The interphotoreceptor matrix in rats with inherited retinal dystrophy

Matthew M. LaVail, Laurence H. Pinto,* and Douglas Yasumura

The retinas of Royal College of Surgeons (RCS) rats with inherited retinal dystrophy and of genetic control RCS-rdy+ rats have been examined histochemically to determine whether the stainable interphotoreceptor matrix (IPM) is abnormal in dystrophic retinas. The mucosubstances that are stained with Alcian blue, metachromatically stained with toluidine blue, or reacted with colloidal iron appear in normal retinas beginning on postnatal day 12 as an intense band of stain at the apical surface of the pigment epithelium and with less intense staining between the outer segments throughout the rest of the outer segment zone. In RCS retinas the distribution of stainable IPM differs from that in normal retinas beginning on day 12. At this time, there is a failure of the intense band of IPM staining to form completely at the apical surface of the pigment epithelium. As whorls of outer segment membranes accumulate due to the phagocytosis defect in RCS pigment epithelial cells, IPM staining almost disappears along the pigment epithelial cell surface and in the debris zone. In addition, the basal outer segment region stains much more heavily in RCS retinas than in normal retinas, a feature that presumably represents an abnormal accumulation of IPM in this region of mutant retinas. Since the abnormal distribution of stainable IPM is evident 6 to 8 days before the first pyknotic photoreceptor cell nuclei are seen, it may play a role in photoreceptor cell death in the RCS rat. Furthermore, since the difference in IPM distribution between mutant and normal retinas is first evident on the same day that disc shedding and phagocytosis begin in the normally developing retina, the abnormal IPM distribution in RCS rats may also be related to the phagocytosis defect in the mutant pigment epithelial cells. (INVEST OPHTHALMOL VIS SCI 21:658-668, 1981.)

Key words: retinal dystrophy, inherited retinal degeneration, interphotoreceptor matrix, mucopolysaccharides, rat

Inherited retinal dystrophy in the rat is characterized by a grossly reduced ability of the pigment epithelial cells to phagocytize rod outer segment membranes, which leads to an accumulation of these membranes at the surface of the pigment epithelium.1-4 By about postnatal day 20 (P20), pyknotic photoreceptor nuclei begin to appear, and by P60 most of the photoreceptor cells have degenerated and disappeared.2, 3, 5 Although the mechanism of photoreceptor cell death is unknown, it appears to be a secondary effect of mutant gene action in the pigment epithelial cell.6, 7

During the course of testing linkage between the retinal dystrophy gene (gene symbol, rdy) and the nonagouti pigmentation gene,8 many dystrophic and normal rat eyes were examined histologically during the pe-
period of active photoreceptor degeneration (P25 to P40). When the sections were stained with toluidine blue, the normal retinas (+/rdy genotype) showed a metachromatic pink stain at the apex of the rod outer segments and pigment epithelium, whereas the dystrophic retinas (rdy/rdy) showed little or no metachromatic staining of this region. Polyanionic mucosubstances such as those making up the interphotoreceptor matrix (IPM)\(^{13}\) stain metachromatically with cationic dyes such as toluidine blue.\(^{15-18}\) Therefore these preliminary findings suggested a possible abnormality in the distribution of the IPM in dystrophic rat retinas. Since the IPM is in a strategic position to mediate the exchange of metabolites between the pigment epithelium and photoreceptors,\(^{14-18}\) and since a change in the IPM might lead to photoreceptor cell death, we decided to examine the retinas of mutant and control animals to (1) characterize histochemically any abnormal distribution of the IPM of the mutants and (2) determine whether an abnormality occurs before photoreceptor cell death. We found that the IPM has a significantly abnormal distribution in the mutant animals at a time substantially before photoreceptor cell death.

**Materials and methods**

Dystrophic animals included both those from the inbred Royal College of Surgeons (RCS) strain and from the congenic albino RCS-c strain.\(^{19}\) Non-dystrophic control animals were obtained from the RCS-rc/y+ congenic strain. Animals ranged in age from P8 to P29. For the developmental analysis, two animals from each strain were taken every other day beginning on P8.

