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Role of exotoxin A in inducing severe *Pseudomonas aeruginosa* infections in mice

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Summary. The effects of exotoxin A (EXA) from *Pseudomonas aeruginosa* on polymorphonuclear leucocytes (PMNLs) were studied in a mouse model and *in vitro*. *P. aeruginosa* PA103, which produced EXA, was 20 times more virulent for normal mice than was its EXA-deficient mutant, PA103-29. EXA was detected in the plasma of mice infected with *P. aeruginosa* PA103, and its presence correlated with increasing numbers of bacteria in the blood and internal organs. A monoclonal antibody (MAb) against EXA prevented the death of the mice if it was given simultaneously with, or 2 h before, infection with strain PA103. The number of PMNLs in murine blood decreased by 50 % within 30 min of intravenous injection of EXA, but this decrease was prevented by simultaneous or prior injection of MAb to the toxin. EXA inhibited *in vitro* phagocytosis and killing of *P. aeruginosa* by human and murine PMNLs and decreased the number of the PMNLs by between 60 and 68 %. Collectively, these results not only confirm that EXA is toxic *in vivo*, but also suggest that this toxin accelerates the growth of virulent *P. aeruginosa* in mice.

Introduction

Pseudomonas aeruginosa is a significant cause of nosocomial infection and has a capacity for tissue invasion, often leading to septicaemia and death. Mortality rate in pseudomonas bacteraemias are higher than in bacteraemias caused by most other gram-negative opportunist pathogens.^{1–3}

Exotoxin A (EXA) is a major toxic product of *P. aeruginosa* and contributes to mortality in experimental animals and patients. Its mechanism of action against mammalian cells has been studied in detail.^{4–11} There are reports that the survival rate among patients with antibodies to EXA or the lipopolysaccharide (LPS) of *P. aeruginosa*, or both, are higher than those of patients without these antibodies.^{5,7} Moreover, it has been reported that 80 % of *P. aeruginosa* isolates from infections produce EXA and that the mortality rate among patients infected with isolate producing EXA is three times higher than that among patients infected by non-producers.¹

We hypothesised that EXA might cause a decrease in the numbers of polymorphonuclear leucocytes (PMNLs), or a disturbance of their function, and that

this might accelerate the proliferation of the bacteria, increasing the likelihood of death. To clarify the action of EXA on PMNLs, the changes in bacterial numbers in the blood and internal organs of mice inoculated intraperitoneally with an EXA-producing strain of *P. aeruginosa* or with its EXA-deficient mutant were examined. In addition, the effects of EXA on the function of PMNLs were examined *in vitro* and *in vivo*. Finally, the effect of a neutralising antibody, MAb 3C7,¹² on the *in vivo* and *in vitro* actions of EXA on PMNLs was investigated.

Materials and methods

Organisms

P. aeruginosa PA103, which is virulent for mice, and its non-virulent derivative, PA103-29, were a gift from Dr Igrewski. Both were stored at –80°C in skimmed milk 20 %.

Exotoxin A (EXA)

Pseudomonas EXA was purchased from the Swiss Serum and Vaccine Institute (Berne, Switzerland). It was > 97 % pure and was free of LPS.

Mouse monoclonal antibodies (MAbs)

MAB 3C7 (IgG1), which binds to EXA, was a gift from the Research Laboratories of Sumitomo Pharmaceuticals Ltd (Osaka, Japan). MAB 1B5 (IgG3), to an outer-membrane component of *Haemophilus influenzae*, was prepared by Dr T. Matsumoto in this laboratory (unpublished).

