliquot was processed without this stimulus in the presence of BFA. Briefly, tears (100 μl) were incubated with 400 μl RPMI to obtain the same volume (500 μl) for each sample. To 500 μl of blood- and tear-activated samples was added 50 μl BFA (final concentration, 10 μg/ml; Sigma), 130 μl RPMI (final concentration, 25 ng/ml; Sigma), 100 μl ionomycin (final concentration, 1 μg/ml; Sigma), and 220 μl RPMI. All samples were then incubated for 4 hours in CO₂ at 37°C. Blood samples from the additional control group were similarly processed but incubated for 4 and 8 hours. Isotype-matched control subjects were prepared with 450 μl RPMI and 50 μl BFA. Ten micrometers of mAb anti-CD4 conjugated with PerCP was added to all samples and incubated for an additional 20 minutes. After washing, samples were fixed (100 μl of component A, Fix and Perm; Caltag, South San Francisco, CA) and incubated for 15 minutes at room temperature. After two washings, 100 μl of fixative (component B; Fix and Perm; Caltag), 20 μl of mAb anti-Hu-IFN-γ-FITC (IgG₂a), and 20 μl of anti-Hu-IL-4-Pe (IgG₁) were added. Isotype-matched control subjects were prepared with IgG₁-Pe and IgG₂a-FITC at the same concentration as the anti-cytokine mAb. After a 30-minute incubation in the dark, samples were washed and then fixed with 500 μl of 1% paraformaldehyde.

To ensure the specificity of the staining procedure, each sample had a control in which the specific binding of the anti-IL-4 and anti-IFN-γ mAbs was blocked with a molar excess of recombinant cytokine (IL-4 and IFN-γ; PharMingen, San Diego, CA). Samples were analyzed on the flow cytometer. Because lymphocytes had not been separated from other leukocytes and epithelial cells in the tear samples, 30,000 events were acquired, reflecting the total number of nonspecific cells in the sample and not the number of CD4 cells. The gating, however, was on only CD4-positive lymphocytes. Three-color dot plots were generated by plotting IL-4 versus IFN-γ fluorescence after gating to exclude dead and/or contaminating non-CD4 + lymphocytes from the analysis. Results are expressed as the percentage of cytokine-producing cells within the CD4 + population. One-parameter histograms demonstrating cytokine staining were created by commercial software (Lysis II; Becton Dickinson, San José, CA), and set markers statistics were performed on the basis of the staining of isotype-matched control subjects.

**Cytokine and Mediator Assay**

In patients with VKC and control subjects, serum and tear levels of IL-4 (by enzyme-linked immunosorbent assay [ELISA]; Endogen, Woburn, MA), serum levels of IFN-γ (Immuno Radio- metric Assay [IRMA]; Biosource-Europe, Fleurus, Belgium), serum levels of IL-2 (ELISA), and IL-2R (by chemiluminescence; Immunolite-ILR2; Euro/DPC, Llanberis, UK) were measured according to their respective standard protocols. The lower detection assay limit for IFN-γ was 1 U/ml; for IL-4, 2 pg/ml; for IL-2, 6 pg/ml; and for IL-2R, 50 U/ml. In serum, the following tests were also performed: total IgE (fluoroenzyme immunoassay [FEIA]; Pharmacia, Uppsala, Sweden) eosinophil cationic protein (ECP), eosinophil protein X/neurotoxin (EPX), and myeloperoxidase (MPO; by radioimmunoassay; Pharmacia).

**Statistics**

Data from the VKC and control groups were compared using the Mann–Whitney test. The Spearman correlation was used to correlate different parameters with the severity of the clinical disease. P < 0.05 was considered significant. All data are expressed as mean ± SD.
RESULTS

Mean total cell number was 88,500 (range, 15,500–180,000) in VKC tear samples and 18,800 (range, 7,500–26,500) in normal samples. The percentage of CD4+ T cells was 2.9% ± 1.6% in VKC tear samples and 2.5% ± 1.0% in normal samples. In VKC tears, the expression of IL-4 within the CD4+ T-cell population was identified by flow cytometry in 9.2% ± 9.5% of lymphocytes (Table 1). Only activated aliquots were positive for intracellular cytokine staining (Fig. 1). Of the 12 patients with VKC, 8 (67%) had tear lymphocytes positive for only IL-4 (Th2); two (17%) had a double population of lymphocytes (one positive to IL-4 and the other to both IL-4 and IFN-γ [Th0]); one (8%) was positive only to IFN-γ (Th1); and one was negative to both ILs. In one tear sample from the normal subject group, a small percentage of Th1 lymphocytes was found. The percentage of IL-4–positive lymphocytes was significantly increased in patients with VKC compared with normal samples (9.2% versus 0%; \( P = 0.001 \)).

No difference in the percentage of Th2 lymphocytes was found between those with the tarsal and those with the limbal form of the disease. The percentage of Th2 lymphocytes was correlated with the total clinical score of the disease \( (P < 0.001) \) and with the degree of corneal involvement \( (P < 0.05) \). In five of the six patients with VKC who were negative for specific serum IgE, local Th2 cells were identified. Cytokine flow cytometry in peripheral blood samples was negative except in one of the normal subjects, in whom 3.9% of lymphocytes were positive for IFN-γ. In the additional control group, in which blood samples were activated for 4 and 8 hours, a lower expression of IL-4 and more IFN-γ–positive cells were detected at 8 hours than at 4 hours' incubation time (IL-4, 0.7% ± 0% versus 0%; IFN-γ, 24.7% ± 6.6% versus 2.1% ± 3%, respectively).

Because of the limited quantity of tear samples, it was possible to measure levels of tear IL-4 only in six patients with VKC and in five normal subjects. Results showed that this cytokine was found only in one patient with VKC (5 pg/ml).

The number of eosinophils and the levels of serum IgE, ECP, and EPX were all significantly increased in patients with VKC compared with control subjects (Table 2). However, these values were not correlated with the severity of the ocular allergic disease expressed by the total score of signs and symptoms. No differences between VKC and control subjects were found in the percentage of peripheral blood lymphocytes CD3+, CD4+, CD8+, and CD16+/CD56+ (natural killer cells). A significantly increased percentage of HLA-DR+ lymphocytes and CD19+ lymphocytes was observed in VKC samples (Table 2). CD4+/CD29+ lymphocytes were also significantly increased in VKC compared with control subjects (15.6% ± 4.5% versus 10.6% ± 2.8%; \( P = 0.002 \)), whereas CD4+/CD45RA+ cells were reduced (14.5% ± 5% versus 20.5% ± 4.1%; \( P = 0.01 \)). None of these values was correlated with the severity of the disease. Only serum levels of sIL-2R were increased in patients with VKC compared with control subjects (Table 2).

After differentiation of tarsal versus limbal VKC, the only statistically significant systemic parameter was the higher number of peripheral blood eosinophils in tarsal \( (731 ± 277 × 10^6/l) \) than in limbal VKC \( (316 ± 234 × 10^6/l; P = 0.02) \).

DISCUSSION

A prevalent Th2 response seems to be involved in the physiopathology of atopic diseases. Among the first descriptions of the cloning of CD4+ cells from allergic tissues, Maggi et al. observed obtained from conjunctival specimens of three patients with VKC T-cell clones that produced mostly IL-4 and little IFN-γ and that supported IgE synthesis in vitro. IL-4 has also been found to be increased in tears from patients with VKC—however, with no evidence of the cell source. An increased expression of IL-3, IL-4, and IL-5 mRNA has also been shown in VKC conjunctival tissues using in situ hybridization histochemistry.

Cytokine flow cytometry is a single-cell technique in which individual cells are analyzed as they pass through the laser in single file. In the present study, this technique was applied to conjunctival-derived lymphocytes freshly collected from a relatively diverse group of patients with VKC. The goal was to identify the functional properties of effector Th cells in vivo by evaluating fresh ex vivo cytokine production at the single-cell level, without the interference of possible in vitro

<table>
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<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Clinical Form</th>
<th>Associated Diseases</th>
<th>Ocular Clinical Score</th>
<th>Total Tear Cell Number x10^3</th>
<th>Eosinophil Cytology (%)</th>
<th>Lymphocyte Cytology (%)</th>
<th>Tear CD4+ (%)</th>
<th>Tear Th1 (%)</th>
<th>Tear Th2 (%)</th>
<th>Tear Th0 (%)</th>
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T, tarsal; L, limbal; E, eczema; As, asthma; R, rhinitis.
artifacts of a cloning procedure. Only after a short polyclonal stimulation, 0% to 28% of the tear CD4\(^+\) lymphocytes of patients with VKC were capable of producing IL-4 and/or IFN-\(\gamma\). The specificity of staining was demonstrated by the positive intracellular staining in aliquots activated with PMA and BFA compared with those to which a molar excess of recombinant cytokine was added. Conversely, this method did not show cytokine expression in T cells from the peripheral blood of young patients with VKC and age-matched control subjects. However, a higher expression of intracellular cytokines was

![Figure 1. Intracellular cytokine-specific staining in VKC tears. Tear samples were stimulated for 4 hours with PMA and BFA. PE-labeled anti-IL-4 antibody and anti-CD4 PerCp were then incubated with either molar excess of the corresponding recombinant cytokine (IL-4) (A) or an equivalent volume of phosphate-buffered saline (B) before staining. Single-color histograms were generated by gating on CD4-positive cells.](image)

### Table 2. Cell Counts, Mediators, and Cytokines in Peripheral Blood of Patients with VKC and Controls

<table>
<thead>
<tr>
<th></th>
<th>VKC ((n = 12))</th>
<th>Control ((n = 10))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgE (kU/l)</td>
<td>213 ± 176</td>
<td>53 ± 22</td>
<td>0.0028</td>
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<tr>
<td>Eosinophils ((\times 10^6/dl))</td>
<td>35 ± 22</td>
<td>2.5 ± 1.2</td>
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<tr>
<td>Lymphocytes ((\times 10^9/dl))</td>
<td>55.4 ± 0.3</td>
<td>55.4 ± 0.3</td>
<td>NS</td>
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<tr>
<td>CD4+ (%)</td>
<td>71.9 ± 5.0</td>
<td>71.9 ± 5.0</td>
<td>NS</td>
</tr>
<tr>
<td>CD19+ (%)</td>
<td>11.8 ± 2.2</td>
<td>11.8 ± 2.2</td>
<td>NS</td>
</tr>
<tr>
<td>HLA-DR+ (%)</td>
<td>15.3 ± 5.3</td>
<td>15.3 ± 5.3</td>
<td>NS</td>
</tr>
<tr>
<td>ECP ((\mu g/dl))</td>
<td>38.6 ± 7.6</td>
<td>38.6 ± 7.6</td>
<td>NS</td>
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<tr>
<td>EPX ((\mu g/dl))</td>
<td>204 ± 5</td>
<td>204 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>MPO ((\mu g/dl))</td>
<td>5.6 ± 1.5</td>
<td>5.6 ± 1.5</td>
<td>NS</td>
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<tr>
<td>IL-4 (pg/ml)</td>
<td>63 ± 28</td>
<td>63 ± 28</td>
<td>NS</td>
</tr>
<tr>
<td>IL-2 (pg/ml)</td>
<td>116 ± 1</td>
<td>116 ± 1</td>
<td>NS</td>
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<tr>
<td>IL-2R (U/ml)</td>
<td>130 ± 16</td>
<td>130 ± 16</td>
<td>NS</td>
</tr>
<tr>
<td>IFN-(\gamma) (U/ml)</td>
<td>298 ± 63</td>
<td>298 ± 63</td>
<td>NS</td>
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</table>

Data are expressed as mean ± SD. NS, not significant.
detected in peripheral blood of adult control subjects when a longer incubation time was used. These latter findings are in agreement with previous studies of peripheral blood cells in which different cloning procedures were implemented, cultured lymphocytes were selected, or longer or different cell stimulations were used, showing that data are influenced by varying methods. However, the great difference between local findings in VKC and normal subjects, and between findings in tears and in peripheral blood in VKC after a short stimulation, may better reflect the physiologic situation of already-activated lymphocytes only at the target organ.

Although 68% of patients with VKC had local Th2 cells, only 17% had both Th2 and Th0 cells. It was notable that most of the patients negative to prick tests and/or serum-specific IgE had local IL-4-positive T-cells. The presence of Th2 cells correlated significantly with the clinical severity of the disease, demonstrating that actively producing effector T-cells play a proinflammatory role in VKC. Whether infiltrating T cells in the inflamed conjunctiva can recognize environmental allergens and thus contribute to the development of a clinical inflammation and to serum and local levels of IgE is still unknown. Several exogenous stimuli, such as aeroallergens and other specific or nonspecific stimuli, may act together in a genetically predisposed host, resulting in a preferential Th2 response. The Th0 cells identified in some patients with VKC may be either a precursor to polarized Th1 or Th2 phenotypes or a stable, differentiated population that is present in conditions in which environmental allergens and antigens induce both cell-mediated and humoral immunity. The presence of IFN-γ, although in a small percentage of cases, may explain why some patients are negative for local IgE, because this cytokine exerts a negative regulatory influence on IL-4-driven IgE synthesis. This finding supports the hypothesis that a delayed-type hypersensitivity reaction may coexist with immediate-type reactions in VKC.

