Interstitial Retinol-Binding Protein (IRBP) in the RCS Rat: Effect of Dark-rearing

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The effect of light- and dark-rearing on the amounts of rhodopsin and interstitial retinol-binding protein (IRBP) in RCS rats and their congenic controls (RCS-rdy+) was determined. Rhodopsin was measured spectroscopically and IRBP by dot-blot enzyme immunoassay utilizing rabbit antibovine IRBP IgG. After P15–20, dark-reared RCS and RCS-rdy+ rats always had more rhodopsin than their light-reared, age-matched counterparts. The rhodopsin in the light-reared RCS rats peaked at about 2 nmol/eye at P20–25. The rhodopsin in the dark-reared RCS rats peaked at about 4 nmol/eye at P60–70. Maintenance of RCS-rdy+ rats in darkness had no effect on their IRBP content, which continued to increase up to P80–110. In both groups of RCS rats, the amount of IRBP reached a peak at P22. In RCS rats maintained in darkness, the amount of IRBP attained at this peak was about twice that in the corresponding light-reared group and in RCS-rdy+ animals at this age. The decline of IRBP after P22 in RCS rats was slowed in darkness by approximately 10 days. This slowed decline of IRBP is associated with a decreased rate of photoreceptor degeneration, and the results are therefore consistent with the hypothesis that the photoreceptors synthesize and secrete IRBP. The layer of membranous debris would restrict the diffusion of IRBP in the subretinal space and could partially exclude this retinol transport protein from access to the zone adjacent to the apical surface of the retinal pigment epithelium (RPE). Invest Ophthalmol Vis Sci 26:1381–1385, 1985

Our findings on the properties, emergence, distribution, and biosynthesis of interstitial retinol-binding protein (IRBP) in RCS and normal rats led us to propose that this glycoprotein was synthesized and secreted by the photoreceptors. Autoradiographic observations on isolated human retinas incubated with 3H-fucose have also supported this suggestion. Gonzalez-Fernandez et al showed that IRBP disappeared rapidly from the subretinal space between postnatal days 22 and 30, ie, during the period of active photoreceptor cell death (see Eisenfeld et al). The rats used in that investigation were maintained under cyclic illumination and were pink-eyed. For reasons that have not been established, the photoreceptor degeneration in this strain is retarded by darkness. In order to obtain more information on this effect, and possibly elucidate it, we undertook to determine the effects of dark-rearing on the levels of rhodopsin and IRBP. The results are consistent with the hypothesis that the photoreceptors are responsible for IRBP synthesis. We relate these findings to observations by other workers on the transport of retinol between the retina and RPE in RCS rats kept in darkness and then light-adapted. We propose that debris accumulation hinders the free diffusion of IRBP in the subretinal space and may partially exclude it from access to the apical surface of the RPE.

Materials and Methods

Animals

Breeding pairs of tan-hooded, pink-eyed RCS rats homozygous for the retinal dystrophy gene (rdy), and of their congenic controls (RCS-rdy+) were obtained from M. M. LaVail. Some animals were maintained on a 12-hr light–dark cycle under fluorescent lights that gave an illumination level in the cages that ranged from 2 to 22 foot-candles. The dark-reared animals were transferred to darkness at P1–5. They were briefly exposed to dim red light during daily cleaning, inspection, watering, and feeding. These investigations were carried out in a manner that conformed to the ARVO Resolution on the Use of Animals in Research.
Rhodopsin

In our previous study, IRBP and rhodopsin were measured in the same eye; in the present work, these measurements were made from the contralateral eyes. Cyclic light-reared rats were first dark-adapted overnight, and subsequent operations were carried out under dim red light. The animals were killed by decapitation, the eyes were enucleated, a slit was made in the cornea of one eye, and the lens was extruded with slight pressure. The eye was then cut in half, and both halves were homogenized in 1 ml of extraction buffer (100 mM hydroxylamine, 200 mM L-1690, 100 mM Na phosphate, pH 7.0) and centrifuged at 16,000 x g for 0.5 hr. This procedure extracted 98% of the rhodopsin, as determined by subsequent treatments with further aliquots of extraction buffer. L-1690, a mixture of long-chain fatty acid esters of sucrose (see Fong et al9), was obtained from the Kyoto Company Ltd (Tokyo, Japan).