The eyes were enucleated under ether anesthesia and the corneas were slit. The eyes were then immersed overnight at 4°C in a fixative that contained 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.5% cetlylpiridinium chloride (CPC)* in 0.1M sodium phosphate buffer.\(^{13}\) After bisec- tion along the vertical meridian, the eyes were rinsed for 2 hr in distilled water, dehydrated with a 1:1 mixture of methanol–methyl Cellosolve and embedded in polyester wax.\(^{20}\) Six and 10 μm thick sections were prepared for histochemical staining and reaction. Sections from some of the older normal and dystrophic animals were mounted on the same microscope slides to obtain as close to identical staining of the two genotypes as possible. It was often difficult to distinguish mutant from control tissues in the youngest eyes, so these were mounted on separate slides, but the slides of mutants and controls of the same age were stained in the same dish.

Metachromatic staining with toluidine blue was done with a 1.0% solution of toluidine blue O (Chroma 1B481) dissolved in a 10 mM potassium phthalate–tartrate (pH 4.5) buffer. The slides were exposed to the stain for 10 to 15 min, rinsed in the buffer for 2 min, dehydrated in tert-butanol (four rinses of 2 min each), and cleared in xylene.\(^{12}\)

Reaction with colloidal iron was generally done according to the method described by Rohlich.\(^{13}\) Dehydrated sections were reacted with Muller’s colloidal iron oxide reagent (pH 1.4) for 60 min, rinsed in an acetic acid solution (35% glacial acetic acid; pH 1.4; four rinses of 2 min each), bathed in mordant stain (a solution made from equal parts of 2% hydrochloric acid and 2% potassium ferrocyanide) for 20 min, washed in running water for 2 min, dehydrated in graded ethanols, and cleared in xylene. Control slides untreated with iron oxide reagent were bathed in the stain to check for the presence of iron-containing substances, but no staining was detected in the pigment epithelium, in outer or inner segments, or in the photoreceptor cell bodies of either dystrophic or normal animals. Some sections were counterstained with either neutral red or the periodic acid–Schiff (PAS) method.

\*The term mucosubstance is used to denote glycoproteins and proteoglycans.\(^{8}\) It is the preferred generic histochemical term for biochemically characterized and uncharacterized carbohydrate-rich constituents of tissues.\(^{10}\) Although the glycoproteins and proteoglycans of the IPM have been characterized fairly specifically by biochemical means (see Discussion), we use the more general term mucosubstance because the IPM of the rat (or of any other species) has not yet been studied histochemically with enough rigor to categorize all the constituents as specific types of mucins or mucopolysaccharides, terms which are used in the most contemporary histochemical classification.\(^{11,12}\)

\*CPC stabilizes mucosubstances and apparently is necessary when formaldehyde alone is used.\(^{12}\) However, the combined aldehyde fixative without the addition of CPC gave substantial IPM staining, albeit somewhat less than with CPC. We should note that combined aldehyde fixative with CPC added causes rapid hemocoagulation and is not suitable for perfusion fixation.
Staining with Alcian blue 8GS (Chroma 1A 288, Chroma-Gesellschaft lots 11.77 or 4.79, but not lot 8.78, which stained nonspecifically) was done with a 1% solution of stain, dissolved in 0.1N or 0.025N HCl (pH 1.0 or 1.6) for 10 min. Staining was preceded by a 30 sec wash in 0.1N HCl and followed by dehydration in 100% ethanol (two rinses of 2 min each) and clearing in xylene.

Some sections were incubated for 3 hr at 37° C in bovine testicular hyaluronidase (type I-S; Sigma, St. Louis) by using the protocol and controls described previously. Sections were then stained with toluidine blue, Alcian blue, or the colloidal iron reaction.

At least four sections from two or three blocks of embedded retinal hemispheres were examined from each animal. We searched for regions of maximal staining of reaction product in both the posterior (from optic nerve head almost to the equator) and far peripheral retinas (near the ora serrata) of each section, and unless otherwise noted, all results will be from areas of maximal staining or reaction. Although the 6 μm thick sections provided better resolution, the 10 μm sections inevitably showed more intense staining and were usually used for analysis and photomicrography. Results from tan-hooded RCS and albino RCS-c rats were indistinguishable, so that reference to mutant or RCS retinas applies to either of these strains.

