The corneal response to

*Pseudomonas aeruginosa:*

histopathological and enzymatic characterization

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The host response to the intrastromal injection of heat-inactivated *Pseudomonas aeruginosa* cells was studied. An extensive polymorphonuclear leukocyte (PMN) infiltration, which progressed to ulceration within 1 week, was observed. In some cases descemetoceles also developed. Only a limited degree of PMN infiltration and no ulcerations were observed at 1 week in eyes treated with corticosteroids. Collagenase and proteolytic enzymes capable of degrading proteoglycans were found in the ulcerated corneas. A correlation was made between the level of these host-derived enzymes and the extent of corneal destruction. It was concluded that corneal destruction by *P. aeruginosa* depends not only on the *Pseudomonas* protease, which rapidly destroys the cornea, but also on host-derived enzymes which are capable of degrading both collagen and proteoglycans.

Key words: *Pseudomonas aeruginosa*, corneal ulcers, host-derived enzymes, collagenase, protease.

*Pseudomonas aeruginosa* has become a leading cause of severe central corneal ulcers. The ulcer is characterized by rapid corneal liquefaction which progresses to perforation if not treated immediately and intensively. Early studies by Fisher and Allen¹ ² have indicated that an extracellular protease, thought to be collagenolytic in nature, is produced by the invading bacteria and is responsible for this rapid destruction of the cornea. Subsequent studies also emphasized the role of the *Pseudomonas* protease in the production of corneal ulcers. It was shown by Brown and co-workers³ that the effective bacterial filtrate fraction contained proteolytic but not collagenolytic activity. Since this fraction was found to readily hydrolyze proteoglycans, the authors concluded that corneal destruction results from enzymatic degradation of
the corneal proteoglycans. This view was supported by Kreger and Griffin, who also demonstrated an absence of collagenase in the bacterial filtrate. Furthermore, the disappearance of polysaccharides from rabbit corneas infected with *P. aeruginosa* was recently demonstrated by the ultrastructural studies of Gray and Kreger.8

In addition to the destructive effects of the *Pseudomonas* protease, it has been recently suggested that host-derived hydrolytic enzymes, including collagenase, may be produced as part of the inflammatory response of the cornea to *Pseudomonas* infections.5, 6 Since such enzymes have not been demonstrated to date, the present studies which focus on the host response to *Pseudomonas* were undertaken. For this purpose, a model system was developed which eliminated the effect of active bacterial infection and bacterial protease production. Corneal ulcers were produced by the injection of heat-inactivated *Pseudomonas* cells into rabbit corneas. The resultant ulcers were characterized histopathologically and were analyzed for the presence of host-derived hydrolytic enzymes.

**Materials and methods**

**Bacteria and preparation of heat-inactivated cells.** A virulent strain of *P. aeruginosa* which was isolated from an active human corneal infection was used throughout this work. Stock cultures were maintained on nutrient agar slants at 4° C. and were retransferred every 3 months. For cultivation, a slant was suspended in 5 ml. of sterile saline, and 1 ml of the suspension was inoculated into 1 liter of tryptic soy broth without dextrose (Difco Laboratories, Detroit, Mich.) placed in a Fernbach flask. The inoculated medium was incubated at 37° C. for 18 hours on a gyratory shaker (Model G-10; New Brunswick Scientific Co., Inc., New Brunswick, N. J.) at 150 cycles/min., yielding an absorbance of 1.7 to 2.25 at 660 nm. The cells were collected by centrifugation (Sorvall RC2-B, 10,000 x g, 15 min.) and washed twice with sterile saline by repeated suspensions and centrifugations. The washed cells were finally suspended in a small volume of sterile saline to yield an approximate final concentration of 3 x 10^11 cells/ml. (based on viable count) and autoclaved at 120° C. for 30 minutes. The heat-inactivated cell suspension was stored at -20° C. prior to use.

