INFECTION AND IMMUNITY, Sept. 1981, p. 927-932
0019-9567/81/090927-06$02.00/0

Vol. 33, No. 3

Characterization of Rabbit Corneal Damage Produced by Serratia Keratitis and by a Serratia Protease

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Received 17 April 1981/Accepted 5 June 1981

The structural alterations elicited in the rabbit corneal stroma by experimental *Serratia marcescens* keratitis and by a highly purified serratia protease preparation were compared by gross observation, biochemical analyses, and electron microscopic examination of the affected tissue. Acute inflammation, liquefactive necrosis of the cornea, and descemetocele formation occurred during the development of the infection and after the intracorneal injection of submicrogram amounts of the protease. In vitro incubation of insoluble corneal stromal tissue with the bacterium or with the protease resulted in solubilization of the stromal proteoglycan ground substance; however, specific collagenase activity was not detected. Electron microscopic examination of corneas damaged by the bacterial infection and by the protease revealed loss of ruthenium red staining of the proteoglycan ground substance and dispersal of ultrastructurally normal collagen fibrils. Thus, our findings indicate that the major corneal damage which occurs during serratia keratitis and after the injection of the serratia protease is caused by solubilization and loss of the ground substance of the tissue. In addition, the observation that the major structural alterations observed during serratia keratitis can be reproduced by the bacterial protease supports the idea that the enzyme is involved, at least in part, with the production of severe corneal damage by the bacterium.

Serratia marcescens is an opportunistic pathogen capable of causing a variety of human ocular diseases, including keratoconjunctivitis, corneal abscess, purulent conjunctivitis, lacrimal duct infections, and endophthalmitis (1, 3, 7, 8, 16, 20, 22-24). Bernstein and Maddox (2) produced keratitis in rabbits by injecting *S. marcescens* intracorneally and reported that the administration of large numbers of bacteria (9×10^7 colony-forming units) produced an infection which caused liquefactive necrosis and destruction of the cornea. They also noted that intracorneal injection of a large amount (150 μg) of the bacterial lipopolysaccharide endotoxin elicited severe corneal opacification but did not cause the extensive liquefactive necrosis characteristically produced by the viable bacterium. In addition to the potential cornea-damaging activity of the bacterial endotoxin, the possible importance of serratia extracellular proteases in the pathogenesis of serratia keratitis was suggested by the observation that intracorneal injection of submicrogram amounts of serratia protease preparations elicited rapid and extensive liquefactive necrosis of and descemetocele formation in the rabbit cornea (15, 17). Although the above-cited studies suggested the possible involvement of serratia endotoxin or extracellular proteases or both in the pathogenesis of serratia keratitis, additional studies were needed to characterize and compare the biochemical and ultrastructural changes produced in corneas infected with the bacterium with those in corneas treated with highly purified preparations of the bacterial products. In the present study, we have investigated the possible role of serratia extracellular proteases in experimental serratia keratitis by characterizing and comparing the biochemical and ultrastructural alterations occurring in the corneal stroma during the infection with those elicited by a highly purified serratia protease preparation.

MATERIALS AND METHODS

Bacterium and bacterial enzyme. *S. marcescens* strain BG was cultivated, and its extracellular metalloprotease was purified as previously described (17).

In vitro digestion of rabbit corneal stromal tissue by bacteria and enzymes. Fourteen corneas were excised aseptically from rabbit eyes obtained from Pel-Freez Biologicals, Inc. (Rogers, Ark.), and the epithelium, Descemet's membrane, and endothe-