Alterations of other systemic parameters considered in the present study involved a systemic B-cell activation and polyclonal IgE production, although no Th2 cells were found in peripheral blood. The observed increase in IL-2R serum levels in patients with VKC after a short stimulation may better reflect the physiologic situation of already-activated lymphocytes only at the target organ. Several exogenous stimuli, such as aeroallergens and other specific or nonspecific stimuli, may act together in a genetically predisposed host, resulting in a preferential Th2 response. The Th0 cells identified in some patients with VKC may be either a precursor to polarized Th1 or Th2 phenotypes or a stable, differentiated population that is present in conditions in which environmental allergens and antigens induce both cell-mediated and humoral immunity. The presence of IFN-γ, although in a small percentage of cases, may explain why some patients are negative for local IgE, because this cytokine exerts a negative regulatory influence on IL-4-driven IgE synthesis. This finding supports the hypothesis that a delayed-type hypersensitivity reaction may coexist with immediate-type reactions in VKC.

Th2-derived cytokines may be the effectors of many of the clinical and histologic aspects of VKC. IL-3 and IL-5 are mast cell and eosinophil differentiation factors and may be responsible for the high number of conjunctival mast cells and eosinophils seen in VKC. An excess of either IL-4 or IL-13, which are essential for IgE production, may result in the high levels of IgE that can be found only in tears. However, other proinflammatory cytokines and abnormalities have been found in VKC, the pathogenesis of which remains far from clear.

Although results of the present study were limited by the small amount of samples, cytokine flow cytometry clearly demonstrated active Th cells from a mixed population of inflammatory cells freshly derived from the site of the reaction. With this technique, the cytokine production of a small T-cell subpopulation was identified without prior cell purification. These results provide further support that Th2- and Th0-type responses predominate in the conjunctival mcosa of patients with VKC.

References

Prevention of Allergic Eye Disease by Treatment with IL-1 Receptor Antagonist

Andrea M. Keane–Myers, Dai Miyazaki, Grace Liu, Iva Dekaris, Santa Ono, and M. Reza Dana

PURPOSE. To determine the impact of interleukin-1 (IL-1) inhibition using IL-1 receptor antagonist (IL-1Ra) in a mouse model of allergic eye disease.

METHODS. A/J mice sensitized and challenged with cat dander in the eye were treated with topical IL-1Ra or vehicle alone. Control mice were treated with IL-1Ra or vehicle but sensitized and challenged with phosphate-buffered saline alone. Immediately after the final allergen challenge, the mice were observed for behavioral changes and assessed for lid injection and chemosis. The animals were then killed, eyes and attached lids were removed for either RNA extraction or histology, and draining lymph nodes were removed for either RNA extraction or in vitro stimulation assays. Differences in chemokine message between experimental and control groups were determined by RNase protection assays.

RESULTS. Treatment with IL-1Ra in allergen-challenged animals significantly reduced allergen-induced changes in photosensitivity (60%, P = 0.0002), chemosis (50%, P = 0.0151), and injection (86.7%, P = 0.0068) compared with vehicle-treated controls. Interleukin-1Ra reduced the number of degranulated mast cells and caused a significant reduction in the number of eosinophils infiltrating the conjunctival matrix (P < 0.001) after allergen challenge. Examination of chemokine mRNA taken from the conjunctiva and draining lymph nodes by RNase protection assay showed a profound decrease in the production of a number of C–C chemokines.

CONCLUSIONS. These findings suggest that IL-1Ra is suppressing allergic eye disease by a down-modulation of the recruitment of eosinophils and other inflammatory cells essential for the immunopathogenesis of ocular atopy. (Invest Ophthalmol Vis Sci. 1999;40:3041–3046)

Allergic conjunctivitis (AC), atopic inflammation of the mucous membrane that lines the eyelid and outer eyeball, affects over 40 million patients per year in the United States.1 Patients with AC experience itching and burning sensations in the eye in response to allergens that are innocuous for normal individuals and show clinical signs of chemosis, tearing, conjunctival hyperemia, and lid edema.1 The diagnosis of AC is a clinical one mainly based on the history and ophthalmologic findings. Conjunctival scrapings, although not commonly used, are of help because eosinophils are not ordinarily found in the conjunctival scrapings form nonallergic individuals, making the presence of even one eosinophil considerable evidence in favor of a diagnosis of AC.1

We have developed an experimental murine model for cat-dander-induced AC, which provides the clinical, cellular, and humoral parameters of allergic disease. Sensitized mice are challenged via eyedrops with cat dander extract containing defined amounts of the major cat allergen, a 35-kDa protein known as Felis domesticus allergen 1 (Fel d 1).2 As the predominant human IgE binding component in cat dander, Fel d 1 is known to elicit the symptoms of perennial AC.2 Sensitization and challenge with cat dander results in significant increases in photosensitivity, itching, chemosis, and conjunctival injection compared with phosphate-buffered saline (PBS)–challenged control animals. These early clinical symptoms correlate with mast cell degranulation observed within 1 hour after challenge, and significant infiltration of inflammatory cells, including eosinophils. 24 hours later. This model enables us to carefully study disease pathogenesis and to evaluate new therapies, for the treatment of AC.

Interleukin 1 (IL-1), a 17.5-kDa cytokine synthesized primarily by activated macrophages, plays a central role in the initiation and coordination of host defenses in response to a range of physiological insults, including trauma, infection, and inflammation. The IL-1 family consists of two agonists, IL-1α and IL-1β, and a structurally related specific receptor antagonist, IL-1Ra. Interleukin-1Ra binds with high avidity to IL-1 receptors but fails to trigger intracellular responses, thus acting as a competitive inhibitor.3 Recombinant human IL-1Ra has been used both in vitro and in vivo to block a range of IL-1–induced biological activities, thereby validating its use in determining the precise role of IL-1 in short-term animal models of disease.

In the study reported in this article, we used recombinant IL-1 receptor antagonist to study the effects of IL-1 inhibition in a mouse model of AC. Interleukin-1 has been found to regulate chemokines and adhesion factors, the upregulation of which has been closely linked to the development of allergic disease.3 Therefore, we hypothesized that treatment with IL-1Ra could suppress ocular allergy. Our results suggest that IL-1Ra can effectively treat allergic eye disease by decreasing the expression of chemokines essential for the recruitment of allergy-inducing inflammatory cells, including eosinophils.

MATERIALS AND METHODS

Animals

Six-week-old A/J mice were obtained from Jackson Laboratory (Bar Harbor, ME). To prevent any potential aerosolization of allergen from affecting PBS-challenged control animals, all mice were kept in cages with filter-topped lids. The studies reported
here conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the National Institute of Health guidelines for the experimental use of animals and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We used 10 mice for each experimental group at each time point studied, unless specified otherwise.

Antigen Challenge Protocols

We sensitized the mice to cat dander by local administration via eye drops of prepared cat dander extract containing quantitated amounts of Fel d1 (ALK; 250 AU/eye) drop-wise in the eye (2.5 μl/eye) with the use of a p10 micropipette man (Eppendorf). Mice were then challenged with the same amount of allergen via eyedrops once per week for the next 4 weeks. Control mice were challenged with a similar volume of PBS (sham challenge).

Administration of IL-1Ra

Two percent IL-1Ra (20 mg/ml) in 0.2% sodium hyaluronate was administered to both cat dander-challenged and sham-challenged (PBS) control mice three times daily for the duration of the experiment (28 days), starting 30 minutes before the initial sensitization. The dosage and administration of IL-1Ra treatment used in these experiments conformed to previous protocols using topical IL-1Ra in models of inflammatory eye disease. To control for any potential response to sodium hyaluronate alone, two additional groups (either cat dander-challenged or sham-challenged) received a similar volume of vehicle (0.2% sodium hyaluronate).

Clinical Evaluation

In all the challenge protocols, the identity of treatment in each of the cages was masked, and mice were observed by two independent investigators every 5 minutes for the first half hour after the final antigen challenge for changes in behavior (i.e., increased photosensitivity as evidenced by squinting or excessive face washing, suggesting a response to itching eyes). The animals were also assessed by two ophthalmologists (DM and ID), using a slit lamp for assessment of injection and chemosis. Representative photographs of mouse eyes were recorded as during each 1-minute timed assessment. The percentage of animals per cage experiencing a particular symptom was then recorded. The animals were then killed at 1, 24, 48, or 72 hours after the final antigen challenge. In all cases, the mice were anesthetized with ketamine/xylazine (200 mg/kg ketamine + 10 mg/kg xylazine) and exsanguinated via cardiac puncture for antibody analysis of their sera.

Histology

To assess the cellular infiltrate in the conjunctiva and surrounding tissue, the eyes were removed with the attached lids and intact conjunctiva, immediately fixed in 4% paraformaldehyde, and embedded in Historesin (Leica Instruments GmbH, Heidelberg, Germany). Serial sections were cut from each eye and stained with Giemsa for mast cells and hematoxylin and eosin (H&E) or Congo red (both from Sigma) for the identification of eosinophils. All slides were masked, and cell counts were made by two independent investigators. For cellular quantitation, five sections were examined from each mouse per treatment group (n = 10 mice per group), and cells were counted in five 400× nonoverlapping fields per eyelid section, for a total of 250 400× fields per treatment group. Eosinophils were identified by red eosin staining of their cytoplasmic granules and their distinctive bilobed nuclei. Mast cells were identified by their oval contour, mean diameter of approximately 12 to 15 μm, and cytoplasm filled with distinctive pink-to-purple granules.

RNase Protection Assay

To determine relative chemokine mRNA levels, we used the Riboquant Multiprobe RNase Protection Assay system (PharMingen, San Diego, CA). Briefly, draining (cervical) lymph nodes and eyeballs containing conjunctival samples were removed from the mice immediately after they were killed. The conjunctiva was dissected from its attachments to the eyelids. The tissues were placed in 2 ml of RNASat 60, homogenized, and immediately frozen on dry ice. The samples were stored at −80 °C until used. For use, separate RNA samples from five mice per experimental group were defrosted on ice, and the RNA was extracted with a phenol chloroform procedure using DEPC-treated materials. Aliquots of the samples were run on a 1% agarose gel to determine concentration and ascertain any potential degradation. The samples were then used as per manufacturer’s instructions with the mCK-5 probe positive for lymphotaxin (Ltn). Regulated on activation normal T cell expressed (RANTES), cotaxin, macrophage inflammatory peptide-1α (MIP-1α), MIP-1β, MIP-2, interleukin-gamma–induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), T cell activation gene-3 (TCA-3), and the housekeeping genes L32 and GAPDH. To semiquantify differences in amounts of RNA, the film was scanned after development, and the bands were subjected to densitometry using Digital Science ID software (Kodak). The numerical values were normalized according to the densitometry of the housekeeping genes GAPDH and L32.

Data Analysis

Data are summarized as mean ± SE. The statistical analysis of the results was performed by ANOVA using Fisher’s least significant differences test for multiple comparisons. P < 0.05 was considered significant.

RESULTS

IL-1Ra Significantly Reduced Clinical Signs of Allergic Eye Disease

To control for any potential effects of either IL-1Ra treatment or vehicle, a second set of mice were sham-challenged with PBS. Previous studies in our laboratory did not find any discernible difference between PBS-challenged and naive control animals. Antigen treatment of sensitized mice caused significant increases in clinical signs of AC, including an increase in photosensitivity (86.6%, P = 0.0001) indicated by marked squinting, itching as evidenced by vigorous face washing and scratching about the eyes (66.6%, P = 0.001), and chemosis (50%, P = 0.001) and injection (100%, P = 0.0001) of the conjunctiva compared with sham-challenged control animals. The allergic symptoms began within 5 minutes after antigen
challenge and peaked 20 minutes after the final challenge (Fig. 1). Control mice sham-challenged with PBS displayed none of these symptoms and appeared physiologically normal. Treatment with IL-1Ra in allergen-challenged animals significantly reduced allergen-induced changes in photosensitivity (60%, P < 0.0002), chemosis (50%, P = 0.0151), and injection (86.7%, P = 0.0068), reducing symptoms to levels indistinguishable from the sham-challenged control groups.

Reduction in Allergen-Induced Eosinophil Infiltration and Mast Cell Degranulation after IL-1Ra Treatment

To assess the cellular infiltration in the conjunctiva and surrounding tissue, the intact eyes and lids were sectioned and stained with either hematoxylin and eosin or Congo red for the assessment of eosinophils, or with Giemsa for the assessment of intact mast cells. The groups were masked, and slides were counted by two independent investigators. There was a significant increase in the numbers of degranulated mast cells in the matrix surrounding the conjunctiva after allergen challenge compared with sham-challenged controls (301%, P = 0.03; Fig. 2A). Treatment with IL-1Ra caused a modest, statistically insignificant decrease in mast cell degranulation in cat dander–challenged animals (P = 0.1846; Fig. 2A).

Sham-challenged control mice had few eosinophils in their eyelids and conjunctiva. At 24 hours after the final allergen challenge, there were significant increases in the total number of eosinophils infiltrating into the matrix surrounding the conjunctiva, and especially in the fornical region, compared with sham-challenged controls (Fig. 2B) (598%, P = 0.0001). Cell counts on conjunctiva from animals treated with IL-1Ra showed a reduction in the numbers of eosinophils to levels similar to that observed in the sham-challenged control animals (P = 0.998), suggesting an ablation of antigen-induced eosinophil influx.

