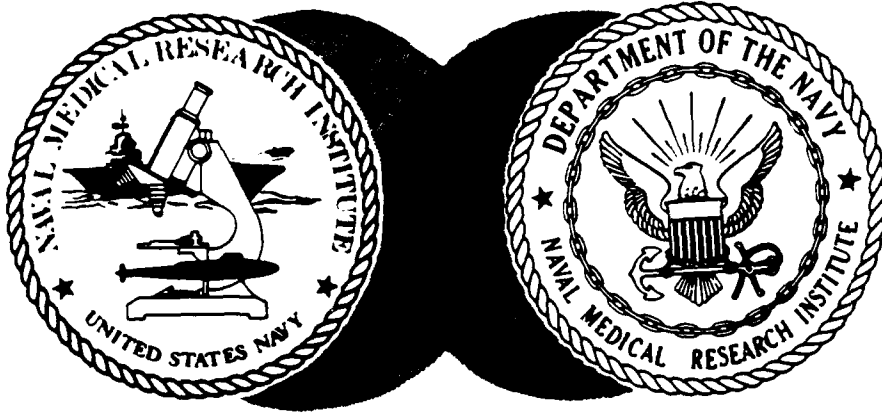


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THE ROLE OF PROTEASES AND EXOTOXIN A
IN THE PATHOGENICITY OF PSEUDOMONAS
AERUGINOSA INFECTIONS

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The Role of Proteases and Exotoxin A in the Pathogenicity of *Pseudomonas aeruginosa* Infections

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ABSTRACT. Most *Pseudomonas aeruginosa* strains produce exotoxin A and two extracellular proteases (elastase and alkaline protease). Exotoxin A is a lethal toxin that inhibits protein synthesis in mammalian cells by the same mechanism as diphtheria toxin. It is generated in clinical and experimental animal infections. Passive or active immunization against this toxin gives significant protection against experimental infections with exotoxin-producing strains. The proteases have tissue-damaging activity and are capable of degrading various plasma proteins such as complement and coagulation factors. Proteases probably play a part in localized pseudomonas infections such as keratitis, pneumonia and burn infection. When invasion and colonization have occurred and septicemia is established, these enzymes probably are less important.

INTRODUCTION

The pathogenicity basis of *Pseudomonas aeruginosa* is still not clearly defined. Several extracellular products such as proteases, phospholipase, hemolysin, exotoxin A and exoenzyme S have been established or implicated as virulence factors (43, 3). The relative contribution of these factors versus cell-bound components, e.g. endotoxin, slime polysaccharide (14, 69) to the virulence is poorly understood. The purpose of this review is to describe the properties of exotoxin A and extracellular proteases produced by *P. aeruginosa* and to evaluate their possible role in various types of infections.

EXOTOXIN A

Liu et al. (41, 46) found and purified a lethal toxin from *P. aeruginosa*. This toxin—exotoxin A—was subsequently purified to homogeneity and characterized (7, 8, 38, 76). A detailed description of purification and assay methods has recently been published (22). The toxin has a molecular weight of 71 500 daltons and an isoelectric point of 5.0. Its mechanism of action is the same as that of diphtheria toxin, although the two toxins are serologically distinct and have different amino acid composition and cell receptors (20, 38, 79). Exotoxin A is produced by 90% of clinical *P. aeruginosa* strains (5). Its formation in vitro is influenced by a number of factors, such as iron content of the medium (4, 11, 42).

Exotoxin A is produced as a proenzyme which is converted to active enzyme by the action of reducing or denaturing compounds (39). The activated enzyme catalyzes the transfer of the adenosine diphosphate ribose moiety from nicotinamide adenine dinucleotide to a ribosomal protein in eukaryotic cells—elongation factor 2 (EF-2). This process inactivates EF-2 and halts protein synthesis (20). The activated enzyme is less toxic for animals or cell cultures than is the proenzyme.

Role of exotoxin A in infections

Exotoxin A has been shown to be the most lethal product of *P. aeruginosa* (1, 8, 38, 43, 45), the mean lethal dose (LD₅₀) being 60 to 80 ng per 20 g mouse (8). The exotoxin is produced in vivo in clinical infections (63, 64, 75) as well as in experimental animal infections (58, 59, 66, 72, 74). Purified exotoxin A markedly affected the liver when injected into mice (61). The microscopic liver lesions following a single injection of 2 LD₅₀ of exotoxin A were characterized by necrosis, cellular swelling

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The animal experiments in our laboratories were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institutes of Laboratory Resources, U.S. National Research Council, DHEW, Pub. No. (NIH) 78-23.

and fatty change within 4 to 8 h and near total hepatocellular necrosis at 48 h. Similar changes have been seen in the liver of mice infected with *P. aeruginosa* (80). Haemorrhagic lungs and necrotic kidneys were further observed in mice injected with exotoxin A (43).

