MICROBIAL PATHOGENICITY

The influence of exo-enzyme S and proteases on endogenous *Pseudomonas aeruginosa* bacteraemia in mice

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Summary. The role of *Pseudomonas aeruginosa* exo-enzymes was evaluated in a murine model of endogenous bacteraemia in which the bacteria invaded the bloodstream after oral dosage. Although an elastase mutant PAO-E64 was as virulent as its parent strain PAO1, an exo-enzyme S-deficient mutant, DG1-ExS5 and alkaline protease mutants PAKS-16, PAKS-17, PAKS-19, were less virulent than their parent strains, DG1 and PAKS-1, respectively (p < 0.01). Thus exo-enzyme S and alkaline protease, but not elastase, appear to contribute to the pathogenicity of *P. aeruginosa* in this model.

Introduction

*Pseudomonas aeruginosa* is an opportunistic pathogen and frequently causes severe sepsis, particularly in immunocompromised patients. Among gram-negative bacteria, *P. aeruginosa* sepsis has the highest mortality rate. Lipopolysaccharide (LPS) is an important virulence factor of many gram-negative pathogens but the LPS of *P. aeruginosa* is not as toxic as that of enterobacteria. Therefore, it is clear that other factors are involved in the virulence of this organism.

*P. aeruginosa* produces a large number of extracellular products that are more toxic than LPS and are important in pathogenesis. We have reported previously that exotoxin A (ETA), which catalyses the transfer of ADP-ribose from NAD to eukaryotic proteins and is the most toxic product of *P. aeruginosa*, is produced in a model of endogenous bacteraemia in mice. Moreover, the lethality for mice of the ETA mutant PAO-PR1 was significantly less than that of the parent strain. We also demonstrated that clinical isolates of *P. aeruginosa* from blood produce large amounts of ETA in vitro and are more lethal for neutropenic mice with endogenous bacteremia than strains isolated from other sources such as sputum and urine. These data indicate that ETA may be an important factor in the development of *P. aeruginosa* bacteraemia and for lethality in mice. However, the role of other extracellular enzymes in sepsis caused by *P. aeruginosa* is not fully understood. To further understand the pathogenicity of this organism, the contribution of several extracellular enzymes to endogenous bacteraemia with *P. aeruginosa* was examined with enzyme-producing strains and their enzyme-deficient mutants.

Materials and methods

Bacterial strains

The bacterial strains used in the present study are described in table I. *P. aeruginosa* PA01, PAO-E64, 388 and 388exs1::Tn1 were kindly provided by Professor B. H. Iglewski, University of Rochester School of Medicine and Dentistry, Rochester, NY, USA. Strain PA01 has been well characterised and produces most of the recognised virulence factors. Its elastase mutant strain, PAO-E64, obtained by nitrosguanidine treatment, produces elastase that is antigenically indistinguishable from that of the parent strain. Strain 388 is a well characterised exo-enzyme S-producing strain that also produces protease but not ETA. Strain 388exs1::Tn1 is a transposon-insertion mutant derived from strain 388, and has no exo-enzyme S detectable either by enzyme activity or by antigenic activity. It is indistinguishable from the parent strain except for its lack of exo-enzyme S and resistance to carbenicillin. *P.
aeruginosa strains DG1\textsuperscript{14} and DG1-ExS5\textsuperscript{15} were kind gifts from Professor D. E. Woods, University of Calgary, Alberta, Canada. Strain DG1 is another well characterised exo-enzyme S-producing strain, that also produces ETA and proteases.\textsuperscript{14} Strain DG1-ExS5 is a transposon-insertion mutant derived from strain DG1. It is deficient in exo-enzyme S but otherwise indistinguishable from the parent strain. Strains PAKS-1, PAKS-16, PAKS-17 and PAKS-19\textsuperscript{16} were kindly provided by Dr B. Wretlind, Karolinska Hospital, Stockholm, Sweden. Strain PAKS-1 is a hyper-producer of alkaline protease, and, in addition, produces elastase. PAKS-16, PAKS-17 and PAKS-19 are mutants of PAKS-1 induced by ethyl methanesulphonate treatment and produce only minute amounts of alkaline protease. Although PAKS-17 produces no elastase, the other mutants produce normal levels of the enzyme.

Animals

Specific-pathogen-free male ddY mice (Japan SLC Co., Shizuoka, Japan) weighing 20–24 g were used. The animals were housed in sterile cages supplied with filter hoods and were fed a sterile diet. They received sterile distilled water except during the period of oral administration of the bacteria. Faecal specimens were obtained before the study and examined to ensure the absence of P. aeruginosa.

