EXPERIMENTAL INFECTIONS WITH PROTEASE-DEFICIENT MUTANTS OF *PSEUDOMONAS AERUGINOSA* IN MICE

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PLATE VIII

THE role of extracellular enzymes such as proteases and lecithinase in the pathogenesis of *Pseudomonas aeruginosa* infection has been the subject of several reports (Mull and Callahan, 1965; Lui, 1974).

Muszyński (1973) found a correlation between protease production in broth cultures and virulence in mice for strains of *P. aeruginosa*, but Kobayashi (1971) found no relation between formation of extracellular products and virulence in experimental mouse infections. Neither Hedberg, Miller and Tompkins (1969) nor Wretlind *et al.* (1973) found any difference in production of extracellular elastase and other extracellular enzymes between strains from bacteraemic infections and those from other sources. However, two studies showed that pseudomonas strains isolated from patients with septicaemia produced more elastase than other strains (Baltch, Griffin and Malatino, 1975; Davis and Doubek, 1975).

The purpose of the present work was to investigate the role of extracellular proteases in experimental *P. aeruginosa* infections. A direct approach to the study of bacterial products is to study the purified enzymes and toxins *in vitro*, in tissue cultures and in animal models (Meinke *et al.*, 1970; Kawaharajo *et al.*, 1975a; Pavlovskis, Voelker and Shackelford, 1976). However, high and unphysiological concentrations of the products have sometimes been employed and this practice is liable to give an exaggerated view of the importance of the products in infections. The role of individual characteristics of micro-organisms can also be analysed if mutants defective in these characteristics are used (Forsgren, 1972). A number of pseudomonas mutants selected for loss of extracellular protease activity have been isolated and their characteristics are described elsewhere (Wretlind, Sjöberg and Wadström, 1977). In this paper we report the results of animal-infection experiments with mutants of a protease-producing strain of *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. *Pseudomonas aeruginosa* strain PAKS-1 was isolated from a urine specimen in 1970 (Wretlind *et al.*, 1973). The strain produced (Wretlind and Wadström, 1977) in broth cultures three proteases corresponding to those described by Morihara (1964)

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and Kreger and Griffin (1974). One of these proteases (protease II), possessing elastase activity, was responsible for more than 75% of the extracellular protease activity. The strain also produced extracellular staphylolytic enzyme, lipase and lecithinase. Mutants were isolated after treatment of strain PAKS-1 with a mutagen (ethyl methane sulphonate), but strain PAKS-10 was a spontaneous protease-deficient mutant.

Table I summarises some phenotypic properties of the strains. One group of protease-deficient mutants (class I), containing strains PAKS-14, PAKS-18 and PAKS-31, had a high activity of cell-bound alkaline phosphatase and staphylolytic enzyme in contrast to the wild-type parent strain, possibly indicating mutations affecting the cell envelope structure and function with decreased ability to release extracellular enzymes. Strains PAKS-16 and PAKS-17 probably also belonged to this group, although PAKS-16 produced extracellular lipase in contrast to the other class I mutants, and PAKS-17 lacked cell-bound staphylolytic enzyme. In class II mutants less than one-third of the total alkaline phosphatase and the staphylolytic enzyme was cell bound. Only one mutant, PAKS-20, possessed a significant amount of cell-bound protease activity. In some of the strains mutation may have affected a common regulatory mechanism for extracellular enzymes (Wretlind *et al.*, 1977).

All strains were prototrophic. The generation time of the mutants in broth cultures was close to that of the wild type (33 min.) except for PAKS-19 (41 min.) and PAKS-23 (42 min.).

Preparation of inoculum. The strains were cultured in 100 ml Brain Heart Infusion Broth (Difco) in 1-litre flasks on a shaking table at 37°C for 16 h. The culture was centrifuged (4000 *g*, 10 min.) and the cell pellet suspended in sodium phosphate-buffered saline (PBS). Dilutions were made in PBS and stored in an ice bath until used. Growth was monitored by viable count.

Animals. Female Swiss white mice of the NMRI strain, weighing 16–18 g, were used for all experiments.