In toluidine blue-stained sections, the pink metachromatic staining was usually difficult to demonstrate convincingly in photomicrographs. Therefore microphotometric readings of optical density were obtained from the 10 μm sections. Figs. 9 and 10. Alcian blue stain; peripheral retina; P16. In the mutant retina (Fig. 10), Alcian blue staining is minimal and confined to the basilar outer segment region, whereas in the control retina, maximal staining is present in the basilar outer segment region (Fig. 9).
Figs. 1 to 14. For legends see facing page.
density were obtained from selected sections by using a Zeiss MPM 03 microscope photometer with a 63X planapochromat lens and a circular measuring aperture 0.8 μm in diameter. Since toluidine blue metachromatic staining has an absorption maximum of 480 to 540 nm,12 a Zeiss BP485/20 filter was used to provide maximal illumination at 485 nm with a 20 nm half-bandwidth.

Results

Colloidal iron reaction

Posterior retina. The distribution and density of colloidal iron reaction product, which was found in the interstices between the developing outer segments, was similar for P8 and P10 animals of both the RCS and RCS-rdy+ genotypes (Figs. 1 and 2). By P12, however, the distribution and density of reaction product differed for RCS and normal animals. In the posterior retina of every normal animal P12 or older, we observed a dense, almost continuous band of reaction product adjacent to the apical surface of the pigment epithelium (Figs. 3, 5, and 7). Beneath this dense band, less dense but relatively uniform staining of the interstices between rod outer segments was observed.

In RCS rats at P12, the first membranous whorls appeared at the pigment epithelial cell surface, and the apical band of reaction product seen in the normal rats was reduced in density (Fig. 4). This became progressively more apparent in older mutant animals. An example of a posterior retina from a P16 RCS rat (Fig. 6) demonstrates patches of reaction product at the apical pigment epithelial cell surface, where most of the surface was clearly free of reaction product. Even less reaction was present at P20 and older (Fig. 8). Reaction product was also found in the IPM between the remaining outer segments located beneath the debris zone, and although some reaction product was present between the membranous whorls as far distally as the pigment epithelial cell surface (Fig. 6), this diminished with age (Fig. 8). The density of reaction product was clearly less in the debris zone than in the outer segment zone of normal rats (cf. Figs. 6 and 8 with 5 and 7). The density of reaction product was maximal between the surviving basal outer segments (Figs. 6 and 8), and it frequently appeared denser than in normal retinas.

In sections counterstained with the PAS method, the outer segments stained the expected pink to magenta,22 and blue colloidal iron reaction product was present between them. The membranous whorls in RCS retinas were also stained pink, confirming that the whorls were composed mostly of outer segment membranes.3 In the counterstained sections, the colloidal iron reaction product was a much more brilliant, deeper blue color than in the noncounterstained material. However, we chose to use noncounterstained sections for most of our analysis because superposition of the deep blue IPM and several thin, pink outer segments within a given section often resulted in a purple color that obscured the blue IPM staining patterns described above.

Peripheral retina. At P8 and P10, the density and distribution of reaction product were about equal in the posterior and peripheral retina of both RCS and control rats.

In control rats P12 and older, the intensity of the reaction product at the apical surface of the pigment epithelium was substantially greater in the far peripheral retina (Fig. 9) than in the posterior retina (Fig. 5). The distribution of reaction product in the peripheral retinas of RCS animals P12 and older (Fig. 10) was also different from that found in the posterior retinas (Fig. 6). In contrast to the reduced reaction product near the membranous whorls in the posterior retina, in the peripheral retina there was a continuous, dense band of reaction product at the apical surface of the pigment epithelial cells, despite the presence of some whorls adjacent to the pigment epithelium. In RCS peripheral retinas at P26 and P29, the apical band of reaction product still formed a continuous band, although it was somewhat reduced in intensity from that found at earlier ages.

The cause of the increased staining at the periphery is unclear. Since it occurred in the control retina, it presumably represented a normal regional difference in distribution of the IPM that was maintained in the mutant.
The increased peripheral staining was not due to the lightly pigmented melanosomes that are found in the peripheral pigment epithelium of pink-eyed \((p/p)\) rats,\(^4\) since the albino RCS-c rats with no melanin showed the same IPM staining distribution as the pink-eyed animals.