**Induction of corneal ulcerations and experimental scheme.** Thirty-seven albino rabbits, weighing between 2 and 4 kg., were anesthetized by intravenous injection of sodium pentobarbital (about 15 mg./kg.) and topical proparacaine hydrochloride (0.5 per cent). The globes were propoioned, and the central corneal epithelium (approximately 6 mm. in diameter) was removed by gentle scraping with a scalpel blade. The heat-inactivated cell suspension (40 μl) was injected intrastromally into the center of the cornea with a 0.25 ml. tuberculin syringe and a 30-gauge needle. All rabbit eyes were treated with topically applied gentamicin sulfate (3 mg./ml.) once daily. The rabbits were divided into the following three groups:

1. **Gross observations.** Ten rabbits were examined daily for 2 weeks and twice weekly for an additional 2 weeks for the development and course of corneal ulcerations. Cultures of the eyes were repeatedly taken and were negative for *P. aeruginosa* or any other organisms. Six additional rabbits received bilateral subconjunctival injections of triamcinolone diacetate (40 mg./eye) in addition to the intracorneal injections of heat-inactivated *Pseudomonas* and were followed by gross observation for 2 weeks.

2. **Histopathological examination.** Six rabbits were sacrificed in pairs on days 2, 5, and 10 after injection. Three additional rabbits received bilateral subconjunctival injections of 40 μg. of triamcinolone diacetate in addition to the intracorneal injections of heat-inactivated *Pseudomonas* and were followed by gross observation for 2 weeks.

3. **Corneas for tissue culture.** Six rabbits were sacrificed 6 days after injection, and the corneas cultured as described below. The corneas from six rabbits which received bilateral subconjunctival injections of corticosteroids in addition to the intracorneal injections were cultured similarly. Six rabbits were sacrificed, and their corneas cultured as controls with only the central epithelium removed and without an intracorneal injection.

**Culture conditions and enzyme preparation.** Rabbits were sacrificed, and their corneas were immediately removed and placed in plates containing sterile gentamicin solution (3 mg./ml.) for 10 minutes. The corneas were transferred into plates containing culture medium (see below) to which 1 to 2 drops of gentamicin solution were added, and incubated for 1 to 3 hours at 37° C. The corneas were then cut into small pieces (2 by 2 mm.) and placed in organ culture plates (Falcon Plastics, Oxnard, Calif.) contain-
ing Dulbecco's modified Eagle's medium (DME, Grand Island Biological Co., Madison, Wis.) diluted 1:1 with Tyrode's salt solution and supplemented with penicillin and streptomycin sulfate at 0.063 and 0.1 mg/ml, respectively. The plates were incubated at 37° C in a humidified atmosphere of 5 per cent CO₂ and 95 per cent air for 7 days. Media were replaced on days 1, 3, 5, and 7 and stored at -20° C prior to use. The media from each group of animals were pooled and centrifuged to remove cell debris. The media from each group of animals were pooled and centrifuged to remove cell debris. The media from each group of animals were pooled and centrifuged to remove cell debris.

The concentrated fractions were stored as a frozen solution in small vials at -20° C.

Preparation of salt-soluble [¹⁴C]-glycine-labeled collagen. Ten guinea pigs each weighing approximately 225 gm. were injected with 100 µCi of [U-¹⁴C]glycine (96 mCi/mole; New England Nuclear, Boston, Mass.) in 1.0 ml of sterile saline. After 6 hours the animals were killed, and the soluble skin collagen was extracted and purified by salt precipitation. The precipitate was dissolved in a mini-
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acrylamide gel electrophoresis in presence of SDS was performed according to the method of Fairbanks and co-workers. Bio-Phore gels (7.5 per cent; Bio-Rad Laboratories, Richmond, Calif.) were pre-equilibrated and used with Bio-Rad Tris-acetate-SDS buffer, pH 6.4, as recommended by the producer's instructions.

Histopathological preparations. Corneas for histopathological examinations were fixed in 10 per cent formalin (in 0.1M phosphate buffer, pH 7.4), dehydrated with alcohol, and embedded in paraffin. Six-micron sections were stained with hematoxylin and eosin or with PAS (periodic acid-Schiff's base reagent) using standard techniques.

