Refractile Bodies in the Inner Segments of Cones in the Aging Human Retina

Gail S. Tucker

Refractile inclusion bodies (~0.80 μm in size) were found in the inner segments of cone photoreceptors in the aging human retina. They were easily resolved with the light microscope. They were never seen in rods, and occurred primarily in retinas from eye donors older than 40 years of age. The incidence of these inclusion bodies is related significantly to age (they occur more frequently with increasing age) and to sex (they are more likely to occur in the aging female than in the aging male). They were often smaller in size and fewer in number in the cones of males compared with females, and in males, fewer cones contained the RB than in females. Electron microscopy revealed that these inclusions are membrane-bound organelles having granular, fibrous, and tubular subcomponents. The occurrence of the RB appears to be unrelated to specific disease processes, medications in use at the time of enucleation, or specimen preparation times. Invest Ophthalmol Vis Sci 27:708–715, 1986

Several types of cellular inclusion bodies have been described in the retinas of animals and man. For example, under certain conditions, visual cells in the cat, rat, frog, goldfish, and ground squirrel contain autophagic vacuoles.1 Remé and Young2 demonstrated that autophagic vacuoles in the photoreceptors of the cone-rich ground squirrel retina are associated, during hibernation, with a pronounced loss of other cellular organelles. These changes were found to be reversible. Autophagic vacuoles have also been reported in the perinuclear cytoplasm of foveal cones in four elderly patients and one younger male patient diagnosed with retinitis pigmentosa.3–6

Villegas7 described the occurrence of small (0.8 μm) organelles in the perinuclear cytoplasm of human foveal cones near the outer limiting membrane. She suggested that these organelles were Stage 3 melanin granules (after Moyer, ref 8). Villegas did not specify the age or sex of the donor(s) in which they were found, other than to say that the six retinas included in her study were from patients “38 years old” and “over 60 years of age.”

The present study (see also ref 9) documents the occurrence of refractile inclusion bodies (RB) in the basolateral inner segment (IS) of cones in the retina of aging human males and females, although they also occur in the perinuclear cytoplasm of cones in males. Their incidence is shown here to be related significantly to both age and sex.

Materials and Methods

Specimen Preparation

Human retinas included in this study were obtained through the Florida Lions Eye Bank Donor Program. In addition, slides were studied which had been prepared from an eye removed surgically from a 70-year-old woman and fixed immediately in the operating room. This patient had diagnosed choroidal melanoma with consequent cystoid macular edema.

Whole donor eyes were injected superiorly at the limbus with 10 cc of buffered glutaraldehyde (5%; 0.1 M PO₄ at pH 7.40) using a disposable syringe equipped with a 19-gauge needle. A second 19-gauge needle just superior to the optic nerve head penetrated into the vitreous cavity, serving as a drain for the vitreous as fixative was injected under pressure into the eye. The eye was then immersed in glutaraldehyde for several hours after which the anterior chamber and lens were cut off and most of the remaining vitreous was removed. The standard procedure in this laboratory is to prepare wedge-shaped specimens cut from the eye-cup superiorly, inferiorly, and nasally, with each piece containing a tab of the optic nerve head. A larger rect-

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Table 1. Refractile body prevalence: Age in decades (years)

<table>
<thead>
<tr>
<th></th>
<th>1 (Birth to 9)</th>
<th>2 (10 to 19)</th>
<th>3 (20 to 29)</th>
<th>4 (30 to 39)</th>
<th>5 (40 to 49)</th>
<th>6 (50 to 59)</th>
<th>7 (60 to 69)</th>
<th>8 (70 to 79)</th>
<th>9 (80 to 89)</th>
<th>10 (90 to 99)</th>
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<tbody>
<tr>
<td>Number of Males*</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>+ (16)</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>− (20)</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Number of Females*</td>
<td></td>
<td></td>
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<td>1</td>
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<td>2</td>
<td>5</td>
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<td>1</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* A plus sign in this table and in Table 3 indicates that the specimen was positive for RB; a minus sign indicates that no RB were seen in that retina.

Angular piece is then cut temporally to 5 mm beyond the macula, and also contains a tab of the nerve head. This precaution during specimen preparation reduces the incidence of artifactual retinal detachment at the macula. The specimens are then post-fixed for 1–2 hr in buffered OsO₄ (2% in 0.1 M PO₄ at pH 7.40) and dehydrated in graded alcohols and propylene oxide for embedment in epoxy resins. Blocks were trimmed and sectioned on glass for light microscopy or on a diamond knife for electron microscopy.

