

Role of *Pseudomonas aeruginosa* Exoenzymes in Lung Infections of Patients with Cystic Fibrosis

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We investigated the role of *Pseudomonas aeruginosa* exoenzymes in cystic fibrosis lung infection in the presence and absence of specific serum antibodies. In sputa of 21 cystic fibrosis patients, concentrations of *P. aeruginosa* proteases and exotoxin A were determined by sensitive radioimmunoassays. In all sputa, detection of exoenzymes was negative (≤ 10 ng). Positive serum antibody titers to bacterial exoenzymes were found in the majority of patients. Purified immunoglobulin G (IgG) preparations from the sera of two patients revealing specific antibody titers to the bacterial proteases neutralized these enzymes at ratios of 1,000:1 to 5,600:1 (wt/wt). Above the neutralizing capacity of IgG, proteases caused cleavage of IgG; below that level, no enzymatic activity was observed. In vitro incubation of *P. aeruginosa* elastase, alkaline protease, or exotoxin A with elastase derived from polymorphonuclear leukocytes showed that polymorphonuclear leukocyte elastase: (i) was cleaved by bacterial elastase, (ii) was not inactivated by alkaline protease, and (iii) inactivated exotoxin A. The results suggest that soon after the onset of *P. aeruginosa* lung infection in cystic fibrosis patients, bacterial proteases, but not exotoxin A, become important virulence factors. The results also suggest that exoenzymes do not directly contribute to lung damage after immune response to bacterial antigens has begun.

Infection of the respiratory tract with *Pseudomonas aeruginosa* is often seen in patients with cystic fibrosis (11, 12, 46). The pathogenic events that contribute to a poorer prognosis for these patients compared with uninfected patients (12) are not fully understood. High proteolytic activity in sputa of these patients, originally reported by Lieberman et al. (25-27), may contribute to severe pulmonary damage. As a result of several studies, there is no doubt that this activity is mainly derived from polymorphonuclear leukocytes (PMNs) (25, 30, 42-44). Thus, lung damage in cystic fibrosis may partly be caused by endogenous proteases, for example, PMN elastase and cathepsin G (18, 39).

It is not yet clear, however, whether extracellular enzymes of *P. aeruginosa*, especially exotoxin A (14) and proteases (28, 32), contribute to lung damage in this disease. Indirect evidence of their in vivo production stems from the detection of specific antibodies in sera and sputa of cystic fibrosis patients (4, 7, 22, 37). However, detection of exotoxin A by direct means in sputa from patients was not accomplished until now, and attempts to detect proteases of *P. aeruginosa* directly in these samples by adding metalloprotease inhibitors were negative (42) or only slightly positive (43). In the latter investigation, the participation of a metalloprotease from alveolar macrophages was not excluded. In another study (7) with sensitive radioimmunoassays, detection of *P. aeruginosa* proteases was only successful in the absence of specific antibodies. Since antibody response to *P. aeruginosa* antigens starts 5 to 15 months after the onset of chronic infection (4), the hypothesis of a synergistic action of proteases from human and bacterial sources (42) may only be valid at the beginning of pulmonary infection. For the remaining major time of lung infection,

which may last 10 years or more, there is no in vivo proof of the presence of free *P. aeruginosa* proteases. Proteases of *P. aeruginosa* may therefore be neutralized by specific antibodies, giving rise to immune complexes. Indeed, immune complexes containing bacterial proteases were found in sputa of these patients (2). However, proof that these proteases were no longer enzymatically active in these immune complexes was lacking until now. Furthermore, nothing is known about whether PMN proteases which are present in high amounts in sputa from patients are able to inactivate bacterial exoenzymes.

Therefore, the aims of the present study were: (i) to investigate the occurrence of proteases and exotoxin A of *P. aeruginosa* in sputa of patients with cystic fibrosis, (ii) to determine antibody levels to these exoenzymes in sera from patients, (iii) to test the neutralizing activity of purified specific antibodies to proteases, and (iv) to investigate the interaction between *P. aeruginosa* exoenzymes with PMN elastase.

