Inherited Retinal Dystrophy: Primary Defect in Pigment Epithelium Determined with Experimental Rat Chimeras

Abstract. *Chimeric rats were produced by the aggregation of embryos of the pink-eyed, retinal dystrophic RCS strain with those of pigmented, normal rats. In the mosaic eyes, patches of neural retina with abnormal and degenerated photoreceptors were present only opposite patches of nonpigmented, mutant pigment epithelium. This indicates that the retinal dystrophy gene acts in the pigment epithelial cell rather than in the photoreceptor cell.*

There has been increasing interest in the RCS rat with inherited retinal dystrophy (gene symbol, *rdy*) as an animal model for the retinitis pigmentosa class of inherited diseases that causes blindness in man. Retinal dystrophy in the rat may be primarily a pigment epithelial cell disorder, since pigment epithelial cells in the mutant rat fail to phagocytize rod outer segments (*1*) as they do in normal animals (*2*). This leads to the accumulation of debris from the outer segment membranes before the overt degeneration of photoreceptor cells which occurs between about days 20 to 60 (*1*, *3*, *4*). Others have suggested that an abnormality in the outer segment of the rod may lead to secondary involvement of the pigment epithelium, which, in turn, causes degeneration of the photoreceptor cell (*5*). Thus the site of mutant gene action has remained a puzzle, since photoreceptor cell degeneration could result from a primary genetic defect in the pigment epithelial cell, the photoreceptor cell, or both, or even from factors extrinsic to the eye. To explore this problem we have produced chimeric rats which contain mixtures of mutant and genetically normal cells. By using different pigmentation genotypes that allow identification of mutant and normal pigment epithelial cells, we have analyzed the interaction of these cells with the underlying retina. The results indicate that the pigment epithelial cell is the primary site of *rdy* gene action.

The strains and genotypes of the embryos used to make the chimeras were as follows: RCS [*rdy*/*rdy* and nonpigmented pigment epithelial cells (*6*)], ACI (normal retina and pigmented pigment epithelium), and CDF × RCS F_1 [+/*rdy*, normal retina and pigmented pigment epithe-

