Effect of Vitamin A Deficiency on the Early Response to Experimental Pseudomonas Keratitis

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Purpose. Vitamin A-deficient humans and animals are more susceptible to infections than are healthy humans and animals. This study compares the early corneal response (within 24 hours) to an experimental Pseudomonas aeruginosa infection between vitamin A deficient and control rats.

Methods. Male WAG/Rij/MCW rats were fed either a vitamin A-deficient diet (A—) or the same diet with retinyl palmitate added back in a nonrestricted manner (N) or under pair-fed conditions (A+) to yield weight-matched rats. Some A— rats were repleted with retinyl palmitate 16 days before being killed and then given free access to the retinyl palmitate-supplemented diet (R). Twenty-four hours before being killed, the corneas of anesthetized rats were scratched and P. aeruginosa organisms were applied to the corneal surface. The rats were killed using an overdose of sodium pentobarbital. Corneas were either processed for light and electron microscopic examination or extracted for proteinase and myeloperoxidase determination. Corneal myeloperoxidase concentrations relative to neutrophil myeloperoxidase concentrations were used to determine the number of neutrophils in the cornea. Zymography was used to study caseinases, gelatinases, and plasminogen activators. Reverse zymography was used to detect proteinase inhibitors. Similar results were noted at early, mid, and late weight plateau stages of vitamin A deficiency.

Results. Ulceration occurred within 24 hours when low numbers of P. aeruginosa (10⁴ cpu) were applied topically onto scratched A— corneas, whereas no ulceration was observed in the A+, R, and N corneas. When higher numbers of P. aeruginosa (10⁷–10⁸) were applied to the scratched corneas, all corneas became ulcerated within 24 hours. The extent of ulceration in the control corneas was greater than that in A— corneas by a factor of two. Only the A— corneas contained inflammatory cells with unusual striated deposits in phagolysosomes. The total number of neutrophils in the cornea and the concentrations of caseinases, plasminogen activators, and gelatinases in the infected corneal extracts were similar; however, the concentrations of cysteine proteinase inhibitors were elevated under A— conditions.

Conclusions. Vitamin A deficiency alters the response of the cornea to a P. aeruginosa infection during the first 24 hours. The alterations observed are probably due to multiple factors: an insufficient tear film for bacterial clearance and migration of neutrophils, epithelial keratinization, alterations in corneal wound healing, and changes in polymorphonuclear function. Invest Ophthalmol Vis Sci. 1996;37:511–522.

Vitamin A deficiency is characterized by progressive ocular changes and increased susceptibility to infection. This deficiency is observed most often in underdeveloped countries; however, low plasma vitamin A concentrations with early clinical symptoms have been observed in some children with measles in the inner cities of the United States, in alcoholics,_b in persons positive for the human immunodeficiency virus (type 1),_c in persons with hepatic dysfunction, and in persons with cystic fibrosis. The progressive ocular changes associated with vitamin A deficiency include night blindness, conjunctival xerosis, Bitot’s spots, corneal xerosis, and corneal ulceration or keratomalacia.

Vitamin A deficiency is associated with an in-
creased rate of infection, greater severity of infections, and increased mortality risk in humans.\textsuperscript{9,10} This increase is observed even in the early stages of the deficiency. The mortality rate in persons infected with the type 1 human immunodeficiency virus who have a mild vitamin A deficiency (plasma retinol < 1 \( \mu M \) compared with a mean value of 2.1 \( \mu M \) for healthy adults) is six times greater than in an infected person with plasma retinol > 1 \( \mu M \).\textsuperscript{11} Forty-nine percent of persons positive for the human immunodeficiency virus have decreased plasma retinol concentrations of less than 1 \( \mu M \); in comparison, only 2% of the healthy population have abnormally low plasma retinol concentrations.\textsuperscript{12} Infants born to mothers with the human immunodeficiency virus and low retinol concentrations are four to five times more likely to become infected with the virus.\textsuperscript{12} The mucosal immune response,\textsuperscript{13} cytotoxic and helper T-cell function,\textsuperscript{14-17} and natural killer cell activity\textsuperscript{18} are altered when vitamin A is deficient. T-cell–dependent antibody responses are impaired, but T-cell–independent antibody responses are not.\textsuperscript{19}

