The opportunistic bacterial pathogen *Pseudomonas aeruginosa* is a common cause of severe corneal infections. Factors that predispose humans to corneal infections with this pathogen include (1) trauma to the cornea with, or implantation of, foreign bodies or substances contaminated with the bacteria; (2) the presence of preexisting ocular disease; (3) immunosuppressive chemotherapy; (4) presumed immunoincompetency in premature infants; or (5) perhaps genetic disposition. The infection is difficult to treat, since it progresses rapidly and may cause dissolution of the corneal stroma within 72 hr.

The pathogenic mechanisms leading to stromal destruction are not clear. On the one hand tissue destruction may be caused by the direct action of *P. aeruginosa* extracellular virulence factors. Thus, purified exotoxin A (ExoA), elastase (Ela), or alkaline protease (AP), when dropped on the incised cornea of experimental animals elicit corneal ulcerations which resemble those caused by *P. aeruginosa* strains. Whereas ExoA acts directly on epithelial, endothelial and stromal cells, inhibiting the protein biosynthesis, both *P. aeruginosa* proteinases degrade the proteoglycan matrix rather than the collagen fibers.

The role of the proteinases in the process of corneal infection is further demonstrated by studies using proteinase-deficient mutants, which seem to be less virulent than the respective wild strains.

On the other hand, host-derived inflammatory processes may cause corneal destruction in *P. aeruginosa* infections. Potential sources of pathogenic factors include keratocytes, corneal epithelial cells, and polymorphonuclear leukocytes (PMN). The latter have been shown to migrate into the corneal stroma in high numbers after the onset of the infectious process. The release of lysosomal enzymes and oxidative substances capable of damaging collagen fibers and the proteoglycan matrix may be relevant in this respect.

The question therefore arises whether the two mechanisms function in sequence or simultaneously, and whether one of them predominates. It was the objective of the present study to determine this by (1) excluding bacterial virulence factors as a possible cause of corneal destruction through active immunization of rabbits and (2) by comparing the pathogenic effects of non-proteolytic and proteolytic *P. aeruginosa* strains in immunized and non-immunized animals.

Light photography, light microscopy, and electron microscopy were used to demonstrate corneal destruction elicited with purified enzymes and viable *P. aeruginosa* strains.
Materials and Methods

**P. aeruginosa** Strains and Enzyme Purifications

Alkaline protease (AP) and elastase (Ela) were purified from *P. aeruginosa* strain no. 1, originally isolated from the sputum of a cystic fibrosis patient, as previously described. Exotoxin A (ExoA) was purified from strain PA 103. The purified enzymes migrated as single bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% spacer gel and a 12% separating gel in the presence of β-mercaptoethanol.

The strains used in the animal experiments were PA01, producing ExoA, Ela and AP, and PA103, producing ExoA and a minute amount of AP. These strains were propagated in a shaken (120 rpm) trypticase soy broth for 24 hr at 30°C and harvested by centrifugation at 8,000 g. Cells were washed three times in 0.9% sodium chloride. AP, Ela and ExoA production of the two strains was determined in the culture supernatant fluids (CSF) using specific radioimmunoassays (RIA). Briefly, the solid phase of flexible polyvinyl chloride U-shaped plates was coated with purified rabbit immunoglobulin G (IgG) antibodies specific for the respective enzymes. After saturation of the solid phase with 0.1 M phosphate-buffered saline (PBS) (pH 7.2) supplemented with 5% bovine serum albumin, 100 μl of the ten-fold diluted CFU was added to the washed wells, and the plates were incubated for 12 hr at 4°C. Serial dilutions (ten-fold) of the purified enzymes in PBS were used as a standard for quantitative measurements on each plate. After washing, 100 μl of a solution of rabbit 1-IgG specific for the respective enzymes was added to the wells, and the plates were again incubated for 12 hr at 4°C. Finally, the bound radioactivity of the washed wells was measured with a gamma counter (Berthold, Wildbad, FRG).

