Numbers of Cortical Vitreous Cells and Onset of Cataracts in Royal College of Surgeons Rats

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Royal College of Surgeons (RCS) rats have hereditary retinal degeneration with associated posterior subcapsular opacities. A link between light, retinal degeneration, and cataracts may consist in peroxidation of polyunsaturated fatty acids of rod outer segment lipids to yield water-soluble toxic aldehydes that can traverse the vitreous and react with bow cells and posterior lens fibers. In an immune reaction to the retinal degeneration, macrophages multiply in the retina and in the cortex of the vitreous. In dystrophies, the cortical vitreous separates readily from attachments to retina, ciliary body and lens, and from the vitreous gel. This web-like structure was stained and spread on a counting chamber. Cells were counted at 15–130 postnatal days in pink- and black-eyed RCS dystrophies and in congenic controls to correlate numbers of cells, temporal and geographic patterns of retinal degeneration, and onset of opacities. Rats were reared in cyclic light (10–40 lux inside the cage) and fed a natural ingredient diet (NIH-07). Cortical vitreous cells increased markedly in pink- and black-eyed dystrophies at 50–53 days when slit-lamp detectable opacities occurred in both. The increase was 4.6-fold in pink- and 2.3-fold in black-eyed rats compared with controls. At 50–53 days, the dystrophy affected all quadrants of the retina severely in pink-eyed RCS but only the inferior periphery in black-eyed RCS. Consequently, severe degeneration in one quadrant may suffice to initiate an opacity. In black-eyed rats, degeneration in the rest of the retina occurs 10–35 days later, but the number of cortical vitreous cells was never as great as the number in pink-eyed rats at 50–53 days. The lower cell count in black-eyed RCS may be related to the fact that less than 3% of them ever develop mature cataracts. In contrast, one fourth of pink-eyed RCS have mature cataracts by 1 yr of age. Invest Ophthalmol Vis Sci 32:200–207, 1991

Royal College of Surgeons (RCS) rats have hereditary defect in phagocytic activity of the retinal pigmented epithelium (RPE), leading to early death of the rod photoreceptor cells of the retina.1 The disease begins at 12 postnatal days when the rod outer segment (ROS) membranes first require phagocytosis. In pink-eyed, tan-hooded RCS, which have been most frequently studied,2–4 the ROS layer is twice normal in thickness by 18 days and maximally thick by 27 postnatal days. The rod nuclei begin to degenerate by 22 days; most are pyknotic by 50 days and have disappeared by 60 days. The retinal degeneration is accompanied by posterior subcapsular opacities (PSO), which occur in all the animals bilaterally5 and can be observed by slit lamp in vivo or by low-power dissecting microscopy in vitro by 7–8 weeks of postnatal age. The opacities appear subsequent to maximal debris accumulation and death of most of the rod cells. Water-soluble toxic aldehyde products indicative of lipid peroxidation were detected in the vitreous at the time of maximal debris accumulation and may to be involved in initiation of the opacities.6 Presumably, the toxic aldehydes originated from peroxidation of the polyunsaturated fatty acids of phospholipids of ROS membranes, which are more highly unsaturated than any other membranes that have been studied.7

Macrophages first appear in the photoreceptor layer at 22 days and actively phagocytize the debris after 4 weeks.8 These cells are capable of peroxidative damage to membranes9 and may themselves contribute to the toxic aldehyde products present at any given time. Furthermore, the rat lens in organ culture has been shown to be damaged by lipid peroxidation products10 and by the presence of macrophages.11

The presence of macrophages in the cortical vitreous of the dystrophic RCS rat at 7–8 weeks of age was brought to our attention by the spontaneous appearance of a sack-like structure adhering to the posterior aspect of the anterior segment when the globe was cut into anterior and posterior halves. On low-power microscopic examination (50–70×), this structure was
found to be the vitreous cortex (and gel) which had separated cleanly from the retina. In the normal rat eye, only small fragments of the vitreous cortex ordinarily are obtained during a dissection, unless a special effort is made to recover it. When examined by higher power microscopy, the vitreous cortex of the RCS rat appeared to contain a dense population of cells, and this subjective impression was confirmed after a technique was developed to stain and count them and compare their numbers with those in normal rats of the same age.

In the present article, the numbers of cells per mm² of cortical vitreous were determined in pink- and black-eyed retinal dystrophic RCS rats and their congenic controls over the age range of 15–130 postnatal days. Rats were reared in cyclic light (10–40 lux inside the cage) and fed a natural ingredient diet (NIH-07). In the controls, no changes were seen during this age interval, but in the two dystrophic strains the increases in numbers of cells per mm² paralleled the course of retinal degeneration. The known slowing of retinal degeneration in black-eyed dystrophies was reflected in lower cell counts in the vitreous cortex of dystrophic rats of the same age.