Clearance of \textit{Pseudomonas aeruginosa} in experimental lung infections\textsuperscript{20} and in \textit{Pseudomonas keratitis}\textsuperscript{21} involves a specific immune-mediated response. Immunization of rats with \textit{P. aeruginosa} significantly increased bacteria clearance from lungs 30 minutes and 4 hours after pulmonary challenge.\textsuperscript{20} The neutrophils from immunized rats phagocytized more bacteria per neutrophil, exhibited increased chemiluminescence, and demonstrated enhanced specific chemotaxis toward live \textit{P. aeruginosa} than did the neutrophils from nonimmunized rats. In the \textit{Pseudomonas keratitis} model, similar numbers of neutrophils were observed in the corneas 18 hours after \textit{P. aeruginosa} was applied topically.\textsuperscript{21} However, the neutrophils of the naive rats were less competent in ingesting the bacteria than were those from the immunocompetent rats. Very little corneal degradation was observed in the naive rat corneas, but the central portions of the immunocompetent rat corneas were destroyed. Although \textit{P. aeruginosa} organisms can be phagocytized by both nonspecific and immune-specific mechanisms, phagocytosis is more efficient when immune-specific mechanisms are used.\textsuperscript{22} Neutrophils recognize this organism by at least two immune-specific mechanisms.\textsuperscript{22} In both, specific antibodies bind to the bacteria through the F\textsubscript{ab} portion of the molecules. One set of neutrophil membrane receptors binds the \textit{P. aeruginosa}-immunoglobulin G (IgG) complex through the F\textsubscript{c} portion of the IgG molecule. A second set of neutrophil receptors binds to the activated complement component, C3b, which in turn is bound to the IgG-P. \textit{Aeruginosa} complex. Potentially, both of these pathways play a role in immune recognition in the cornea because both IgG\textsuperscript{21,23} and complement components are present.\textsuperscript{24}

The purpose of this study was to determine, using an established model, the scratch model of \textit{Pseudomonas keratitis}, the effect of vitamin A deficiency on the cornea’s ability to mount an inflammatory response within the first 24 hours after infection.\textsuperscript{21} This study directly examined the ability of vitamin A-deficient rats (A–) to handle ocular infections compared with the same ability in weight-matched, pair-fed rats (A+), retinyl palmitate repleted rats (R), and rats given free access to food (N).

**MATERIALS AND METHODS**

All reagents, unless specifically noted, were from Sigma Chemical Company (St. Louis, MO).

\textit{Pseudomonas aeruginosa} Cultures and Quantification

The corneal-derived \textit{P. aeruginosa} strain 107 (from S. Davis, Medical College of Wisconsin, Milwaukee, WI) was grown in Mueller-Hinton broth (DIFCO Laboratories, Detroit, MI) supplemented with 50 mg/ml CaCl\textsubscript{2} and 20 mg/ml MgCl\textsubscript{2} for 18 hours at 37°C on a rotary shaker. Serial dilutions of the bacteria were grown on Mueller-Hinton plates (DIFCO Laboratories) and the number of colony-forming units was determined.

\textit{P. aeruginosa} strain 107 is a nonmucoid bacterial strain originally isolated from a human corneal ulcer by G. M. Bohigian. It produces very small amounts of proteinases,\textsuperscript{25} average amounts of endotoxin, and several other unidentified low molecular weight chemotactants (Twining et al, unpublished observations). Infections by this strain induce an inflammatory response.\textsuperscript{25}

**Rats**

WAG/Rij/MCW rats were raised in a defined flora colony, where they were exposed to a restricted number of bacterial species but not to mycoplasma or viruses. Females with litters were fed an autoclavable casein-based vitamin A-deficient diet (Harlan-Teklad, Madison, WI) 16 days after birth. At day 21, male pups were divided into three dietary groups. Ninety-eight animals. And fifty-three healthy control (N) rats were safflower oil 16 days before exsanguination. These rats
were then given free access to the retinyl palmitate-supplemented diet. The animals were weighed every 3 days to monitor the progress of the deficiency.

Because *P. aeruginosa* is not part of the normal flora of this rat colony, the rats were made immunocompetent to this organism using a phenol-killed *P. aeruginosa* 107 vaccine.²¹ The vaccine was injected intraperitoneal when the rats were 6, 7, 8, and 12 (if required) weeks old. Immunocompetency was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis²² and Western blots for specific IgGs that bound *P. aeruginosa* antigens. Use of these animals adhered to Association for Research in Vision and Ophthalmology standards for the use of animals in ophthalmic and vision research.

**Pseudomonas Keratitis**

*Pseudomonas* keratitis was induced in the rats in the early (9 weeks: 4A−, 4A+, 4R, 4N), mid (12 weeks: 4A−, 4A+, 4R, 4N), mid-late (13 weeks: 4A−, 36A+, 23R, 36N), and late (14 week: 4A−, 4A+, 4R, 4N) weight plateau stages of vitamin A deficiency. Light and electron microscopic examination of randomly selected nonexperimental corneas showed that at the early weight plateau stage the outer epithelial cells in the A− corneas had fewer microvilli and the superficial cells were edematous. At the mid through the late weight plateau stages (12 to 14 weeks), the epithelial cells had typical characteristics observed with vitamin A deficiency. Light and electron microscopic examination of randomly selected nonexperimental corneas showed that at the early weight plateau stage the outer epithelial cells in the A− corneas had fewer microvilli and the superficial cells were edematous. At the mid through the late weight plateau stages (12 to 14 weeks), the epithelial cells had typical characteristics observed with vitamin A deficiency. The outer epithelial cells were flattened, devoid of microvilli and organelles, and attached to neighboring cells through desmosomes. The basal epithelial cells were irregular and had lost their columnar appearance.

