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Pathogenic Capacity of Proteases from *Serratia marcescens* and *Pseudomonas aeruginosa* and Their Suppression by Chicken Egg White Ovomacroglobulin

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The pathogenicities of three proteases from *Serratia marcescens*, two proteases from *Pseudomonas aeruginosa*, and one thermolysin from *Bacillus stearothermophilus* were examined. All proteases tested caused acute liquefactive necrosis of the cornea and descemetocele formation in guinea pig eyes after intrastromal injection, with the exception of the 60-kilodalton protease from *S. marcescens*, which produced only an opaque lesion. When injected into guinea pig skin, the proteases also enhanced vascular permeability, which was followed by edema formation. The permeability-enhancing activity of the proteases increased in parallel with the concentration of the enzymes. When tested in vitro for its effect on these bacterial proteases, chicken egg white ovomacroglobulin (ovoM) inhibited the enzymatic activity of all the proteases after a short incubation period at an enzyme/inhibitor ratio (molar) of 1:1 to 1:4 or at a lower concentration after a longer incubation period. Such treatment of the proteases with chicken egg white ovoM before injection intrastromally into the eyes or intradermally into the clipped flanks of guinea pigs protected the cornea from destruction or completely prevented the permeability reaction and edema formation. No inhibitory effects of plasma protease inhibitors against these bacterial proteases were noted. Since the proteases are critical in the pathogenic processes caused by the bacteria, these results suggest a beneficial effect of ovoM against bacterial infections.

Pseudomonas aeruginosa elastase and alkaline protease and the *Serratia marcescens* 56-kilodalton (kDa) protease cause opaque lesions and ulcer formation in the cornea (8, 10), suggesting that they play an important role in bacterial infections (2–4, 6, 8–10, 12, 13, 15, 18, 22, 24). We have isolated three different secretory proteases from *S. marcescens*. The protease with a molecular mass of about 56 kDa (56K protease) is considered to be the most important because of its level of production and proteolytic activity (8, 17), and we therefore characterized it first. During our investigations we demonstrated its enhancement of the vascular permeability reaction via activation of the Hageman factor (9, 18, 19). We also found that it could degrade various human plasma proteins and was cytotoxic for fibroblasts (20).

The 56K protease induced ulceration when injected into rabbit or guinea pig corneas at a dose of 1 μg; this phenomenon was prevented by antibody to the 56K protease (8). However, corneal ulcer formation by the serratial 60- and 73-kDa proteases (60K and 73K proteases) or vascular permeability reaction enhancement possibly caused by the pseudomonal and serratial (60K and 73K) proteases remains to be clarified.

In another series of experiments, we found that the 56K protease was inhibited only about 60% by human α_2-macroglobulin (α_2M) after a short incubation period; after prolonged incubation more than 90% of its activity was restored (20, 21). In contrast, we found that the homologous protein of α_2M in chicken egg white ovomacroglobulin (ovoM) almost completely inhibited protease activity as well as cytotoxicity (20). In addition, Ikai and co-workers (1, 7, 11) demonstrated that ovoM inhibited trypsin, thermolysin, subtilisin, and papain in vitro. The goal of the present study was to compare the serratial 56K protease with other purified proteases for the ability to cause corneal ulcer vascular permeability and for susceptibility to inhibition by ovoM.

In this communication we describe three major findings. (i) Serratial and pseudomonal proteases as well as thermolysin from *Bacillus stearothermophilus* cause corneal lesions, including ulceration. (ii) These bacterial proteases also cause a vascular permeability reaction in guinea pig skin. (iii) Chicken egg white ovoM prevents both the ulceration and the permeability-enhancing reaction induced by these proteases.

MATERIALS AND METHODS

Substances. Chicken egg white ovoM was a gift from Otsuka Pharmaceutical Co., Tokushima, Japan. *S. marcescens* (kums 3958) was isolated from a patient with severe keratitis. It produced three different proteases, 56K, 60K, and 73K proteases, which were purified to homogeneity as described previously (17). Crystallized *P. aeruginosa* elastase (PE) (39 kDa) and alkaline protease (PAP) (48 kDa) were purchased from Nagase Biochemicals, Ltd., Osaka, Japan. Thermolysin (37 kDa) produced by *B. stearothermophilus* was obtained from Amano Pharmaceuticals Co. Ltd., Nagoya, Japan. All proteases and proteins were homogeneous in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. All other reagents were purchased from Wako Pure Chemical Industry, Ltd., Osaka, Japan.

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Protein concentration. The method of Lowry et al. (14), with bovine serum albumin as the standard, was used to determine protein concentrations.

Animals. Albino Hartley guinea pigs of both sexes and weighing 300 to 400 g were used.

Protease activity. The proteolytic activity of serratial proteases was determined by the fluorescence polarization (FP) method (16) with fluorescein isothiocyanate-labeled gelatin as the substrate. One FP unit was defined as one arbitrary unit of decrease in the fluorescence polarization value measured by a fluorescence spectropolarimeter (model MAC II; Japan Immunoresearch Inc., Ltd., Takasaki, Japan).

The proteolytic activities of PAP and PE were determined either by the FP method (16) or by the method of Fisher and Allen (4) with casein as the substrate; in the latter method an increase in the level of the trichloroacetic acid-soluble fraction was correlated with protease activity.