Animals

Four week-old male ICR mice, weighing 18–20 g (Japanese Charles River, Atsugi, Japan) were used in groups of 10, unless otherwise indicated. Leucopenia was induced by treatment with a single intraperitoneal (i.p.) dose of cyclophosphamide (Sigma) of 250 mg/kg, given 4 days before bacterial challenge.¹³

Preparation of inocula for infection

Bacteria were cultured overnight on Nutrient Agar (Difco) at 35°C, then harvested, washed twice and resuspended in saline to an OD₆₆₀ of 0.2, corresponding to *c.* 3×10^8 cfu/ml. Doubling dilutions of this suspension were employed for i.p. injection. To confirm the inoculum size, plate counts were determined in duplicate on serial dilutions of the suspensions.¹⁴

LD50 determinations

Mice were challenged with a single i.p. injection of 0.5 ml of the bacterial suspension. Eight dilutions, containing different numbers of viable bacteria, were used to determine LD50 values, and mortality was assessed daily for 5 days after infection.

Protection by MAb to EXA against *P. aeruginosa* infection in mice

The method of Kohzuki *et al.* was followed.¹⁵ Groups of mice were given MAB 3C7 intravenously (i.v.) at a dose of 10 µg/mouse either simultaneously with, 2 h before or 2 h after, infection with 1.5×10^6 cfu/mouse of *P. aeruginosa* cells. A control group of mice was challenged with the same inoculum of bacteria but without MAB 3C7.

Quantitation of bacteria in mice and estimation of EXA and LPS

Mice, in groups of five, were challenged by i.p. injection with 0.5-ml portions of bacterial suspensions. The challenge dose was 1.6×10^7 cfu/mouse of the virulent strain PA103 or 1.3×10^8 cfu/mouse of the avirulent strain PA103-29. Samples were obtained at 2, 4 and 6 h after infection; the mice were anaesthetised with diethyl ether. Blood was obtained by heart puncture with a disposable heparinised syringe and, simultaneously, the liver, spleen, kidneys and lung were removed, homogenised (Homogenizer, Hongoikakikai, Tokyo, Japan) and diluted in saline. The samples and their dilutions were plated in dupli-

cate on nutrient agar for viable counts.¹⁴ Portions of the blood samples also were centrifuged, and the supernates were assayed for EXA and LPS. EXA was measured by ELISA (according to Kohzuki *et al.*¹⁵), which has a lower detection limit of 2 ng/ml, and LPS was assayed by the chromogenic Limulus test (Toxicolor system; Seikagaku Co., Tokyo, Japan),¹⁶ which has a lower detection limit of 0.3 ng/ml.

Influence of purified EXA on PMNLs

Mice, in groups of five, were inoculated i.v. with either 0.1 ml of EXA 1 µg/ml in saline or with a mixture containing EXA 1 µg/ml plus either MAB 3C7 or MAB 1B5 100 µg/ml. At 0.5, 1, 2 and 24 h after injection, the mice were lightly anaesthetised with diethyl ether and 30-µl samples of blood were removed from the retro-orbital plexus with disposable, heparinised, capillary tubes. These samples were stained with Turk solution for determination of white blood cell numbers or with May-Grünwald-Giemsa stain for PMNL counts.

Interactions of PMNLs and EXA in vitro

Mouse PMNLs were prepared as described elsewhere,¹⁷ and suspended at a density of 1.0×10^7 /ml in Eagle's Minimal Essential Medium (MEM; Gibco Laboratories, Grand Island, NY, USA). Human PMNLs were purified from human peripheral blood by Hypaque-Ficoll (Flow Laboratories, Irvine)¹⁸ and resuspended in MEM at the same density as the mouse PMNLs. A 0.1-ml-portion of EXA solution, 2 µg/ml in MEM, with or without MAB 3C7 at 200 µg/ml, was added to 0.9 ml of the human or mouse PMNLs and the mixture was rotated at 5 rpm for 1 h at 35°C. One ml of bacterial suspension (1.0×10^6 cfu/ml) in MEM, containing fresh human or mouse serum 10%, was then added, and after rotation for a further 2 h, each sample was plated in duplicate on nutrient agar for viable counts.¹⁴ In addition, the PMNLs were counted in a haemocytometer and their morphology was examined by microscopy after staining by the May-Grünwald-Giemsa method.