The corneas of rats anesthetized with methoxyflurane (Fitman-Moore, Mundelein, IL) were scratched centrally with a sterile 21-gauge needle in an X pattern (one per rat). The scratch cut through both the epithelium and basal lamina into the stroma. Immediately after scratching, one 10−μl aliquot of Mueller-Hinton broth (DIFCO Laboratories) containing 1 × 10⁴, 2 × 10⁷, or 5 × 10⁸ *P. aeruginosa* 107 colony-forming units (cfu) was applied topically onto the scratched cornea. Representative corneas were scratched without applying *P. aeruginosa* and then processed for light and electron microscopic examination, to confirm that the depth of the scratch was below the basal lamina but within the upper 20% of the stroma.

Twenty-four hours after infection, each rat was anesthetized with methoxyflurane and bled by heart puncture into a 10-ml syringe containing 100 units sodium heparin (Elkins-Sinn, Cherry Hill, NJ) in 100 μl rat phosphate-buffered saline (RPBS; 4.36 mM sodium phosphate, 1.46 mM potassium phosphate, 2.68 mM KCl, and 0.15 mM NaCl pH 7.4). The animals were then killed using an overdose of pentobarbital (Abbott Laboratories, North Chicago, IL). The eyes were removed and either the whole globe with the posterior segment cut open was placed immediately into 2.67% glutaraldehyde in 0.1 M PBS buffer, pH 7.3, for fixation and then the cornea was processed for light and electron microscopic examination; or the cornea was dissected from the globe and the entire cornea homogenized and used for bacterial counts and then extracted for zymography of proteinases and inhibitors. Rat livers were removed and stored at −80°C until extracted for retinoids. The animals were bled, killed, and dissected under yellow lights to preserve the retinoids. All samples were stored either in foil or orange tubes for protection from light.

**Light and Electron Microscopic Examination**

Fixed whole corneas (27 A−, 27 A+, 27 R, and 27 N infected; 5 A−, 5 A+, 5 R, and 5 N scratched but uninfected; 20 A−, 12 A+, 12 R, and 12N contralateral nonscratched eyes) were embedded in Spurr's medium (Polysciences, Warrington, PA) and processed for light and transmission electron microscopic examination using standard methods.²⁸ Thick sections (0.5 μm) were stained with toluidine blue plus basic fuchsin.²⁹ Thin sections (70 nm) for transmission electron microscopic examination were stained with uranyl acetate and lead citrate.²⁸

**Corneal Extractions**

Entire infected (26 A−, 21 A+, 8R, 21N) and uninfected and unscratched contralateral (8A−, 8A+, 8R and 8N) corneas were minced and homogenized in RPBS (1 ml/cornea). A portion of the homogenate (100 μl) was used for bacterial counts; the remaining portion was mixed with hexadecyltrimethylammonium bromide to a final concentration of 0.5%. The latter homogenate was frozen and thawed three times in an ethanol dry-ice bath, centrifuged (13,000g), and the supernatant fraction stored at −80°C until used for neutrophil counts by myeloperoxidase activity (50 μl), for proteinase/inhibitor studies (500 μl), and for protein determination (100 μl).

**Neutrophil Isolation and Extraction**

Neutrophils were isolated under sterile conditions at room temperature from heparinized blood by dextran sedimentation followed by separation on a density gradient. The blood from each animal was mixed with an equal volume of 3% Dextran T 500 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in RPBS for 10 seconds. Sediment from erythrocytes was allowed to settle at 1 g for 40 minutes. The straw-colored upper phase was layered over 5.5 ml 1.119 density Histopaque (Sigma, St. Louis, MO), which in turn sat on 3.5 ml 1.119 density Histopaque. The cell preparation was centrifuged for 40 minutes at 400g at room tem-
perature. After centrifugation, the neutrophils were found at the Histopaque interface. The neutrophils were removed, suspended in RPBS, and centrifuged at 400g, 4°C, to form pellets of the cells. The pelleted cells were washed three times with cold RPBS, suspended in 1 ml RPBS, and counted with a hemacytometer. Cell smears were stained with LeukoStat (Fisher Diagnostics, Orangeburg, NY) and the neutrophil purity was determined. The purified neutrophils were homogenized and extracted in the same manner as described for corneas.

**Myeloperoxidase Assay**

To determine the number of neutrophils present in a given *P. aeruginosa*-infected cornea, the myeloperoxidase activity of the cornea was compared with a standard myeloperoxidase curve generated from neutrophil extracts from the same animal. The myeloperoxidase assay used here is an adaptation of that by Williams et al. Briefly, the sample (10 μl) was mixed with the assay solution (190 μl), which contained 0.53 mM o-dianisidine, 0.5% hydrogen peroxide, and 50 mM potassium phosphate, pH 6.0. The change in absorption was measured at 460 nm using a ThermoMAX microplate reader (Molecular Devices, Menlo Park, CA). Myeloperoxidase from Athens Research Technology, Inc., (Athens, GA) was used as a standard.