Virulence tests. The degree of virulence for mice was measured by three methods. (1) Intraperitoneal (i.p.) injections of bacterial suspensions with 1% (w/v) mucin (Sigma Chemical Co., St Louis, Mo., USA) were made. (2) Intravenous (i.v.) injections of bacteria were made 4 days after pretreatment of the mice with cyclophosphamide (Pharmacia, Uppsala, Sweden). Cyclophosphamide was used as Grogan (1975) and Hazlett, Rosen and Berk (1977) demonstrated profound leucopenia and increased sensitivity to pseudomonas

TABLE I

Phenotypic properties of protease-deficient mutants of Pseudomonas aeruginosa strain PAKS-1

Strain	Mutant class*	Extracellular protease† (units per mg cell dry weight)	Lecithinase‡	Exotoxin A§ (LD50 per ml)
PAKS-1	WT	9.4	+++	4
PAKS-14	I	0.9	—	2
PAKS-16	I	0.1	+	2
PAKS-17	I	0.1	+++	2
PAKS-18	I	0.2	—	<2
PAKS-31	I	0.3	—	<2
PAKS-10	II	0.4	+++	10
PAKS-15	II	0.1	+	5
PAKS-19	II	0.1	—	2
PAKS-20	II	1.0	+++	4
PAKS-23	II	1.7	+++	<2
PAKS-41	II	2.3	+	10

* For definition of class I and class II, see *Materials and methods*; WT = wild-type strain.

† Extracellular protease was determined according to Kunitz (1946–47) after growth in broth.

‡ Lecithinase was estimated from agar-plate assays.

§ Exotoxin was determined by a mouse lethality test.

infections in mice treated with this drug. Cyclophosphamide (200 mg per kg body weight) was given i.p. (3) Intraperitoneal injections of bacteria were made 4 days after cyclophosphamide pretreatment.

Dilutions of the inoculum (3.2-fold) were prepared to obtain two dilution steps for each $\log_{10}$ (number of bacteria). Six mice were inoculated with each dilution, and the animals were observed for 7 days. The LD₅₀ values were calculated by the Spearman-Kärber method described by Finney (1964). Other statistical methods were Student's *t*-test and analysis of variance (Armitage, 1971).

Examination of mice. Mice were killed for post-mortem examination 1, 2 and 3 days after infection. Samples from the peritoneal cavity, liver and heart blood were streaked on blood agar plates which were then incubated aerobically and anaerobically. Isolated bacteria were identified as *P. aeruginosa* by ability to grow on cetrimide agar and were checked for protease production on agar plates containing 15% (v/v) skimmed milk (Wretling *et al.*, 1973). Material for histological examination was fixed in 10% (v/v) neutral formalin. The lung, liver and kidney of every mouse were examined histologically. In several mice of each group the spleen, urinary bladder, myocardium and brain were also examined. Frozen sections of myocardium, liver and kidney were stained with scarlet red. All embedded sections were stained with haematoxylin and eosin.

Exotoxin assay. Exotoxin A was determined by a mouse-lethality test as described by Callahan (1976) except that the preparations were given i.p. The strains were cultured in dialysed Trypticase Soy Broth (BBL), supplemented with 0.1M glycerol and 5mM nitrilotriacetate (Sigma) in 1-litre flasks containing 50 ml broth, on a shaking table for 16 h at 30°C. The culture supernates were sterilised by filtration through a membrane filter (porosity 0.22 μ m; Millipore, Bedford, Mass., USA). Supernates from cultures of strains PAKS-1, PAKS-10 and PAKS-18 were tested by counter-immunoelectrophoresis (CIE) against a monospecific exotoxin-A antiserum. CIE was performed at room temperature for 60 min. (Callahan, 1976). Culture supernates from the three strains were concentrated 10 times. Samples (0.4 ml) of these concentrates were mixed with either 0.2 ml of antiserum (sheep) against exotoxin A or 0.2 ml of normal rabbit serum. The mixtures were incubated at 6°C for 48 h. The final volume was adjusted to 0.8 ml with saline and three mice were inoculated i.v. with 0.2 ml of each mixture.

RESULTS

Virulence of the strains

Strain PAKS-1 and three protease-deficient strains, PAKS-10, PAKS-16 and PAKS-18, were injected intraperitoneally with mucin into mice. As may be seen from table II, PAKS-1, PAKS-10 and PAKS-18 had LD₅₀ values in the same range, but PAKS-10 was possibly less virulent.

Three strains, PAKS-1, PAKS-10 and PAKS-18 were chosen for i.v. injection into cyclophosphamide-treated animals. The LD₅₀ values for these strains are shown in table II. No significant differences between the strains were observed.