Protease inhibitory assay. The proteases were preincubated with various concentrations of ovoM for 20 min to 24 h at either 25 or 37°C in 50 mM Tris hydrochloride buffer (pH 7.5), and the remaining proteolytic activity was assayed as described above.

Tissue-destroying activity of proteases in the cornea and its inhibition. The left eye of each guinea pig was injected intrastromally with 1.5 μg of protease in 10 μl of saline. The same amount of protease mixed with ovoM was similarly injected into the right eye. A 28-gauge needle connected to a 10-μl syringe was used for injection. Corneal changes were observed macroscopically until 24 to 48 h after injection. At that time corneal damage was also scored by the method of Ohman et al. (23).

Histopathological examination. Biopsies of all the corneas were taken for histopathological examination at 24 and 48 h after injection. The excised corneas were fixed with 10% Formalin, embedded in paraffin, and stained by a conventional method (hematoxylin-eosin or Azan-Mallory).

Vascular permeability assay. Saline (100 μl) containing 1 to 8 μg of protease was injected intradermally into the clipped flanks of guinea pigs immediately after an intravenous injection of 25 mg of Evans blue (2.5% solution in saline) per kg. Permeability was quantitated by the dye extraction method of Udaka et al. (25). The effect of ovoM on the permeability-enhancing activity of the proteases was evaluated by injecting proteases after preincubation with various amounts of ovoM for 20 min at room temperature.

RESULTS

Inhibition of proteases by ovoM in vitro. Figures 1A and B show the inhibitory effect of ovoM against serratial 56K, 60K, and 73K proteases as well as PAP, PE, and thermolysin after a short (20-min) incubation period at 37°C. The dose-dependent inhibitory profiles were biphasic for serratial and pseudomonal proteases, suggesting that there are two binding sites on ovoM with different association constants. When a 2.5- to 3-fold molar excess of ovoM (molecular weight, 720,000) was added to the serratial proteases, they were almost completely inhibited within 20 min (Fig. 1A). A fourfold molar excess of ovoM completely inhibited PAP, whereas only a twofold molar excess of ovoM was required for complete inhibition of PE (Fig. 1B). A monophasic inhibitory relationship was obtained in the case of thermolysin versus ovoM until the molar ratio reached 1.0, suggesting that 1 mol of ovoM inhibits 1 mol of thermolysin (Fig. 1B). The observation of one binding site for thermolysin is similar to a previous observation (11).

When the mixtures were incubated for 24 h at 25°C, lower concentrations of ovoM were sufficient for complete inhibition of the proteases. Table 1 shows that 1 mol of ovoM added to 1 mol of protease inhibited all the proteases tested by about 55 to 75% within 20 min; thereafter, about 95% inhibition was gradually reached during 24 h of incubation at 25°C.

Corneal damage caused by bacterial proteases and its inhibition by ovoM. When injected intrastromally (1.5 μg), serratial 60K protease produced corneal opacity, whereas serratial 73K protease (1.5 μg) rapidly led to ulceration of the cornea and descemetocele formation within 5 h (Fig. 2). Intracorneal injection of serratial 56K protease, PAP, or PE (1.5 μg) caused ulceration of the cornea within 5 h (Fig. 2).

When each protease was mixed with ovoM at an enzyme/inhibitor (E/I) ratio (molar) of 1:1 to 1:4 and then injected into guinea pig corneas, corneal ulcer or opaque lesion formation caused by the three serratial proteases was completely prevented at an E/I ratio (molar) of 1:2.5 or less (Fig. 2). Corneal ulceration caused by PAP was also blocked at an E/I ratio (molar) of 1:4. In contrast, in the case of PE, ovoM delayed the appearance of detectable melting by approximately 8 h, but once the melting started it progressed rapidly and ulceration occurred within 1 to 2 h. Thermolysin (1.5 μg) also caused corneal ulceration within 6 h; this ulceration was completely prevented by ovoM at an E/I ratio (molar) of 1:1 (Fig. 3). The cornea-damaging capacity of these proteases and the inhibitory effect of ovoM are clearly shown in Table 2 as corneal damage indices (23).

Histopathological examination. Corneal tissues were extensively destroyed, with liquefactive necrosis of the stroma by these proteases, and ulceration was clearly observed, except in the case of serratial 60K protease (Fig. 3 and Table 2). The response of the inflammatory cells which infiltrated the cornea and surrounding conjunctiva was relatively weak. When the proteases were injected along with ovoM, ovoM had a very potent protective effect. This effect of ovoM in vitro was confirmed in vivo. Biopsies of the cornea were also taken after intrastromal injection of saline or ovoM alone as a control experiment (data not shown); the result was the same as that shown in Fig. 3.

Enhancement of vascular permeability by bacterial proteases and its inhibition by ovoM. The bacterial proteases tested in this study caused enhanced vascular permeability resulting in edema formation in guinea pig skin (Fig. 4 and 5). The most intense extravascular leakage of dye was observed at 10 min after intradermal injection of the proteases (1 to 8 μg) (Fig. 4 and 5). PAP, PE, and serratial 56K protease caused a stronger permeability reaction than did thermolysin and serratial 60K protease. Serratial 73K protease caused the strongest permeability reaction of all the proteases tested: within 10 min it produced severe swelling around the injection site.

The effect of ovoM in vivo was then tested. The vascular permeability and edema formation caused by these proteases were inhibited by ovoM when it was injected along with the proteases into the skin (Fig. 4 and 5).