Statistical Analyses

In order to determine whether the RB occurred preferentially in specific groups of the study population, their prevalence was analyzed statistically using the Wilcoxin Rank Sum Test and Fisher’s Exact Test (two-tailed). Age and sex were tested for significance as independent variables in a logistic regression model. Table 1 gives a tally of the specimens studied and the distribution of the donors by age and sex.

Medical Histories

The medical histories were examined to determine whether any finding might be associated consistently with the prevalence of the RB in the affected eyes. Medical histories obtained for most of the donors included a questionnaire responded to by the physicians of record, hospital charts, and the death certificate. When available, autopsy reports were also studied. The course of illness and therapy prior to death were evaluated, and included the final diagnosis; duration of the final hospitalization; surgical history; kind, dosage, and duration of administration of medications; medical and ocular diseases. In some cases, the physicians of record were interviewed directly to obtain additional information about their patients. The time of death and the elapsed times before enucleation and fixation were recorded and corroborated when possible by careful examination of these records.

Light Microscopy

Sections were taken of 89 blocks (73 eyes from 63 donors) from the temporal retina at the macula (73), in the mid-peripheral retina (superiorly, 5 blocks; inferiorly, 2; nasally, 5), or in the far peripheral retina (4 blocks). The trimmed face of the block was 1–2 mm wide. Ten to 15 slides containing contiguous pairs of 1-μm and 5-μm thick sections were prepared from each block, encompassing about a 0.5-mm wide field of retina at each site examined. The mounted sections were stained on a hotplate using 1% toluidine blue in aqueous 1% sodium borate, or 1% aqueous paraphenylenediamine. The sections were then cover-slipped, and examined and photographed using a Zeiss (Carl Zeiss, Inc., Oberkochen, West Germany) light microscope (Neofluor objectives; ×25, ×40, and ×100).

The retinas were scored for the presence or absence of refractile inclusion bodies in the inner segments. For ease of discussion, those specimens in which RB were observed are referred to as “affected”, whereas those which lack refractile bodies are referred to as “unaffected.” At no time during scoring of the light microscope material did the observer know the age or sex of the coded specimen being examined. However, relative rod and cone distributions or thinning of the retina at the foveola made some retinal locations apparent in the specimens examined. Otherwise, retinal location was also unknown at the time of the light microscope evaluation.

Electron Microscopy

Following light microscopy, 21 blocks from affected (8 eyes) and unaffected (8 eyes) specimens were selected for ultrastructural analyses so that those observed in the electron microscope included a cross-section of donors of different age (6–92), race (3 blacks, 13 whites) and sex (8 males, 8 females). Retinas from all quadrants of the eye were studied. Thin sections were stained with ethanolic (50%) saturated uranyl acetate and with lead citrate, and examined in a JEOL 100CXII electron microscope set at 60 kV.
Table 2. Medical Histories*

<table>
<thead>
<tr>
<th>Causes of death (number)</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholism (2)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aneurisms (1)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Atherosclerotic heart disease (11)</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Automobile accident/trauma (7)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cancer (10)</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Diabetes (4)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Emphysema (2)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Endocarditis (1)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pneumonia/respiratory failure (5)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Sepsis post-surgery (1)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Other (Parkinson's, gunshot wounds, trauma, Guillaume barre, brain hemorrhage; 8)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Other heart disease (7)</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

* Medical Histories were incomplete for the remainder of the specimens studied.

Results

General

The presence of RB in the IS is unrelated to the cause of death, medications administered either chronically or acutely, or the known systemic disease(s) of the donor (Table 2). The presence of the RB is unrelated to the time of death (Table 3), enucleation time postmortem or fixation delays post-enucleation.

Statistical Analyses

The prevalence of the RB with increasing age is statistically significant \(P = 0.003\) by the Wilcoxon Rank Sum Text. The RB clusters were seen in 32 of 47 retinas from donors older than 40 years of age, but in only 4 of 12 retina from donors younger than 40 years of age. The median age of donors with RB was higher (67 years) than the median age of donors lacking RB (41 years).

The difference in prevalence of the RB in males (44%) and females (74%) was also statistically significant \(P = 0.035\). The retinas from 16 of 36 males contained refractile bodies, while the retinas from 20 of 27 female donors contained RB.

The significance of these findings was confirmed using a logistic regression model. When age and sex are both taken into account in that model, they are both statistically significant.

Light Microscopy

The photoreceptors were classified as rods or cones based on the appearance of their IS and other features. The IS containing RB were broader and longer than neighboring granule-free IS in the same location. These findings, together with the observation that proportionately more cells in and near the foveola (except in the deepest portion of the pit) contain the RB, allow one to conclude that they are present only in the cone photoreceptors.