MATERIALS AND METHODS

Subjects, sera, and sputa. Sputum and serum samples were obtained from 21 cystic fibrosis patients attending the Cystic Fibrosis Clinic, Rigshospitalet, Copenhagen, Denmark, and the Fachklinik für Kinder und Jugendliche, Wangen im Allgäu, Federal Republic of Germany. Diagnosis of cystic fibrosis was based on accepted criteria (46), including a typical history of cystic fibrosis with markedly elevated sweat electrolyte levels in repeated tests and altered lung function. All patients suffered from chronic *P. aeruginosa* lung infection (11, 12). Sputum samples were solubilized with dithiothreitol as described previously (7). Sera and sputa were stored at -20°C .

Enzyme assays. Activity of *P. aeruginosa* alkaline protease and elastase in incubations with PMN elastase was deter-

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mined by azocasein assay (38). Incubation was carried out for 20 min at 30°C. One unit of enzyme was defined as the amount per milliliter which causes one optical density change per min at 430 nm. Activity of PMN elastase in incubations with *P. aeruginosa* exoenzymes was measured by photometric assay (34) with methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-*p*-nitroanilide (Bachem Feinbiochemika AG, Bubendorf, Switzerland) as a substrate. Reaction mixtures were incubated for 1 h at room temperature (22°C). Enzyme units were calculated from a standard curve of *p*-nitroaniline. One unit was defined as the release of 1 μ mol of *p*-nitroaniline per min per ml of reaction mixture at room temperature. Protein was estimated by the Lowry method (29) with bovine serum albumin as a standard. Exotoxin A activity was measured by the method of Collier and Kandel (1). Elongation factor 2 was purified from untreated wheat germ (Sigma AG, München, Federal Republic of Germany) as described by Iglewski and Sadoff (15). [14 C]NAD was purchased from Amersham, Braunschweig, Federal Republic of Germany. Incubation was carried out for 30 min at 24°C. One unit was defined as the counts per minute per minute of incubation and per milliliter of test solution. The nonspecific binding (NSB) of [14 C]NAD in the assay was 150 cpm. The limit of sensitivity of the assay was arbitrarily set at three times the NSB.

Radioimmunoassays. Detection of alkaline protease, elastase, and exotoxin A of *P. aeruginosa* in sputa from patients was accomplished by specific radioimmunoassays. The assays for detection of alkaline protease (6) and elastase (36) have been described previously. The newly developed assay for exotoxin A detection follows a similar procedure. Briefly, the solid phase of flexible polyvinyl chloride U-shaped plates was coated with purified rabbit immunoglobulin G (IgG) antibodies specific for exotoxin A. Procedures for raising antibodies to exotoxin A in rabbits and purification of the IgG fraction were the same as those described for raising antibodies to alkaline protease and elastase (6, 36). Highly purified exotoxin A (see below) was used for injections. The purified antibodies showed no cross-reaction with alkaline protease, elastase, or PMN elastase in the assay. After saturation of the solid phase with phosphate-buffered saline (pH 7.4) supplemented with 5% bovine serum albumin, 100 μ l of the test solutions were added to the washed wells and the plates were incubated for 12 h at 4°C. Serial dilutions (10-fold) of the purified exotoxin A in phosphate-buffered saline were used as a standard for quantitative measurements on each plate. After washing, 100 μ l of a solution of rabbit 125 I-IgG specific for exotoxin A was added to the wells and the plates were again incubated for 12 h at 4°C. Radiolabeling was performed as described previously (6). Finally, the bound radioactivity of the washed wells was measured with a gamma counter (Berthold GmbH, Wildbad, Federal Republic of Germany). The NSB of the tracer was in the range of 1,000 to 2,000 cpm. The exotoxin A standard curve was linear in the concentration range of 10^{-4} to 10^{-6} mg/ml. The lower level of sensitivity was set arbitrarily to three times the NSB. Titers of antibody to alkaline protease, elastase, and exotoxin A of *P. aeruginosa* in sera of cystic fibrosis patients were determined by radioimmunoassays which are modifications of the assays described above and have been previously described for antibodies to alkaline protease and elastase (7). The newly developed assay for detection of antibodies to exotoxin A follows a similar procedure.