DISCUSSION

Many strains of *S. marcescens* and *P. aeruginosa* isolated from patients with serratial and pseudomonal keratitis in our hospital show multiple drug resistance. Therefore, it is important to clarify their mechanism of pathogenesis and to develop a method for protection against these pathogens. We previously found that injection of *S. marcescens* pro-

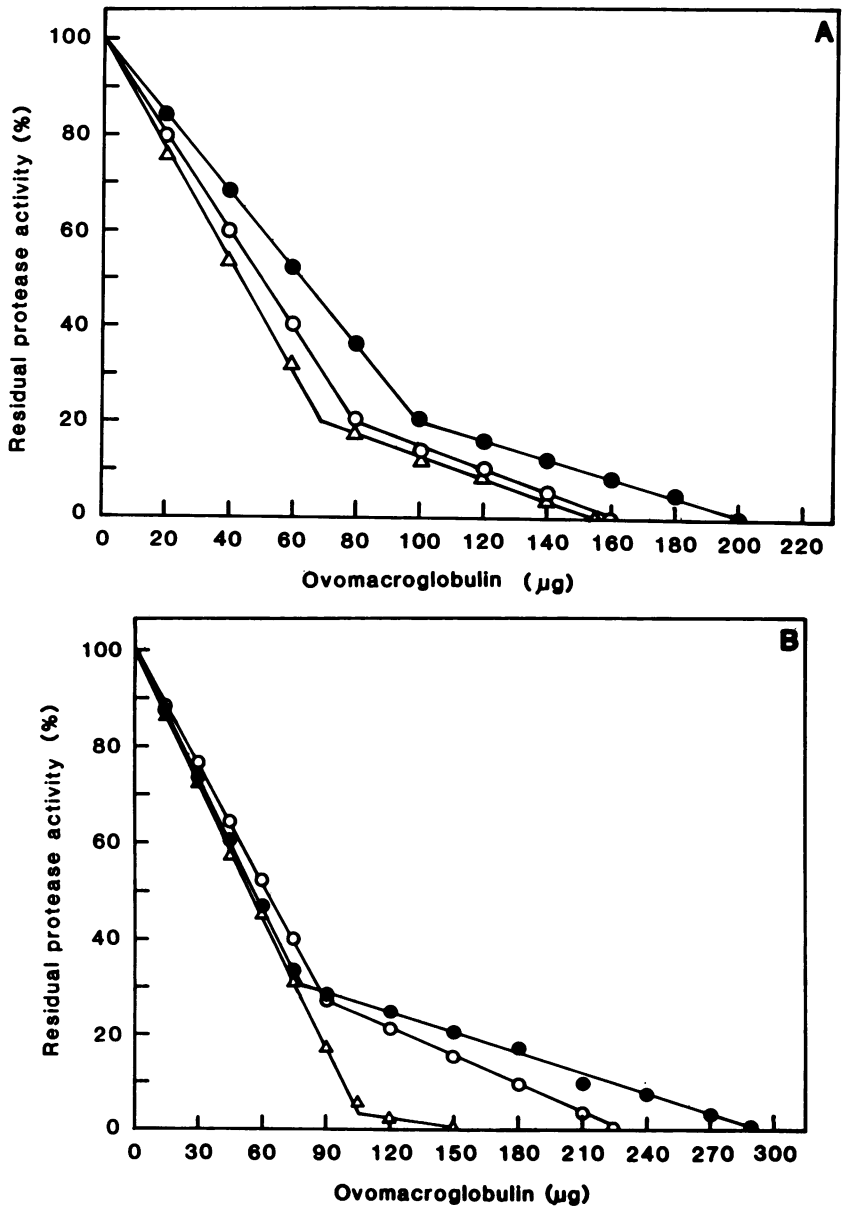

FIG. 1. Effect of ovoM on the proteolytic activity of serratial and pseudomonal proteases and *B. stearothermophilus* thermolysin. Each protease (5 μg) was incubated with various concentrations of ovoM in 50 mM Tris hydrochloride buffer (pH 7.5) at 37°C for 20 min. Residual protease activity was measured with either casein or fluorescein isothiocyanate-labeled gelatin as the substrate. (A) Serratial 56K (●), 60K (○), and 73K (△) protease. (B) PAP (●), PE (○), and thermolysin (△).

duced keratitis in experimental animals (8). Lyerly and Kreger (15) and we (8) reported that the metalloprotease (56 kDa) secreted from *S. marcescens* rapidly caused keratitis when injected at a dose of 0.1 to 1.0 μg. However, the pathogenesis of corneal ulceration caused by two other proteases from *S. marcescens* remained unclear. Our present results show that the serratial 73K protease rapidly caused liquefactive necrosis and ulceration in guinea pig corneas, whereas the serratial 60K protease produced only corneal opacity (Table 2 and Fig. 2 and 3). There are other reports of corneal ulcer formation caused by PAP and PE (3, 4, 10, 12, 13). Our results here are consistent with these previous data. We also found in the present study that thermolysin at a dose of 1.5 μg caused severe keratitis in guinea pig corneas.

TABLE 1. Inhibition of proteases by ovoM at an E/I ratio (molar) of 1:1 with different incubation times[a]

Protease	% Inhibition at:			
	20 min	3 h	12 h	24 h
56K	65	78	88	95
60K	67	82	87	96
73K	64	79	89	95
PAP	55	72	85	90
PE	62	78	82	89
Thermolysin	75	87	94	98

[a] Based upon the activity of each protease incubated for the same period of time in 50 mM Tris hydrochloride buffer (pH 7.5) at 25°C. Within 24 h the protease activity without ovoM was 97 to 98% of that at zero time. FP was the assay method.