IL-1Ra Treatment Reduces the Transcription of Allergen-Induced Chemokines

The comparatively small amount of tissue available from the mouse conjunctiva resulted in relatively low total concentrations of RNA and therefore weaker overall signals than what could be observed from other tissues, including the draining lymph nodes (Fig. 3). However, we were able to observe a reduction in eotaxin and RANTES mRNA in the conjunctiva after IL-1Ra treatment in both the allergen-challenged and sham-challenged control groups (Fig. 4A). The levels of gene expression for eotaxin and RANTES in cat dander– and IL-1Ra–treated conjunctival samples were comparable to levels seen in sham-challenged control animals.

Because there are a large number of articles showing that chemokine expression in the draining lymph node of an inflamed organ affects the expression of immunity and inflammation in the organ itself; we also examined the level of chemokine expression in the draining (cervical) lymph nodes. Cat dander challenge caused significant increases in the gene expression of the chemokines RANTES, eotaxin, MIP-1α, and IP-10 in the draining lymph nodes (Figs. 3 and 4B) compared with sham-challenged PBS controls. Interleukin-1Ra treatment substantially reduced the expression of these chemokines in both the cat dander–challenged mice and in the sham-challenged (PBS) mice. We used densitometric analysis of the autoradiogram with Digital Science ID software to approximate differences in chemokine mRNA. RANTES production was decreased 15% (Fig. 4) in samples taken from draining lymph nodes of mice that had been treated with IL-1Ra compared with control animals, although the actual differences may have been greater because the bands in the cat-dander + vehicle group appeared to have reached saturation. Differences in eotaxin and MIP-1α expression between allergen-challenged animals treated with IL-1Ra and controls were even more striking, with a 41% and a 47% decrease, respectively, in allergen-challenged IL-1Ra–treated animals compared with controls. Levels of IP-10 after IL-1Ra treatment showed the biggest
differences, with a 50.7% decrease compared with vehicle-treated animals.

**DISCUSSION**

Increased levels of IL-1 have been found in late phase reactions in the skin after allergen skin challenge in sensitive humans, suggesting an important role for IL-1 in allergic disease. More-over, a critical element of all immunoinflammatory responses, including allergic disease, is the recruitment of leukocytes by chemokines and upregulation of adhesion factors. Interleukin-1 increases chemokine production, increases adhesion factors, increases macrophage infiltration and activity, and increases lymphocyte proliferation, all of which play a role in the immunopathogenesis of allergic disease.

The administration of IL-1Ra used in the present study proved effective in significantly reducing the clinical stigmata
of allergy in response to cat dander. In contrast, treatment with vehicle alone did not significantly alter responses in either sham-challenged or cat dander–challenged mice. The reduction in clinical symptoms after IL-1Ra treatment coincided with the ablation of the allergen-induced eosinophilia, because the number of eosinophils in allergen-challenged IL-1Ra–treated animals was similar to that seen in the sham-challenged control animals. A number of studies in recent years have shown the infiltration of eosinophils and the subsequent release of eosinophil-derived granular proteins and lipid metabolites to be central in the pathogenesis of allergic disease. Initially, we thought the decrease in eosinophil infiltration into the conjunctiva after IL-1Ra treatment was due to a modification in cytokine levels. A number of articles have stressed the importance of T_{H2} cytokines, especially IL-4 and IL-5, in the induction of eosinophilia. However, although we did see an allergen-induced increase in the concentrations of IL-4 and IL-5 in lymphocyte cultures from draining lymph nodes after allergen challenge, blockade of IL-1 did not significantly alter IL-4 and IL-5 cytokine production in this model (data not shown).

The traffic of eosinophils to the sites of allergic reactions is presumed to be regulated at several levels, including the expression of chemokines. Increased levels of RANTES, eotaxin, and MIP-1α have been detected in the nasal secretions of atopic patients exposed to allergen challenge and have been reported to be increased in nasal washings of ragweed-sensitive subjects during the pollen allergen season. In this model of AC, several chemokines including RANTES and eotaxin are upregulated in the conjunctiva of sensitized mice after allergen challenge. Treatment with IL-1Ra substantially decreases the concentrations of these chemokines, both of which have been found to be important in eosinophil chemotaxis.

Additionally, examination of chemokine profiles in the draining lymph nodes shows a substantial reduction in the

**Figure 4.** Densitometry for IL-1Ra data in conjunctiva (A) and draining lymph nodes (B). To assess semi-quantitative differences in amounts of RNA, the film was scanned after development, and the bands were subjected to densitometry using Digital Science ID software (Kodak). The numerical values were normalized according to the densitometry of the housekeeping genes GAPDH and L32. The amount of chemokines produced in mice given the maximal stimulus (cat dander + vehicle) was set as 100%. Interleukin-1Ra significantly reduced the amount of RANTES, eotaxin, MIP-1α (MIP-1a), and IP-10 in both allergen- and sham-challenged control groups. In addition, IL-1Ra treatment reduced the concentrations of eotaxin and RANTES found in conjunctival samples in both cat dander-treated and sham-challenged control groups.
allergen-induced chemokines RANTES, MIP-1α, eotaxin, and IP-10. All of these chemokines are thought to play an important role in the allergic inflammatory process, because they are chemotactic for activated T cells, eosinophils, basophils, and monocyte/macrophages. We do not contend that chemokine patterns in the draining lymph nodes directly influence cell traffic into the conjunctiva. However, the importance of lymph node chemokine data is underscored by the central role lymph nodes play in bringing together mature antigen presenting cells and naive T cells whose priming in the nodes leads to clonal proliferation of effector T cells. Accordingly, suppression of RANTES and MIP-1α gene expression in the lymph node as a result of IL-1Ra treatment may affect immune responses by the down-modulation of the activation and eventual recruitment of T cells to the eye, which could, in turn, influence the infiltration of histamine-releasing cells such as eosinophils into the conjunctiva. Interestingly, the reduction in RANTES and eotaxin production was even more striking in the IL-1Ra-treated, sham-challenged groups in both the conjunctiva and the draining lymph nodes. This finding suggests that IL-1Ra was able to down-modulate both the endogenous and the antigen-induced gene expression of chemokines.

Interleukin-1 has been shown to regulate the expression of eotaxin in epithelial and endothelial cells in vitro, again suggesting a strong association between IL-1 production, eosinophil infiltration, and allergic disease. Unlike the more general effects of the other chemokines examined, eotaxin exclusively attracts eosinophils when applied in vivo, and its expression is enhanced in animal models of allergic inflammation and in tissue cells at sites of eosinophil accumulation. Because of this preferential and potent action on eosinophils and its occurrence in different species, eotaxin is considered perhaps the most relevant chemokine in the pathophysiology of allergic conditions and asthma. The major cellular sources of eotaxin are thought to be the epithelia and activated infiltrating leukocytes like eosinophils. The localization of eotaxin production is important not only to our understanding of the basic mechanisms of tissue eosinophilia and tissue damage but also to the design of drug delivery, which can now target the specific cells that generate the signal responsible for the selective recruitment of eosinophils. IL-1Ra treatment yielded a substantial decrease in eotaxin levels in conjunctiva and draining lymph nodes compared with allergen-challenged animals that received vehicle alone.

Conjunctiva tends to be a relatively fragile tissue to work with, particularly in small rodent species. It is for this reason that we were not able to extract chemokine proteins themselves from our murine samples. However, because we observed such a striking decrease in clinical symptoms as well as an ablation of eosinophil infiltration into the conjunctival matrix after IL-1Ra treatment, we propose that the differences in mRNA assayed were biologically relevant and that they explain, at least in part, the observed striking suppression of clinical and histologic parameters of allergic eye disease.

The highly variable efficacy and myriad side effects of topical and systemic nonspecific antiinflammatory pharmaceuticals including corticosteroids are well known to clinicians who use these agents to arrest allergic disease. The observations implicating eosinophils in the pathogenesis of allergy make it likely that understanding the mechanisms of eosinophil recruitment into the conjunctiva will offer new therapeutic approaches for the treatment of this disease. Because chemokine production appears to be essential for the recruitment of eosinophils in the allergic response, strategies that block chemokine production may be effective in treatment of allergic disease. Using an in vivo model of allergic eye disease, we have shown that antagonism of IL-1 activity by the topical administration of IL-1Ra offers an effective means of suppressing eosinophilia and the resultant allergic response in the eye. Our data strongly suggest that specific molecular targeting strategies, such as the use of IL-1Ra to suppress IL-1 activity, may eventually offer a novel approach to the management of AC.

References

Responses of Intraocular Pressure and the Pupil of Feline Eyes to Prostaglandin EP<sub>1</sub> and FP Receptor Agonists

Parimal Bhattacherjee, Billy Shawn Williams, and Christopher A. Paterson

**Purpose.** Previous studies suggested that FP receptors do not mediate the relaxation of the ciliary muscle and reduction of intraocular pressure in cats by prostaglandin (PG) F<sub>2α</sub>. The present study was undertaken to determine whether the reduction of intraocular pressure in cats induced by PGF<sub>2α</sub>, is mediated by FP or other prostaglandin receptors.

**Methods.** One eye of each cat was treated topically with prostaglandin F<sub>2α</sub>, fluprostenol (FP receptor agonist), or 17-phenyl trinor PGE<sub>2</sub> (EP<sub>1</sub> receptor agonist) in a dose range of 12.5 to 50 μg. The effects of SC19220 and SC51089 (EP<sub>1</sub> receptor antagonists), BWA868c, and SQ29548 (DP and TP receptor antagonists, respectively) on the intraocular response to PGF<sub>2α</sub>, were also examined. At intervals up to 6 hours after treatment, intraocular pressure was measured with a pneumotonometer, and pupil diameters were measured with a millimeter ruler.

**Results.** In the dose ranges used, PGF<sub>2α</sub>, and 17-phenyl trinor PGE<sub>2</sub> decreased intraocular pressure and pupil diameter. The greatest reduction of intraocular pressure by 50.0 μg PGF<sub>2α</sub>, was 5.0 ± 1.4 mm Hg, whereas by 50 μg 17-phenyl trinor PGE<sub>2</sub>, was 6.2 ± 1.5 mm Hg. The isopropyl ester of PGF<sub>2α</sub> at a dose of 1.25 μg reduced intraocular pressure by 3.75 ± 0.25 mm Hg at 2 hours. At doses up to 100 μg, fluprostenol did not decrease intraocular pressure but did reduce pupil diameter. SC19220, a weak but selective EP<sub>1</sub> receptor antagonist, inhibited the intraocular pressure response to both PGF<sub>2α</sub>, and 17-phenyl trinor PGE<sub>2</sub>. The more potent EP<sub>1</sub> receptor antagonist SC51089 had a greater inhibitory effect than SC19220 on the intraocular pressure response to PGF<sub>2α</sub>. Both of these antagonists had a small but non-dose dependent and statistically insignificant effect on the pupil response to PGF<sub>2α</sub>. These observations suggest that in cats, intraocular pressure and pupil responses to PGF<sub>2α</sub>, are mediated by EP<sub>1</sub> and FP receptors, respectively. However, SC19220 significantly and dose-dependently inhibited the pupil response to 17-phenyl trinor PGE<sub>2</sub>, suggesting that EP<sub>1</sub> receptors mediate pupil response to this agonist. DP and TP receptor antagonists at doses 5- to 20-fold greater than the IC<sub>50</sub> values had no effect on the ocular hypotensive response to PGF<sub>2α</sub>. The concurrent administration of 12.5 μg of each of PGF<sub>2α</sub>, and 17-phenyl trinor PGE<sub>2</sub>, did not produce an additive effect on intraocular pressure, indicating that in cats PGF<sub>2α</sub>, and 17-phenyl trinor PGE<sub>2</sub>, act on the same receptor type.

**Conclusions.** These results suggest that a significant proportion of the ocular hypotensive action of PGF<sub>2α</sub>, in cats is mediated by EP<sub>1</sub>, but not by FP receptor. Evidence was also provided to show that 17-phenyl trinor PGE<sub>2</sub>, is an ocular hypotensive agent in cats. (Invest Ophthalmol Vis Sci. 1999;40:3047–3053)

Prostaglandins (PGs), administered topically in appropriate concentrations, reduce intraocular pressure (IOP) in rabbits, cats, and humans. Prostaglandin F<sub>2α</sub> in particular is an extremely potent ocular hypotensive agent and is currently thought to reduce intraocular pressure by increasing uveoscleral outflow, perhaps through the mediation of discrete FP receptors in the ciliary muscle. Recently, one report suggests that the ocular hypotensive action of PGF<sub>2α</sub>, in the cat is not mediated by FP receptors. Also, PGF<sub>2α</sub>, has been reported to have a weak relaxant effect on cat ciliary muscle, whereas the more selective FP receptor agonist 17-phenyl trinor PGF<sub>2α</sub>, was without effect. These observations suggest that increased uveoscleral outflow in response to PGF<sub>2α</sub>, may not be mediated by FP receptors. Recently, a study on the generation of second messengers reported that 17-phenyl trinor PGE<sub>2</sub>, but not PGF<sub>2α</sub>, stimulated the formation of inositol phosphates in cat ciliary muscles. To examine whether the ocular hypotensive action of PGF<sub>2α</sub>, is mediated by EP<sub>1</sub>, or other PG receptors, we investigated the response of IOP and pupil diameter in cats to PGF<sub>2α</sub>, and fluprostenol, FP receptor agonists, and an EP<sub>1</sub> receptor agonist, 17-phenyl trinor PGE<sub>2</sub>.