Visual pigment concentration was determined by measuring the absorbance loss at the \( \lambda_{\text{max}} \) of rat rhodopsin (498 nm; see Bridges10) when the extract was bleached. A molar absorbance coefficient of 42,000 M\(^{-1}\) cm\(^{-1}\) was used.11

Preparation of Interphotoreceptor Matrix (IPM)

The IPM from the contralateral eye was prepared in approximately 5 ml of phosphate-buffered saline (PBS; 150 mM NaCl, 5 mM Na phosphate, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) essentially as described.3 The sector anterior to the limbus (together with the lens) was cut away and the bulk of the vitreous desribed. 3 The sector anterior to the limbus (together with the lens) was cut away and the bulk of the vitreous was removed. The opened eye was immersed in about 0.5 ml of PBS and the retina was gently detached with a small glass hook. The retina and the fluid in which the eye had been immersed were combined with a subsequent 1.0 ml PBS rinse of the eyecup and an additional 2.5 ml of PBS. The mixture was placed on ice for 10 min with occasional swirling and centrifuged at 500 x g for 2 min. The supernatant was then centrifuged at 100,000 x g for 1 hr. The amount of IRBP in the supernatant IPM was determined by a dot-blot enzyme immunoassay.

Dot-blot Enzyme Immunoassay of IRBP

Antibovine IRBP IgG was prepared from rabbit antitobovine IRBP serum15 by chromatography on Bio-Rad DEAE-Affi-Gel Blue (Bio-Rad; Richmond, CA), according to the manufacturer's directions. Stock solutions of purified bovine apo-IRBP were made up at concentrations of 0.4 mg/ml, as determined from an \( E_{1%} \) cm of 9.89 for the apoprotein at 280 nm.12 Fifteen-\( \mu l \) aliquots were stored at \(-70^\circ\)C until required. It was established that freezing and thawing did not alter the concentration or antigenic properties of the solute. Two hundred microliter volumes of IPM samples or of solutions containing 0.5–9.9 ng of purified bovine IRBP were applied to sheets of Bio-Rad Zeta-Probe membrane using a Bio-Rad multi-well dot-blot chamber. Zeta-Probe was used in preference to nitrocellulose because of its greater protein-binding capacity.13 Each IPM sample was assayed in eight dilutions that ranged from 0.001–0.013 parts of one eye. After the samples in each well had drained by gravity, the membrane was removed from the apparatus and placed in a solution of 10% bovine serum albumin in Tris-buffered saline (20 mM, 50 mM NaCl, 0.02% merthiolate, pH 7.5) at 45°C for 12 hr on a mixer plate. This solution was filtered before use and was not used more than three times. The membrane was then incubated at room temperature for 3 hr in a 1:175 dilution of antibovine IRBP IgG in 2% bovine serum albumin. This was followed by two washings at 40°C in Tris-buffered saline containing 0.05% Tween 20 (10 min each). The membrane was then incubated for 1 hr in a 1:2,500 dilution of affinity purified goat antirabbit IgG horseradish peroxidase conjugate (Bio-Rad) and washed as before. Color development was carried out according to the manufacturer's instructions. The densities of the dots were quantitated by scanning the membrane on a Kontes (Vineland, NJ) or Joyce Loeb (Vickers Instruments, Inc.; Malden, MA) reflectance densitometer at a wavelength of 560 nm. Under these conditions, the plot of reflectance of each dot against the amount of bovine IRBP or IPM dilution was a straight line.

Rat IRBP is not serologically identical with bovine IRBP (unpublished observations; compare human IRBP14). In the present work, therefore, rat IRBP is expressed as equivalent amounts of the bovine protein. The maximum amount of IPM protein15 delivered in a volume of 200 \( \mu l \) per well was 0.6–5.1 \( \mu g \) (0.013 parts of an eye). It was established that the presence of up to 5 \( \mu g \) of another protein (bovine serum albumin) per well did not interfere with the determination of bovine IRBP.

Results

In the upper part of Figure 1, the effects of cyclic light- and dark-rearing on the quantities of rhodopsin extracted from RCS rats and their congenic controls are compared. In the control animals kept under cyclic illumination, rhodopsin was first measurable at P6. The amount of rhodopsin in all four groups of animals was...
very similar until P15, but differences became apparent as the animals aged beyond this point. In the cyclic light-reared controls, the rhodopsin content reached its adult level of about 1.6 nmol/eye at P50. The dark-reared controls, on the other hand, always had more rhodopsin after P10–P20, and their eyes contained as much as 50% more rhodopsin than their cyclic light-reared counterparts at P140.16,17

In the RCS rats, there was no difference between the light and dark groups up to P20–P25, when they contained about 2 nmol/eye, a level that exceeded that found in the controls. The rhodopsin in the cyclic light-reared RCS rats then proceeded to fall, until none was detectable at P94. In contrast, the rhodopsin in the dark-reared RCS rats continued to increase after P25, until at P70 the amount obtained per eye had doubled to over 4 nmol. Thereafter, the level slowly fell.