Purification of *P. aeruginosa* exoenzymes, PMN elastase, and human IgG. Alkaline protease and elastase of *P. aeru-*

ginosa were purified from strains PA 78 and PA 92, which were originally isolated from the sputum of cystic fibrosis patients as described previously (36). Exotoxin A was purified from strain PA 103 (15, 24). The purified enzymes migrated as single bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% spacer gel and a 12% separating gel in the presence of β -mercaptoethanol (23). Molecular weights were 57,000 for alkaline protease, 39,000 for elastase, and 69,000 for exotoxin A. The specific activities of the proteases determined by azocasein assay were 0.43 U/mg for alkaline protease and 0.61 U/mg for elastase, and 16.5×10^7 U/mg for exotoxin A, measured by the assay of Collier and Kandel. PMN elastase was purified from 500 ml of pooled sputum of cystic fibrosis patients as described by Martodam et al. (30). Purity of the enzyme was checked by SDS-PAGE. Bands of isoenzymes were detected between 25,000 and 30,000. The specific activity of the purified enzyme was 243.2 U/mg, measured with the above-mentioned peptide nitroanilide substrate. No activity of cathepsin G was found in this preparation with *N*-succinyl-L-phenylalanyl-L-leucyl-L-phenylalanine-*p*-nitroanilide (Bachem) as a substrate (45). Serum IgG from two patients with cystic fibrosis was purified separately from 20 ml of serum by affinity chromatography (10). Purity of the IgG preparations was checked by immunoelectrophoresis (9) with polyspecific rabbit anti-human serum protein and monospecific rabbit anti-human IgG (Boehringer Ingelheim, München, Federal Republic of Germany), as well as with SDS-PAGE. The antibody titers to alkaline protease and elastase of the two IgG preparations were determined by radioimmunoassay (7).

Neutralization of alkaline protease and elastase by IgGs. One milliliter each of the purified IgG preparations, no. 1 (4.1 mg/ml) and no. 2 (4.2 mg/ml) from two patients with cystic fibrosis, was incubated with various concentrations of alkaline protease and elastase (10, 1, and 0.1 μ g) for 3 h at 37°C. Afterward, the reaction mixtures were analyzed by SDS-PAGE and radioimmunoassay for detection of alkaline protease and elastase.

Recovery of exoenzymes from pooled sputum. To 1 ml of pooled sputum from 10 patients with cystic fibrosis which showed low antibody titers to alkaline protease, elastase, and exotoxin A, 100 μ l each of alkaline protease, elastase, and exotoxin A was added at various concentrations (10^{-1} to 10^{-6} mg/ml). Incubation was carried out for 3 h at 37°C. Afterward, recovery of exoenzymes was checked by radioimmunoassay for detection of the exoenzymes.

Incubation of *P. aeruginosa* exoenzymes with PMN elastase. *P. aeruginosa* alkaline protease and elastase (1 mg/ml) were incubated with purified human PMN elastase (2 mg/ml) to yield bacterial protease-PMN elastase ratios (wt/wt) of 10:1, 7.5:1, 5:1, 2.5:1, 1:1, 0.1:1, 0.05:1, and 0.001:1. Incubation was carried out for 3 h at 37°C. Reaction mixtures were analyzed by azocasein assay. This substrate was cleaved only by the *P. aeruginosa* proteases, and not by PMN elastase, under the present experimental conditions. Since the lower sensitivity limit of the assay was 1 μ g of alkaline protease or elastase, even lower concentrations of the respective enzymes were analyzed by radioimmunoassay. PMN elastase activity in incubation mixtures was analyzed by photometric assay (34). This substrate was not cleaved by the *P. aeruginosa* proteases. Exotoxin A (0.18 mg/ml) was incubated with PMN elastase (2.7 mg/ml) to yield PMN elastase-exotoxin A ratios (wt/wt) of 100:1, 50:1, 10:1, 2:1, 1:1, 0.1:1, and 0.01:1. Incubation was carried out for 3 h at 37°C. A sample of the 2:1 incubation mixture was then

subjected to SDS-PAGE. The reaction was stopped by adding a molar excess of soybean trypsin inhibitor. Control experiments revealed that PMN elastase was 100% inactivated by this amount of inhibitor. The activity of exotoxin A was then determined by the described assay (1).

