Deficiencies of Vitamins E and A in the Rat: Lipofuscin Accumulation in the Choroid

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The effects of vitamin E and A deficiencies on the formation of lipofuscin in the melanocytes and fibroblasts of the choroidal stroma and in the endothelial cells of the choriocapillaris were studied. Weanling female albino rats (Sprague-Dawley) were divided into three groups and fed purified diets adequate or deficient in vitamins E and A: +E, +A; "E, +A; "E, ~A. After 35 weeks, vitamin E deficient rats (~E, + A) exhibited increased lipofuscin-specific autofluorescence in the choroid compared to the controls (~E, +A). By electron microscopy and morphometric methods the choroidal stroma of vitamin E deficient rats displayed an increase in lipofuscin content as measured by the number of lipofuscin granules and their size, if compared with the controls. However, in vitamin E deficiency, only animals with a supply of vitamin A (~E, +A) showed higher amounts of lipofuscin in the choroidal stroma; animals deficient in both vitamins (~E, ~A) stayed on the same level as the controls (~E, +A). In the endothelial cells of the choriocapillaris, on the other hand, no significant increases in lipofuscin content were observed in either group of vitamin E deficient animals (~E, +A), (~E, ~A). Apparently vitamin E deficiency affects the choroid by increasing lipofuscin formation only in the melanocytes and fibroblasts. Vitamin A appears to play a role in lipofuscin formation. Invest Ophthalmol Vis Sci 25:429–433, 1984

Marked increases in the amounts of lipofuscin that accumulate in the retinal pigment epithelium of rats deficient in vitamins E and A have been well documented.1,2 Lipofuscin accumulations due to antioxidant deficiency and to aging have been reported for many tissues and have been reviewed recently.3 The possible cellular sources of this "wear-and-tear" pigment and various dietary influences on its accumulation have been subject to numerous investigations.3 Most of the studies dealing with lipofuscin accumulation in ocular tissues have been centered on the retinal pigment epithelium.1,2,4 The choriocapillaris, however, also displays lipofuscin-specific autofluorescence as a result of vitamin E deficiency.1 The source and precise pathogenetic mechanism leading to lipofuscin formation in the choroid remain unclear. The purpose of this study was to elucidate the influences of deficiencies in vitamins E and A on lipofuscin formation in the choroid. Using morphometric procedures the authors quantified the increase in lipofuscin granules separately for stromal tissue and endothelial cells, which led to some unexpected findings and a differential response of these tissue components to the dietary treatments.

Materials and Methods

Weanling, female, albino rats (Sprague-Dawley) were fed a purified diet that was free of vitamins E and A, yet high in polyunsaturated fatty acids.2 One week after this basal diet, 18 such animals were divided into three groups and fed diets adequate or deficient in vitamins E and A: +E, +A; "E, +A; "E, ~A. The +E, +A group received a supplement of 250 mg a-tocopherol-acetate (General Biochemicals, Cleveland, OH) and 2 mg of retinol per kilogram of diet which was in the form of stabilized microencapsulated retinyl palmitate (Nopco Chemical Co., Louisville, KY); the ~E, +A animals received only the retinol; the group deficient in vitamin E and in retinol (~E, ~A) was supplemented with retinoic acid (2 mg/kg diet), which maintains most tissues in a healthy state, but results in a vitamin A deficient retina.5,6 After 35 weeks on the diets all animals were killed with an overdosage of ether vapors.

The left eye of each rat was processed for tissue autofluorescence,1 whereas the right eye was prepared for ultrastructural studies.1,2 Lipofuscin specific autofluorescence was determined in the retina and in the attached choroid including the choroidal vessels by studying frozen sections 10 μm in thickness, mounted

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on slides in glycerol. Examination was carried out employing a Leitz Orthoplan microscope equipped with a mercury vapor lamp, a GB 38 heat filter, a 360 nm excitation filter, and a 530 nm emission cutoff filter. Sections (1.0 \mu m thick) of the Epon embedded tissue were stained with toluidine blue. Ultrathin sections were taken of the inferior nasal region of the right eye of each animal, frequently changing the section depth to obtain representative samples of the tissue. Transmission electron microscopy was performed with a JEM 100 B electron microscope following staining with uranyl acetate and lead citrate.

The final magnification of the micrographs was 15,000. Morphometric analysis was performed using a grid of squares printed on transparent plastic sheets with a distance among the intersections of 0.5 cm for the counts of lipofuscin granules and 0.75 cm for the determination of the area of cytoplasm. Counts were made separately for (1) melanocytes and fibroblasts as major elements of the stromal tissue of the choroid and for (2) endothelial cells of the choriocapillaris. When applied to the micrographs, the number of grid intersections overlaying the tissue component of interest is proportional to the area covered by this tissue component. The number of lipofuscin granules per animal, the number of grid intersections overlaying cytoplasm, and the number of grid intersections overlaying lipofuscin granules were counted. From these values, we calculated the number of lipofuscin granules per 100 square micrometers of cytoplasm and the average size of the granules. Statistical significance of the results was checked among the groups of treatment using analysis of variance and the Newman-Keuls pairwise comparison test. The utilization of animals in this experiment conformed to the ARVO Resolution on the Use of Animals in Research.

