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Cleavage of Immunoglobulin G (IgG) and IgA around the Hinge Region by Proteases from *Serratia marcescens*

AKHTERUZZAMAN MOLLA,[1] TADASHI KAGIMOTO,[2] AND HIROSHI MAEDA[1]*

Departments of Microbiology[1] and Internal Medicine,[2] Kumamoto University Medical School, Kumamoto 860, Japan

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Seven clinical and two nonclinical isolates of *Serratia marcescens* were examined for their ability to produce extracellular enzymes that cleave immunoglobulin G (IgG) and IgA molecules. All seven clinical isolates excreted a large amount of a 56-kilodalton (kDa) protease (56K protease) and small amounts of a 60-kDa and a 73-kDa protease (60K and 73K proteases, respectively) in culture medium during growth. All purified proteases cleaved IgG and IgA effectively if the level of protease production exceeded 2 to 5 μg/ml. The proteolytic activity in the culture supernatant was inhibited by about 85% by a chelating agent (EDTA), which indicated that the major immunoglobulin-cleaving enzyme is the metalloprotease(s) reported previously. Immunological quantification of proteases by single radial immunodiffusion showed similar results: the amount of 56K protease was about 65% and those of the 60K and 73K proteases were about 20 and 5%, respectively. Incubation for 3 h at 37°C was required to generate immunoreactive Fab and Fc fragments. Further analysis of the cleavage products of IgG or IgA demonstrated that the 56K protease, as well as the 60K and 73K proteases, cleaves only the heavy chain of these immunoglobulins near the hinge region to generate Fab and Fc fragments. The susceptibilities of the subclasses of IgG and IgA to the 56K protease were as follows: IgG3 > IgG1 > IgG2 > IgG4 and IgA1 > IgA2. IgG2, IgG4, and IgA2 were relatively resistant to the 56K protease.

Immunoglobulins G and A (IgG and IgA) are involved in the complement system, opsonization, and phagocytosis in bacterial infections. Serum IgA is also involved in the clearance of antigen from the circulation (23).

Serratia marcescens is a pathogen that often causes severe keratitis (7, 9), urinary infections (15), and pulmonary infections that cause pneumonia (11). *S. marcescens* secretes nucleases (28), superoxide dismutase (14), a number of proteases (10, 17, 20), hemolysin, lecithinase, and lipase (22). The organism is becoming multidrug resistant although it has been classified as fairly benign.

We previously demonstrated that *S. marcescens* produces three different proteases (17), and one of them, the 56-kilodalton (kDa) protease (56K protease), causes severe keratitis at amounts as low as 0.1 to 1.0 μg (7). The protease activates the Hageman factor, followed by prekallikrein activation, resulting in the bradykinin-generating cascade (8, 18). We have recently showed that the 56K protease was also able to cleave human IgG, IgA, and lysozyme, which are potentially important in host defense against microbial infections (19). Virca et al. (25) and we (19) also showed previously that this protease inactivated human serum α_1-protease inhibitor. α_2-Macroglobulin (α_2M) had been shown to inhibit serratial protease up to 60% (19, 21). Recently, we examined its inhibitory kinetics more thoroughly and found that α_2M indeed is gradually degraded by the 56K protease after a transitory inhibitory state. In parallel, proteolytic activity is restored to more than 90% after several hours (19, 21). In contrast, the homologous protein of α_2M present in chicken egg white, ovomacroglobulin, blocked protease activity and cytotoxicity almost completely (19–21). The potent cytotoxicity of the 56K protease in fibroblasts is mediated by uptake of a complex of the 56K protease and α_2M after binding to a specific α_2M receptor on the fibroblast (13). Very recently we demonstrated that the 60- and 73-kDa proteases (60K and 73K proteases, respectively) from *S. marcescens* and *Pseudomonas aeruginosa* alkaline protease and elastase enhanced vascular permeability in guinea pig skin and produced corneal ulcers in guinea pigs. These effects were completely blocked by ovomacroglobulin (20).

In our previous paper, we showed the IgG and IgA were degraded by the 56K protease, but we did not characterize the cleavage products of each immunoglobulin subclass. The aims of the present investigation were (i) to determine whether only the protease or other exoproducts in the culture supernatant of *S. marcescens* cleave immunoglobulins, and if so, which protease has the greatest activity, and (ii) to identify the cleavage products of these immunoglobulins.