RESULTS

The examination of sputa of cystic fibrosis patients for the occurrence of extracellular enzymes of *P. aeruginosa* is an important step toward understanding the mechanisms of pathogenicity in this disease. The results of tests on 21 sputa from patients with chronic *P. aeruginosa* lung infections are shown in Table 1. The concentrations of *P. aeruginosa* alkaline protease, elastase, and exotoxin A were determined by sensitive radioimmunoassays. These enzymes were not found in the sputa of any of the patients. To determine whether the sputum material inhibits the detection of the exoenzymes by nonspecific adsorption and thus leads to negative detection, various concentrations of alkaline protease, elastase, and exotoxin A were added to a sputum pool in vitro and the recovery of the enzymes was determined after 3 h of incubation. No significant reduction in enzyme detection was seen when enzyme amounts ranging from 10 to 0.5 µg were added to sputum. Between 500 and 10 ng, enzyme detection was reduced; below this concentration, detection of the enzymes was negative. This control experiment suggests that in exoenzyme-negative sputa, up to 10 ng of enzyme may be present, which is not detectable by radioimmunoassay. However, a more likely explanation of the reduction in enzyme detection from the sputum pool are the low but significant levels of free antibodies positive to the

TABLE 1. Reciprocal titers of antibodies to alkaline protease, elastase, and exotoxin A of *P. aeruginosa* in sera of 21 patients with cystic fibrosis

Patient no.	Reciprocal titer of serum antibodies to ^a :		
	AP ^b	Ela ^c	ExoA ^d
1	13	31	
2	55	22	2
3		18	
4	29	330	43
5	45	13	
6	22	22	12
7	20		135
8	295	270	80
9	25	49	12
10	64	61	16
11	90	36	
12	18		21
13	135		
14	180	20	
15	5	55	9
16	47	65	68
17	47	54	79
18	47	45	
19	50	135	
20	620	240	36
21	530	135	

^a Serum antibody titers were determined by radioimmunoassay (7); for details, see the text. Empty cells indicate a lack of detectable antibody levels in serum (>75% of the maximal binding of the tracer in the radioimmunoassays). Antibody titers are the means of three determinations; the standard deviation was <20% of the mean value.

^b AP, Alkaline protease.

^c Ela, Elastase.

^d ExoA, Exotoxin A.

TABLE 2. Neutralization of alkaline protease and elastase of *P. aeruginosa* by IgG preparations of two patients with cystic fibrosis^a

IgG preparation	Reciprocal titer of antibody to:		Protein concn (mg/ml)	100% neutralization (µg) of:	
	AP ^b	Ela ^c		AP ^b	Ela ^c
1	500	72	4.1	4.1	1.0
2	100	400	4.2	0.74	2.7

^a IgG preparations were purified by affinity chromatography (10). Titers of antibody to alkaline protease and elastase were determined by radioimmunoassay (7). Values represent the means of three determinations. The standard deviation was <20% of the mean. For determinations of the enzyme amounts which were 100% neutralized by the IgG preparations, 1 ml of each purified IgG preparation was incubated with 10, 1, and 0.1 µg of alkaline protease and elastase for 3 h at 37°C. The reaction mixtures were analyzed by radioimmunoassay for detection of free alkaline protease or elastase (6, 35). The intersection of the alkaline protease and elastase titration curves with the level of NSB of the tracer gave the enzyme concentration that was 100% neutralized.

^b AP, Alkaline protease.

^c Ela, Elastase.

exoenzymes in this pool (anti-alkaline protease, 1:1.1; anti-elastase, 1:2.5; anti-exotoxin A, 1:2.4).

To investigate the hypothesis that negative exoenzyme detection is due to the occurrence of specific antibodies in the sera of patients, which may reach the inflamed lungs by passive transudation and neutralize the exoenzymes, we determined the serum antibody titers to alkaline protease, elastase, and exotoxin A of these patients (Table 1). The majority of patients had antibody titers to one or more of the enzymes. Twenty patients had antibody titers to alkaline protease ranging from 1:5 to 1:620, 18 patients had titers to elastase ranging from 1:13 to 1:330, and 12 patients had titers to exotoxin A ranging from 1:2 to 1:135.

