An RCS-Like Retinal Dystrophy Phenotype in Mer Knockout Mice

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PURPOSE. To determine whether mice that are homozygous for a targeted disruption of the Mer receptor tyrosine kinase gene (mer1) manifest a retinal dystrophy phenotype similar to RCS rats, which carry a mutation in the orthologous gene Merk.

METHODS. Eyes of mer1 and C57BL/6 wild-type (WT) mice were examined by light and electron microscopy, whole-eye rhodopsin measurement, and Ganzfeld electroretinography (ERG).

RESULTS. The mer1 mice showed rapid, progressive degeneration of the photoreceptors (PRs). Features of the phenotype common to mer1 and RCS rats included the absence or near absence of phagosomes in the retinal pigment epithelium (RPE) at the peak of outer segment (OS) disc shedding, accumulation of debris and whorls of membranes at the RPE-OS interface, transient supernormal rhodopsin content and OS lengths, the presence of OS vacuoles beginning at early ages, and a relatively slow removal of pyknotic PR nuclei. Most PRs were missing, and OS debris was removed by approximately postnatal day (P)45. Scotopic ERG responses were lower than age-matched WT responses and declined with PR loss. Photopic responses were preserved better than scotopic responses, corresponding with preferential cone preservation as judged histologically. ERG amplitudes were usually unmeasurable beyond P40, although a small-amplitude scotopic threshold response (STR) could still be elicited at P253 in some mice when only scattered PR nuclei remained.

CONCLUSIONS. Ablation of Mer function in mer1 mice results in a retinal phenotype almost identical with that of RCS rats. The similarity in phenotypes between the two rodent models suggests that an RPE phagocytic defect is a feature of all types of retinal degeneration caused by loss of function of Mer tyrosine kinase, perhaps including mutations in human MERTK. (Invest Ophthalmol Vis Sci. 2003;44:826–838) DOI:10.1167/iovs.02-0438

Inherited and age-related retinal degenerations are a group of diseases that share progressive dysfunction and death of photoreceptors (PRs). Retinitis pigmentosa affects approximately 1 in 3500 to 4000 people worldwide. A more common condition, age-related macular degeneration (AMD), affects an estimated 6 million people in the United States alone, and this number is expected to increase in the future, because the elderly population will double over the next 50 years.1 AMD shares other features with some inherited retinal degenerations. For example, abnormalities of the retinal pigment epithelial (RPE) cells are a feature of AMD,2–4 and specific RPE defects are seen also in several inherited degenerations such as Best disease,5 Stargardt disease,7 Sorsby fundus dystrophy,8,9 childhood-onset severe retinal dystrophy,10 and Leber congenital amaurosis due to mutations of RPE65.11–14 RCS rats have been studied extensively as a model of retinal degeneration15,16 and PR-RPE cell interactions.16–17 The primary defect responsible for the RCS phenotype is failure of the RPE to phagocytize shed rod outer segments (OS).22 The unphagocyctized OS membranes form membranous whorls at the RPE surface, and the rod OS grow abnormally long.20,21 Eventually, the OS layer degenerates into a debris zone with subsequent PR cell death.20

Recently, positional cloning was used to identify a null mutation in the receptor tyrosine kinase Merk gene in RCS rats.22 Mer is the official designation for the murine gene that is orthologous to rat and human Merk. Mer is a member of the Axl/Mcer/Tyro5 receptor tyrosine kinase subfamily. The growth-arrest–specific protein 6 (Gas6) is a ligand for all three members of this subfamily.23–25 Gas6 binds to the receptors through its carboxyl-terminal half,26 whereas an N-terminal domain of Gas6 that is rich in γ-carboxylated glutamic acid residues mediates binding to phosphatidylserine.27 Phosphatidylserine molecules normally are localized to the inner membrane leaflet, but are exposed on the exterior of apoptotic cell membranes.28 It has been suggested that Gas6 may mediate RPE phagocytosis of OS by binding to and serving as a bridge between older OS membranes and Mer.22 In support of this model, purified Gas6 has been shown to stimulate phagocytosis of OS by cultured RPE cells.29 Moreover, Mer localizes to the sites of OS uptake and appears to trigger the ingestion step of RPE phagocytosis in cell culture.30 Several other RPE cell surface proteins have been implicated in the binding or internalization of OS,31,32 such as ανβ5 integrin,33 CD36,34 and the mannose receptor;35,36 but there is no current evidence that these molecules are causally related to the degeneration of PRs. A viral vector was used recently to transfer wild-type (WT) Mer to the RPE of RCS rats. This resulted in reversal of the RPE phagocytosis defect and PR rescue,57 which conclusively demonstrated that mutation in Merk is the cause of retinal degeneration in RCS rats. Mutations in human MERTK have
been identified in patients with retinitis pigmentosa. Mer defects in other species could help to elucidate the role of Mer in RPE cell phagocytosis and retinal degeneration. A mouse strain with targeted disruptions of the genes encoding Tyro3, Axl, and Mer has been produced. These mice were described as blind due to postnatal degeneration of rods and cones, but no retinal histologic or electrophysiological results were presented. A mouse strain carrying only the targeted disruption of Mer (mer^df) has also been produced that exhibits hypersensitivity to endotoxins and a generalized defect in phagocytosis of apoptotic thymocytes by macrophages. The retinal phenotype of these mice has not been previously investigated. Herein we present structural and functional characteristics of the retinal phenotype of mer^df mice. Because the gene mutated in these mice is orthologous to the mutant gene responsible for retinal dystrophy in RCS rats, we examined mer^df mice for all the key retinal phenotypic characteristics of RCS rats.

