Rabbits vaccinated with lipopolysaccharide endotoxins or with purified protease preparations from *Pseudomonas aeruginosa* and *Serratia marcescens* before corneal challenge with the viable bacteria exhibited significantly less corneal damage than rabbits not vaccinated with the bacterial products. However, the rabbits vaccinated with the lipopolysaccharide endotoxin preparations were significantly better protected than rabbits vaccinated with the bacterial proteases. Rabbits vaccinated with antisera raised against the proteases showed significantly less corneal damage than rabbits vaccinated with normal rabbit serum, and the passive protection was not significantly different than that elicited by active immunization against the bacterial proteases. The ability of the antiserum raised against the pseudomonas elastolytic protease to passively protect against severe corneal damage produced by experimentally induced pseudomonas keratitis was confirmed in mice. These findings support the idea that the bacterial endotoxins and proteases are virulence factors during the development of pseudomonas and serratia keratitis.

**Materials and Methods**

**Bacteria**

*P. aeruginosa* strain 5-31 (Fisher immunotype 2; Habs immunotype 11), isolated from a human corneal infection, was originally studied and kindly supplied by John Gerke. *S. marcescens* strain BG (bio-type A1a) was obtained from the culture collection of the Department of Microbiology and Immunology of the Bowman Gray School of Medicine. The bacteria were cultivated as previously described.

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Submitted for publication: August 2, 1985.

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Table 1. Grossly observable corneal disease in nonimmunized and immunized rabbits challenged with P. aeruginosa 5-31

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Number of corneas exhibiting corneal disease grade of*</th>
<th>Average corneal damage ± SD†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-incomplete FA (control) [10]†</td>
<td>0 0 0 5 4 1</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Pseudomonas elastase-incomplete FA [13]</td>
<td>2 4 3 0 2 0</td>
<td>2.9 ± 0.4 (&lt;0.001)</td>
</tr>
<tr>
<td>Heptavalent pseudomonas LPS endotoxin [20]</td>
<td>14 5 1 0 0 0</td>
<td>0.4 ± 0.1 (&lt;0.001)</td>
</tr>
<tr>
<td>Normal rabbit serum (control) [10]</td>
<td>0 0 0 5 5 0</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Anti-pseudomonas elastase antiserum [12]</td>
<td>0 0 2 3 4 3</td>
<td>3.7 ± 0.3 (&lt;0.01)</td>
</tr>
</tbody>
</table>

* Results shown are 9 days after challenge. Corneal disease was graded according to the descriptions in the legend for Figure 1.† P values in parentheses. Because of multiple-comparison corrections, the 95% significance level for the active immunization studies is P = 0.025. The 95% significance level for the passive immunization study is P = 0.05. † Numbers in brackets indicate the number of corneas challenged.

Vaccines and Active Immunization Protocols

All investigations involving animals reported in this study conformed to the ARVO Resolution on the Use of Animals in Research. The heptavalent pseudomonas lipopolysaccharide (LPS) vaccine (Pseudogen®, lot #43152; Warner-Lambert/Parke, Davis & Co., Detroit, MI) was kindly provided by George Cole and Robert Brackett. The vaccine has been previously described15,16 and contains the Fisher immunotype 2 LPS, which corresponds to our P. aeruginosa challenge strain 5-31. Twenty New Zealand white rabbits, each weighing ca. 2.7 kg, were bled prior to vaccination (for preimmunization sera) and were injected intramuscularly on days 0, 4, 9, and 14 with 135 µg of LPS vaccine in 0.25 ml of sterile 0.9% saline (50 µg LPS per kg). The animals tolerated this regimen well, with no local abscesses, illness, or unexpected deaths occurring. A phenol-killed, heat-treated serrattia LPS vaccine was prepared as described by McMeel et al.17 for a pseudomonas LPS vaccine, and six rabbits were injected intravenously with the vaccine over a 3-wk period as described by McMeel et al.17. Agglutinating antibody titers of the preimmunization sera and of the sera obtained from rabbits 7 to 10 days after the last injections with the LPS vaccines were determined by a tube agglutination method (against heat-killed bacteria) as described by Garvey et al.18. The rabbits were challenged 7 to 10 days after the last injections with the LPS vaccines. The elastolytic protease of P. aeruginosa 5-31 and the nonelastolytic, neutral metalloprotease of S. marcescens BG were purified as previously described.6,19

Groups of rabbits (13 rabbits per group) were vaccinated subcutaneously every 2 wk for a total of four vaccinations with 0.5 ml of water-in-oil emulsions containing 1 mg of purified protease in incomplete Freund adjuvant (FA). Twenty-four control animals were vaccinated with an emulsion composed of 0.02 M phosphate-buffered saline (PBS, pH 7.4) and incomplete FA. Rabbits were bled 7 to 10 days after the last vaccinations and precipitating antibody titers (optimal proportions zones) of the sera from the control animals and from the animals vaccinated with the proteases were determined against the purified proteases as described by Garvey et al.20. The rabbits were challenged 7 to 10 days after the last vaccinations with the proteases.

