Localization of lysosomal enzymes in retinal pigment epithelium of rats with inherited retinal dystrophy

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Four acid hydrolase activities are demonstrable by light microscopy in pigment epithelial cell lysosomes of rats (Royal College of Surgeons-RCS) with inherited retinal dystrophy and in control (Fischer) rats. The enzymes include acid phosphatase, aryl sulfatase, N-acetyl-β-glucosaminidase, and esterase activities. No marked differences are observed in distribution or staining intensity of lysosomes in the two strains of rat. Acid hydrolase activities are not localized in sites other than lysosomes. Acid phosphatase and aryl sulfatase activities are also demonstrable by electron microscopy. In both strains, acid phosphatase reaction product is localized to various forms of lysosomes in pigment epithelial cells. A diffuse precipitate, considered to be nonspecific in origin, is seen in the cytoplasm, apical processes, outer segments (control), and outer segment debris (RCS). The precipitate is probably due to adsorption of lead from the incubation medium or of lead phosphate that diffuses from heavy accumulations in nearby lysosomes. Aryl sulfatase reaction product, in contrast to acid phosphatase, is localized to far fewer lysosomes and there is virtually no nonspecific precipitate. The findings indicate that lysosomes of RCS pigment epithelial cells possess several cytochemically demonstrable acid hydrolase activities. There is no evidence for the localization of acid phosphatase (or aryl sulfatase) activities in sites other than lysosomes.

Key words: inherited retinal dystrophy, retinal pigment epithelium, lysosomes, enzyme activity, acid hydrolases, Royal College of Surgeons rats

The Royal College of Surgeons (RCS) strain of rat is characterized by progressive degeneration of photoreceptor cells. The disorder, which is evident by the third postnatal week, leads to disintegration of the visual cell layer by 2 to 3 months of age. Although the nature of the defect is not known it is thought to reside in the pigment epithelial cell. In normal animals, the pigment epithelial cell phagocytoses groups of rod outer segment disks that are discarded by the photoreceptor cells. However, in pigment epithelial cells of RCS rats phagocytosis is markedly reduced, and phagosomes containing outer segment material are rarely seen. As a result, outer segment debris accumulates in massive amounts in the interphotoreceptor space.

Several investigators have proposed that the degeneration of photoreceptor cells in dystrophic rats may be due to an alteration in the lysosomes of the pigment epithelial cells. According to Reading, the concentration of retinol (vitamin A alcohol), an agent known...
to cause lysosomal instability and release of lytic enzymes in other tissues,12 is higher in the pigment epithelium of dystrophic rats than in normal animals. He suggested that the lysosomes of pigment epithelial cells, destabilized by excessive, local concentrations of vitamin A, may release acid hydrolases into the interphotoreceptor space, where they diffuse into the adjoining photoreceptor cell layer and cause degeneration of the visual cells. Burden et al.13 reported that the concentrations of total and “free” acid protease (catheptic) activities were higher in the retina of dystrophic rats than in controls. They considered that this increase in “free” proteolytic activity could be due, at least in part, to the breakdown of lysosomes in the pigment epithelium. These investigators, as well as Yates et al.,14 also found evidence for decreased lysosomal “stability,” using a histochemical procedure to estimate the “fragility” of the lysosomal membrane.

Enzyme release from unstable lysosomes was also inferred by Ansell and Marshall15 from observations on the electron microscopic localization of acid phosphatase reaction product. They reported that enzyme reaction product was demonstrable in the lysosomes of normal pigment epithelium but not in those of dystrophic rats. However, relatively light deposits were also observed “along the apical villi and within the debris of the spent disks,” and these were interpreted as “positive” reactions for acid phosphatase activity. On the other hand, acid phosphatase reaction product has been demonstrated by electron microscopy in lysosomes of RCS pigment epithelial cells.16

This communication is an attempt to clarify these conflicting observations. We show that acid phosphatase, together with three other hydrolase activities, are demonstrable by light microscopy in lysosomes of RCS pigment epithelial cells; two of these activities are localized by electron microscopy. Also included is evidence that the precipitates seen in sites other than lysosomes are nonspecific and do not result directly from enzyme activity.

Materials and methods

A total of 10 RCS17 (tan-hooded, pink-eyed; F41 generation) and 34 control (Fischer) rats, 17 to 70 days of age and young adults, were used in this study. The two strains were matched in age as closely as possible. The rats were maintained in a 12 hr light–12 hr dark environment at a room illumination of about 10 to 15 footcandles from overhead fluorescent lamps. Cage illumination varied with the position of the cage, but did not exceed 15 footcandles.