Data analysis

The LD50 of the bacteria was calculated by probit analysis from the survival rates of the mice after 5 days of infection.¹⁹ Numbers of organisms and PMNLs in different groups of animals were compared by Student's *t* test.

Results

Virulence of *P. aeruginosa* PA103 and PA103-29 in mice

The LD50s of *P. aeruginosa* PA103 and PA103-29 in normal mice were 6.82×10^5 and 1.36×10^7 cfu/mouse,

Fig. 1. Survival of mice infected with *P. aeruginosa* PA103 or PA103-29. a, In normal mice the challenge dose of *P. aeruginosa* PA103 was 1.26×10^7 (●), 2.52×10^6 (▼), 5.04×10^5 (■) or 1.01×10^5 (▲) cfu/mouse and that of *P. aeruginosa* PA103-29 was 2.72×10^7 (○), 5.44×10^6 (□) or 1.09×10^6 (△); b, in neutropenic mice that of *P. aeruginosa* PA103 was 5.04×10^5 (●), 1.01×10^5 (▼), 2.02×10^4 (■) or 4.03×10^3 (▲) and that of *P. aeruginosa* PA103-29 was 2.72×10^7 (○), 5.44×10^6 (□) or 1.09×10^6 (△). Ten mice were used in each group.

Fig. 2. Viable *P. aeruginosa* cells in the blood of normal mice after infection with *P. aeruginosa* PA103 (■) or PA103-29 (▨). The challenge dose of strain PA103 was 1.6×10^7 cfu/mouse and that of PA103-29 was 1.3×10^8 cfu/mouse. * Indicates a significant difference ($p < 0.05$) between the results for the two organisms; bars indicate SD.

Fig. 3. Viable counts of *P. aeruginosa* in a: lungs; b: livers; c: spleens; d: kidneys of normal mice after infection with *P. aeruginosa* PA103 (▨) or strain PA103-29 (■). The challenge doses were as shown in fig. 2. *Indicates a significant difference at $p < 0.01$ and **, at $p < 0.05$, between the results for the two organisms; bars indicate SD.

respectively, whereas those in neutropenic mice were 8.40×10^3 and 6.03×10^6 , respectively (fig. 1). Thus, the virulence of *P. aeruginosa* PA103 in the neutropenic mice was 151-fold greater than in normal mice, whereas the virulence of *P. aeruginosa* PA103-29 was only double that in normal mice. Normal and neutropenic mice infected with *P. aeruginosa* PA103 died between 1 and 4 days after infection, whereas deaths among mice infected with strain PA103-29 occurred only on the first 2 days after infection.

When MAb 3C7 was given simultaneously with, or 2 h before, infection with 1.50×10^6 cfu/mouse of *P. aeruginosa* PA103, the survival rates of the mice were 70% and 80%, respectively. However, only 40% of the mice survived when the antibody was given 2 h

post-infection, no more than when the mice were not given the antibody.

Localisation of bacteria in infected mice

The bacterial numbers in blood samples taken at various times after infection were examined. At 2 and 4 h after infection, the mean numbers of *P. aeruginosa* PA103-29 cells were more than those of *P. aeruginosa* PA103, reflecting the fact that the challenge dose of the former, avirulent, organism was about 10-fold greater than that of the latter, virulent, strain (fig. 2). However, by 6 h after infection the mean bacterial numbers in the blood of mice infected with *P. aeruginosa* PA103 were > 10-fold greater than those from mice infected

Fig. 4. Levels of: a, EXA; b, LPS in the plasma of mice after infection with *P. aeruginosa* PA103 (○) or PA103-29 (●). The challenge doses were as shown in fig. 2. Numbers in brackets indicate when identical results were obtained for more than one mouse.

with *P. aeruginosa* PA103-29. This difference was significant ($p < 0.05$).