**Methods**

**Materials**

All natural and synthetic prostaglandins and SQ29548 were purchased from Cayman Chemical (Ann Arbor, MI). The EP<sub>1</sub> receptor antagonists SC19220 and SC51089 were a gift from Searle Research and Development (Skokie, IL). DP receptor antagonist BWA868c was supplied by Glaxo–Wellcome (Hertfordshire, UK). Alcaine (0.5% proparacaine) was purchased from Alcon Laboratories (Fort Worth, TX).

**Measurement of IOP and Pupil Diameter**

All animals used were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty cats (2-3 kg) were domesticated for 4 months and trained to accept topical administration of saline or local anesthetic drops and the application of a pneumotonometer tip to the cornea to measure IOP. These cats were randomly divided into groups of three to six cats depending on the experimental design of each experiment. A washout period of 15 to 30 days was allowed between separate experiments. Before the experiments with prostaglandins, IOPs of the eyes treated with the vehicle and untreated contralateral eyes of all the cats were measured for a 6-hour period using a pneumot-
It is obvious that feline IOP remains stable during a 6-hour experimental period with minor variations. At the maximal concentration, none of the agonists induced a contralateral effect. Furthermore, SC19220 alone at 100 μg had no effect on IOP.

Prostaglandin F₂α, its isopropyl ester 17-phenyl trinor PGE₂, and fluprostenol all dose-dependently induced miosis. The time course and duration of the pupil response to these agonists were similar to those of the IOP responses (Figs. 3A, 3B, and 3C). As shown in Table 1, the selective EP₁ receptor antagonist SC19220 inhibited the IOP response to PGF₂α, or 17-phenyl trinor PGE₂ as well as isopropyl PGF₂α. The highest concentration of SC19220 blocked the IOP response to PGF₂α by 38%, compared with the 80% and 94% reductions of the responses to 17-phenyl trinor PGE₂ and isopropyl PGF₂α, respectively. SC51089, a more potent EP₁ receptor antagonist than SC19220, confirmed the above observation on PGE₂ response. The results (Table 1) show that SC51089 is more effective than SC19220 in inhibiting the IOP response to PGF₂α. Inhibition of the pupil response to PGE₂ by SC19220 or SC51089 was not dose-dependent or statistically insignificant; whereas the inhibition of 17-phenyl trinor PGE₂ response by SC19220 was dose-dependent and significant (SC51089 was not tested against 17-phenyl trinor PGE₂). The highly potent DP receptor antagonist BWA868c (IC₅₀, 1 nM) at 0.5 ng (25 nM) or 1.2 ng (100 nM) and TP receptor antagonist SQ29548 (IC₅₀, 10 nM) at 0.48 ng (50 nM) or 0.96 ng (100 nM) did not affect the ocular hypotensive response to PGF₂α (Table 1). We performed one classic pharmacological experiment to determine whether PGF₂α, and 17-phenyl trinor PGE₂ are acting on the same or different receptor type. Figure 4 shows that 12.5 μg of each of these agonists administered together did not produce a greater hypotensive effect than when administered alone. This observation suggests that these two agonists activated the same EP₁ receptors. If they had been acting on different receptor types, the IOP response would have been additive.

**Results**

The effects of PGF₂α, fluprostenol, and 17-phenyl trinor PGE₂ on IOP and the pupil diameters of cats are summarized in Table 1. In the dose range of 12.5 to 50.0 μg, PGF₂α reduced IOP without an initial phase of ocular hypertension. All doses of PGF₂α lowered IOP, beginning 1 hour after the treatment. The greatest reduction, 5.0 ± 0.4 mm Hg, occurred between 1 and 2 hours after treatment with 50.0 μg of PGF₂α (Fig. 1A). The IOP returned to baseline value within 6 hours after treatment with all doses of PGF₂α. In contrast, the response of IOP to 1.25 μg isopropyl ester of PGF₂α, was significantly greater than that to PGF₂α (Fig. 1A). This was not unexpected because the ester forms of prostaglandins achieve greater intraocular concentrations than do their acidic forms.¹⁸ 17-Phenyl trinor PGE₂ also reduced intraocular pressure in a dose-dependent manner, with an onset and duration of action similar to those of PGF₂α (Fig. 1B). The greatest reduction of IOP by 50.0 μg of 17-phenyl trinor PGE₂ was 6.2 ± 1.5 mm Hg at 2 hours after treatment. Fluprostenol, in doses up to 100 μg, did not reduce IOP (Fig. 1C). Intraocular pressures of the eyes treated with vehicle or 100 μg SC19220 and the contralateral eyes of the 50 μg PGF₂α-treated group are shown in Figure 2. It is obvious that feline IOP remains stable during a 6-hour

**Discussion**

In the present study, we demonstrated for the first time that a single dose of 17-phenyl trinor PGE₂, an EP₁ receptor agonist, reduced IOP and pupil diameter in cats. Prostaglandin F₂α also reduced IOP and pupil diameter in a dose-dependent manner. At a low dose of 1.25 μg, the isopropyl ester of PGF₂α also reduced IOP. To maintain consistency, we primarily used prostaglandins instead of their isopropyl esters because two of the agonists used, fluprostenol and 17-phenyl trinor PGE₂, are not available as isopropyl esters.

Intraocular pressure responses to PGF₂α, and its isopropyl ester are consistent with previous reports that these compounds are potent ocular hypotensive and miotic agents in cats. Prostaglandin F₂α, a more selective FP receptor agonist than PGF₂α, did not lower IOP but was as potent as PGF₂α and 17-phenyl trinor PGE₂ as a miotic agent. The EP₁ receptor antagonist SC19220 at a 100-μg dose significantly inhibited the reduction in IOP by PGF₂α, its isopropyl ester, and 17-phenyl trinor PGE₂. The doses of the antagonist used in the present study appear to be high. However, it should be emphasized that, although this EP₁ antagonist is highly selective, it is not potent, particularly in vivo. Therefore, it is not surprising that high doses were needed to exert an inhibitory effect. The more
potent EP<sub>1</sub> receptor antagonist SC51089, at a dose of only 10 μg, inhibited 80% of the IOP response to PGF<sub>2α</sub>, suggesting that the ocular hypotensive action of PGF<sub>2α</sub> is mediated by EP<sub>1</sub> but not by FP receptors in the cat. These observations were further supported by the fact that fluprostenol, a more selective FP receptor agonist than PGF<sub>2α</sub>, did not lower IOP while contracting the sphincter muscle to induce miosis. If FP receptors were present in the ciliary body, fluprostenol would have reduced IOP as it contracted the sphincter muscles that are known to express FP receptors. Furthermore, the observation that the concurrent administration of PGF<sub>2α</sub> and 17-phenyl trinor PGE<sub>2</sub> was not additive in lowering IOP indicated that these two agonists were acting primarily on the same EP<sub>1</sub> receptors. If these two agonists were acting on separate receptor types, then the response to the combined treatment would have been greater than the response to either of the agonists alone. The order of potency of prostaglandins for EP<sub>1</sub> receptors is PGE<sub>2</sub> > PGF<sub>2α</sub>, prostacyclin > PGD<sub>2</sub> and TXA<sub>2</sub>. Therefore,
in tissues in which FP receptors are lacking, PGF$_{2\alpha}$ is most likely to activate EP$_1$ receptors. Our pharmacological study in vivo only suggests that PGF$_{2\alpha}$ is acting via EP$_1$ receptors. To determine whether this is precisely the situation, additional in vitro studies on the contractile or relaxing response of the feline ciliary muscles to FP and EP$_1$ receptor agonists are needed.

Ligand binding assays and functional studies suggest that PGF$_{2\alpha}$ has affinity for DP and EP$_3$ receptors.\textsuperscript{21-23} Also, stimulation of DP or EP$_3$ receptors reduces IOP in rabbits.\textsuperscript{22,23} and stimulation of TP receptors reduces IOP in beagle dogs.\textsuperscript{24} Thus, to exclude the possibility that the IOP-lowering effect of PGF$_{2\alpha}$ was due to the stimulation of either EP$_3$ or DP receptors or both, we tested a potent and selective DP receptor antagonist, BWA868c.\textsuperscript{25} At 25- to 100-fold greater concentration than its IC$_{50}$, the DP receptor antagonist did not significantly modify PGF$_{2\alpha}$ response, suggesting that DP receptors are not involved in the ocular hypotension induced by PGF$_{2\alpha}$. Although PGF$_{2\alpha}$ has a poor affinity for TP receptors, to rule out the extreme possibility that PGF$_{2\alpha}$ also stimulated these receptors, the ef-

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**Figure 2.** Intraocular pressure of vehicle-treated eyes (A; n = 8) and contralateral eyes (B; ■) of 50 µg PGF$_{2\alpha}$-treated groups (n = 8) and IOP of 100 µg SC19220-treated eyes ( Thief; n = 4). Vertical bars are ±SEM.
The effect of the TP receptor antagonist SQ29548 on the responses to PGF$_{2\alpha}$ was examined. Even at high concentrations, this antagonist did not block PGF$_{2\alpha}$ response. We could not test the effect of an EP$_3$ receptor antagonist on PGF$_{2\alpha}$ response because no such antagonists are available. Therefore, we suggest that PGF$_{2\alpha}$ does not stimulate DP or TP receptors to lower IOP in cats.

Previous studies have suggested that the reduction of IOP and relaxation of ciliary muscle of cats by PGF$_{2\alpha}$ are not mediated by FP receptors.\textsuperscript{11–13} Also, it has been reported that PGF$_{2\alpha}$ does not increase inositol phosphate turnover in the feline ciliary muscles ex vivo.\textsuperscript{14} All these studies suggest a lack of FP receptors in the feline ciliary body but do not describe prostaglandin receptors in cats that are activated by PGF$_{2\alpha}$ to lower IOP. The data we obtained with fluprostenol, EP$_1$ receptor antagonists, and concurrent administration of PGF$_{2\alpha}$ and 17-phenyl trinor PGE$_2$ suggest that FP receptors are not expressed in the feline ciliary muscles. However, studies on the expression of mRNA of FP receptors in the target tissues, such as the feline ciliary muscles, need to be performed to confirm our pharmacological observations.

The concentrations of prostaglandins used in the present study appear to be high, and it could be argued that other prostaglandin receptors, particularly EP$_1$ receptors, were stim-

**Figure 3.** The reduction of pupil diameter in cats by PGF$_{2\alpha}$ (A), 17-phenyl trinor PGE$_2$ (B), and fluprostenol (C) administered topically. The values are the net of those for control minus treated eyes. Each experiment at each concentration used three animals, and each experiment was done at least twice. Vertical bars are ±SEM. *Significant difference between controls and treated at $P < 0.05$ level.
ulated by such high concentrations. In fact, the doses of prostaglandins used in the present study were smaller than those used in previous studies.2,11,18 It should also be pointed out that a dose of a compound administered topically to the eye is diluted by tears and that only a fraction of the dilution permeates the cornea. Thus, the intraocular prostaglandin concentration will probably range only from 0.25% to 0.5% of the administered dose.27–29 Therefore, the intraocular concentrations of the agonists at the doses used in the present study were probably in the range of 0.03 to 0.25 \( \mu \text{g} \). Furthermore, the actual concentration of the agonist in the tissues of the uveoscleral pathway or the site of action of \( \text{PGF}_{2\alpha} \) or 17-phenyl trinor \( \text{PGE}_2 \) is likely to be less than the total concentration in the intraocular tissues. Therefore, prostaglandin agonists, ad-

### Table 1. IC\(_{50}\) Values of Prostaglandin Receptor Antagonists, SC19220 (EP\(_1\)), SC51089 (EP\(_1\)), BWA868c (DP), and SQ29548 (TP), for the Inhibition of IOP and Pupil Response to \( \text{PGF}_{2\alpha} \) and 17-phenyl Trinor \( \text{PGE}_2 \) in Cats

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Antagonist</th>
<th>IOP Decrease (mmHg)*</th>
<th>Pupil Diameter Decrease (mm)*</th>
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<tbody>
<tr>
<td>50 ( \mu \text{g} ) ( \text{PGF}_{2\alpha} )</td>
<td>0</td>
<td>5.0 ± 1.4 (4)</td>
<td>5.0 ± 1.5 (4)</td>
</tr>
<tr>
<td>SC19220 (( \mu \text{g} ))</td>
<td>25</td>
<td>4.2 ± 0.4 (8)</td>
<td>3.3 ± 1.0 (8)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.5 ± 0.4 (8)</td>
<td>3.6 ± 0.7 (8)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.1 ± 1.0 (8)†</td>
<td>4.0 ± 0.4 (8)</td>
</tr>
<tr>
<td>SC51089 (( \mu \text{g} ))</td>
<td>0</td>
<td>6.3 ± 0.3 (4)</td>
<td>5.75 ± 1.2 (4)</td>
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<td></td>
<td>2.5</td>
<td>6.3 ± 0.4 (4)</td>
<td>4.8 ± 0.9 (4)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.3 ± 0.4 (4)†</td>
<td>4.9 ± 0.6 (4)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.3 ± 1.5 (4)†</td>
<td>4.8 ± 1.0 (4)</td>
</tr>
<tr>
<td>BWA868c (ng)</td>
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<td>4.5 ± 1.0 (4)</td>
<td>4.2 ± 1.1 (4)</td>
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<td></td>
<td>0.3</td>
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<tr>
<td></td>
<td>1.2</td>
<td>3.7 ± 0.2 (4)</td>
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<tr>
<td>SQ29548 (ng)</td>
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<td>4.1 ± 0.7 (4)</td>
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<td></td>
<td>0.48</td>
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<td>3.3 ± 1.1 (4)</td>
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<td></td>
<td>0.96</td>
<td>3.3 ± 0.6 (4)</td>
<td>3.3 ± 1.4 (4)</td>
</tr>
<tr>
<td>SC19220 (( \mu \text{g} ))</td>
<td>100</td>
<td>0.2 ± 0.1 (5)</td>
<td>3.6 ± 0.7 (5)</td>
</tr>
<tr>
<td>1.25 ( \mu \text{g} ) ( \text{PGF}_{2\alpha} ) (isopropyl ester)</td>
<td>0</td>
<td>3.5 ± 0.1 (5)</td>
<td>5.2 ± 0.6 (5)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.2 ± 0.1 (5)†</td>
<td>3.6 ± 0.7 (5)</td>
</tr>
<tr>
<td>50 ( \mu \text{g} ) 17-phenyl trinor ( \text{PGE}_2 )</td>
<td>0</td>
<td>6.2 ± 1.5 (8)</td>
<td>3.5 ± 0.8 (8)</td>
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<td></td>
<td>25</td>
<td>5.8 ± 0.9 (8)</td>
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<tr>
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<td>50</td>
<td>5.0 ± 1.2 (8)</td>
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</tr>
<tr>
<td></td>
<td>100</td>
<td>1.3 ± 1.1 (8)†</td>
<td>0.5 ± 0.4 (8)†</td>
</tr>
</tbody>
</table>

Decreases in IOP and pupil diameter were measured 2 hours after administration of the agonist and antagonist.

