Reduction of Phagosomes in the Vitiligo (C57BL/6-mi<sup>vit</sup>/mi<sup>vit</sup>) Mouse Model of Retinal Degeneration

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Purpose. The vitiligo (C57BL/6-mi<sup>vit</sup>/mi<sup>vit</sup>) mouse has a slowly progressing retinal degeneration, in which photoreceptor cell nuclei are gradually lost and the retinal pigment epithelium (RPE) is unevenly pigmented. The purpose of the present study was to assess the phagocytic ability of the RPE in the vitiligo mouse by determining whether and when a phagocytic burst occurs in affected mice and whether the number of phagosomes varies between control and affected animals.

Methods. Eyes of control and vitiligo mice 4 to 20 weeks of age were embedded in Spurr. Thin sections were cut and examined by electron microscopy to confirm the presence of phagosomes, particularly in the affected animals. Thick (1 μm) sections were cut, and quantitative morphometry was performed at the light microscope level. The length of RPE was determined, and phagosomes were counted in RPE cytoplasmic and microvillous areas. Data were expressed as phagosomes per 1000 μm.

Results. The vitiligo mouse has a peak phagocytic episode approximately 2 hours after light onset. The number of phagosomes in 4-week-old affected mice was significantly less than that in controls (13 phagosomes per 1000 μm compared to 30 phagosomes per 1000 μm). By week 8, the number was reduced to approximately 5 per 1000 μm. Phagosome number was not reduced further between weeks 8 and 20 in the affected animal. Macrophage-like cells containing pigment granules and phagosomes were observed in the subretinal space in areas where the rod outer segments had been separated from the RPE.

Conclusions. The vitiligo mouse RPE contains phagosomes, but there are significantly fewer than in controls. It is not known whether a defect in RPE phagocytosis is the direct cause of the retinal defect in this model. Invest Ophthalmol Vis Sci. 1994;35:3625–3632.

This article describes the assessment of the number of phagosomes in the retinal pigment epithelium (RPE) of the vitiligo (C57BL/6-mi<sup>vit</sup>/mi<sup>vit</sup>) mouse model of retinal degeneration. The vitiligo mouse has progressive depigmentation of the pelage and unevenly pigmented RPE. Hyperpigmented RPE cells frequently lie adjacent to cells with little or no pigment. Interestingly, pigmentation does not necessarily decrease with age in the RPE of affected animals, as it does in the skin. The vitiligo mouse also has a slowly progressing retinal degeneration. Photoreceptor cell nuclei are lost at a rate of approximately one row per month beginning 8 weeks after birth; rod outer segments (ROS) become severely disrupted and deteriorate much more rapidly than the inner segments. ROS are not measurable after approximately 4 months, whereas inner segments are similar in length to those of controls until 6 months of age; at 8 months they are reduced to half the length of control inner segments. Rhodopsin levels gradually decrease in the affected animal. Electoretinographic studies indicate that the b-wave amplitude is lost gradually in the mi<sup>vit</sup>/mi<sup>vit</sup> mouse in a manner that is strongly correlated with loss of the photoreceptor cells and rhodopsin.

The cause of retinal degeneration in the vitiligo mouse is not known, nor is it certain whether the...
pigment epithelium or photoreceptor cell is the primary site of the defect. The unusual RPE pigmentation has caused some researchers to hypothesize that a defect in this cell type precedes the photoreceptor cell degeneration. The numerous biochemical and physiological functions of the RPE cell are well known. One of the most important of these functions is the phagocytosis of rod and cone outer segments. Outer segments, or shed discs, are enveloped by the cytoplasmic extensions of the pigment epithelium and are then withdrawn into the main body of the RPE cytoplasm as membranous inclusion bodies, termed phagosomes. Mammalian photoreceptor cells typically renew their outer segments approximately every 10 days; thus, each RPE cell must phagocytize approximately 10% of the adjacent rod outer segments per day. An impairment of the phagocytic process can lead to retinal degeneration as seen in the Royal College of Surgeons (RCS) rat. It is not known if phagocytosis in the mi<sup>1<sup>/mi<sup>4<sup> mouse is normal. The uneven pigmentation of the RPE may be indicative of abnormal melanization alone, or it may suggest that several biologic functions of the RPE, including phagocytosis, have gone awry. The purpose of the present study was to determine whether phagosomes were present in the RPE of the mi<sup>1<sup>/mi<sup>4<sup> mouse, and if they were, to determine whether they differed in number from controls and from increasingly older affected animals.