Bacterial numbers in the liver, lungs, spleen and kidneys were also examined (fig. 3). At 2 h after infection, the mean bacterial counts from the livers of mice infected with *P. aeruginosa* PA103 were significantly ($p < 0.05$) greater than in those of mice infected with *P. aeruginosa* PA103-29 (fig. 3b). *P. aeruginosa* PA103 proliferated rapidly, reaching $c. 10^5$ cfu/g in all tissues examined by 4 h after infection, but with little further change by 6 h after infection. However, with *P. aeruginosa* PA103-29, the number of organisms in each organ peaked around 4 h after infection, at $c. 10^4$ cfu/g, and tended to decline over the next 2 h, especially in the spleen and kidneys where the counts at 6 h reduced to 10^2 cfu/g of tissue (fig. 3c,d). These 6-h counts were significantly below those recorded in mice infected with *P. aeruginosa* PA103 ($p < 0.01$).

Concentrations of EXA and LPS in plasma

Only one of five mice infected with strain PA103 had a measurable EXA level at 2 h after infection (fig. 4a), but four of five infected mice had measurable levels by 6 h after infection, ranging from 6.9 to 13 ng/ml. EXA was not detected in mice infected with *P. aeruginosa* PA103-29. The plasma concentrations of LPS were almost identical in mice infected with either *P. aeruginosa* PA103 or its mutant by 6 h after infection (fig. 4b).

The effects of EXA on PMNLs in vivo

The mean number of PMNLs in blood from normal mice was 1420 (SD 117) cells/mm³, but this was significantly reduced ($p < 0.05$) by 0.5–2 h after injection of EXA alone (fig. 5). This reduction did not

occur in mice given a mixture of EXA and the homologous antibody MAb 3C7, but was still observed when EXA was given with the non-homologous antibody, MAb 1B5. No significant differences in the numbers of PMNLs in the blood remained by 24 h after injection of EXA.

Effect of EXA on bacterial phagocytosis and killing by PMNLs

When *P. aeruginosa* PA103 cells, at an initial density of 5×10^5 , were incubated for 2 h with fresh human serum, with or without human PMNLs, the bacterial counts were 1.82×10^4 and 4.79×10^5 , respectively (table I). Addition of EXA reduced the extent of bacterial killing by the PMNLs, but killing was partially regained if MAb 3C7 was also added. *P. aeruginosa* PA103-29 was also killed by human PMNLs and, once again, this killing was reduced if EXA was added ($p < 0.01$), but substantially recovered when MAb 3C7 also was present ($p < 0.01$).

The decreases in bacterial numbers achieved by murine PMNLs in murine serum were smaller than those obtained by human PMNLs in human serum, but the pattern of the results was almost identical. After incubation with serum-treated bacteria and EXA for 2 h, the numbers of human and murine PMNLs were decreased to 60% and 68% of their starting numbers, respectively. The destroyed PMNLs, and their swollen nuclei, were observed by microscopy after staining by the May-Grünwald-Giemsa method.

Discussion

Woods and Iglewski⁸ found significantly higher LD₅₀ values for the toxin-deficient *P. aeruginosa* mutants, PA103-29 and PAO-T1, than for their

Fig. 5. Mean number (and SD) of PMNLs in peripheral streaming blood in mice. □, mice given EXA alone; ■, mice given EXA plus MAb 3C7; ▨, mice given EXA plus MAb 1B5. * Indicates $p < 0.01$, compared to control mice and those given EXA plus MAb 3C7.

