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Proteolytic Enzymes: a New Treatment Strategy for Prosthetic Infections?

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Among the different mechanisms of bacterial resistance to antimicrobial agents that have been studied, biofilm formation is one of the most widespread. This mechanism is frequently the cause of failures in the treatment of prosthetic device infections, and several attempts have been made to develop molecules and protocols that are able to inhibit biofilm-embedded bacteria. We present data suggesting the possibility that proteolytic enzymes could significantly enhance the activities of antibiotics against biofilms. Antibiotic susceptibility tests on both planktonic and sessile cultures, studies on the dynamics of colonization of 10 biofilm-forming isolates, and then bioluminescence and scanning electron microscopy under seven different experimental conditions showed that serratiopeptidase greatly enhances the activity of ofloxacin on sessile cultures and can inhibit biofilm formation.

It is known that bacteria can grow both while floating in a liquid environment (planktonic conditions) and while forming adherent microcolonies and biofilms at a solid-liquid interface (sessile conditions) (5). Among the different mechanisms bacteria possess for resisting antimicrobial agents, biofilm formation is one of the most important and widespread (4, 5, 10, 15).

Biofilm formation enables cells to "glue" themselves to inert surfaces and can also induce an enhancement of other mechanisms of bacterial resistance (7). Biofilm formation is frequently involved in bacterial adhesion to mucosal surfaces, catheters, and other prosthetic devices (2). Recent reports have also shown that the resistance of a biofilm to antibiotics is directly correlated to the age of the biofilm itself (2), probably as a consequence of chemical stabilization of the exopolysaccharides produced in the process of biofilm formation (1, 3, 6).

Proteolytic enzymes and protease inhibitors have been shown in vitro to have the capacity to depress or inhibit some fundamental activities of bacteria (8).

Clinical observations (4a) suggest that the administration of proteolytic enzymes could enhance therapeutic outcomes in the treatment of contact lens and endo-ocular prosthetic device infections.

The present study aimed to evaluate in vitro the effects of some proteases on bacterial biofilm formation and on the susceptibilities of biofilms to antibiotics.

MATERIALS AND METHODS

Strains. Ten bacterial strains were used; of these, five were *Pseudomonas aeruginosa* and five were *Staphylococcus epidermidis*. All the strains were freshly isolated from prosthetic device infections and were identified at the species level by the API 20NE and API Staph systems (API System, La Balme les Grottes, Montalieu Vercieau, France), respectively.

Procedures. The MICs and MBCs for all tested strains were determined under both planktonic and sessile conditions of growth by a previously published method (14). Ofloxacin (Sigma Tau, Italy) alone (dilution range, 200 to 0.1 μg/ml) and ofloxacin in combination with different proteases were tested. The following proteases were used: clostridiopeptidase A (Catalog no. C9891, Sigma Chemical Co., St. Louis, Mo.), fibrinolysin (catalog no. P7911; Sigma), streptokinase (Kabipharmacia, Stockholm, Sweden), and serratiopeptidase (Takeda Italia, Rome, Italy).

Tests under planktonic conditions were performed in 5 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) by inoculating 10^6 CFU of each tested strain per ml. Test tubes were incubated overnight at 37°C, and after determination of MIC, 10 μl of each culture showing no growth was transferred into tubes containing 5 ml of the same medium without ofloxacin and protease, and the tubes were incubated for 7 days at 37°C to determine the MBC. Tests under sessile conditions were performed as described above for those under planktonic conditions, but with a polystyrene bead (diameter, 7 mm; The Plastic Ball Co.) colonized for 1 h in a suspension of 10^6 CFU of the tested strain per ml; the bead was used as the inoculum. Preliminary observations showed that this colonization procedure allowed us to obtain an inoculum of 10^6 CFU per bead. After overnight incubation at 37°C and MIC determinations, the beads in all tubes showing no growth were transferred steriley into 5 ml of fresh medium (after extensive rinsing in sterile saline), and the mixture was incubated for 7 days at 37°C to determine the MBC.