**Immunization**

The rabbits were immunized as follows: 1 mg per ml of the respective purified enzymes emulsified in an equal volume of Freund’s complete adjuvant were injected intramuscularly and subcutaneously. After 3 weeks the injection was repeated four times at 2 week intervals using 1 mg per ml of the respective purified enzymes suspended in Freund’s incomplete adjuvant. Serum antibody response to the enzymes was checked using Ouchterlony’s method and specific RIA. Occurrence of specific antibodies to the enzymes was also checked in the tear fluids of two rabbits immunized against AP or Ela using the method of Knopf. Briefly, one sterile Schirmer strip (Dr. Mann, Berlin, FRG) was placed in the unanesthetized lower fornix of each eye. The saturated strips were then dropped into 6 ml of PBS, pH 7.2, and stored at 4°C. Tears were collected once daily for 2 weeks. All strips taken from a single animal were pooled. Two non-immunized rabbits served as controls. After 2 weeks the strips were removed, and 60% saturated ammonium sulfate solution was added. After centrifugation (10,000 rpm, 4°C, 20 min) the pellets were dissolved in 1 ml of PBS and the solution analyzed for antibodies to AP or Ela with RIA.

**Preparation of the Corneal Pocket**

Rabbits aged approximately 12 months and weighing 2–4 kg were used. For the inoculation of *P. aeruginosa* enzymes or strains the animals were anaesthetized intravenously with 5 mg Diazepam (Valium®, Hoffman-La Roche, Basel, Switzerland); additionally the cornea was anaesthetized with several drops of Benoxinate® 0.4% (Thilo, Sauerlach, West Germany). A pocket was then prepared in the left cornea of the animals using Oggel’s method: the cornea was incised horizontally in the central area, and a sterile spatula was cautiously inserted into the cornea to form the pocket. Then 0.1 ml of various *P. aeruginosa* enzymes was injected with a 20-gauge needle. For the controls, 0.1 ml PBS was used.

**Corneal Inoculation of Infective Agents**

For the animal experiments AP was diluted in 0.1 M PBS (pH 7.4) and Ela in 0.9% saline, supplemented with 0.01 M sodium acetate and 0.05 M calcium chloride (pH 7.4). Microgram amounts of AP or Ela were inoculated into the prepared corneal pockets of rabbits immunized against the respective protease and of non-immunized animals. For each enzyme three immunized rabbits and one control were used.

For experiments with bacterial strains the bacteria were suspended in 0.9% sodium chloride and 10⁶ colony-forming units (CFU) were inoculated into the rabbit corneal pockets. Twelve rabbits were immunized against AP, Ela and ExoA; six rabbits were used as non-immunized controls. Six immunized and three non-immunized rabbits were used for each of the two *P. aeruginosa* strains.

**Documentation**

Rabbit eyes were monitored by light photography (LP) after 5, 12 and 24 hr and then every 24 hr up to 14 days after inoculation. For light and electron microscopy (LM, EM) the animals were killed after 48 h with pentobarbital (Nembutal®, Abbott, Wiesbaden, West Germany). For LM corneal specimens were embedded in paraffin. Semithin sections were stained...
with PAS (Periodic-Acid-Schiff-reaction). For EM corneal specimens were fixed in 2.5% buffered glutaraldehyde solution for 4 hr, then refixed in 1% OsO₄ solution, dehydrated and embedded in Epon. Semithin sections were prepared using an ultramicrotome (Ultracut, Reichert and Jung, Nussloch, FRG), and stained according to the method of Richardson et al. All ultrathin sections were analyzed with the EM 10 electron microscope (Zeiss, Oberkochen, FRG).

All experiments with these animals were carried out in accordance with the ARVO Resolution on the Use of Animals in Research.

Results

Immunization

After three booster injections of the various enzymes all rabbits revealed specific serum antibody titers, as shown by Ouchterlony double diffusion analysis and RIA (reciprocal serum titers: mean AP: 107; mean Ela: 133; mean ExoA: 93). The tear fluids of one rabbit immunized against AP and one rabbit immunized against Ela which were assayed by RIA were both positive for antibodies to the respective proteinases, ie, inhibition of the tracer in the assay was 33% for AP and 31% for Ela compared to 1–2% for the non-immunized control tear fluids.