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blots for Plasma IgG Specificity**

Phenol-killed *P. aeruginosa* was sonicated and solubilized in Laemmeli sodium dodecyl sulfate sample buffer without a reducing agent. Particulate matter was pelleted by centrifugation at 13,000g. The supernatant fraction was separated on sodium dodecyl sulfate–polyacrylamide gels under nonreducing conditions. The separated proteins were electroblotted onto nitrocellulose and the blots were blocked, washed, reacted with serum from individual rats and then peroxidase-conjugated goat anti-rat IgG antibodies, and visualized by the enhanced chemiluminescence method according to the method recommended by the ECL Kit (Amersham, Arlington Heights, IL).

**Zymography To Detect Proteinases**

Proteinases in the corneal supernatant fractions were resolved on either 9% or 11% sodium dodecyl sulfate–polyacrylamide gels containing 0.2% casein or 0.1% gelatin. Plasminogen activator gels contained both 0.2% casein and 16 μg/ml plasminogen (gift from Dr. Richard Marlar, University of Colorado, Denver). Samples, normalized for corneal protein, were mixed with sample buffer without reducing agents. The molecular weight protein standards (low and high) were electrophoresed in the presence of 20 mM dithiothreitol. After electrophoresis, the gels were washed in 2.5% Triton X-100 (Sigma) for 1 hour and then incubated overnight at 37°C in either casein gel incubation buffer (0.05 M Tris, pH 7.6, 0.1 M NaCl) or gelatin gel incubation buffer (0.05 M Tris, pH 8.0, 0.01 M CaCl2, 1 mM ZnCl2, 0.15 M NaCl). The gels were stained with Coomassie brilliant blue R-250.

To determine the type of proteinases observed on the zymograms, the samples were mixed with inhibitors or the solvent used for the inhibitor and incubated for 60 minutes at room temperature before electrophoresis. After electrophoresis, the gels were washed and incubated in buffers that also contained the inhibitors. The final concentrations of inhibitors used were 1 mM phenylmethylsulfonyl fluoride, 100 μM chymostatin, 100 μM tosylleucyl chloromethyl ketone, 1 μM pepstatin, 10 μM E-64 (L-trans-epoxysuccinyl-leucyl amide-(4-guanidino)-butane-N-[N-L-3-trans-carboxytheidine-2-carboxyl-L-Leucyl]-agmatine), and 4 mM ethylenediaminotetraacetate. Control samples and gels were incubated with the solvents used for the inhibitors (1 mM HCl for tosylleucyl chloromethyl ketone, isopropanol for phenylmethylsulfonyl fluoride, H2O for ethylenediaminetetraacetate and E-64, dimethyl sulf oxide for chymostatin, and methanol for pepstatin).

**Reverse Zymography to Detect Proteinase Inhibitors**

Reverse zymography on casein gels was performed in the same manner as zymography, but during the incubation period either chymotrypsin (4 μg/100 ml; Worthington Biochemical Corp., Freehold, NJ) or papain (20 μg/100 ml; Sigma Chemical Co.) plus 1 mM diithiothreitol and 0.1 mM ethylenediaminetetraacetate were added to the incubation buffer. The density of the bands was determined using an AMBIS imaging system (San Diego, CA).

**Serum and Liver Retinoid Concentrations**

Serum retinoids were extracted using hexane (Sigma-Aldrich Chemical Co., Milwaukee, WI) in the presence of 0.1% ascorbic acid (J.T. Baker, Phillipsburg, NJ). The entire liver was weighed, homogenized, saponified, and extracted using hexane in the presence of 1% pyrogallol. Retinyl acetate (Sigma Chemical Co.) was added as an internal standard. The extracts in ethanol (50 μl) were separated by high-performance liquid chromatography (Dionex, Sunnyvale, CA) on a Whatman (Hillsboro, OR) Partisil ODS-10 column (4.6 mm ID × 25 cm) using acetonitrile: 1% ammonium acetate (80:20) at a flow rate of 1.1 ml/min monitored at 325 nm.
Statistical Analysis

One-way analysis of variance was used to determine whether an overall difference existed among the A−, A+, R, and N groups of data. The Scheffe comparison procedure was used to determine individual differences among the groups.

