

Influence of Dietary Vitamin A on Autofluorescence of Leupeptin-induced Inclusions in the Retinal Pigment Epithelium

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Substantial evidence indicates that the major precursors for retinal pigment epithelial (RPE) lipofuscin are molecular components of the photoreceptor outer segments. Previous experiments have demonstrated that the retinoids required for transduction by the photoreceptors promote RPE lipofuscin fluorophore formation. Animals deficient in these retinoids accumulate lipofuscin in the RPE at greatly reduced rates. The protein components of the photoreceptor outer segments also appear to be involved in the formation of RPE lipofuscin fluorophores. When degradation of phagocytosed photoreceptor outer segment proteins by the RPE is blocked, this tissue rapidly becomes engorged with phagosome-derived inclusions that have fluorescence properties similar to lipofuscin. Experiments were conducted to determine whether the development of lipofuscin-like fluorescence in these inclusions was dependent on the availability of retinoids. Rats were fed diets containing vitamin A either in the form of retinyl palmitate (+A), which can be metabolically converted into the retinoids involved in vision, or retinoic acid (–A), which does not support visual function. After the retinas of the –A animals had been depleted of retinoids involved in vision, animals from both groups were given intraocular injections of the protease inhibitor leupeptin. Two days later, the amounts of lipofuscin-like autofluorescence from the RPEs of rats in each group were determined. Leupeptin treatment produced an increase in this fluorescence in RPEs of the +A animals, but not in the RPEs of the rats fed the –A diet, despite the fact that phagosome-like inclusions accumulated in the RPE in both dietary groups. This finding suggests that retinoids involved in the visual process are probably directly involved in RPE lipofuscin fluorophore formation.

Key words: lipofuscin; aging; protease inhibitor; retinoids; phagocytosis; photoreceptors; fluorescence; retina.

1. Introduction

An apparently universal feature of aging in mammalian species is the progressive accumulation of the autofluorescent pigment lipofuscin in the retinal pigment epithelium (RPE) (Wing, Blanchard and Weiter, 1978; Feeney-Burns, Hilderbrand and Eldridge, 1984; Katz and Robison, 1984), as well as in numerous other post-mitotic cell types (Sohal, 1981). Neither the mechanism for lipofuscin formation, nor the precise chemical composition of this pigment has been determined for any tissue. A variety of evidence indicates that the major precursors for RPE lipofuscin are molecular components of the photoreceptor outer segments. Ultrastructural examination of the RPE reveals the presence of inclusions that have morphological features of both phagocytosed outer segments and lipofuscin granules (Katz et al., 1982). The presence of these inclusions of intermediate structure suggests that some components of the phagosomes may be converted into the contents of lipofuscin bodies. Additional evidence supporting this possibility was provided by the discovery that when the source of outer segment material for phagocytosis was absent, the age-related accumulation of lipofuscin in the RPE

was greatly reduced (Katz et al., 1986; Katz and Eldred, 1989a). In the RCS rat, outer segment material accumulates adjacent to the RPE due to a phagocytic defect in these cells. This material was found to develop a lipofuscin-like fluorescence (Katz et al., 1986), further suggesting that lipofuscin fluorophores are derived directly from outer segment components.

Experiments have indicated that both outer segment proteins and photoreceptor retinoids may be involved in lipofuscin pigment and fluorophore formation. When rats were maintained on a diet lacking in the retinoid precursors of the visual pigment, RPE lipofuscin accumulation was greatly reduced (Katz, Drea and Robison, 1986). Retinoid deficiency also largely prevented the development of lipofuscin-like autofluorescence in the photoreceptor outer segment debris in RCS rat retinas (Katz, Eldred and Robison, 1987). Thus, vitamin A compounds appear to have a significant influence on RPE lipofuscin autofluorescence. Outer segment proteins also appear to be involved in the development of this pigment. When the degradation of outer segment proteins by the RPE was blocked by a protease inhibitor, the RPE rapidly accumulated outer segment-derived inclusions that displayed a lipofuscin-like fluorescence (Katz and Shanker, 1989). The present experiments were undertaken to determine whether the fluorescence of the

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RPE inclusions induced by protease inhibitor treatment was dependent on the availability of the retinoids involved in the visual process.