METHODS

Mice
All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mer^df mice were bred from the same stock as previously described and compared with age-matched C57BL/6 WT mice raised in the same facility. Mice were maintained in a 12-hour light-dark cycle at an in-cage illuminance of less than 10 ft-c.

Immunoblot Analysis
Retina/sclera and kidney were dissected from 4-month-old mer^df and C57BL/6 mice and homogenized in 1% NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40 [pH 8.0]) containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Glycoproteins were enriched by wheat germ agglutinin (WGA)-Sepharose beads (Sigma, St. Louis, MO) as described, separated by 6% SDS-PAGE and transferred to a nitrocellulose membrane (Fisher Scientific, Pittsburgh, PA). Mer tyrosine kinase protein was detected by a polyclonal antibody directed against the C-terminal part of rat MerTK or by a polyclonal antibody directed against the ectodomain of murine Mer (R&D Systems, Minneapolis, MN), followed by a horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Super Signal West Pico Chemiluminescent Substrate system; Pierce, Rockford, IL).

Histologic Analysis
At different ages (Table 1) mice were killed with carbon dioxide inhalation and immediately perfused intracardially with a mixture of aldehydes (2% paraformaldehyde and 2.5% glutaraldehyde). Eyes were removed, bisected along the vertical meridian, postfixed in osmium tetroxide, and embedded in an Epon-Araldite mixture. Sections of the retina were cut 1-μm thick and stained with toluidine blue, as described elsewhere. The thickness of the outer nuclear layer (ONL) was taken as a measure of PR number, and the mean ONL and rod OS thickness were obtained from 54 measurements around the retina, as described elsewhere. Tissue sections were chosen where the rod OS and Müller cell processes crossing the inner plexiform layer were continuous in the plane of the section, or nearly so, to assure that the photoreceptor process was not oblique. Electron microscopy was used for ultrastructural evaluation of the retinas at selected ages by previously described methods. To study rod OS disc shedding and phagocytosis by the RPE, we perfusion fixed four mer^df and four WT mice (two each at postnatal day [P]15 and P20 for each genotype) between 1 and 1.5 hours after the onset of light in the morning. We quantified the number of phagosomes in the RPE cell processes and cell bodies by light micro-

Rhodopsin Measurements
Mice were dark adapted overnight, and all procedures were performed in dim red light. Both eyes from each mouse assayed (Table 1) were pooled after lens removal and homogenized in 0.85 ml 20 mM (5-N-morpholino) propane sulfonic acid (MOPS) containing 1.5% lauryl maltoside and 0.2 M hydroxylamine hydrochloride. The homogenate was extracted on ice for 3 hours and centrifuged for 15 minutes at 135,000g at maximum rotation (r max) in a centrifuge (TL100; Beckman Instruments, Carlsbad, CA). The change in absorbance at 498 nm was measured with a spectrophotometer (Genesys 5; Spectronics, Westbury, NY) after bleaching for 60 seconds at a distance of 10 cm with a 60-W bulb and used to calculate the whole-eye rhodopsin content (absorbance coefficient of rhodopsin at 498 nm = 42,000 M/cm).

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P, postnatal day; numbers in parentheses indicate numbers of eyes in which ONL thickness was quantified.

* Age of each animal was ± 1 day at P20 and older.