The RB refract (Fig. 2b) violet to reddish blue with phase optics in tissue stained with toluidine blue, but are deep blue with ordinary transmitted light (Fig. 1); the RB are refractile and stain brown with paraphenylenediamine (Fig. 2a).

The position of the RB may be different in males and females. In males, the RB are usually localized in the proximal IS at the outer limiting membrane (Figs. 2b, d), or more proximally in the perinuclear cytoplasm. In 19 of the 20 affected females the RB were located in the myoid of the cell, nestled amongst the ribosomes and RER but well away from the outer limiting membrane (Figs. 2a, c). In only one female were the RB located nearer the outer limiting membrane. Thus, the granules are positioned more proximally in the males than in the females. Except in the very center of the foveola, where cone profiles are relatively free of the inclusions (Fig. 1), every cone in a field contained RB in the retinas from female donors (Figs. 1, 2a, 2c). In the retinas from affected male donors however, many cone cell profiles were free of the RB (Figs. 2b, 2d).

Table 3. Refractile body prevalence: Times of death

<table>
<thead>
<tr>
<th>Time of Day</th>
<th>Number of Males</th>
<th>Number of Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:00-9:59 AM</td>
<td>+ (16)</td>
<td>+ (19)*</td>
</tr>
<tr>
<td>10:00-11:59 AM</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Noon to 1:59 PM</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2:00-5:59 PM</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6:00-7:59 PM</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>8:00-11:59 PM</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Midnight to 5:59 AM</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

* The eye enucleated for clinical reasons is not included in this Table.
The cones of the females tend to have more and larger RB than the cones of the males. However, the number and size of the RB appear to be unrelated to age. That is, the retina from an affected female in her forties might have more or larger RB in a cluster than the retina from an affected 60-year-old female.

In the 73 eyes studied here there is a remarkable uniformity in the distribution of RB throughout the retina of a particular donor. In a retina having many and large RB, the RB seem to occur in the same number, size, and location in all affected cells, in both eyes, and in several different regions from a single eye.

**Electron Microscopy**

In female donor retinas the RB are nearly always in the lateral aspect of the myoid in the IS near the rough endoplasmic reticulum, and in intimate association with the plasma membrane of the cell (Fig. 3), but the membrane of the RB is not continuous with the cisternae of the RER. Individual RB vary in size in different specimens. Occasionally, electron transparent or granular vacuoles are present in the cytoplasm near the RB (Figs. 3, 4). Free ribosomes occur throughout the cytoplasm near the RB.

Several RB may become segregated within a discontinuous membrane in the cytoplasm. The discontinuity of the enclosing membrane is thought to be a postmortem fixation artifact. These membrane-bound clusters are often surrounded by an organelle-free, granular halo (Fig. 4a). RB clusters were not observed in any of the eight males studied in the electron microscope.

**Discussion**

**General**

The presence or absence of RB in the inner segments of retinal cones is unrelated to the cause of death of the donor. Similarly, postmortem enucleation and fixation delays are not implicated as causative in their formation since they varied in the specimens examined, and are not relevant in the eye enucleated for clinical reasons, which was fixed immediately. The RB are often absent from cone cells in the center of the fovea.
Thus, the RB are commonly occurring organelles that are seen in aging females and males and which appear to form unassociated with specific pathologic states or enucleation or other delays.

The RB may form as part of degenerative physiological processes or by hormonal or other as yet undefined aging changes occurring in retinal cones. Even though the incidence of the RB is age-related, the relative number of RB in each cell, or whether or not they form clusters is unrelated to the age of the donor. Individual RB were not seen in retinas containing clusters of RB, and the membrane-bound clusters of RB were never seen in males. The RB appear to form unassociated with the circadian rhythms postulated for photoreceptor disc shedding in several animals and the human.15
Fig. 3. Retinal cone inner segment from a 68-year-old white female. Electron micrographs (Figs. 3, 4) demonstrate the ultrastructural appearance of the RB and the surrounding cytoplasmic organelles in the inner segments of affected cone cells. Calibration bars: in microns. Key: C, Cords of Refractile Bodies; GV, Granular Vesicles; IS, Inner Segment; M, Mitochondria; MD, Membrane Density; MT, Microtubules; N, Nucleus; OLM, Outer Limiting Membrane; RB, Refractile Body; RER, Rough Endoplasmic Reticulum; Small Arrowheads, RB Membrane; V, Clear Vesicles. a, RB adjacent to well-defined cisternae of the RER. The cytoplasm also contains clear vesicles (V) and granular vesicles (GV). b, A small cluster of RB that are free in the cytoplasm is shown. They are positioned several microns distal to the OLM.