2. Materials and Methods

Animals and Diets

Male Fischer 344 albino rats were obtained at 21 days of age from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Upon arrival, the animals were divided into two treatment groups. One group was fed a synthetic diet that contained adequate levels of all nutrients known to be required by the rat (Katz, Drea and Robison, 1987); this diet (+A) contained vitamin A in the form of retinyl palmitate, which can be metabolized to all of the retinoids involved in the visual process, as well as the retinoids utilized by other tissues. The remaining rats were fed an identical diet, except that retinoic acid was substituted for retinyl palmitate (-A) (Katz, Drea and Robison, 1987). Retinoic acid can satisfy the metabolic requirements of most tissues for vitamin A, but cannot be metabolically converted into the retinoids involved in vision.

The animals were all housed in the same room under 12 hr/12 hr cyclic illumination. Illumination was provided by 75 W incandescent bulbs. Light levels were determined with a Lutron model LX-101 light meter with the probe placed face-up on the bottom of the animal cages. During the light phase of the lighting cycle, average illuminance measured at the cage bottoms was 10–20 lx. This level of illumination was low enough so that no apparent light-induced damage to the retinas occurred during the course of the experiments (Katz and Robison, 1986; Katz and Eldred, 1989b). Total darkness was maintained during each 12-hr dark cycle. The room in which the animals were housed was maintained at a constant temperature of 20°C. All investigations were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

Protease Inhibitor Treatment

The effect of protease inhibitor treatment on the RPE was evaluated after the animals had been fed the experimental diets for 26 weeks. After 26 weeks, the retinal rhodopsin content of rats fed the -A diet was less than 15% of that in animals fed the +A diet (Katz et al., 1991), although retinoid deficiency had not reduced the photoreceptor content of the retina (Katz et al., 1986). Between 1 and 2 hr after the onset of the light phase of the daily cycle, the rats were each anesthetized with a combination of ketamine (80 mg kg⁻¹ body weight) and xylazine (12 mg kg⁻¹ body weight). Each animal was then given an intravitreal injection of 150 mM NaCl in one eye and 0.6 µmol of the protease inhibitor leupeptin (synthetic hemisulfate: Sigma, St Louis, MO) dissolved in 150 mM

NaCl in the other eye. The total volume injected into each eye was 3.0 µl.

Quantitation and Photomicrography of RPE Lipofuscin-specific Fluorescence

Between 53 and 54 hr after administration of the intraocular injections, rats from each dietary group were killed via carbon dioxide inhalation and their eyes were immediately enucleated. Following enucleation, the corneas, irides, lenses and retinas were dissected from the eyes to be used for RPE autofluorescent pigment quantitation. The RPE lined the interior surface of the remaining eye tissue. This tissue was prepared immediately for examination and quantitation of RPE autofluorescent pigment content by fluorescence microscopy as described previously (Katz and Eldred, 1989a). Fluorescence measurements were performed with the assistance of a Zeiss Photomicroscope I equipped for epi-illumination. Fluorescent emissions were stimulated by light from a 100-W high-pressure mercury vapor source. The light employed for excitation was passed through Zeiss filter set 487705, which consisted of a 400–440-nm bandpass excitation filter, an FT 460 dichromatic beam splitter and an LP 470 barrier filter to block emissions below 470 nm from reaching the measuring device or camera. In addition, a 515-nm barrier filter was also placed in the emission light path. All measurements were performed using a Zeiss Neofluor ×40 objective with a numerical aperture of 0.75.