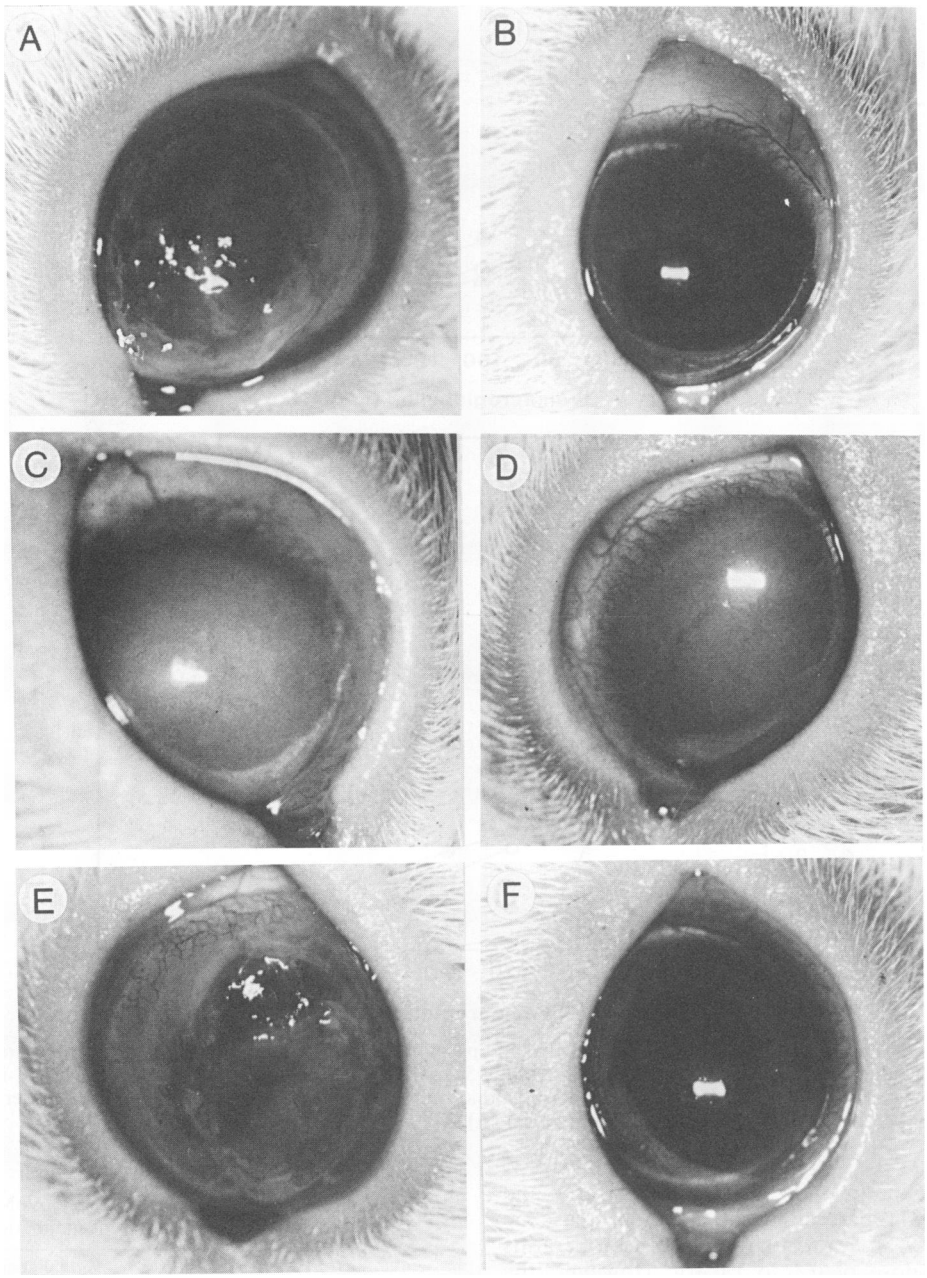

FIG. 2. Corneal ulceration and descemetocele formation in guinea pig eyes caused by pseudomonal and serratial proteases and protection against these damages by ovoM. (A, E, G, and I) 24 h after intrastromal injection of 56K protease, 73K protease, PAP, and PE (all at 1.5 μg), respectively. In all cases the cornea is ulcerated. (C) 24 h after injection of 60K protease (1.5 μg). Opacification of the cornea can be seen. (B, D, F, H, and J) 56K protease, 60K protease, 73K protease, PAP, and PE (all at 1.5 μg) were mixed with 61, 57, 47, 95, and 107 μg of ovoM, respectively, and then injected into corneas, which were observed at 48 h after injection (B, D, F, and H) and at 8 h after injection (J). A minimum alteration of each cornea can be seen; all corneas were completely protected from damage by proteases. (K) PE with ovoM 24 h after injection. Liquefactive necrosis of the cornea can be seen. ovoM protected the eyes against PE for about 8 h after injection.