Tissues were prepared for study as follows. After decapitation, the eyes were enucleated, and the globes were cut in half while immersed in either (1) cold 4% formaldehyde–1% calcium chloride18 (overnight) or (2) cold 1% to 3% glutaraldehyde–0.2M cacodylate buffer,19 pH 7.4 (1 to 4 hr). After being washed in cold 0.2M cacodylate buffer + 5% sucrose, the tissues were processed for light and electron microscopy as follows.

Light microscopy. For demonstration of enzyme activities, frozen sections 10 to 15 μ thick were cut on a Sorbortius-freezing microtome. In order to obtain as large an area of tissue as possible, tangential sections were generally prepared from the posterior portion of the pigment epithelium. The sections were incubated, free-floating, in the following media: acid phosphatase (Gomori medium, cytidine-5’ monophosphate as substrate,20 and Barka and Anderson22 medium, naphthol AS-TR phosphate as substrate), aryl sulfatase (Goldfischer medium, p-nitrocatechol sulfate as substrate), IV-acetyl-/3-glucosaminidase (Hayashi24 medium, naphthol AS-BI-IV-acetyl-/3-D-glucosaminide as substrate) and nonspecific esterase (Gomori medium, a-naphthol acetate as substrate). Media in which naphthol derivatives were used as substrates also contained freshly hexazotized pararosanilin as the coupler.25 Sections incubated for acid phosphatase (Gomori medium) or aryl sulfatase activities were rinsed in water and briefly exposed to dilute ammonium sulfide. All sections were mounted on glass slides in glycerin jelly. Sections that were incubated in substrate-free media served as controls.

Electron microscopy. For the demonstration of acid phosphatase and aryl sulfatase activities, nonfrozen sections, 40 μm thick, were cut with a Sorvall TC-2 tissue sectioner (DuPont Instruments, Sorvall Operations, Newton, Conn.) as suggested by Smith and Farquhar.26 Sections incubated for acid phosphatase (Gomori medium) or aryl sulfatase activities were rinsed in water and briefly exposed to dilute ammonium sulfide. All sections were mounted on glass slides in glycerin jelly. Sections that were incubated in substrate-free media served as controls.
Figs. 1 to 4. See facing page for legends.
for aryl sulfatase activity as described above. After incubation, the sections were washed in 0.2M cacodylate buffer, pH 7.4, containing 5% sucrose and fixed in 1% osmium tetroxide for 1 hr. Some sections were stained en bloc with 0.5% uranyl acetate in 0.05M acetate buffer, pH 5, for 1 hr at room temperature. The sections were then either dehydrated in alcohols and propylene oxide and embedded in Epon or were dehydrated in alcohols only and embedded in Spurr’s27 mixture. Thin sections, cut on a diamond knife, were examined either unstained or stained with lead citrate, in a Philips 301 electron microscope equipped with a 70 μm objective aperture.

**Results**

All four acid hydrolase activities were demonstrable by light microscopy in the lysosomes of RCS rats and control pigment epithelial cells (Figs. 1a to 8). Lysosomes were not visualized when sections were incubated in media without substrate. No significant differences were observed in distribution or staining intensity of lysosomes in the two strains of rat. Acid phosphatase activity was demonstrable with both the Gomori and pararosanilin (not illustrated here) methods. There was some indication that acid phosphates–rich lysosomes are more numerous in RCS pigment epithelial cells than in controls (Figs. 1a and 2b), but this point was not studied further.

In control rats, the aryl sulfatase reaction visualized lysosomes that were smaller and more uniform in size than those seen in acid phosphatase preparations. Apparently, many of the larger lysosomes did not stain for aryl sulfatase activity. This is consistent with the electron microscope observations, discussed below, which showed that the larger lysosomes have acid phosphatase but not aryl sulfatase reaction product. In RCS rats this difference in lysosome size was not evident, since the large lysosomes that contain outer segment material were not present. In both strains, N-acetyl-β-glucosaminidase and esterase activities were inhibited more by glutaraldehyde than by formol-calcium fixation. In the retinas of RCS rats, many macrophages were seen in the interphotoreceptor space; acid hydrolase activities were demonstrable in the large lysosomes of these cells (Figs. 2a and 8).