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Materials and Methods

Animals

The animals we used were: (1) pink-eyed, tan-haired dystrophic and congenic control RCS rats (rdy/rdy, p/p and rdy⁺, p/p, respectively) and (2) black-eyed, black-haired dystrophic and congenic control RCS rats (rdy/rdy, p⁺ and rdy⁺/p⁺, respectively). The use of rats in these investigations adhered to the ARVO Resolution on the Use of Animals in Research.

Housing and Lighting

All rats were maintained in plastic cages under a 12-hr on/12-hr off cyclic light schedule. The cages were placed on metal racks that cut off much of the light, and the light inside the cages was 10–40 lux.

Diets

The rats were fed either a standard NIH-07 natural ingredient rodent-pellet diet or a custom-pelleted diet consisting of a commercial diet (R-M-H #3500; Charles River Farms) supplemented with 25% ground sunflower kernels (ICN, Cleveland, OH). In our conventional laboratory animal quarters, better reproduction was attained, and the growth of pups to weaning was more consistent with the second diet.

Dissection to Obtain the Vitreous Cortex

At different ages, from 15–130 days, rats were killed in an atmosphere of carbon dioxide (from dry ice in a closed vessel), and the eyes were excised and placed in Dulbecco’s phosphate-buffered saline, pH 7.4. All excess tissue was removed from the globe, especially at the area of the optic nerve, and the eye was divided into anterior and posterior segments by cutting about 1 mm behind the ora serrata with scissors having 8-mm long blades.

The web-like vitreous cortex was removed from the retinal cup by one of two methods. In older dystrophics with advanced degeneration of the retina, the web was loose and often separated cleanly from the retina when the anterior and posterior segments were gently pulled apart; then it only had to be separated from its attachments at the ciliary body and lens. In younger dystrophics with less advanced retinal degeneration, and in controls, the stronger attachments between web and retina made it necessary to do a more careful dissection. The attachment at the optic nerve area was especially strong. First, the web was detached from the lens and ciliary body, then it was pulled firmly but gently off the retina. The transparency of the web made it difficult to observe well until it was removed from the cup. In the youngest rats, where the retina was not fully differentiated, a second method was used. After division of the eye into anterior and posterior segments, the sclera (with RPE) was peeled off the retina, and the retina was punctured at the optic nerve and gently stripped off the vitreous cortex from the optic nerve to the ora serrata. The retina was peeled back in consecutive sections around the eye, as the web was held in place beneath it. This left the web attached to the lens and ciliary body. The web could then be separated by grasping it near its attachment to the lens and pulling it away from the ciliary body.

Enumeration of Cells in Vitreous Cortex

The isolated web was stained with a concentrated solution of brilliant green made by dissolving a few crystals in Dulbecco’s buffered saline in a small plastic petri dish (60 × 15 mm). The vitreous gel separated from the web during the staining period. The stained web was spread on the grid of a hemocytometer and covered with a special micro cover slip which trapped a single layer of cells. The cover slip (6 × 6 mm) was cut from a thin microscope slide cover slip by means of a diamond pencil. The cells were counted in a 1-mm² area using the conventions for counting white blood cells. Two or more areas of the web were counted in most cases, and the results were averaged.
Measurement of Area of Vitreous Cortex

At ages from 10 days–1 yr, the web of the vitreous cortex was dissected as described, laid on a Sedgewick Rafter counting cell (Graticules, Tonbridge, England), which was ruled in a 1-mm grid. A cover slip (7 × 7 mm) was cut with a diamond pencil and placed over the web. This smoothed the web to a single thickness. If necessary, slits were made with microscissors to allow the web to flatten. The area was estimated by counting the number of 1-mm squares occupied by the web, under low-power microscopy.

Microscopic Observations

The anterior segment obtained by the initial dissection was observed with a Zeiss stereo (Carl Zeiss Instrument Inc., Hanover, MD) dissecting microscope to study the posterior and lateral aspects of the lens as it floated in saline. In pink-eyed RCS dystrophies reared in cyclic light, an opacity was readily visible by this technique and was photographed at 7–8 postnatal weeks and older. At certain ages (49, 50, 51, 60, 80, and 85 days), one eye was dissected and the lens observed in this fashion; the other eye was removed for histopathologic study; and a cut was made at the area of the limbus to facilitate entrance of fixative (phosphate-buffered 4% glutaraldehyde, pH 7.2 for 30 min, followed by 10% buffered neutral formalin). The eye was embedded in plastic. Sections were cut at 2 μm and stained with hematoxylin and eosin.