Table I. Effects of EXA on the phagocytosis and killing *P. aeruginosa* by murine and human PMNLs

Organism	Additions to the mixture of bacteria and serum	Viable bacteria* after incubation for 2 h ($\log_{10}$ cfu/ml)	
		mouse PMNLs	human PMNLs
<i>P. aeruginosa</i> PA103	None	5.42 (0.25)	5.68 (0.05)
	PMNLs	5.09 (0.04)††	4.26 (0.03)††
	PMNLs + EXA	5.39 (0.03)	5.29 (0.03)†
	PMNLs + EXA + MAb 3C7	5.12 (0.04)††	4.88 (0.09)††
<i>P. aeruginosa</i> PA103-29	None	5.51 (0.12)	5.30 (0.05)
	PMNLs	5.06 (0.08)§	4.81 (0.49)§
	PMNLs + EXA	5.41 (0.04)	5.27 (0.09)
	PMNLs + EXA + MAb 3C7	5.15 (0.21)§	5.04 (0.02)§

* Mean (SD) of three separate experiments.

† $p < 0.01$ as compared to None versus *P. aeruginosa* PA103.

‡ $p < 0.01$ as compared to PMNLs + EXA versus *P. aeruginosa* PA103.

§ $p < 0.01$ as compared to None or PMNLs + EXA versus *P. aeruginosa* PA103-29.

corresponding toxigenic parent strains, PA103 and PAO-1. In the case of strain PA103-29, EXA was absent, or produced at a vastly lower level, than in strain PA103.²⁰ It is well known that EXA inhibits protein synthesis in mammalian cells by transferring the ADP-ribose moiety of NAD⁺ to elongation factor II, leading to tissue damage and cell death.¹² EXA is one of the most powerful exotoxins of *P. aeruginosa*⁶ and levels of antibody to EXA were higher in patients who survived *P. aeruginosa* bacteraemias than in those who died of their infection.¹ Pollack and Young⁵ showed that the amount of antibody to EXA correlated well with the survival rate among patients with *P. aeruginosa* bacteraemia.

The present study confirmed that the virulence of *P. aeruginosa* PA103 was greater than that of strain PA103-29 in both normal and neutropenic mice. Mice infected with strain PA103-29 only died if at all from bacteraemia within 2 days, whereas those infected with strain PA103 died in greater numbers not only within the first 2 days but also later. The presence of neutropenia enhanced the virulence of *P. aeruginosa* PA103 much more than that of *P. aeruginosa* PA103-29. This result concurs with the higher mortality rate for infections caused by EXA-producing *P. aeruginosa* strains in patients with leucopenia than in those without this condition.¹ The greater virulence of *P. aeruginosa* PA103 seems likely to be due to the in-vivo

production of EXA itself, considering that production of this toxin is the main difference from strain PA103-29.

We postulated that the greater virulence of strain PA103 might involve the action of EXA against PMNLs. EXA decreased the number of murine PMNLs *in vivo*,²¹ and killed murine and human PMNLs *in vitro*. Also, EXA inhibited the phagocytosis and killing of *P. aeruginosa* cells by PMNLs *in vitro*. Administration of MAb 3C7 antibodies against EXA inhibited the decrease in PMNL numbers caused by EXA *in vivo* and prevented death of the mice, whereas the non-homologous antibody, MAb 1B5, had no effect. Kohzuki *et al.*¹⁵ reported previously that administration of MAb 3C7 did not protect mice against bacteraemic death caused by *P. aeruginosa* 103. However, these authors only gave the antibody 2 h post-infection and, as the present study showed, protection against killing required that MAb 3C7

was administered simultaneously with, or 2 h before, infection.

P. aeruginosa does make another toxin, termed cytotoxin or leucocidin, that is harmful to PMNLs,²² but this component was not detected in strains PA103 and PA103-29 by the methods of Hayashi²³ and Hirayama in the present study.²⁴ Furthermore the gene (*ctx*) encoding this toxin was not found by the colony hybridisation method (Hayashi, personal communication).

The toxicity of *P. aeruginosa* LPS is 2000 times lower than that of EXA in mice.⁶ In this study, LPS was detected at similar levels in the plasma of mice infected with either strain PA103 or PA103-29. These data argue against any role for LPS in the greater virulence of strain PA103, but an indirect relationship remains possible since the toxicity of the purified EXA in mice is apparently enhanced by simultaneous injection of LPS.²⁵

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