Lipofuscin in Vitamin E Deficiency and the Possible Role of Retinol

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ABSTRACT

This study was designed to determine if the vitamin A status of rats could affect the degree of lipofuscin formation in vitamin E deficient rats, inasmuch as an earlier report proposed a retinoyl complex in human brain lipofuscin pigment. Female rates were depleted of vitamin E from weaning while being maintained on different intakes of vitamin A (0, 0.8 and 8.0 mg/kg diet). The amount of lipofuscin present in the uterus was estimated at intervals between 2 and 8 months by visual observations, by histological fluorescence and by organic solvent extractable fluorescence. There was no difference in pigment deposition by any of the three criteria used, whether the animals were made retinol deficient and maintained on retinoic acid or were fed a low or high intake of retinol. Organic solvent extractable fluorescence was a poor indicator of the degree of pigment deposition in the uterus. It appears unlikely that retinol is a significant component of lipofuscin pigment in this tissue.

INTRODUCTION

Interest in the inert fluorescent pigment, termed lipofuscin or ceroid, that accumulates in animal tissues with age or at an accelerated rate in vitamin E deficiency, has recently been focused on its chemical and physical properties. The pigment is generally considered to be a product of lipid peroxidation involving in part a Schiff's base-type structure (1), but a recent report proposed that human brain pigment contained a moiety derived from vitamin A or retinol (2). If confirmed, this would add a new consideration to the origin and structure of the fluorescent pigment. In this report, we have investigated the possible involvement of vitamin A in the formation of the lipofuscin pigment which accumulates in the uterus of vitamin E deficient rats.

EXPERIMENTAL PROCEDURES

Animals and Diets

Weanling female rats of the Sprague Dawley strain were obtained from Taconic Farms (Germantown, NY) and placed in individual stainless steel suspended cages with food and water provided ad libitum. Temperature was controlled at 24 ± 1 C with a fixed 12 hr light-dark cycle. The basal diet was free of all forms of vitamin A and vitamin E and had the following composition in percent: vitamin-free casein, 20.0; mineral mixture with trace minerals (3), 3.6; vitamin mixture (3) with no A or E, 2.0; vitamin E-stripped corn oil with 0.02% BHT, 10.0; cellulose, 4.0; sucrose, 60.4. To this diet were added retinol as retinyl palmitate in the form of a stabilized powder, or retinoic acid in ethanol; control diets had vitamin E added as RRR, α-tocopheryl acetate. The amounts of the various supplements are indicated below. After 2, 5, and 8 months, the animals were sacrificed and tissues taken for histology or frozen at -20 C until analyzed.

Three experiments were carried out with vitamin E deficient rats comparing the effects of various levels of retinol or retinoic acid on lipofuscin formation: (1) a minimal intake of retinol, 0.8 mg/kg diet vs. a high intake, 8.0 mg/kg; (2) a minimal retinol level, 0.8 mg/kg vs. no retinol but with retinoic acid, 4 mg/kg, to maintain normal growth and (3) a moderate level of retinol, 2 mg/kg vs. no retinol but with retinoic acid, 4 mg/kg.

Histological Fluorescence

Tissue was fixed for 30 min at room temperature in a solution of 2.5% glutaraldehyde and 6% sucrose buffered to pH 7.2 with 50 mM sodium cacodylate, then fixed for another 30 min in 10% phosphate-buffered formalin. Frozen sections 10 μm thick were prepared and mounted on slides in glycerol. They were examined for autofluorescence under a Leitz Orthoplan microscope equipped with a mercury vapor lamp and a GB 38 heat filter. A 2 mm UGI excitation filter (peak transmission 360 nm) and a 460 nm barrier filter were used to localize and characterize the autofluorescence typical of lipofuscin.