Antisera and Passive Immunization Protocols

Monospecific rabbit antisera were raised against the purified proteases by the same vaccination protocol used to actively immunize rabbits, and the antisera were lyophilized and stored at 4°C. The monospecificity of the antisera was confirmed by crossed immunoelectrophoresis against crude protease preparations as previously described.21 In addition to estimating the precipitating antibody titers of the antisera (as mentioned in the preceding paragraph), the ability of the IgG fractions of the control serum and the antisera to inhibit the cornea-damaging activity of the pseudomonas and serrattia proteases was determined. The IgG fractions were obtained by chromatography on a column of DEAE Affi-Gel Blue (Bio-Rad Laboratories, Inc.; Richmond, CA) as recommended by the manufacturer. Mixtures (1 ml) containing the purified pseudomonas or serrattia protease (1 protease unit) and IgG (3 mg) from either control rabbit serum or monospecific antiprotease antisera were incubated in 0.05 M Tris-HCl buffer (pH 7.5) at 25°C for 60 min. After incubation, samples (30 to 40 µl) of the mixtures were injected intracorneally into rabbits (as described in the Experimental keratitis and statistical analysis section of the Materials and Methods), and the corneas were observed for 8 hr to note any reduction in grossly observable cornea-damaging activity of the proteases.
Fig. 1. Corneal disease observed in nonimmunized and actively immunized rabbits challenged with P. aeruginosa 5-31 or S. marcescens BG. Results shown are 9 days after challenge with viable bacteria suspended in PBS. A, Normal cornea (grade 0) typically observed in rabbits fully protected by vaccination with the preparation of heptavalent pseudomonas lipopolysaccharide endotoxins. B, Mild conjunctivitis and faint opacity (grade 1) typically observed in rabbits partially protected by the vaccination protocols. C and D Dense opacity over part of pupillary area and dense opacity over entire pupillary area (grades 2 and 3, respectively) typically observed in rabbits partially protected by the vaccination protocols. E through H, Extensive opacity and central liquefactive necrosis (grade 4), and extensive liquefactive necrosis and corneal protrusion (descemetocele formation) (grade 5) typically observed in nonvaccinated rabbits and in rabbits not protected by the vaccination protocols. The lesions shown in E and G (grades 4 and 5, respectively) were produced by P. aeruginosa 5-31, and the lesions shown in F and H (grades 4 and 5, respectively) were produced by S. marcescens BG. Grade 6 corneal damage includes corneal perforation.

three different groups of rabbits (10 to 14 rabbits per group) were vaccinated intramuscularly, 24 hr prior to and 48 hr after corneal challenge, with 5 ml of 2X concentrated preparations (150 mg/ml) of (1) control rabbit serum, (2) monospecific anti-pseudomonas elastase antiserum, and (3) monospecific anti-serratia protease antiserum. In the second series of experiments, 6-wk-old, female C57BL/6J mice (Jackson Laboratories; Bar Harbor, ME) were injected intraperitoneally, 1 to 3 hr prior to and 72 hr after corneal challenge, with 0.2 ml of 2X concentrated control rabbit serum or monospecific anti-pseudomonas elastase antiserum.

Experimental Keratitis and Statistical Analysis

The control rabbits and the rabbits that were vaccinated with the LPS and protease preparations and with the anti-protease antisera were anesthetized by intramuscular injections of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (3 mg/kg) and by topical application of 0.5% tetracaine hydrochloride. The central portion of the left cornea of each rabbit challenged with P. aeruginosa 5-31 was abraded with a sterile 18-gauge needle in a crosshatching manner and the challenge dose (ca. 10^9 viable organisms in 50 μl of 0.9% saline) was immediately dropped onto the traumatized cornea. The central portion of the left cornea of each rabbit challenged with S. marcescens BG was injected (30-gauge, 0.5-in needle) with the challenge dose (ca. 6 x 10^4 viable organisms in 40 μl of 0.9% saline). The subsequent course of the keratitis was examined visually each day for 9 days, and the severity of the grossly observable lesions was scored according to the criteria described in the legend for Figure 1.