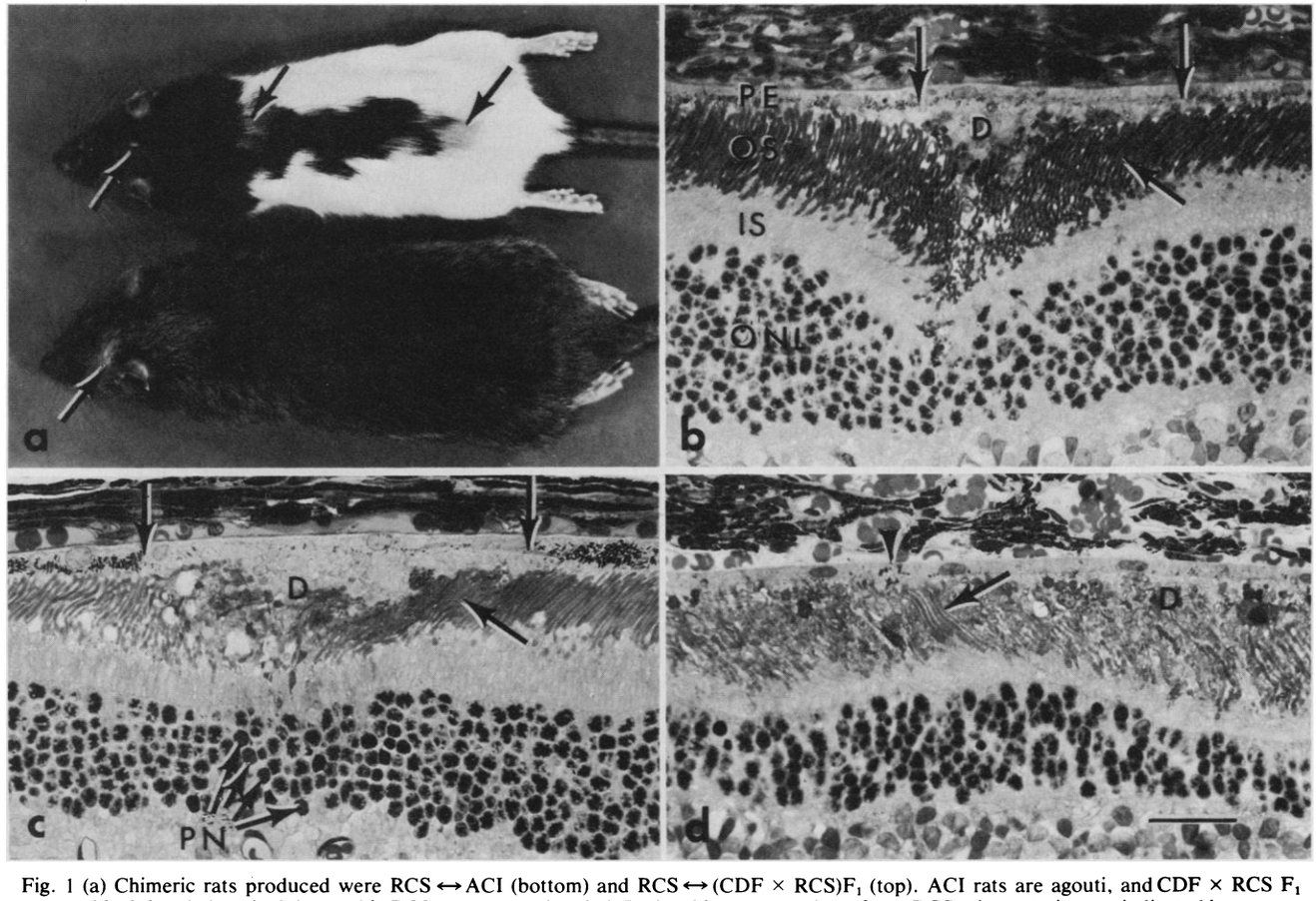

Fig. 1 (a) Chimeric rats produced were RCS ↔ ACI (bottom) and RCS ↔ (CDF × RCS)F_1 (top). ACI rats are agouti, and CDF × RCS F_1 rats are black-hooded; retinal dystrophic RCS rats are tan-hooded. In the chimeras, patches of tan (RCS) pigmentation are indicated by arrows in the otherwise agouti coat of the RCS ↔ ACI and the otherwise black hood of the RCS ↔ (CDF × RCS)F_1. (b to d) Photomicrographs of plastic sections (1 to 2 μm thick) from the eyes of the rat chimeras. (b) From RCS ↔ (CDF × RCS)F_1 chimera at 111 days of age. (c and d) From RCS ↔ ACI chimera at 127 days of age. In (b) and (c) nonpigmented (*rdy*/*rdy*) pigment epithelium is present between the wide vertical arrows, and outer segment debris (*D*) and degenerating or abnormal photoreceptors (or both) are present opposite the *rdy*/*rdy* pigment epithelium; the outer nuclear layer (*ONL*) in these patches is reduced to three to five rows of photoreceptor nuclei from the normal eight to ten rows. Only normal photoreceptors are opposite pigmented (normal) pigment epithelium. Narrow arrows indicate some relatively normal photoreceptor outer segments opposite *rdy*/*rdy* pigment epithelium at the periphery of the patches. In (d), all the pigment epithelium is *rdy*/*rdy* except for a single pigmented (normal) cell (arrowhead), adjacent to which are some relatively normal photoreceptor outer segments (narrow arrow). The melanosomes in the pigmented pigment epithelial cells in (b) are slightly refractile and are just out of the level of focus. Furthermore, these cells are less heavily pigmented than those in (c) because of differences in eye pigmentation of different regions of rat eyes [see figure 12 in (*4*)]. Abbreviations: *PN*, pyknotic nucleus; *IS*, inner segments; *OS*, outer segments; and *PE*, pigment epithelium. The bar in (d) represents 25 μm; (b) and (c) have the same magnification.