The results of the i.p. inoculation of cyclophosphamide-treated mice are shown in tables III and IV. Table III shows the results of repeated determinations of LD₅₀ for PAKS-1, PAKS-10 and PAKS-18. Analysis of variance showed no statistically significant difference in virulence between these strains ($F[2/11 \text{ degrees of freedom}] = 1.30$; $P > 0.05$; standard deviation = 0.25). Based on the data in table III, a $\log_{10}$ LD₅₀ of 6.13 may be calculated for PAKS-1, with a standard deviation of 0.25. The LD₅₀ values in table IV, except for those of strains PAKS-1, PAKS-10 and PAKS-18, were based on single determinations. From the formula for Student's *t*-test for unpaired

TABLE II

Virulence of P. aeruginosa strain PAKS-1 and of its protease-deficient mutants for mice

Strain	Log ₁₀ LD50 when bacteria were injected	
	i.p. with mucin into mice	i.v. into cyclophosphamide-treated mice
PAKS-1	6.68	5.41
PAKS-10	6.40	5.43
PAKS-16	7.30	...
PAKS-18	6.65	5.84

i.p. = Intraperitoneally; i.v. = intravenously.

TABLE III

Results of repeated determination of LD50 for P. aeruginosa strain PAKS-1 and two protease-deficient mutants in cyclophosphamide-treated mice

Determination number	Log ₁₀ LD50 for strain		
	PAKS-1	PAKS-10	PAKS-18
1	6.27	6.22	6.30
2	5.85	5.89	6.38
3	6.10	6.44	6.30
4	6.28	6.85	...
5	6.08	...	...
6	6.33	...	...
7	5.97	...	...
Mean	6.13	6.35	6.33
SD*	0.18	0.40	0.05

*SD = Standard deviation.
The bacteria were injected intraperitoneally.

TABLE IV

Virulence of P. aeruginosa strain PAKS-1 and mutants in cyclophosphamide-treated mice

Wild-type strain PAKS-1 and protease-deficient mutants (class I)		Protease-deficient mutants (class II)	
Strain	Log ₁₀ LD50	Strain	Log ₁₀ LD50
PAKS-1	6.13	PAKS-41	6.23
PAKS-17	6.19	PAKS-10	6.35
PAKS-18	6.33	PAKS-20	6.70
PAKS-14	6.71	PAKS-23	6.84
PAKS-31	6.86	PAKS-15	7.16
PAKS-16	6.89	PAKS-19	8.95

The bacteria were injected intraperitoneally.

data, it was calculated that strains with $\log \text{LD}_{50} > 6.78$ were probably less virulent than the wild-type strain ($P < 0.05$). Accordingly, five of the 11 mutants were considered to have reduced virulence.

Exotoxin production

The results of the exotoxin-A assays of culture supernates are shown in table I. The protease activity as determined by the method of Kunitz (1946-47) was less than 1 unit per ml for all strains except PAKS-1 (4 units per ml). Corresponding levels of activity in purified proteases from PAKS-1 have been found not to be lethal for mice (B. Wretling, unpublished). The lethal factor was inactivated by heating at 60°C for 15 min. as described for exotoxin A (Vasil, Liu and Iglewski, 1976). All the mice that were inoculated with a mixture of 10-fold concentrates of culture supernates of PAKS-1 or PAKS-10 and normal rabbit serum died within 24 h, but no mice died in the exotoxin-A antiserum groups. Of the mice that received supernate of PAKS-18 only one died, this animal having been inoculated with supernate and normal rabbit serum. Counter-immunoelectrophoresis showed precipitation lines between culture supernates of strains PAKS-1, PAKS-10 and PAKS-18 and mono-specific exotoxin-A antiserum.

Post-mortem examination

Mice pretreated with cyclophosphamide were examined after i.p. or i.v. infection, as follows: seven mice, two mice and one mouse were examined 1, 2 and 3 days respectively after infection with strain PAKS-1; eight, two and two mice were examined 1, 2 and 3 days respectively after infection with strain PAKS-10; six and two mice were examined 1 and 3 days respectively after infection with strain PAKS-18. In all, 30 infected mice were examined. No differences were observed between the mice that received PAKS-1 and those given the mutant strains PAKS-10 and PAKS-18, nor were differences observed between the mice inoculated i.v. and those inoculated i.p.

In most mice, the liver was yellowish-brown owing to fatty change. The livers of several mice that were examined on the second and third days after infection showed very small, greyish-white foci.

Only one mouse showed macroscopical changes in the lung; this animal was examined 2 days after infection with PAKS-1. A large part of the lungs was swollen, dark-red, and moderately firm.