Effect of Purified Ela and AP on Corneas of Immunized and Non-Immunized Rabbits

After preparation of the pocket in the corneal stroma one immunized rabbit received 38 μg Ela and the other two immunized rabbits received 19 μg in 0.1 ml of buffer (one of the rabbits which had received 19 μg Ela developed an infection with Staphylococcus aureus and was excluded from the study). Five hours after the injection of Ela, the area around the corneal pocket of the two remaining immunized rabbits became opaque. The rest of the corneas remained unchanged. The opacity disappeared within 5–8 days and the wound area slowly healed, leaving a scar at the pocket incision (Fig. 1).

Ocular damage developed as follows in the non-immunized rabbits: opacity appeared around the pocket area 5 hr after the injection of Ela and the entire cornea became opaque after 24 hr. After 2 days the cornea had melted; a protruding descemetocele was observed, and the lids and conjunctiva were markedly inflamed. The rabbits were killed at this point. The other rabbit, which had received 19 μg of Ela, revealed similar, but less severe corneal damage. After 2 weeks corneal necrosis was seen only in the central wound area (Fig. 1).

The three rabbits immunized against AP received 41.6 μg AP in 0.1 ml of buffer in the corneal pocket. Five hours after injection of the proteinase, opacity in the pocket area was observed. In all three animals this opacity disappeared within 9 days (Fig. 1). When the same concentration of AP was injected into the corneal pocket of a non-immunized rabbit, the opacity appearing 5 hr after injection expanded within 24 hr to cover the whole cornea. After 2 days, as with Ela, descemetoceles were observed, and the necrotic cornea melted (Fig. 1). Figure 2 A–D shows the eyes of immunized and non-immunized rabbits 48 hr after challenge with AP or Ela.

Effect of P. aeruginosa Strains PA01 and PA103 on Corneas of Immunized and Non-Immunized Rabbits

Rabbits immunized against AP, Ela and ExoA were inoculated with 10⁶ CFU of either the high proteolytic strain PA01 or the low AP-producing strain PA103. Both strains were positive in vitro for ExoA production. After 12 hr only slight corneal opacity was observable in the pocket area (Fig. 3, Table 1). Thereafter, rapid corneal deterioration with abundant pus developed, indicating a high inflammatory response. Between 24–48 hr corneal transparency was entirely lost, and severe ulcerations had appeared. No improvement in the corneal status was noticed in the following 14 days; this was documented in four animals. No significant differences were observed in the corneal damage caused by PA01 and PA103. Corneal opacification in non-immunized rabbits was more intense during the first 24 hr than in immunized rabbits (Figs. 3, 4). Significant differences between the two strains in non-immunized animals were first noticed 12 hr after inoculation; PA103 was followed by severe opacity in the pocket area, whereas PA01 led only to grade 2 destruction (Table 1, Fig. 3). After 48 hr there were no
detectable differences between the corneal statuses of the above-mentioned animal groups.

**LM and EM Findings**

Forty-eight hours after inoculation of bacteria of either strain, LM revealed necrosis of the corneal stroma in both immunized and non-immunized animals. In addition, a massive PMN invasion was visible (Fig. 5A, B). There was a large epithelial defect with necrosis of the underlying superficial stroma and loss of endothelial cells. No differences in tissue damage or PMN numbers were observed between immunized and non-immunized animals.

EM corneal specimens from all animals infected with the two *P. aeruginosa* strains showed a massive inter- and intracellular edema in the periphery of the basal epithelial layer. In addition, cells of the basal lamina were partly dissolved and surrounded by large numbers of PMN. In the underlying stroma the lamellar network of collagen fibers showed a slight edema (Fig. 6A), but the collagen structure itself was unaltered (Fig. 6B). Bacteria of both strains were visible among these regularly arranged collagen fibers in the corneas of both immunized and non-immunized animals, with no electrolucent halo (Fig. 6).

The epithelial layer had disappeared in the pocket area of all infected animals. The corneal stroma revealed many activated PMN. In addition, free lysosomes were visible among destroyed collagen material (Fig. 7). This amorphous collagen material was phagocytized by PMN (Fig. 8). Degenerative alterations of keratocytes within the corneal stroma were evident (Fig. 9).