lium (*7*)]. The embryos were collected at the eight-cell stage on day 3.5 of pregnancy (day 0 was the day of mating). The technique used for aggregation of embryos was that described for mouse embryos (*8*). Briefly, it consisted of flushing the eight-cell embryos from the oviducts, removing the zona pellucida with Pronase, placing embryos of two different genotypes together, and culturing them overnight. The next day (day 4.5), chimeric morulae or blastocysts were surgically transplanted for the remainder of gestation to the uteri of day-3.5 pregnant females. Primarily because of difficulties in culturing rat embryos in "mouse medium," the yield of chimeric rats was low; two chimeras, identified by mosaicism in coat color, were produced (Fig. 1a). To our knowledge, only two other rat chimeras have been produced (*9*). One of our chimeras was an RCS ↔ ACI, the other an RCS ↔ (CDF × RCS)F_1. The first eye of the RCS ↔ ACI chimera was removed on day 26 after birth, the second on day 127; those of the RCS ↔ (CDF × RCS)F_1 were taken on days 111 and 160. The eyes of the chimeras were grossly pigmented, and, since eye pigmentation slows the rate of retinal dystrophy (*4, 10*), the eyes of pigmented RCS rats [RCS-*p*/+ strain (*11*)] of the same ages were used as controls. The eyes were fixed, embedded in plastic, and sectioned at 1 to 1.5 μm (*4*). A total of 21 regions of retina extending from the optic nerve head to the ora serrata were examined.

The four eyes from the two rat chimeras displayed mosaicism in both the pigment epithelium and neural retina (*12–14*). In the pigment epithelium, nonpigmented (*rdy*/*rdy*) cells were interspersed with pigmented (normal) cells (Fig. 1, b to d), with patches of both types ranging from single cells to several hundred micrometers in length. In the neural retina, patches of abnormal and degenerated (missing) photoreceptor cells were interspersed with patches of normal photoreceptors (Fig. 1, b to d). Abnormal photoreceptors were defined by the presence of outer segment debris, pyknotic nuclei, or both. Most strikingly, however, patches of abnormal and degenerated photoreceptors were present only opposite *rdy*/*rdy* pigment epithelial cells and not opposite normal pigment epithelial cells (Fig. 1, b to d). Furthermore, of the more than 200 patches of mutant pigment epithelium examined that were greater than two cells in length, we found degenerated and abnormal photoreceptors opposite all. Less disorganized outer segments and little apparent cell death were usually seen opposite patches of single (and sometimes two) *rdy*/*rdy* pigment epithelial cells.

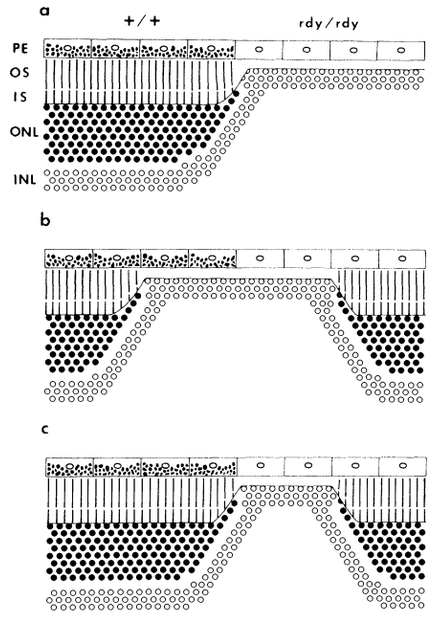

Fig. 2. Hypothetical models of degeneration patterns that would be expected in chimeras if photoreceptor cell death results from the retinal dystrophy (*rdy*) gene acting in (a) the pigment epithelial cell, (b) the photoreceptor cell (or in some other cell in the neural retina), or (c) both sites. The mutant (*rdy*/*rdy*) pigment epithelial cells are drawn as unpigmented and the normal (+/+) ones as pigmented. See text for further details. Abbreviations: *INL*, inner nuclear layer; *IS*, inner segments; *ONL*, outer nuclear layers; *OS*, outer segments; and *PE*, pigment epithelium.

The disease process probably starts at the usual time in chimeras, for at 26 days of age the amount of debris was similar in degenerating regions in the chimeras and in the controls. The chimeric constitution of these animals, however, modified the severity of the disease. At 26 days, the proximal regions of the outer segments appeared less disorganized, and there were only about $^1/_5$ as many pyknotic nuclei as in control animals. In the chimeras at 111 and 127 days, long after almost all cells have disappeared in pigmented RCS rats (*4*), 30 to 60 percent of the photoreceptor nuclei were still present opposite patches of *rdy*/*rdy* pigment epithelium, although some were degenerating [as evidenced by pyknotic nuclei (Fig. 1c)]. At 160 days, the degenerating patches were similar to those at 111 and 127 days, but the disease process may have been further slowed or arrested in the chimeras; the debris zone was somewhat thinner and few pyknotic nuclei were present. Only one patch of retina was found degenerated to the extent expected for the age of the animal, and it was under a long stretch of mutant pigment epithelium near one ora serrata.