lium were removed by scraping. The stromal tissue thus obtained was cut into small pieces (about 2 by 2 mm) and uniformly dispersed at 4°C in 20 ml of sterile 0.02 M phosphate buffer (PB; pH 7.0) by treatment (six 1-min pulses at full power, with 1-min rest periods between pulses) with a VirTis model 45 homogenizer (The VirTis Co., Inc., Gardiner, N.Y.) equipped with midi turboshear blades. The preparation was centrifuged (25,000 × *g*, 20 min, 4 °C), the supernatant fluid was discarded, and the pellet was suspended in 5 ml of sterile PB. Portions (1 ml) of the sterile suspension of dispersed stromal tissue were incubated, with gentle agitation in sterile plastic tubes (17 by 100 mm), with sterile PB (100 μl), with a washed suspension of *S. marcescens* strain BG (10^5 colony-forming units), or with sterile solutions (0.5 mg in 100 μl of PB) of papain (Sigma Chemical Co., St. Louis, Mo.) activated with 0.1 M cysteine hydrochloride, collagenase (Worthington Biochemical Corp., Freehold, N.J.), or serratia protease. The tissue suspension inoculated with *S. marcescens* was incubated for 20 h at 37°C. The tissue suspensions containing the various enzyme preparations or the control PB were supplemented with penicillin G (125 μg/ml) and streptomycin sulfate (200 μg/ml) and incubated for 6 h at 37°C. After 6 h, a second portion of either sterile buffer (100 μl) or the various enzyme preparations (0.5 mg) was added to the appropriate mixtures, and the mixtures were incubated for an additional 6 h. After incubation, the sterility of the tissue suspensions incubated with the control PB and with the various enzyme preparations was determined, and the bacteria in the tissue suspension inoculated with *S. marcescens* were quantitated by plate counts on Columbia agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% (vol/vol) sheep blood. The supernatant fluids of the digestion mixtures (solubilized stromal tissue) were obtained by centrifugation (25,000 × *g*, 20 min, 4°C), one PB wash (1 ml) of the pellets was pooled with the supernatant fluids, and the solutions were stored at −80°C until analyzed. The pellets from the digestion mixtures (nonsolubilized stromal tissue) were suspended in 4.5-ml portions of 4 N hydrochloric acid and incubated at 70°C for 5 h to produce homogeneous suspensions. The suspensions were adjusted to pH 7.0 and stored at −80°C until analyzed. The ability of the growing bacteria and the protease preparation to solubilize the corneal proteoglycan ground substance and collagen was examined by analyzing the supernatant fluids and pellets for glucuronic acid (4) and total hexosamines (5) (markers for ground substance) and for hydroxyproline (25) (marker for collagen).

Production of corneal damage by bacteria and enzyme preparations. New Zealand white rabbits (ca. 2.3 kg) anesthetized with ketamine hydrochloride, ether, and topical tetracaine hydrochloride were injected intracorneally (30-gauge needles), in groups of ca. 16 rabbits per group, with portions (40 μl) of the following: (i) suspensions containing various numbers (6×10^2 to 6×10^5 colony-forming units) of viable *S. marcescens* strain BG (right eyes in group 1); (ii) sterile 0.85% saline or heat-killed (100°C for 15 min) bacteria (control left eyes in group 1); (iii) filter-sterilized preparations of serratia protease (250 to 2,000 ng) in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5; right eyes in group 2); and (iv) Tris-hydrochloride buffer or heat-inactivated (100°C for 15 min) protease preparations (control left eyes in group 2).

Electron microscopy. Experimental animals were sacrificed by intravenous injection of sodium pentobarbital, their corneas were immediately excised without disturbing the central corneal lesions, and the tissues were processed for and examined by electron microscopy as previously described (14), except that the thin sections were stained with phosphotungstic acid (9) and examined in a Zeiss 10-A microscope with an accelerating voltage of 60 kV.

Electron microscopic visualization of the stromal proteoglycan ground substance in corneas injected with 0.05 M Tris-hydrochloride buffer (pH 7.5), serratia protease (500 ng), or *S. marcescens* (6×10^4 colony-forming units) was accomplished by infiltrating the corneas (by injection) with ruthenium red-containing fixative (11), excising and cutting the corneas into wedge-shaped sections, fixing with the ruthenium red-containing fixative for 2 h at 4°C, washing the tissue sections with cacodylate buffer (0.2 M, pH 7.4), and postfixing for 3 h at room temperature in a solution of osmium tetroxide-ruthenium red (11). Further processing of the tissues for electron microscopy was done as described in the preceding paragraph, except that thin sections were subsequently stained with uranyl acetate and lead citrate (9).

