Relevance of Host-Derived and Bacterial Factors in \textit{Pseudomonas aeruginosa} Corneal Infections

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Two pathogenic mechanisms of \textit{Pseudomonas aeruginosa} corneal infections are discussed, one involving bacterial exoenzymes, the other involving polymorphonuclear leukocyte (PMN)-derived lysosomal enzymes. The objective of the present study was (1) to show the relative importance of the two mechanisms and (2) to evaluate the effect of active immunization against \textit{P. aeruginosa} exoenzymes on ocular damage. Rabbits were immunized against \textit{P. aeruginosa} alkaline protease (AP) or elastase (Ela) and challenged with the respective enzymes. Corneal damage was studied by light photography (LP). In another group, rabbits were immunized against AP, Ela and exotoxin A (ExoA) and challenged with \textit{P. aeruginosa} strains PA01 or PA103. Corneal damage was studied with LP, light microscopy, and electron microscopy. Immunized animals were totally protected against intracorneal inoculation of \textit{P. aeruginosa} proteases. Twelve hr and 24 hr after challenge with whole bacteria, immunized rabbits revealed less corneal damage than non-immunized animals. However, after 48 hr corneal damage (ie severe corneal ulceration) was comparable in both groups. The study suggests that corneal damage involving lysosomal enzymes from stimulated PMN is more important after bacterial infection than direct damage by \textit{P. aeruginosa} exoenzymes. Invest Ophthalmol Vis Sci 28:1559–1568, 1987

The opportunistic bacterial pathogen \textit{Pseudomonas aeruginosa} is a common cause of severe corneal infections.\textsuperscript{1} 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.\textsuperscript{1} The infection is difficult to treat, since it progresses rapidly and may cause dissolution of the corneal stroma within 72 hr.\textsuperscript{2}

The pathogenic mechanisms leading to stromal destruction are not clear. On the one hand tissue destruction may be caused by the direct action of \textit{P. aeruginosa} extracellular virulence factors. Thus, purified exotoxin A (ExoA),\textsuperscript{3–5} elastase (Ela),\textsuperscript{6,7} or alkaline protease (AP),\textsuperscript{7} when dropped on the incised cornea of experimental animals elicit corneal ulcerations which resemble those caused by \textit{P. aeruginosa} strains.\textsuperscript{8} Whereas ExoA acts directly on epithelial, endothelial and stromal cells, inhibiting the protein biosynthesis,\textsuperscript{3} both \textit{P. aeruginosa} proteinases degrade the proteoglycan matrix rather than the collagen fibers.\textsuperscript{9,10}

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.\textsuperscript{11–13}

On the other hand, host-derived inflammatory processes may cause corneal destruction in \textit{P. aeruginosa} infections.\textsuperscript{1} 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.\textsuperscript{14,15} The release of lysosomal enzymes and oxidative substances capable of damaging collagen fibers and the proteoglycan matrix may be relevant in this respect.\textsuperscript{16,17}

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 \textit{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 \textit{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 shaker (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 I-gG 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 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, Saarbruecken, 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 proteinase 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 10⁵ 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 glu-
taraldehyde solution for 4 hr, then refixed in 1% OsO4
solution, dehydrated and embedded in Epon. Se-
mithin sections were prepared using an ultramicro-
tome (Ultracut, Rechert and Jung, Nussloch, FRG),
and stained according to the method of Richardson et
al.26 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 en-
zymes 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 re-
ceived 19 µg Ela developed an infection with Staphy-
llococcus 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 re-
main 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-immu-
nized rabbits: opacity appeared around the pocket
area 5 hr after the injection of 38 µg 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 cor-
neal 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 cor-
nea 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 pro-
teolytic 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 abun-
dant pus developed, indicating a high inflammatory
response. Between 24–48 hr corneal transparency
was entirely lost, and severe ulcerations had ap-
ppeared. No improvement in the corneal status was
noticed in the following 14 days; this was docu-
mented in four animals. No significant differences
were observed in the corneal damage caused by PA01
and PA103. Corneal opacification in non-immu-
nized rabbits was more intense during the first 24 hr
than in immunized rabbits (Figs. 3, 4). Significant
differences between the two strains in non-immu-
nized animals were first noticed 12 hr after inocula-
tion; PA103 was followed by severe opacity in the
pocket area, whereas PA01 led only to grade 2 de-
struction (Table 1, Fig. 3). After 48 hr there were no

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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
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 μg) and Ela (neg.) by PA103 compared to the production of AP (0.2 μg) and Ela (4.7 μg) 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 \textit{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 \textit{P. aeruginosa} keratitis induced experimentally in the trauma model by Kahawarojo and Homma,11 who reported that proteolytic \textit{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 al12 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 or ExoA, 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 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 demonstrated a pronounced ef-

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**Fig. 5.** Light photographs of corneal rabbit tissue 48 hr after inoculation with whole *P. aeruginosa* strains. Note the extensive PMN infiltration, especially in the pocket area (P). (A) Immunized rabbits inoculated with PA01; (B) non-immunized rabbits inoculated with PA01. A and B, magnification X570.
fect 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, 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. 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 collagenic 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 ×9,200).

As in the present study, Van Horn et al also observed no proteolytic alterations of the corneal matrix associated with bacteria. 14

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 seems 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 16 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

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References