Results

In pink-eyed, tan-hooded dystrophic rats from 28–127 postnatal days of age, counts of cells/mm² in vitreous cortex were done as described. Three to 20 eyes were counted, and the results are shown in detail in Figure 1, containing a scattergram, which also shows results for the pink-eyed tan-hooded congenic control strain. Similar results are shown for the black-eyed dystrophic, from 20–130 days, in Figure 2. Here, three to eight eyes were counted, and the results for the black-eyed congenic control strain are also given. Cortical vitreous cells increased markedly at 50–53 postnatal days in both pink- and black-eyed dystrophics, and it was at this time that slit-lamp detectable PSO was observed in both strains.
Fig. 3. Mean values for macrophage concentrations in vitreous cortex at different ages in cyclic light reared pink- and black-eyed RCS dystrophic rats. Points represent values for 3–20 eyes for pink- and 3–8 eyes for black-eyed rats.

In Figure 3, mean values for the cell concentrations at the different ages in the pink- and black-eyed dystrophies are shown. In Table 1 the results are summarized for the two most outstanding periods. The first is at 50–53 days, when the sudden increase in cells occurred in both strains, but especially in the pink-eyed dystrophic. The second is at 60–80 days, when the black-eyed dystrophic showed a broad peak of cells, greater than the number of cells at 50–53 days. These distributions are related to the temporal and geographic patterns of retinal degeneration in the two strains. The single large peak of cells at 50–53 postnatal days in the pink-eyed rat may represent the immunologic response to severe degeneration in the whole retina. On the other hand, in the black-eyed dystrophic the mean number of cells at 50–53 days was significantly greater than at 60–80 days (P < 0.001); in the black-eyed dystrophic the mean at 60–80 days was significantly greater than that at 50–53 days (P < 0.001).

In comparison with the respective controls, the pink-eyed dystrophies had a 4.6-fold increase in numbers of cells at 7–8 weeks, while the black-eyed dystrophies had only a 2.3-fold increase. The mean number of cells at 60–80 days in black-eyed dystrophies represented an increase of 2.9-fold compared with the black-eyed control.

Statistical analysis (Table 1) showed that over the age range from 20–80 days, the cells/mm² were significantly greater in the vitreous cortex of the pink-eyed congenic control than in that of the black-eyed congenic control (P < 0.001). Greater significance (P < 0.001) was found between the cell concentrations in pink- and black-eyed dystrophics, both at 50–53 days and at 60–80 days. Also, in the pink-eyed dystrophic the mean number of cells at 50–53 days was significantly greater than at 60–80 days (P < 0.001); in the black-eyed dystrophic the mean at 60–80 days was significantly greater than that at 50–53 days (P < 0.001).

Figure 3 illustrates the growth in area of the vitreous cortex from 10 days–1 yr in pink-eyed dystrophic RCS rats. The maximal rate of growth was in the first 7 weeks, and the area at 1 yr was approximately 49 mm². Using these data, the numbers of cells in the vitreous cortex at ages between 20–80 days in pink- and black-eyed dystrophics were estimated (Table 2). The number of cells that could be obtained readily by harvesting the vitreous cortex in dystrophic rats is of the order of 5000–7000 in black- and 10,000–12,000 in pink-eyed rats over the age range from 50–80 days. In black- and pink-eyed RCS controls the yield of cells would be less than the 2300–2800 that can be obtained.

Table 1. Cortical vitreous cells in pink- and black-eyed RCS dystrophic and congenic control rats reared in cyclic light

<table>
<thead>
<tr>
<th>RCS rat strains</th>
<th>Postnatal days 50–53</th>
<th>Postnatal days 60–80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pink-eyed dystrophics</td>
<td>359 ± 16.8 (52)*</td>
<td>266 ± 12.3 (36)*</td>
</tr>
<tr>
<td>Black-eyed dystrophics</td>
<td>155 ± 4.17 (24)</td>
<td>191 ± 6.5 (31)</td>
</tr>
<tr>
<td>Pink-eyed controls</td>
<td>78.2 ± 3.08 (44)¹</td>
<td>19–83 postnatal days</td>
</tr>
<tr>
<td>Black-eyed controls</td>
<td>66.2 ± 3.08 (19)</td>
<td>17–73 postnatal days</td>
</tr>
</tbody>
</table>

Numbers of eyes studied are indicated in parentheses.
Statistical comparisons by Student’s t test: *Pink- and black-eyed dystrophics, P < 0.001; ¹Pink- and black-eyed controls, P < 0.01.
Table 2. Estimated total numbers of cells in vitreous cortex at different ages in pink- and black-eyed dystrophic RCS rats, reared in cyclic light