The mice vaccinated with normal rabbit serum and with anti-pseudomonas elastase antiserum were challenged with P. aeruginosa 5-31 by the previously described procedure of Hazlett et al. Briefly, mice were anesthetized with ether, placed beneath a stereoscopic microscope, and the left eye of each animal was wounded by gently scratching the corneal surface with a sterile 26-gauge needle. Five microliters of bacterial cell suspension (ca. 1 x 10^8 viable organisms) were delivered onto incised corneas with a micropipet having a sterile disposable tip, and the subsequent course of the infection was examined visually at 3-day intervals for 4 wk. The corneas also were examined by light microscopy. The grades of grossly observable corneal damage used for the mice were similar to those used for the rabbits, except grades 2 and 3 were combined into grade 2, and grades 4, 5, and 6 were designated 3, 4, and 5, respectively.

The average corneal damage indicated in Tables 2, 3, and 4 is a weighted mean estimated after determining the number of corneas exhibiting the various grades of corneal disease, and is expressed as

\[
\text{Average corneal damage} = \frac{\sum (\text{corneal damage grade} \times \# \text{ of corneas in the grade})}{\text{total} \# \text{ of corneas challenged}}
\]

The average corneal damage values were analyzed for statistical significance by generalized categorical data analysis using weighted least squares. A mul-
Table 2. Grossly observable corneal disease is nonimmunized and immunized rabbits challenged with S. marcescens BG

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Number of corneas exhibiting corneal disease grade of *</th>
<th>Average corneal damage ± SD**†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-incomplete FA (control) [14]‡</td>
<td>0 0 0 5 8 1</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>Serratia protease-incomplete FA [13]</td>
<td>0 1 5 5 1 0</td>
<td>2.7 ± 0.3 (&lt;0.001)</td>
</tr>
<tr>
<td>Phenol-killed S. marcescens BG [6]</td>
<td>0 2 4 0 0 0</td>
<td>1.7 ± 0.2 (&lt;0.001)</td>
</tr>
<tr>
<td>Normal rabbit serum (control) [13]</td>
<td>0 0 0 5 7 1</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>Anti-serratia protease antiserum [14]</td>
<td>0 0 2 8 3 1</td>
<td>3.2 ± 0.2 (&lt;0.001)</td>
</tr>
</tbody>
</table>

* Results shown are 9 days after challenge. Corneal disease was graded according to the descriptions in the legend for Figure 1.
† P values are in parentheses. Because of multiple-comparison corrections, the 95% significance level for the active immunization studies is P = 0.025. The 95% significance level for the passive immunization study is P = 0.05.
‡ Numbers in brackets indicate the number of corneas challenged.

Light Microscopy

Mice were killed by intraperitoneal injection of sodium pentobarbital. The eyes were enucleated, washed briefly with sodium phosphate buffer (0.2 M, pH 7.4), and fixed intact for 3 hr at 4°C in sodium phosphate buffer containing 1% osmium tetroxide and 2.5% glutaraldehyde. After fixation, specimens were rinsed in sodium phosphate buffer (0.1 M, pH 7.4) and dehydrated with ethanol and propylene oxide. Specimens were infiltrated with a mixture of Epon-araldite and propylene oxide (1:1 for 24 hr, followed by 3:1 for 24 hr) and were infiltrated with Epon-araldite for 1 wk before embedding. Thick sections (1.5-μm) were stained with a modified Richardson stain and were examined and photographed with a Zeiss (Oberkochen, West Germany) automatic photomicroscope with bright-field optics.

Results

Active and Passive Immunization of Rabbits Against Pseudomonas and Serratia Keratitis

The four groups of rabbits vaccinated with the LPS preparations and protease preparations before corneal challenge with the viable bacteria exhibited significantly less corneal damage than rabbits not vaccinated with the bacterial products (Fig. 1, Tables 1 and 2). However, the rabbits vaccinated with the pseudomonas LPS preparation were significantly better protected than rabbits vaccinated with the pseudomonas elastolytic protease (P = <0.001), and rabbits vaccinated with the serratia LPS preparation were significantly better protected than rabbits vaccinated with the serratia protease (P = 0.002). The main difference between the results obtained with the LPS and protease preparations was that vaccination with the proteases protected against corneal melting (liquefactive necrosis) but not against corneal opacification, but vaccination with the LPS preparations protected against both corneal melting and opacification. The sera of the LPS-vaccinated rabbits had agglutination titers of 300 to 600; the titers of the preimmunization (control) sera were <5.

