An electron microscopic study of macrophages in rats with inherited retinal dystrophy

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In retinas of rats (RCS) with inherited retinal dystrophy, cells interpreted as macrophages infiltrate the outer nuclear layer and subsequently appear in the interphotoreceptor space, where they accumulate during the course of the disease. The morphology and distribution of these cells and their relations to the pigment epithelial cells were investigated. Macrophages, regardless of their location, possessed morphological features that distinguished them from the pigment epithelial cells. Premelanosomes and melanosomes, typical of pigment epithelial cells, were never observed in macrophages. There was no evidence to indicate that, during the period studied, the pigment epithelial cells had become dedifferentiated or had migrated from Bruch's membrane. Macrophages, like pigment epithelial cells, phagocytized little or no outer segment material. The findings indicate that, at least during the interval studied, the cells that infiltrate the retina and interphotoreceptor space are macrophages rather than pigment epithelial cells.

Keywords: macrophages, inherited retinal dystrophy, lysosomes, differentiation, acid hydrolases

Rats of the Royal College of Surgeons (RCS) strain are characterized by an autosomal recessive retinal dystrophy that results in progressive degeneration of the photoreceptor cells. The site of the defect is not known but appears to involve primarily the pigment epithelial cells. In the normal rat, an important function of these cells is to phagocytize portions of the outer segments as they are shed from the photoreceptor cells. This process is virtually absent from pigment epithelial cells of dystrophic rats, and consequently the discarded outer segment material accumulates in massive amounts in the interphotoreceptor space.

Further indication that the primary site of mutant gene action is in the pigment epithelial cells is provided by two recent studies using different approaches. Mullen and LaVail, using chimeras derived from normal, pigmented rats and pink-eyed RCS rats, showed that in the resulting mosaic eyes, degenerated photoreceptor cells appeared only opposite areas of mutant pigment epithelium. This suggests that the site of the mutant gene is in the pigment epithelial cell rather than in the photoreceptor cell. Using a culture technique, Edwards and Szamier demonstrated that pigment epithelial cells from the RCS rat rarely phagocytize outer segment material from either RCS or normal rats, although they readily ingest latex spheres.

Another interesting aspect of the pigment epithelial cells in dystrophic rats, one that has received relatively little attention, is...
Fig. 1. Light micrographs of frozen sections that were incubated for enzyme activities. A, Normal rat retina incubated 35 min in esterase medium. Numerous small, esterase-positive granules (lysosomes) are present in pigment epithelial cells (PE). Some stained granules, possibly lysosomes, are seen in outer nuclear layer (ONL) and choriocapillaria (C); none is in interphotoreceptor space (S). (×300.) B, Retina of a 14-day-old RCS-p+ rat, incubated 20 min in acid phosphatase medium. Reactive lysosomes are visualized in pigment epithelial cells (PE), but there are none in interphotoreceptor space (S). (×300.) C, Choroid. (×300.) C, Retina of a 42-day-old RCS-p+ rat, incubated 40 min in acid phosphatase medium. Acid phosphatase—continued
their apparent migration into the retina. Bourne et al.,\(^1\) in their original description of this dystrophy (which included pigmented rats), observed that by about the twelfth week, pigment epithelial cells had migrated toward the retina along fiber tracks that formed between the pigment epithelium and retina. Dowling and Sidman\(^2\) studied this process in greater detail and observed that by the fortieth day, partially dedifferentiated pigment epithelial cells migrated through the outer segment debris toward the retina. In late stages of the disease some of these cells also appear to return to their original location on Bruch’s membrane. Bok and Hall\(^1\) also noted that at 58 days of age cells of "undetermined origin" were present in the "area of compacted debris near the outer limiting membrane."

In a recent study of the RCS retinal dystrophy,\(^12\) we observed that migratory cells in the retina and interphotoreceptor space contained relatively large lysosomes that were strongly stained in frozen sections incubated for the cytochemical demonstration of acid hydrolase activities. On this basis, the cells were considered to be macrophages. In the present communication, the ultrastructure of these cells and their relations to the pigment epithelium were studied in greater detail. As an aid in tracing their origin, we took advantage of a recently developed strain of pigmented RCS rat\(^13\) in which the premelanosomes and melanosomes served as a "marker" for determining the location of the pigment epithelial cells and their state of differentiation.

