Xerophthalmia in vitamin A–deficient rabbits
Clinical and ultrastructural alterations in the cornea

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Xerophthalmia developed in the eyes of rabbits maintained on a vitamin A–deficient diet for 4 to 6 months. The earliest clinical change, a lusterless graying of the central corneal epithelium, was noted after 16 to 18 weeks on the diet. Multiple small punctate epithelial erosions appeared in the interpalpebral fissure zone within 7 to 10 days after the lusterless graying became evident. The erosions gradually became confluent, and a striking dry, glazed, peau d'orange appearance was noted. Polycystic microbullae appeared in the epithelium in some eyes. Thick keratinized epithelial plaques developed in all eyes 1 to 2 weeks after the appearance of severe peau d'orange. Electron microscopy of corneas with lusterless graying of the epithelium revealed swelling of the most superficial epithelial cells with flattened and shorter microvillous projections. In corneas with punctate epithelial erosions and keratinized plaques, microvilli were absent or decreased in number on superficial cells, and multilayered, keratinized epithelial cells were present on the surface of the cornea. The stroma appeared essentially normal with minimal edema at all stages when examined by electron microscopy. Intercellular edema was present in the endothelium in early- and late-stage xerotic corneas but could not be detected clinically. No significant clinical or microscopic alterations were seen in the corneas of control rabbits on normal diet or in rabbits on the vitamin A–deficient diet supplemented with vitamin A. The alterations seen in the corneas of vitamin A–deficient rabbits are similar to those which have been described in vitamin A–deficient humans. Rabbit therefore appears to be a good model for further studies of xerophthalmia.

Key words: cornea, electron microscopy, rabbit, vitamin A, vitamin A deficiency, xerophthalmia
peripheral epithelial change which caused some of the surface cells in the central area of the cornea to appear "opaque and slightly greasy." Microscopic examination demonstrated that these cells were epithelial cells undergoing metaplasia into squamous and keratinized cells. Keratinization of the conjunctiva occurred later.

The purpose of this study was to correlate clinical changes with light and electron microscopic alterations occurring in the cornea of vitamin A–deficient rabbits.

Materials and methods

Twenty-one New Zealand white rabbits, weighing 2.5 to 3.0 kg at the start of the experiment, were maintained on a diet deficient in vitamin A (test diet No. 77227; Teklad Test Diets, Madison, Wisc.). The bulk of the diet consists of casein, corn starch, sucrose, and cellulose supplemented with dl-methionine, a mineral mix, and vitamins other than A. Eight control rabbits were maintained on the same diet which differed only in that it was supplemented with 500,000 IU/gm dry vitamin A palmitate and an inert red food color for identification (test diet No. 78403; Teklad). Eight other rabbits were maintained on Purina Rabbit Chow Checkers (Ralston Purina Co., St. Louis, Mo.) as additional controls. Approximately half of the animals in each group were used for sequential clinical observations, determination of growth curves, and subsequent therapeutic studies (to be reported elsewhere). The other rabbits in each group were observed clinically and sacrificed at appropriate times for microscopic studies of corneal alterations present at the time of sacrifice.

The fatality rate in the vitamin A–deficient group was 52% (11 rabbits, nine with enteric symptoms, two of unknown etiology). In the vitamin A–supplemented group, the fatality rate was 25% (two rabbits, both with enteric symptoms). One of the eight (12.5%) control rabbits on Purina Rabbit Chow Checkers died of an unknown cause.

The average daily food intake of the animals on the supplemented diet (vitamin A+) was restricted to that of the animals on the deficient diet (vitamin A–). The food intake of the rabbits on Purina Rabbit Chow Checkers was not restricted. All rabbits were weighed weekly, and serum protein was assayed monthly. Serum vitamin A levels were assayed monthly with a modification of Neeld and Pearson's colorimetric procedure. Internal standards were used to correct for interference with the color reaction.