Fig. 4. a, Low power view of the IS from a 72-year-old white female. Two adjacent cone IS contain clusters of membrane-bound RB enclosed within a second membrane (filled arrowheads, upper right and upper left of Figure) and surrounded by a moderately electron dense granular matrix. b, Higher power view of RB from a 60-year-old white female. Each RB is enclosed within a membrane (small arrowheads), but the cluster of RB is not surrounded by the granular halo shown in 4a. The dense contact here is distal to the OLM (not shown in this figure).
Light Microscopy

The RB are not located more distally in the IS of the older donor retinas when compared with their position in younger donor retinas. Therefore, progressive displacement of the RB toward the distal end of the cone cell IS does not occur with age. The variability in location of the RB in the male and female donor retinas appears to be a sex difference. In addition, membrane-bound clusters of RB were only seen in females. Because of these observed differences in their compartmentalization, it may be that the RB function differently in males and females. In affected males there were generally fewer and smaller RB than in affected females, and fewer of the cones in an affected male contained RB. The significance of these differences in the incidence, frequency, size, and location of RB in males and females is unclear at this time.

Rod cells never contained RB in any of the specimens examined. Except at the center of the foveola, there are more affected cells in the macula (which is known to be rich in cone cells) than elsewhere in the retina. As one might predict, in the periphery where there are fewer cones, fewer cells contain RB. These facts, taken together with the appearance of the affected IS (their size and shape, and the large number of mitochondrial clusters in a single IS profile), allow one to conclude that RB occur only in the IS of cone cells.

In the 70-year-old white female patient with a choroidal melanoma and cystoid macular edema, the photoreceptors appeared normal in tissue samples examined in pathology-free portions of the retina (Fig. 2a). Every cone in a field in this retina had a cluster of RB in the proximo-lateral portion of the IS near the plasma membrane. From the positive findings in this eye, one may conclude that enucleation and fixation delays in the Eye Bank donor eyes are unlikely to have initiated RB formation. Feeney et al.16 also found that fixation delays (2–9 hr) had no effect on the complement of specific organelles in the RPE of human donors.

The number and size of RB in an affected cone cell may be related to the race of the donor. There is a tendency in blacks for affected IS to contain more and larger RB than whites. Although this may represent a metabolic difference such as is known to occur in the formation of pigment granules in the RPE (there are more pigment granules in the RPE of blacks than whites), additional specimens from blacks need to be examined to determine the significance of this trend. Even considering this difference, cones from aging blacks and whites appear equally likely to contain RB with advanced age.

Electron Microscopy

The primary subunits of the RB are electron dense cords surrounded by a discontinuous membrane. Similar organelles were considered by Villegas to be immature melanin granules.7 In some specimens, clusters of RB were found in the present study to be embedded in a granular matrix and enclosed within a second discontinuous membrane. This heterogeneity of RB structure is reminiscent of descriptions given for autophagic vacuoles.3 Thus, the resemblance of the RB at the ultrastructural level to widely different organelles described in human cones points up the need for careful histochemical analyses of these structures.

The RB are localized in a portion of the inner segment containing many prominent cytoplasmic microtubules (Fig. 4b). The association of these microtubules with the RB may provide the structural basis for the localization of the RB within the inner segment. Understanding how they are localized is relevant to understanding the different position of RB in males and females.

Functional Significance

Autophagy occurs in response to cellular injury in the form of trauma, infectious disease, chemical toxicity, and as part of changing metabolic processes that accompany stress and/or aging, including hormonal changes.1 Nuclear and cytoplasmic organelles and specific chemical reactions in the cell may be affected by autophagy. The rough endoplasmic reticulum (RER) and Golgi may be swollen and distended; mitochondria may swell with an associated loss of normal cristal ultrastructure; and autophagic vacuoles may form in the Golgi Complex17 to associate with lysosomes in focal cytoplasmic digestion.1,2,17,18 RB clusters occur near profiles of both mitochondria and the RER, but the appearance and number of the energy-producing and synthesizing organelles in the IS is as normal as can be expected in postmortem retinal donor tissue. Therefore, the involvement of the RB in an autophagic process involving these organelles seems unlikely.

Cytoplasmic inclusions observed in the cone cells of donor retinas from retinitis pigmentosa patients3–6 have been described as autophagic vacuoles. The perinuclear localization of these organelles is different from that of most of the RB, and they are also ultrastructurally different from the RB (see especially ref 3, Figure 2b). Therefore, the RB are probably different from these organelles.

The anatomic findings suggest only that the RB are organelles that arise in retinal cones following some
change with age. The chemical nature of the RB is currently under investigation in this laboratory.

**Key words:** cones, aging, inner segments

**Acknowledgments**

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**References**