After demonstration of the presence of potential neutralizing antibodies in these patients, we purified the serum IgG

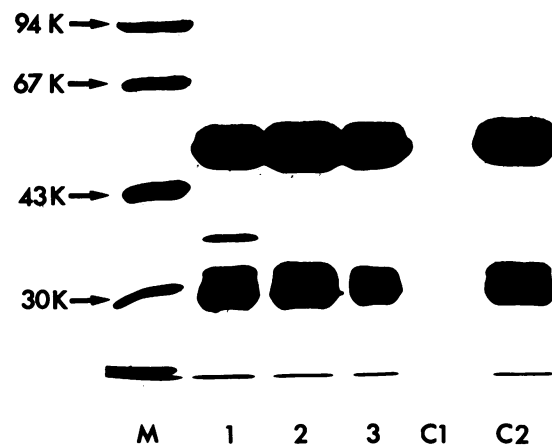


FIG. 1. Incubation of IgG preparations from a cystic fibrosis patient with various concentrations of *P. aeruginosa* alkaline protease and elastase. For SDS-PAGE, 4.1 mg of IgG was incubated with 10, 1, or 0.1 µg of alkaline protease and elastase (lanes 1 through 3, respectively). Electrophoresis was carried out by the method of Laemmli (23) with a 4% spacer gel and a 12% separating gel in the presence of β-mercaptoethanol. Lane M, marker proteins: phosphorylase b (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), and soybean trypsin inhibitor (20.1K). C1, enzyme control (10 µg of alkaline protease and elastase without IgG). C2, IgG control (4.1 mg of IgG without enzymes).

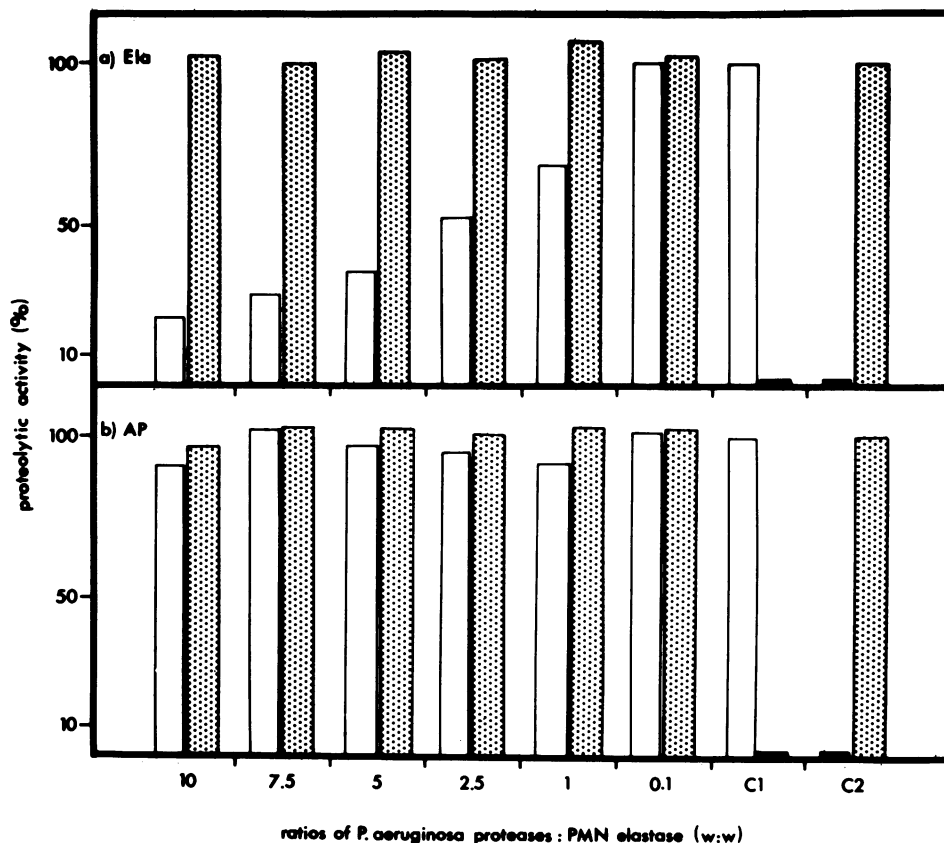


FIG. 2. Incubation of elastase (a) or alkaline protease (b) of *P. aeruginosa* with elastase derived from PMNs. Equal concentrations of PMN elastase were incubated with decreasing concentrations of alkaline protease and elastase for 3 h at 37°C. Proteolytic activities of bacterial proteases were measured by azocasein assay (38); proteolytic activities of PMN elastase were measured by photometric assay (34). Activities are given as the percentages of the respective controls. Values represent the means of three determinations. The standard deviation was less than 5% of the mean. ▨, Alkaline protease or elastase activity measured by azocasein assay. □, PMN elastase activity measured by photometric assay. C1, Activity of PMN elastase alone in the photometric and azocasein assays. C2, Activity of alkaline protease or elastase alone in the photometric and azocasein assays.