MATERIALS AND METHODS

Animals

The C57BL/6-mi<sup>1<sup>/mi<sup>4<sup> mice used in this study were the offspring from our colony of breeding pairs. Age-matched, wild-type controls (C57BL/6 +/+ ) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were maintained in clear plastic cages and subjected to standard light (12-hour light/12-hour dark) cycles. Light levels measured from the bottom of cages ranged from 1.2 to 1.5 foot-candles (12.9 to 16.1 lx). Temperature in the room was 24° ± 1°C. Mice were fed a Purina High Fat rodent diet (Teklad, Madison, WI; minimum crude protein, 16.9%; minimum crude fat, 10.8%; maximum crude fiber, 2.18%). Care and use of mice adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Although Besharse and Hollyfield<sup>12</sup> reported that the peak phagocytosis occurs approximately 2 hours after light onset in normal (C57BL/6) mice, it is possible that mi<sup>1<sup>/mi<sup>4<sup> mice do not have a phagocytic peak or that they demonstrate a peak of phagocytosis at a different time in the light cycle. One group of 6-week-old mi<sup>1<sup>/mi<sup>4<sup> mice were killed 1 hour before light onset, at light onset, and at 1, 2, 3, 4, 7, 9, and 11 hours after light onset. Because this study indicated that the peak phagocytosis time had not shifted (Fig. 1), a second group of C57BL/6 and mi<sup>1<sup>/mi<sup>4<sup> mice were killed 2 hours after light onset. At 4, 8, 12, and 20 weeks of age, six eyes were obtained from mice in the affected group and six from mice in the control group.

Histologic Procedures

Animals were killed by CO<sub>2</sub>, and eyes were pierced through the cornea and fixed by immersion in a solution containing 2% paraformaldehyde and 3% glutaraldehyde in phosphate buffer, pH 7.4. While the eyes were submerged, the cornea, iris, and lens were removed. After overnight fixation, the eyes were post-fixed in 1% osmium tetroxide, dehydrated in a series of ethanol concentrations, and processed for embedding in Spurr (Electron Microscopy Sciences, Fort Washington, PA). Eyes were oriented so that 1-μm thick sections included a full length of retina approximately along the horizontal meridian that passed through the ora serrata and optic nerve in both temporal and nasal hemispheres of the eye. Sections were stained with toluidine blue. Thin sections were obtained and stained with uranyl acetate and lead citrate for electron microscopic confirmation that structures counted at the light microscopic level were indeed phagosomes. These observations were made using the Phillips 400 electron microscope (Einhoven, The Netherlands).

Microscopic Evaluation and Measurement Procedures

Measurements were made using a Zeiss WZ microscope (Oberkochen, Germany) equipped with a 63×
objective, a 10X eyepiece and a reticle calibrated with an American Optical Micrometer (Buffalo, NY). The total length of RPE was determined using the reticle, and phagosomes were counted along the full length of RPE and reported as the number of phagosomes per 1000 μm of RPE. The rationale for reporting phagosome counts in this manner was to allow comparison of the data to that of Besharse and Holyfield,12 who examined photoreceptor outer segment turnover rates in C57BL/6 mice, the same animals used as controls in the present work. Phagosomes were defined as densely staining bodies, located either in the pigment epithelium somas or in the microvilli, with a minimum diameter of at least 1 μm in the smallest dimension. Light microscopic assessments of phagocytosis adhering to criteria similar to these have been reported in studies of other models of retinal degeneration.13–16 To prevent investigator bias, the slides were coded before counting, and measurements were performed using a “single-blind” design. Each slide was counted twice, and an average number of phagosomes was obtained per eye. One group of coded slides, selected at random, was counted by a second investigator to make certain that the criteria for counting structures as phagosomes was employed uniformly.