Extraction of Tissue Fluorescence

Initially, the procedure of Csallany and Ayaz (4) was used to quantify the lipid soluble fluorescent material. In this procedure, a chloroform-methanol extract of tissue (0.3-0.5 g of uterus) was washed with relatively large volumes of water (50 ml five times) and the chloroform soluble material chromatographed

on a column of Sephadex LH-20. Two ml fractions of the column effluent were collected and their fluorescence determined in an Aminco Bowman spectrophotofluorometer at the predetermined wavelengths for maximum activation (350 nm) and emission (440 nm). Standardization was with 0.1 μg/ml quinine sulfate. Because we encountered spurious fluorescent peaks which were traced to the water used in washing the extracts, and which persisted even with glass-distilled or ion-exchange-treated distilled water, we modified the procedure by reducing the number of washings to two with one-third volume of water each time. This eliminated the spurious peaks which emerged from the Sephadex column. In addition to these two procedures for measuring lipid soluble fluorescence, the procedure of Flectcher et al. (5) was also used. This is similar to the above modified procedure but has only one equal volume water wash of the chloroform-methanol extract.

RESULTS

Lipofuscin Deposition in Uterus

At 2 months the uteri of all rats had the normal white appearance. After 5 months the uteri from vitamin E deficient animals had become brown, while those from controls remained unpigmented. Figure 1 compares one horn of a control uterus (center) flanked by uteri from rats deficient in vitamin E for 5 months (experiment 3). Although the control uterus was white in visible light (Fig. 1A) and non-autofluorescent in ultraviolet light (Fig. 1B), the uteri of vitamin E deficient rats had accumulated pigment which appeared brown in visible light (Fig. 1A) and autofluoresced bright yellow when excited by ultraviolet light (Fig. 1B). No differences in pigmentation or in autofluorescence were seen whether the rats had received retinol (left uterus of Fig. 1A and 1B) or had received retinoic acid instead (right uterus). Likewise, rats fed 10-fold different amounts of retinol (0.8 and 8.0 mg/kg of diet) showed no visible differences in uterine pigmentation or autofluorescence even after 8 months, when all vitamin E deficient uteri had become dark brown. The low dietary retinol content (0.8 mg/kg) provided barely enough of the vitamin to promote a normal growth rate and gave little liver storage, whereas the high level (8.0 mg/kg) gave very high storage (16 μg/g vs. 538 μg/g, respectively, for 3 rats after 8 months). Differences in plasma retinol levels, however, were not so great (23 vs. 34 μg/dl, respectively).

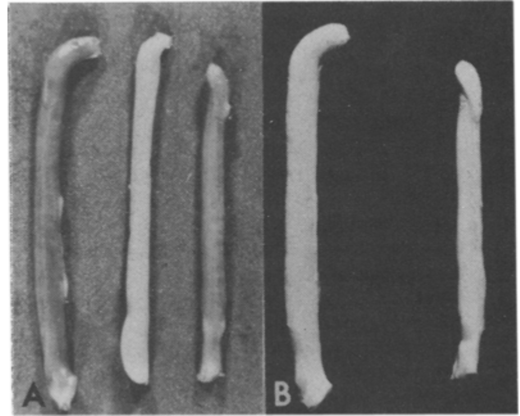

FIG. 1. Left horns of uteri from rats killed after 5 months on a control diet (center of each micrograph), or on vitamin E free diets with 2.0 mg retinol/kg of diet (left) and no retinol but 4.0 mg retinoic acid/kg of diet (right), photographed under white light (Fig. 1A), and under light of ca. 360 nm (Fig. 1B) to elicit autofluorescence of lipofuscin pigment. The control uterus appeared white to light pink in visible light and nonfluorescent in the ultraviolet light. Both vitamin E deficient uteri appeared brown in visible light and bright yellow in ultraviolet light. (X1.4).

Plasma and liver retinol in the retinoic acid-fed rats were undetectable at 5 months. It should be noted that retinoic acid does not accumulate in any tissue, including liver, and has a rapid turnover (6). In none of the three experiments was there evidence that the presence or absence of dietary retinol had any effect on the amount of lipofuscin in the uterus as determined by gross examination or by ultraviolet autofluorescence of histological preparations (Fig. 2). The number of lipofuscin granules counted in the muscle cells of the uterine wall corresponded to the intensity of fluorescence. All vitamin E deficient rats after 5 and 8 months showed more than a 100-fold increase in number of lipofuscin granules over E-adequate rats, regardless of vitamin A status. Control rats receiving dietary α-tocopherol with low, high or no retinol (retinoic acid-supplemented) had normal appearing uteri with little histological fluorescence and very few lipofuscin granules.