After 48 hr neither LM or EM showed significant differences between immunized and non-immunized animals or between proteolytic or non-proteolytic *P. aeruginosa* strains.

**Discussion**

The results suggest that the pathogenic mechanisms activated by the bacteria and bacterial enzymes are less important for tissue destruction than host-derived factors in corneal *P. aeruginosa* infection. Several lines of evidence support this notion: (1) after exclusion of bacterial virulence factors by immunization, no differences were noted between immunized and non-immunized animals following challenge with viable *P. aeruginosa* strains (Fig. 3) 48 hr after inoculation. That immunization was in fact effective

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>PA01</th>
<th>PA03</th>
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<tbody>
<tr>
<td>12</td>
<td>1.0 ± 0</td>
<td>1.67 ± 0.58</td>
</tr>
<tr>
<td>24</td>
<td>3.0 ± 0.63</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>48</td>
<td>4.83 ± 0.98</td>
<td>4.67 ± 0.58</td>
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*Grade of corneal destruction ± SD: 1: Clear (only scars due to the pocket preparation); 2: slight opacity in the pocket area; 3: severe opacity in the pocket area; 4: opacity of the cornea; 5: melting of the cornea with hypopyon; 6: perforation.*
in excluding bacterial virulence factors in corneal damage was clearly demonstrated by the application of purified exoproteinases in high doses in immunized animals (Figs. 1, 2): all animals were totally protected—a result which had also been achieved earlier by Hirao and Homma.\(^27\) (2) No differences in corneal damage were found between high proteolytic and low proteolytic strains in immunized and non-immunized animals (Fig. 3). Regarding the minute production of AP (0.1 \(\mu\)g) and Ela (neg.) by PA103 compared to the production of AP (0.2 \(\mu\)g) and Ela (4.7 \(\mu\)g) by PA01, differences would have been visible if AP or Ela had actually played an important role in the pathogenicity of corneal infection. Although AP and Ela have been shown in vitro to inhibit pronounced effects on PMN chemiluminescence and chemotaxis,\(^28,29\) so the possibility cannot be excluded that they inhibited these functions to some degree in the non-immunized rabbits in vivo as well, the high numbers of PMN in both immunized and non-immunized animals and the course of corneal destruction (which did not differ significantly in either group 48 hr after inoculation) make this very unlikely. (3) Electron micrographs from all animals challenged with either strain show bacteria surrounded by intact corneal stroma, suggesting that the release of bacterial exoproteinases, and thus tissue damage, are negligible (Fig. 6). Intact stroma in immunized rabbits may also be due to neutralization of bacterial exoproteinases by specific antibodies; this cannot, however, be the case in non-immunized animals. (4) Massive dissolution of the stromal tissue and the loss of the regular network of the extracellular matrix in the neighborhood of activated PMN and free lysosomes is shown by EM (Figs. 7, 8). These PMN digest amorphous collagen material.

The discrepancies between these results and those of other investigators may be best explained by the use of different experimental eye infection models. In the present study the pocket model, which is similar to the intracorneal injection model,\(^14,15\) was chosen. In both models the bacteria are placed directly into the corneal stroma. In the trauma model, on the other hand, a scratch is made through the epithelial layer of the cornea into the stroma, and the bacteria are dropped onto the traumatized corneal surface. Whereas the establishment of infection in the trauma model requires the virulence of \(P.\) aeruginosa, this seems less important when the bacteria have already reached the stroma, as in our model.

These considerations may explain the results of \(P.\) aeruginosa keratitis induced experimentally in the trauma model by Kahawarajo and Homma,\(^11\) who reported that proteolytic \(P.\) aeruginosa strains caused corneal ulcers in contrast to non-proteolytic strains which were totally avirulent. The same considerations apply to the results of Ohman et al\(^12\) and Howe and Iglewski,\(^13\) who demonstrated the importance of...
AP for the establishment of corneal infection, since AP-deficient *P. aeruginosa* mutants were unable to colonize the traumatized cornea. Furthermore, other virulence factors of *P. aeruginosa*, such as hemolysins\(^3\) or ExoA,\(^5\) may also be important in the early stages of keratitis. In the present study a role for AP, Ela and ExoA within the first 24 hr after inoculation is also indirectly implied by the delay of corneal destruction in immunized in contrast to non-immunized rabbits.