RESULTS

In vitro effect of *S. marcescens* and serratia protease on solubility of rabbit corneal stromal tissue. Neither the growing bacteria, which reached a population density of ca. 10^8 bacteria per ml, nor the bacterial enzyme preparation possessed significant collagenolytic activity; however, both caused extensive solubilization of the stromal proteoglycans (Table 1). Collagenase and papain served as positive controls to demonstrate the solubilization of stromal collagen and proteoglycan ground substance, respectively.

Gross corneal damage elicited by experimental serratia keratitis and by the serratia protease. Intracorneal injection of ca. 6×10^4 bacteria caused a rapidly progressing, destructive infectious process characterized by conjunctivitis, corneal opacification, hypopyon formation, the presence of a mucopurulent exudate, liquefactive necrosis of the cornea, descemetocele formation, and corneal perforation (Fig. 1a and b). Intracorneal injection of submicrogram amounts of the highly purified serratia protease preparation caused rapid liquefactive necrosis of the cornea and descemetocele formation (grossly observable within 6 h) which closely resembled the lesion produced by the bacterial infection (Fig. 1c and d). However, the protease usually did not elicit as extensive corneal opacification and mucopurulent exudate as

TABLE 1. *Effect of S. marcescens and a highly purified serratia protease preparation on the solubility of corneal proteoglycans (as glucuronic acid and hexosamines) and collagen (as hydroxyproline)*

Treatment	Amt solubilized (%)		
	Glucuronic acid[a]	Hexosamines[b]	Hydroxyproline[c]
PB control	9	21	1
S. marcescens	39	49	3
Serratia protease	100	61	8[d]
Papain	49	53	19[d]
Clostridium histolyticum collagenase	100[e]	74[e]	99

[a] Determined by the method of Blumenkrantz and Asboe-Hansen (4).

[b] Determined by the method of Blumenkrantz and Asboe-Hansen (5) after hydrolysis of the samples with 4 N hydrochloric acid for 30 min at 120°C.

[c] Determined by the micromethod of Woessner (25) after sample hydrolysis with 6 N hydrochloric acid for 18 h at 130°C and removal of insoluble residue by centrifugation.

[d] This effect is typical of that produced by noncollagenolytic proteases which possess limited nonspecific activity against peptide bonds in the nonhelical, terminal, telopeptide regions of the collagen molecule, rather than that produced by specific collagenases which cleave peptide bonds in the helical region of the molecule (13).

[e] The solubilization of the stromal proteoglycans by the commercially obtained *C. histolyticum* collagenase preparation was caused by contamination of the preparation with nonspecific clostridial proteases.

the infection. Injection of more than 1 μg of the protease preparation usually resulted in corneal perforation.

Electron microscopic characterization of structural alterations in corneal stromata damaged by experimental serratia keratitis and by the serratia protease. Electron microscopic examination of corneas 3 h, 1 day, and 7 days after the injection of control preparations showed no obvious structural alterations in the extracellular matrix of the corneal stromata (Fig. 2a, d, and e).

Structural alterations in the extracellular matrix of corneal stromata infected with *S. marcescens* were studied 1 day and 7 days after the injection of ca. 6×10^4 bacteria. Only small numbers of infiltrating polymorphonuclear leukocytes (PMNL) were observed in the corneal stroma at 1 day postinfection, thus suggesting that the bulk of the structural alterations observed at that time were not attributable to substances released from the PMNL. Stromal alterations observed 1 day postinfection consisted of the dispersal of ultrastructurally normal collagen fibrils (Fig. 2b) and the loss of ruthenium red-staining of the proteoglycan ground substance, especially in areas adjacent to bacteria (Fig. 2f and g). In addition, electron-dense beads indicative of degraded proteoglycan ground substance (21) were visible on many of the collagen fibrils (Fig. 2g). Corneal stromata examined late in the infection (7 days postinfection) showed structural changes which were qualitatively similar to, but quantitatively more extensive than, those seen at 1 day postinfection. In addition to the dispersed collagen fibrils and the loss of proteoglycan ground substance, numerous deposits of fibrin and necrotic PMNL were observed.

The structural changes elicited in the extracellular matrix of the corneal stromata by the serratia protease were identical to those observed during the bacterial infection (Fig. 2c and h), except that the enzyme preparation elicited less PMNL infiltration of the stromata than the infection.