* Values are mean ± SEM of \((n)\) number of experiments.

† Significantly different from antagonist untreated eye at \( P < 0.05 \) level.

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**Figure 4.** The response of IOP to 12.5 \( \mu \text{g} \) of \( \text{PGF}_{2\alpha} \) and 17-phenyl trinor \( \text{PGE}_2 \) administered alone or in combination. The IOP values are the net of those for control minus treated eyes. Each experiment consisted of six animals. Vertical bars are ±SEM.
ministered topically, most likely stimulated the receptors for which the agonist has primary affinity. This suggestion is supported by the fact that fluprostanol, at large doses of 25 to 50 μg, stimulated only FP receptors in the iris sphincter muscle, not EP1 or other prostaglandin receptors.

At present, the mechanism of the ocular hypotensive action of the EP1 receptor agonist 17-phenyl trinor PGE2 on IOP is not known. In a recent study, Krauss et al. observed that novel TP receptor agonists reduce IOP and increase aqueous humor outflow in dogs. TP receptor activation results in mobilization of intracellular calcium, leading to the contraction of smooth muscle cells in the trabecular meshwork and ciliary muscles. These events may be the underlying mechanism of facility increase by TP receptor agonists. Stimulation of EP1 receptors also mobilizes intracellular calcium, thus it can be speculated that 17-phenyl trinor PGE2 has the same mechanism of action as TP receptor agonists in lowering IOP. However, such speculation needs to be explored in studies on aqueous humor dynamics.

References
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The Effect of Intraocular Pressure on Human and Rabbit Scleral Permeability

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PURPOSE. The purpose of this study was to evaluate the effects of intraocular pressure on the permeability of human and rabbit sclera to water, dexamethasone, and carboxyfluorescein.

METHODS. Scleral sections excised from moist-chamber-stored human globes or eyes obtained from euthanatized New Zealand White rabbits were mounted in a perfusion chamber that can create a transscleral pressure that simulates an intraocular pressure. A small depot of drug (100 μl) was added to the episcleral surface while perfusing an irrigating solution slowly across the choroidal side. The perfusate was collected and scleral permeability calculated. Experiments were performed at 0, 15, 30, and 60 mm Hg for each compound in human and rabbit tissue.

RESULTS. Analysis of variance showed a significant effect of intraocular pressure on both human and rabbit scleral permeability. Human scleral permeability was decreased by as much as a factor of two for water (P = 0.0004), dexamethasone (P < 0.0001), and carboxyfluorescein (P = 0.0064) at elevated intraocular pressures. Rabbit scleral permeability was similarly affected by elevated intraocular pressure for water (P = 0.0039), dexamethasone (P = 0.0001), and carboxyfluorescein (P = 0.0016).

CONCLUSIONS. This study shows that simulated intraocular pressure ranging from 15 to 60 mm Hg can decrease scleral permeability to small molecules by one half when compared with the sclera with no pressure applied. (Invest Ophthalmol Vis Sci. 1999;40:3054–3058)

R etinal disease is one of the major causes of blindness. The treatment of retinal disease is to some degree limited by the difficulty of delivering drugs to target tissues in the posterior eye. Traditional routes of local ophthalmic delivery (i.e., topical) do not yield therapeutic drug levels in the posterior tissues of the eye. Although systemic administration can deliver drugs to the posterior eye, the large systemic doses necessary are often associated with side effects. The sclera offers another vector to obtain therapeutic vitreous and retinal drug concentrations by either subconjunctival or retrobulbar injection.1–3 Delivering drugs across the permeable sclera would be safer and less invasive than intravitreal devices yet could provide a more effective retinal dose than systemic or topical delivery.

Although past in vitro studies have reported scleral permeability for compounds with a wide range of molecular weights, the experiments were performed using chambers that do not impose a transscleral pressure to simulate the intraocular pressure observed in an intact eye.4,5 For this study, we designed a chamber that emulates depot delivery from the scleral surface. The chamber design allows the experimenter to impose a transscleral pressure through a water column (Fig. 1). The simulated intraocular pressure can be controlled by varying the height of the water column, measured by an attached pressure transducer. The choroidal hemichamber—representing the choroidal tissues—is perfused at a slow rate, whereas the episcleral hemichamber is held static, similar to a drug added to Tenon’s space and directly exposed to the sclera.

The purpose of this study was to determine the effect of intraocular pressure on scleral permeability of low-molecular-weight compounds, including water, after a depot application to the episclera. Scleral permeability constants (Ktrans) for water, dexamethasone, and carboxyfluorescein were measured at simulated intraocular pressures of 0, 15, 30, and 60 mm Hg.

METHODS

Scleral tissue was obtained from 66 human donor eyes (Georgia Eye Bank, Atlanta) that had been stored in moist chambers for an average ± SD of 4.5 ± 1.9 days (mean age, 55.0 ± 15.8 years). For rabbit studies, sclera was obtained from 55 eyes of New Zealand White rabbits weighing 5.0 to 5.5 kg, which were anesthetized and then killed by intracardiac injection of sodium pentobarbital (97.2 mg/kg). All animal protocols for these experiments conformed to the Guiding Principles in the Care and Use of Animals (Department of Health, Education and Welfare Publication, NIH 80-23) and the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research. The eye was enucleated, and the extraocular tissues, including conjunctiva and extraocular muscles, were carefully removed. The episclera and uvea were removed with a cotton swab to isolate the bare sclera. Scleral disks of 15 to 20 mm in diameter were excised from the superior temporal section of globe, 12 to 15 mm posterior to the limbus.5

The excised sclera was mounted choroid side down in a specially designed Lucite perfusion chamber, in which the sclera was mounted horizontally (Fig. 1). The sclera was clamped between two 2.5-mm-wide (and approximately 1-mm-thick) cylindrical rings (Sylgard; Dow Corning, Midland, MI) cut to the size of the chamber opening to prevent lateral leakage and scleral edge damage. Chambers with a 7-mm aperture were used for rabbit experiments, whereas at times a 10-mm aperture was used for human experiments and at others a 7-mm aperture was used. BSS Plus (Alcon Laboratories, Fort Worth, TX) was perfused through the lower hemichamber (500 μl volume) at a rate of 0.03 ml/min. Fluid mixing was
achieved in the lower hemichamber with a magnetic microstir bar, with the chamber resting on a magnetic stir plate. The tissue was perfused for 15 to 30 minutes to verify that no leaks were present before applying a test compound to the surface. At times, particularly with rabbit experiments, the cylindrical rings would not form a complete seal around the circumference of the exposed region, causing fluid to leak laterally along the surface of the sclera. Thus, carboxyfluorescein (or Evan’s Blue when measuring carboxyfluorescein permeability) was added to the upper hemichamber to help visualize leakage. Experiments in which leakage occurred were not included in the results.

The test compound, adjusted to a total volume of 100 μl with BSS Plus, was added to the episcleral surface 15 to 30 minutes after the sclera was mounted in the chamber. The upper hemichamber containing the test compound was covered with paraffin and sealed with silicone grease (Dow Corning) along the edges of the exposed area of the chamber to prevent evaporation. This provided a flexible seal that did not alter transscleral pressure. The temperature of the water-jacketed perfusion chamber was maintained at 37°C by a circulating water bath.

Permeability to three compounds was evaluated: 10⁻⁴ M 5(6) carboxyfluorescein (Eastman Kodak, Rochester, NY) diluted in BSS Plus, 2.37 × 10⁻⁷ M ³H-dexamethasone-acetate (42.2 Ci/millimole; NEN, Boston, MA) packaged in ethanol and diluted to 10% (vol/vol) in BSS Plus, and ³H-water (1 mCi/g; NEN) diluted to 10% (vol/vol) in BSS Plus. For experiments with carboxyfluorescein, the perfusate passed through a flow-through quartz cuvette (NSG Precision Cells, Farmingdale, NY), and measurements of total fluorescence in the cuvette were taken at 60-second intervals using a spectrofluorometer (Photon Technology, New Brunswick, NJ). Time-based fluorescence concentration was calculated using a standard dilution curve generated from a sample of the donor solution for each experiment.²

For the experiments with ³H-water and ³H-dexamethasone, samples of the perfusate were collected by a fraction collector (ISCO, Lincoln, NE) at 10- to 15-minute intervals. At the completion of the perfusion, 50 μl of each fraction was added to 10 ml Aquasol (Packard, Meriden, CT), and tritium disintegrations were measured using a liquid scintillation counter (model LS 5801; Beckman, Irvine, CA). Disintegrations per minute (dpm) were calculated based on quenched standards of tritium. Samples (10 μl) were taken from the upper hemichamber at the beginning and end of the experiment to verify the initial donor concentration and to measure donor drug depletion over the course of the experiment.

Separate experiments were performed at transscleral pressures of 0, 15, 30, or 60 mm Hg. Pressure was applied across the tissue by raising the height of the outflow tube and was determined by measuring the distance between the tissue and the outflow tube as it flowed into the collector receptacle (e.g., 15 mm Hg was equivalent to a 22-cm water column). The pressure across the tissue was verified using a pressure transducer (Statham, Oxnard, CA) connected to the lower hemichamber.

Steady state permeability constant (Ktrans) was calculated from the spectrofluorometry (carboxyfluorescein) or scintillation spectroscopy (³H-water, ³H-dexamethasone) data as:

\[
K_{\text{trans}} = \frac{R_{\text{total}}}{(I/A)} \times \frac{1}{[D]}
\]

where \( R_{\text{total}} \) is the total amount of drug in the receiver effluent per collected fraction (measured as radioactive dpm or fluorescent counts), and \( I \) is the fraction collection time (in seconds). \( A \) is the area of exposed sclera (in square centimeters). This value—\( R_{\text{total}}/(O(A)) \)—is equal to the flux across the tissue. \( D \) is the concentration of drug in the donor hemichamber (dpm per cubic centimeter or counts per second per cubic centimeter). Permeability thus represents the steady state flux normalized by donor concentration. The area of exposed sclera was 0.385 cm² for the 7-mm chamber and 0.785 cm² for the 10-mm chamber.

Mean permeability values (±SD) were calculated from three to eight experiments performed for each compound at each pressure in both human and rabbit sclera. Analysis of variance (ANOVA) was calculated to compare the permeabilities at different pressures for each compound in both human and rabbit sclera. Tukey-Kramer multiple comparisons were then used to compare differences between pairs of pressure-dependent permeability measurements of each compound in human and rabbit sclera.

RESULTS

Human and rabbit scleral permeabilities to the compounds studied at different simulated intraocular pressures are shown in Figure 2 and Table 1. ANOVA showed human and rabbit scleral permeability to water (molecular weight: 18 Da, molecular radius: 2.0 Å) to be significantly affected by transscleral pressure (\( P = 0.0004 \) across human sclera and \( P = 0.0039 \) across rabbit sclera). The greatest difference in permeability was observed between 15 mm Hg and 30 mm Hg. The steady state Ktrans of human sclera to water was measured as (mean ± SD) 5.18 ± 0.95 × 10⁻⁵ cm/sec at 15 mm Hg and 2.57 ± 0.95 × 10⁻⁵ cm/sec at 30 mm Hg. Rabbit scleral permeability to water was 5.43 ± 1.28 × 10⁻⁵ cm/sec at 15 mm Hg and 1.90 ± 0.91 × 10⁻⁵ cm/sec at 30 mm Hg. These differences represent a significant reduction in scleral permeability for both human and rabbit tissue at the higher pressure (\( P < 0.01 \)).
Tukey–Kramer multiple comparisons test.

of data at a level of $P < 0.05$ respectively, determined by the Tukey–Kramer test. Permeability was similar at 0 and 15 mm Hg for both tissues. Permeability was also similar at 30 and 60 mm Hg for both tissues. At 0, 15, and 30 mm Hg, human scleral permeability was similar to rabbit scleral permeability (see Fig. 2A). However, at 60 mm Hg, rabbit sclera was significantly more permeable to water than human sclera ($P = 0.002$; Student's $t$-test).