Results

Gross and histopathological examination of corneal damage induced by injection of heat-inactivated Pseudomonas. Twenty-four hours after intracorneal injection of heat-inactivated Pseudomonas cells, an opaque white stromal ring, surrounding the injection site, began to develop in 10 out of the 20 eyes studied. Forty-eight hours after injection, the ring was complete, or nearly complete, in all eyes (Fig. 1, A). In addition, the conjunctiva was markedly hyperemic, and the cornea hazy around the injection site. The brownish, granular character of the injected material became obscured by a white infiltrate as early as 24 hours after injection. The infiltrate became progressively more extensive and expanded beyond the ring, masking it completely within 4 days. By the end of the first week, all corneas developed grossly visible ulcers (Fig. 1, B), and during the second week, four out of the 20 corneas developed descemetoceles (Fig. 1, C). Hypopyon formation was noted in 16 of the eyes. In the third and fourth weeks after injection, corneal neovascularization extended into the ulcerated areas which healed, leaving dense leukomas.

In the six rabbits receiving bilateral intrastromal injections of heat-inactivated Pseudomonas cells combined with a sub-
conjunctival injection of corticosteroids, opaque rings surrounding the injection sites were not detected. In addition, the corneal haze around the injected material and conjunctival hyperemia were less than in the nonsteroid-treated rabbits. Infiltration of the injection site was slow to develop and was never as extensive on a given day as in the nonsteroid-treated rabbits. By the end of the first week, no ulcerations were observed. In the second week, ulcerations developed which were comparable to those seen in the nonsteroid-treated rabbits in the first week after injection.

Histopathologic examination of corneas taken from non-steroid-treated rabbits 2 days after injection revealed an extensive infiltrate of polymorphonuclear leukocytes (PMN's) anterior to the injected material. Examination of the areas corresponding to the opaque rings also revealed an accumulation of PMN's. At 5 days, the infiltrate was more extensive, became localized to the injected material, and again consisted predominantly of PMN's (Fig. 2). At 10 days, the anterior stroma was necrotic and ulcerated (Fig. 3). The steroid-treated rabbits demonstrated significantly less PMN infiltration on day 2 and on day 5.

Collagenase and protease production by cultured corneas. The media from cultures of control corneas and corneas injected with inactivated *Pseudomonas* cells (with and without corticosteroid treatment) were processed as described in Methods and analyzed for collagenase and protease activities as well as for protein contents. As seen in Table I, no collagenase activity and only a basal level of proteolytic activity could be detected in the media from the control corneas. On the other hand, high levels of collagenase activity and a 20- to 30-fold increase in the protease level were found in media from the corneas injected with heat-inactivated *Pseudomonas* cells.
Fig. 3. Histopathological appearance of corneal necrosis and ulceration 10 days after injection of heat-inactivated Pseudomonas cells. (Hematoxylin and eosin; original magnification ×100.)

Table I. Collagenase and protease production by corneas injected with heat-inactivated Pseudomonas cells with and without corticosteroids

<table>
<thead>
<tr>
<th>Cultured corneas*</th>
<th>Collagenase</th>
<th>Protease</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total protein (mg.)</td>
<td>Total activity c.p.m./hr.</td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>22.2</td>
<td>0</td>
</tr>
<tr>
<td>Intracorneal injection (unsteroid-treated)</td>
<td>20.2</td>
<td>27,140</td>
</tr>
<tr>
<td>Intracorneal injection (steroid-treated)</td>
<td>15.4</td>
<td>12,613</td>
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</tbody>
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*Each group represents the values obtained for 12 corneas.
†Protein concentrations were determined according to Lowry and co-workers,11 with bovine serum albumin as the standard.

The level of collagenase activity in the corneas which were injected with heat-inactivated organisms was found to be twice as high in the rabbits which did not receive subconjunctival injections of corticosteroids as in those which did. In addition, the specific activity of the collagenase was 60 per cent higher in the nonsteroid- than in the steroid-treated corneas. The difference in protease levels was less striking. The protease activity in media from corneas not treated with steroids was only 50 per cent higher than that found in media from corneas treated with steroids, and the specific activity was nearly the same in both groups.