MATERIALS AND METHODS

Bacteria. Various clinical isolates of *S. marcescens* were obtained from patients at Kumamoto University Hospital (see Table 1).

Production of protease. Each strain of *S. marcescens* was grown and kept in a tryptic soy agar slant culture, inoculated by touching of a loop to 2 ml of tryptosoy broth (Eiken, Tokyo, Japan), and cultured for 6 to 8 h at 37°C for the seed culture. After adjustment of the optical density at 600 nm of each seed culture, 0.15 ml was inoculated into 200 ml of the same broth in a 1-liter round-bottom flask. After cultivation at 30°C for 24 h with reciprocal shaking, the culture was centrifuged at 8,000 × *g* for 45 min at 4°C. The supernatant was removed and concentrated to 1/5 to 1/10 by ultrafiltration under positive pressure by using an Amicon PM-10 membrane (Amicon Corp., Lexington, Mass.). The concentrate was stored at −70°C and designated as the crude preparation.

Proteases and antisera. The proteases were purified to homogeneity from a clinical strain of *S. marcescens* 3958 as described previously (17). Antibodies against the 56K, 60K, and 73K protease preparations were raised in rabbits with Freund complete adjuvant. Commercial rabbit antisera against human IgG or IgA or its subclasses, Fab and Fc

* Corresponding author.

fragments of human IgG, and κ-type light chain were obtained from Bio-Yeda (Weizmann Institute of Science, Rehovot, Israel) and Meloy Laboratories, Inc. (Springfield, Va.).

Chemicals. Tetraethylenepentamine and fluorescein isothiocyanate (FITC) were from Dojin Chemical Laboratories, Kumamoto, Japan. All other chemicals were from local suppliers.

Immunoglobulin preparation. IgG was purified from human serum by ammonium sulfate precipitation and column chromatography using DEAE-cellulose and Sephadex G-200 superfine (4, 5). It was further separated into its subclasses by protein A-Sepharose (Pharmacia, Uppsala, Sweden) and ion-exchange chromatography with DEAE-cellulose (2, 24). Human serum myeloma IgA was purified by salt-mediated hydrophobic chromatography on L-phenylalanine-conjugated Sepharose 4B (Pharmacia) (1).

Measurement of degradation of immunoglobulins by culture supernatant. The immunoglobulin-degrading activity of each bacterial culture supernatant was determined by the fluorescence polarization (FP) method using FITC-conjugated IgG or IgA as the substrate (12). The protein concentrations of the crude preparations were determined by the Lowry method with phenol reagent. Immunoglobulins and different concentrations of the crude preparations were mixed and incubated in 0.05 M Tris hydrochloride buffer, pH 7.5, at 37°C for 30 min to 24 h during measurement by FP. Protease activity using FITC-gelatin as the substrate was also measured by the FP method as described above.

Inhibition of protease activity. Crude preparations or purified proteases were preincubated with various concentrations of different inhibitors or the respective anti-protease antibodies at 37°C for 30 min before addition of IgG or IgA as a substrate. After addition of the substrate, inhibition of proteolytic activity was measured as described above.

SDS-PAGE. Digestion of IgG or IgA by purified protease was done under conditions similar to those of the FP method described above. After digestion with proteases, all immunoglobulins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed by the method of Weber and Osborn (26). Approximate molecular weights were estimated by using standard proteins. Reduced samples were also analyzed by treatment with 0.1 M 2-mercaptoethanol at 100°C for 2 to 3 min before electrophoresis. Some gels stained with Coomassie blue were scanned by densitometry.

RESULTS

Immunoglobulin-degrading enzymes in the culture supernatant. Reaction mixtures containing different concentrations of the crude preparation and IgG or IgA were analyzed by the FP method and by immunoelectrophoresis for proteolytic activity after different incubation periods. We found that culture supernatants from seven of nine strains degraded IgG and IgA extensively within the 3-h incubation periods. Two culture supernatants from nonclinical isolates (strains 30 and 632) did not cleave IgG or IgA as revealed by the FP method (Table 1). Immunoelectrophoresis of IgG or IgA after 3 h of incubation with the crude preparation of two strains (3958 and 514) showed that IgG or IgA cleaved into corresponding Fab and Fc fragments (data not shown).