Stromata damaged by the experimental infection or by the serratia protease did not show tactoid formation, which is thought to be at first view evidence of collagen breakdown and faulty fibrillar accretion (19) or of fragmented collagen fibrils, both of which are produced by intracorneal injection of clostridial collagenase or concentrated PMNL lysosomal preparations (10, 19).

DISCUSSION

The extensive grossly observable liquefactive necrosis and descemetocele formation which occurs in rabbit corneas during serratia keratitis and after the intracorneal injection of a highly purified serratia protease preparation suggests that the major cause of the observed corneal destruction is the alteration or loss of the major structural component of the cornea, i.e., the extracellular stromal matrix composed of collagen and proteoglycan ground substance. The observations described in this study indicate that both viable *S. marcescens* and the serratia protease preparation severely damage the rabbit cornea by causing the solubilization and loss of the corneal proteoglycan ground substance. The proteoglycan is believed to maintain the order and interfibrillar attachments of the corneal collagen fibrils (18, 21); thus, its loss could result in dispersal of undegraded collagen fibrils, weakening of the cornea, and subsequent descemetocele formation and corneal perforation by the anterior chamber pressure.

The strong similarity between the tissue damage produced by the infectious process and that

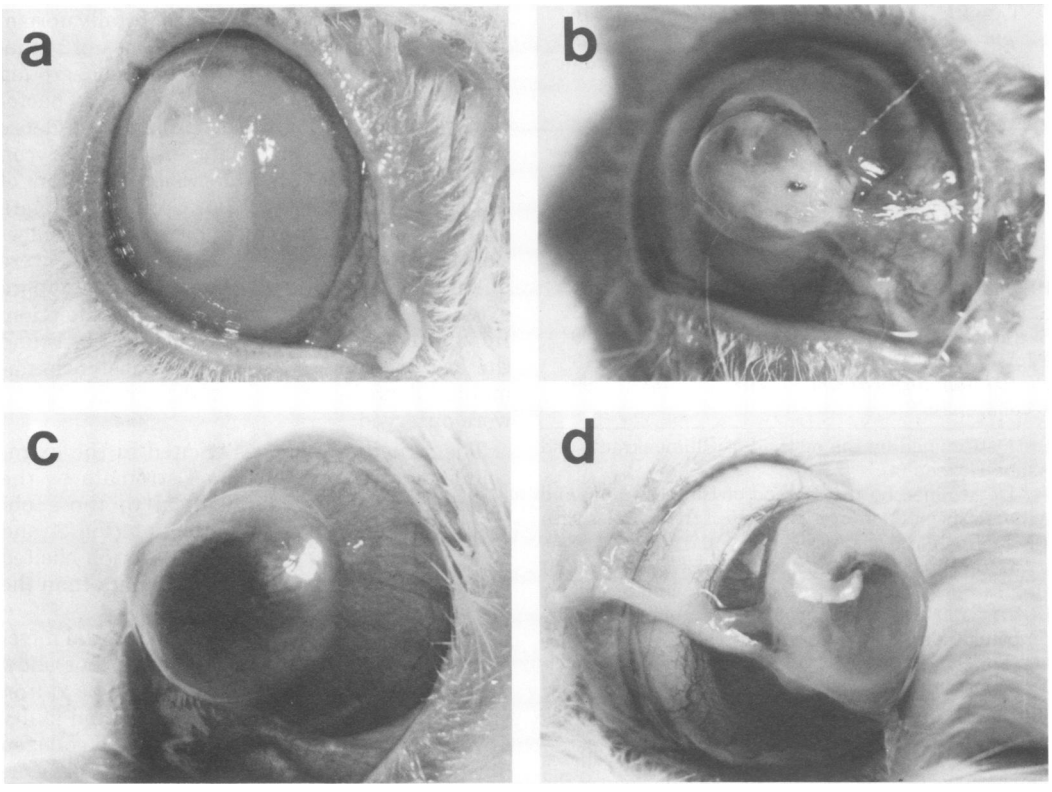
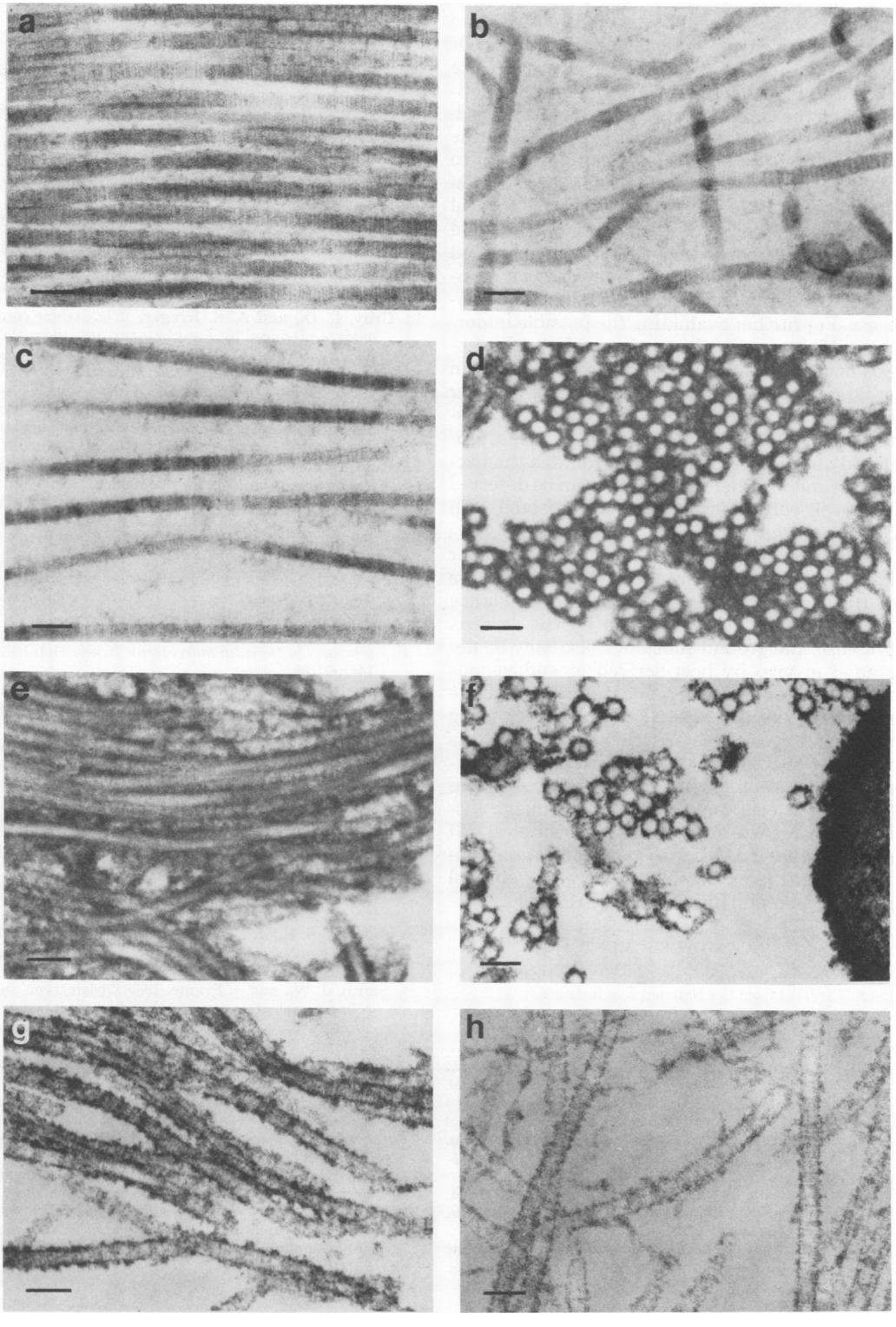