Outer segments of photoreceptor cells lying opposite patches of *rdy*/*rdy* pigment epithelium were often less disorganized at the periphery of the patches than those at the center of the patches (Fig. 1, b and c). Furthermore, the degree of disorganization of the outer segment appeared to be a function of the position of the outer segment tips rather than the position of the cell nucleus with respect to the pigment epithelial cell type. Photoreceptor cells whose nuclei were apparently opposite normal pigment epithelial cells but whose outer segment tips leaned into mutant pigment epithelial cells tended to have disorganized outer segments (Fig. 1, b and c). Likewise, opposite patches of *rdy*/*rdy* pigment epithelium, those outer segments leaning toward normal pigment epithelial cells were often relatively normal (Fig. 1, b to d).

If, in the rat, the *rdy* gene acts solely in the photoreceptor cell or other cell type in the neural retina, then, in chimeric rats, we would expect a pattern similar to that in mouse *rd*/*rd* ↔ +/+ chimeras (*15*), that is, patches of degeneration opposite both genetically normal and mutant pigment epithelium (Fig. 2b). If the gene acts solely in the pigment epithelial cell and secondarily results in photoreceptor cell death, degeneration of photoreceptors (regardless of their genotype) should be seen wherever and only where the pigment epithelium is genetically mutant (Fig. 2a). If the gene acts in both cell types, we would expect degeneration only opposite patches of mutant epithelium but not opposite all patches; in other words, there should be degeneration only in those areas where both photoreceptor and pigment epithelial cells were genetically *rdy*/*rdy* (Fig. 2c).

The direct correspondence of mutant pigment epithelium with degenerating retina observed in the chimeric rat eyes best fits the diagram of Fig. 2a and indicates that, although it is the photoreceptor cell that degenerates, the *rdy* gene actually acts in the pigment epithelial cell. Although only two chimeric rats were produced, we base our conclusion on the consistent association of mutant pigment epithelium with degenerating photoreceptors in more than 200 patches of degenerating retina. This pattern of degeneration also eliminates the possibility that the mechanism of retinal dystrophy in the rat is a systemic or circulating factor. Furthermore, the observation that outer segments leaning into *rdy*/*rdy* pigment epithelium were disorganized while

those leaning into +/+ pigment epithelium were relatively normal suggests that the site of aberrant interaction between the defective pigment epithelial cell and the photoreceptor is at or by way of the tip of the rod outer segment, the site of contact between the two cell types.

Survival of some photoreceptor cells under mutant pigment epithelium may be mediated by adjacent, normal pigment epithelial cells. This is consistent with the finding that outer segments underlying patches of mutant pigment epithelium are more normal at the periphery of the patch than those under the center of the patch. If amelioration of the disease proves to be mediated by a diffusible substance, it may be possible in future work to isolate the substance from normal pigment epithelial cells and provide it exogenously to the mutant retina or pigment epithelium or both, and thereby retard or alleviate the degenerative disorder.

This study and others (*14, 16*) demonstrate the usefulness of experimental chimeras in determining whether particular neurological mutations are acting intrinsically, within the most obviously affected cell type, or extrinsically, within either an adjacent or distant interacting cell (for example, by a circulating factor). The rat chimeras are particularly informative, for we can conclude not only that the *rdy* gene is acting extrinsically to the photoreceptor cell, but also that the pigment epithelial cell is the actual site of the gene action (*17*). As far as we are aware, this is the first neurological mutation in mammals in which a primary site of mutant gene action can be assigned to a particular cell type.