Materials and methods

Pigmented, dystrophic rats (RCS-\(p^+\)) were sacrificed at 14, 17, 32, 42, 48, 50, 52, 56, 60, 70, and 101 days of age. Long Evans Blue rats were used as controls. Pink-eyed (RCS) dystrophic rats and normal Fischer rats were also used in a few experiments.

Tissues were prepared as follows. After decapitation, a portion of the pigment epithelium was removed and fixed in (1) cold, 4% formaldehyde-1% calcium chloride\(^14\) overnight, (2) 2% or 4% glutaraldehyde in 0.1M cacodylate\(^15\) or Millonig's phosphate buffer\(^16\) (pH 7.4) for 1 to 2 hr at 4° C, or (3) Karnovsky's fixative\(^17\) diluted 1:1 with 0.1M cacodylate buffer for 1 to 2 hr at 4° C. After being washed in cold buffer containing 5% sucrose, the tissues were processed for light and electron microscopy as described below.

For the light microscopic demonstration of enzyme activities, frozen sections, 10-15 \(\mu\)m thick, were cut on a Sartorius freezing microtome. To localize acid phosphatase activity, free-floating sections were incubated in Gomori medium\(^18\) with cytidine-5'-monophosphate\(^19\) as substrate. After incubation the sections were rinsed in water, briefly exposed to dilute ammonium sulfide in order to convert sites of lead phosphate deposition into lead sulfide, and mounted in glycerin jelly on glass slides. For the localization of nonspecific esterase activity, free-floating sections were incubated in Gomori medium\(^18\) with \(\alpha\)-naphthol acetate as substrate and hexazotized pararosanilin\(^20\) as the coupler. After rinsing, the sections were mounted in glycerin jelly on glass slides. Sections incubated in substrate-free media served as controls for the two enzyme reactions.

For routine electron microscopy, glutaraldehyde or Karnovsky-fixed tissues were rinsed in buffer, fixed in 1% \(\mathrm{OsO}_4\) for 1 hr, dehydrated in alcohols, stained en bloc with 2% uranyl acetate in 100% ethanol for 30 min, and embedded in Spurr's mixture.\(^21\) For the electron microscopic localization of acid phosphatase and aryl sulfatase activities, nonfrozen sections, 40 \(\mu\)m thick, were prepared with a Sorvall TC-2 tissue sectioner (Dupont Instruments, Sorvall Operations, Newton, Conn.) as suggested by Smith and Far-
Fig. 2. For legend see facing page.
Sections were incubated for acid phosphatase activity in Gomori medium as described above or for aryl sulfatase activity in the medium of Goldfischer with \(p\)-nitrocatechol sulfate as substrate. Sections from the latter incubation were visualized in 2% ammonium sulfide (10 min). Afterward, sections were rinsed in cacodylate buffer, pH 7.4, containing 5% sucrose, fixed in 1% \(\text{OsO}_4\) for 1 hr, and dehydrated and embedded as described above. Semithin (1 \(\mu\)m) sections were stained with toluidine blue and examined by light microscopy. Thin sections, prepared on a diamond knife, were stained with lead citrate and viewed with a Phillips 301 electron microscope.

**Results**

The main purpose of this study was to determine whether the cells that migrated into the outer nuclear layer and interphotoreceptor space were macrophages or altered pigment epithelial cells. Although observations were made on animals of various ages, representing different stages of the degeneration, no attempt was made to arrange the findings in strict chronological order. This appeared to be unnecessary, since the features that served to distinguish the migrating cells from pigment epithelial cells were observed throughout the period studied.

The results of the light microscopic studies are as follows. Control sections incubated in substrate-free media showed no enzyme activity. In the retina of the normal rat (Fig. 1, A), nonspecific esterase activity was localized in a few small granules in the outer nuclear layer, in small punctate lysosomes of the pigment epithelial cells, and in various cells of the choroid. No enzyme activity was present in the interphotoreceptor space. Acid phosphatase preparations of normal rat retina (not illustrated) showed a similar type of enzyme distribution. In 14-day-old RCS rat retinas, the distribution of acid phosphatase (Fig. 1, B) (and esterase) activities was similar to that of controls. At about 21 days, a few acid-phosphatase–positive cells could be detected in the outer nuclear layer and interphotoreceptor space. By about 40 days of age, greater numbers of these cells were evident (Fig. 1, C). The cells were irregular and often stellate in form and contained pleomorphic, acid phosphatase–rich lysosomes, some of which exceeded in size the largest ones seen in the pigment epithelial cells (Fig. 1, D). As the degenerative changes progressed, large numbers of these cells accumulated in the interphotoreceptor space (Fig. 1, E).