Copy with ×100 oil-immersion optics at ×1000 magnification (Axio-phot; Zeiss, Thornwood, NY), according to previously described criteria. These required that the phagosome, either in the RPE cell processes or in the RPE cell body, be greater in any dimension than 0.75 μm (half the rod OS diameter) and stain more densely than the OS. Thus, smaller intensely staining structures (e.g., residual bodies, lipid droplets, and mitochondria) were not counted. The rationale for including in the phagosome counts the densely staining shed packets of rod OS discs located in the RPE cell processes is discussed elsewhere. The entire retinal length was examined (i.e., > 4 mm) in a single, midsgalittal section from each animal.
A 10-μL injection of NMA was given with a 30-gauge beveled needle attached to the posterior retinas of merkd mice to prevent an estimated final vitreous concentration of 5 mM NMA. The contralateral eye received a 0.5-μL intravitreous injection of normal saline to serve as the control. After the NMA was injected and the mice were prepared for ERG under dim red light, the mice were kept in darkness for an additional 45 minutes before the ERGs were recorded.

Statistical Analysis

The unpaired two-tailed Student’s t-test with the Welch correction for unequal variance was performed on computer (Prism, ver. 3.00 for Windows, GraphPad Software, San Diego, CA) to compare data from merkd mice and age-related WT mice. The Pearson correlation coefficient was used to calculate the coefficient of determination ($r^2$), to correlate rates of structural and functional retinal degeneration.

RESULTS

Assessment of Mer Protein

The merkd allele was generated by targeted deletion of an exon encoding an essential portion of the Mer tyrosine kinase domain, presumably leading to premature termination of translation and ablation of the receptor’s kinase activity. It is not certain that this mutation results in a complete loss of function. Indeed, the mutation was not intended to be a null allele. The abundance of the mutant mRNA is similar to that of the WT mRNA, which indicates that the merkd allele has the potential to produce a truncated protein. Merkt protein cannot be detected in RCS rat tissues, indicating that the retinal dystrophy gene rd1 is a null allele. We tested protein samples from the retinas and kidneys of WT and merkd mice for the presence or absence of Mer protein, using polyclonal antisera directed against the receptor’s kinase activity. Both antibodies detected Mer proteins of the expected sizes in tissues from WT animals (Fig. 1). By contrast, Mer protein was not detected in tissues from merkd mice, and there was no evidence of a truncated protein. These data indicate that merkd is a null allele. Comparison of the retinal phenotype of merkd mice and RCS rats is therefore warranted.

General Histologic Characteristics of PR Degeneration

As in RCS rats, PR degeneration in merkd mice led to progressive thinning of the ONL over time (Fig. 2). The ONL thickness in merkd mice was indistinguishable from WT at P20 and younger but was significantly less than in age-matched WT mice.

Electroretinographic Analysis

Electroretinograms were recorded as described previously on merkd mice and WT mice at several ages (Table 1). Photopic a-waves were not quantitated, because WT mice have negligible photopic a-waves. Threshold criterion amplitudes were 20 μV for scotopic b-waves and 10 μV for photopic b-waves. Thresholds and implicit times for a-waves were not measured because of interference from the cornea-negative scotopic threshold response (STR) observed between b-wave thresholds and the normal a-wave threshold.

N-methyl-DL-aspartic acid (NMA; Sigma) suppresses synaptic transmission by acting primarily on third-order neurons and is used to distinguish between the a-wave and the STR, both of which are cornea-negative ERG waveforms, in cats and RCS rats. A doublet of Mer bands (~150 and ~170 kDa) is detected in WT tissues, but is absent from merkd tissues. The two bands are differentially glycosylated forms of the receptor.

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Absence of Mer protein in tissues from merkd mice. Protein lysates from the retina/sclera and kidney of merkd mutant (mu) and B6 (WT) mice were tested for Mer protein by immunoblot analysis, with polyclonal antisera directed against the C-terminal 100 amino acids of the rat Mer tyrosine kinase (left) or against the ectodomain of mouse Mer (right). A doublet of Mer bands (~150 and ~170 kDa) is detected in WT tissues, but is absent from merkd tissues. The two bands are differentially glycosylated forms of the receptor.

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The degree of PR cell loss was observed, with a few even at the oldest ages. Although but at least a few surviving nuclei were found in each section, (Table 1), there were progressively fewer PR surviving nuclei, retaining a single row of ONL cell nuclei in some parts of the retina. The percentage of surviving cone PRs increased as the rod PRs degenerated. During PR degeneration in RCS rats, this phenomenon is not typically seen in animal models of retinal degeneration. When we examined a line of mutant rhodopsin transgenic rats that has a rate of PR degeneration comparable to that of merkd mice, we found less than 5% of the ONL nuclei to be pyknotic at any given age. At P30 to P40, some of the pyknotic nuclei in the ONL of merkd mice coalesced and formed large masses of residual heterochromatin. In merkd mice, a feature also observed in RCS rats.