Results of these preliminary experiments led us to choose serratiopeptidase for further studies. The dynamics of establishment of a biofilm and the effect of antimicrobial treatment were simulated in a specially designed glass container (50-ml volume) with a screw cap and two openings (3 mm in diameter) for silicone tubes, through which a constant flow rate of 120 ml/h was maintained. The container was inoculated with 12 polystyrene beads, which were colonized for 1 h at 37°C in a suspension of each test strain of 10^4 CFU/ml (inoculum equal to 10^4 CFU per bead). The flow was maintained for up to 5 days under the following seven different conditions: A, Trypticase soy broth (TSB; Difco); B, TSB with serratiopeptidase at 10 U/ml; C, TSB with ofloxacin at a concentration equal to the MIC under planktonic growth conditions for each test strain; D, TSB with

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serratiopeptidase at 10 U/ml and ofloxacin as described above for condition C; E, TSB with serratiopeptidase at 10 U/ml incubated for 24 h at 37°C and then heated to 60°C for 15 min to inactivate the enzyme before introducing the inoculated beads and ofloxacin as described above for condition C; F, TSB with ofloxacin at the MBC that was determined in the presence of serratiopeptidase for sessile bacteria; and G, TSB with serratiopeptidase at 10 U/ml and ofloxacin as described above for condition F.

At predetermined times (0, 24, 48, 72, 96, and 120 h), two beads were removed from the container and vigorously vortexed 10 times in sterile saline (20 ml); one bead was processed for scanning electron microscopy (SEM), and the other one was used to determine the number of adherent bacterial cells by bioluminescence.

Samples for SEM were fixed in 2.5% glutaradheyde in Millonig's phosphate buffer (MPB; pH 7.4) for 4 h and were then rinsed in MPB and placed in 2% osmium in MPB for 90 min. The specimens were then rinsed in MPB and dehydrated through graded water-ethanol and then ethanol-acetone series. The dehydrated specimens were dried to the critical point, mounted on stubs, sputter coated with gold, and examined in a Cambridge S240 scanning electron microscope at an accelerating voltage of 25 kV.

The number of adherent bacterial cells colonizing each bead was determined by using a bioluminescence apparatus (Lumac M2500; Lumac bv, Landgraaf, The Netherlands), which allowed for the quantitation of bacterial ATP by the luciferin-luciferase reaction as relative light units (9). Results of these assays were expressed as relative light units per bead, and the values that were obtained were related to the number of adherent bacterial cells, expressed as CFU per bead, by the criteria proposed by Selan et al. (13) and considering the value of 250 CFU per bead as the lowest number of accurately detectable bacteria; lower values were not considered or plotted.

RESULTS

Among the different proteases tested, serratiopeptidase showed the greatest activity (data not shown) and was thus used in more extensive studies.

The MICs and MBCs of ofloxacin alone and ofloxacin with serratiopeptidase for the 10 test strains under both planktonic and sessile conditions of growth are shown in Table 1. Serratiopeptidase enhanced the activity of ofloxacin under sessile conditions of growth, particularly when MBCs were considered, and did not influence either MICs or MBCs under planktonic conditions of growth.

The activity of serratiopeptidase was confirmed in the experiments on the dynamics of colonization (Fig. 1). In fact, while all test strains grown under conditions A, B, C, and F showed a progressive increase in the number of CFU per bead at all sampling times, the strains grown under condition D showed a significant reduction in the number of CFU per bead (Fig. 1) and the strains grown under condition G showed a very significant reduction in the same values, which decreased rapidly below the detection limit of the assay. The strains grown under condition E, performed to evaluate whether the activity of serratiopeptidase was due to proteolytic activity on medium components or on bacterial cells, showed that with incubation of the medium with serratiopeptidase for 24 h at 37°C and then inactivation of the enzyme by heating to 60°C for 15 min, no significant difference from the data obtained with strains grown under condition C could be observed. These data suggest an active interaction of the peptidase with biofilm-forming bacteria.