FIG. 1. *Liquefactive necrosis and descemetocele formation produced in rabbit corneas by S. marcescens keratitis and by a highly purified serratia protease preparation. (a) and (b) 3 and 7 days, respectively, after the intracorneal injection of ca. 6 × 10^4 bacteria. Injection of heat-killed bacteria produced only mild conjunctivitis and corneal opacification, which resolved by 3 to 4 days postinjection. (c) and (d) 6 and 16 h, respectively, after the intracorneal injection of ca. 500 ng of serratia protease preparation. Injection of the diluents or heat-inactivated enzyme produced immediate opacification at the site of injection; however, the cornea appeared normal by 3 to 4 h postinjection.*

caused by the purified bacterial enzyme supports the idea that the ability of the bacterium to elicit corneal destruction is dependent, at least in part, on the in vivo production by the bacterium of cornea-damaging proteases. However, our observations should not be construed as suggesting that the bacterium may not synthesize other cornea-damaging toxins or enzymes capable of playing a role in the pathogenesis of serratia keratitis. One difference we noted between the in vivo response elicited by the bacterium and that elicited by the protease was that more mucopurulent exudate and more PMNL infiltration of the corneal stroma was produced by the

FIG. 2. *Ultrastructural corneal alterations produced by experimental S. marcescens keratitis and by the serratia protease. Line markers on all micrographs represent 0.1 μm. (a) Normal structure and ordered arrangement of collagen fibrils (longitudinal section) in control cornea. (b) Longitudinal section of extensively dispersed but ultrastructurally normal collagen fibrils observed 1 day after the intracorneal injection of ca. 6 × 10^4 bacteria. (c) Longitudinal section of extensively dispersed but ultrastructurally normal collagen fibrils observed 6 h after the intracorneal injection of ca. 500 ng of serratia protease. (d) and (e) Normal staining pattern of corneal proteoglycan ground substance by the ruthenium red technique. Note the electron-dense, ruthenium red-stained ground substance surrounding and coating the collagen fibrils. (d) Cross section. (e) Longitudinal section. (f) and (g) Cross section and longitudinal section, respectively, demonstrating the reduction of ruthenium red staining of the proteoglycan ground substance 1 day after the intracorneal injection of ca. 6 × 10^4 bacteria. The glycocalyx of a bacterium in the field of (f) is also stained with ruthenium red, thus confirming the penetration of the stain into the tissue. Electron-dense beads indicative of degraded proteoglycan ground substance (21) are visible on many of the ultrastructurally normal collagen fibrils. (h) Longitudinal section demonstrating the reduction of ruthenium red staining of the proteoglycan ground substance 6 h after the intracorneal injection of ca. 500 ng of serratia protease.*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infection than by the enzyme. This observation suggests that chemotactic factors, which are not present in the purified protease preparation, are produced by the bacterium during the development of the infectious process. In this regard, intracorneal injection of the lipopolysaccharide endotoxin of the bacterium was reported (2) to elicit severe corneal edema and opacification and intense PMNL infiltration of the corneal stroma. However, the substance was not found to cause the extensive liquefactive necrosis and descemetocele formation characteristically produced by the bacterium and the bacterial protease. For further evaluating the possible importance of the bacterial protease in the pathogenesis of serratia keratitis, studies are currently in progress in our laboratory to determine whether active immunization against the purified protease can reduce or prevent the damage produced after intracorneal challenge with the bacterium and whether the protease can be detected in situ in corneas experimentally infected with *S. marcescens*.

Although the data presented in this paper indicate that the observed solubilization and loss of the stromal proteoglycan ground substance result from direct degradation by the serratia protease, the ground substance also may be degraded in vivo by host-derived or endogenous enzymes. For example, the presence of large numbers of necrotic PMNL in the tissue during the later stages of the infectious disease process and after injection of the bacterial protease suggests the possibility that enzymes released from the necrotic cells may enhance the damage elicited by the bacterial protease. Brown et al. (6) reported that PMNL lysosomes possess an enzyme or enzymes capable of degrading corneal proteoglycan at neutral pH in vitro.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant EY-01104 from the National Eye Institute.