Some hemispheric differences and gradients of PR degeneration in RCS rats were either less pronounced or not observed in merkd mice. For example, in pigmented RCS rats a significant difference in the rate of degeneration has been found between the superior and inferior hemispheres, with ONL cell loss in the inferior hemisphere occurring more rapidly than in the superior hemisphere. In merkd mice studied between P30 and P40, a similar pattern was observed, with a trend toward more rapid degeneration in the inferior retina. However, the difference was only approximately 5 μm, or one cell nucleus, and was statistically significant only at P36 and P40. RCS rats also show a distinct central–peripheral gradient of cell loss. Degeneration in the peripheral retina is slowed by approximately 10 days compared with the degeneration in the posterior pole. In contrast, the merkd mice showed little, if any, consistent difference in the rate of degeneration in the posterior and peripheral retina.

Evidence of an RPE Phagocytic Defect

Before PR cell death during retinal degeneration, RCS rats show a failure to phagocytize rod OS. There are several presumptive consequences of this defective phagocytosis, including the formation of membranous whorls adjacent to the RPE surface, longer than normal OS, and increased rhodopsin content in the eye. Each of these features of RCS rats was seen in merkd mice. Early in the degeneration, some membranous whorls were present at the RPE surface (Fig. 5B). Electron microscopic examination showed the whorls consisted of disorganized OS membranes and RPE cell processes (Fig. 6A). At P21, a few OS were present that reached the RPE surface, nearly so (Fig. 6A), and although some OS were relatively intact (Fig. 6A), OS structure was not as orderly as in WT mice. At later ages, the OS degenerated further, and a debris zone nearly replaced the inner segment and OS layer (Figs. 2B, 2C). However, electron microscopic examination showed that some fragments of OS still were present, and some still reached the RPE cell surface at P37 (Fig. 6B). By P45, the whorls, abnormal OS, and membranous debris had disappeared in most merkd mice (Fig. 2D), although small pockets of debris membranes were seen in a few animals after this age.

By P20, the rod OS were significantly longer in merkd mice than in WT mice (Fig. 5). This abnormal increase in OS length (Fig. 7A) was accompanied by an increase in whole-eye rhodopsin concentration in merkd mice (Fig. 7B) at young ages, a feature also of RCS rats. At P25, whole-eye rhodopsin concentration was 67% greater in merkd than in WT mice (P < 0.005), whereas OS length was increased by 35% (P < 0.02). At
In contrast, few if any phagosomes were observed, and some vacuoles are seen in either WT or merkd mice, although no typical phagosomes were seen by light microscopy, but any such phagosomes. Electron microscopic examination also did not reveal any typical phagosomes in merkd mice, although a few profiles were seen in which some apparent disc membranes were surrounded by RPE cytoplasm. None of these profiles showed highly condensed OS membranes characteristic of typical phagosomes, and it is therefore possible that the packets of disks may not have been internalized by the RPE cells.

### OS Vacuoles

At many ages in merkd mice, vacuoles were observed in the OS zone in well-fixed tissue (Figs. 2B, 2C). Similar vacuoles have been seen in RCS rats at all ages when OS or debris membranes were present (LaVail MM, unpublished observations, 2000), but have not been described previously. Retinas of merkd and WT mice were examined from P2 to P60, and the size and number of vacuoles were quantified. No vacuoles were identified in either WT or merkd retinas younger than P8. However, as early as discrete OS could be seen (beginning at P8 and P10), many vacuoles were found in merkd retinas (Fig. 9A), and they were seen at early age examined (Figs. 2B, 2C, 9B) up to P60. Most of the vacuoles in merkd mice were greater than 5 μm in diameter (Fig. 9B), and some were extremely large, reaching up to 35 μm in length. These structures were found in tissues fixed either by vascular perfusion or immersion, and very few vacuoles of this type were found in WT retinas at comparable ages. In some cases, none were seen in WT retinas (Fig. 9B). Most of the vacuoles in merkd retinas appeared to contain delicate fragments when viewed by light microscopy (Figs. 2B, 2C, 9A). When examined by electron microsc-
copy, the vacuoles almost always appeared to contain fragments of OS membranes (Fig. 6B), which were observed even when the vacuoles were present in or near the degenerating inner segment zone.

### Invading Cells

A few invading macrophages or microglia in each retinal section were found in the ONL as early as P15, although in the absence of specific stains, these were difficult to distinguish from developing cone nuclei. By P20, a few invading cell nuclei could clearly be seen in the ONL, and occasionally in the OS zone. By P25, distinctly more cells were found in both layers, and the number peaked at P33 to P45 (e.g., Figs. 2C, 4B), where 25 to 60 invading cells per section could be seen in each layer. During this peak period and through approximately P60, cytoplasmic inclusions and lipid droplets occasionally were seen in the invading cells (Figs. 2C, 4B), but almost none of the cells appeared engorged with ingested material, similar to the appearance at these stages of degeneration in RCS rats.