The localization of acid phosphatase and aryl sulfatase activities was confirmed by electron microscopy. Acid phosphatase reaction product was localized to lysosomes in pigment epithelial cells of both RCS rats (Figs. 11 to 13) and controls (Figs. 9 and 10). In both strains, the lysosomes varied in their morphology and distribution. Some lysosomes were found within the slender apical processes that projected into the interphotoreceptor space (Figs. 9 and 10). In pigment epithelial cells of control rats, phagosomes containing rod outer segment material fuse with lysosomes to form secondary lysosomes (residual bodies) in which the material is digested. Acid phosphatase reaction product was demonstrable in these residual bodies (Figs. 9 and 10). Since phagocytosis of rod outer segments was markedly reduced in pigment epithelial cells of RCS rats, residual

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**Figs. 1 to 4. Light micrographs of 10 μ frozen sections from pigmented epithelium of control (Fisher) and RCS rats.**

**Figs. 1a and 1b. Control rat, 52 days old. Incubated in acid phosphatase medium for 60 min. Lysosomes (arrows) show acid phosphatase activity. IP, Interphotoreceptor space; C, choroid. (1a, ×600; 1b, ×1,200.)**

**Figs. 2a and 2b. RCS rat, 70 days old. Incubated in acid phosphatase medium for 60 min. Numerous lysosomes (arrows) show acid phosphatase activity. Those with rim of activity are seen at arrow (lower left). M, Macrophages with acid phosphatase–positive lysosomes; IP, interphotoreceptor space; C, choroid. (2a, ×650; 2b, ×1,300.)**

**Fig. 3. Control rat, 189 days old. (A positive reaction was also seen in a 52-day-old rat.) Incubated in aryl sulfatase medium for 3 hr. Many lysosomes (left arrow) show aryl sulfatase activity. Right arrow indicates one with rim of activity. OS, Outer segments. (×1,400.)**

**Fig. 4. RCS rat, 60 days old. Incubated in aryl sulfatase medium for 2.5 hr. Activity is seen in numerous lysosomes (arrow). (×1,300.)**
Figs. 5 to 8. See facing page for legends.
bodies containing outer segment material were rarely encountered. When seen, however, these bodies also showed acid phosphatase reaction product (Fig. 12).

Pigment epithelial cells of RCS rats (Figs. 12 to 14) and controls (Fig. 10) frequently showed a light, randomly distributed precipitate in the cytoplasm and on the apical processes; it was also present on the rod outer segments in control rats (Fig. 10) and on the outer segment debris in RCS rats (Fig. 13).

As was evident in frozen sections, the lysosomes in macrophages in the interphotoreceptor space of RCS rat retinas showed acid phosphatase activity (Fig. 14).

 Aryl sulfatase reaction product was also localized in some, but not all, lysosomes of control (Fig. 15) and RCS (Fig. 16) pigment epithelial cells. Many bodies, including residual bodies containing degraded rod outer segment material, did not show aryl sulfatase reaction product (Fig. 15). Aryl sulfatase reactions were virtually free of the randomly distributed precipitates seen in acid phosphatase preparations.

**Discussion**

The results presented in this communication indicate that lysosomes of RCS pigment epithelial cells (and those of controls) display acid phosphatase as well as three other acid hydrolase activities. The findings are consistent with previous light microscopic studies on the localization of acid phosphatase and naphthylamidase activities in dystrophic pigment epithelial cells. In these studies, however, it was also reported that, in unfixed frozen sections, the lysosomes of dystrophic eyes stained more rapidly for the two enzyme activities than did those of controls. This difference was presumably due to changes in the permeability of the lysosome membrane which resulted in an increase in the accessibility of the enzymes to their respective substrates. On this basis, an increase in staining intensity of lysosomes over that of the controls was interpreted as evidence of lysosome "instability." In the present report, no significant differences in staining intensities of the several enzyme activities were noted between the two strains of rat. However, in order to obtain better cytologic preservation and to minimize diffusion artifacts, we used fixed, rather than unfixed, frozen sections. The relatively subtle changes in staining intensity described above were probably not present in our material, since the permeability of the lysosome membrane is undoubtedly altered by fixation.

Our findings on the ultrastructural localization of acid phosphatase reaction product in lysosomes of RCS pigment epithelial cells confirm the preliminary observations of Bok. The absence of a positive reaction in lysosomes of dystrophic eyes reported by Ansell and Marshall may be due to differences in methodology. These investigators did not assess acid phosphatase localizations by light microscopy, a procedure that helps to

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**Figs. 5 to 8.** Light micrographs of 10 μm frozen sections from pigmented epithelium of control (Fisher) and RCS rats.

**Figs. 5a and 5b.** Control rat, 52 days old. Incubated for 40 min in N-acetyl-β-glucosaminidase medium. Numerous lysosomes (arrows) in pigment epithelial cells (PE) show enzyme activity (red, in original preparations). OS, Outer segments; C, choroid; N, nucleus. (5a, X600; 5b, X1,000.)

**Figs. 6a and 6b.** RCS rat, 70 days old. Incubated for 40 min in N-acetyl-β-glucosaminidase medium. Tangential section through pigmented epithelium (PE). Lysosomes (arrows) in pigmented epithelial cells show activity. Lysosomes of macrophages (MA) in choroid are also stained. IP, Interphotoreceptor space. (6a, X350; 6b, X1,100.)