**Figs. 2, left; 3, right.** Numbers and relative sizes of lipofuscin granules in the choroidal stroma. Counts and measurements were made of 14 to 110 granules within a minimum of 400 \mu m² of fibroblasts and melanocyte cytoplasm per animal for 6 rats in each experimental group. Bars represent SEM.
Results

Fluorescence Microscopy

The choroid showed lipofuscin-specific yellow autofluorescence that was most striking in the vitamin E deficient animals ("E, + A") (Fig. 1). Doubly deficient animals ("E, - A") and the control animals ("E, + A"), by contrast, both displayed relatively weak autofluorescence. This observation, which gave a merely qualitative impression, has been confirmed by quantifying the lipofuscin granules on an ultrastructural level (see below).

Electron Microscopy

Stroma of the choroid. In the vitamin E deficient animals ("E, + A"), the number of lipofuscin granules per unit cytoplasmic cross-sectional area increased by threefold in the melanocytes and the fibroblasts of the choroid (Fig. 2) as compared with the control animals ("E, + A"), and the average size of the lipofuscin granules was doubled (Fig. 3). No significant increases in lipofuscin content were observed in the animals that were kept on a diet deficient in both vitamins E and A ("E, - A") (Figs. 2, 3). Lipofuscin content of this ("E, - A") group, although unchanged when compared to the controls ("E, + A"), was found significantly lower than the ("E, + A") group.

Endothelial cells of the choriocapillaris. Vitamin E deficient animals ("E, + A") showed no significant change in the number of lipofuscin granules per 100 square micrometers and also no change in their size, if compared to the controls ("E, + A") (Figs. 4, 5). Animals deficient in both vitamins ("E, - A") showed an elevated number of lipofuscin granules and a somewhat increased size of the granules, but none of these values was significantly higher than the controls ("E, + A"). Examples of lipofuscin granules in both the choroidal stroma and the endothelial cells of the choriocapillaris are given in Figure 6.

Discussion

Vitamin E deficiency has an influence on the formation of lipofuscin in the choroidal stroma, but not in the endothelial cells of the choriocapillaris. There was increased lipofuscin content in the melanocytes and fibroblasts of the choroidal stroma in animals kept on a vitamin E deficient diet ("E, - A"). This, however, holds true only when vitamin A is supplied; doubly deficient animals ("E, - A") were found to have significantly lower lipofuscin content in the choroidal stroma than animals with vitamin E deficiency alone ("E, + A") (Figs. 2, 3). This suggests an influence of vitamin A on lipofuscin formation in vitamin E deficient animals.

In the endothelial cells of the choriocapillaris, on the other hand, there was no clear influence of vitamins E and A on the formation of lipofuscin. No significant differences in endothelial lipofuscin granule size or number were observed among any of the dietary groups.

Various tissues accumulate lipofuscin granules in response to vitamin E deficiency.39 Although the origin and formation of lipofuscin is not understood fully, most of the available evidence suggests that lipid compounds become highly peroxidized, forming residual deposits that are essentially inert. Recent work10,11 indicates that vitamin A might contribute to lipofuscin formation. In the retinal pigment epithelium, dietary vitamin A levels influence the extent of lipofuscin accumulation in vitamin E deficiency.10 Our results in-
Fig. 6. Sample electron micrographs of lipofuscin granules (arrowheads) in the choroid: (The lipofuscin granules were defined ultrastructurally as bodies, ranging in size from 0.2 μm to 1.5 μm in diameter that were more electron-opaque than lipid droplets and contained nonhomogeneous material.) a, Choroidal stroma of a control rat ("E, "A). b, Choroidal stroma of a vitamin E deficient rat ("E, "A). c, Endothelial cell (E) of the choriocapillaris of a control rat ("E, "A). d, Endothelial cell (E) of the choriocapillaris of a doubly deficient rat ("E, "A) showing the only large lipofuscin granule found in this group. (×15,000).

dicate that lipofuscin formation in the melanocytes and fibroblasts of the choroid can be accelerated as the result of vitamin E deficiency only if retinol is present in the diet. Apparently vitamin A is able to influence lipofuscin formation in either a direct or an indirect way. In fact, retinol itself may be incorporated into lipofuscin.

Key words: eye, choroid, vitamin E, vitamin A, lipofuscin, polyunsaturated fatty acids
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