<table>
<thead>
<tr>
<th>Postnatal days</th>
<th>Area, sq. mm.</th>
<th>Pink-eyed Cells/sq. mm.</th>
<th>Total</th>
<th>Black-eyed Cells/sq. mm.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>24</td>
<td>81</td>
<td>1940</td>
<td>73</td>
<td>1750</td>
</tr>
<tr>
<td>40</td>
<td>31</td>
<td>113</td>
<td>3500</td>
<td>71</td>
<td>2200</td>
</tr>
<tr>
<td>50–53</td>
<td>34</td>
<td>359</td>
<td>12210</td>
<td>155</td>
<td>5270</td>
</tr>
<tr>
<td>60–80</td>
<td>37.5</td>
<td>266</td>
<td>9980</td>
<td>191</td>
<td>7160</td>
</tr>
</tbody>
</table>

Area of vitreous cortex was measured as described in Methods. Results were rounded to the nearest tens of cells.

estimated, respectively, because of the difficulty in recovering the vitreous cortex. Since postmortem hydrolysis can release the attachments to the retina in normals, the recovery of vitreous cortex could be improved by delaying the dissection. The numbers of cells of vitreous cortex that can be obtained for study were sufficient for many biochemical, immunologic, and other investigations.

Histopathologic studies of the retinas agreed with the report of LaVail et al.,4 who found a delay in the rate of degeneration of the retina in the black-eyed RCS dystrophic rat compared with the pink-eyed rat.

Discussion

The cells we studied were all derived from the vitreous cortex, not from the vitreous gel. We did not study cells of the vitreous gel in RCS rats. We photographed some of the fresh, stained preparations of RCS cortical vitreous cells. Typically, the cells had long pseudopods, consistent with their being macrophages. The vitreous cortex was studied in adult cows and in calves.14 It consists of a collagenous network separating the central vitreous gel from the retina, ciliary body, and lens. The collagen fibrils are attached to the basement membranes of: (1) retina glial cells (Muller cells), (2) ciliary epithelium, and (3) lens fiber cells (capsule). Normally, the posterior portion of the vitreous cortex contains hyalocytes, which are resident macrophages, and the anterior area behind the lens is free of cells. The hyalocytes are scattered evenly in a monolayer and do not touch one another or the basement membranes of the neighboring tissues. In adult cows, the density of hyalocytes was greatest near the ciliary body (457 cells/mm³) and lowest at the equator of the retina (51 cells/mm³); at the optic disc, the density was slightly higher than at the periphery. In calves, the cell density was 60–100% greater than in cows, since the cells migrate to the cortex during embryonic life, but not later, meaning that the density would decrease with growth.

The mean numbers of cortical vitreous cells per mm² in normal pink- and black-eyed RCS rats (78 compared with 66) were similar to the numbers of cells reported per mm² in bovine vitreous cortex at the retinal equator.14 Since the hyalocytes form a monolayer, the numbers per mm² and per mm³ can be considered equivalent (Fig. 4).

Clinical human studies have not dealt specifically with the cells of the cortical vitreous, but rather with total cells (presumably of cortex and gel) available from cataract operations. In one study, biopsy specimens of human vitreous were obtained from six retinitis pigmentosa patients during cataract surgery. Macrophage-like cells, uveal melanocytes, retinal astrocytes, and RPE cells were found.15 In a second study, monoclonal antibodies were used to detect immune system cells in vitreous obtained: (1) by vitrectomy techniques from three patients with end-stage retinitis pigmentosa and (2) by dissection from

![Fig. 4. Measured area (sq. mm.) of vitreous cortex in RCS rats during development.](image-url)
potmortem eyes of eight donors with retinitis pigmentosa and eight control donor eyes. In these samples of vitreous from patients, about two thirds of the cells reacted with monoclonal antibodies that detected various lymphocyte subsets, B cells, and macrophages. In the eight control samples, only 7% of the cells reacted with monoclonal antibodies, detecting macrophages and occasional T cells. These findings suggest it would be interesting to examine total cells of the vitreous (cortex plus gel) in RCS dystrophic rats at various ages to try to detect immune system cells other than macrophages.

Postmortem autolysis will release the basal lamina of the Muller cells and permit removal of the vitreous cortex if the latter is then dissected free of its other attachments. Similarly, in RCS dystrophies, a complete separation of the posterior vitreous from the retina was seen at about 50 postnatal days when the cortical cells were most numerous and before the final stage of retinal degeneration. Hydrolytic enzymes of macrophages may be a significant factor in these separations. Also, in human retinitis pigmentosa, the posterior vitreous cortex populations were different. The results for the two eyes of a rat were often different, suggesting an environmental factor, such as light, rather than a general systemic pathophysiologic factor.