Apart from the exceptions noted below, the ultrastructure of the migratory cells remained essentially the same regardless of their location or the degree of photoreceptor degeneration. Several morphological features served to distinguish these cells from the pigment epithelial cells. In addition to a relatively high nuclear/cytoplasm ratio, the nuclei contained prominent clumps of marginated chromatin (Figs. 2, A, 3, and 4, A and B) which stained strongly in semithin, plastic-embedded sections treated with toluidine blue. The cells contained rough endoplasmic reticulum and polysomes (Fig. 2, B and C) but little smooth endoplasmic reticulum. Also present were polymorphic, dense bodies (Fig. 2, A) that tended to be larger and of more irregular shape in later stages of the disorder (Figs. 4 and 7). Reaction products of acid phosphatase (Fig. 4, B) and aryl sulfatase (Fig. 5, A and C) activities were demonstrable in these organelles, suggesting that they were lysosomes; presumably, they correspond to the acid hydrolase–positive lysosomes demonstrated by light microscopy (Fig. 1).

**Fig. 2.** A, Electron micrograph of outer nuclear layer (ONL) from retina of 49-day-old RCS-\(p^+\) rat. Two macrophages (M) have nuclei (N) with marginated chromatin, cytoplasmic dense bodies (DB), and cytoplasmic extensions (arrows), one of which partially envelops a pyknotic nucleus (PN). IS, Degenerating inner segments; D, outer segment debris. (\(\times 5,000\).) B, Enlargement of area marked by arrows in A. Cytoplasmic extensions (arrows) of macrophage (M) are evident. (\(\times 13,000\).) C, Enlargement of pyknotic nucleus (PN) from A. Macrophage cytoplasm (arrows) partially envelops a pyknotic nucleus. (\(\times 13,000\).)
Fig. 3. Electron micrograph of retina from 52-day-old RCS-η⁺ rat. Three macrophages (M) are seen amidst debris (D) in the interphotoreceptor space. Note nuclei with marginated chromatin (arrows) and cytoplasmic dense bodies. Pigment epithelial cells (PE) appear to be well-differentiated and contain numerous melanosomes (ME). C, Choriocapillaris. (×3000.)
Fig. 4. A, Electron micrograph of interphotoreceptor space from retina of 101-day-old RCS-+ rat. A macrophage is seen containing polymorphic dense bodies (DB), Golgi apparatus (GA), and outer segment material (P) that appears to have been phagocytosed. Note outer segment debris (D) that is closely applied to plasma membrane of macrophage. (×14,000.) B, Electron micrograph from outer nuclear layer retina of 49-day-old RCS-+ rat, incubated 20 min for acid phosphatase activity. Enzyme reaction product is localized to the dense bodies (L). D, Outer segment material. (×12,000.)
Fig. 5. For legend see facing page.
or melanosomes were never observed in these cells, whether in the outer nuclear layer or the interphotoreceptor space.

In the outer nuclear layer, migratory cells displayed conspicuous cytoplasmic extensions that sometimes encircled pyknotic nuclei or other portions of degenerated photoreceptor cells (Fig. 2). In the interphotoreceptor space, the cells also showed cytoplasmic processes that sometimes encircled portions of outer segment debris. Despite these images, phagosomes containing outer segment debris were rarely seen in these cells. Furthermore, the cells in the interphotoreceptor space were enmeshed in massive amounts of outer segment material (Figs. 3, 4, and 5, A and C), and this material frequently appeared to protrude into the cytoplasm. Thus even the few images of apparent phagocytosis (Fig. 4, A) may actually have represented sections through “pockets” of extracellular material.