TABLE 1. MICs and MBCs of ofloxacin alone and ofloxacin plus serratiopeptidase for 10 bacterial strains isolated from prosthetic device infections and grown under both planktonic and sessile conditions

Strain	MIC (μg/ml)[a]				MBC (μg/ml)			
	OFX		OFX + SP		OFX		OFX + SP	
	PL	SE	PL	SE	PL	SE	PL	SE
P. aeruginosa 1	0.78	12.5	0.78	3.12	0.78	100	0.78	12.5
P. aeruginosa 2	0.39	6.25	0.39	3.12	0.78	100	0.78	12.5
P. aeruginosa 3	1.56	6.25	1.56	3.12	1.56	200	1.56	12.5
P. aeruginosa 4	0.78	3.12	0.78	1.56	1.56	100	1.56	6.25
P. aeruginosa 5	1.56	12.5	1.56	6.25	1.56	200	1.56	25
S. epidermidis 1	0.19	3.12	0.19	1.56	0.39	50	0.39	6.25
S. epidermidis 2	0.39	3.12	0.39	1.56	0.78	100	0.78	12.5
S. epidermidis 3	0.78	6.25	0.78	3.12	0.78	100	0.78	6.25
S. epidermidis 4	0.78	6.25	0.78	3.12	1.56	200	0.78	12.5
S. epidermidis 5	0.19	3.12	0.19	1.56	0.39	100	0.39	6.25

[a] OFX, ofloxacin alone; OFX + SP, ofloxacin plus serratiopeptidase at 100 U/ml; PL, test performed under planktonic conditions of growth, SE, test performed under sessile conditions of growth.

The data presented above were confirmed by SEM observations, showing that for all strains tested, beads taken from strains grown under conditions A, B, C, E, and F were largely colonized by bacterial cells organized in biofilms (enlarging with time, and reaching, in the case of *P. aeruginosa* 4, a density of about 3.25×10^5 CFU/mm^2 after 5 days of growth under condition C [Fig. 2]), while beads taken from the culture grown under condition D showed a minor number of adherent cells (decreasing with time, reaching a concentration of about 6.5 CFU/mm^2 after 5 days under condition D [Fig. 3]). We found no evidence of microcolony formation or even cell-to-cell aggregation in the samples taken after 72, 96, and 120 h of incubation, and we found that

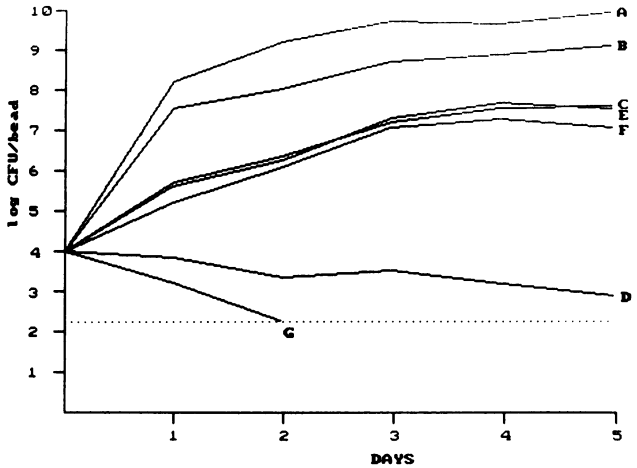

FIG. 1. Bacterial counts, expressed as log CFU per bead, obtained by bioluminescence from polystyrene beads taken from cultures of *P. aeruginosa* 4 with a flow rate of 120 ml/h maintained for 5 days under seven different experimental conditions (conditions A to G; see text). The dotted line indicates the detection limit of the bioluminescence counting method, corresponding to a value of 250 CFU per bead.