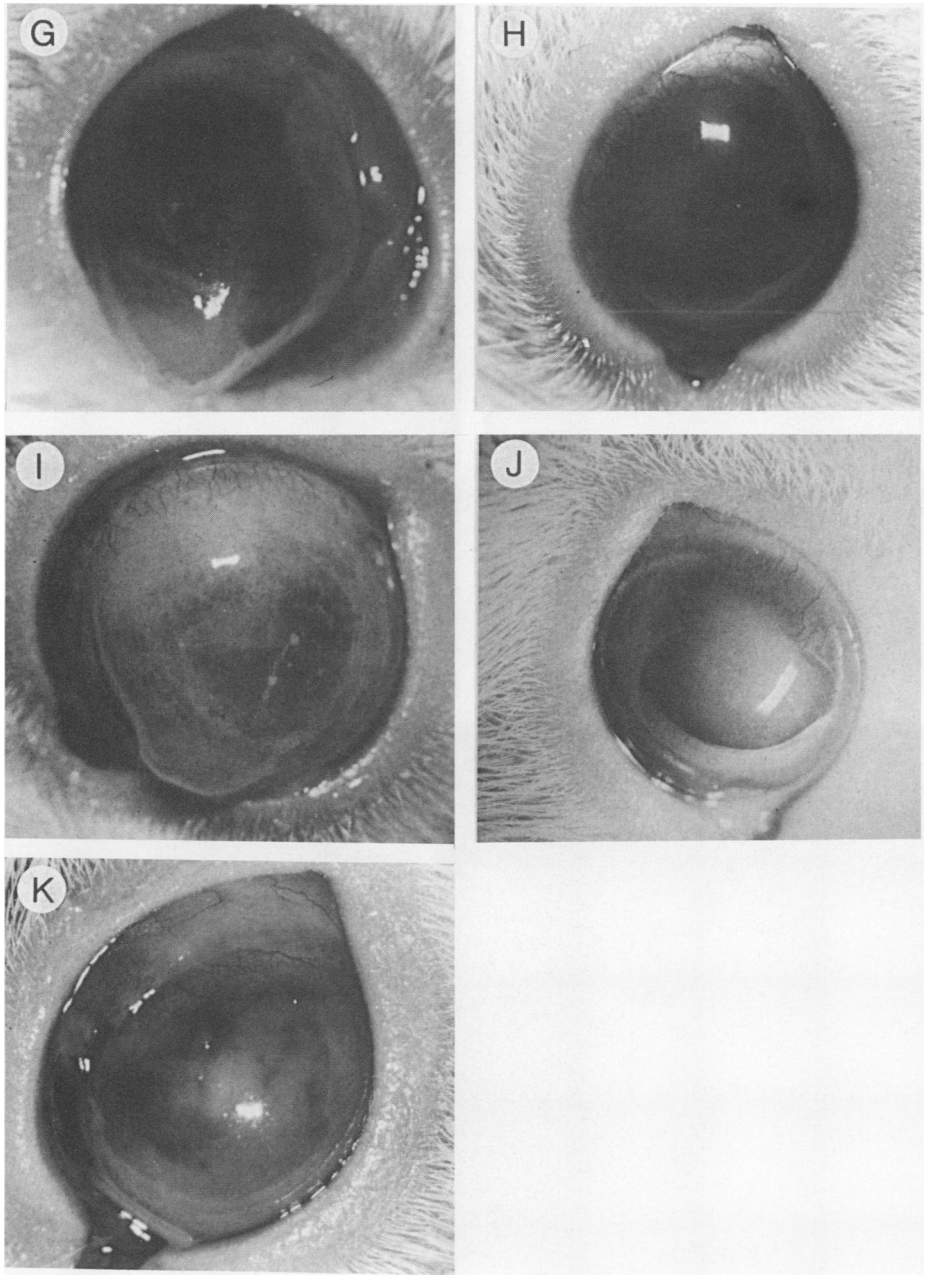

FIG. 2—*Continued*

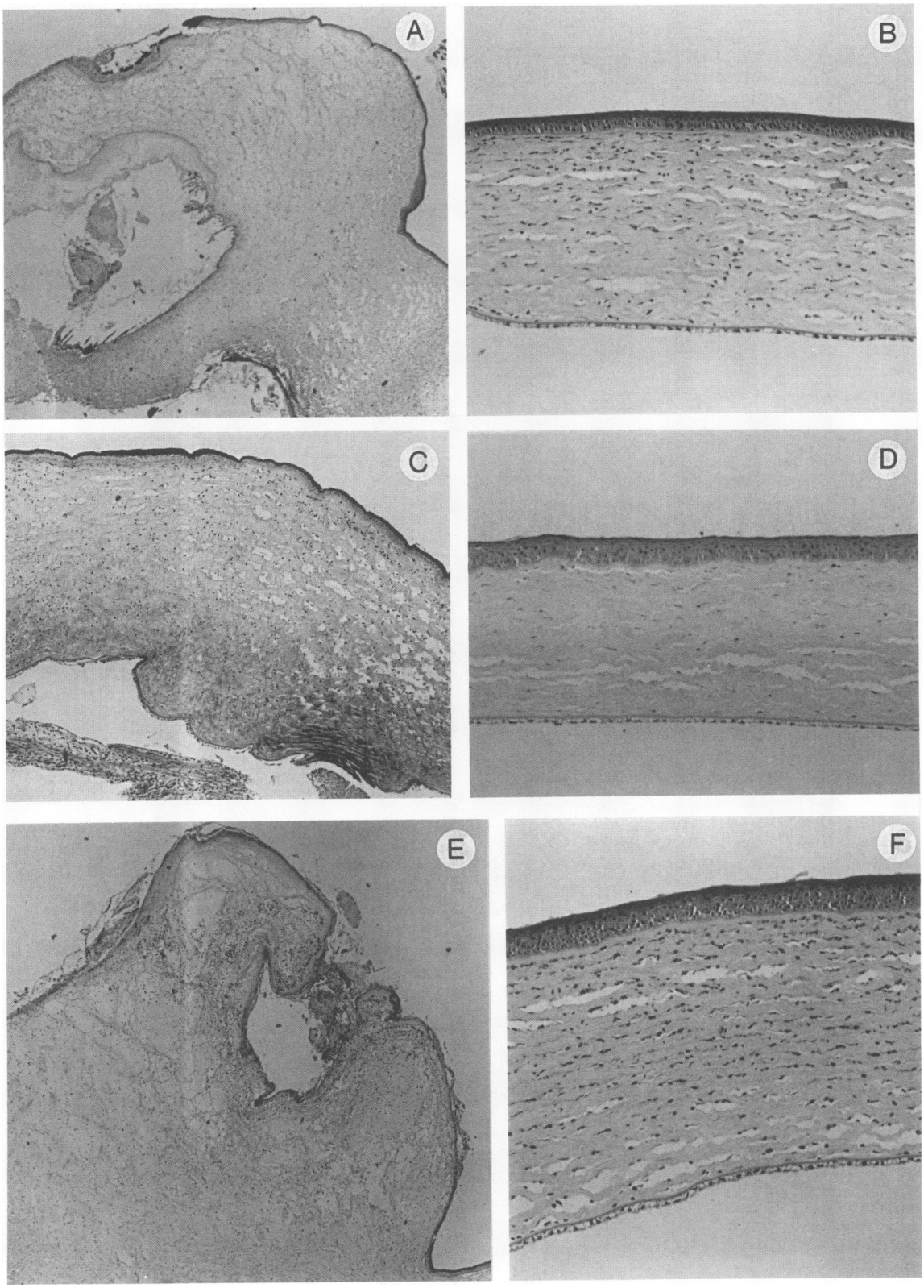

FIG. 3. Histopathological examination of ulcerated corneas and protection by ovoM. (A, C, E, and G) Serratial 73K protease, serratial 60K protease, PAP, and thermolysin, respectively, all at 24 h after intrastromal injection. Panels A, E, and G represent sections of corneas showing extensive liquefactive necrosis of the stromal collagens, ulceration of the cornea, and infiltration of polymorphonuclear leukocytes, and panel C represents a section showing edema formation, polymorphonuclear leukocyte infiltration, and epithelial abrasion. (B, D, F, and H) 73K protease, 60K protease, PAP, and thermolysin, respectively, after treatment with ovoM. Sections were examined 48 h after intrastromal injection. Complete prevention by ovoM of corneal destruction can be seen. Hematoxylin-eosin staining was used in panels A, B, D, F, G, and H; Azan-Mallory