Light emitted from the specimens which has passed through the barrier filters was either viewed through the ocular lenses, transmitted to a photomultiplier mounted on the vertical viewing port of the microscope, or was photographed by diverting the emitted light to the camera port of the microscope. All micrographs were made using a uniform exposure time of 45 sec with Kodak EPD-200 film. Fluorescence intensity measurements were performed with a photometer consisting of a Hamamatsu R636 photomultiplier controlled by an American Instrument Co. (Silver Spring, MD) controller and amplifier. The amplified photomultiplier outputs were recorded on a Kipp and Zonen model BD-41 strip-chart recorder. Between ten and 15 separate measurements were made from different locations in the central region of each specimen. Each region from which a measurement was taken, was focused by viewing the specimen through the microscope ocular lenses. Immediately preceding the measurements on each specimen, a blank intensity measurement was made by recording the photomultiplier output with a portion of the slide not containing the specimen placed in the field of the objective. The emission intensity of a Perkin-Elmer solid fluorescence standard was also measured prior to analysis of each tissue sample. Using this standard, the relationship between the photon flux emanating from the specimens and the photometer response was

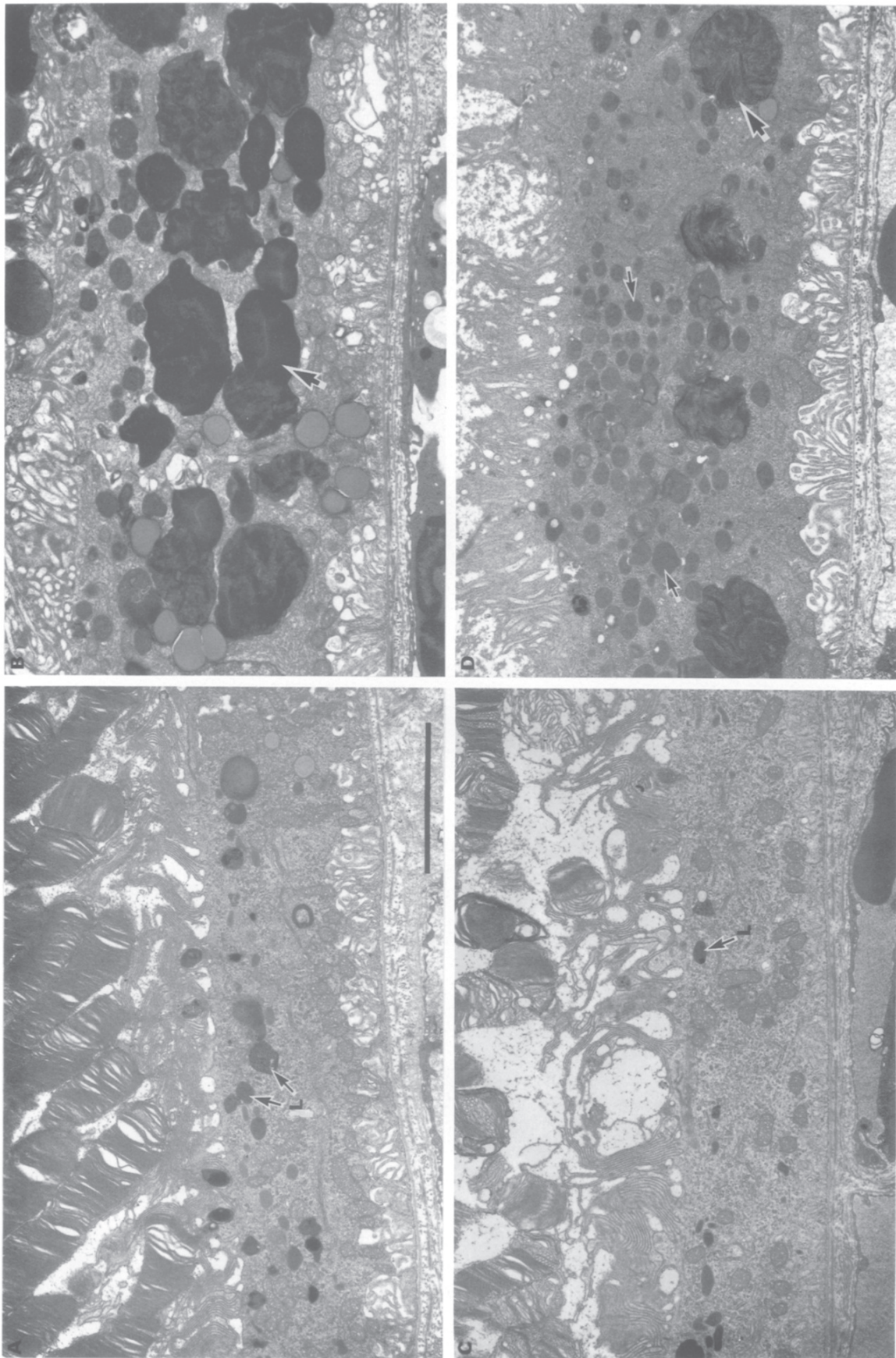


FIG. 1. Electron micrographs of the RPE from rats in +A, vehicle injected (A); +A, leupeptin injected (B); -A, vehicle injected (C); and -A, leupeptin injected (D) treatment groups. Leupeptin-induced inclusions in the +A rats were usually quite large [arrow in (B)], whereas both large and small leupeptin-induced inclusions were observed in the -A rats [arrows in (D)]. RPE lipofuscin (L) was more abundant in +A (A) than in -A (C) vehicle-treated eyes. Bar represents 3.5 μ m