Action of collagenase on soluble collagen. The viscosity of a collagen solution incubated at 25° C. with collagenase-containing media from corneas injected with inactivated Pseudomonas was reduced by approximately 50 per cent (Fig. 4). Under the same conditions trypsin (50 µg/ml.) reduced the viscosity by no more than 17 per cent. Samples taken from the collagen-collagenase reaction solution after 0, 2, 5, and 20 hours of incubation were subjected to SDS-polyacrylamide electrophoresis, and the results are presented in Fig. 5. A time-dependent disappearance of the collagen α- and β-chains, accompanied by the formation of increasing amounts of the tropocollagen A and B (TCα and TCβ) fragments, which is typical of the action of other mammalian collagenases on collagen, was evident.
Fig. 4. Effect of collagenase produced by the ulcerated corneas on the viscosity of a collagen solution at 25°C. Enzyme aliquots (75 µl) were added to 0.925 ml of collagen solution in 0.05 M Tris-HCl, pH 7.5, 5 mM in CaCl₂, and containing 1.8 mg. of collagen. , Collagen control; A, trypsin (50 µg) control; and B, medium from cultures of ulcerated corneas (250 µg of protein).

Fig. 5. Progress of collagen hydrolysis by the collagenase from ulcerated corneas as followed by SDS-polyacrylamide gel electrophoresis. The reaction solution and incubation conditions were identical with those described for Fig. 4. The reaction was stopped at each time interval by adding an aliquot of 0.1M ethylenediamine tetraacetic acid (EDTA) solution to a final concentration of 10 mM and freezing. An aliquot containing initially 40 µg of collagen was applied on each gel. A, Collagen alone; B, enzyme alone; C, D, E, and F, collagen-collagenase reaction solution after 0, 2, 5, and 20 hours’ incubation, respectively.

Fig. 6. Effect of concentrated media from cultures of (+) control and (°) ulcerated corneas on the viscosity of a proteoglycan solution. Enzyme aliquots (50 µl) containing approximately 160 µg protein were added to 950 µl of a 4 mg./ml proteoglycan solution in 0.05 M Tris-HCl, pH 7.5, 5 mM in CaCl₂, and incubated at 37°C. The curve obtained with the medium from control corneas coincided with that obtained with the PGS control (a solution of proteoglycan alone), the experimental points of which are not shown.

Degradation of the proteoglycan subunit (PGS). The effect of culture media from control corneas and corneas injected with heat-inactivated Pseudomonas on proteoglycans was examined, with a reduction in viscosity used as an indication of hydrolysis. As shown in Fig. 6, no change in viscosity was obtained with media from the control corneas, but a rapid drop in viscosity which leveled off at approximately 50 per cent of the initial viscosity was observed with media from the corneas with intracorneal injections. In order to determine what portion of the proteoglycan molecule was attacked, the reaction solution was analyzed for sugar-reducing ends and for the appearance of new ninhydrin-positive spots when subjected to thin-layer chromatography (TLC). The results were compared to a control proteoglycan solution of the same concentration. No change in the level of reducing ends could be distinguished, indicating that the polysaccharide chains remained intact. On the other hand, new ninhydrin-positive spots were detected by TLC when compared to the control. This indicates that
the observed decrease in viscosity resulted from breakdown of the polypeptide portion of the molecule by one or more proteolytic enzymes which were present in the media.

**Discussion**

The object of this investigation was to examine the response of the cornea to *P. aeruginosa* and to demonstrate and characterize the host-derived enzymes involved in this response. In order to study the enzymes produced by the host, it was necessary to develop a system which eliminates the effects of active infection and protease production by *P. aerugi nosa*. This was achieved by the intracorneal injection of autoclaved *Pseudomonas* cells in which toxic, heat-stable components such as endotoxin (lipopolysaccharides of the cell wall) and slime (polysaccharides adsorbed to the cell surface) survive.\(^\text{14, 15}\)

The host response to the intracorneal injections involved an extensive infiltration of PMN's and the production of ulcerations in all eyes within 1 week. Typically, an opaque ring which surrounded the injection site and which was composed of PMN's was observed 1 to 2 days after injection. Similar rings are occasionally observed in *Pseudomonas* infections of the human cornea,\(^\text{9}\) but their significance is not clear.