staining was used in panels C and E. Magnifications: ×76 in panels A and E, ×380 in panels B, D, F, and H, ×152 in panel C, and ×38 in panel G. Protease and ovoM concentrations are the same as those in Fig. 2.

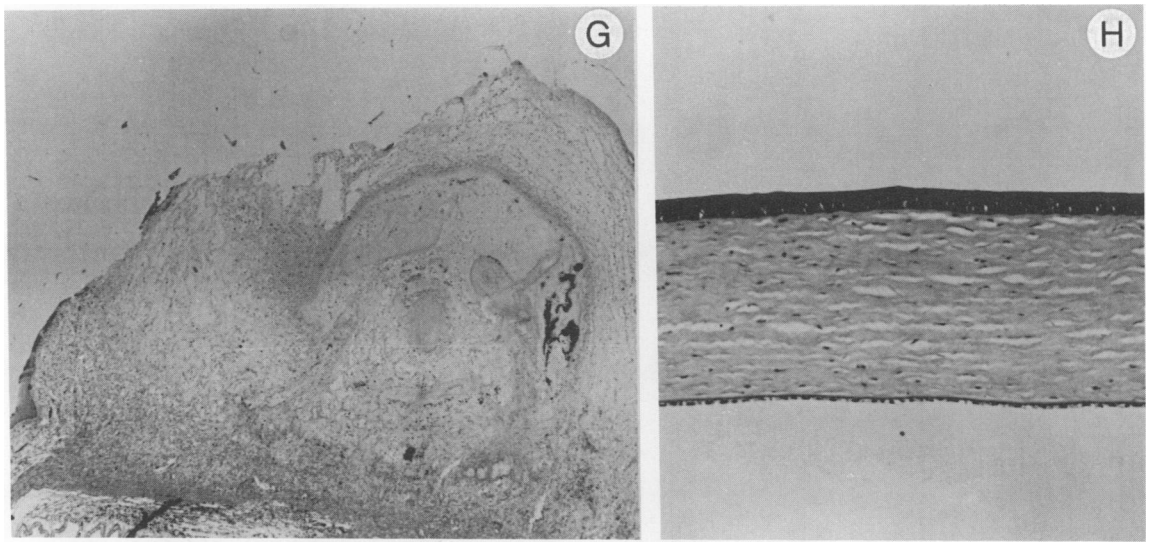

Fig. 3—*Continued*

The ability of the serratial and pseudomonal proteases to cause extensive liquefactive necrosis and descemetocele formation in guinea pig corneas supports the hypothesis that these proteases play a major role in the pathogenesis of these bacterial infections. Solubilization and loss of stromal ground substances may facilitate damage in the tissue. In this context, degradation of fibronectin and collagens by the serratial 56K protease and pseudomonal proteases is also involved (5, 20, 27). An important aspect of these proteolytic lesions is that many of the proteases are resistant to serum protease inhibitors such as α_1-protease inhibitor and α_2M (20, 22, 26).