RESULTS

Alterations in the early corneal response to a \textit{P. aeruginosa} infection due to vitamin A deficiency were determined using corneas from vitamin A-deficient (A−), pair-fed (A+), vitamin A-deficient repleted with retinyl palmitate (R), and normal rats (N). Their corneas were scratched and several different concentrations of \textit{P. aeruginosa} strain 107 were applied topically to the cornea. When low numbers of bacteria (10^4 cfu) were applied to the corneas at 13 weeks (mid to late weight plateau stage of vitamin A deficiency), the five A− but not the five A+ or five N corneas (Fig. 1A compared with Fig. 1B) were mildly infected by 24 hours. An average of 2 × 10^5 cfu bacteria/cornea was present (Table 1). Most of the corneal destruction occurred in the central epithelium and anterior stroma (Fig. 1A). Inflammatory cells were associated with all three layers, including the endothelial cell layer. The major inflammatory cells present in this area were neutrophils (Fig. 1C) that contained typical secretory granules. An average of 4.2 × 10^5 neutrophils was present per A− cornea. In contrast, when 10^4 \textit{P. aeruginosa} cfu was applied topically to the scratched corneas of A+ (Fig. 1B) and N (not shown) rats, infection did not occur. After 24 hours, the epithelial layer was intact and the scratched area in the anterior stroma was filled with epithelial cells. The basal lamina of the corneas was intact except in the scratched area. No bacteria or neutrophils were detected in homogenates of these corneas using bacterial counts or myeloperoxidase assays, respectively. Nor were they noted in sections examined using light and electron microscopes.

When higher concentrations of bacteria were applied to the corneas topically (10^7 and 10^6 cfu), the corneas from all groups of rats became infected within 24 hours. Surprisingly, the extent of ulceration was not as great in the A− corneas at 24 hours (Figs. 2A and B) as in the A+ (Fig. 2C) and N (not shown) rats. This pattern was observed for A− corneas from rats at the early (9 weeks), mid (12 weeks), and late (14 weeks) plateau stages of weight. The A+ and N corneal ulcers were consistent in size, which was about twice that developed in the A− corneas when either 10^7 and 10^6 cfu was applied topically. To confirm that the difference in ulcer size was related to the deficiency, A− rats were fed retinyl palmitate (500 μg in 200 μl safflower oil) 16 days before they were killed; after the retinyl palmitate, they were given free access to the A+ diet. The size of the ulcerated area of these corneas with experimental \textit{Pseudomonas} keratitis was also about twice as large as that of the A− corneas.

Twenty-four hours after \textit{P. aeruginosa} infection, the number of viable bacteria present in the A− corneas was 2 logs greater than the number found in the A+ and N corneas when 10^7 organisms was applied topically (Table 1). The number of neutrophils present in the A− corneas compared with the number found in the A+ and N corneas was 2.6 fold less when 10^7 cfu of bacteria was applied topically. These results suggest that there may be a problem under A− conditions in recruiting neutrophils to the infection site.
TABLE 1. Quantification of *P. aeruginosa* and Neutrophils in Vitamin A Deficient (A—), Pair-fed Control (A+), and Normal Corneas (N) 24 Hours After Infection

<table>
<thead>
<tr>
<th>Bacteria Applied</th>
<th>Rat*</th>
<th>Number of Corneas</th>
<th>Bacteria (log 10)/Cornea</th>
<th>Neutrophils/Cornea (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^4</td>
<td>A—</td>
<td>8</td>
<td>5.30± (3.0–5.85)†</td>
<td>4.2 ± 0.4 × 10^5</td>
</tr>
<tr>
<td></td>
<td>A+</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10^7</td>
<td>A—</td>
<td>10</td>
<td>5.40± (4.90–5.69)†</td>
<td>3.5 ± 0.2 × 10^5†</td>
</tr>
<tr>
<td></td>
<td>A+</td>
<td>8</td>
<td>3.78 (3.90–4.38)</td>
<td>9.9 ± 0.3 × 10^5</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>8</td>
<td>3.92 (3.82–4.03)</td>
<td>9.2 ± 0.1 × 10^5</td>
</tr>
<tr>
<td>10^8</td>
<td>A—</td>
<td>8</td>
<td>5.74 (5.00–6.13)</td>
<td>1.8 ± 2.05 × 10^7</td>
</tr>
<tr>
<td></td>
<td>A+</td>
<td>8</td>
<td>5.70 (5.08–6.19)</td>
<td>3.0 ± 1.4 × 10^7</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>8</td>
<td>5.57 (5.30–5.79)</td>
<td>4.41 ± 1.34 × 10^7</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>8</td>
<td>5.78 (5.26–6.06)</td>
<td>2.4 ± 0.8 ± 10^7</td>
</tr>
</tbody>
</table>

* Serum retinol concentrations were 9.3 ± 4.0, 275 ± 51, 473 ± 74, and 498 ± 105 ng/ml serum for the A—, A+, R, and N rats, respectively. The total liver retinol concentrations were 4.7 ± 2.4, 110 ± 22, 45.2 ± 9.0, and 105 ± 18 μg/g liver for the A—, A+, R, and N rats, respectively.  
† Significant difference at the *P* < 0.01 level from A+ and N for the overall comparison and the individual contrasts (Scheffe comparison) but not between the A+ and N values.  
‡ The range of bacterial counts is given for comparison.

No significant differences were observed in the number of bacteria or neutrophils present in the A— relative to the control rat corneas 24 hours after infection when 10^4 cfu of bacteria was applied topically to the scratched corneas (Table 1). These latter results suggest that the difference in the amount of corneal degradation observed between the A— corneas and the control corneas is not simply due to the number of neutrophils present but may also be related to the activation state of the neutrophils.