The pigment epithelial cells showed no signs of dedifferentiation and retained many of the morphological and topological features characteristic of normal pigment epithelium. The morphology, number, and distribution of the major cytoplasmic organelles appeared to be unchanged. Premelanosomes, melanosomes, and intermediate forms were evident throughout the period studied (Figs. 3, 4, 6, A and B, and 7). Numerous slender processes (Fig. 5, A), some containing melanosomes (Figs. 6, A and 7), could be traced from the apical surface of the pigment epithelial cells for a distance of about one-third the width of the interphotoreceptor space, where they became indistinguishable from the processes of the migratory cells. Occasionally an apical process appeared to encircle outer segment debris (Fig. 5, B) as though about to ingest it, but phagosomes containing this material were not observed within the pigment epithelial cells.

At 101 days, the pigment epithelial cells, although still well differentiated, showed certain changes. Sometimes, the main portion of the cell, including the nucleus, became rounded and protruded into the interphotoreceptor space, whereas in other regions the cytoplasm appeared to be attenuated (Fig. 6, B). The number of dense bodies (lysosomes) appeared to have increased (Fig. 7), and there was more rough endoplasmic reticulum and much less smooth endoplasmic reticulum than in normal pigment epithelial cells. Premelanosomes, melanosomes and intermediate forms were still abundant (Fig. 7), but their normal, apical distribution (Fig. 6, C) was less apparent (Fig. 6, B). The basal infoldings were somewhat more irregular at this stage (Fig. 6, B), but there was no indication that the cells were separating from Bruch’s membrane, and no detached cells were seen in the interphotoreceptor space.

Discussion

The present findings indicate that the cells which infiltrate the outer nuclear layer and interphotoreceptor space are macrophages rather than pigment epithelial cells. Dowling and Sidman observed that on about the fortieth day, coincident with the total loss of the inner segments and reduction of the photoreceptor nuclei to two or three layers, cells appeared throughout the outer segment debris. On the basis of “nuclear pattern” and the presence of “inclusion bodies,” these
Fig. 6. For legend see facing page.
cells were interpreted as "partially dedifferentiated" pigment epithelial cells that had separated from Bruch's membrane. We observed, however, that the morphology of the nuclei and of the inclusion (dense) bodies differed in the two populations of cells. Many of these dense bodies appear to contain the remnants of undigested materials and show cytochemically demonstrable acid hydrolase activities. Thus they correspond to secondary lysosomes \(^{25}\) such as those found in many other types of cells.\(^ {19}\) In pigment epithelial cells of normal animals, many of these dense bodies contain the residues of phagocytized outer segment material. In the RCS rat, however, the dense bodies presumably do not originate in this manner, since the pigment epithelial cells rarely phagocytize outer segment material.

As a further means of identifying pigment epithelial cells in the RCS rat, we took advantage of a recently developed strain of pigmented rat \(^ {13}\) rather than the pink-eyed strain studied previously by Dowling and Sidman.\(^ {2}\) The migratory cells, regardless of their location in the retina, were never seen to contain premelanosomes or melanosomes. It is possible, however, that these organelles might have disappeared if the pigment epithelial cells had proliferated and become dedifferentiated prior to their separation from Bruch’s membrane. Indeed, Dowling and Sidman\(^ {2}\) considered the pigment epithelial cells in their study to have undergone partial dedifferentiation and cited changes in the "vesicular" (smooth) endoplasmic reticulum, basally located mitochondria, and apical processes as evidence to support this conclusion. We observed somewhat similar alterations, particularly in the smooth endoplasmic reticulum. Such changes, however, show considerable variation, and it is possible that they are caused more by adverse affects of the environment than by changes in the cell's specialized functions. Melanogenesis, on the other hand, is regulated by a series of discrete and measurable biochemical reactions.\(^ {26}\) The melanosome, which represents the end point of this process, provides a more convenient and reliable means of judging the degree of cell differentiation.\(^ {27}\) Over the entire period studied, the pigment epithelial cells contained typical premelanosomes and melanosomes, and there was no other morphological evidence to indicate that they had become dedifferentiated.

In the present study, the onset of macrophage infiltration into the retina occurred at about 21 days when a few acid hydrolase-positive cells were detected in cytochemical preparations, in the outer nuclear layer, and in the interphotoreceptor space. Although the cause of the migration is not known, the degeneration of inner and outer segments may produce substances that initiate macrophage migration in a manner analogous to the infiltration of mononuclear cells into an inflammatory site in response to specific mediators.