**Statistical Analysis**

Statistical analysis used analysis of variance to determine whether the number of phagosomes differed between the mi^vlt/mi^vlt and control mice. A P value <0.01 was considered significant. Tukey’s paired comparison test was the post hoc statistical test.

**RESULTS**

Before determining the number of phagosomes over the course of the retinal degeneration, it was essential to determine whether a peak phagocytic burst occurred in the affected animals. Animals were studied at week 6 because the number of photoreceptor cell nuclei and the levels of rhodopsin are still similar to those of controls.4 Figure 1 provides the data obtained in a pilot study in which phagosomes were counted at various times during the light cycle. It is clear that the control mice demonstrated an increase in phagosome number within 2 hours of light onset. Similarly, vitiligo mice demonstrated a small peak in phagosome number 2 hours after light onset. The peak was not as pronounced in the mutant mice as it was in the controls. Nevertheless, the finding that the vitiligo mice had observable phagosomes, and in fact had a phagocytic peak, permitted systematic assessment of RPE phagocytic function in mutant mice of varying ages.

The data obtained by counting phagosomes in mi^vlt/mi^vlt and C57BL/6 mice at 4, 8, 12, and 20 weeks are shown in Figure 2. Based on the findings of the pilot study, animals were killed 2 hours after light onset. The control mice typically exhibited between 25 and 30 phagosomes per 1000 μm, regardless of the age examined. Statistical analysis indicated that there was not a significant difference in phagosome number among any of the control ages (ANOVA, F = 0.61, P = 0.62). Comparison of data obtained in the affected group with the control data indicated a significant difference in number of phagosomes between the two groups (ANOVA, F = 285.6, P < 0.0001). Within the vitiligo group, there were approximately 13 phagosomes per 1000-μm length of RPE in 4-week-old mice. By week 8, the number of phagosomes in the affected animals decreased to approximately 4 to 5 phagosomes per 1000 μm. There was not a marked decline in phagosome number in the mi^vlt/mi^vlt mice between weeks 8 and 20. There was, instead, a plateau in number of phagosomes detected in mice with the more pronounced disruption of ROS.

Figure 3 shows a light micrograph of the RPE–ROS region of a 20-week-old control animal. The ROS are attached to the RPE. The RPE is uniformly pigmented, and nuclei tend to be oval. Phagosomes are clearly seen in the area of the microvillous processes and are more prominent in the apical RPE than in the basal areas. Regardless of age, phagosomes were present and easily counted in control animals. Figure 4 provides an electron micrograph of the RPE–ROS region from a control retina, in which the microvillous process of the RPE has engulfed a recently shed disc, thus forming a phagosome. The inset of the figure provides an even higher magnification of the process.

Figure 5 shows the ROS–RPE area of a 4-week-old mi^vlt/mi^vlt mouse. The outer segments are adjacent to the RPE, although they do not appear to be juxta-

![Figure 2](image-url)
FIGURE 3. Light photomicrograph of ROS and RPE of a C57BL/6J control mouse 20 weeks of age. Arrow points to a phagosome (original magnification, X1512). ROS = Rod outer segments, RPE = retinal pigment epithelial cells.

posed as tightly to RPE as in controls. It should be mentioned that in retinas of some of the 4-week-old mice, portions of ROS were actually separated from the RPE. At week 4, the RPE nuclei appear somewhat rounded and some RPE areas have lighter pigmentation than others. There are phagosomes present in the RPE that are observed in the microvillous area as well as in the basal area of RPE cytoplasm. An electron micrograph of phagosomes in the microvillous region of the RPE of a 4-week-old vitiligo mouse is shown in Figure 6.

Figure 7 shows the ROS-RPE region of a 20-week-old mi"/mi" mouse. In some instances, the nuclei of the RPE are round, but in others, they appear cre- nated. The RPE pigmentation is variable but is not noticeably less than that of younger animals. In many areas of the vitiligo retina, particularly in the posterior region, the ROS are disrupted severely by week 20, preventing their orderly attachment to the RPE. In spite of the ROS-RPE separation, phagosomes are present, although their numbers are greatly reduced. Often, the phagosomes are located more basally in the RPE cells, rather than apically located as in controls.