The major inflammatory cell noted in all corneas was the neutrophil (Fig. 1C). Infrequently, other inflammatory cells were also observed in the ulcerating corneas. These include the neutrophil precursor, the band cell, eosinophils, and mast cells (Fig. 3). The inflammatory cells were observed within the corneal matrix between the limbus and the ulcerating area (Fig. 2A), associated with the epithelial layer (Fig. 1A), and the endothelial layer (Fig. 1A). Neutrophils containing phagocytized *P. aeruginosa* organisms at different stages of degradation were observed in the corneas of all types of animals (data not shown). Often the cells contained multiple organisms.

In the A— infected corneas, but never in the A+, R, or N infected corneas, cells with unusual granules were noted (Figs. 4 and 5). These cells were found in A— corneas from rats at early (9 weeks old), mid (12 weeks old), and late (14 weeks old) weight plateau stages. These cells were observed in areas of major matrix destruction (Fig. 4D) and in areas where the collagen matrix was essentially intact (Fig. 4A). Approximately 10% of the inflammatory cells present contained abnormal granules. The amount of nuclear...
Pseudomonas Keratitis in Vitamin A Deficiency

FIGURE 3. Examples of inflammatory cells found in rat corneas infected with *P. aeruginosa*. (A) Band cell. (B) Eosinophil and polymorphonuclear leukocyte. (C) Mast cell.

Material present in the sections varied, with some cells being obviously multilobed (Fig. 4A) whereas others showed little nuclear material (Figs. 4B, 4D, and 5A) even with consecutive sections. All cells contained much glycogen. Rough endoplasmic reticulum was observed in many sections (Figs. 4B, 4D, and 5A). Mitochondria were also observed in some sections (Figs. 4B and 5A). Few secretory granules were noted in these cells, but occasionally some were observed (Figs. 4A, 4D, and 5).

The shape, size, and content of the unusual granules varied both within a given cell and between cells (Figs. 4 and 5). The most characteristic feature of these granules was the presence of parallel electron-dense striations in all or part of the granules. Some of the granules resembled autophagic vesicles (Figs. 4C and 4E), whereas others were attached to rough endoplasmic reticulum (Fig. 4E). The striated material was characterized by regular spacing of approximately 80 nm between parallel lines (Fig. 5C). The same spacing was observed between the starlike clusters (Figure 5B). When observed at higher magnification, the parallel lines were composed of globular material. These parallel lines were connected by linear materials (Fig. 5C).

One possible reason for the differences observed in the corneal response to *P. aeruginosa* is the inability to produce specific opsonic antibodies to this organism. To explore this possibility, the distribution of specific antibodies toward *P. aeruginosa* present in the serum of rats from the four dietary groups was examined using Western blots. The serum of all rats contained IgG that recognized multiple *P. aeruginosa* antigens (data not shown). This indicates that all four dietary groups of rats synthesize specific IgG molecules that recognize *P. aeruginosa* and are potentially able to opsonize the bacteria.

Because the number of neutrophils present in the various types of corneas was approximately the same,

FIGURE 4. Inflammatory cells, containing granules with unusual deposits, present in *P. aeruginosa*-infected A—corneas. (A,B,D) Arrows indicate unusual deposits. Bar = 750 nm. (C) Enlargement of B showing the autophagiclike vesicle. Bar = 250 nm. (E) Enlargement of D showing a phagolysosome-like granule and two vesicles containing striated material. Arrows indicate endoplasmic reticulum attached to vesicles. Bar = 500 nm.
when 10⁶ cfu of *P. aeruginosa* was applied, the lesser amount of corneal destruction in A− rats compared with that observed in the A+, R, and N rats could reflect lower concentrations of proteinases or increased amounts of proteinase inhibitors in the A− corneas compared with that in the control corneas. Zymography of corneal extracts from infected corneas was performed on casein (Fig. 6B), casein plus plasminogen (Fig. 6A), and gelatin (Fig. 6C) gels. The pattern of caseinases, plasminogen activators, and gelatinases from the various animals showed cleavage patterns that depended more on the individual animal than on the vitamin A status of the rats. All of the infected corneal extracts contained a 27/28-kDa phenylmethylsulfonyl fluoride-sensitive band (Fig. 7) as well as less prominent bands at higher molecular weights. A 22-kDa band was observed in most of the corneal extracts (Figs. 6 and 7). This band comigrated with the major caseinase band found in uninfected rat corneas (Fig. 7, Control) and a 22-kDa band found in neutrophils (Fig. 7, Control) of control rats. This band, like the other caseinases, was inhibited by the serine proteinase inhibitor phenylmethylsulfonyl fluoride (Fig. 7, phenylmethylsulfonyl fluoride). The 22-kDa band of the infected and uninfected corneas, and the polymorphonuclear cells were specifically inhibited by the inhibitor chymostatin (Fig. 7, Chymostatin). No caseinases were inhibited by the metal chelator, ethylenediaminetetraacetate; the cysteine proteinase inhibitor, E-64; or the aspartic proteinase inhibitor, pepstatin (Gels not shown).