Scleral permeability to dexamethasone (molecular weight: 392 Da, molecular radius: 5.2 Å) was also significantly affected by transscleral pressure (Fig. 2B). $P < 0.0001$ with human tissue and $P = 0.0001$ with rabbit tissue (ANOVA). The greatest difference was observed between 0 and 15 mm Hg. At 0 mm Hg, permeability was measured at $1.82 \pm 0.58 \times 10^{-5}$ cm/sec across human sclera and $1.27 \pm 0.23 \times 10^{-5}$ cm/sec across rabbit sclera. Permeability to dexamethasone at 15 mm Hg was $8.94 \pm 1.5 \times 10^{-6}$ cm/sec and $7.12 \pm 2.3 \times 10^{-6}$ cm/sec for human and rabbit sclera, respectively. Scleral tissue was significantly less permeable to dexamethasone at 15 mm Hg than at 0 mm Hg ($P < 0.01$ for human and rabbit sclera; Tukey–Kramer test). There was also a significant decrease in permeability between 30 and 60 mm Hg for rabbit sclera ($P < 0.05$; Tukey–Kramer test). However, no difference was observed between 15 and 30 mm Hg in either species. Human scleral permeability to dexamethasone may be greater than rabbit scleral permeability to dexamethasone at comparable pressures ($0.08 < P < 0.25$; Tukey–Kramer test).

Figure 2C shows the permeability of human and rabbit sclera to carboxyfluorescein (molecular weight, 317 Da; molecular radius, 5.2 Å) was also significantly affected by transscleral pressure across human sclera and $1.27 \pm 0.23 \times 10^{-5}$ cm/sec across rabbit sclera. Permeability was similar at 30 and 60 mm Hg for both tissues. At 0, 15, and 30 mm Hg, permeability was measured by the Tukey–Kramer test. Permeability was similar at 0 and 15 mm Hg for both tissues. Permeability was also similar at 30 and 60 mm Hg for both tissues. At 0, 15, and 30 mm Hg, human scleral permeability was similar to rabbit scleral permeability (see Fig. 2A). However, at 60 mm Hg, rabbit sclera was significantly more permeable to water than human sclera ($P = 0.002$; Student's $t$-test).

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Figure 2C shows the permeability of human and rabbit sclera to carboxyfluorescein (molecular weight, 317 Da; molecular radius, 5.2 Å). The permeability of human and rabbit sclera to carboxyfluorescein was significantly affected by intraocular pressure ($P = 0.0064$ and 0.0016 for human and rabbit sclera, respectively; ANOVA). In general, permeability measurements between neighboring pressure values were similar, determined by the Tukey–Kramer test, with the exception of a difference in permeability across rabbit sclera at 15 and 30 mm Hg, when a significant decrease at the higher pressure was observed ($P < 0.05$). However, there was an overall trend of decreased permeability with increased pressure in both human and rabbit sclera. Moreover, scleral permeability at 60 mm Hg was significantly different from 0 mm Hg ($P < 0.01$ for human and $P < 0.01$ for rabbit sclera; Tukey–Kramer test).

**Figure 2.** Permeability (mean ± SD) of human and rabbit sclera. (A) $^3$H-water, (B) $^3$H-dexamethasone, and (C) carboxyfluorescein at 0, 15, 30, and 60 mm Hg. Each histogram represents the mean ± SD of three to eight experiments (Table 1). *, †: statistical difference between pairs of data at a level of $P < 0.01$ and $P < 0.05$, respectively, determined by the Tukey–Kramer multiple comparisons test.

**Table 1.** Permeability Constant ($K_{trans}$) for Water, Dexamethasone, and Carboxyfluorescein across Human and Rabbit Sclera

<table>
<thead>
<tr>
<th>Transscleral Pressure</th>
<th>Human</th>
<th>Rabbit</th>
<th>Human</th>
<th>Rabbit</th>
<th>Human</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$H_2O$</td>
<td></td>
<td>Dexamethasone</td>
<td></td>
<td>Carboxyfluorescein</td>
<td></td>
</tr>
<tr>
<td>0 mm Hg</td>
<td>$44.6 \pm 13$</td>
<td>$54.4 \pm 19$</td>
<td>$18.2 \pm 5.8$</td>
<td>$12.7 \pm 2.3$</td>
<td>$11.8 \pm 1.37$</td>
<td>$13.0 \pm 3.4$</td>
</tr>
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<td>(n = 8)</td>
<td>(n = 5)</td>
<td></td>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>15 mm Hg</td>
<td>$51.8 \pm 18$</td>
<td>$54.3 \pm 12$</td>
<td>$8.94 \pm 1.5$</td>
<td>$7.12 \pm 2.3$</td>
<td>$9.93 \pm 3.46$</td>
<td>$11.4 \pm 2.1$</td>
</tr>
<tr>
<td>(n = 7)</td>
<td>(n = 5)</td>
<td></td>
<td>(n = 5)</td>
<td>(n = 4)</td>
<td>(n = 6)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>30 mm Hg</td>
<td>$25.7 \pm 9.5$</td>
<td>$19.0 \pm 9.1$</td>
<td>$8.65 \pm 1.9$</td>
<td>$7.07 \pm 2.2$</td>
<td>$6.81 \pm 0.76$</td>
<td>$6.97 \pm 1.2$</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(n = 4)</td>
<td></td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 4)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>60 mm Hg</td>
<td>$14.0 \pm 3.3$</td>
<td>$36.7 \pm 8.0$</td>
<td>$3.92 \pm 1.3$</td>
<td>$2.33 \pm 0.94$</td>
<td>$6.15 \pm 1.59$</td>
<td>$7.66 \pm 1.8$</td>
</tr>
<tr>
<td>(n = 4)</td>
<td>(n = 4)</td>
<td></td>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
</tr>
</tbody>
</table>

Data are means ± SD × 10$^{-6}$. n represents number of eyes.
Increased intraocular pressure (Fig. 2).

Across human sclera, and Maurice and Polgar2 studied the range from 130 to 70,000 Da (radii ranging from 3.3 to 65 Å) from the earlier studies are comparable to our data at 0 mm Hg of different sized compounds without varying pressure. Data weight compounds under the influence of a simulated intraocular pressure. This study shows that the sclera is permeable to low-molecular

FIGURE 3. Comparison of human and rabbit scleral permeability. Measurements determined from the present study at 0 mm Hg were compared with those in two previous studies2,3. The data of Maurice and Polgar2 have been converted to permeability as described by Olsen et al.3. The data collected from this study closely agree with previous work. However, note that lower scleral permeability occurred with increased intraocular pressure (Fig. 2).

DISCUSSION

This study shows that the sclera is permeable to low-molecular-weight compounds under the influence of a simulated intraocular pressure. Other studies have evaluated scleral permeability of different sized compounds without varying pressure. Data from the earlier studies are comparable to our data at 0 mm Hg (Fig. 3). Olsen et al.3 measured the permeability of compounds ranging from 130 to 70,000 Da (radii ranging from 3.3 to 65 Å) across human sclera, and Maurice and Polgar2 studied the diffusion of compounds from 23 to 69,000 Da (radii ranging from 1.0 to 64 Å) across bovine sclera. As shown in Figure 3, the zero-pressure permeability values measured in this study were comparable to those previously described. The current permeability values were slightly lower for human sclera than those in the previous report of human sclera. However, the chambers with constant mixing used in previous studies may yield a higher apparent permeability than that determined in the current study by using an unmixed depot on the surface of the sclera in which static boundary layers could form.

Although our results indicated that intraocular pressure could affect permeability, we found that the effect of pressure was small for the compounds tested. For all three compounds tested, the largest difference due to pressure was approximately a factor of two—that is, an increase of 15 mm Hg pressure causes scleral permeability to be reduced to approximately half its value at the lower pressure. Although intraocular pressure has a statistically significant effect on scleral permeability, it may not be sufficient to have clinical importance in a depot delivery system. In this study, the pressure was applied with the high pressure on the inner, choroidal side of the sclera and the low pressure on the outer, episcleral side where the drug solution was applied. For drug delivery into the eye, this is the physiologically correct orientation.

Elevated transscleral pressure could reduce rates of transport across sclera in two principal ways: It could induce flow of water across the sclera, which could carry molecules with it by convection, and it could directly reduce scleral permeability by altering the microanatomy of sclera. Considering the first mechanism, the role of water flow across the sclera can be addressed by comparing the rate of transport by flow-induced convection with that of diffusion. The Peclet number (Pe) characterizes this:

\[ Pe = \frac{v}{D/d} \]

where \( v \) is the velocity of the water flow, \( d \) is scleral thickness, and \( D \) is diffusivity in the sclera. The ratio of \( D/d \) gives a characteristic velocity of diffusion. Thus, the Peclet number can be thought of as the ratio of the velocity of convection to the velocity of diffusion. Assuming that the sclera is effectively homogeneous, which is consistent with its ultrastructure, then a Peclet number greater than 1 indicates that transport by pressure-induced flow is important. If it is much less than 1, then transport by flow is not important, and diffusion through the tissue should be the dominant mode of transport.

A representative value for the Peclet number can be calculated as follows. The sclera’s hydrodynamic permeability is known to be 1.34 nm² at a pressure of 60 mm Hg and a temperature of 37°C.8 This means that the velocity of water convection is \( v = 2.6 \times 10^{-6} \text{ cm/sec} \). A characteristic human scleral thickness can be taken as \( d = 0.4 \text{ mm at 12 to 15 mm posterior to the limbus} \), and effective diffusivity in the sclera for small molecules can be taken as \( D = 1.1 \times 10^{-6} \text{ cm²/sec} \). Combining these values yields a diffusion velocity of \( 2.8 \times 10^{-5} \text{ cm/sec} \), which is an order of magnitude greater than the velocity of water convection. This yields a Peclet number of \( Pe = 0.095 \).

Because the Peclet number is less than 1, the rate of transport by diffusion must be much greater than that by convection. This shows that water flow induced by high transscleral pressure is not sufficient to affect rates of diffusion across sclera significantly. As a result, increased pressure probably reduces scleral permeability by changing tissue microanatomy. This could be in the form of compressing the tissue, which in turn reduces the spaces between the collagen fibers and extracellular matrix molecules that define the pathways for diffusion. Smaller pathways hinder diffusion and thus lower scleral permeability. Measurements of tissue thickness and hydration as a function of pressure in recent experiments have verified this hypothesis (data not shown).

This report shows that scleral permeability to small compounds is a weak function of transscleral pressure. Past reports2,3 have shown that scleral permeability, in the absence of an applied pressure, is a strong function of molecular weight. It is likely that these two effects are synergistic, rather than simply additive, when diffusion of macromolecules in the presence of a transscleral pressure is considered. Because the Peclet number analysis indicates that pressure reduces scleral permeability by compressing fibers (e.g., collagen, extracellular matrix) within the sclera, narrowing intracollagen pathways should affect diffusion of macromolecules more than small molecules because of the molecular size (i.e., nanometers) of the pathways.10 Thus a narrowing of intramolecular fibers within the sclera slows diffusion of small molecules yet may completely block transport of macromolecules. Future studies are needed to test this hypothesis. Although the permeability of the sclera to larger compounds as a function of pressure has not yet been determined, the data presented in this report indicate that intraocular pressure is an important consideration in developing a practical model of drug delivery across the sclera.
Modulation of Retinal Pigment Epithelial Cell Behavior by Agaricus Bisporus Lectin

Hartmut Wenkel,1,2,5 David Kent,1 Paul Hiscott,1 Mark Batterbury,1 Carl Groenewald,1 Carl M. Sheridan,1 Lu-Gang Yu,4 and Jeremy Milton4

PURPOSE. To determine whether Agaricus bisporus lectin (ABL) binds retinal pigment epithelial cells (RPEs), to conduct a preliminary viability study of RPEs exposed to ABL, and to evaluate the effects of ABL on RPE proliferation and RPE-mediated matrix contraction in vitro.

METHODS. Using cultured bovine RPEs, immunohistochemistry was used to study ABL binding. Morphologic and trypan blue exclusion techniques were used for toxicity studies. The effect of ABL on RPE proliferation was investigated by [methyl-3H]–thymidine incorporation. The effect of ABL on RPE-mediated matrix contraction was evaluated with RPE-populated three-dimensional collagen matrices.

RESULTS. ABL bound to RPE cells. This binding was inhibited by asialomucin. No change in RPE morphology or trypan blue exclusion compared with controls was observed in RPEs incubated with 5 to 60 μg/ml ABL for 3 days. Twenty-four-hour incubations of RPEs with ABL significantly inhibited RPE proliferation in a dose-dependent way. 40 μg/ml ABL inhibited proliferation by 83% (SE 14, P < 0.05). ABL showed a dose-dependent significant inhibition of RPE-mediated collagen matrix contraction over 3 days, with 93% inhibition compared with controls by 40 μg/ml lectin (P < 0.05). The inhibitory effect of ABL on proliferation and gel contraction was partly reversible after eliminating ABL from the culture medium.