**Metachromatic staining with toluidine blue.** Several features distinguished the toluidine blue-stained sections from those reacted with colloidal iron. In addition to the obvious orthochromatic blue staining with toluidine blue, the outer segments in both genotypes and the whorls of outer segment debris in RCS rats stained blue to aqua in heavily stained sections (although they were virtually unstained in lightly stained sections), and the IPM stained metachromatically pink. In control retinas the pink band at the apical surface of the pigment epithelium was the most prominent metachromatically stained feature (Fig. 11). Unlike the appearance after the colloidal iron reaction, however, little pink metachromatic staining was seen in other parts of the outer segment zone unless the long axes of the rod outer segments were perfectly aligned in the plane of section. In such regions the IPM stained light pink along the entire length of the outer segment zone, but the staining was always more intense at the apical surface of the pigment epithelium. In sum, the overall staining of the IPM in normal retinas was less intense after toluidine blue staining than after the colloidal iron reaction.

Despite these general differences after the two staining procedures, the pink metachromatic staining at the apical pigment epithelial cell surface displayed almost the same developmental sequence as seen with the colloidal iron reaction in RCS and control retinas in both the posterior and peripheral retina. For example, retinas of the two genotypes were indistinguishable at P8 and P10, but by P12 in the posterior retina, the band of pink staining at the apical surface of the pigment epithelium in RCS rats was greatly reduced in intensity from that seen in control rats. At older ages, the band was virtually missing in RCS retinas (Fig. 12). In the peripheral RCS retinas at P26 and P29, the apical band was also missing, unlike the colloidal iron reaction in which the staining there was only reduced in intensity.

Metachromatic staining with toluidine blue demonstrated one difference between RCS and control retinas more clearly than did the colloidal iron reaction. In rats P12 and older, pink IPM staining was conspicuous in the basal outer segment region in virtually every RCS retina that we examined (Fig. 12), whereas little was ever present in the same region in control retinas (Fig. 11). The staining in the basal region of RCS retinas may also have been on some inner segment surfaces, but resolution of this issue was impossible in the wax-embedded material and will require further work using electron microscopy cytochemistry.

The visual impression of pink metachromatic staining was confirmed by microphotometric readings of absorbance. In the normal retinas (Figs. 15, A, and 16, A), the apical band of pink staining showed a high absorbance, whereas the remainder of the outer segment zone showed a low absorbance, except near the junction of the inner and outer segments. In the mutant retinas (Figs. 15, B and 16, B), the absorbance was lowest at the apical surface of the pigment epithelium and outer half of the outer segment debris zone and was highest in the basal outer segment region.

To check for a possible heterozygote (gene dosage) effect, we re-examined the retinas of \(190\; +/rdy\) rats from linkage-testing stocks\(^8\) that previously had been stained with warm, 0.125\% toluidine blue in sodium benzoate (pH 4.4).\(^9\) The staining pattern of the heterozygous \(+/rdy\) rats was the same as that of the homozygous wild-type RCS-\(rdy^+\) rats in every case.

**Alcian blue staining.** The pattern of Alcian blue staining was similar to that of the pink metachromatic staining of toluidine blue at each age, for both genotypes of rats and in both the posterior and peripheral retina. A selected control retina showing intense staining at the apical pigment epithelial cell surface is shown in Fig. 13. A dystrophic retina showing the virtual absence of Alcian blue
staining at the pigment epithelial cell surface but staining of the basal region of the outer segment zone is illustrated in Fig. 14. In normal retinas, this basal band of staining was either just perceptible or invisible. By contrast, it was always the most prominently stained feature in dystrophic retinas beginning about P14. The one feature of Alcian blue staining that differed somewhat from the metachromatic toluidine blue staining was a diminution rather than a complete loss of stain at the apical pigment epithelial cell surface in the peripheral retinas at P26 and P29 RCS rats.

**Hyaluronidase extraction.** Pretreatment of sections with hyaluronidase revealed no differences between mutant and normal retinas. The enzymatic digestion abolished all pink metachromatic staining of toluidine blue, only partially reduced the amount of colloidal iron reaction product, and caused no detectable loss of Alcian blue staining in the retinas of both genotypes. The partial reduction of colloidal iron reaction product is similar to that reported previously in normal rats.\(^21\) Furthermore, the differential staining of the IPM by the three staining procedures after hyaluronidase digestion underscores the somewhat different specificity of the three stains (see Discussion).

