BACTERIAL PATHOGENICITY

Impairment of host defence by exotoxin A in *Pseudomonas aeruginosa* pneumonia in mice

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Exotoxin A (P-ExA) is considered to be a major virulence factor of *Pseudomonas aeruginosa*. Neutrophils, cytokines and nitric oxide (NO) have been implicated as important components of an effective host defence against bacterial respiratory tract infection. To study the role of P-ExA in the pathogenesis of *P. aeruginosa* pneumonia, C57Bl/6 mice were inoculated intranasally with wild-type PA103 or a mutant *P. aeruginosa* strain that did not produce P-ExA, PA103-29. P-ExA facilitated the outgrowth of *P. aeruginosa* in lungs, as reflected by an increasing number of cfu during pneumonia with strain PA103, whereas the number of cfu decreased during pulmonary infection with strain PA103-29. Influx of neutrophils was similar in broncho-alveolar lavage fluids (BALF) during pneumonia with strains PA103 and PA103-29. Lung levels of cytokines (tumor necrosis factor- α , interleukin-6) and chemokines (macrophage inflammatory protein-2, KC) were higher in mice inoculated with strain PA103, whereas BALF concentrations of NO were similar in mice treated with strains PA103 and PA103-29. These data suggest that P-ExA impairs host defence during pneumonia caused by *P. aeruginosa* by a mechanism that does not involve effects on neutrophil influx, cytokines, chemokines or NO formation.

Introduction

Pseudomonas aeruginosa is the most frequent gram-negative pathogen causing nosocomial pneumonia [1, 2]. Ventilator-associated pneumonia caused by *P. aeruginosa* has a mortality rate of 40–68% and treatment with antibiotics does not improve survival [3, 4]. Moreover, treatment is hampered by increasing resistance against antibiotics. A growing line of evidence suggests that *P. aeruginosa* exotoxin A (P-ExA) is a major virulence factor involved in pseudomonal infections. The toxicity of this single-chain 66-kDa polypeptide is due to its ability to inhibit protein synthesis after translocation to the cytosol of susceptible cells [5]. Mortality in mice challenged with an intraperitoneal injection of P-ExA-producing *P. aeruginosa* is higher than in mice challenged with P-ExA-deficient mutant bacteria, and anti-P-ExA monoclonal antibodies exert a protective effect during *P. aeruginosa*

infection in mice [6, 7]. A single injection of P-ExA alone significantly reduces the number of neutrophils in peripheral blood, suggesting a direct effect of P-ExA on neutrophils [7].

Immune host defence plays an important role in the outcome of bacterial pneumonia. Alveolar macrophages release various mediators upon invasion of pathogens, including cytokines and chemokines, leading to a vigorous inflammatory response, involving the recruitment of neutrophils and the production of nitric oxide (NO). Local production of pro-inflammatory cytokines makes an important contribution to host defence against pneumonia with pathogens like *Streptococcus pneumoniae* [8–10], *Klebsiella pneumoniae* [11–13] and *P. aeruginosa* [14–16].

The present study investigated the different elements involved in host defence during *P. aeruginosa* pneumonia, and the influence of P-ExA. Therefore, it compared bacterial outgrowth, the local production of cytokines and chemokines, neutrophil recruitment and NO production in a mouse model of respiratory tract infection with either wild-type *P. aeruginosa* or a

hypotoxigenetic *P. aeruginosa* mutant strain, with a chemically derived mutation in *regA*, that did not produce P-ExA [17].

Materials and methods

Animals

Female C57Bl/6 mice (8–10 weeks) (Harlan, Horst, The Netherlands) were used in all experiments. The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Induction of pneumonia

Strains PA103 and PA103-29, provided by Dr B. Iglewski (Iglewski Laboratory, Rochester, NY, USA), were grown to mid-logarithmic phase in Luria broth for 6 h at 37°C, harvested by centrifugation at 1500 *g* for 15 min, washed twice in pyrogen-free NaCl 0.9% and resuspended in 10 ml of NaCl 0.9%. The number of bacteria was determined by serial dilution in sterile isotonic saline and culture on blood agar plates for 16 h. Mice were lightly anaesthetised with inhaled isoflurane (Forene®; Abott, Queensborough, Kent) and an inoculum of 50 µl of the solution was administered intranasally. Some mice were inoculated with 50 µl of sterile NaCl 0.9% alone (control mice).