CONCLUSIONS. Bovine RPE cells bind ABL, and preliminary evaluations suggest that levels of ABL that are nontoxic to the cells potently inhibit RPE proliferation and RPE-mediated matrix contraction. ABL deserves further investigation as a potential inhibitor of RPE proliferation and cell-mediated matrix contraction in anomalous reparative processes such as proliferative vitreoretinopathy and as a laboratory tool for RPE behavioral studies. (Invest Ophthalmol Vis Sci. 1999;40:3058–3062)

Proliferative vitreoretinopathy (PVR) is an anomalous wound repair process typified by the formation of scar-like membranes on the retinal surfaces.1 The membranes may contain abundant retinal pigment epithelial cells (RPEs).1 RPE proliferation and RPE-mediated tissue contraction are thought to be fundamental to PVR membrane development. Drug prevention of PVR membrane formation has been based chiefly on antiproliferative and anti-inflammatory agents, but the results of such treatments have been disappointing, largely because these agents tend to be ineffective and toxic to the retina.1 There is a need for nontoxic agents that will specifically block cellular activities such as RPE proliferation and RPE-mediated membrane contraction in PVR.

Lectins are ubiquitous carbohydrate-binding non-immunoglobulin proteins. They bind noncovalently to carbohydrates and are readily purified from a wide variety of sources. A range of carbohydrates occurs on all cell surfaces, and lectins have been used to explore cell membranes and to distinguish different cell types, because cells express distinct carbohydrates that can be detected by specific lectins.2 Furthermore, lectins binding to cell surface carbohydrates may affect the behavior of the cell. Thus, lectins such as peanut agglutinin and the lectin of the edible mushroom (Agaricus bisporus lectin; ABL), which both bind to the carbohydrate structure galactosyl β-1,3-N-
acellularly, modulate the proliferation of malignant epithelial cells. Peanut agglutinin increases colonic carcinoma cell division, whereas ABL inhibits proliferation of these cells. Moreover, ABL inhibits proliferation of a range of other cells including Tenon’s capsule fibroblasts, and it inhibits contraction of collagen matrices by Tenon’s fibroblasts. These effects occur without apparent cytotoxicity. Because cell-mediated membrane contraction and proliferation are key RPE activities in PVR, we evaluated the effect of ABL on these RPE activities in vitro, having first determined whether ABL binds to (or affects the viability of) cultured RPEs.

Materials and Methods
All reagents were of analytical grade, all lectin and peroxidase-conjugated lectin were obtained from EY Laboratories Ltd (San Diego, CA) and [methyl-3H]-thymidine was supplied by Amersham International (Amersham, UK).

RPE Culture
Bovine RPEs were obtained and cultured as previously described. Established cultures were maintained with minimal essential medium (MEM) containing glutamine and fungizone, penicillin and streptomycin, and 15% newborn calf serum (NCS) (GIBCO Europe Ltd., Paisley, UK). The cultures were kept at 37°C in the presence of 5% CO2 and air. The RPEs reached confluence within 1 to 2 weeks, and subcultures between the fourth and seventh passages were used in the present study. The purity of the cultures was confirmed on the basis of cytokeratin staining as described previously.

All experiments were conducted in MEM and 2% NCS. Some serum components in NCS bind ABL (e.g., IgA, fetuin), neutralizing its activity, but 2% serum was required to maintain RPE viability.

Lectin Histochemistry
Bovine RPEs were grown on eight chamber tissue culture glass slides (LabTeks; Nalge Nunc International, Life Technologies Limited, Glasgow, UK) as reported previously. Peroxidase- or fluorescein isothiocyanate (FITC)-labeled ABL was added to the chambers at a concentration of 30 µg/ml for 1 or 8 hours, respectively. Controls consisted of preincubation of the labeled ABL with 10 mg/ml asialomucin for 5 minutes before the addition of the labeled lectin to the cells and preincubation of the chambers with 100 µg/ml unconjugated ABL for 1 hour before adding the conjugated lectin. The slides were washed with phosphate-buffered saline (PBS), and either peroxidase was developed with diamine benzidine or slides were mounted after fixation with methanol for 7 minutes at 20°C. Slides were dehydrated, mounted, and evaluated using DIC optics (Polyvar, Reichert-Jung, Austria) or epifluorescent photography, respectively.

Morphologic Evaluation and Trypan Blue Staining
RPEs were seeded in 24-well plates (Corning Costar, High Wycombe, UK) at a concentration of 2 × 10^4 cells/well. After 1 day, the cells were washed three times with MEM without serum (to remove the serum transferred with the maintenance medium). ABL was added in concentrations ranging from 5 to 60 µg/ml in MEM with 2% NCS. Controls were kept in MEM with 2% NCS. Three wells were used for each concentration, and the experiment was repeated twice. Cell morphology was evaluated daily for 3 days by phase contrast microscopy. Representative preparations were selected each day and stained with 2% trypan blue for 5 to 10 minutes. Stained and unstained cells were counted in each well.

Cell Proliferation Assay
Cells were seeded in 24-well plates at a density of 1 × 10^4/well. After incubation for 48 hours, the wells were washed three times with PBS after which ABL was added (20, 30, 40 µg/ml in 0.5 ml MEM with 2% NCS). Cells were incubated for a further 24 hours. The cells then received a 1-hour pulse with 0.5 µCi/well [methyl-3H]-thymidine. Each well was washed twice with PBS before cell precipitation with 0.5 ml/well of 5% trichloroacetic acid at 4°C. The precipitate was washed once with 5% trichloroacetic acid at 4°C and twice with 0.5 ml/well of 95% ethanol at 4°C and left to dry at room temperature. After solubilization in sodium hydroxide (NaOH), 0.3 ml of the precipitate was added to 1 ml Optima Gold MV scintillation cocktail (Packard, Pangbourne, UK), and the cell-associated radioactivity was determined using a Packard scintillation counter.

Collagen Matrix Contraction
For collagen matrix contraction experiments, the method published by Mazure and Grierson was adapted to 24-well plates. Briefly, rat tail type I collagen (Sigma, Poole, UK) was dissolved in 0.1% (vol/vol) acetic acid in sterile distilled water. RPEs were counted after harvesting from maintenance cultures and then resuspended in MEM at a volume of 1.23 ml containing 5.76 × 10^6 cells, sufficient for one 24-well plate. The cell suspension was mixed with 4.91 ml of 5 mg/ml collagen and with 2.86 ml of concentrated serum-free MEM containing glutamine, antibiotics, and NaOH. The collagen-cell mixture was then transferred in 350-µl aliquots to 24-well plates, ensuring that the matrix covered the bottom of the wells. The solution polymerized rapidly when incubated at 37°C in the presence of 5% CO2, thus trapping the cells (at a density of 2.4 × 10^5 RPEs per matrix) within the three-dimensional matrix. The matrices were detached from the edges and allowed to float in the wells by the addition of 1 ml of MEM with 2% NCS. Mushroom lectin was added at concentrations of 5, 10, 20, 30, and 40 µg/ml medium, and the preparations were incubated at 37°C in 5% CO2 in air for at least 3 days. The surface area of each matrix was recorded photographically daily. Four wells were used for each concentration, and experiments were repeated three times. After 3 days, medium containing ABL was removed and gels were washed twice and then were incubated for an additional 4 days with medium containing 15% of serum.
Statistical Analysis
ANOVA was used to explore between-group significance. Dun- can’s multiple comparison test was used to detect homoge- neous subsets.

RESULTS

Binding of RPEs by ABL In Vitro
Lectin histochemistry demonstrated specific binding of ABL to bovine RPEs, equally distributed by two different techniques (FITC and peroxidase). After 8 hours’ incubation, there was perinuclear accumulation of labeled ABL, best seen with FITC- labeled ABL (Fig. 1A). This binding was abolished by preincu- bation with 100 μg/ml of unlabeled lectin and by 10 mg/ml asialomucin (Fig. 1B). After only 1 hours’ incubation, labeled ABL was seen to be adherent to cell surfaces, best visualized by the peroxidase technique (Fig. 1C). This binding and uptake also were abolished by preincubation with 100 μg/ml of unlabeled lectin (Fig. 1D) and by 10 mg/ml asialomucin.

Cell Viability Study
No cell morphologic change was noticed during the 3 days of incubation with ABL (5-60 μg/ml) or in controls (Fig. 2). Staining with trypan blue revealed more than 95% RPE viability after 3 days’ incubation at all concentrations of ABL from 5 to 60 μg/ml and in the controls (exact data not shown).

Inhibition of RPE Proliferation by ABL In Vitro
ABL caused a significant dose-dependent inhibition of proliferation as assessed by thymidine incorporation (one-way ANOVA, \( P = 0.0002; F = 66; \) Fig. 3 A). Compared with wells incubated with media without ABL, 20 μg/ml ABL produced a 40% (SE 10) inhibition of thymidine incorporation in RPEs, whereas 40 μg/ml ABL caused an 83% (SE 14) inhibition. Duncan’s test for multiple comparisons indicated that each treatment group was homogeneous (\( P < 0.05 \)).

After removal of ABL from the medium, an increase in cell number was demonstrated for all ABL concentrations (Fig. 3B). However, cells exposed to higher ABL doses exhibited a slower proliferation rate. Thus, by day 6, although the control cells (incubated without ABL) reached a density of 17.04 ×10^4 ± 0.92 cells/well, cells incubated with 40 μg/ml ABL had reached 10.40 ×10^4 ± 1.12 cells/well (\( P < 0.05 \)).

Inhibition of Contraction of RPE-Populated Collagen Matrices by ABL
ABL caused a significant concentration-dependent inhibition of contraction (one-way ANOVA, \( P < 0.001, F = 14; \) Fig. 4). Duncan’s test for multiple comparisons indicated that 5 to 10 μg/ml ABL was moderately inhibitory (12% matrix contraction at day 3) and that 20 to 40 μg/ml ABL caused greater inhibition (6.2%—2.5% contraction at day 3; \( P < 0.05 \)). Calculating inhibition as \( [1 - (\text{test contraction}/\text{control contraction})] \times 100 \), ABL in the range 5 to 40μg/ml produced inhibition of 43% to 93% (Fig. 4A).

After elimination of ABL from the medium, gel contraction was partly restored (Fig. 4B). Percentage of gel contraction was calculated as \( [\text{gel size (day 3)} - \text{gel size (day 7)}]/\text{gel size (day 3)} \times 100. \) Compared with control gels that were not preincu- bated with lectin, the ability to contract collagen gels was restored to 90.9% for 5 μg/ml ABL, to 83.4% for 10 μg/ml ABL, to 64.7% for 20 μg/ml ABL, and to 23.2% for 40 μg/ml ABL.

DISCUSSION
Our results demonstrate that ABL binds to RPE. RPEs adhere a number of lectins, including peanut agglutinin, which binds...
to galactosyl β-1,3-N-acetyl-galactosamine (the oncofetal Thomsen Friedenreich antigen TFα). Because ABL also binds this antigen, we were not surprised to observe ABL binding to RPEs. The changes in the pattern of binding with time indicate that bound ABL is internalized, a finding consistent with observations concerning the fate of ABL in other cell types.9

The binding and uptake of ABL by RPE cells do not appear to alter RPE viability in vitro. RPEs incubated for 3 days with ABL concentrations up to 60 μg/ml (the highest studied level) show normal trypan blue exclusion and no discernible light microscopic change in morphology compared with control cultures. In addition, our data indicate that the effects of ABL on RPE proliferation and cell-mediated matrix contraction are recoverable after removal of the lectin from the media. Taken together, these results suggest that ABL has little or no toxicity for cultured RPEs. Indeed, evidence from studies of other cell types suggests that ABL generally is noncytotoxic at doses up to 200 μg/ml.5

Despite the apparent lack of effect of ABL on RPE viability in vitro, the lectin markedly inhibits RPE proliferation and RPE-mediated collagen matrix contraction in vitro even at concentrations of less than 30 μg/ml. The mechanism by which ABL modulates cell activities in general is not clear, although there is evidence that it interferes with nuclear protein import.9 Whatever the mechanism of action, the lectin is known to influence the behavior of a variety of cell types. For example, the proliferative activity of colonic and breast carcinoma cells and mammary and Tenon’s fibroblasts3,4 is, like that of RPEs, inhibited by ABL. However, in contrast to our observation that ABL inhibits RPE-mediated collagen matrix contraction and to a finding that ABL inhibits matrix contraction by...


Transplantation of retinal cell suspensions, of retinal pieces, or of particular cell types to the subretinal space are some of the strategies being tested to improve vision in certain forms of outer retina degeneration. Most cell types and synapses are seen to develop in the transplants, and structural proteins and neurotransmitters found in normal retinas also are expressed by the transplanted cells. However, for in oculo retinal transplant to be functional, it is required that they integrate and form relevant connections with the host retina. Subretinally transplanted photoreceptors cells expressing the lacZ reporter gene product, β-galactosidase, have been found to exhibit well-developed synaptic terminals and to contact host bipolar cells. Processes projecting from the graft into the host retina also have been observed after epiretinal and subretinal transplantation of the entire neuroretina. Using electron microscopy, prelabeling of the donor tissue, or cell markers to identify distinct retinal cell types, it has been shown that graft–host contacts also can involve cells of the inner retina, for example, contacts between amacrine and bipolar cells.

However, the anatomic demonstration of graft–host connections based on synaptic contacts or by using structural markers does not provide information about the functional potential of the connecting cells. Therefore, in the present study we looked at the ability of a chemically and functionally defined retinal cell type to project into the host retina. Nitric oxide is a recognized retinal neuromodulatory compound. It is synthesized by subpopulations of wide-field amacrine cells in the rabbit retina and has been shown to be expressed by transplanted retinal cells. The processes of cells expressing the neuronal form of its synthesizing enzyme, nitric oxide synthase (NOS), can be followed for long distances, thus providing an effective tool for studying connectivity. We show in the present report that graft cells potentially capable of producing nitric oxide can project to and reach target areas in the host retina.