We previously reported that the serratial 56K protease enhanced vascular permeability by activating the Hageman factor-dependent pathway in guinea pigs (9, 18, 19). However, enhanced vascular permeability caused by other proteases remained to be clarified. We reported here that other bacterial proteases also enhanced the vascular permeability reaction. The mechanism of permeability enhancement by the proteases used in this study is under investigation. Lyerly and Kreger (15) reported that a serratial protease play a major role in serratial pneumonia with extensive pulmonary edema. Our present results support the possible involvement of the various proteases in pneumonia in a manner similar to the enhancement of vascular permeability observed in the skin. When the 73K protease was injected into the periphery of the cornea or into the subconjunctiva, it caused a permeability reaction at the injection site which resulted in corneal swelling and chemosis. This phenomenon may also contribute to the pathogenesis of edema during corneal keratitis. Dose-response curves showed a stronger enhancement of the permeability reaction by the serratial 73K and 56K proteases and PE than by PAP, *B. stearothermophilus* thermolysin, and the serratial 60K protease (Fig. 4 and 5). We previously showed that lipopolysaccharide from *S. marcescens* did not enhance vascular permeability (9).

TABLE 2. Cornea-damaging capacity of various proteases and the inhibitory effect of ovoM

Protease	Corneal damage index[a] at indicated time (h) after intrastromal injection of:						
	Protease alone[b]			Protease + ovoM[c]			
	5	8	24	5	8	24	48
56K	4.0	4.0	4.0	0	0	0	0
60K	1.0	2.0	2.0	0	0	0	0
73K	4.0	4.0	4.0	0	0	0	0
PAP	4.0	4.0	4.0	0.5	0	0	0
PE	4.0	4.0	4.0	0	0.5	3.0	3.0
Thermolysin	2.0	3.0	4.0	0	0	0	0

[a] Corneal damage indices (23) were as follows: 0, normal; 0.5, small area of opacity or neovascularization; 1.0, light or partial opacity; 2.0, extensive opacity; 3.0, central necrosis and opacity; 4.0, perforation of cornea.
[b] Each protease was injected at 1.5 μg per site.
[c] Each protease of the complex was injected at 1.5 μg per site. The doses of ovoM are indicated in Fig. 2.

In this article we described the inhibitory effect of ovoM on both the destruction of the cornea and the enhancement of vascular permeability in guinea pig skin, both of which are caused by various bacterial proteases after in situ injection (Table 2 and Fig. 2 to 5). ovoM is known to bind and inhibit a wide variety of endopeptidases, such as seryl, cysteinyl, aspartyl, and metal proteases, as does α_2M (1, 7, 11, 20, 21). Since both α_2M and ovoM are tetrameric and contain two identical binding subunits linked by a disulfide bond, they can bind a maximum of two molecules of protease per inhibitor molecule. Thus, the mechanism of protease inhibition by these two macroglobulins is assumed to be similar. However, several different characteristics have been reported for these two inhibitors. α_2M has a thioester bond, whereas ovoM does not (1), and ovoM has a higher association constant for the 56K protease than does α_2M (21). The molecular size of the enzyme-inhibitor complex is larger for ovoM than for α_2M (as judged by fluorescence polarization values [1, 21]). During complex formation with the 56K protease, ovoM suffers only slight proteolysis, as demonstrated by the appearance of a 360-kDa band in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, while α_2M is degraded into several fragments of 180 and 90 kDa and smaller sizes (20, 21).

We previously showed that the serratial 56K protease was inhibited by about 75% within 20 min at 37°C when incubated with ovoM at an E/I ratio (molar) of 1.2:1.0, and when incubation was continued, about 90% inhibition was