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6. RCS rats are homozygous for the pink-eyed dilution (*p*) pigmentation gene [R. L. Sidman and R. Pearlstein, *Dev. Biol.* **12**, 93 (1965)], which reduces eye pigmentation to only a few melanosomes in peripheral pigment epithelial cells during the first few months of age (*4*), and these cells appear virtually unpigmented in 1- to 2-μm-thick plastic sections (*3, 4*).
7. CDF is the abbreviation for inbred cesarian-derived Fischer rats obtained from Charles River Breeding Laboratories. The rats are albinos (*c/c*) and are +/+ at the *p* and *rdy* loci; therefore, the CDF × RCS F_1 hybrids are +/*c*, +/*p*, and +/*rdy*.
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15. In studies of inherited retinal degeneration (*rd*) in mouse *rd/rd* ↔ +/+ chimeras, the distribution of patches of degenerating photoreceptor cells was independent of the genotype of the adjacent pigment epithelial cells. This was interpreted to mean that the pigment epithelium is not primarily involved in the mouse disease (*14*) and that the developmental lineages of adjacent photoreceptor and pigment epithelial cells are independent of one another although both of them are derived from the optic vesicle (*13*); B. Mintz, *Annu. Rev. Genet.* **8**, 411 (1974).
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17. The possibility exists that the *rdy* gene also acts in cells remote from the eye. This problem of gene pleiotropism is discussed in detail by M. M. La Vail and R. J. Mullen, in *Retinitis Pigmentosa: Clinical Implications of Current Research*, M. B. Landers, M. L. Wolbarsht, J. E. Dowling, A. M. Laties, Eds. (Plenum, New York, in press).
18. We thank J. Handke, D. Silber, and C. O. Gerhardt for technical assistance. Supported by a Basil O'Connor starter research grant from the National Foundation–March of Dimes (R.J.M.) and NIH grant EY-01202 and career development award EY-70871 (M.M.L.).

12 January 1976; revised 27 February 1976

Lysergic Acid Diethylamide– and Mescaline-Induced Attenuation of the Effect of Punishment in the Rat

Abstract. *At a dose as low as 1 microgram per kilogram of body weight, lysergic acid diethylamide (LSD) significantly decreased the suppressive effect of electric shock on licking behavior of the rat. Attenuation of punishment was also obtained with mescaline, but neither dimethyltryptamine nor* Δ^9*-tetrahydrocannabinol was active in this test. Cyproheptadine and* α*-propyldopacetamide, drugs that interfere with the function of neurons that contain serotonin, have a behavioral effect similar to that of LSD and mescaline, which suggests that the attenuation of punishment produced by these hallucinogens may result from decreased activity of such neurons.*

Pharmacological interference with the function of neurons that contain serotonin (5-hydroxytryptamine) restores behavior suppressed by electric shock. Such attenuation of the effect of punishment occurs after the administration of antagonists of serotonin receptors (*1, 2*). A similar effect is obtained after depletion of serotonin by *p*-chlorophenylalanine, an inhibitor of tryptophan hydroxylase (*3*). Benzodiazepines and barbiturates also diminish the effect of punishment on responding (*4*). Among their many neuropharmacological effects, these "antianxiety" drugs decrease serotonin turnover (*5*). This effect on serotonin metabolism is also produced by several drugs with hallucinogenic properties, including lysergic acid diethylamide (LSD) (*6*), mescaline (*7*), dimethyltryptamine (DMT) (*8*), and Δ^9-tetrahydrocannabinol (THC) (*9*). Therefore, I tested these hallucinogens in a procedure sensitive to benzodiazepines (*10*). I found that LSD and mescaline significantly reduce the effect of punishment.

The procedure was identical to that used by Vogel *et al.* (*10*). The apparatus was a clear Plexiglas box (38 by 38 cm) with a black Plexiglas compartment (10 by 10.5 cm) attached to one wall and an opening 5 by 7.5 cm between the two. The entire apparatus had a stainless steel grid floor. A metal water tube extended 1.5 cm into the small box 4 cm above the grid. A drinkometer circuit (Grason-Stadler, model E4690A) was connected between the drinking tube and the grid floor. An unscrambled shock (0.75 ma) was administered by switching the connection to the tube and grid from the drinkometer to a shock generator (Aim BioSciences, model 507).

After 48 hours of water deprivation, each rat (200 g, male, albino, Sprague-Dawley) was placed in the apparatus 30 minutes after the drug or saline was injected. It was allowed to find the drinking tube and to lick 20 times, after which shocks were administered for each tube contact during a 2-second period. The cycle of 20 licks without shock followed by 2 seconds of vulnerability to shock was repeated until 3 minutes after the first shock was delivered. During this 3-minute session, the total number of shocks was recorded automatically. Each daily experiment included one group treated with saline and four groups treated with a drug (there were eight rats in each group). Drugs were either dissolved in saline or suspended in distilled water with one drop of Tween 80, and they were ad-