Protease activity of bacteria. We then determined the protease activity of culture supernatants as a whole by the FP method by using FITC-gelatin as the substrate (Table 1). The amounts of 56K and other proteases in culture supernatant were determined by single radial immunodiffusion (6). Strains 632 and 30 produced no detectable amount of protease, whereas the remaining seven strains produced high amounts of the 56K protease and low amounts of the 60K or 73K protease (Table 1). This indicates that the gelatinolytic activity of the bacteria correlates with proteolysis of IgG or IgA.

We then tested whether, like the crude preparation, purified proteases from strain 3958 cleave IgG and IgA. Incubation of FITC-IgG or FITC-IgA with purified 56K, 60K, or 73K protease resulted in degradation of both immunoglobulins, as with the crude preparation (Table 1).

Inhibition of protease activity. Inhibition of cleavage of IgG and IgA was tested after preincubation of the crude preparation or three purified proteases with different inhibitors or the respective anti-protease antibody. EDTA (5 mM) or tetraethylenepentamine (5 mM), a zinc-specific chelator (16), inhibited degradation of IgG by the crude preparation by

TABLE 1. Relative proteolytic activity of serratial culture supernatant or purified protease on IgG, IgA, or gelatin and quantification of proteases by single radial immunodiffusion

Strain or protease	Source	% Decrease in FP value with the following substrate[a]:			Amt of protease (μg/ml) detected by antibodies to[b]:		
		IgG	IgA	Gelatin	56K	60K	73K
3958	Eye	55	59	68	120	23	6.1
514	Sputum	51	58	71	109	16	6.8
1247	Sputum	45	48	64	96	22	6.0
534	Sputum	38	42	55	84	14	5.3
303	Sputum	36	39	46	82	12	5.0
382	Eye	39	38	52	87	12	5.0
580	Urine	32	35	44	58	6	0
632	Urine[c]	0	0	5	0	0	0
30	Urine[c]	0	0	4	0	0	0
56K[d]		60	65	76			
60K[d]		26	21	34			
73K[d]		51	49	71			

[a] Determined by the FP method with 3 h of incubation (37°C), using FITC-conjugated protein substrates. Each supernatant was diluted appropriately to give 140 μg of protein per ml in an assay system.

[b] Quantitation was by the single radial immunodiffusion method using corresponding anti-protease antibodies. The numbers indicate micrograms of protease per milliliter of culture supernatant.

[c] Isolated from healthy adults with no clinical symptoms.

[d] The 56K, 60K, and 73K proteases were purified from strain 3958 and used at 5 μg/ml.

TABLE 2. Inhibition of IgG proteolytic activity of serratial culture supernatant or purified proteases by different inhibitors[a] or anti-protease antibodies[b]

Strain or protease[c]	Residual proteolytic activity (%) with IgG as a substrate and treatment with[d]:						
	EDTA	TEP	Iodoacetamide	NEM	56K antibody	60K antibody	73K antibody
3958	15	16	86	87	36	80	85
514	16	18	86	85	35	82	87
1247	15	17	83	84	35	78	86
534	16	16	86	87	35	76	88
56K	0	0	98	100	4	100	100
60K	0	0	98	98	100	3	100
73K	98	97	38	32	100	100	1

[a] Used at a concentration of 5 mM.
[b] Used at 0.01 mM IgG.
[c] The protease or protein concentration in the supernatant was as described in Table 1.
[d] Measured by the FP method (12). The substrate concentration was 50 μg/ml. TEP, Tetraethylenepentamine; NEM, *N*-ethylmaleimide.

about 85% as determined by the FP method (Table 2). When thiol protease inhibitors were used, only 10 to 15% inhibition resulted (Table 2). Rabbit antibody to the 56K, 60K, and 73K proteases inhibited degradation of IgG about 65, 20, and 13%, respectively, when crude preparations were incubated with these antibodies (Table 2). The results of inhibition of degradation of IgA by different inhibitors and antibodies were very similar to those obtained for IgG (data not shown). We concluded that the 73K and 60K proteases can cleave IgG or IgA, although the activity in the culture supernatant is low (Table 1). Thus, the major protease in the culture supernatant for the cleavage of IgG or IgA is the 56K protease, a metalloprotease.