LITERATURE CITED

1. **Atlee, W. E., R. P. Burns, and M. Oden.** 1970. *Serratia marcescens* keratoconjunctivitis. Am. J. Ophthalmol. **70**:31–33.
2. **Bernstein, H. N., and Y. T. Maddox.** 1973. Corneal pathogenicity of *Serratia marcescens* in the rabbit. Trans. Am. Acad. Ophthalmol. Otolaryngol. **77**:432–440.
3. **Bigger, J. R., G. Meltzer, A. Mandell, and R. M. Burde.** 1971. *Serratia marcescens* endophthalmitis. Am. J. Ophthalmol. **72**:1102–1105.
4. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54**:484–489.
5. **Blumenkrantz, N., and G. Asboe-Hansen.** 1976. An assay for total hexosamine and a differential assay for glucosamine and galactosamine. Clin. Biochem. **9**:269–274.
6. **Brown, S. I., C. W. Hook, and M. P. Tragakis.** 1972. Presence, significance, and inhibition of lysosomal proteoglycanases. Invest. Ophthalmol. **11**:149–152.
7. **Cooper, R. L., and I. J. Constable.** 1977. Infective keratitis in soft contact lens wearers. Br. J. Ophthalmol. **61**:250–254.
8. **Davis, J. T., E. Foltz, and W. S. Blakemore.** 1970. *Serratia marcescens*. A pathogen of increasing clinical importance. J. Am. Med. Assoc. **214**:2190–2192.
9. **Dawes, C. J.** 1971. Biological techniques in electron microscopy. Barnes and Noble Books, New York.
10. **Gray, L. D., and A. S. Kreger.** 1975. Rabbit corneal damage produced by *Pseudomonas aeruginosa* infection. Infect. Immun. **12**:419–432.
11. **Hayat, M. A.** 1972. Basic electron microscopy techniques, p. 85. Van Nostrand Reinhold Co., New York.
12. **Huber, J. D., F. Parker, and G. F. Odland.** 1968. A basic fuchsin and alkalinized methylene blue rapid stain for epoxy-embedded tissue. Stain Technol. **43**:83–87.
13. **Kessler, E., H. E. Kennah, and S. I. Brown.** 1977. *Pseudomonas* protease. Purification, partial characterization, and its effect on collagen, proteoglycan, and rabbit corneas. Invest. Ophthalmol. Visual Sci. **16**:488–497.
14. **Kreger, A. S., and L. D. Gray.** 1978. Purification of *Pseudomonas aeruginosa* proteases and microscopic characterization of pseudomonal protease-induced rabbit corneal damage. Infect. Immun. **19**:630–648.
15. **Kreger, A. S., and O. K. Griffin.** 1975. Cornea-damaging proteases of *Serratia marcescens*. Invest. Ophthalmol. **14**:190–198.
16. **Lazachek, G. W., G. L. Boyle, A. L. Schwartz, and I. H. Leopold.** 1971. *Serratia marcescens*, an ocular pathogen. Arch. Ophthalmol. **86**:599–603.
17. **Lyerly, D., and A. Kreger.** 1979. Purification and characterization of a *Serratia marcescens* metalloprotease. Infect. Immun. **24**:411–421.
18. **Maurice, D. M.** 1969. The cornea and sclera, p. 489–600. *In* H. Davson (ed.), The eye, vol. 1. Academic Press, Inc., New York.
19. **Rowsey, J. J., R. M. Nisbet, J. L. Swedo, and L. Katona.** 1976. Corneal collagenolytic activity in rabbit polymorphonuclear leukocytes. J. Ultrastruct. Res. **57**: 10–21.
20. **Salceda, S. R., J. Lapuz, and R. Vizconde.** 1973. *Serratia marcescens* endophthalmitis. Arch. Ophthalmol. **89**:163–166.
21. **Smith, J. W., and J. Frame.** 1969. Observations on the collagen and proteinpolysaccharide complex of rabbit corneal stroma. J. Cell Sci. **4**:421–436.
22. **Stenderup, A., O. Faergeman, and M. Ingerslev.** 1966. *Serratia marcescens* infections in premature infants. Acta Pathol. Microbiol. Scand. **68**:157–160.
23. **Tokunga, T., T. Fujino, K. Yamamoto, and A. Ideda.** 1969. A case of serpiginous corneal ulcer caused by *Serratia* of *Enterobacteriaceae*. Folia Ophthalmol. Jpn. **20**:862–868.
24. **Von Graevenitz, A., and S. J. Rubin.** 1980. The genus *Serratia*. CRC Press, West Palm Beach, Fla.
25. **Woessner, J. F., Jr.** 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys. **93**:440–447.