**Fig. 7.** Control rat, adult. Incubated in esterase medium for 10 min. Reactive lysosomes (arrows) (reddish brown in original preparations) are seen in pigment epithelial cells. OS, Outer segments. (X1,100.)

**Fig. 8.** RCS rat, 70 days old. Incubated in esterase medium for 10 min. Arrows point to reactive lysosomes in pigment epithelial cells. Cytoplasm of cells is dark because of diffuse staining. Note macrophages (MA) in the interphotoreceptor space (IP). C, Choroid. (X1,000.)
Fig. 9. Control rat, 52 days old. Incubated in acid phosphatase medium for 40 min. Acid phosphatase reaction product is localized in numerous lysosomes (L) of pigmented epithelial cells. Some lysosomes are located in apical processes (L, above). Arrows indicate two large lysosomes that contain outer segment (OS) material. B, Basal infoldings; BM, Bruch's membrane. (×11,000.)

Fig. 10. Control rat, 52 days old. Incubated in acid phosphatase medium for 40 min. Enlargement of left side of Fig. 9. The large lysosome (arrow) with degraded outer segment material shows rim of acid phosphatase reaction product. Several smaller lysosomes (L), two of which are located in apical processes (AP) (L, upper right), also show reaction product. Random precipitate is scattered over apical processes (AP) and outer segments (OS). (×43,000.)
In agreement with Ansell and Marshall, we also observed electron-dense precipitates on the apical processes and outer segment debris in RCS rats. However, such deposits do not accumulate in amounts sufficient to be regarded as due to enzyme activity, nor are they evident by light microscopy. Furthermore, the precipitates occur not only in RCS
Figs. 13 to 14. Electron micrographs of tissue that had been incubated for acid phosphatase or aryl sulfatase activities.

Fig. 13. RCS rat, 14 days old. Incubated in acid phosphatase medium for 20 min. Acid phosphatase reaction product is localized to several lysosomes (L). Randomly scattered precipitate is seen in cytoplasm. Somewhat heavier amounts are present on apical processes (A). Precipitate is also seen on outer segment debris (D). (×15,000.)

Fig. 14. RCS rat, 60 days old. Incubated in acid phosphatase medium for 45 min. Acid phosphatase reaction product is localized in lysosomes (L) of macrophages (M) in interphotoreceptor space. Note precipitate on outer segment debris (D). (×11,000.)

In both control and RCS rats, electron microscopy revealed differences in the distribution of acid phosphatase and aryl sulfatase activities. Acid phosphatase reaction product was localized to a variety of bodies, including those containing outer segment material. In contrast, aryl sulfatase reaction product was absent from these latter bodies and was found in a smaller, more homogeneous population of bodies whose precise identification requires further study. Whether the more limited distribution of aryl sulfatase activity reflects differences in levels of the enzyme or is due to technical problems associated with demonstrating this activity is not known.

Burden et al. reported that, beginning at 4 weeks of age, the total and “free” acid protease activity of RCS rat retinas was higher than that of controls. They attributed the increase to the infiltration of enzymes that had been released from lysosomes in the pigment epithelium. In the present study, numerous macrophages containing large, acid hydrolase-positive lysosomes were seen in the interphotoreceptor space of 10-week-old RCS rat retinas. In other experiments, we have noted a similar infiltration of macrophages in the interphotoreceptor space as well as in the outer nuclear layer of a 6-week-old pigmented RCS rat (rdy-p). These findings may have a bearing on the biochemical data reported by Burden et al. It is possible that the increased levels of acid hydrolases demon-
Figs. 15 and 16. Electron micrographs of tissue that had been incubated for acid phosphatase or aryl sulfatase activities.

Fig. 15. Control rat, adult. Incubated in aryl sulfatase medium for 1 hr (25°C). Narrow rim of aryl sulfatase reaction product encircles lysosomes (L). Note absence of reaction product in residual body (arrow). OS, Outer segments; A, apical processes; M, mitochondria; G, Golgi apparatus; B, basal infoldings; BM, Bruch’s membrane. (x28,000.)

Fig. 16. RCS rat, adult. Incubated in aryl sulfatase medium for 4.5 hr. Lysosomes (L) are encircled, completely or partially, by rim of reaction product. M, mitochondrion; D, outer segment debris in interphotoreceptor space. (x52,000.)
strated by these investigators were due to the presence of acid hydrolase-rich macrophages in the outer nuclear layer and/or the debris in the discarded outer segment disks if this material, which tends to adhere to both neural retina and pigment epithelium, is not completely removed during dissection.

We are grateful to Dr. Matthew M. LaVail for supplying us with breeding animals of the RCS strain. We thank Georgean Grubb for typing the manuscript.

REFERENCES