A 50% inhibition of protein synthesis in the liver was found within 4 h after intravenous injection of exotoxin A (2 LD₅₀) into mice (60). Subsequent studies showed in vitro (20) and in vivo (21) that the toxin catalyzes ribosylation of the ribosomal protein EF-2, resulting in inhibition of protein synthesis.

The role of the toxin in *P. aeruginosa* infections has been indicated by significant inactivation of EF-2 in the organs of mice experimentally infected with *P. aeruginosa* (58, 66, 72). A mouse burn-infection model mimicking clinical infection was used to study the role of exotoxin A in experimental infections (59, 73). Organs from mice infected with the toxigenic *P. aeruginosa* strain PA 103 displayed considerably less EF-2 activity than did organs from uninfected controls or from mice infected with a non-toxigenic strain. Though EF-2 activity was reduced in all the examined organs from the PA 103 infected mice, the greatest decrease was found in the liver, where the levels of active EF-2 were reduced by 70 to 90%. Treatment of mice with monospecific antitoxin prior to the infection prevented inactivation of EF-2. Protein synthesis was consistently inhibited in the liver, but not in other organs, of mice infected with toxigenic *P. aeruginosa* strains.

The role of exotoxin A has also been illustrated by the protective action of intravenously administered rabbit antitoxin in experimentally infected, traumatized mice. Subcutaneous injections of viable *P. aeruginosa* into a small, non-lethal burn area resulted in these experiments in a lethal infection associated with bacteremia and systemic invasion (59). All of the employed strains produced a lethal infection at a strain-characteristic dose. Different levels of protection were conferred by antitoxin serum on groups of mice infected with different toxigenic strains. After infection with 2 LD₅₀ of toxigenic, low protease-producing strain (PA 103), survival was considerably enhanced in antitoxin-treated mice (100% survival) as compared with controls that had received anti-bovine serum albumin serum ($p=0.0004$). With other toxigenic, protease-producing strains (PA 86, PA 220) there was

likewise significant increase in survival time, though not in survival rate ($p=0.0003$, $p=0.01$, respectively). By contrast, antitoxin had no protective effect in mice infected with lethal doses of a non-toxigenic strain.

The different levels of survival found in antitoxin-treated, infected mice may reflect the different biological and biochemical properties of the micro-organisms. PA 103 was serum sensitive and, as mentioned, produced only trace amounts of proteases, whereas PA 86 and PA 220 were serum-resistant protease producers. Antiserum to exotoxin A has also been raised in ponies and shown to provide protection against toxigenic *P. aeruginosa* infections in mice (45).

The importance of exotoxin A for the pathogenicity of the micro-organism was further demonstrated in mice immunized with a toxoid prepared from this exotoxin (72, 40, Pavlovskis & co-workers, unpublished results). Survival time was prolonged in mice immunized with either glutaraldehyde or formalin-prepared toxoids.

Another relevant point is that serum antibodies to exotoxin A in septicemic patients can be correlated with a protective effect (65), indicating a role of exotoxin A in clinical *P. aeruginosa* infections.

The available data thus indicate that exotoxin A is an important pathogenic factor in *P. aeruginosa* infections and that its effects after in vivo release can be blocked by passive immunization with antitoxin and possibly also by active immunization with biologically inactive forms of exotoxin A.

PROTEASES

P. aeruginosa strains usually produce more than one extracellular protease. A protease with elastolytic activity (elastase) and an alkaline protease have been purified and characterized by several investigators.

Elastase

More than 85% of *P. aeruginosa* strains produce a protease with elastase activity. This elastase can be separated from other proteases by ion exchange chromatography or isoelectric focusing, and it can be purified by precipitation with ammonium sulfate or gel chromatography (53, 36, 82, 24). More recently, affinity chromatographic methods have also been used (50, 56). The isoelectric point is 5.7-6.6 and the protease shows marked heterogeneity, with

multiple forms differing in charge properties, probably as a result of autodigestion (82, 67). The molecular weight has been reported as 39 500 (53), 23 000 (36, 67, 82) or 33 000 (35), depending on the method used. The reason for this discrepancy is not known.