The major plasminogen activator found in infected corneal extracts was observed at 41 kDa, with other forms at 63 kDa and 50 kDa occurring at much lower amounts (Fig. 6A). The 63-kDa form migrated on these gels to the same extent as the human tissue plasminogen activator. No major differences in the patterns of plasminogen activators were observed between the extracts from the A−, A+, R, and N corneas.

The major gelatinases were observed at 82 and 95 kDa (Fig. 6C). Minor bands were observed at 71, 57, and 50 kDa. None of these bands corresponded to the 63-kDa alkaline protease band observed in *P. aeruginosa* 10⁷-conditioned Mueller-Hinton broth. No *Pseudomonas* elastase was detected. Although there were major differences between individual animals, no differences in the overall pattern or amounts of gelatinases were observed for the extracts from the A−, A+, R, and N corneas.

Reverse zymography was used to determine the relative amounts of inhibitory activity present toward the cysteine proteinase papain and the serine proteinase chymotrypsin in the corneal extracts of uninfected corneas. Twofold higher concentrations of cysteine proteinase inhibitors with molecular weights of 63 and 45 kDa were observed in the A− corneal extracts (Fig. 8). No differences were detected in the concentrations of the serine proteinase inhibitor observed at 55 kDa between the extracts from the different dietary groups. These results suggest at least one reason for the decreased degradation of the A− corneas during *P. aeruginosa* infection: The host tissue is protected from proteolytic degradation by increased amounts of cysteine proteinase inhibitors in the A− corneas.

**DISCUSSION**

Vitamin A-deficient corneas are more susceptible to corneal ulceration when low numbers (10⁶ cfu) of *P. aeruginosa* are applied topically to scratched corneas than are corneas from A+, A−, R, and N rats. Given these conditions, the control corneas, A+, R, and N, did not become ulcerated within 24 hours. The control animals mounted an effective defense against this level of infection, both by efficient clearing of the bacteria and by effective wound healing. The total clearance of the bacteria from the anterior segment of the control animals within 24 hours probably involved killing of the bacteria by tear antibacterial proteins such as lysozyme and by neutrophil-mediated mechanisms, and clearance in the tear film. These results are consistent with studies in which the central corneal epithelium of vitamin A-deficient and control rabbits was scraped and wound healing was monitored. No bacteria
similar *P. aeruginosa* proteins. This indicates that the availability of opsonic antibodies is not involved in the differences observed with A— conditions.

Other possible reasons for the differences observed in corneal ulceration, at low numbers of bacteria (10^4 cfu), between the A— and control rats, include increased adherence of *P. aeruginosa* to the keratinized epithelium, alterations in wound healing allowing increased penetration of the bacteria into the stromal matrix, decreased access of neutrophils to the corneal scratch through the tear film route, and changes in the basic functions of neutrophils, which are the initial cells that respond to corneal infections. All four factors probably contribute. After partial removal of the epithelial layer, several differences in the basement membrane area of A— corneas have been noted. No fibronectin or plasminogen activator is present in the scraped area in the A— corneas, in contrast to control corneas. Epithelial wound healing is delayed even in early vitamin A deficiency. The migration of neutrophils to the corneal surface in the tear film probably is not as efficient in the A— animals as in the control animals because of insufficient tears. Although neutrophils in A— animals reach the corneal surface in this way, their ability to phagocytize the bacteria and then be cleared themselves from the corneal surface probably is impeded by the lack of a competent tear film. In the corneas used in the present study, neutrophils were observed from the limbus to the scratched area. This indicates that the limbus and the tear film are major routes taken by neutrophils to the infection. Study of the in vitro functions of neutrophils were added in these studies. Only the deficient animals’ corneas became infected, and the control animals experienced effective wound healing.

Because immunity plays a role in the early response to *P. aeruginosa*, one possible reason for the difference in susceptibility of the A— corneas to *P. aeruginosa* is the inability of the A— rats to synthesize specific opsonic IgG for the organism. Both the A— and control rats synthesized IgG, which recognized...
were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels with and without casein. After electrophoresis, the gels were incubated overnight in the presence of either papain (A) or chymotrypsin (B).

Figure 8. Comparison of proteinase inhibitors present in the uninfected corneas visualized by reverse zymography using papain and chymotrypsin. Representative samples were electrophoresed on sodium dodecyl sulfate–polyacrylamide gels with and without casein. After electrophoresis, the gels were incubated overnight in the presence of either papain (A) or chymotrypsin (B).

from A−, A+, R, and N animals revealed that chemotaxis of neutrophils and adhesion of *P. aeruginosa* to neutrophils are altered by the deficiency (unpublished data).