### Late-Stage Changes

At late stages of degeneration after most PRs and the OS debris zone have been lost, a number of features seen in RCS rats were observed in merkd mice. The RPE showed small focal regions of thinning as early as P45, with several such regions in both hemispheres at P77 and older (Fig. 10A), yet some regions of RPE appeared thicker than normal. Retinal capillaries invaded the RPE beginning at approximately 6 months of age (Figs. 10B, 10C), and pigmented RPE cells invaded the retina and localized along retinal blood vessels beginning at approximately 8 months of age (Figs. 10B–D). Some of the pigmented cells or their processes were found to cuff small capillaries (Fig. 10C), and some migrated to the nerve fiber layer of the retina (Fig. 10C). Also, beginning at approximately 6 months of age, strands of nuclei that clearly had been displaced from the inner nuclear layer began to appear crossing the inner plexiform layer toward the ganglion cell layer (Fig. 10D). These displaced cells have been shown in RCS rats to result from vascular tortuosity.

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**Figure 6.** Electron micrographs of merkd mouse retinas. (A) At P21, some OS reach nearly to the RPE cell surface (arrowhead) and are relatively intact, although less organized than OS in age-matched WT mice. Large rounded and elongated whorls (w) of membranes are formed by disorganized OS and RPE processes. (B) At P37, the OS zone is mostly a debris zone, although some fragments of OS are still present, and some reach the RPE cell surface (arrowheads). A large vacuolated profile (v) is shown with fragments of OS disc membranes (arrows) in its periphery. Bar, 2 μm.

**Figure 7.** Rod OS grow abnormally long, and whole eye rhodopsin concentration is increased early in the degeneration in merkd mice. (A) Rod OS lengths are greater in merkd than in WT mice at young ages. Each point represents the mean of two to seven eyes measured (Table 1). (B) At P25, whole-eye rhodopsin concentration is increased by 67% compared with WT (P < 0.005). Bars represent the mean ± SEM of results in 8 to 20 eyes (Table 1).
Correlations between Retinal Structure and Function

A rapid and progressive loss of retinal function with age was apparent in mer<sup>kd</sup> mice (Fig. 11). Representative examples of scotopic and photopic ERG recordings from a WT mouse at P30 and from mer<sup>kd</sup> mice of different ages are shown in Figures 11A and 11B, respectively. At the youngest age examined (P20), mer<sup>kd</sup> scotopic a- and b-wave amplitudes were significantly lower than normal (P ≤ 0.001 and P ≤ 0.0005, respectively; Figs. 11A, 11D, 12A). In contrast, photopic b-wave amplitudes were not significantly different from normal until mer<sup>kd</sup> mice reached P33 (Figs. 11B, 11D, 12A, 12B). At this age and beyond, photopic amplitudes were significantly lower than normal (P ≤ 0.04; Figs. 11B, 12B–D). Compared with WT, scotopic and photopic implicit times after P35 were delayed by approximately 75% (P < 0.0001) and 30% (P < 0.02), respectively. Interpretation of the scotopic a-wave implicit time was complicated at advanced stages of degeneration (P56 and beyond), because of interference from the corneal-negative STR (Fig. 11A). Both scotopic a- and b-wave and photopic b-wave amplitudes declined rapidly, and implicit times increased with age. No clear ERG a- or b-waves were detectable beyond P40 in most mice (Figs. 11A, 11B, 11D, 12D), although small-amplitude scotopic and photopic b-waves were seen in a few mice at ages P58 to P65 (Fig. 12).

As in most retinal degenerations, scotopic ERG amplitudes decreased with age and PR loss in mer<sup>kd</sup> mice. The ONL thickness decreased with scotopic a-wave (r<sup>2</sup> = 0.83, P < 0.0001) and b-wave (r<sup>2</sup> = 0.82, P < 0.0001) amplitudes (Fig. 11D). However, as seen in Figure 11D, the positive correlation between ERG amplitudes and ONL thickness in mer<sup>kd</sup> mice was not as strong as that which has been observed in other retinal degenerations<sup>35</sup>: ERG amplitudes declined significantly earlier than ONL thickness. We considered that the persistence of pyknotic nuclei may have resulted in artificially thick ONL measurements. In an effort to correct for this possibility, we subtracted from the ONL thickness (Fig. 11D) the percentage of pyknotic nuclei at each age (Fig. 4A). When the estimate of ONL thickness that included only nonpyknotic cells was compared with the scotopic a- and b-wave and photopic b-wave amplitudes, a much stronger positive correlation was evident (Fig. 11D). The corrected ONL thickness, which excluded the percentage of pyknotic cells, decreased with scotopic a-wave (r<sup>2</sup> = 0.96, P < 0.005) and b-wave (r<sup>2</sup> = 0.93, P < 0.01) and photopic b-wave (r<sup>2</sup> = 0.82, P < 0.05) amplitudes (Fig. 11D). These correlation coefficients are comparable to those reported by others for light-damaged rats<sup>68</sup> and transgenic rats with rhodopsin mutations.<sup>55</sup> Thus, in those instances in which pyknotic nuclei persist, a correction in the ONL thickness must be made to obtain an accurate estimate of the number of viable PR cells for structural-functional correlations.