All eyes were examined at appropriate intervals by slit-lamp biomicroscopy with the Haag-Streit 900. Tear secretion was measured by the Schirmer
Fig. 3. Sequential clinical stages observed during development of xerophthalmia in vitamin A-deficient rabbits. A, Punctate epithelial keratopathy with lusterless, peau d'orange appearance. B, Early keratinization with epithelial edema and polycystic microbullae. C, Keratinization with early plaque formation. D, Advanced keratinization of the epithelium with stromal edema.

test without topical anesthesia. Rose Bengal and fluorescein staining were used to aid in detecting epithelial defects. Rabbits for microscopic studies were sacrificed with an intracardiac overdose of sodium pentobarbital. The eyes were immediately enucleated, and the corneas excised with a 1 mm scleral rim. The corneas were fixed in 2.67% glutaraldehyde in phosphate buffer for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). One half of each cornea was embedded in a low-viscosity epoxy resin for light microscopy (LM) and TEM. The other half was critical point-dried for SEM.

Results

Growth rates, serum protein, and serum vitamin A levels. The growth rate of rabbits on the vitamin A-deficient diet was significantly less than that of animals on both the normal diet and the vitamin A-supplemented diet (Fig. 1) in spite of the fact that the food intake of the supplemented rabbits was limited to that of the rabbits on the vitamin A-deficient diet. Serum protein levels in the vitamin A-deficient and supplemented rabbits remained within the normal range throughout the experiment (6 months). Serum vitamin A levels in the rabbits on the vitamin A-deficient diet, however, decreased by 50% within 1 month (Fig. 2). No further significant decrease in mean levels was measured after 4 to 5 months when clinical alterations were apparent in the corneas (Fig. 2). Serum vitamin A levels did not change significantly with time in the animals on the normal diet or in those on the vitamin A-supplemented diet (Fig. 2).

Clinical observations. No significant clinical abnormalities were observed in the eyes of rabbits on either the normal or the vitamin A-supplemented diets during the 6 months of observation. Small evanescent epithelial defects were sometimes seen, but these are
Fig. 4. Scanning electron micrograph of the corneal epithelium from rabbit on vitamin A-deficient diet supplemented with vitamin A for 6 months. No clinical alterations were noted in the cornea. The epithelium has a patchwork quilt-like appearance of light, medium, and dark cells. (x500.)

Fig. 5. Higher magnification of center of Fig. 4. Light cells have many microvilli. Medium cells have fewer, shorter microvilli. Dark cell (lower left) has no microvilli. (x2000.)
known to occur in normal rabbits. In contrast, significant and progressive corneal abnormalities were observed in rabbits on the vitamin A-deficient diet beginning after 16 to 18 weeks. Although the corneas appeared dry in the animals at various stages of the disease, no significant decrease in tear production was detectable at any stage as measured with the Schirmer test.

The earliest corneal change detectable with the slit lamp was a lusterless graying of the central corneal epithelium, often with an accompanying subepithelial haze. At this time there was no evidence of keratinization.

Fig. 6. Light microscopic photograph of a cross-section of corneal epithelium from the same rabbit. The epithelium is five to six cell layers in thickness, and the basal epithelial cells tend to be columnar. (×470.)

Fig. 7. Transmission electron micrograph of the superficial epithelial cells in same rabbit. Note presence of numerous microvilli on surface cells and small, desmosome-like junctional complexes between cells. (×8500.)
Fig. 8. Transmission electron micrograph of superficial corneal epithelial cells from vitamin A-deficient rabbit with earliest detectable clinical alteration—lusterless graying of corneal epithelium. The most superficial cells are edematous, resulting in flattened and shorter microvillous projections on their bulging anterior surface. (×8500.)

Fig. 9. Scanning electron micrograph of epithelial surface from cornea with punctate epithelial erosions illustrated in Fig. 3, A. Dark and medium cells predominate. (×500.)
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Fig. 10. Higher magnification of center of Fig. 9. Microvilli are few in number on the dark and medium cells but still normal on the remaining light cells. (×2000.)

of the conjunctiva or nictitating membrane. The corneal stroma and endothelial mosaic were unremarkable. The application of topical glycerine (Ophthalgan; Ayerst Labs., New York, N. Y.) cleared much of the epithelium at this stage.