The elastase is a neutral proteinase containing zinc. It is active against casein, elastin, hemoglobin and other proteins, but not against collagen (47, 82, 53, 67, 2, 35). It has specificity for hydrophobic and bulky amino acids at the amino side of the splitting point (49, 56). Plasma α_2 -macroglobulin and phosphoramidon inhibit the enzyme (52, 56, 16). Elastase is produced as an inactive proenzyme which is activated by limited proteolysis, either by other proteases or by the elastase itself (23).

Alkaline protease

Most stains of *P. aeruginosa* produce alkaline protease, but its formation is repressed in media containing free amino acids (47, 82, 20). The enzyme has been purified (48). Its isoelectric point is 4.1–4.5. A molecular weight of 48 400 has been reported (51), though other investigators have found values around 20 000 (36; B. Wretlind, unpublished data). The enzyme has broad specificity, which is determined by amino acids distant from the splitting point rather by those on either side of it (49). The protease contains 1–2 atoms of calcium per molecule, but cobalt ions are more effective in promoting hydrolysis.

Biological properties of pseudomonas proteases

The toxicity of proteases is low in comparison with bacterial toxins such as exotoxin A. The mean lethal dose of elastase for mice varies from 60 to 400 μ g, depending on the route of inoculation—intravenous, intraperitoneal, intrapulmonary (30, 82). The lethal effect is probably produced by hemorrhages in internal organs. The elastase is inactivated by the serum protease inhibitor α_2 -macroglobulin, which may explain the low toxicity.

Leake et al. (37) demonstrated cytotoxic effects of proteases, with agglutination and vacuolization of rabbit alveolar macrophages but no significant decrease in cell viability. Wretlind & Wadström (82) found no damage to cell membranes of HeLa cells or human fibroblasts treated with elastase or alkaline protease. Morphological changes similar to those produced by trypsin were detected, however. In *pseudomonas* infections, therefore, the cytotoxic

activity of proteases is probably less important than their effects on intercellular substance.

Mull & Callahan (54) suggested that the destruction of arterial elastic lamina in the vasculitis associated with *pseudomonas* septicemia is caused by elastase. In experimental models, intrapulmonary or intrapleural injection of 10–100 μ g purified proteases caused lung damage with hemorrhages and necrosis of alveolar septal cells, indicating a role for proteases in *pseudomonas* pneumonia (30, 13, 71). The hemorrhages probably arose from degradation of fibrin and other coagulation factors. Elastase and alkaline protease produce dermonecrosis after injection of less than 10 μ g enzyme, which suggests that they may play a part in skin and wound infections (82, 26).

Kreger & Griffin (36) found three cornea-damaging proteases in culture supernatant from a *P. aeruginosa* strain. Less than 1 μ g of protease caused corneal damage. Light and electron microscopic examination of elastase-treated rabbit cornea showed degradation of the proteoglycan, but not of collagen fibrils (6, 25, 31, 35).

Schulz & Miller (68) reported that elastase inactivated several complement factors (C1, C3, C5, C8 and C9) in vitro. If such inactivation occurs also in vivo, it would explain the lack of inflammatory response in *pseudomonas* vasculitis.

Inactivation of human α_1 -proteinase inhibitor by *pseudomonas* elastase has also been demonstrated (52). If this applies to in vivo conditions during infections, the loss of the protease inhibitor could permit serine proteases from leukocytes to cause tissue damage.

Role of pseudomonas proteases in infections

P. aeruginosa infections of the human cornea are not common, but they usually result in loss of vision in the infected eye. Various investigations have implicated *pseudomonas* proteases in such infections. The proteoglycan ground substance of the cornea is dissolved, resulting in dispersal of undamaged collagen fibrils (12). Similar observations were made after intracorneal injection of *pseudomonas* proteases (31, 35). Two protease-producing strains were found by Kawaharajo & Homma (27) to cause severe keratitis in mice, whereas two protease-negative strains did not damage the cornea. Passive or active immunization against elastase, alkaline protease and endotoxin protein (OEP) protected against experimental eye infection (15, 28). Other

studies have indicated a role also for host-derived proteases and collagenase in pseudomonas keratitis (32, 78).