Preparation of lung homogenates

At 6 and 24 h after inoculation, mice were anaesthetised with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, The Netherlands) and blood was collected from the inferior vena cava in heparin-containing vacutainer tubes. Whole lungs were harvested and homogenised at 4°C in 5 volumes of sterile NaCl 0.9% in a tissue homogeniser that was carefully cleaned and disinfected with alcohol 70% after each homogenisation. Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates (and blood), and 50-µl volumes were plated on to sheep blood agar plates and incubated at 37°C in air with CO₂ 5%; cfu were counted after 24 h. For cytokine measurements, lung homogenates were centrifuged at 1500 *g* for 15 min at 4°C, supernates were filtered through a 35-µm filter (Becton Dickinson, Lincoln Park, NJ, USA) and frozen at –20°C until cytokine measurement.

Broncho-alveolar lavage

The trachea was exposed through a mid-line incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). Broncho-alveolar lavage (BAL) was performed by instilling two 0.5-ml volumes of isotonic saline; 0.9–1 ml of BAL fluid (BALF) was retrieved per mouse, and total cell numbers and differential cell counts were determined from cytopspins of each sample. BALF differential cell

counts were done on cytopspin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL, USA). BALF was then centrifuged at 750 *g* for 5 min at 4°C and supernates were frozen at –20°C until measurements were performed.

Histological examination

For histopathological examination, lungs were fixed in buffered formalin 10% and embedded in paraffin; 4-µm sections were stained with haematoxylin and eosin.

Assays

Cytokine and chemokine levels were measured by ELISAs according to the manufacturer's recommendations: tumor necrosis factor- α (TNF- α ; Genzyme, Cambridge, MA, USA), interleukin-6 (IL-6; Pharmin-gen), macrophage inflammatory protein-2 (MIP-2; R&D Systems, Minneapolis, MN, USA) and KC (R&D systems). NO₂[–]NO₃[–] concentrations in BALF were determined with a commercially available NO assay, according to the manufacturer's recommendations (R&D Systems).

Statistical analysis

Comparisons of survival data were made by the log-rank test. All other data are expressed as means and SEM. Comparisons between means were made by the Wilcoxon test. Significance was set at $p \leq 0.05$.

Results

Survival

Intranasal inoculation with either strain PA103 or strain PA103-29 led to a dose-dependent mortality (Fig. 1). While inoculation with 10⁴ cfu did not cause death, mortality rates increased to 40% and 80% after administration of 10⁵ and 10⁶ cfu, respectively. Although mortality tended to be lower after inoculation with strain PA103-29, differences from strain PA103 did not reach statistical significance (Fig. 1). Further experiments comparing host responses to pulmonary infection with strains PA103 and PA103-29 were performed with a bacterial inoculum of 10⁵ cfu.

Histology

Inoculation with strains PA103 and PA103-29 induced signs of pneumonia in all mice. At 24 h after inoculation, lungs of mice displayed perivascular inflammatory infiltrates with endothelialitis. A slight interstitial infiltrate was also present (Fig. 2). At this time point there were no differences in the intensity and composition of the inflammation between mice inoculated with strain PA103 and mice inoculated with strain PA103-29.

Fig. 1. Survival of mice infected with strain PA103 (■) and mice inoculated with strain PA103-29 (□). Mice were challenged with (a) 10^4 , (b) 10^5 or (c) 10^6 cfu of *P. aeruginosa*; $n = 10$ mice group for each dose. Differences were not statistically significant.

Fig. 2. Histopathology of lungs. Representative lung pathology 24 h after inoculation with either strain PA103 or strain PA103-29 (b) showing perivascular inflammation and slight interstitial inflammatory infiltrates compared with normal lung histology (a). Haematoxylin and eosin staining (bar = 1 mm). Sections are representative for the groups; $n = 5$ per group.

Bacterial clearance

As the importance of early events after induction of *P. aeruginosa* pneumonia was underscored in the survival curves, the study compared outgrowth of strains PA103 and PA103-29 in lungs at 6 and 24 h after inoculation (Fig. 3). After 6 h the number of cfu recovered from lungs was similar in mice challenged with strain PA103 or strain PA103-29, and none of the animals was bacteraemic. From 6 to 24 h after induction of pneumonia, strain PA103, but not strain PA103-29, demonstrated an increase in the number of cfu in lungs ($p < 0.05$). After 24 h mice inoculated with strain PA103 had $>1 \log_{10}$ more cfu in lungs than mice inoculated with strain PA103-29 ($p < 0.05$), while the number of cfu recovered from blood was similar – 5.0 (SEM 2.7) $\times 10^2$ and 2.9 (SEM 2.1) $\times 10^2$ cfu/ml blood; NS.

