Mortality rates amongst mice with endogenous septicaemia caused by *Pseudomonas aeruginosa* isolates from various clinical sources

N. FURUYA, Y. HIRAKATA*, K. TOMONO†, T. MATSUMOTO, K. TATEDA*, M. KAKU and K. YAMAGUCHI*

Department of Laboratory Medicine, Nagasaki University School of Medicine, Nagasaki 852; * Department of Microbiology, Toho University School of Medicine, Tokyo 143 and † Department of Pulmonary Medicine, Jichi Medical School, Tochigi 329-04, Japan

Summary. Mice that had been treated with cyclophosphamide and ampicillin were fed with *Pseudomonas aeruginosa*. These procedures induced an endogenous septicaemia under conditions mimicking the pathophysiology of the disease in man. This model was used to compare the mortality rates in mice infected with *P. aeruginosa* isolates from various clinical sources. Mortality rates in mice given isolates from blood cultures had a broad range (0–100%), but the mean rate was significantly higher than with isolates from other infection sites. Moreover, blood isolates persisted in the intestines of mice after oral inoculation, whereas most isolates from other sources were gradually eliminated. Most *P. aeruginosa* isolates from blood culture produced significantly higher levels of exotoxin A and total proteases than isolates from other infection sites. Amongst the blood isolates, all but one of the lethal strains produced large quantities of exotoxin A or total proteases or both. Taken together, the results suggest that the ability of *P. aeruginosa* to adhere to the intestinal tract and to produce high levels of exo-enzymes may contribute to the development of fatal septicaemia.

Introduction

The mortality rate for septicaemia caused by gram-negative bacteria remains high in immunocompromised patients, despite the availability of various potent broad-spectrum antimicrobial agents.^{1,2} Most septicaemias in immunocompromised patients arise as a result of invasion by the endogenous microflora, particularly that of the gastro-intestinal tract.^{3,4}

Amongst gram-negative bacilli causing septicaemia, *Pseudomonas aeruginosa* has been associated with the highest mortality rates.^{5,6} We reported previously that *P. aeruginosa* also gives a high mortality rate in leucopaenic mice with endogenous bacteraemia.⁷ *P. aeruginosa* also causes severe infections in patients with burns, malignant tumours, cystic fibrosis and other diseases.⁸

P. aeruginosa has several toxic cellular components, notably lipopolysaccharide, and manufacturers extracellular products such as proteases and exotoxins, that account for its virulence in these patients.^{9,10} Recent investigations have indicated that proteases may play an important role in the establishment and maintenance of various types of pseudomonal infections.¹¹

However, the significance of these exo-enzymes in septicaemia remains unclear.

In this study, septicaemia was caused in leucopaenic mice fed with *P. aeruginosa* cultures. Unlike conventional infections models, which usually employ intraperitoneal or intravenous infection routes, this model incorporated the steps of bacterial colonisation, overgrowth and invasion, and consequently closely mimicked the pathophysiology of septicaemia in man.^{12,13} With this model, the mortality rates for *P. aeruginosa* strains obtained from various clinical sources were evaluated, as was the relationship between the mortality rate and the ability of the bacteria to survive in the intestinal tract and produce exotoxin A, proteases, elastase and phospholipase C *in vitro*.

Materials and methods

Bacteria

Thirty-five *P. aeruginosa* isolates obtained at Nagasaki University Hospital, Nagasaki, Japan were used (table I); 17 isolates were from blood, 12 from sputum, three from urine and one each from ascites, pus and other secretion. *P. aeruginosa* D4,⁷ which was isolated from the cardiac blood of a mouse with

Table I. Clinical sources and serotypes of *P. aeruginosa* isolates

Source	Serotype	Strain no.
Blood	A	B2, B4, B5, B8, B11, B16
	B	B9, B12, B13, B14, B17
	E	B10
	G	B6, B15
	M	B3
	Non-typable	B1, B7
Sputum	A	S5, S7 (mucoid), S8, S10 (mucoid), S11
	B	S2
	E	S1, S12
	F	S6
	G	S4
	M	S3
Urine	Non-typable	S9 (mucoid)
	A	U1
	I	U2
	Non-typable	U3
Other sites	A	O1, O2
	E	O3

systemic bacteraemia, was also used. This strain consistently caused a 70–90% mortality rate in the model of endogenous septicaemia used.

Bacteria were identified with Vitek Gram-Negative Identification Cards (Vitek System Inc., Hazelwood, MO, USA). Serotypes were determined by agglutination with monoclonal antibodies (Mel-Assay; Meiji Seika Kaisha, Ltd, Tokyo, Japan).