However, immunization was not effective in improving the situation 48 hr after inoculation with the bacterial strains. Although Hirao and Homma\(^2\) have stated that active and passive immunization against *P. aeruginosa*, AP, Ela and the common antigen (OEP) exhibited remarkable therapeutic effect on corneal ulcers in mice due to viable *P. aeruginosa* inoculation, their effects were achieved only in combination with antibiotic chemotherapy. In a recent study, Kreger et al\(^3\) demonstrated a pronounced ef-
Fig. 6. Electron micrograph of PA01 in immunized (A) and non-immunized (B) animals taken about 6 mm from the limbus. Note the bacteria within regularly arranged collagen fibers 48 hr after inoculation. Only slight stromal edema is apparent (E). No electrulcent halo could be observed around the bacteria. A, magnification ×70,000; B, ×19,000.

Effect of active and passive immunization against *P. aeruginosa* lipopolysaccharide or purified proteinase preparations in rabbits and mice. Again, the use of the trauma model may explain the divergence between their results and ours. On the other hand, Twining et al,32 using the same trauma model, reported that rats immunized against phenol-killed *P. aeruginosa* revealed massive corneal degradation in contrast to mild stromal degradation in non-immunized animals 18 hr after challenge with *P. aeruginosa* organisms. The latter study corroborates our findings that the PMN may contribute significantly to corneal degradation.

This hypothesis was also supported by Kessler et al and Hazlett et al. Both groups noticed extensive tissue damage due to massive PMN infiltrates after elimination of bacterial virulence factors by heat inactivation of *P. aeruginosa* prior to inoculation.16,33 Furthermore, several authors have reported that after application of agents that reduce PMN infiltration (ie, corticosteroids, cyclophosphamide) or after C3 depletion ulceration of the cornea was markedly re-
Fig. 7. Electron micrograph showing free lysosomes (L) within the corneal collagen. The outer membrane of the lysosomes is partly destroyed (arrow). Note the unusual arrangement of the collagen fibers (arrowheads). (Pocket area of an immunized rabbit 48 hr after challenge with strain PA103. Magnification ×18,200).

Fig. 8. Polymorphonuclear leukocyte within amorphous collagentic material. The amorphous material is partly adherent to the cell membrane (arrows). Inset: higher magnification of the amorphous collagen material (magnification ×14,000; inset, ×75,000).
Fig. 9. Alteration of keratocytes in the periphery of the cornea is shown by chromatin margination (N) and the partial lysis of the cell membrane (arrow). (Electron micrograph taken from an immunized animal 48 hr after challenge with strain PA103. Magnification x9,200).

As in the present study, Van Horn et al also observed no proteolytic alterations of the corneal matrix associated with bacteria.

Summarizing the results from this and other studies on experimentally-induced *P. aeruginosa* keratitis, pathogenic events in the early stage of bacterial colonization seem to involve several *P. aeruginosa* virulence factors which facilitate the establishment of infection and bacterial multiplication. As soon as the host responds to the invasion with high numbers of PMN, tissue destruction increases rapidly due to enzymes released from stimulated PMN, and the clinical situation deteriorates significantly. This may occur as soon as 24–48 hr after successful bacterial colonization. Thus it seem that the two pathogenic mechanisms function in sequence and that the latter quickly predominates. Concerning therapeutic aspects of *P. aeruginosa* corneal infection, immunization against *P. aeruginosa* exoenzymes or inhibition of AP or Ela by metalloproteinase inhibitors seems to be less effective in preventing corneal damage than chemotherapy with the aim of reducing PMN infiltration or direct inhibition of PMN lysosomal enzymes.

**Key words:** *Pseudomonas aeruginosa* exoenzymes, corneal infections, immunization, polymorphonuclear leukocytes

**Acknowledgments**

The authors wish to thank Ingrid Gutbrod and Maria Haug for technical assistance and Thomas Rice for preparation of the manuscript.

**References**