When more *P. aeruginosa* organisms (10⁷ to 10⁸ cfu) were applied topically to the corneas, all corneas became infected within 24 hours. The controls, A+, R, and N corneas, were more degraded at 24 hours after the application of the bacterium than were the A− corneas. This phenomena was noted consistently at all stages of the deficiency (early, mid, and late weight plateau stages). These observed differences may be true only in the early stages of infection. By 48 hours, the A− corneas may be degraded to the same or greater extent. Time-course studies with 10⁷ to 10⁸ *P. aeruginosa* strain 107 applied to scratched normal rat corneas revealed descemetocele formation and perforation in 75% of the corneas by 36 hours and in 100% of the corneas by 48 hours (Twining et al, unpublished data). Future experiments will compare the time course of *Pseudomonas* keratitis in the A− and control corneas.

The estimated numbers of total neutrophils present were about 2.6 times higher in the control corneas compared with the A− corneas when 10⁷ *P. aeruginosa* cfu were applied topically to the scratched corneas; however, when 10⁸ *P. aeruginosa* cfu were applied topically, no significant differences were observed in the number of neutrophils present in the corneas of the different groups of animals. In addition, no differences were observed in the concentrations of gelatinases, cascinases, or plasminogen activators in the infected corneal extracts by zymography. This technique detects both active and inactive forms of matrix metalloproteinases. These forms of the enzymes are observed as different molecular weight bands on the zymograms. However, this technique does not distinguish between released and granule-sequestered neutrophil proteinases. This suggests the neutrophils in the control corneas are more actively secreting oxidative and proteolytic molecules than are neutrophils in the A− corneas. The major difference in the amount of proteinases present in the A− compared with A+, R, and N control neutrophils is a deficiency in a cathepsin G-like enzyme that has a molecular weight of 22 kDa (unpublished data). Cathepsin G can activate matrix metalloproteinases both from the neutrophil (neutrophil progelatinase and procollagenase) and host tissues (stromelysin and MMP-2). This enzyme also has antimicrobial activity independent of its proteolytic activity. Because neutrophil cathepsin G comigrates with a similar enzyme in the cornea, the cascin zymograms of the infected A− corneal extracts do not show this deficiency when compared with those of control corneal extracts. In addition, the observed increase in cysteine proteinase inhibitory activity in the A− uninfected corneas compared with the A+, R, and N uninfected corneas may account for part of the decreased ulceration observed with A− conditions.

Neutrophils were the major inflammatory cells present in the infected corneas, although some others, such as band cells, eosinophils, and mast cells, were observed 24 hours after applying *P. aeruginosa* to the scratched corneas. Only A− corneas contained cells with unusual striated or pseudostriated granules. These were found in areas of high corneal destruction and in areas with intact collagen. They were approximately the size of polymorphonuclear leukocytes (neutrophils, eosinophils, and basophils) and were too small for macrophages or keratocytes. In sections observed using the electron microscope, the number of visible lobes varied, which may reflect the location of the granules in relation to the nuclei. The starlike clusters found in some of the granules are similar to nuclear pore complexes. This suggests that some of the granules might contain degraded nuclear components, which would account for the low number of visible nuclear lobes.

The cells that contain the striated deposits are probably abnormal neutrophils or basophils. The presence of mitochondria and prominent rough endoplasmic reticulum suggests that these cells are immature. If these cells are neutrophils, most of the en-
zyme granules have fused with phagosomes, the contents have been secreted into the extracellular matrix, or both. The shape of the granules are irregular and can vary in size. Striated material can also be found in phagolysosomal-like vesicles and in autophagic-like vesicles, with some of these granules attached to rough endoplasmic reticulum–like structures. The identity of the abnormal striated material is not known. One possibility is that the abnormal deposits are remnants of abnormally degraded P. aeruginosa organisms. Other possibilities include type VI collagen or an unusual form of type I collagen, such as “segment-long-spacing crystallites” of collagen. The granular material observed at high magnification, however, would suggest that this material is not type I collagen but more likely type VI collagen. The presence of the deposits in inflammatory cells suggests a possible deficiency in a degradative enzyme(s) in these inflammatory cells. As noted before, the only enzyme found to be deficient in the neutrophils of the A— animals is cathepsin G.

If these cells are basophils rather than neutrophils, the phagosome-like vesicles could be partially degranulated granules. Many of the granules are larger than those in normal basophils, which would suggest that the cells are abnormal. Basophil granules often contain membranous structures such as those observed in some of the cells. Additional studies will be required to determine the exact identity of these cells and the contents of the vesicles. Acid phosphatase reactivity will be useful to determine whether these are neutrophils (positive) or basophils (negative).

These results suggest that the early response of the vitamin A deficient cornea to experimental P. aeruginosa infections differs from that of control corneas. Future experiments will explore the time course and the cellular and molecular basis for these differences.

Key Words
cornea, electron microscopy, proteinases, Pseudomonas aeruginosa, rats, vitamin A deficiency

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