fractions of two patients and incubated the preparations with different concentrations of purified proteases. Titration was analyzed by radioimmunoassay and revealed that microgram amounts of bacterial proteases were neutralized by milligram amounts of total IgGs (Table 2). SDS-PAGE analysis of the incubation of IgG preparation no. 1 with the proteases showed that IgG was partly cleaved when more than the maximal amount of protease which can be neutralized was added to the IgG (Fig. 1, lane 1). However, no cleavage products of IgG were detected after incubation of the preparation with smaller amounts of proteases than the maximal neutralization capacity of the IgG (lanes 2 and 3). This shows that the *P. aeruginosa* proteases were no longer enzymatically active when sufficient antibodies were present to bind all of the proteases in immune complexes. Analysis of the other IgG preparation after incubation with the bacterial proteases gave the same results (data not shown). It may therefore be concluded that, after onset of the immune response, *P. aeruginosa* enzymes are bound by specific human antibodies to immune complexes and subsequently play no important role in the pathogenesis of lung infection in cystic fibrosis patients.

In the beginning of *P. aeruginosa* lung infection, however, specific antibodies to bacterial antigens are not present and exoenzymes may conceivably be directly involved in pathogenicity. As shown in a previous study (7), alkaline protease

and elastase were detectable in nanogram amounts in bronchial secretions of cystic fibrosis patients in the absence of specific antibodies to the enzymes. An interaction may occur in this early phase of lung infection between bacterial exoenzymes and proteases released from PMNs, which are present in large amounts. To study this interaction, we incubated *P. aeruginosa* exoenzymes with purified PMN elastase. PMN elastase had no inactivating effect on either bacterial protease at any incubation ratio (Fig. 2). The azocasein assay, which is specific for alkaline protease and elastase under the described experimental conditions, revealed 100% activity of both proteases after the incubation period. At bacterial protease-PMN elastase incubation ratios of 0.05 and 0.01, incubation mixtures were analyzed by radioimmunoassay; these assays also revealed 100% activity of alkaline protease and elastase (data not shown). In contrast, the enzymatic activity of PMN elastase measured with a specific substrate was markedly reduced when an excess of *P. aeruginosa* elastase was incubated with PMN elastase (Fig. 2a). Alkaline protease had no inactivating effect on PMN elastase at any ratio (Fig. 2b).

Figure 3 shows the result of incubation of PMN elastase with exotoxin A. At PMN elastase-exotoxin A ratios of 100:1 to 2:1, 100% of the toxin was inactivated, as shown by NAD ribosylation assay. At lower ratios, the cleavage effect of PMN elastase on the toxin decreased, and 100% activity of

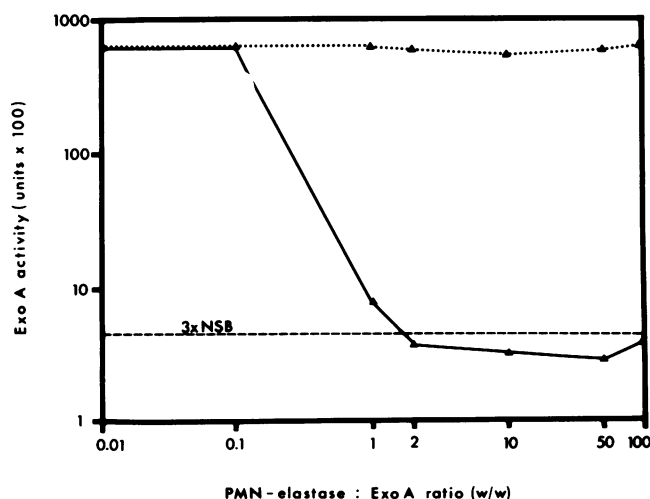


FIG. 3. Incubation of exotoxin A (ExoA) of *P. aeruginosa* with elastase derived from PMNs. ExoA was incubated with PMN elastase at different ratios (wt/wt) for 3 h at 37°C. The protease was inactivated by adding soybean trypsin inhibitor, and ExoA activity was determined as described by Collier and Kandel (1). Values represent the means of three determinations. The standard deviation was less than 20% of the means. ····, ExoA control without PMN elastase. —, ExoA incubated with PMN elastase. The limit of sensitivity of the assay was arbitrarily set at three times the NSB of the NAD used in the assay procedure.