The infiltrated and ulcerated corneas were analyzed for the presence of enzymes capable of degrading connective tissue components, and an attempt was made to correlate their levels with the extent of corneal destruction observed grossly and histopathologically. It was found that the inflammatory response to the autoclaved *Pseudomonas* cells is indeed accompanied by the production of collagenase(s) and protease(s), which are either absent or present at a basal level in the control corneas (Table I). The collagenase activity found in the ulcerated corneas was typical of that of mammalian collagenase, cleaving the native collagen molecule at a single site to yield the characteristic TC\(_A\) and TC\(_B\) fragments (Fig. 4). The degradation of proteoglycans was shown to be proteolytic in nature. It was therefore attributed to the proteases produced in the ulcerated corneas.

It is of interest that the level of both collagenase(s) and protease(s) in media from ulcerated corneas injected with heat-inactivated *Pseudomonas* is significantly higher than that found in media from the corticosteroid-treated corneas (Table I) which, although injected with inactivated organisms, exhibited less infiltration and no ulceration at 6 days. This difference is more pronounced for collagenase and is reflected also in the specific activity. Although the specific activity of protease is approximately the same in culture media from corneas with and without steroid treatment, the specific activity of collagenase is 60 per cent higher in media from the nontreated than from the steroid-treated corneas. This indicates that the rate of collagenase production relative to the other proteins synthesized is higher in the ulcerated corneas which did not receive corticosteroids than in those which did. It is possible, therefore, that the extent of corneal destruction which results from the inflammatory response of the host is related to the collagenase level.

The corneal cells which are possible sources of the aforementioned enzymatic activities are the invading PMN's, the stromal keratocytes, and the corneal epithelial cells. The PMN's are known to contain in their granule fraction connective tissue-degrading enzymes such as collagenase\(^\text{16}\) and proteases which have been shown to attack proteoglycans from both cornea\(^\text{17}\) and cartilage.\(^\text{18, 19}\) The keratocytes and the epithelial cells undergo degeneration in an active *Pseudomonas* infection and have been postulated as a possible source of various hydrolytic enzymes.\(^\text{5}\) The correlation between the enzyme levels and extent of PMN infiltration found in this study suggests that the PMN's are the most likely source of these activities. However, further characterization of the system and of the enzymes is required before a final
Conclusion regarding their origin can be drawn.

Corneal ulceration often continues in human infections despite conversion of cultures for *Pseudomonas* from positive to negative by intensive antibiotic treatment. One of the explanations for this phenomenon is that *Pseudomonas* cells may persist deep in the stroma and may therefore be inaccessible to superficial culturing techniques. These cells may account for the continuing destruction and for recurrences. In addition, it has been recently demonstrated that the *Pseudomonas* protease may persist for at least 2 days following intrastromal injection in the rabbit cornea and may therefore continue to degrade the cornea after the elimination of viable organisms. It is also possible, however, that the collagenase(s) and protease(s), which are produced by the cornea as part of its inflammatory response to *P. aeruginosa*, also contribute to the corneal degradation which continues after the elimination of viable bacteria. As shown in this work, the inflammatory response of the host to *Pseudomonas*, including the production of hydrolytic enzymes, does not depend exclusively on the presence of viable organisms but may also develop as a reaction to the cellular and surface components of the dead *Pseudomonas* cells.

In conclusion, the present studies indicate that two processes are involved in the destruction of the cornea by *P. aeruginosa*. The first process involves the *Pseudomonas* protease which rapidly degrades and perforates the cornea. The second process depends on the host response to heat-stable components of *Pseudomonas* and is characterized by the production of both collagenolytic and proteoglycanolytic enzymes, which may be an additional factor in the corneal destruction caused by *P. aeruginosa*.

REFERENCES