**Discussion**

**Source of abnormal distribution of IPM in RCS retinas.** Proteoglycans and glycoproteins of the IPM are thought to be produced normally by the photoreceptor inner segments, Müller cells, and pigment epithelial cells.\(^{15, 15, 21, 23-28}\) Transfer of these macromolecules into the extracellular space may involve sloughing of the cell coat and/or secretion by these cells.\(^{25-27}\) In light of our present histochemical findings, it is possible that an abnormality exists in RCS retinas in the rate of synthesis of the IPM by one or more of the cell types that produce it. For example, if the \(rdy\) gene abolished the IPM synthetic capability of the pigment epithelial cells, then a loss of the apical band of IPM staining might result. The IPM in the basal outer segment region might accumulate as a result of continued synthesis by the photoreceptor and Müller cells.

Another possibility is that the apparent accumulation of IPM in the basal outer segment zone of dystrophic retinas could be due to an abnormal rate of IPM turnover. Details of IPM turnover are sketchy, even in normal animals. Ocumpaugh and Young\(^{21}\) have calculated a quite rapid biologic half-life of about 2.5 days for sulfated components of the IPM of normal rats. Feeney\(^{26, 27}\) has sug-
Fig. 16. Microphotometric readings of absorbance (OD units) at 485 nm taken from a single, representative retinal section stained with toluidine blue from either a normal RCS-rdy⁺ (A) or dystrophic RCS (B) rat at P26. Each point represents the mean ± S.E.M. of 10 readings at a given position in the retina taken at approximately 40 μm intervals along the posterior retina. The positions in the retina where readings were taken correspond to those shown in Fig. 15. In the normal retina (A), position 1 is the pigment epithelial cell soma; 2 is the apical band of pink metachromatic staining; 3 to 5 are the outer segment zone; 6 is at the irregular basal border of the outer segments, so it consists of a mixture of inner and outer segments; and 7 is the inner segment zone. In the mutant retina (B), positions 1 and 2 are the same as in A; 3 to 8 are the outer segment debris zone; and 9 is the residual and thinned inner segment zone. Absolute absorbance of the different sections should not be compared directly, due to slight differences in section thickness, overall staining intensity, etc. However, relative comparisons show that in the normal retina (A), the highest absorbance is that of the apical band of pink-staining (position 2), and it is substantially greater than the absorbance of the pigment epithelium. The outer segment zone (positions 3 to 5) shows the lowest absorbance. In the dystrophic retina (B), by contrast, the position corresponding to the apical band in the normal retina (position 2) has the lowest absorbance and equals that of the outer half of the outer segment zone (positions 3 to 5). The absorbance is highest in the basal half of the outer segment zone (positions 6 to 8), where it is equal to or greater than the absorbance of the pigment epithelium.

Suggested that removal of some of the IPM may occur during phagocytosis of shed rod outer segment discs. The grossly reduced rate of outer segment disc phagocytosis in the RCS rat²⁹ might therefore be a contributing factor in the basal accumulation of IPM. A possible relevant factor is that an excessive amount of vitamin A may exist in the outer segment debris zone of RCS rats (reviewed in ref. 29). In other systems, excess vitamin A causes an increased rate of turnover of mucopolysaccharides.⁹ An understanding of the abnormal distribution of IPM in RCS rats clearly will require studies on synthesis and turnover of the IPM in the mutant animals.

Role of IPM abnormality in photoreceptor...
cell death and phagocytosis defect. Many workers have suggested functions for the IPM in the normal retina, including retinal adhesion to the pigment epithelium, optical refraction, cell (or outer segment) recognition for phagocytosis, and facilitation of diffusion of metabolites and vitamin A between photoreceptors and the pigment epithelium. These functions, except for optical refraction, indirectly implicate the IPM in the viability of the photoreceptor cell. The fact that the IPM distribution in the dystrophic rat retina is abnormal suggests that one or more of the functions of the IPM might be disturbed in the mutant. The most obvious possibility is that the abnormal IPM distribution causes abnormal metabolite diffusion which results in photoreceptor cell death. Indeed, proteoglycans such as those in the IPM have been found to influence the diffusion of ionic and possibly nonionic species. Such a cause of cell death would be more plausible than the membranous debris itself acting as a diffusional barrier, since the extracellular space between the debris membranes presumably should still be patent.