Closer inspection of the disrupted ROS-RPE area by electron microscopy (Fig. 8) revealed varying lengths of outer segments that were lying on the processes of the RPE. These structures were clearly not phagosomes but were outer segments that had not been phagocytosed. The outer segments were adjacent to RPE processes that were rounded or vesiculated rather than within elongated microvilli typical of normal RPE.

One feature observed as the degeneration progressed in the vitiligo mouse retina was the influx of macrophage-like cells in the subretinal space (Fig. 9). These cells occasionally contained phagosomes that were not included in the counts for this study. The cells were of varying size and often contained pigment granules. Whether they were from the RPE, the neural retina, or the circulation is not known. Interestingly, areas of RPE cells that were close to the areas where the macrophages were present frequently contained nuclei that were crenated.

DISCUSSION

There are three important findings of the present study. First, phagosomes are present in the mi"/mi" RPE. There is a measurable peak of phagocytosis in the mi"/mi" mice that appears during the first 2 hours of light onset, the time characteristic of peak phagocytosis in normal mice. Second, the number of phagosomes in vitiligo mice is less than in controls. Even as early as 4 weeks, the number of phagosomes in vitiligo mice is only approximately half the number in controls. This is an age at which the number of photoreceptor cell nuclei and the level of rhodopsin are comparable to those in normal mice. Third, between 4 and 8 weeks, the number of phagosomes is reduced markedly in the mi"/mi" mice to approximately 16% that of controls, but between 8 and 20 weeks the number of phagosomes does not diminish.

The observation that phagosomes are present is significant, because it distinguishes the vitiligo mouse retinal degeneration from the retinal dystrophy of the RCS rat, the hallmark of which is virtual absence of phagocytosis of shed outer segment discs by RPE.10,11,17
FIGURE 4. Electron micrograph of apical area of RPE at the junction with ROS in a C57BL/6 control mouse. In the upper right corner, there is a phagosome (large arrow) being engulfed by the microvillous processes of the RPE. It is centered between the apical processes of two ROS (R). The inset provides a higher magnification (original magnification, X34,375) of the phagosome and the enveloping microvillous processes. There is another phagosome (arrow) present in the lower left corner immediately above the nucleus (N) (original magnification, X18,750).

firmed that the structures counted in the vitiligo mouse retina were indeed phagosomes. The uneven pigmentation of the RPE in vitiligo mice is striking and may prompt consideration of this cell as the primary site of the retinal defect. It is possible that the RPE cell is defective in one of its many functions, but clearly it is capable of phagocytizing shed outer segment discs, albeit at a reduced rate.

Given that the number of photoreceptor cell nuclei in the mi"/mi" retina decreases over a period of months and that the ROS become disrupted, the decrease in number of older phagosomes was not surprising. However, it was not anticipated that at week 4, when the number of photoreceptor cells is similar to that in controls and ROS appear fairly normal at the light microscopic level, the number of phagosomes would be so greatly reduced. Three hypotheses are offered here to explain this observation: that RPE phagocytosis is partially impaired or that photoreceptor cell disc shedding is impaired; that the attachment between RPE and ROS is compromised; and that there is a systemic problem in this mouse, and the defect affects the retina secondarily.

With respect to the first hypothesis, one of the limitations of any assay that examines phagocytosis is that it is impossible to separate this process from disc shedding. That is, phagocytosis is always a reflection of the separation of the shed outer segment tips and their uptake as disc packets. Although the two processes are inextricably linked, there is a real possibility that the defect lies in only one of the two cell processes. Thus, RPE cells may be less efficient in phagocytizing shed discs, and this inefficiency may worsen with age in the mutant mouse. The presence of numerous macrophage-like cells in the subretinal space may be the physiological response to poorly functioning RPE cells. They may be assuming much of the RPE phagocytic function because they are mobile and can migrate to the area of the shed discs. Although not included in the phagosome count, these macrophage-like cells frequently contained phagosomes. However, a decrease in phagosome number could reflect an abnormality in photoreceptor cells, particularly in the shedding of outer segment discs. Clearly, a reduction in shed discs would result in fewer phagosomes. The elongated rod outer segments reported between weeks 4 and 12 may be indicative of such a defect. The processes of outer segment renewal and disc shedding have not been systematically evaluated in this model. Recently, photoreceptor cells of mi"/mi" mice were assessed for their ability to synthesize opsin in vitro; they were capable of this task for many weeks.