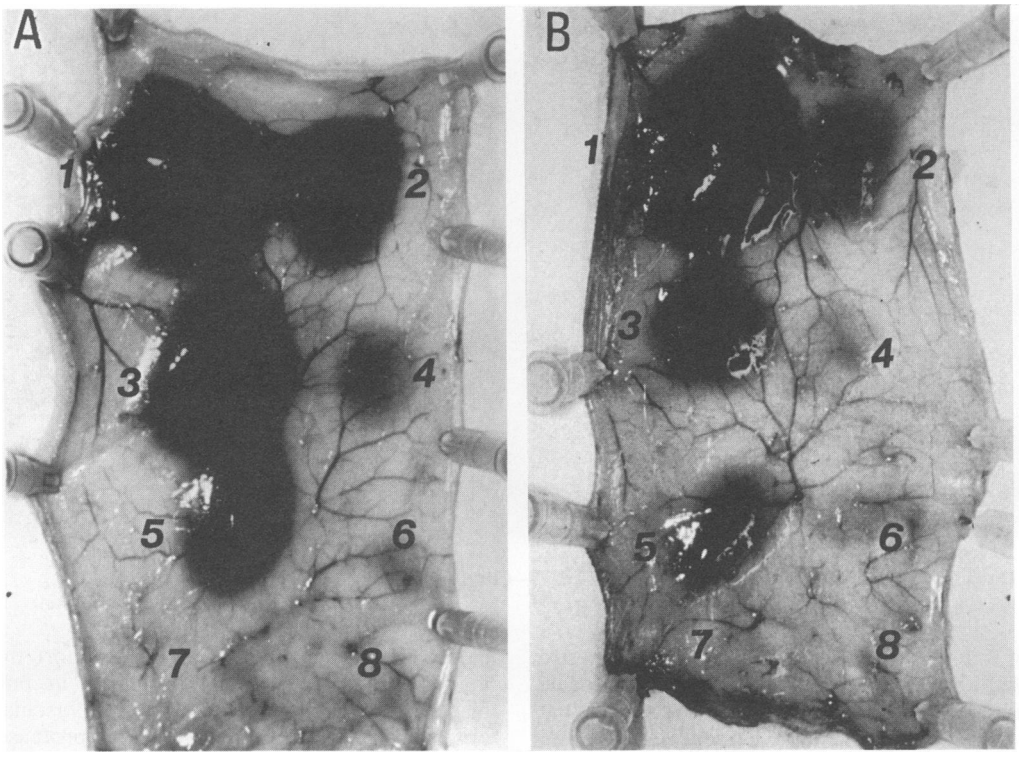

FIG. 4. Vascular permeability-enhancing activity of 73K protease and PE and the effect of ovoM. Aliquots (0.1 ml) of various concentrations of proteases with or without ovoM were injected into guinea pig skin after intravenous injection of the animal with Evans blue. (A) 73K protease injected at 8, 3, or 1 μg alone (1, 3, and 5, respectively) or with 166 μg of ovoM (2, 4, and 6, respectively); 7, saline; 8, ovoM alone. (B) PE injected as described for 73K protease in panel A, except that 312 μg of ovoM was used.

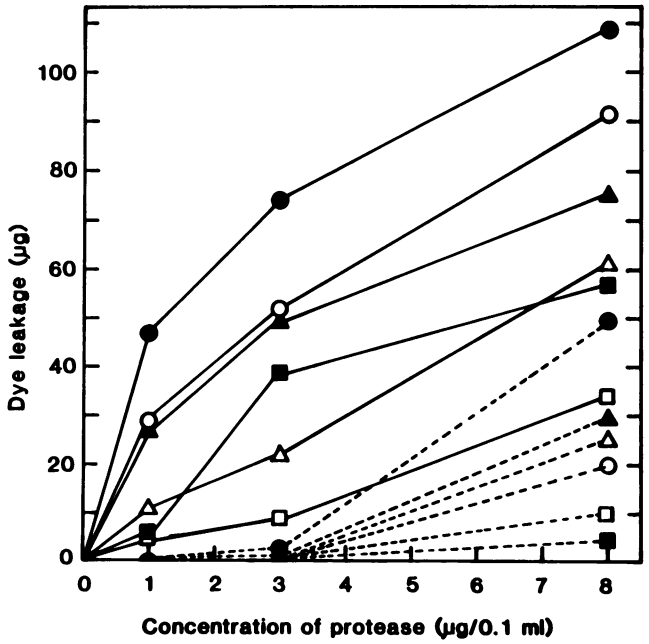

FIG. 5. Quantification of the enhancement of the permeability reaction by proteases and the inhibitory effect of ovoM on dye leakage in guinea pig skin. Various concentrations of proteases with or without ovoM were injected into the skin of dye-treated guinea pigs. After 30 min, the skin was excised. Permeability activity was measured quantitatively after extraction of the Evans blue dye from the stained area with formamide for 48 h at 60°C. Symbols: ●—●, 73K protease alone; ●---●, 73K protease with 156 μg of ovoM; ○—○, 56K protease alone; ○---○, 56K protease with 205 μg of ovoM; □—□, 60K protease alone; □---□, 60K protease with 203 μg of ovoM; △—△, PAP alone; △---△, PAP with 380 μg of ovoM; ▲—▲, PE alone; ▲---▲, PE with 313 μg of ovoM; ■—■, thermolysin alone; ■---■, thermolysin with 163 μg of ovoM.

achieved in 24 h (21). Here, we showed that when excess amounts of ovoM were added to the proteases, protease activities were completely inhibited within 20 min at 37°C. In comparison, α_2M had a completely different inhibitory profile: a low association constant, fewer binding sites and, most importantly, a loss of inhibition during prolonged incubation (21) (more than 90% of the enzyme activity was regained within 24 h [20, 21]). These observations suggested that α_2M was not the optimal inhibitor for these bacterial proteases. Similarly, α_1-protease inhibitor was rapidly degraded by the serratial 56K protease, PAP, and PE (20, 22, 26). Thus, the protective effect of plasma protease inhibitors against these bacterial proteases is much lower than expected. This is in clear contrast to the potent protective efficacy of ovoM in vivo. Corneal ulcer caused by PE manifested slowly in the presence of ovoM, and its permeability-enhancing activity in guinea pig skin was completely inhibited by ovoM. The reason for this difference in inhibition is not clear. Whatever the mechanism of permeability enhancement or ulceration by these different proteases, ovoM may be beneficial in the treatment of pseudomonal and serratial infections. Experiments to test this possibility are under way.

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