Stock bacterial cultures were suspended in Mueller Hinton Broth (Difco) containing glycerol 30%, and stored at -80°C until required.

Establishment of endogenous septicaemia

A previously described model of endogenous septicaemia was used, but with some modification.¹³ Pathogen-free male ddY mice (Japan SLC Inc. Ltd, Shizuoka, Japan) weighing 20–24 g were housed in sterile plastic cages with filter hoods and given a sterile diet and distilled water *ad libitum*. They were given sodium ampicillin (Vicillin; Meiji) 200 mg/kg daily by intraperitoneal injection on days 1–5 to disturb the normal intestinal flora and aid colonisation by *P. aeruginosa*. Cyclophosphamide (Endoxan; Shionogi and Co., Ltd, Osaka, Japan) was given intraperitoneally at a dose of 250 mg/kg on days 6 and 9. This dose of cyclophosphamide induced leucopaenia (<1000 leucocytes/mm³) without lethality in the absence of infection.

Bacteria were grown overnight at 37°C on Trypticase Soy Agar (BBL Microbiology Systems), then suspended in sterile saline 0.45%. The density of the suspension was adjusted to 10^7 cfu/ml by reference to a standard curve relating optical density (OD) and bacterial count. The bacterial suspension was given to the mice in their drinking water on days 2–4. The mice were examined twice daily, and deaths were recorded until day 16.

Quantitation of faecal P. aeruginosa in mice

Before the start of the study, faecal specimens from the mice were examined to confirm the absence of *P. aeruginosa*. Fresh faecal pellets were also collected from each mouse on days 6 and 10. Each sample was weighed, suspended and diluted in saline, then plated on agar containing nalidixic acid 15 mg/L and cefrimide 200 mg/L (NAC Agar; Eiken Chemical Co. Ltd, Tokyo, Japan) for selection of *P. aeruginosa*. After incubation for 24 h at 37°C , the plate counts were recorded as cfu of *P. aeruginosa*/g of faeces.

Exo-enzyme assays

Exotoxin A. Trypticase Soy Broth (BBL) dialysate (TSBD-C) was used as the growth medium for the measurement of exotoxin A.^{14,15} The iron content the medium after treatment with Chelex (BioRad) was 0.1 mg/L, as determined with bathophenanthroline.¹⁶

Bacteria were incubated in TSBD-C for 24 h at 32°C with vigorous shaking. After centrifugation at 3000 *g*, the supernates were passed through a 0.22- μm pore filter and then assayed by an enzyme-linked immunosorbent assay (ELISA) similar to that described previously.¹⁶ Briefly, microtitration plates were first coated with goat anti-exotoxin A antiserum (List Biological Laboratories Inc., CA, USA). Culture supernates, or serial dilutions of purified *P. aeruginosa* exotoxin A (List) (0.0031–12.5 mg/L in TSBD-C broth), were then added. Rabbit antiserum against *P. aeruginosa* exotoxin A (a gift from Sumitomo Chemical Co. Ltd, Tokyo, Japan) was used as a second antibody. Alkaline phosphatase-labelled goat anti-rabbit IgG antibodies (E-Y Laboratories Inc., San Mateo, CA, USA) were then added, followed by the reaction substrates detailed previously.¹⁶

Protease assays. Total proteolytic activity was measured by the method of Woods *et al.*¹⁷ Organisms were grown for 24 h at 37°C in Brain Heart Infusion Broth (Difco) with vigorous mixing. Subsequently, 0.5-ml amounts of the culture supernates were diluted 1 in 5 in 10 mM Tris, pH 7.5, and added to 15 mg of hide powder azure (Sigma) as a substrate. The mixture was incubated at 37°C for 1 h, with vigorous shaking. Undissolved substrate was removed by centrifugation at 3000 *g* for 10 min. OD₅₉₅ values of the supernates were then compared with a standard curve prepared by treating the substrate for the same period with purified *P. aeruginosa* protease (Nagase Biochemicals Ltd., Kyoto, Japan) at enzyme concentrations of 0.0075–15.63 mg/L.

The assay method for elastase activity was identical to that for total protease, except that: (i) the culture supernates were diluted three-fold; (ii) 10 mg of elastin Congo red (Sigma) was used as a substrate and (iii) OD₄₉₅ was recorded. Values were compared with those of a standard curve derived with purified *P. aeruginosa* elastase (Nagase) at concentrations of 0.49–125 mg/L.