With respect to the second hypothesis, disruption of the attachment between ROS and RPE may be responsible for decreased phagosomes. Williams and Fisher demonstrated that shedding and phagocytosis is a contact-dependent process. One of the most noticeable features of mi"/mi" retinas between 2 and 4 months of age is the gross disruption of ROS. The contact between ROS and RPE is fragile when standard histologic fixation methods are used. The separation is more pronounced in the posterior retina, whereas the peripheral retina is frequently spared. There is a progressive detachment of the outer segments as the disease worsens. Interestingly, the electron microscopic evaluation of areas of gross disruption revealed that in some areas, variable lengths of rod outer segments (not phagosomes) lay on the disrupted microvillous processes. This observation may suggest that contact between rod outer segments and the RPE microvillous process is not sufficient to induce phagocytosis in the vitiligo mouse.

Finally, a hypothesis regarding a systemic defect in this mouse must be considered to account for phagosome reduction. The mi"/mi" mutation maps mouse chromosome 6 at or near the microphthalmia locus. Recently, mutations at this locus have
been shown to be associated with a defect in a gene encoding a novel basic–helix–loop–helix–zipper protein,22 which is part of a family of DNA transcription factors. It may be that such a mutation affects a process common to several cells, including those of the skin and the retina. Hence, the expression of the microphthalmia mutation may be far-reaching in its effects. If this is the case, then the retinal pigment epithelial cells and the photoreceptor cells could react secondarily to a primary defect elsewhere in the body. Recent work in our laboratory has demonstrated that retinoid metabolism is perturbed in the vitiligo mouse.23 The initial work was performed in retina, and it was clear that interphotoreceptor retinoid-binding protein and retinyl palmitate were elevated. The elevation of retinyl palmitate occurred in the RPE and was not due to some aberrant accumulation in the neural retina. However, further studies of retinoid levels in plasma and liver revealed that retinyl palmitate levels were also higher in liver of the affected mice, although
Figure 8. Electron micrograph of RPE microvillous area in mi<sup>vc</sup>/mi<sup>vc</sup> retina. The microvillous processes of the RPE are not elongated; instead, they are small vesiculated structures (small arrow points). Furthermore, they do not surround the ROS. Various lengths of outer segments (wide arrows) lie adjacent to the microvillous area (original magnification, ×11,550).

If the plasma levels were normal. These findings may support the hypothesis that the defect in the vitiligo mouse is systemic rather than specific to the retina. It is possible that the elevation of retinyl palmitate in the eye has an effect on rod outer segment disc production rate, although this has not been investigated. Furthermore, the finding that retinyl palmitate is elevated may implicate a defect in lecithin retinyl acyl transferase, because retinyl palmitate is the lecithin retinyl acyl transferase substrate for the production of 11-cis-retinal. No systematic study of this enzyme has been reported in the mi<sup>vc</sup>/mi<sup>vc</sup> mouse.

Figure 9. Photomicrograph of macrophage-like cells (wide arrows) in the area of disrupted subretinal space of a mi<sup>vc</sup>/mi<sup>vc</sup> mouse 20 weeks of age. Proper outer segments in this area are nonexistent. Some RPE cells contain crenated nuclei (thin arrows) (original magnification, ×846).

Which, if any, of these hypotheses is correct remains open for investigation. Clearly the present work has demonstrated that the vitiligo mouse RPE cells can phagocytize shed outer segment discs, although at a considerably reduced rate.

**Key Words**
retinal degeneration, phagocytosis, retinal pigment epithelium, vitiligo, microphthalmia

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**References**