Specificity for different subclasses of IgG and IgA. Four subclasses of IgG and two subclasses of IgA were digested with the 56K protease at an enzyme/substrate (E/S) ratio (molar) of 1:10 for 3 h at 37°C and analyzed by SDS-PAGE. The amounts of degraded IgG or IgA were quantified by densitometric scanning after staining of the gels. IgG3, IgG1, and IgA1 were more sensitive to the 56K protease than were IgG2 and IgG4 (Fig. 1). IgA2 was almost resistant to proteolysis.

Degradation of IgG3 and γ and α heavy chains. The proteins used in this study were subjected to SDS-PAGE with or without the 56K protease under reducing and nonreducing conditions. IgG3 was degraded extensively within 3 h, even at an E/S ratio (molar) of 1:200 (Fig. 2A). The protease cleaved IgG3 into different fragments of 130, 87, 55, and 30 kDa.

A reduced sample of undigested IgG or IgA migrated as two bands of approximately 55 (H-chain) and 23 (L-chain) kDa, as expected. Stepwise increases in the concentration of purified 56K protease with IgG1 resulted in generation of a band of 32 kDa; with more protease, an additional band of 45 kDa was generated (Fig. 2B). Increasing the concentration of the 56K protease against IgA1 resulted in 51-, 44-, and 33-kDa fragments (Fig. 2C). Further addition of protease resulted in disappearance of the 51-, and 44-kDa bands. Degradation of IgG and IgA by the 60K and 73K proteases was similar to that by the 56K protease (data not shown). Digestion of IgG with *Pseudomonas* elastase, which is known to cleave at the hinge region of IgG, resulted in an electrophoretic pattern upon SDS-PAGE very similar to that obtained with the 56K protease (data not shown) (3).

Immunological identification of cleavage products. Purified IgG1, IgG3, and IgA1 were digested with the 56K protease at an E/S ratio (molar) of 1:25 to 1:200 at 37°C for 3 to 16 h and then applied to a column of Sephadex G-150 (superfine) to obtain each fragment (data not shown). All peak fractions were pooled, and Fab, Fc, and residual undigested immunoglobulin fractions were further separated by an anti-light-chain immunoadsorbent (27). Fragments were tested by Ouchterlony immunodiffusion using anti-IgG, anti-IgA, and anti-light-chain (κ-type) antibodies. The Fab fragment reacted with anti-light-chain (κ) antibody, whereas the Fc fragment did not react with anti-light chain antibody (Fig. 3A and B). The isolated Fab and Fc fragments exhibited a common partial identity to intact IgG or IgA, but they were not identical to one another (Fig. 3C and D).

DISCUSSION

This study demonstrated that *S. marcescens* produces proteases capable of cleaving both IgG and IgA molecules. The enzymatic activity was found in the culture supernatant, which indicates that *S. marcescens* excreted the enzyme into the medium during growth. There was an appreciable amount of protease activity produced by the bacteria in the culture medium within 24 h (Table 1). Proteolytic activity in

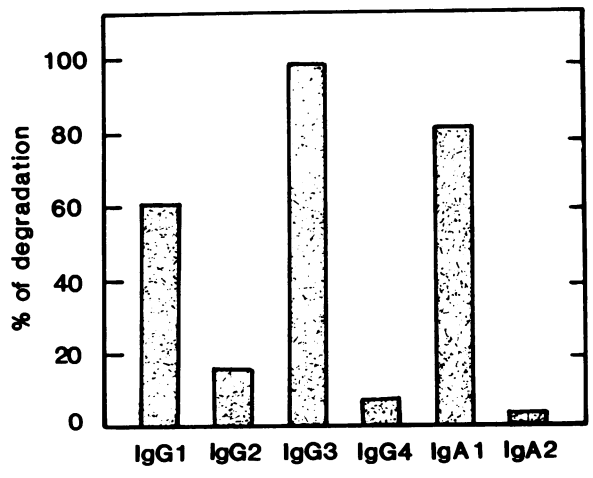

FIG. 1. Effect of the 56K protease on the degradation of IgG or IgA subclasses revealed by a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. The E/S ratio (molar) of 1:10, with 50 μg of substrate per tube, was incubated in 50 mM Tris hydrochloride buffer, pH 7.5, at 37°C for 3 h. The extent of degradation of intact immunoglobulin bands was determined by densitometric scanning of the polyacrylamide gel after staining with Coomassie blue. The amounts of intact immunoglobulins were calculated from the relative intensities of the Coomassie blue stain in the areas under the scanned peaks and expressed as percentages of the degraded bands.