the toxin was seen at a ratio of 0.1:1. Since PMN elastase was totally inhibited by soybean trypsin inhibitor after incubation with exotoxin A, a possible influence of the protease on the assay system was excluded. The enzymatic inactivation of exotoxin A was due to cleavage into small fragments by PMN elastase, as demonstrated by SDS-PAGE analysis of the reaction. The 69-kilodalton band of the toxin was totally absent in the reaction mixture, and only fragments of 20 kilodaltons or less were detected in the gel (not shown).

DISCUSSION

Lung infection with *P. aeruginosa*, which occurs in the majority of patients with cystic fibrosis, may be divided into two stages which may be totally different with regard to pathogenicity. In the early stage, after the onset of lung infection, proteases of *P. aeruginosa* may contribute to the persistence of the organism by impairing the antimicrobial defense system through cleavage of immunoglobulins (3, 5) and complement components (41), as well as inactivation of phagocytic PMNs by inhibition of chemotaxis (20) and chemoluminescence (21). Furthermore, the main protease inhibitors of the lung, α -1-antitrypsin and bronchial mucosal inhibitor, are inactivated by *P. aeruginosa* elastase (19, 33), and α -2-macroglobulin, the only effective inhibitor of this enzyme, is normally absent, or if present, exists in only very low concentrations in the lung (8, 13, 43).

In the present study, an additional potentially pathogenic effect of *P. aeruginosa* elastase is revealed by the considerable inhibition of PMN elastase observed in vitro. However, it must be considered that this and other pathogenic effects markedly depend on the in vivo concentrations of bacterial enzymes, as well as human enzymes and cells. PMN elastase is only cleaved by *P. aeruginosa* elastase if the latter is present in molar excess or in equivalent molar concentrations with respect to PMN elastase. On the other hand, *P.*

aeruginosa exotoxin A is cleaved by PMN elastase if the protease is present in excess or in equivalent concentrations to the toxin. Thus, to extrapolate the present in vitro results to the in vivo situation, further efforts should be made to determine concentrations of *P. aeruginosa* proteases and exotoxin A, as well as that of PMN elastase, in the initial phase of lung infection.

There is now experimental proof that in the second phase of *P. aeruginosa* lung infection in cystic fibrosis patients—which is characterized by the occurrence of high PMN counts (43) and increasing antibody titers to many *P. aeruginosa* antigens, including proteases and exotoxin A (4, 7, 22)—bacterial proteases and exotoxin A are no longer present as free enzymes. Microgram amounts of proteases are neutralized by specific antibodies in titers commonly found in sera of cystic fibrosis patients. These serum IgG antibodies can reach the infected lungs of patients by passive transudation as previously shown by Schiøtz et al. (40). Additional neutralization may occur in vivo through IgA antibodies of the local secretory immune system. The observation that free antibodies specific for *P. aeruginosa* proteases are detectable in several sputa of cystic fibrosis patients even suggests that a surplus of antibodies over enzymes is present (2). This hypothesis of effective neutralization of bacterial virulence factors by the human immune system is further substantiated by the studies of Jacquot et al., who showed that the concentrations of human airway lysozyme, a substrate for *P. aeruginosa* elastase in vitro (17), were not significantly different in cystic fibrosis sputa with various levels of *P. aeruginosa* infection (16). From this and other studies therefore, it is rather unlikely that *P. aeruginosa* proteases or exotoxin A are directly involved in lung damage in the second phase of lung infection. It appears to be more likely that high amounts of immune complexes in cystic fibrosis sputa, some of which incorporate *P. aeruginosa* proteases as antigens (2), continuously stimulate the excretion of PMN proteases, which, in turn, may be responsible to a great extent for tissue damage in such patients (26, 30, 42–44).

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