In addition to an abnormal distribution of the IPM in the dystrophic retina, there could also be qualitative changes in the IPM that contribute to photoreceptor cell death. The histochemical stains that we and others have used to demonstrate the IPM have only limited specificity and can only partially characterize the complex mixture of proteoglycans and glycoproteins that make up the IPM. The Alcian blue dye reacts mainly with uronic acid and sulfate groups; metachromasia of toluidine blue indicates only the presence of free electronegative surface charges of a certain minimum density; and colloidal iron is bound by carboxyl and sulfate groups (and by neuraminic acid residues on the cell surface in some systems). These stains clearly show the presence of the acidic polysaccharides that, in part, make up the IPM, but the stains are unable to distinguish certain changes in the composition of the IPM. For example, solely by histochemical means we presumably would fail to recognize the complete absence of one or even two of the different species of chondroitin sulfate present in the IPM. The possibility that differences in IPM mucosubstances might exist between RCS and control rats is plausible when one considers the recent findings of McLaughlin and Wood, who used various lectins to demonstrate potential differences in the carbohydrates of the outer segment debris membranes and pigment epithelial cell processes of RCS and RCS-rdy + rats. Even subtle changes might be important in the mechanism of retinal dystrophy. For example, elevation of hyaluronic acid decreases the rate of phagocytosis in some cell types. Such a change in the IPM might help explain the enigmatic phagocytosis defect in RCS rats.

The temporal sequence of IPM changes and photoreceptor cell death is compatible with a mechanism of cell death involving disruption of normal IPM function. The first abnormality in the IPM staining pattern in dystrophic retinas is seen at P12 in the posterior retina, a full 6 to 8 days before photoreceptor cell death begins. Furthermore, the normal band of IPM staining at the pigment epithelial cell surface almost completely disappears by about P20, the time when pyknotic photoreceptor nuclei are first seen.

The temporal sequence of IPM changes may also have an important bearing on phagocytosis of outer segment membranes by the pigment epithelium. The intense band of IPM staining at the rod outer segment–pigment epithelial cell interface appears in normal rats on about day 12, precisely the time when disc shedding and phagocytosis begin to occur in the developing rat retina. A similar temporal correlation occurs in the developing mouse retina, where the apical band of IPM staining is first seen on P9 and P10 and disc shedding begins substantially on about P11. If the IPM in this zone is necessary for phagocytosis, then the failure of the intense band of IPM to form completely in the RCS retina could explain the failure of the RCS pigment epithelial cells to phagocytize an appreciable number of outer segment membranes. Moreover, the IPM demonstrated as small foci of apical staining (Fig. 6).
might account for the small amount of phagocytosis by the mutant pigment epithelial cells. 

Comparison of IPM alterations in retinal dystrophy to other retinal disorders. As far as we are aware, retinal dystrophy in the rat is the first form of inherited (or any other type of) retinal degeneration that shows an abnormality in the IPM preceding photoreceptor cell degeneration. For example, in the retinal degeneration (rd) mouse, the prominently staining IPM adjacent to the apical surface of the pigment epithelium remains unaltered even after photoreceptor degeneration is well underway.  

It is well known that most forms of the inherited mucopolysaccharidoses in man are accompanied by a pigmentedary retinal degeneration. However, little is known about the histochemical localization of the IPM in these diseases, since most histochemical work has centered on ocular sites such as the cornea that do show conspicuous alterations in mucous substance localization. Furthermore, specimens with very early stages of the disease, when it may be necessary to observe critical changes in the IPM, generally are unavailable for study. Nevertheless, the fact that retinal degenerations occur in cases of abnormal systemic mucopolysaccharide metabolism in man points out the need to determine whether histochemical changes in the IPM occur in early stages of these and other forms of inherited retinal degeneration in man and laboratory animals.

We thank Bonnie A. P. Lord and Gregg M. Corrin for technical and photographic assistance, Nancy Lawson for maintenance of the animal colony, and Carson Optical Co. for the use of the Zeiss microscope photometer.

REFERENCES
8. LaVail MM: Assignment of retinal dystrophy (rd) to linkage group IV of the rat. J Hered (in press).
24. Berman ER: The biosynthesis of mucopolysaccharides and glycoproteins in pigment epithelial