Histopathological examination

Liver. On the first day after infection, fatty infiltration of hepatic cells was seen in 17 of 21 mice examined. This infiltration was diffuse and varied between slight and severe. In 15 of the mice there were masses of bacteria in the sinusoids, and hepatic cells lying close to the bacterial masses showed signs of degeneration characterised by swelling and vacuolisation. On the second day after infection severe and diffuse fatty infiltration was seen in all mice. There were numerous well-developed circular foci of necrosis. The foci were filled

with varying amounts of debris consisting of broken-down hepatic cells and masses of bacteria. Inflammatory cells could not be seen (fig. 1). On the third day after inoculation signs of regeneration were seen in the hepatic parenchyma and there was fatty infiltration (fig. 2).

Lungs. Pathological changes were seen in only two mice. In one mouse, infected with PAKS-1, areas with acute and subacute inflammatory changes were observed 2 days after infection. The acute changes consisted of severe hyperaemia, oedema, haemorrhages and severe necrotic vasculitis with numerous bacteria within and outside the vascular walls. The endothelial cells were swollen. Areas with subacute inflammatory changes showed numerous interstitial histiocytes that appeared sometimes as single cells and sometimes as accumulations of various size. Hyperaemia, haemorrhages and bacteria were not seen. Vascular changes consisted of swelling of endothelial cells. The other mouse—infected with PAKS-10—showed the subacute interstitial changes 3 days after infection.

Spleen. In 13 of 16 mice examined 1, 2 or 3 days after infection multiple necrotic foci filled with cell debris and masses of bacteria were seen.

Myocardium and brain. No changes were observed.

Kidney. Except for solitary clusters of bacteria located mainly in the lumen of glomerular capillaries, no changes were observed.

Urinary bladder. Five of eight mice examined 1, 2 or 3 days after infection showed severe haemorrhagic cystitis, sometimes with necrotic changes. The lesions were most pronounced in the lamina propria where there was oedema and hyperaemia, with haemorrhages and necrotic vasculitis; numerous bacteria occurred within and outside the vascular walls and endothelial cells were swollen. There were also varying degrees of necrosis and desquamation of the epithelium, the remaining layers seeming not to be affected.

Bacteriological examination

Cultures from the peritoneal cavity, liver and heart blood of infected mice yielded *P. aeruginosa* only. The protease-deficient strains produced only faint, opaque precipitates around colonies on agar plates containing skimmed milk, indicating that the mutants did not revert to protease production during passage *in vivo*.

Control mice

The mortality among uninfected mice treated with cyclophosphamide (200–400 mg per kg body weight) was about 2%. Six mice, treated with cyclophosphamide (200 mg per kg) were inoculated i.v. with 8.2×10^7 strain PAKS-1 organisms killed by heating at 80°C for 10 min. This dose of bacteria would, in the living state, have represented 300 LD₅₀. All mice survived for the 7-day observation period. Three mice received cyclophosphamide 200 mg per kg, and three mice 400 mg per kg. They were killed after 6 days. Post-mortem examination revealed no macroscopical changes. Histological examination of lung, spleen, liver and kidney showed no abnormalities, but in the urinary bladder changes similar to those described for the infected mice were observed.

However, no bacteria were present, and the necrotic vasculitis seemed to be much less pronounced.

DISCUSSION

P. aeruginosa produces several extracellular enzymes and toxins that may contribute to virulence. Exotoxin A and elastase are two factors capable of causing tissue damage and death when injected into experimental animals. These proteins differ greatly in toxicity, the LD₅₀ for exotoxin A in mice being 0.1 µg and for elastase 100–500 µg, depending on the route of injection (Meinke *et al.*, 1970; Liu, 1974; Kawaharajo *et al.*, 1975a; Callahan, 1976). However, it is difficult to estimate the relative importance of these proteins in the pathogenesis of pseudomonas infections. Elastase is produced *in vitro* by more than 80% of clinical isolates (Wretling *et al.*, 1973) and evidence for the production of the enzyme *in vivo* is provided by the presence of antibodies against elastase, and another protease, in sera from patients and animals with pseudomonas infections (Homma *et al.*, 1975). The regulation of formation of extracellular protease *in vitro* is poorly understood, but several factors such as carbon source, pH and oxygen tension are of importance (B. Wretling, unpublished data). Very little is known about the formation of extracellular enzymes *in vivo*, nor do we know if proteases are released in amounts sufficient to cause significant tissue damage. Purified elastase caused corneal damage and dermonecrosis in doses of 1–10 µg and 10–100 µg respectively, after local injection (Kawaharajo *et al.*, 1974; Kreger and Griffin, 1974; Kawaharajo *et al.*, 1975b; Wretling and Wadström, 1977).