**Materials and Methods**

Animals were handled according to the guidelines set by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, the Declaration of Helsinki, and the local animal experimentation and ethics committee (Djurförsöksetiska nämnden i Lund).

The preparation of donor tissue and the transplantation procedure have been described previously in detail. Briefly, outbred pigmented rabbits of a mixed strain (embryonic stage [E] 15, normal gestation 31 days) were used as donors for the transplantation. Embryos were placed in Ames solution at 4°C, and the eyes were enucleated. Retinas were dissected free from the retinal pigment epithelium and were stored at 4°C in fresh Ames solution until used for transplantation (4 hours at most). Eleven adult rabbits of the same breed as the donors were used as recipients. Thirty minutes before surgery, the right pupil of the recipients was dilated with 1% cyclopentolate-HCl (Cyclogyl; Alcon-Couvreur, Puurs, Belgium,) and one drop of 10% phentylephrine-HCl (Sanofi Winthrop Pharmaceuticals, New York, NY). The recipient rabbits were anesthetized with 1 ml/kg Hypnorm (10 mg/ml fluanison and 0.2 mg/ml fentanyl; Janssen Pharmaceutica, Beerse, Belgium) and topical tetracaine-HCl (0.5%, Alcon-Couvreur) was applied. Two to four embryonic retinas (in up to 10 μl total volume) were drawn up into a thin polyethylene capillary mounted on a special instrument that was connected to a precision microsyringe. A small scleral incision was made in the recipient eye 2 to 4 mm behind the limbus. The capillary was advanced through the vitreous to the posterior pole of the eye where the retina was penetrated. The embryonic retinas were deposited slowly in the subretinal space in the central retina, below the myelinated streak. The animals were kept in light–dark cycles (12 hours each) and were allowed to survive for 21 to 118 days after the surgery. The transplants thus reached ages equivalent to postnatal days (PN) 5 (n = 2), 12 (n = 2), 20 (n = 3), 45 (n = 1), 90 (n = 1), and 102 (n = 2). No immunosuppressive drugs were used.

Eyes carrying transplants were quickly enucleated and immersed in a solution of 4% formaldehyde in Sörensen’s buffer (0.1 mM; pH 7.2). After 30 minutes, the eyes were hemisected, and the anterior segment, the lens, and the vitreous were discarded. The remaining eyecups were kept in the same fixative for an additional 90 minutes, after which they were rinsed and cryoprotected in Sörensen’s buffer containing sucrose. The area containing the transplant was cut out, embedded, and frozen. Cryostat sections were incubated with sheep antineuronal NOS serum, followed by incubation with Texas red sulfonyl chloride–conjugated donkey anti-sheep IgG (Jackson Immunoresearch, West Grove, PA). The NOS antiserum used (gift from Ian G. Charles and Piers C. Emson) was raised against purified rat recombinant neuronal NOS protein and was found to be specific for neuronal NOS in control experiments.

**Results**

In all grafts, cells of the outer nuclear layer were organized as rosettes, and in between the rosettes, cells of the inner retina were observed (Fig. 1A). The outer host retina degenerated with time in most areas adjacent to the graft (see below), whereas the innermost layers appeared to have retained their normal structure (Figs. 1A, 1C, 2, 3). With the antibody used, NOS immunoreactivity was found in normal adult rabbit retinas restricted to a few cell bodies in the innermost cell row of the inner nuclear layer, to the inner plexiform layer (Fig. 1B), and to some cells in the ganglion cell layer (not shown). This distribution pattern was preserved in the host retinas even in the areas adjacent to the graft (Figs. 1C, 2, 3). The number of labeled cells found in the host retina also was not different in areas adjacent to the graft or when compared to normal retinas. Further, there were no indications that after transplantation, cell types other than those seen in a normal rabbit retina expressed NOS immunoreactivity in the host retina. The only difference was noted in the host inner plexiform layer, where stronger and denser labeling often was seen compared with nonoperated animals. Only sections in which no morphologic disturbance of the host inner retina could be observed in the areas adjacent to the graft were analyzed. The presence of labeled amacrine cells in the proximal inner nuclear layer and of immunoreactivity in the inner plexiform layer as a continuous plexus, running parallel to the vitreal border of the host retina and the graft–host border, were used as an indication of the relative integrity of the inner host retina. NOS-immunoreactive cells were judged to belong to the host or to the transplant, depending on their location.
NOS-immunoreactive cells and processes were seen in all grafts examined. Immunolabeled processes occasionally could be found in the grafts, close to the graft-host border. However, in regions where one or more photoreceptor cell rows of the host outer nuclear layer remained, such processes were seen to run parallel to the border, without entering the host retina (Fig. 1C). Rows of photoreceptor cells could be observed in the host retina at the shorter survival times and at the edges of the bleb created in the host retina by the graft (Fig. 1C).

Nevertheless, labeled fibers originating in the graft could at times be seen crossing the graft-host border. Such bridging was observed at all survival times, but not in all specimens examined (in 8 of 11), and only in regions where the host photoreceptor layer was absent. In fortuitous cases, it was possible to follow a labeled process from the graft all the way into the host inner plexiform layer. Examples of this are given in Figures 2 and 3. A long thin bridging process is seen originating in a graft corresponding to PN 12 (28 days after transplantation; Fig. 2A) and a shorter one is seen in Figure 2B in a transplant corresponding to PN 20 (36 days after transplantation). As a result of the degeneration of the host outer layers, NOS-containing cells and fibers occasionally were found in the grafts relatively near the host inner plexiform layer. In Figure 2B, a bridging process is seen to run between the host inner plexiform layer and a more distal area where several immunolabeled structures are observed. Because no NOS-immunoreactive fibers were seen external to the inner plexiform layer in a normal rabbit retina (Fig. 1B), the bridging process seen in Figure 2B is also likely to connect structures within the graft with the host retina. In Figure 3A, several stained processes are seen within a graft corresponding to PN 45 (61 days after transplantation). In addition, a process arising from a NOS-immunolabeled cell located in the graft is seen to extend toward the host inner plexiform layer. Serial sections confirmed the location of this cell. Further, a stained cell body also is seen in the amacrine cell layer of the host retina, and strong and continuous labeling is noted in the host inner plexiform layer, reflecting the relative organization of the inner host retina. The immunolabeled fiber, seen in Figure 3A to reach almost perpendicularly the host inner plexiform layer, can thus be judged as a bridging process that originates in the graft. In Figure 3B, a stained cell body is seen in the host retina 106 days after transplantation. One strongly labeled cell body also is seen in the transplant (corresponding to PN 90) to project to a region within the graft where faintly stained fibers are seen. In addition, one large NOS-immunoreactive cell body is seen to project to the same region within the graft and to emit a more weakly labeled process toward the host inner plexiform layer. As mentioned above, a progressive loss of the outer host retina is normally observed, which with time brings the graft closer to the host inner layers. Again, a labeled cell is seen in the host retina in its expected position, next to the inner plexiform layer. The connecting cell could therefore, judging from its position, belong to the transplant. However, it is not possible to determine conclusively in this case whether the bridging cell indeed belongs to the graft.

Figure 1. (A) Bright field micrograph (hematoxylin–eosin) showing a transplant (T) corresponding to PN 20 (36 days after transplantation). Cells belonging to inner retinal layers (IR) are located between photoreceptor cells [ONL(t)], which are organized in rosettes (R). The host outer nuclear layer and part of the host inner nuclear layer [INL(h)] have degenerated. (B) Fluorescence micrograph showing the distribution of NOS immunoreactivity in normal adult rabbit retina. Labeled cells (small arrows) are seen in the proximal inner nuclear layer (INL) and their processes in the inner plexiform layer (IPL) (small arrowheads). (C) Distribution of NOS immunoreactivity in a retinal transplant corresponding to PN 12 (28 days after transplantation). A few cell rows are present in the host outer nuclear layer at the edges of the bleb created by the graft. Immunoreactive fibers in the transplant run parallel to the graft-host border (arrowbeads). Scale bars, 30 μm.
DISCUSSION

The present study demonstrates that a subpopulation of graft cells with the ability to express neuronal NOS extend processes into the host retina in rabbit-to-rabbit transplants. Furthermore, it was seen that the NOS-containing processes are capable not only of projecting into the host retina, but also of reaching the host inner plexiform layer, the appropriate target in the host retina. It was not determined here whether synaptic contacts are established by the projecting NOS-accumulating cells. However, previous studies have shown that synaptic terminals of transplanted cells exhibit normal morphology, even after long survival times.\textsuperscript{5,7,14} Further, there is evidence that neurites projecting from retinas transplanted to the brain are capable of forming synaptic contacts on reaching the host target tissue.\textsuperscript{15} In normal rabbit retinas, NOS is expressed by subpopulations of wide-field amacrine cells,\textsuperscript{10–12} and it is likely that the NOS-containing cells found in the grafts also are of this type. It has been suggested that nitric oxide produced by amacrine cells may modulate light-induced inward currents in ON cone bipolar cells.\textsuperscript{10} Thus, it is possible that the NOS-

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption{(A, B) Fluorescence micrographs showing extension of NOS-immunoreactive fibers from the transplant (T) to the host retina (H). The host retina exhibits immunoreactive cells located within the inner plexiform layer [IPL(h)] and ganglion cell layer [GCL(h)] (arrows), and an immunoreactive plexus (arrowheads) in the inner plexiform layer. (A) PN 12 rabbit retinal transplant (28 days after transplantation) with a rosette (R). A long immunoreactive process (open arrowhead) is seen to cross over from the transplant to the host inner plexiform layer. (B) PN 20 retinal transplant (36 days after transplantation) showing a short process (open arrowhead) connecting an immunoreactive plexus in the transplant (large arrowheads) to the immunoreactive plexus in the host inner plexiform layer (small arrowheads). Scale bars, 30 μm.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure3}
\caption{(A, B) Fluorescence micrographs showing NOS-immunoreactive cells connecting the transplant (T) to the host retina (H). (A) Specimen taken 61 days after transplantation showing several stained processes (large arrowheads) in a transplant, corresponding to PN 45. One of the processes (open arrowhead) belonging to a NOS-immunolabeled cell located in the transplant (arrow) is seen to project toward the immunoreactive plexus in the host inner plexiform layer [IPL(h)] (small arrowheads). A stained cell body is also seen in the host inner nuclear layer [INL(h)] (small arrow), next to the host inner plexiform layer. (B) PN 90 retinal transplant (106 days after transplantation) showing a strongly labeled cell body (large arrow) connecting the inner plexiform layer of the host (small arrowheads) to an immunoreactive plexus in the transplant (large arrowheads). An immunoreactive cell is seen in the inner nuclear layer of the host (small arrow). Scale bars, 30 μm.}
\end{figure}
containing cells found in the grafts contact cone bipolar cells at the level of the host inner plexiform layer.

At times, the origin of the bridging fiber could not be established. We also have found examples of NOS-immunoreactive neurons projecting to the graft and to the host retina. In the case illustrated in Figure 3B, the possibility that the contacting cell belonged to the host cannot be ruled out. Whatever the origin of the projecting neuron might be, it appears to connect the host inner plexiform layer to an equivalent region within the graft. If so, information from the graft could be conveyed to the host retina not only by those neurons that directly project to the host, but also indirectly.

Bridging fibers were more often seen at long survival times. This is as expected, considering that also in normal developing rabbit retinas, outgrowth of NOS-containing fibers is a relatively slow process. Further, a better graft–host fusion was observed in older transplants, which conceivably should also favor the formation of connections. Bridging, however, was never observed in areas where one or more cell rows were left of the host outer nuclear layer. Labeled processes located next to the graft–host border were in these cases seen to run parallel to the graft–host border, without crossing over, in spite of the fact that the host outer nuclear layer was thinned and that the host inner plexiform layer was located near the graft. This does not seem to be unique for rabbit-to-rabbit transplants or a limitation of NOS-containing fibers only, because the same has been observed in rat-to-rat transplants using additional cell markers.

Furthermore, it was noted that even when present, the number of NOS-immunoreactive fibers crossing the graft–host border was not very large and varied between specimens and also between sections from the same specimen. It may be noted that even in normal retinas, NOS is expressed in only a small population of cells, and high numbers of bridging fibers are not necessarily expected. The variable and small number of NOS-containing cells and fibers within the grafts may be explained in part also by the random organization of the transplants, which results from transplantation of pieces of embryonic retina. However, it has been consistently difficult to demonstrate graft–host connections, irrespective of the transplantation method used. The number of connections found has been low also when transplanting photoreceptors alone or large sheets of properly laminated and oriented neuroretina, suggesting that there may well be other factors involved than tissue orientation. After transplantation, a rapid loss of the outer layers of the rabbit host retina is observed in areas adjacent to the graft. Numerous dying cells are also found in the grafts during the first weeks after transplantation, mostly next to the host retina. As a result of neuronal cell death, a gliotic response is normally seen, and factors associated with reactive glia have been identified that inhibit neurite outgrowth. Glial cell activation is observed in the host rabbit retina and with time also in the grafts. It is thus possible that a glia-associated factor(s) may, at least in part, influence negatively the formation of connections between graft and host retinas.

In summary, the present study demonstrates that at least some graft–host contacts involve graft cells capable of synthesizing a neuronal messenger, disclosing the functional potential of these connections.

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