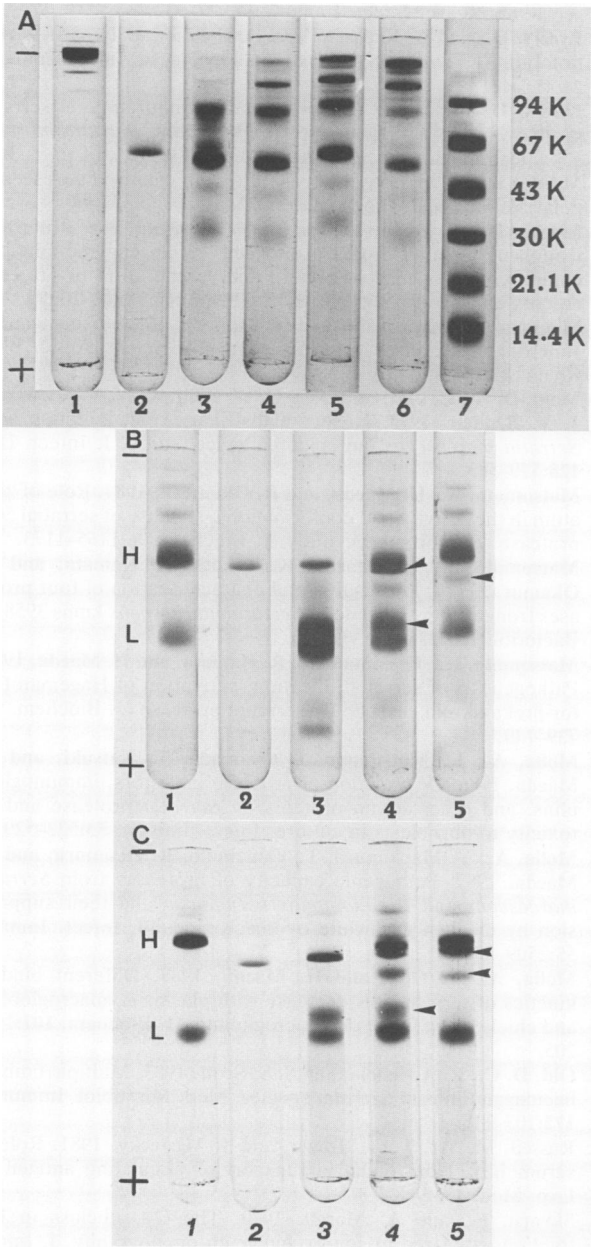

FIG. 2. Effect of the 56K protease on the degradation of IgG3 (A), γ heavy chain (B), and α heavy chain (C). (A) The gel was 7.5% polyacrylamide. Lanes: 1 and 2, IgG3 (57 μg) and the 56K protease (2 μg), respectively; 3, 4, 5, and 6, E/S ratios (molar) of 1:10, 1:50, 1:100, and 1:200, respectively; 7, standard protein markers. (B) Purified IgG1 digested by the 56K protease at 37°C for 3 h and then reduced with 2-mercaptoethanol and electrophoresed in a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. Lanes: 1 and 2, IgG1 (58 μg) and the 56K protease (2 μg), respectively; 3, 4, and 5, E/S ratios (molar) of 1:10, 1:50, and 1:100, respectively. (C) Same as in panel B, except that IgA1 was used. Arrowheads indicate main cleavage products. H, Heavy chain; L, light chain.

the culture supernatant was inhibited by about 85% by chelating agents, such as EDTA and tetraethylenepentamine, a zinc-specific chelator, whereas anti-56K protease (metalloprotease) antibody inhibited proteolytic activity by about 65%. The remaining proteolytic activity was from another metalloprotease secreted by *S. marcescens*: the 60K protease. This was confirmed by use of anti-60K protease antibody (Table 2). Thiol protease inhibitors, such as *N*-ethylmaleimide and iodoacetamide, inhibited the proteolytic activity by only 10 to 15% in a crude preparation when determined by the FP method (Table 2). Thus, the major enzyme excreted into the medium is the 56K protease (Table 1). In addition, recently we demonstrated that the 56K protease was the major pathogenic factor in serratial keratitis in vivo (7). Accordingly, we focused our subsequent work on the 56K protease.