Pseudomonas pneumonia is characterized by intra-alveolar hemorrhage, necrosis of alveolar cells and mononuclear cell infiltration. These changes suggest protease-induced damage (13, 71, 57, 77). A vaccine containing toxoids of pseudomonas elastase, alkaline protease and OEP had a protective effect against hemorrhagic pneumonia in mink (17). That patients with pseudomonas pneumonia or cystic fibrosis have serum antibodies against elastase and alkaline protease is evidence that these enzymes are produced during infections (18, 33).

Conflicting results have been reported regarding the role of proteases in experimental septicemia in mice. Kobayashi (34) found no relationship between protease production and virulence, but Muszynski & Kedzia (55) reported a positive correlation. However, Liu & Hsieh (44) found that protease-producing strains were less virulent than protease-deficient strains. Wretlind & Kronevi (80) studied the virulence of protease-deficient mutants in mice. These mutants were derived from a protease-producing clinical strains (PAKS-1). The mutants were defective in the formation of extracellular proteases, lipase and staphylolytic enzyme. Some of the mutants produced less exotoxin A than did the wild type strain (81). The bacteria were intraperitoneally or intravenously injected into cyclophosphamide-treated mice. No significant difference was found between LD₅₀ for the wild-type strain and for six out of eleven protease-deficient mutants. These results indicate that proteases are of little importance in experimental septicemia. Histologic examination showed fatty infiltration and focal necrosis of the liver, suggesting exotoxin A-induced damage.

Sezen et al. (70) observed some small protective effect after active immunization of mice against elastase in experimental pseudomonas septicemia. Antiserum against elastase gave no protection.

Kawaharajo & Homma (29) showed that immunization against elastase was protective in mouse burn infection. Snell et al. (72) and Holder & Haidaris (16) reported that injection of 10 µg elastase, alkaline protease or *Bacillus thermoproteolyticus* protease (thermolysin) together with a protease-deficient strain (PA 103) resulted in a thousand-fold reduction of LD₅₀ values as compared with controls injected with bacteria only.

This effect was specific for strains of *P. aeruginosa*. The serum protease inhibitor α₂-macroglobulin elicited a significant protection against protease-positive strains after injection at the burn site.

Pavlovskis & Wretlind (62) studied the role of proteases in mouse-burn infection, using two protease-deficient mutants of the protease-producing strain PAKS-1. The virulence of the mutants was equal to that of the wild type strain in cyclophosphamide-treated mice (log LD₅₀=6.1-6.4). In the burned-mouse model, however, there was a ten-fold difference in LD₅₀ between PAKS-1 and its protease-deficient mutants (log LD₅₀: PAKS-1=3.8; mutants 4.8-4.9; *p*<0.02). Passive immunization with a specific anti-elastase serum protected significantly against the wild type strain, but not against the mutants. Injections of purified elastase (5-45 µg) together with either of the mutants reduced the survival time and the number of surviving mice. Anti-elastase serum gave limited protection when 5-10 µg of elastase was injected. The results indicated that the elastase produced by strain PAKS-1 contributed to the invasiveness of the micro-organism.

According to the above-cited studies, proteases—especially elastase—contribute to the invasiveness of *P. aeruginosa* in experimental burn infections. The role of these enzymes in the septicemia model probably is negligible, which indicates that proteases exert their effect before the bacteria have invaded the blood stream. Cicmanec & Holder (9) suggested that the proteases supply nutrients to the bacteria in the burned area by degrading proteins in the burned skin. Proteases are tissue-damaging and may also destroy anatomical barriers and facilitate spread of micro-organisms from the port of entry. Once septicemia is established, the role of proteases as virulence factors decreases, whereas other factors such as endotoxin become more important.

CONCLUSIONS

Most strains of *P. aeruginosa* produce exotoxin A and at least two extracellular proteases with broad substrate specificities. Exotoxin A is a lethal protein that inhibits protein synthesis in mammalian cells via ribosylation of a ribosomal protein (EF-2). The greatest toxic effect in mice occurs in the liver. The toxin is produced in vivo during infections, and antibodies against the protein have a protective ac-

tion. Proteases are also toxic to various tissues and are capable of degrading complement and coagulation factors. Good evidence has been presented that proteases play a significant part in the pathogenicity of *P. aeruginosa* in keratitis, pneumonia and burn infections. The presented data indicate that active or passive immunization against these proteins may be beneficial to susceptible patients.

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