The two groups of rabbits vaccinated with the antiprotease antisera exhibited significantly less corneal damage than rabbits vaccinated with normal rabbit serum (Tables 1 and 2), and the protection was not significantly different than that elicited by active immunization against the bacterial proteases. The ability of the antisera to passively immunize against corneal melting was associated with high titers of precipitating antibodies against the proteases (optimal proportions

Table 3. Passive immunization of mice against corneal disease produced by P. aeruginosa 5-31

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Clear (Grade 0)</th>
<th>Slight opacity (Grade 1)</th>
<th>Corneal perforation and eye shrinkage (Grade 5)</th>
<th>Average corneal damage ± SD**†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rabbit serum [51]‡</td>
<td>0</td>
<td>0</td>
<td>51</td>
<td>5</td>
</tr>
<tr>
<td>Anti-pseudomonas elastase antiserum [105]</td>
<td>0</td>
<td>17</td>
<td>68</td>
<td>3.4 ± 0.2 (&lt;0.001)</td>
</tr>
</tbody>
</table>

* Results shown are 4 wk after challenge.
† P value is in parentheses. The 95% significance level is P = 0.05.
‡ Numbers in brackets indicate the number of corneas challenged.
zones of 0.05 to 0.1% antigen) and with the ability of the IgG fractions of the antisera to inhibit the corneal damaging activity of the proteases. Intracorneal injections of protease preparations preincubated with IgG from normal rabbit serum elicited extensive corneal melting and opacification by 3 hr postinjection, but injections of protease preparations preincubated with IgG from the antisera did not cause corneal melting or opacification by 8 hr postinjection.

Passive Immunization of Mice Against Pseudomonas Keratitis

The ability of the anti-pseudomonas elastase antiserum to passively protect against severe corneal dam-

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age produced by experimentally induced pseudomonas keratitis was confirmed in mice (Table 3 and Fig. 2).

**Discussion**

Various investigators have observed that rabbits vaccinated with phenol- and heat-killed *P. aeruginosa* with sterile culture filtrates and slime extracts of *P. aeruginosa* and with gamma globulin obtained from rabbits vaccinated with the killed bacteria are protected against severe corneal disease by the homologous bacterial strains. Also, mice have been actively immunized against pseudomonas keratitis by oral or intraperitoneal administration of phenol-killed suspensions of the homologous challenge strain. Studies with purified antigens and their antisera have shown that vaccination with a multicomponent vaccine containing the common protective antigen (OEP) of *P. aeruginosa* and the elastolytic protease and alkaline protease of the bacterium partially protects rabbits and horses against pseudomonas keratitis and acts synergistically with the antibiotic dibekacin to protect mice against severe corneal damage by a heterologous strain of *P. aeruginosa*. Also, rabbit antiserum containing antibodies against the pseudomonas OEP and proteases (detected by a passive hemagglutination assay) has been reported to act synergistically with dibekacin to passively protect mice against severe corneal damage by a heterologous strain of *P. aeruginosa*. In addition, Spierer and Kessler recently observed that the combined topical application of 2-mercaptoacetyl-L-phenylalanine-L-leucine (an inhibitor of the *P. aeruginosa* elastase) and suboptimal doses of gentamicin to rabbit corneas infected with *P. aeruginosa* inhibits corneal melting during the early stages of the developing keratitis (first 28 hr) significantly better than treatment with gentamicin alone. The results in our previous publications by demonstrating that the severity of experimentally induced corneal disease by *P. aeruginosa* and *S. marcescens* is significantly reduced by immunization against either the LPS endotoxins or the proteases of the bacteria. These findings further support the previously proposed idea that the bacterial products are virulence factors during the development of pseudomonas and serratia keratitis.