Neutrophil influx

There was no difference in neutrophil influx in lungs in mice inoculated with either strain PA103 or strain PA103-29 (6 h after inoculation: 4.9 SEM 0.2×10^5 /ml

Fig. 3. Clearance of bacteria is enhanced in mice inoculated with strain PA103-29 compared with mice inoculated with strain PA103: mean (SEM) *P. aeruginosa* cfu in lungs 6 and 24 h after intranasal inoculation with 10^5 cfu of strain PA103 (■) and strain PA103-29 (□). $n = 8$ per time point for each bacterial strain. * $p < 0.05$ versus 6 h after inoculation, † $p < 0.05$ versus strain PA103.

versus $5.0 \text{ SEM } 0.4 \times 10^5/\text{ml}$; 24 h after inoculation: $7.0 \text{ SEM } 0.5 \times 10^5/\text{ml}$ versus $6.8 \text{ SEM } 0.9 \times 10^5/\text{ml}$, NS), and no difference in the number of alveolar macrophages (6 h after inoculation: $0.6 \text{ SEM } 0.2 \times 10^5/\text{ml}$ versus $0.3 \text{ SEM } 0.1 \times 10^5/\text{ml}$; 24 h after inoculation: $0.5 \text{ SEM } 0.1 \times 10^5/\text{ml}$ versus $0.5 \text{ SEM } 0.1 \times 10^5/\text{ml}$, NS).

Cytokines and chemokines

Local production of cytokines and chemokines within the pulmonary compartment can influence host defence mechanisms in pneumonia [8–14, 18]. Therefore, the concentrations of TNF- α , IL-6, MIP-2 and KC in lung homogenates after inoculation with strains PA103 and PA103-29 were measured (Fig. 4). Pneumonia with either *P. aeruginosa* strain was associated with markedly elevated lung levels of each of these inflammatory mediators ($p < 0.05$ versus control). At 6 h after inoculation, cytokine and chemokine levels in lung homogenates of mice inoculated with strain PA103 or strain PA103-29 were similar. At 24 h, TNF- α , IL-6, MIP-2 and KC concentrations were higher in lungs of mice inoculated with strain PA103 than in mice inoculated with strain PA103-29 (all $p < 0.05$).

Nitric oxide

As the production of NO is important for host defence against pneumonia [19], the study compared NO levels in BALF after inoculation with strain PA103 or strain PA103-29 (Fig. 5). NO concentrations increased significantly during pneumonia with either bacterial strain ($p < 0.05$ versus control). Although, at 24 h, NO levels tended to be higher in mice inoculated with strain PA103, the difference from strain PA103-29-treated animals did not reach statistical significance.

Discussion

P. aeruginosa induces a necrotising pneumonia and contributes to the morbidity and mortality of underlying diseases. P-ExA is considered to be a major virulence factor in pseudomonas infections. To determine the influence of P-ExA on different elements of host defence against pneumonia, the present study used a mouse model of respiratory tract infection with strains PA103 and PA103-29, two *P. aeruginosa* strains differing only in their ability to produce P-ExA. It was found that P-ExA facilitated the outgrowth of *P. aeruginosa* in lungs, as reflected by an increasing number of *P. aeruginosa* cfu during pneumonia with the wild-type strain PA103, whereas the number of cfu

Fig. 4. Mean (SEM) cytokine and chemokine concentrations in lung in mice inoculated with strain PA103-29 (□) or strain PA103 (■). Control mice were inoculated with sterile saline and killed after 24 h. $n = 8$ time point for each bacterial strain. * $p < 0.05$ versus strain PA103.

Fig. 5. Mean (SEM) NO concentrations in BALF of mice inoculated with strain PA103 (■) and strain PA103-29 (□). Control mice were inoculated with sterile saline and killed after 24 h. $n = 8$ time point for each bacterial strain. Differences between mice inoculated with strains PA103 and PA103-29 were not statistically significant.

decreased during pulmonary infection with the P-ExA-deficient strain PA103-29.

Strain PA103-29 was derived from the parental PA103 strain by chemical mutagenesis [20]. Strain PA103-29 carries a mutation in the *regA* gene, which results in a profoundly reduced ability to produce P-ExA [6, 20]. Quantitative assays, both enzymic and immunological, have indicated that strain PA103-29 produces P-ExA *in vitro* at levels of $\leq 0.3\%$ of the parental strain PA103 levels [20]. Stability of *regA* has been verified by *in vitro* toxin assay [6, 21] and *in vivo* experiments [7]. Several animal studies have shown that deficient production of P-ExA by strain PA103-29 is the major determinant of the difference in virulence between strains PA103 and PA103-29 [6, 7]. Thus, it is highly likely that the enhanced pulmonary clearance of strain PA103-29 in the present study was related to P-ExA deficiency rather than to other mutations that may influence the outcome of pneumonia.