Some of the protease-deficient mutants had the same virulence as the wild-type strain. Less virulent strains, such as PAKS-15 and PAKS-19, had probably undergone changes in addition to loss of protease that reduced their ability to cause lethal infections. The results show that there was no correlation between the amount of protease produced by a *P. aeruginosa* strain *in vitro* and virulence for mice.

The histopathological examination of infected mice revealed no significant differences between the strains investigated. The most striking findings were fatty changes of the liver, with focal necrosis. These effects are not produced by injection of purified elastase or endotoxin of *P. aeruginosa*, but resemble closely the lesions found after i.v. injection of purified exotoxin A (Kawaharajo *et al.*, 1975a; Pavlovskis, *et al.*, 1976). The histopathological changes are not specific for the toxin but are produced, for example, by agents interfering with protein synthesis of the liver (Lombardi, 1966). Exotoxin A and diphtheria toxin inhibit protein synthesis in mammalian cells by the same mechanism (Iglewski and Kabat, 1975). Similar changes in liver histology have been described for bacteraemic *P. aeruginosa* infections in man (Wöckel, 1967). Strains PAKS-1 and PAKS-10 produced a lethal toxin *in vitro*, that was identified as exotoxin A. Strain PAKS-18 produced a smaller amount of exotoxin A, that was detectable by counter-immunoelectrophoresis. It seems probable that the histopathological changes in the liver were caused by this toxin.

Haemorrhagic pneumonia was found in one mouse infected with the wild-type strain PAKS-1. This lesion may have been caused by proteases, as similar histopathological changes have been described after injection of purified proteases (Kawaharajo *et al.*, 1975a).

The urinary bladder showed changes in the lamina propria with secondary necrosis of the epithelium. These changes were also observed in mice that received only cyclophosphamide, but were more pronounced in the infected mice. Haemorrhagic cystitis is a well-known side-effect of cyclophosphamide treatment in man (Foad and Hess, 1976).

Although purified protease is toxic when injected by a number of routes, some of the protease-deficient mutants had the same virulence as the parent strain. There are at least two possible explanations. The small amounts of proteases produced by the mutants may have been sufficient to cause tissue damage and thus contribute to the death of the animals. A more likely explanation might be that the extracellular proteases played very little part in the virulence of *P. aeruginosa*. The elastase is inhibited by α_2 -macroglobin in serum (Hochstrasser, Theopold and Brandl, 1973), and Sezen, Scharmann and Blobel (1975) found that although active immunisation against elastase possibly protected against experimental mouse infections, passive immunisation did not. The present investigation confirms that proteases are of little importance in the pathogenesis of *P. aeruginosa* infections in mice. The results would rather indicate a role for exotoxin A in systemic infections. However, proteases may be important in localised infections, e.g., infections of the lungs, eyes and skin, in which serum protease inhibitors may be present in amounts insufficient to inactivate the enzymes. Kawaharajo and Homma (1975) reported that strains of *P. aeruginosa* that did not produce protease were, in contrast to protease-producing strains, unable to cause corneal damage in mice.

SUMMARY

The virulence of a protease-producing strain of *Pseudomonas aeruginosa* was compared with that of mutants that had lost the ability to produce proteases and other extracellular enzymes. Lethal infections were produced by inoculating mice intraperitoneally with bacteria in mucin, or by inoculating mice intraperitoneally or intravenously with bacteria 4 days after treatment with cyclophosphamide, 200 mg per kg body weight. No significant difference in virulence between the wild-type parent strain and some of its protease-deficient mutants was found. Histopathological examination of different organs in the cyclophosphamide-treated and infected mice showed striking fatty infiltration and focal necrosis of liver, multiple necrotic foci in the spleen and haemorrhagic cystitis with necrosis. The cystitis was produced by cyclophosphamide alone but was aggravated by the infection. In conclusion, no correlation between the production of protease in broth culture and the ability to produce lethal septicaemia in mice was found, and extracellular proteases probably did not contribute to the virulence of *P. aeruginosa*. However, the histopathological changes in the liver suggested a role for exotoxin A in systemic infections.

INFECTIONS WITH *P. AERUGINOSA* MUTANTS

FIG. 1 (*above*).—Liver of mouse 2 days after infection with *P. aeruginosa* strain PAKS-1 (wild type). Foci of necrosis (N) containing cell debris and bacteria are seen. Note vacuoles (V) in cytoplasm of the hepatic cells (as seen after removal of fat droplets). Haematoxylin and eosin (HE). $\times 250$.

FIG. 2 (*below*).—Liver of mouse 3 days after infection with strain PAKS-18. Regenerative process (R) is seen in hepatic parenchyma. Vacuoles in hepatic cells are still present. HE. $\times 400$.

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