The progressive loss of retinal function in mer<sup>kd</sup> mice was also apparent from a plot of amplitude versus intensity for scotopic and photopic responses at different ages (Fig. 12). At...
the youngest age tested, P20, the maximum scotopic b-wave amplitudes were significantly lower than WT, whereas photopic amplitudes were normal (Fig. 12A). As PRs were lost with time, both scotopic and photopic amplitudes were significantly lower than WT at P33 (Fig. 12B) and P36 (Fig. 12C). At these ages, the uncorrected ONL thickness was reduced 45% (P < 0.01) and 60% (P < 0.0001) below WT, respectively. By P40, neither scotopic nor photopic b-waves were measurable in most mice (Fig. 12D).

Others have found that in RCS and light-damaged rats the scotopic b-wave threshold is more closely related to PR degeneration (i.e., the number of surviving PR nuclei) than is the maximum b-wave amplitude.52,68 We investigated the relationship between the threshold intensity to elicit scotopic and photopic b-waves and PR degeneration in merkd mice. At the youngest ages examined, scotopic b-wave thresholds were significantly higher than normal (P ≤ 0.001), and b-wave thresholds increased with age in merkd mice, until no b-wave was elicited at the highest stimulus intensities after P65 (Figs. 11D, 13A). In contrast, photopic thresholds were normal in merkd mice until P36, then increased rapidly with age (Fig. 13B).

Photopic responses, both amplitudes and thresholds, were better preserved than scotopic responses in merkd mice. Consistent with this, histologic analysis showed a preferential preservation of cones (Fig. 3B). In addition, mice heterozygous for the merkd mutation had normal retinal structure and function at a wide range of ages (data not shown), as is also seen in rats heterozygous for rdy.79

**Scotopic Threshold Response**

The STR, a cornea-negative waveform elicited at flash intensities below the b-wave threshold, has been observed in a number of animals, 59 including RCS rats 52 and WT mice.71 We elicited the STR at low flash intensities in WT mice at all ages tested (data not shown). In young merkd mice, cornea-negative waveforms were elicited by low flash intensities similar to those observed in WT mice (Fig. 11C, arrows; Fig. 13C). This response was markedly attenuated after intravitreal injection with NMA in both WT and merkd mice (data not shown), consistent with the STR response observed in RCS rats.72 At the youngest ages examined, the threshold to elicit the STR was not significantly different between merkd and WT mice, but at P33 and older, the STR threshold was significantly higher in merkd mice than in age-matched WT mice (P < 0.005; Fig. 13C). The STR threshold increased with age in merkd mice (r² = 0.80, P < 0.0001; Fig. 13C).

Usually after P40, and always after P65, neither scotopic nor photopic b-waves were recordable in response to the brightest stimulus intensity (+2.4 log cd sec/m²) in merkd mice. However, the STR could be elicited even at advanced stages of PR degeneration in some merkd mice (Fig. 13D) up to P253. Thus, the STR was measurable at bright stimulus intensities when only scattered PR nuclei remained.

**Discussion**

The retinal phenotype of merkd mice displays a striking similarity to the well-described retinal dystrophy phenotype of RCS rats. As is the case with rdy in RCS rats,79 the merkd retinal phenotype is completely recessive, because mice heterozygous for the mutation have normal retinal structure and function.

**Phagocytosis Defect in the RPE and PR Cell Death**

The hallmark of the retinal dystrophy phenotype in RCS rats is the failure of rod OS phagocytosis by the RPE.18,19,44 In vitro assays indicate a small but quantifiable amount of phagocytosis by the RPE of RCS rats.72–74 An even lower level of phagocytosis has been reported in RCS rats in vivo,75–77 but most of the illustrated inclusions in the RPE in these studies did not meet the criteria of phagosomes as they were originally defined,79,50 and some appeared to be whorls of membranes shown previously to be formed within the RPE layer by the RPE cells.44 Thus, it is unclear whether the defect in RCS rats results in total absence or gross reduction of OS phagocytosis by the RPE in vivo.