The immediate source of the macrophages observed in this study is not known. These cells could come from the blood vessels or from cells (histiocytes) that are normally present in the retina. Sanyal,\(^ {28}\) using cytochemically demonstrable N-acetyl-β-glucosaminidase activity as a "marker" for macrophages in the developing mouse retina, concluded that these cells enter through the...
Fig. 7. Electron micrograph of retina from 101-day-old RCS-p rat. Pigment epithelial cell contains premelanosomes (PM) and melanosomes in various stages of development and dense bodies (DB₂). Note also irregularity of basal infoldings (BI) and numerous apical processes (AP), some of which contain melanosomes (ME). A macrophage (M) located near the apical processes has large polymorphic dense bodies (DB₁). Note dense structures of unknown nature in cell cytoplasm (arrows) and in debris. (×11,000.)
vascular route and ultimately aggregate in the inner nuclear layer where they transform into small cells that persist in the adult retina (see ref. 28 for additional studies). Similarly, in rats subjected to light-induced retinal degeneration, O'Steen and Karcioğlu\(^1\) observed that the debris from degenerated cells was removed by mononuclear cells of vascular origin and by proliferated pigment epithelial cells. On the other hand, in mice with inherited retinal degeneration, the cells that invaded the outer nuclear layer in response to the degeneration of photoreceptor cells were considered to be macrophages that had migrated from their normal location in the outer plexiform layer. Shakib and Ashton\(^3\), studying focal retinal ischemia in the pig, also concluded that the phagocytes they observed were of local rather than hematogenous origin. They suggested that the blood vessels may be a potential source of macrophages in conditions in which the blood-retinal barrier is disrupted. The factors that determine whether the immediate macrophage response will be of vascular or local origin or will include active participation by proliferated pigment epithelial cells are not known. Further studies of the inherited retinal degeneration in the RCS rat may help to clarify these questions.

Since the migration of macrophages in the RCS retina appears to occur at a particular stage of photoreceptor degeneration, factors that alter the pace of the degeneration may also affect the onset of infiltration. LaVail and Battelle\(^3\), identified two such factors, regional and pigmentary, that influence significantly the rate of photoreceptor degeneration. In black-eyed, dystrophic rats, for instance, the rate of photoreceptor degeneration in the superior hemisphere of the eye, along and above the horizontal meridian was found to be slower than that in the inferior hemisphere along the vertical meridian, by 30 to 35 days. Similarly, in black-eyed and pink-eyed rats reared in cyclic light, the rate of photoreceptor degeneration in the posterior retina of the black-eyed rat was slowed from that in pink-eyed rats by about 10 days and in the superior portion of the far periphery by about 30 to 35 days. These observations suggest that the pace of photoreceptor degeneration in our black-eyed animals may have been considerably slower than in the pink-eyed animals studied by Dowling and Sidman.\(^2\) Whether such a difference is sufficient to account for the absence of pigment epithelial cell migration in our 101-day-old specimens is not clear. It would be of interest to study this problem in black-eyed animals in later stages of photoreceptor degeneration than those used in the present work.

The macrophages observed in the interphotoreceptor space were virtually embedded in outer segment debris and showed cytoplasmic extensions that sometimes appeared to encircle this material. Nevertheless, apart from a few equivocal images, the cells did not appear to ingest the debris. This raises the question as to whether the same defect that interferes with phagocytosis in pigment epithelial cells also occurs in macrophages. Such a situation could arise if the mutant gene affects a crucial step in the phagocytic process that is common to both cell types. It would be of interest to further assess the ability of these macrophages to ingest outer segment material, perhaps by exposing the cells to this material in vitro.

Our findings on macrophages may also have a bearing on the studies of Reading\(^3\) and colleagues\(^3\),\(^4\) in which they proposed that "lytic" enzymes, released from lysosomes of the pigment epithelial cells, infiltrated the retina and caused degeneration of the photoreceptor cells.\(^3\) This suggestion is based on the observation that lysosomes of dystrophic rat pigment epithelial cells were less "stable" than those of normal cells and that, beginning at 4 weeks of age, the levels of "free" and total acid protease activity were increased over that of the normal retina.\(^3\) Our findings demonstrate that during the initial stages of degeneration, macrophages containing large acid hydrolase-positive lysosomes accumulate in the outer nuclear layer and interphotoreceptor space. These lyso-
somes, if included in the homogenates, could affect the assays of enzyme activities in the dystrophic retina.

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REFERENCES

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