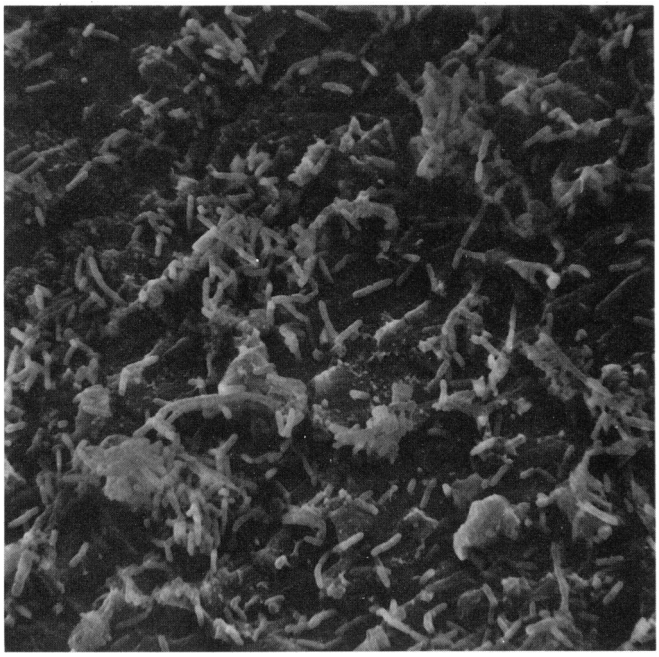

FIG. 2. SEM image of a polystyrene bead incubated for 5 days at 37°C in a culture of *P. aeruginosa* 4 under condition C at a flow rate of 120 ml/h. Magnification, × 1,550.

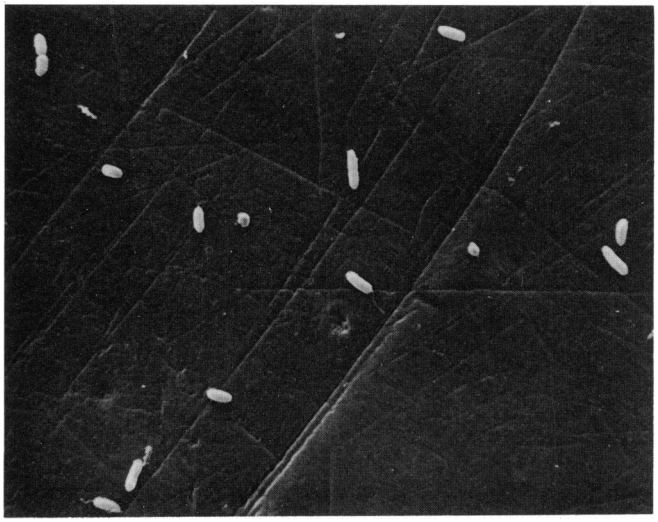

FIG. 3. SEM image of a polystyrene bead incubated for 5 days at 37°C in a culture of *P. aeruginosa* 4 under condition D at a flow rate of 120 ml/h, Magnification, × 2,220.

the beads taken from the culture grown under condition G were almost cell free at both 96 and 120 h. The results were comparable for all strains tested.

DISCUSSION

The treatment of prosthetic device infections and infections caused by biofilm-forming bacteria can be considered one of the biggest problems of antimicrobial therapy today (2). Results of the present study suggest the possibility that some proteolytic enzymes, and particularly serratiopeptidase, could be useful for this purpose.

Serratiopeptidase is a 50.6-kDa metalloprotease (containing zinc) that shows a strong proteolytic activity with a substrate specificity somewhat similar to that of the thermolysin produced by *Bacillus thermoproteolyticus* (12); it has been widely used in therapy as an anti-inflammatory drug because of its ability to enhance the penetration of antibiotics in infected sites (16). It is only partially inhibited by protease inhibitors acting in vivo and is totally inhibited by heating at 55°C for 15 min (11).

Serratiopeptidase was shown to be able to enhance the activity of ofloxacin on sessile cultures of the most common pathogens in prosthetic device infections (*P. aeruginosa* and *S. epidermidis*). Incubation of precolonized beads in a chemostat system with a high flow rate was done with the intent of removing planktonic cells as much as possible so that we could evaluate the dynamics of establishment of biofilms over a 5-day cycle under different cultural conditions; serratiopeptidase was shown to enhance the activity of ofloxacin against the development of bacterial biofilms, using both inhibiting and bactericidal concentrations, whereas biofilms became progressively larger when they were developed in the presence of ofloxacin alone at the same concentrations. Evaluation of biofilm mass on beads was performed by bioluminescence assays, and the results were confirmed by SEM.

The enhancing activity of serratiopeptidase discussed above has not been reported previously, and its molecular mechanism is under investigation. In the experiments reported here, ofloxacin was used since quinolones are widely used in the treatment of prosthetic device infections, but the results of nonreported experiments show that serratiopeptidase enhances the activities of many different antibiotics. The reported mechanism seems to be the result of the activity of the protease on sessile bacteria rather than on the antibiotic or the medium.

Clinical studies on selected prosthetic device infections in humans are in progress to verify the effectiveness of a combined therapy with antibiotics and serratiopeptidase in both prophylactic and therapeutic protocols.

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