The black-eyed control value was significantly lower than that of the pink-eyed control (P < 0.01) suggesting that even at low-light levels retinal damage is occurring in the pink-eyed rat, although this was not evident by light microscopic examination of the retina. Susceptibility of pink-eyed normal rats to light damage is well established, and damage does occur at low light levels.

The normal rat lens in organ culture has been shown to be susceptible to damage by lipid peroxidation products and by addition of macrophages. Similarly, membrane damage was detected in lenses of pink-eyed dystrophic RCS rats aged 40–80 days, by observing their increased leakiness to 86Rb. After 80 postnatal days, the incidence of leakiness was lower, and this may be correlated with “internalization” of the PSO in the 75% of rats that do not develop mature cataracts. Internalization is produced when normal lens fibers are laid down between PSO and capsule (at 9–10 weeks) as though toxic material were no longer being produced to damage lens fibers and cells. This can be observed microscopically in dissected lenses floating in saline. Presumably the healthy fibers restore permeability to normal, and mature cataracts do not occur.

The fact that black-eyed RCS dystrophics develop PSO at the same time as pink-eyed RCS may mean that degeneration in the inferior periphery is sufficient to initiate an opacity. Quantitatively, the concentration of retinal degeneration products (toxic aldehydes) may never be as great in black- as in pink-eyed RCS. This may explain why PSO rarely progress to mature cataracts in black-eyed RCS (3% or less compared with 25% in pink-eyed RCS) by 9–12 months of age.

Recently, we found that when the pink-eyed RCS dystrophics were reared in darkness, the initial PSO were prevented, and the cortical vitreous macrophage concentration was lower than in the black-eyed dystrophics. This suggests that the PSO in black-eyed rats might also be prevented by dark rearing. These results indicate that light is essential for initiation of the RCS cataract.

Retinal damage by light in RCS rats, like that in normal pink-eyed rats has the action spectrum of
rhodopsin. This may occur because retinaldehyde, freed from rhodopsin by light, can act as a photosensitizer to generate singlet oxygen, a highly reactive excited species of molecular oxygen. It oxidizes polyunsaturated fatty acids more actively than ordinary oxygen does. These reactions of retinaldehyde and singlet oxygen have been demonstrated in vitro. For a number of reasons, the RCS rat may be more likely than normal pink-eyed rats to suffer this type of lipid peroxidation in the retina. By 37 days of postnatal age, the conversion of retinaldehyde to retinol in pink-eyed RCS dystrophics is much slower than normal. At this age, the activity of retinaldehyde reductase is decreased. However, this enzyme had normal activity at earlier ages and is not a causative factor in the RCS disease. Presumably, the enzyme was damaged during the earlier stages of degeneration.

Macrophages accumulating in the vicinity of the lens may play some role in cataract formation, but their role may be secondary rather than primary. They may affect the severity of the original opacity and its likelihood of becoming a mature cataract. Activated macrophages generate active species of oxygen: superoxide anion radical, hydrogen peroxide, hydroxyl radical, and perhaps singlet oxygen, which are crucial for intracellular digestive functions of macrophages and for their secretory functions. These oxidizing species can be released extracellularly, and macrophages near the lens could cause lens membrane damage similar to that shown by macrophages introduced into a lens organ-culture system. Further work will be necessary to investigate the possible role of such cells in the RCS rat cataracts in vivo.

Whether toxic oxygen species originate in the retina or in vitreous cortex macrophages, or both, the lens can be protected from damage by nutritional means. When pink-eyed dystrophics were fed 1) a purified diet of the American Institute of Nutrition (AIN-76) or 2) rather than natural-ingredient diets free of animal products such as fishmeal, only 2–5% of the rats developed mature cataracts by 9–12 months of age compared with 25–30% of rats fed natural-ingredient diets (NIH-07 or Charles River #3500) containing animal products such as fishmeal. The protective diets were high in antioxidant or low in prooxidant factors, or both. When the AIN-76 diet was supplemented with beta-carotene (0.4%) and 0.01% BHT, no mature cataracts occurred, and most of the lenses were clear. These nutritional results are consistent with the hypothesis that RCS cataracts are secondary to lipid peroxidative processes in degenerating retina.

Key words: butylated hydroxytoluene vitreous cells, macrophages, hyalocytes, RCS rats, posterior subcapsular cataracts, cataracts

References