Antibacterial host defence within the pulmonary compartment is regulated by a complex interplay between cellular and humoral effector mechanisms. The recruitment of neutrophils is essential for the clearance of bacteria when virulent bacteria enter the lower airspaces. The acute inflammatory response to invasion by *P. aeruginosa* consists of the emigration of neutrophils from pulmonary capillaries into the alveolar space. Importantly, this study did not find a difference in the extent of neutrophil influx in mice inoculated with strain PA103 or strain PA103-29, suggesting that the increased clearance of strain PA103-29 compared with strain PA103 was not the result of differences in the recruitment of neutrophils to the site of infection. Also, the density of alveolar macrophages, another cell population important in bacterial lung clearance [22], was not different in

lungs from mice inoculated with strain PA103-29 or the wild-type *P. aeruginosa* strain. However, as the present study did not assess morphologic integrity and functionality of the neutrophils and alveolar macrophages, it remains possible that these cells are a target for cytotoxicity of P-ExA, eventually causing the differences found in the present study.

Inflammation during pneumonia is orchestrated by locally produced cytokines. The role of pro-inflammatory cytokines in pneumonia has been demonstrated in several experiments. Anti-TNF treatment has been found to hamper host defence mechanisms in mouse models of pneumococcal and klebsiella pneumonia [9, 13, 23]. Also, treatment of granulocytopenic mice with low doses of TNF significantly diminished mortality and enhanced pulmonary clearance of *P. aeruginosa* during severe pneumonia [14, 16]. Evidence for the importance of IL-6 in host defence is derived from studies on pneumococcal pneumonia in IL-6-deficient mice, demonstrating an increased bacterial outgrowth in lungs and enhanced mortality in these mice [8]. MIP-2 and KC, cytokines with chemotactic activities, like TNF- α and IL-6 seem to be important for resistance against respiratory tract infections. Treatment of mice with *K. pneumoniae* pneumonia with an anti-MIP-2 antibody caused a 60% decrease in lung neutrophil influx, a significant increase of outgrowth of bacteria from lungs and a decrease in early survival [11]. Similar results were found in a *P. aeruginosa* pneumonia model [22]. Finally, KC-transgenic mice with selective overexpression of KC in the lung showed increased bacterial clearance and improved survival, in association with enhanced influx of neutrophils to the lung, during *K. pneumoniae* pneumonia [24]. From these studies it can be concluded that endogenously produced TNF- α , IL-6, MIP-2 and KC are important for host defence during pneumonia. Interestingly, P-ExA has been found to inhibit the production of 'protective' cytokines, including TNF- α and IL-6, in whole blood or peripheral blood mononuclear cells [25, 26]. Furthermore, P-ExA suppresses LPS-induced production by alveolar macrophages *in vitro* and dose-dependently reduced TNF- α release in BALF after intratracheal administration of lipopolysaccharide to mice *in vivo* [27]. Therefore, lower concentrations of TNF- α and IL-6 were anticipated in lung homogenates of mice inoculated with strain PA103 than in lungs of mice treated with strain PA103-29, which might at least in part have explained the difference in clearance of bacteria from the lungs. However, the present study found higher concentrations of TNF- α , IL-6, KC and MIP-2 in lungs of mice inoculated with strain PA103, compared with mice inoculated with strain PA103-29, at 24 h after inoculation. It is conceivable that cytokine levels in lung tissue are directly proportional to the number of bacteria present at that moment. Hence, a possible negative effect of P-ExA on cytokine production may have been masked by the higher number of strain PA103 cfu

relative to the number of strain PA103-29 cfu recovered from lungs 24 h after the induction of pneumonia.

Although there was no significant difference in overall mortality between mice infected with strain PA103 and mice infected with strain PA103-29, the presence of P-ExA may have an impact on survival in infections in man. In this model, mice were challenged with bacteria only once, whereas in patients with cystic fibrosis, or mechanically ventilated patients, airways may be colonised with *P. aeruginosa*, leading to more chronic exposure to bacteria. Also, there are differences in the physiological state of the bacterial inoculum in this model compared with bacteria colonising patients during mechanical ventilation; in this model, washed bacteria in a logarithmic phase were used, whereas patients are exposed to aspiration of stationary phase, biofilm-grown bacteria. Furthermore, P-ExA may influence the host response against other pathogens present in the lungs at the same time in these patient groups. Studies in immunocompromised mice or mice with chronically infected airways or mixed pulmonary infections are needed to address the issue. The data from the present study are the first to suggest that P-ExA impairs host defence during respiratory tract infections by *P. aeruginosa*.

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