Phospholipase C. Organisms were grown in tryptose

Fig. 1. Survival of leucopaenic mice in the presence or absence of an oral bacterial challenge. Groups of 10 mice were given ampicillin 200 mg/kg by intraperitoneal injection daily on days 1–5 (▲), and cyclophosphamide 250 mg/kg on days 6 and 9 (△). *P. aeruginosa* D4 was given orally with drinking water on days 2–4 (↔). ○, mice with bacterial challenge; ●, unchallenged control mice.

minimal medium¹⁸ at 32°C for 24 h, whilst being shaken vigorously. Phospholipase C activity was measured by the method of Berka *et al.*¹⁹ with minor modifications. Ten mg of decolourising carbon (Nacalai Tesque Inc., Kyoto, Japan) was added to 1 ml of culture supernate to remove interfering pigment. After centrifugation to deposit the carbon, 20- μ l amounts of supernate were added to 180- μ l amounts of a solution containing 250 mM Tris-HCl buffer (pH 7.2), glycerol 60%, 1.0 μ M ZnCl₂ and 10 mM *p*-nitrophenylphosphorylcholine (Sigma) in the wells of a microtitration plate. The plate was incubated at 37°C for 90 min and the OD₄₀₅ values were read with a microplate reader. Values were compared with those of a standard curve prepared with purified *Clostridium perfringens* phospholipase C (Sigma) at concentration of 0.625–80 U/mL.¹⁶

Statistical tests

Student's *t* test was used to compare means; $p < 0.05$ was considered to be significant.

Results

Mortality rates among mice with experimental endogenous septicaemia

Without an oral challenge with bacteria, no deaths occurred up to day 16 amongst mice treated with cyclophosphamide and ampicillin. By contrast, the mortality rate amongst leucopaenic mice given *P.*

Table II. Comparison of mortality rates amongst mice with experimental endogenous septicaemia

Source	Mortality rate* (%)	Strain no.
Blood	0	B1, B2, B3, B4
	20	B5, B6
	30	B7
	70	B8
	80	B9, B10, B11
	90	B12
	100	B13, B14, B15, B16, B17
Sputum	0	S1, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12
	10	S2
Urine	0	U1, U2, U3
Other sites	0	O1, O2, O3

*Results are for groups of 10 mice.

aeruginosa D4 was eight out of 10. These mice died between days 11 and 13 with systemic bacteraemia caused by the challenge strain (fig. 1).

Table II shows the mortality rates for groups of 10 mice challenged with each of 35 *P. aeruginosa* isolates. The mortality rates ranged widely amongst isolates from blood cultures: with isolates B1, B2, B3 and B4, no deaths were observed up to day 16, whereas isolates B13, B14, B15, B16 and B17, gave 100% mortality, with deaths occurring on days 11–14. Deaths were associated with systemic bacteraemia caused by the challenge strain. In contrast, no deaths occurred in 17 of 18 groups of mice given isolates from other infection sites besides blood. In the one group given a sputum isolates (S2) one of 10 mice died. The mean mortality rates amongst mice given isolates from blood, sputum,

Fig. 2. Quantification of faecal *P. aeruginosa* in mice with experimental endogenous septicaemia after oral inoculation. Mice were treated as described in fig. 1. Results are for **a**, 17 groups each comprising five mice inoculated with different blood isolates; **b**, 18 groups each of five mice inoculated with isolates from other sources. The points show the means within each group and the horizontal bars show the means for all groups.

Table III. Comparison of in-vitro exo-enzyme production by *P. aeruginosa* isolates from various clinical sources

Source	Mean (SEM) concentration in culture supernate of			
	exotoxin A (mg/L)	total protease (mg/L)	elastase (mg/L)	phospholipase C (U/ml)
Blood	1.40 (0.58)*	32.07 (10.81)*	12.30 (3.38)*	33.34 (5.63)
Sputum	0.05 (0.01)	1.67 (0.48)	25.31 (7.81)*	20.91 (3.83)
Urine	0.11 (0.02)	1.81 (0.54)	4.47 (0.11)	28.11 (5.67)
Other sites	0.09 (0.04)	0.97 (0.64)	6.12 (0.59)	55.40 (11.87)

* Significantly higher than for isolates from other sources ($p < 0.05$).

urine and other infection sites were 57, 0.8, 0 and 0%, respectively. The higher mortality rate amongst mice given blood isolates was significant ($p < 0.01$).