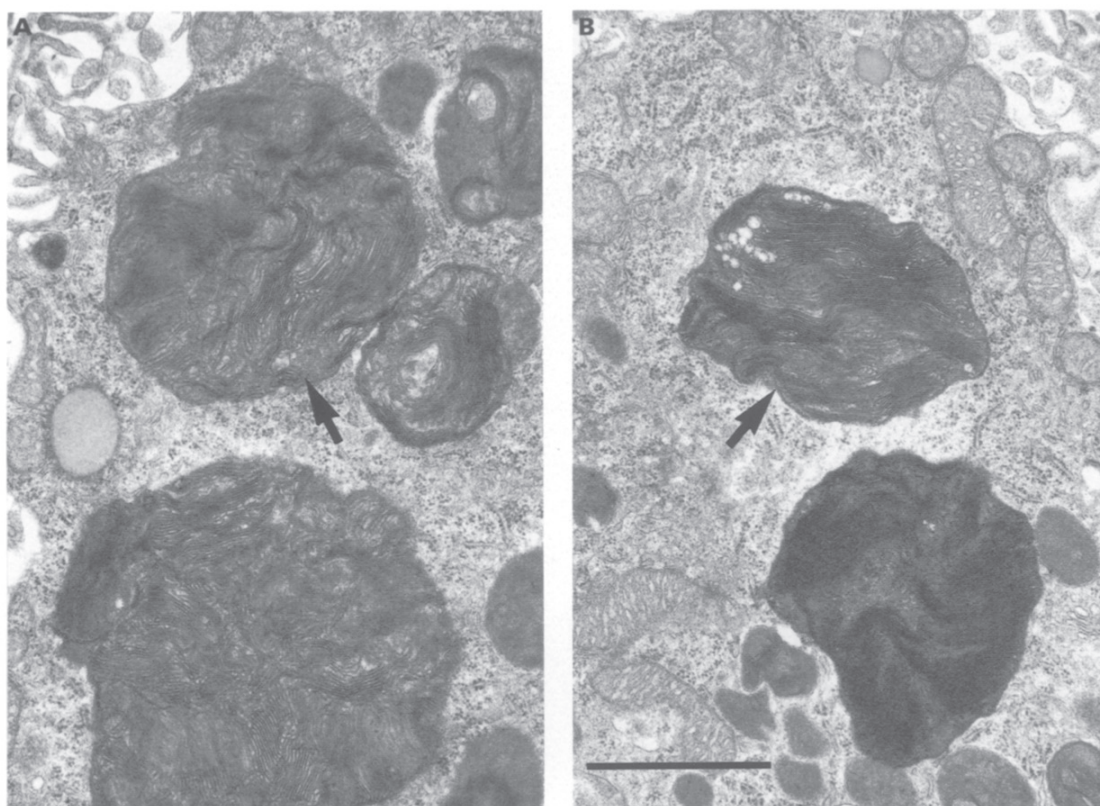


FIG. 2. Electron micrographs of large leupeptin-induced inclusions in the RPEs from +A (A) and -A (B) animals. Many of these inclusions could clearly be seen to contain material with the appearance of photoreceptor disc membranes (arrows). Bar represents 1.0 μm .

determined as described previously (Katz and Eldred, 1989a). This relationship was employed to convert the photometer readings obtained from measurements of the samples, blanks, and standard into relative emission intensities. In order to correct for any possible fluctuations in the intensity of the mercury source or in the sensitivity of the photometer, all blank-corrected fluorescence intensities were expressed relative to the standard fluorescence intensity. RPE fluorescence measurements were performed on eyes from five animals from each dietary group.

Ultrastructural Examination

Eyes from rats that had received the treatments described above were prepared for examination by electron microscopy. Animals were killed via carbon dioxide inhalation and their eyes were immediately enucleated and placed in a fixative consisting of 1.25% glutaraldehyde, 1% paraformaldehyde, 130 mM sodium cacodylate and 130 μM CaCl_2 at pH 7.40. While maintaining the eyes immersed in fixative, the corneas, irides and lenses were removed by dissection. The remaining portions of the eyecups were fixed for 24 hr at room temperature with gentle agitation. The tissues were then dissected to obtain regions each having one edge extending approxi-

mately 2 mm superior from the optic nerve head along the superior-inferior meridian. Sections for microscopic study were cut from these edges of the tissue after they had been post-fixed in OsO_4 and embedded in plastic. Post-fixation, embedding, sectioning and staining of the tissue samples were performed as described previously (Katz and Robison, 1984).

Statistical Analyses

Determination of whether leupeptin treatment and diet had significant influences on RPE lipofuscin-like fluorescence was accomplished using analysis of variance. Comparisons between individual treatment groups were performed using the Newman-Keuls procedure (Winer, 1971).

3. Results

Leupeptin-induced RPE Inclusions

Two days after the intraocular injections, the RPE cells of leupeptin-treated eyes from the +A rats contained many large inclusion bodies [Fig. 1(B)]. The ultrastructural appearances of these inclusions clearly indicated that they were derived from phagocytosed photoreceptor outer segments (Fig. 2). These large inclusions were never observed in the RPE of eyes from