Within 7 to 10 days after the initial appearance of lusterless graying of the epithelium, multiple focal punctate epithelial erosions became evident in the cornea (Fig. 3, A). The epithelial changes were most prominent centrally and progressed to a central dry glazed peau d'orange (orange peel-like) appearance. During this stage the stroma maintained normal thickness with no evidence of edema. The central endothelium was poorly visualized by specular reflection due to the epithelial changes, but the peripheral endothelial mosaic appeared normal.

After the development of the dry, peau d'orange appearance, one third of the rabbits developed polycystic microbullae in the corneal epithelium (Fig. 3, B). The polycystic microbullae were confined to the central region of the cornea and, when present, were accompanied by stromal edema beneath the
area of the microbullae. The microbullae spontaneously resolved as the stage of keratinizing xerosis appeared.

Thick, keratinized plaques appeared in the interpalpebral fissure in all eyes approximately 1 to 2 weeks after the initial appearance of severe punctate epithelial erosions (Fig. 3, C). The xerotic plaques often spontaneously sloughed from the corneal surface and could be easily removed with a moist cotton swab. Keratinization in the bulbar conjunctiva and nictitating membrane became apparent at this stage and stained with rose bengal. The keratinization progressed to a lusterless graying and wrinkling of the bulbar conjunctiva and nictitating membrane.

As the corneal keratinization progressed, stromal edema became evident in the central cornea and usually extended to the limbus (Fig. 3, D). At this stage blood vessels extended approximately 2 mm into peripheral edematous deep stroma. Approximately half of the eyes remained in the keratinized xerotic stage for 2 to 4 weeks without progressing to a more severe stage (Fig. 3, D).

Four eyes developed necrotizing stromal infiltrates beneath keratinized plaques with eventual ulceration of the stroma and hypopyon formation. Microbiologic and microscopic studies of the late stages of corneal necrosis (keratomalacia) will be reported elsewhere.

Light and electron microscopic observations. No microscopic alterations were noted in corneas from rabbits on the vitamin A-supplemented diet when compared to those eating normal rabbit Chow. The epithelial surface as seen by SEM had a patch-
Fig. 13. Transmission electron micrograph of boxed area in Fig. 12. Keratinohyaline granules can be seen in wing cell (arrows). Thickening of cell membranes in superficial cells is obvious (compare to wing cell membrane). Prominent desmosome-like attachment bodies and an electron-dense material are present between some of the cells. The surfaces of cells are smooth due to lack of microvilli. (×34,000.)
work quiltlike appearance due to a normal complement of light, medium, and dark cells (Figs. 4 and 5). Light cells predominated, and their surfaces were covered with densely packed microvilli. Medium cells had fewer and shorter appearing microvilli. Dark cells had almost none. No attempt was made to determine if "microvilli" were microplicae and/or microvilli. In addition, our classifications of light, medium, and dark cells are different from those of Pfister. He reported that dark cell surfaces had twice as many (but shorter) microvilli per square micrometer than light cells.

LM and TEM of the corneas from vitamin A-supplemented animals also revealed normal epithelial (Figs. 6 and 7), stromal and endothelial morphology. As in SEM, the anterior surface of the superficial epithelial cells was seen to be covered with microvillus-like projections in TEM (Fig. 7).

In contrast, ultrastructural alterations could be seen even in corneas with early epithelial graying in vitamin A-deficient animals. The most superficial epithelial cells were edematous with flattened and shorter microvillus projections (Fig. 8). SEM of corneas with punctate epithelial erosions revealed that medium and dark cells with few microvilli predominated (Figs. 9 and 10). Abnormal surface epithelial cells and microcysts could be seen with LM and TEM (Figs. 11 and 12). Abnormal cells contained keratohyalin granules and densely packed filaments and had thickened cell membranes characteristic of keratinized cells (Fig. 13). Prominent desmosome-like attachment bodies appeared to hold the smooth-surfacet keratinized cells together. Wing and basal epithelial cells appeared normal (Figs. 11 and 12) as did the stroma (Fig. 11). The endothelium, however, appeared to be vacuolated (Fig. 11).