Our observation that vaccination against the pseudomonas and serratia LPS endotoxins protects rabbits significantly better against experimental keratitis than does vaccination against the bacterial proteases is consistent with previous observations made with a burn wound sepsis model. Cryz et al found that *P. aeruginosa* LPS is a very effective immunogen against pseudomonas burn wound sepsis in mice and that anti-LPS IgG protects mice against burn wound sepsis better than anti-pseudomonas elastase IgG. It has also been reported that mice immunized against burn wound sepsis by vaccination with pseudomonas LPS clear the bacteria from their tissues faster than nonimmunized mice, and that protective anti-LPS antibodies act as opsonins to increase the efficiency of phagocytosis and killing of *P. aeruginosa* by host phagocytes. We have found during our studies that antibodies against the pseudomonas and serratia proteases inhibit the cornea-damaging activity of the enzymes; however, there is no published data to indicate that the antibodies inhibit in vivo growth or enhance clearance of the bacteria from the cornea. Thus, the pseudomonas and serratia LPS preparations may be more effective immunogens than the bacterial proteases in our rabbit keratitis model because anti-LPS antibodies enhance clearance of the bacteria from infected corneal tissue, whereas anti-protease antibodies only inhibit a tissue-damaging bacterial virulence factor without aiding elimination of the bacteria.

An important question that should be examined in future studies is whether the pseudomonas elastolytic protease and the serratia neutral metalloprotease used in our studies are produced in vivo during the development of corneal infections. Kawaharajo and Homma found that *P. aeruginosa* strain PA-103, a strain that does not produce detectable amounts of the elastolytic protease in vitro but produces the alkaline protease in vitro, causes less severe corneal disease in mice than elastolytic isolates of the bacterium. However, Ohman et al did not observe a significant difference between the severity of corneal damage produced in mice by strain PA-103 and by elastase-producing strains of *P. aeruginosa*. Also, Ohman et al did not observe a significant difference in the mouse corneal virulence of *P. aeruginosa* mutant strain PAO-E64, a strain that produces an elastase with reduced enzymatic activity, and the parental strain. Ohman et al suggested that if proteolytic activity is required for corneal damage by *P. aeruginosa* strains PAO-E64 and PA-103, then it is supplied by the alkaline protease of the strains rather than the elastolytic protease. Intracorneal injection of very small amounts of the pseudomonas elastase (ie, 0.03 protease units; 30 μl of a solution containing 1 unit per ml) produces rapid and extensive liquefactive necrosis of the cornea. Therefore, an alternative explanation for the observations of Ohman et al03 is that strains PA-103 and PAO-E64 may produce an amount of elastolytic protease activity in vivo that is sufficient to cause severe corneal damage, even though strain PA-103 does not produce detectable amounts of elastase in vitro and strain PAO-E64 produces a mutant enzyme with reduced activity. The elastolytic protease has been detected, by indirect immunofluorescence tests, in lung tissue sections of rats.
having an experimentally induced chronic pseudomonas pneumonia caused by strain PA-103. Another possible explanation for the apparent lack of correlation between in vitro elastase production and mouse corneal virulence for strain PA-103 is based on the idea that if a virulent strain does not produce a particular factor, that does not mean the factor is not important for disease production, only that it is not essential for the particular strain examined. Thus, strain PA-103 may not require the elastase for disease production as long as it can produce the alkaline protease. Howe and Iglewski recently reported that (1) strain PA-103 mutants that are markedly deficient in alkaline protease production in vitro are less virulent for the mouse cornea than is the parental strain, and (2) the addition of subdamaging amounts of either alkaline protease or elastolitic protease to corneas infected with the alkaline protease-deficient mutants results in infections that are indistinguishable from infections caused by the parental strain. Their data suggest the possibility that although an extracellular protease is required for maximal mouse corneal virulence of strain PA-103, a single specific protease is not required (ie, either the elastolitic protease or the alkaline protease will suffice). The observation that toxin A-deficient mutants derived from either strain PA-103 or PAO are less virulent for the mouse cornea than are their toxigenic parental strains underscores the possible multifactorial requirements for corneal damage by *P. aeruginosa*.

**Key words:** immunization, keratitis, *Pseudomonas aeruginosa*, *Serratia marcescens*, endotoxin, proteases

**Acknowledgments**

The authors thank George Cole and Robert Brackett (Warner-Lambert/Parke; Davis & Co., Detroit, MI) for supplying the heptavalent pseudomonas lipopolysaccharide vaccine and for immunotyping the *P. aeruginosa* strain used in this study, and thank Patrick A. D. Grimont (Pasteur Institute, Paris) for biotyping the *S. marcescens* strain used in this study.

**References**

28. McMeel JW and Wood RM: Passive immunization against