We previously showed that the 56K protease degraded various serum proteins (19). Here, we demonstrated that a crude preparation cleaved IgG and IgA when the protease concentration in the medium was 2 to 5 μg/ml. The potent proteolytic activity of seven clinical isolates demonstrated that they cause complete degradation of IgG and IgA. The nonpathogenic isolates, which had no gelatinolytic activity, did not cleave the immunoglobulins, which indicates that the proteases, not other excreted products, are responsible for destruction of IgG and IgA (Table 1). We previously demonstrated that a virulent strain produced more protease and caused severe keratitis, whereas a less virulent strain produced a very small amount of protease and caused a mild corneal lesion (7). The association of these protease-producing bacteria with disease strongly suggests a pathogenic role of proteases that cleave IgG and IgA. Consequently, secondary infection may be facilitated by degradation of immunoglobulins.

The limited proteolysis of IgG and IgA by serratial proteases is intriguing as a pathogenic mechanism for this organism. We report here that IgG1, IgG3, and IgA1 are the most sensitive to the 56K protease (Fig. 1). Coincidentally, these susceptible immunoglobulins are known to play a vital role in complement-dependent killing of bacteria. Therefore, it is conceivable that the pronounced susceptibility of IgG1, IgG3, and IgA1 makes the host more vulnerable to subsequent bacterial infections.

Evidence that the cleavage site is near the hinge region is as follows. (i) The Fab fragment obtained by isolation on Sephadex G-150 produced two fragments of molecular weights 29,000 (heavy chain) and 23,000 (light chain) under reducing condition on SDS-PAGE (data not shown) and

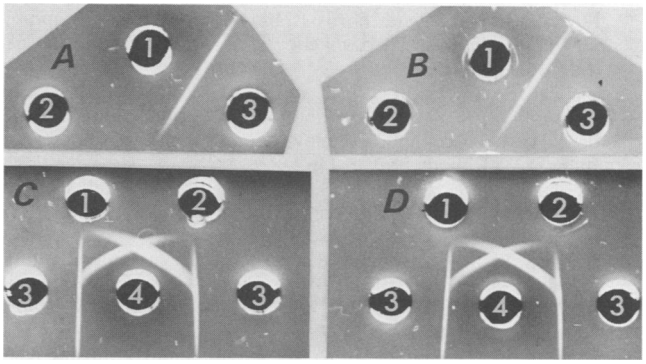

FIG. 3. Ouchterlony immunodiffusion analysis of isolated IgG and IgA fragments. (A and B) Fragments from IgG1 and IgA1, respectively. Wells: 1, anti-light-chain (κ-type) antibody; 2 and 3, isolated Fc and Fab, respectively, from gel filtration. Fab reacted with κ-type antibody. (C and D) Fragments from IgG1 and IgA1, respectively. Wells: 1 and 2, Fc and Fab, respectively, from gel filtration; 3, intact IgG (C) or IgA (D); 4, anti-human IgG (C) or anti-human IgA (D) rabbit antiserum. Fab (well 2) and Fc (well 1) show crossing of the precipitin band, indicating immunological nonidentity; both fragments are immunologically related to the parent IgG or IgA, indicating partial immunological identity (well 3).

reacted with anti-κ chain-specific antiserum (Fig. 3). (ii) The Fc fragment, which was unreactive to anti-κ-chain antiserum (Fig. 3) but reactive to anti-Fc antibody (data not shown), was converted into about half size (molecular weight, 27,000) upon disulfide reduction (data not shown) and shown to be different from Fab. (iii) Both fragments were antigenically different from intact IgG or IgA, showing a reaction of partial identity, and were nonidentical to one another on gel diffusion analysis (Fig. 3). (iv) Although the Fc fragment showed no immunological relationship to Fab, when both fragments were combined they seem to make up the molecular size of an intact immunoglobulin.

On the basis of the above results, it seems possible that colonization of *S. marcescens* results in a focal release of proteases, activating the kinin-generating cascade (8, 18) and forming an enzyme-inhibitor complex that is internalized in the fibroblasts that accompany cell killing (13). Simultaneously, degradation of immunoglobulins at the focal lesion would progress. Thus, multiplication of *S. marcescens* and/or another secondary infection would be facilitated. The proteases also degrade fibronectin (19) and proteoglycan (9). Thus, penetration and spread by potentially toxic and lytic enzymes, as well as bacteria, become easier. The end result may be an enhanced lesion.

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