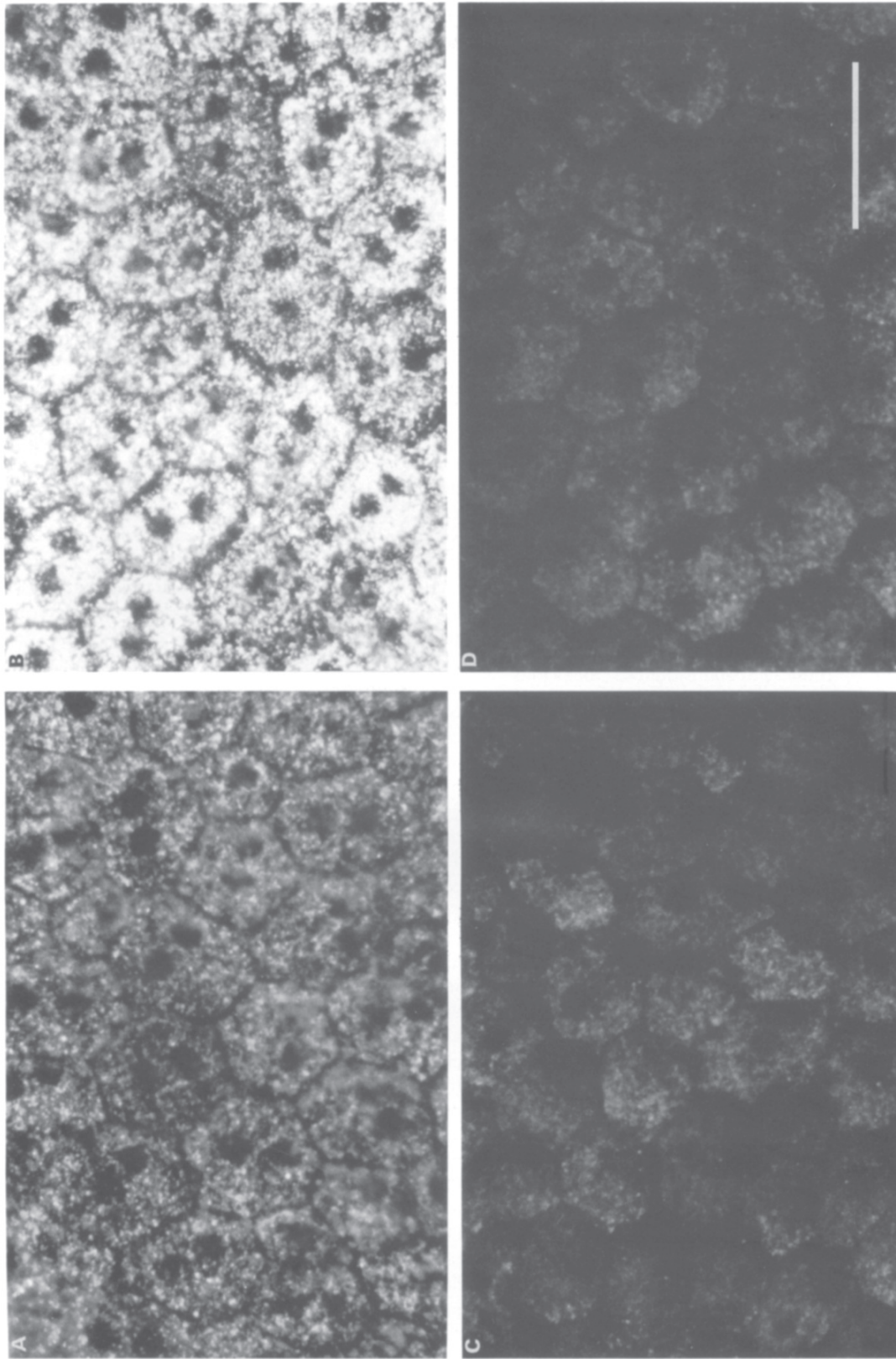


Fig. 3. Fluorescence micrographs of the RPE from rats in +A, vehicle injected (A); +A, leupeptin injected (B); -A, vehicle injected (C); and -A, leupeptin injected (D) treatment groups. Bar represents 60 μ m.

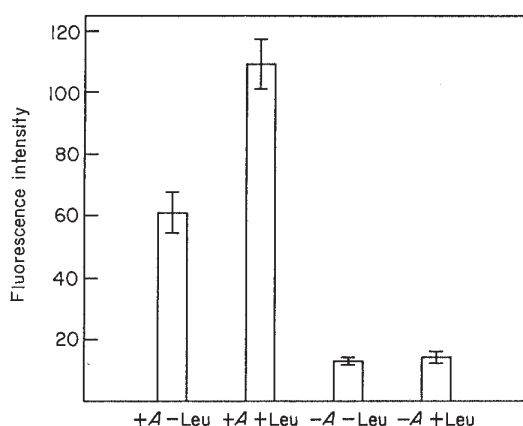


FIG. 4. Effect of dietary vitamin A (+A and -A) and of the protease inhibitor leupeptin (+Leu and -Leu) on RPE lipofuscin-like fluorescence intensity.