Extractable Fluorescence in Uterus

Initial analyses of lipid extracts of uteri (and other tissues) by Sephadex chromatography according to Csallany and Ayaz (4) gave a fluorescent peak, designated A, in column fractions 10-16 which these investigators attributed to lipofuscin. Several other peaks usually appeared in later fractions. In contrast to expectation, however, in the first experiment the primary peak A from control vitamin

E-adequate uteri was of similar magnitude to that obtained from vitamin E-deficient uteri, even though the latter were heavily pigmented. Control extracts with reagents only revealed that much of peak A, and most of the later peaks, were artifacts from the large volume of water used to wash the chloroform-methanol. This water had been distilled and passed through an ion exchange column. A subsequent pass through activated charcoal eliminated most of the fluorescence after fraction 20 and greatly reduced the fluorescence in peak A. To further reduce this artifactual fluorescence, we modified the procedure by greatly reducing the volume of water to two washes of 6 ml each. With this modification, only peak A appeared in E-deficient tissues and either a small or no peak was found in E-sufficient tissues.

In the second experiment, retinol and retinoic acid were fed with and without vitamin E for 14-16 weeks. The uterine extracts were washed only twice with small volumes of water, as described above, prior to being chromatographed on Sephadex. In Table I are shown the relative fluorescence of the whole chloroform extracts and also of peak A from the Sephadex column. There was no significant difference in the extractable fluorescence, either in the whole extract or in the primary peak A, from uteri of E-deficient rats fed retinol or markedly depleted of retinol (maintained on retinoic acid). The fluorescence of the total extracts and also of the Sephadex peaks A for both E-deficient groups were significantly greater than for the E-adequate groups ($p<0.05$). Thus, there does not appear to be any advantage in chromatographing the total lipid extract in order to distinguish between tissues deficient or adequate in vitamin E. The exception may be liver, where the presence of vitamin A can give excessive fluorescence (4). A noteworthy observation in this experiment is that even though counts from histological preparations of uteri would indicate a 100-fold difference or more in lipofuscin pigment granules between E-deficient and E-adequate animals (Fig. 2), the lipid soluble fluorescence indicates only a 2- to 3-fold difference. It is obvious that only a very small fraction of the pigment is extractable, an observation in agreement with the historical definition of this material (7).

A small but detectable amount of retinol was found in rats fed retinoic acid with vitamin E, but no retinol was detected in rats withou' vitamin E. This confirms the well-kno' n sparing action of α-tocopherol on tissue vit- .nin A stores (Table I).

In the third experiment, uterine extracts were extracted by the procedure of Fletcher et al. (5) and the relative fluorescence of both the chloroform and water phases determined. As noted by Desai et al. (8), the water phase of extracts from vitamin E-deficient uteri had more fluorescence than the chloroform phase (data not shown). As in experiment two, there was no difference in relative fluorescence whether the rats were fed no retinol (retinoic acid) or adequate retinol.

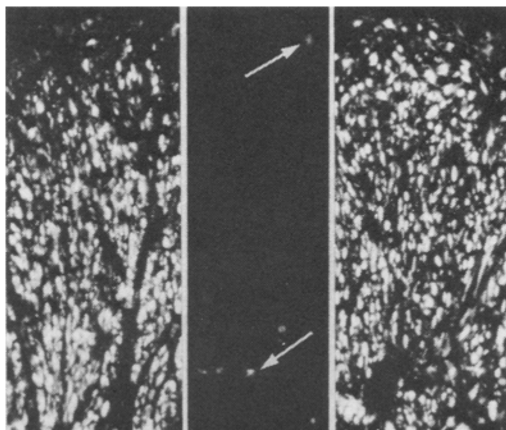

FIG. 2. Lipofuscin fluorescence of frozen transections from the outer (longitudinal) muscle wall of the uteri that are shown grossly in Figure 1. Only a few lipofuscin deposits (arrows) are seen in the control tissue. (X170).