Quantification of faecal *P. aeruginosa*

In the mice given blood-culture isolates or strain D4, the number of *P. aeruginosa* cells persisted at $> 10^5$ cfu/g faeces on day 10 (fig. 2a). In contrast, the numbers of faecal pseudomonas tended to decrease with time when isolates from other sources were used (fig. 2b). However, exceptions to this latter pattern arose with isolates S2, S3, S6, S9 and U3. When averaged, the faecal counts for blood isolates were significantly higher than those for isolates from other sources on day 10 (5.0×10^7 versus 5.5×10^5 ; $p < 0.01$).

Quantification of in-vitro production of *P. aeruginosa* exo-enzymes

Differences in mean exo-enzyme levels were found between *P. aeruginosa* strains isolated from different infection sites (table III). On average, *P. aeruginosa* isolates from blood produced higher levels of exotoxin A and total proteases than strains from any other sites ($p < 0.05$ for both enzymes). Moreover, strains from blood and sputum produced significantly higher levels of elastase than those from other infection sites ($p < 0.05$).

Amongst blood-culture isolates, those causing 100% mortality tended to produce higher levels of one or more exoenzymes than those causing zero mortality (fig. 3). In particular, strains B13 and B15 produced

Fig. 3. Comparison of extracellular enzyme production between blood isolates of *P. aeruginosa* causing 100% mortality in mice with experimental endogenous septicaemia (●) and isolates causing 0% mortality (○). Bars indicate the mean enzyme production levels within each group.

high levels of exotoxin A and strain B16 produced all four exo-enzymes copiously. However, strain B14 gave 100% mortality yet produced relatively low levels of the exo-enzymes examined (fig. 3).

The serotypes of the isolates were not related to sites of isolation, mortality rates in mice, numbers of faecal bacteria or exo-enzyme levels.

Discussion

P. aeruginosa is frequently responsible for septicaemia in patients with malignant diseases, particularly leukaemic patients undergoing chemotherapy.^{4,20} Intestinal colonisation rates with *P. aeruginosa* have been reported as > 50% amongst patients with leukaemia, 20% amongst those with solid tumours, and 6–12% amongst healthy adults.²¹ Tancrede and Andremon⁴ showed that 81% of patients with bacteraemia caused by *P. aeruginosa* were intestinal carriers of the same strain. These data suggest that the *P. aeruginosa* strains causing septicaemia in patients with malignant diseases frequently originate from the intestinal flora. Routes of acquisition of *P. aeruginosa* include patient-patient or staff-to-patient contact, water, food and disinfectants,^{3,22} but often remain uncertain. In many cases, the patient may have been colonised before admission with small numbers of *P. aeruginosa*.²¹ Factors that promote development of endogenous septicaemia include bacterial colonisation and overgrowth in the intestine, compromised immune defences and disruption of the mucosal epithelium.²³

In the present study, an experimental model of endogenous septicaemia was developed. This model

used oral inoculation of the bacteria to mimic the pathophysiology of septicaemia in man. In this model, *P. aeruginosa* strains isolated from blood cultures frequently caused septicaemia, whilst isolates from other sites did not do so. Moreover, blood isolates mostly persisted longer in the gut than those from other sources. These results suggest that blood isolates may carry additional virulence factors that assist colonisation, invasion, or resistance to host defences.

P. aeruginosa exo-enzymes may contribute to corneal damage associated with ocular infections and haemorrhagic and necrotic changes in the skin and internal organs,^{10,23–28} but their contribution in septicaemia is still unclear. Therefore, we examined whether the ability of *P. aeruginosa* to produce exo-enzymes *in vitro* was related to the mortality rates amongst mice with endogenous septicaemia.

The results indicated that *P. aeruginosa* isolates from blood tended to produce higher levels of exotoxin A and total protease *in vitro* than isolates from other infection sites. These data suggested that exotoxin A and total protease may be important exo-enzymes in the development of endogenous septicaemia. Nevertheless, correlation was not total and strain B14 caused 100% mortality despite minimal production of these exo-enzymes. Similarly, amongst blood isolates causing mortality rates between 10 and 90%, some that were highly virulent formed little of the exo-enzymes *in vitro* whereas others that gave low mortality formed relatively higher levels. Further studies with mutant strains will help to understand the role of individual exo-enzymes in septicaemia.

In conclusion, some differences were demonstrated between *P. aeruginosa* strains that caused endogenous

septicaemia with high mortality rates and those causing no septicaemia, with respect to the production of exo-enzymes and the ability of the bacteria to survive within the intestinal tract. Exotoxin A and total proteases may play some role in the pathogenesis of endogenous septicaemia; but it is necessary to examine

the multifactorial nature of *P. aeruginosa* pathogenesis since virulence did not seem to depend on any single determinant studied.

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