the +A rats that had been injected with the saline vehicle alone [Fig. 1(A)]. The vehicle-injected eyes did contain occasional phagosomes that were much smaller in size than the leupeptin-induced inclusions. In rats that had been fed the -A diet, leupeptin treatment also resulted in the appearance of inclusions in the RPE that appeared to be derived from phagocytosed photoreceptor outer segments [Fig. 1(D)]. Many of these leupeptin-induced inclusions were similar in size and ultrastructural appearance to those observed in the +A animals (Figs 1 and 2). Numerous smaller inclusions also appeared in the RPE cells of the -A rats treated with leupeptin [Fig. 1(D)]. The vehicle-treated eyes from the -A rats, like those from the +A animals, contained relatively few phagosomes or other electron-dense inclusions in the RPE [Fig. 1(C)]. In vehicle-treated eyes, less RPE lipofuscin was present in the -A animals than in the +A rats (Fig. 1). In both the +A and -A animals, the accumulation of leupeptin-induced inclusions in the RPE was accompanied by an increase in the height of the RPE cells (Fig. 1).

RPE Lipofuscin-like Fluorescence

As reported previously (Katz et al., 1986), dietary deficiency in the retinoids involved in the visual process substantially reduced the amount of lipofuscin-like fluorescence in the RPE (Figs 3 and 4). In vehicle-treated eyes, the lipofuscin-specific fluorescence intensity from the RPE of the -A rats was an average of 79% less than that from the RPE of the +A animals ($P < 0.001$). In the rats receiving the +A diet, the accumulation of leupeptin-induced inclusions in the RPE was accompanied by an increase of 80% in the mean RPE lipofuscin-specific fluorescence intensity ($P < 0.01$) (Figs 3 and 4). By contrast, leupeptin treatment in the -A animals produced no change in the intensity of lipofuscin-specific fluorescence from the RPE (Figs 3 and 4).

4. Discussion

Aging is the single most important risk factor for retinal pathology in developed countries (Ganley and Roberts, 1983). Age-related retinopathies, particularly macular degeneration, are responsible for significant visual impairment in numerous elderly individuals. It has been estimated that approximately 11% of Americans between 65 and 74 years of age suffer from age-related macular degeneration (Kini et al., 1978), and the incidence of this disease increases with advancing age. The mechanisms underlying age-related retinopathies are not understood, but it is believed that primary changes in the RPE might be responsible for degenerative changes in the adjacent neural retina and the supporting choroid (Hogan, 1972; Sarks, 1976; Young, 1981). One of the most pronounced changes undergone by the RPE during senescence is the accumulation of the autofluorescent pigment lipofuscin (Wing et al., 1978; Feeney-Burns et al., 1984; Katz and Robison, 1984). It is not known whether lipofuscin accumulation is detrimental to RPE cell function. However, developing an understanding of the mechanisms involved in lipofuscin formation may provide the means to determine whether this pigment, or the processes leading to its formation, are involved in age-related retinal disease.

Substantial evidence now suggests that photoreceptor outer segments that are phagocytosed by the RPE contain the major molecular precursors for RPE lipofuscin. In both animals and humans lacking photoreceptors, due either to light damage or to a genetic defect, RPE lipofuscin accumulation is drastically reduced (Katz et al., 1986; Katz and Eldred, 1989a; Katz and Rodrigues, 1991). In addition, RPE cells in culture that are not exposed to photoreceptor outer segments accumulate very little lipofuscin (Boulton et al., 1989). However, if these cells are allowed to phagocytose exogenously added outer segments, they quickly accumulate large amounts of lipofuscin-like pigment (Boulton et al., 1989). It appears that precursors for RPE lipofuscin fluorophores are probably components of the outer segments themselves, and that nothing from the RPE need necessarily be added for lipofuscin-like fluorescence to develop in the outer segments. In the RCS rat, the photoreceptor cells degenerate, and the remnants of the degenerating outer segments accumulate adjacent to the RPE. This outer segment material acquires the fluorescence properties of lipofuscin without being taken up by the RPE (Katz et al., 1987). The latter finding suggests that endogenous components of the outer segments react to form lipofuscin fluorophores, and that these fluorophores accumulate if the precursor molecules are not degraded by the RPE. This possibility is supported by the finding that blocking protein degradation by the RPE in vivo results in the rapid accumulation in these cells of phagosome-derived inclusions that develop lipofuscin-like fluor-