Our findings in the rod OS disc shedding experiments at the peak of rod OS shedding with the merkd mice were virtually identical with those in RCS rats—a gross reduction in the level of rod OS disc shedding and phagocytosis by the RPE cells, with the same degree of uncertainty in the identification of the
very few possible phagosomes seen. Thus, in both RCS rats and \( \text{merkd} \) mice, it remains to be determined whether any residual normal phagocytosis of rod OS membranes by the RPE occurs in vivo. Despite this ambiguity, it is clear from the absence or near absence of phagosomes in the RPE of \( \text{merkd} \) mice and RCS rats that the tyrosine kinase Mer plays a major role in rod OS ingestion by the RPE in vivo.

A cascade of cytopathologic events occurs in RCS rats that ultimately leads to PR cell death. This sequence of events has been assumed to result from defective phagocytosis by the RPE. First, membranous whorls form in the OS layer adjacent to the RPE surface\(^1\)\(^9\),\(^4\)\(^4\); second, OS disc synthesis continues\(^1\)\(^9\),\(^4\)\(^4\) transiently producing longer than normal OS\(^2\)\(^9\),\(^2\)\(^0\),\(^2\)\(^1\),\(^7\)\(^6\) and increased rhodopsin content in the eye\(^2\)\(^0\),\(^2\)\(^1\),\(^7\)\(^6\); third, the OS degenerate into a membranous debris zone; and fourth, PRs degenerate (for as yet unknown reasons), with preferential

**Figure 11.** Time course of ERG amplitude changes in \( \text{merkd} \) mice. (A) Representative scotopic and (B) photopic ERG recordings from a WT mouse at P30 (top waveform) and from \( \text{merkd} \) mice at different ages are shown. Recordings were made in response to a standard flash of 0.4 log cd sec/m\(^2\). Scotopic a- and b-wave amplitudes are lower than normal at the earliest age tested (P20) and rapidly decline with age. Photopic amplitudes are normal until P50 before rapidly declining with age; (C) Representative scotopic ERG waveforms from WT (left) and \( \text{merkd} \) (right) mice at P56. The STR (large arrows) and b-wave (\( \star \)) thresholds are elevated 1.5 and 2.5 log units, respectively, and b- and a-wave amplitudes are reduced in the \( \text{merkd} \) mouse. Arrows: stimulus onset. (D) Horizontal hatched zones: delineation of WT mean ± SEM amplitudes for scotopic b-waves (top zone), scotopic a-waves (middle zone), and photopic b-waves (bottom zone). Scotopic b-wave and photopic amplitudes are measured in response to a standard flash of 0.4 log cd sec/m\(^2\). Scotopic a-waves are in response to a bright flash of 2.4 log cd sec/m\(^2\). Mean amplitudes for (\( \star \)) scotopic b-waves, (\( \nabla \)) scotopic a-waves, and (\( \blacklozenge \)) photopic b-waves for \( \text{merkd} \) mice. Whereas scotopic amplitudes are lower than WT at the youngest ages tested, photopic responses remain normal until P50 before rapidly declining. The ONL thickness (\( \odot \)) decline in \( \text{merkd} \) mice at a rate slower than the rate of loss of ERG amplitude. The modified ONL thickness (\( \bigcirc \)) reflects only nonpyknotic nuclei, which decline at a rate comparable to the rates of loss of ERG amplitude.

**Figure 12.** Amplitude versus stimulus intensity curves for scotopic (diamonds) and photopic (circles) b-waves in WT (filled symbols) and \( \text{merkd} \) (open symbols) mice at different ages. (A) At P20, the maximum scotopic b-wave amplitude is significantly lower than WT, whereas photopic amplitudes are normal. At P53 (B) and P56 (C) both scotopic and photopic amplitudes are lower than WT. (D) By P40 neither scotopic nor photopic responses are measurable in most \( \text{merkd} \) mice. Mean ± SEM amplitudes are shown.
Retinal Dystrophy in *Mer* Knockout Mice

Certain temporal aspects of the retinal degenerations differ between the two types of rodent models. Pigmented RCS rat retinas degenerate at a slower rate than those of pink-eyed RCS rats. The onset and rate of degeneration in the *mer* mice, which are pigmented, are similar to and even slightly more rapid than those of pigmented RCS rats (Fig. 3A). The temporal relationship between PR cell loss, debris membrane loss, and rhodopsin loss also differs between *mer* mice and RCS rats. In pink-eyed RCS rats, some OS debris persists for 2 to 3 weeks after the loss of most PR cell nuclei. When the pink-eyed RCS rats are dark-reared or when pigmented rats are raised in cyclic light, an even greater amount of debris remains for several months after the loss of most PR nuclei. As noted, pigmented *mer* mice showed a rate of PR loss similar to that of pink-eyed RCS rats. However, the OS debris was lost concomitantly with the PR nuclei and did not persist for any substantive period, at least in *mer* mice raised in cyclic light.