In corneas that were xerotic and completely keratinized, microscopic alterations in the epithelium were also more severe. SEM revealed few cells that had even remnants of microvilli, and many cells appeared to be sloughing. In light microscopic sections, the entire epithelium was seen to be involved (Fig. 14). The basal cells were flattened in some areas, and large epithelial bulbae or cysts were present. Normal wing cells could be seen between flattened basal cells and multilayered keratinized cells in LM and TEM (Figs. 14 and 15). Stromal edema was present in some areas, but stromal cells and collagen usually appeared normal. The "vacuoles" seen in the endothelium in early stages (Fig. 11) were also seen in late-stage xerotic corneas. TEM revealed that the vacuoles were due to dilation of the intercellular space (Fig. 16) rather than the presence of intracytoplasmic vacuoles. Except for distortion of nuclear shape, cytoplasmic organelles in the endothelial cells appeared normal.

Discussion

In the human, active xerophthalmia is classified into the following sequential stages: X1A, conjunctival xerosis; X1B, conjunctival xerosis with Bitot's spot; X2, corneal xerosis; X3A, corneal xerosis with ulceration; and X3B keratomalacia. The role of vitamin A deficiency in the genesis of these conditions
remains unclear, in part due to the limited clinical observations available, the imprecision with which the various stages have been described and classified, complexities arising from the frequent association of these conditions with protein-calorie malnutrition, exanthematous diseases, and systemic infections, and the presence of secondary corneal changes. Kuming and Politzer reviewed 100 cases of active corneal xerophthalmia among 1166 malnourished persons and concluded that "protein malnutrition rather than vitamin A deficiency was the important factor." Two cases of well-documented isolated vitamin A deficiency in young adults indicate that vitamin A deficiency alone can result in corneal xerosis which is reversible with treatment with vitamin A. In contrast, two adult patients with severe protein deficiency who developed keratomalacia in the absence of a severely decreased serum level of vitamin A have been reported.

In the rabbit, xerophthalmia due to vitamin A deficiency developed in the following sequential stages in the cornea: (1) lusterless graying of central corneal epithelium, (2) punctate epithelial erosions which gradually become confluent, (3) early keratinization with epithelial edema and microbullae, (4) advanced keratinization with plaque formation, and (5) corneal stromal necrosis and hypopyon with or without subsequent corneal ulceration. None of these alterations were observed in the corneas of rabbits consuming similar amounts of the same diet supplemented with vitamin A. These results confirm that vitamin A deficiency alone causes xerophthalmia. All of the corneal alterations in rabbit, except necrosis, are reversible with treatment with systemic vitamin A and/or topical retinoic acid.

Sommer et al. recently reported that punctate epithelial keratopathy appears to be the earliest corneal manifestation of xerophthalmia and is already present in the vast majority of cases classically considered free of corneal involvement. In rabbit, punctate epithelial erosions were also noted at an early stage, but central lusterless epithelial graying due to edema of the superficial epithelial...
cells could be observed in the slit lamp 7 to 10 days before the appearance of the punctate epithelial erosions.

Keratinization of the superficial epithelial cells and lack of microvillous projections were prominent histological and ultrastructural alterations in the eyes of vitamin A-deficient rabbits. The apparent corneal dryness seen in xerophthalmia may be due not only to the loss of mucin from goblet cells but also to the inability of the smooth-surface keratinized cells to hold the tear film in place. The desmosomes also appear to be better developed between the keratinized cells, and this may account for their tenacity in remaining on the corneal surface. Keratinization of the corneal epithelium has been described in vitamin A-deficient humans, rats, guinea pigs, and rabbits. The similarities between the corneal changes described in the human and in the rabbit indicate that the rabbit is an excellent model for further studies of xerophthalmia.

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