escence properties within a relatively short time (Katz and Shanker, 1989). The fact that fluorophore development occurred as a consequence of protease inhibitor treatment indicated that the protein components of the outer segments probably participate in RPE lipofuscin fluorophore formation.

It is doubtful that proteins alone are responsible for RPE lipofuscin fluorescence. What other type of compounds do the outer segments contain that could participate in the formation of lipofuscin fluorophores? Previous experiments have indicated that the age-related accumulation of RPE lipofuscin was substantially reduced when animals were fed diets lacking precursors of the retinoids involved in the visual process (Katz et al., 1986). In addition, the development of lipofuscin-like fluorescence in the outer segment debris in retinas of RCS rats was greatly reduced if the animals were fed diets lacking all forms of vitamin A that support the visual process (Katz, Eldred and Robison, 1987). Thus, it appears that outer segment retinoids probably participate directly in the formation of RPE lipofuscin fluorophores. If this is indeed the case, one would predict that development of fluorescence in the outer segment-derived RPE inclusions that form in response to protease inhibitor treatment would be dependent on the presence of these retinoids in the outer segments. As shown by the results of the current study, this is the case. After 26 weeks of retinoid deficiency, retinal rhodopsin content was reduced by over 85% (Katz et al., 1991). The retinoid-deprived animals did not show any increase in RPE lipofuscin-specific fluorescence in response to protease inhibitor treatment, despite the fact that outer segment-derived inclusions accumulated in the RPE. Animals that were fed a diet containing sufficient retinyl palmitate to maintain normal rhodopsin levels responded to protease inhibitor treatment with both the appearance in the RPE of inclusions derived from the outer segments, and with a substantial increase in RPE lipofuscin-like fluorescence.

The effect of retinoid deficiency on the development of fluorescence in the protease inhibitor-induced RPE inclusions did not appear to be due to a lowering of outer segment protein content. In a recent investigation, it was found that deficiency of the retinoids involved in vision did not reduce the density of the major outer segment protein opsin in the disc membranes (Katz et al., 1991). Thus, it appears that the retinoids involved in vision play a direct role in forming RPE lipofuscin fluorophores, perhaps by reacting with outer segment proteins to give fluorescent products. Experimental evidence suggests that such reactions between outer segment proteins and retinoids may involve oxidative processes. Rats fed diets deficient in antioxidant nutrients accumulate large amounts of a lipofuscin-like autofluorescent pigment in the RPE (Katz, Stone and Dratz, 1978; Robison, Kuwabara and Bieri, 1979; Katz et al., 1982). As with lipofuscin, the accumulation of this

pigment is greatly reduced when the availability of retinoids involved in vision is restricted (Robison, Kuwabara and Bieri, 1980; Katz et al., 1986). Based on the available data, it now appears that oxidation products of photoreceptor retinoids may react with outer segment proteins to generate the fluorophores characteristic of RPE lipofuscin. Since opsin is the most abundant protein in the outer segments, it is likely that this protein is involved in RPE lipofuscin fluorophore formation. However, other outer segment proteins, such as those involved in the transduction cascade, could also participate in the generation of RPE lipofuscin fluorescence. Further studies will be required to identify the specific protein precursors of RPE lipofuscin.

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