The level of IRBP in both groups of controls increased roughly in parallel with that of rhodopsin from P10 onwards. Unlike the situation with rhodopsin, we could not detect a difference in amounts between the cyclic light- and dark-reared groups. However, IRBP increased in both groups beyond P50, the point at which rhodopsin had stabilized at the adult amount in the cyclic light-reared controls.

In both groups of RCS rats, the amount of IRBP reached a peak at about P22, then declined. However, the maximum amount of IRBP attained in the dark-reared animals was double that observed in RCS rats kept in the light, as well as in control animals at this age. The decline in IRBP after P22 was slowed in darkness by approximately 10 days.

Discussion

Gonzalez-Fernandez et al.2,3 concluded that the loss of IRBP in RCS rats could be partly explained by their observation that its rate of synthesis in the isolated retinas was reduced as the photoreceptors degenerated. Since other retinal cells are not affected by the disease during this time, this finding provided the first evidence that IRBP was synthesized by the photoreceptors, a conclusion that has been supported by studies on isolated human retinas.4 In pink-eyed RCS rats kept in darkness, the degeneration is slowed, and photoreceptor function (as assessed by the ERG threshold9) continues for many days beyond the time when nearly total loss of visual cells (with the possible exception of cones18–21) has occurred in the light. The retarded degeneration of visual cells and the prolongation of visual function in the dark is reflected by the slowed disappearance of IRBP under the same conditions. Although the level of IRBP in the subretinal space is also governed by its rate of removal (a process that has not been elucidated), this observation is consistent with the hypothesis that this protein is synthesized and secreted by the photoreceptors. In this connection, it is interesting that the calculated values of Delmelle et al.8 for the amount of rhodopsin in the surviving visual cells of dark-reared RCS rats also peaks at P22 and subsequently declines on a time course that is almost identical with that found for IRBP in the present study.

The role of light in potentiating photoreceptor cell death in the RCS rat has led to suggestions that retinol released by rhodopsin bleaching may play a role in the degeneration.3,22–24 Delmelle et al.8 who also used dark-reared animals, clearly demonstrated that in the RCS rat there were serious deficiencies in the transfer of
retinol from bleached rhodopsin into the RPE, as well as in rhodopsin regeneration. Although a variety of factors may be responsible for the latter observation, these findings suggest that extracellular retinoid transport is impaired in this mutant. Delmelle et al. noted that the progressively restricted transfer of retinol into the RPE observed from P17 to P35 seemed to relate to the degree of debris accumulation. As shown by the present findings, this effect is not obviously correlated with the total amount of IRBP in the subretinal space of dark-reared RCS rats. In the experiments of Delmelle et al., 82% of the retinol in the eyes of control animals was found in the RPE after 1 hr of light-adaptation. In RCS rats at P25, they found that the proportion of retinol in the RPE was only 41%. At this age, however, we found that the amount of IRBP was approximately double that of the controls. At P35, when the amount of IRBP in our dark-reared RCS rats was still more than half that in the controls, the proportion of retinol in the RPE had dropped to 29%. It is possible, therefore, that the debris forms a barrier that partially excludes IRBP from access to apical surfaces of the RPE and hinders this putative retinol transport protein from diffusing freely in the subretinal space. In support of this supposition, the intense band of IRBP-like immunoreactivity adjacent to the RPE seen in normal rats could not be detected by Gonzalez-Fernandez et al. in RCS rats at P18. Abnormal staining properties of this zone may also correlate with the immunohistochemical observations.

Failure to remove retinol from the photoreceptor outer segments, lamellar debris, and the subretinal space in general could lead to cell death, possibly by its destabilizing effect on membranes (for review, see Roels). The degenerating photoreceptors would progressively lose their ability to synthesize and secrete IRBP, which would accentuate the problem of eliminating the toxic accumulations of retinol. The reason for the immunity of the Müller cells, which also border the subretinal space, is an interesting question. It has been shown that cellular retinoid-binding protein (CRBP) occurs in the Müller cells of the rat retina but is not detectable in the photoreceptors. Although the function of CRBP within cells is not understood, in the Müller cells it could transport excess retinol to their basal surfaces, where it would be purged via the retinal circulation.

Key words: RCS rat, interstitial retinol-binding protein, retinol toxicity, rhodopsin

References