The reasons for the temporal differences between the mice and rats are unclear, and in fact, given the similarity of the other phenotypic characteristics, a significant persistence of the OS debris would have been expected in *mer* mice because of their eye pigmentation. Some possible explanations include (1) species differences between rats and mice such as more rapid development and degeneration in mice, (2) differences in retinal irradiance due to eye size or to the influence of eye pigmentation, (3) differences in background genes that may regulate the degenerative response to light, (4) differences in stability of rhodopsin in the degenerating membranes, (5) differences in the ability of invading phagocytes to remove the OS debris, or (6) a combination of these factors.

**Persistent Pyknotic Nuclei**

A remarkable feature of *mer* mice is the persistence of pyknotic nuclei at all stages of retinal degeneration, with some coalescing and forming large masses of heterochromatin in the ONL. This unusual phenomenon is also seen in RCS rats, but not in other hereditary retinal degenerations, including rd/rd, *nr/nr*, *pcd/pcd*, *rds/rds*, or transgenic rats with rhodopsin mutations (Fig. 4A), or in light-induced degenerations.

Thus, the persistence of pyknotic PR nuclei appears to be unique to retinal degenerations caused by mutations in *Mer* tyrosine kinase. In all PR degenerations examined to date, including that in RCS rats, PR cell death occurs through the process of apoptosis. It is generally accepted that the half-life of apoptotic nuclei is relatively short, and therefore it is surprising that pyknotic nuclei persist in rodents with mutations in *Mer* tyrosine kinase.

It is likely that delayed clearance of pyknotic PR cells from the ONL is a direct consequence of a generalized defect in phagocytosis of postnatal apoptotic cells in *mer* mice. The *mer* mutation has already been shown to cause abnormal clearance by macrophages of dying thymocytes and lymphocytes. Pyknotic PR cells are most likely cleared by migrating phagocytes, either blood-derived monocytes or activated microglia, and *Mer* is known to be expressed in the monocyte/macrophage lineage, as well as a number of other cell types throughout the body. Defective clearance of apoptotic cells in *mer* mice results in increased reactivity to self...
antigens. Humans with retinal degenerations due to MERTK mutations may have as yet unrecognized systemic defects in the clearance of apoptotic cells, which could predispose toward autoimmune disease.

Earliest Phenotypic Expression of the Mer Mutation

Large vacuoles in the OS zone were seen at most ages during the course of retinal degeneration in mer<sup>rd</sup> mice. This feature also has been observed in RCS rats (LaVail MM, unpublished observations, 2000) and is evident in several well-fixed histologic preparations of RCS retinas. Such vacuoles are not regularly observed in several other retinal degenerations, including P<sup>cal/P<sup>cal</sup>, rd<sup>nr/nr</sup>, rd<sup>rd</sup>, or rd<sup>rds</sup>/rd<sup>rds</sup> mutant mice or in transgenic rodents with rhodopsin mutations (LaVail MM, unpublished observations, 2001). The vacuoles in mer<sup>rd</sup> mice and RCS rats appear similar to “exploded” OS—fixation artifacts that result from abnormal osmotic conditions—but the vacuoles were observed in well-fixed mer<sup>rd</sup> tissues and very few were seen in WT tissues. Similar OS vacuoles have been demonstrated in mice that have no interphotoreceptor retinoid-binding protein (IRBP) at as early as 11 days of age and as late as 6 months of age. However, the vacuolated rod OS found at very early ages in RCS rats (and presumably mer<sup>rd</sup> mice) are apparently not due to a deficit in IRBP, because abnormalities in concentration or distribution of IRBP do not appear in RCS rats until P15 to P18, or later.

These facts suggest that complete loss of Mer function may lead to an abnormal osmotic environment around OS and that the vacuoles may result from interphotoreceptor matrix abnormalities, either in osmotic composition or in abnormal transport and recycling of fatty acids. The vacuoles were present at the earliest time when discrete OS were observed (P8) and, as such, they represent the earliest histologic abnormality that has been reported in animals with Mer mutations, occurring before other OS abnormalities and significantly before PR cell death. It remains to be determined precisely how Mer defects lead to vacuoles in the outer retina and what role, if any, the vacuoles play in PR cell death.

Conclusion

The mer<sup>rd</sup> mouse strain displays a retinal degeneration phenotype almost identical with that of the RCS rat. This mouse model may be particularly useful for studying the role of Mer in PR-RPE cell interactions, phagocytosis, and retinal degeneration, given the powerful tools available for forward and reverse genetic studies in mice.

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References