DISCUSSION

The primary purpose of this study was to determine if the vitamin A status of rats could affect the degree of lipofuscin formation in vitamin E-deficient rats. The rationale was a report by Wolfe et al. (2) that the abnormal lipid in nerve tissue from a patient with Batten's disease had a component tentatively identified as a retinoyl complex. In this disease, nerve tissue accumulates an autofluorescent pigment with the characteristics of lipofuscin or ceroid. We selected the uterus as a model because this organ is the most susceptible to lipofuscin accumulation in vitamin E-deficient rats. Under our conditions of depleting weanling rats, the onset of lipofuscin, as noted by gross visual examination or by histological autofluorescence, began after three to four months. By these two criteria and also by measuring the extractable fluorescence, we found no evidence that vitamin A status affected lipofuscin accumulation. Vitamin A status varied widely in the different experiments, from essentially total depletion of

TABLE I

Relative Fluorescence of Organic Solvent Soluble Fluorescent Material from Rat Uterus

Diet[c]	No. rats	Relative fluorescence[a] Total extract[d]	Relative fluorescence[a] Sephadex peak[e]	Tissue retinol[b] Plasma µg/dl	Tissue retinol[b] Liver µg/g
- Vit. E, + retinol	8	9.7 ± 0.8[1]	1.5 ± 0.3[1]	26.8 ± 8.2	154 ± 8
- Vit. E, + retinoic acid	8	10.1 ± 2.3[1]	1.8 ± 0.3[1]	0	0
+ Vit. E, + retinol	6	2.0 ± 0.5[2]	0.7 ± 0.2[2]	40.3 ± 6.1	186 ± 19
+ Vit. E, + retinoic acid	5	4.1 ± 1.3[2]	0.7 ± 0.2[2]	2.8 ± 2.2	8.8 ± 4.6

[a]Tissues extracted with $CHCL_3$/MeOH and the extract washed twice with water as described in Methods. Values are means ± SEM of relative fluorescence per g tissue after 14-16 weeks. Values in same column with different superscript are significantly different ($P<0.05$).

[b]Values are means ± SEM.

[c]Supplements to the vitamin E deficient diet were: retinol, 2 mg/kg; retinoic acid, 4 mg/kg; α-tocopheryl acetate, 250 mg/kg.

[d]Chloroform extract before chromatography.

[e]Peak A, fractions 10-16, from Sephadex column chromatography of the chloroform extract.

retinol by two months when retinoic acid was fed, to low and high tissue levels as determined from plasma and liver analyses. The uterus is not a storage organ for retinol and the very low content which must be present from circulating blood is not detectable by usual analytical methodology.

After this study was begun, Nelson and Halley (9) questioned the interpretation of the data of Wolfe et al. (2) and suggested that the physical evidence indicated that cholesterol rather than some form of retinol could have generated the data. This controversy has not been resolved to date.

Another aspect of our study was to determine if the amount of organic soluble fluorescence obtained from uterus correlated with the degree of lipofuscin pigmentation seen visually or by histological fluorescence. Although normal uteri from rats fed vitamin E had less extractable fluorescence than did E-deficient uteri, within any group of the latter there was a 10-fold range in extractable fluorescence even though the tissues appeared to have similar pigmentation. Even after chromatography on Sephadex, the peak reported to be lipofuscin (4) varied 5-fold in both normal and E-deficient tissues. As noted by Desai et al. (8) in analyses of E-deficient uteri, there was more fluorescence in the water phase obtained from a chloroform-methanol extract than in the organic phase. Neither phase, however, indicated the magnitude of the difference in pigmentation between E-adequate and E-deficient uteri. For quantitative studies of lipofuscin formation in this tissue, extracts appear to have limited value.

As a corollary of this work, the formation of lipofuscin-like granules in the pigment epithelium of the retina was examined. In this tissue, the observed relationship between vitamins A and E was more complex than in the uterus (10), probably because of the central role of retinol in the visual process.

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