Endogenous bacteraemia model

Endogenous bacteraemia was produced as described previously.\textsuperscript{17} Bacteria were grown on Trypticase Soy Agar (BBL Microbiology Systems, Cockeysville, MD, USA) at 37°C for 18 h, suspended in sterile saline, 0.45% and adjusted to a density of 10^9/ml by spectrophotometry. The bacterial suspension was given as the drinking water during days 1–4. To facilitate colonisation of the mice with P. aeruginosa, daily intraperitoneal injections of sodium ampicillin (Vaccillin; Meiji Seika Kaisha, Tokyo) 200 mg/kg were administered on days 1–4 to reduce the normal intestinal flora of the animal. Mice were then given cyclophosphamide (Endoxan; Shionogi and Co., Osaka, Japan) 200 mg/kg by intraperitoneal injection on days 5 and 8. At least 20 mice were used in each group and their survival was monitored until day 18.

Statistical analysis

The $\chi^2$ test was used to compare survival rates. A probability level of 5% was accepted as statistically significant.

Results

The survival kinetics of mice given P. aeruginosa strains orally and treated with cyclophosphamide are shown in the figure. The mortality of mice given PAO1 orally was 78.8%. These mice died between days 11 and 13 due to a systemic bacteraemia caused by the challenge strain. In the absence of an oral bacterial challenge, no deaths occurred in mice treated with cyclophosphamide and ampicillin. Bacterial culture was negative for P. aeruginosa in all control mice killed at the end of the experiment. In mice given the elastase-deficient mutant strain PAO-ExE4, mortality was 80% and did not differ from that of mice given the parent strain.

The mortality induced by exo-enzyme S-producing strain 388 and its exo-enzyme S-deficient mutant 388exs1::TnI was 20% in both cases. In contrast, another exo-enzyme S-producing strain, DG1, caused a high mortality of 80%. The mortality of mice given its exo-enzyme S-deficient mutant, DG1-ExS5, was 20%, which was significantly lower than that of mice given the parent strain (table II).

The mortality of mice given the parent strain PAKS-1 was 70%. On the other hand, the mortalities of mice
Figure. Survival kinetics of mice with endogenous bacteraemia due to P. aeruginosa PA01 (△—△) and its elastase-deficient mutant PA01-64 (■—■). Mice (20–33 per group) were given ampicillin (A) 200 mg/kg and cyclophosphamide (C) 200 mg/kg on the days indicated.

Table II. Mortality of mice given P. aeruginosa exo-enzyme S-producing strains and their mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mortality (%)</th>
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<tbody>
<tr>
<td>388</td>
<td>20</td>
</tr>
<tr>
<td>388ex1::Tn1</td>
<td>20</td>
</tr>
<tr>
<td>DG1</td>
<td>80</td>
</tr>
<tr>
<td>DG1-ExS5</td>
<td>20</td>
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</tbody>
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Table III. Mortality of mice given P. aeruginosa strain PAKS-1 and its alkaline protease mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mortality (%)</th>
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<tbody>
<tr>
<td>PAKS-1</td>
<td>70</td>
</tr>
<tr>
<td>PAKS-16</td>
<td>20</td>
</tr>
<tr>
<td>PAKS-17</td>
<td>20</td>
</tr>
<tr>
<td>PAKS-19</td>
<td>0</td>
</tr>
</tbody>
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given its alkaline protease mutants ranged from 0 to 20%, and were all significantly lower than that of mice infected with the parent strain (table III).

Discussion

A number of P. aeruginosa strains can produce various exo-enzymes in vitro, including ETA, exo-enzyme S, alkaline protease, elastase and phospholipase C. These exo-enzymes seem to be important virulence factors in several P. aeruginosa infections. Woods et al. reported that blood isolates of P. aeruginosa produced a large amount of total protease, ETA, and phospholipase in vitro, compared with isolates obtained from other infection sites. Clinical bacteraemia isolates of P. aeruginosa caused higher mortality in mice in our model of endogenous bacteraemia than did strains obtained from other infection sites and our assays of exo-enzymes in the culture supernates of P. aeruginosa strains were in broad agreement with those of Woods et al. We have also reported that ETA could be detected in murine serum in the endogenous bacteraemia model. Although mice given strain PA01 had a high mortality, strain PA0PR1, which was chemically-derived from strain PA01 and produces a non-toxic, cross-reactive ETA, showed a significantly lower lethality. Such data indicate that ETA may be an important factor in the occurrence of P. aeruginosa bacteraemia and death of the animal.

Proteases produced by P. aeruginosa may also play a role in the virulence of the organism. Two distinct P. aeruginosa proteases have been isolated and purified. These enzymes, alkaline protease and elastase, are metalloproteases, although their substrate specificities and pH optima are different. P. aeruginosa proteases produce corneal ulcers, necrotic skin lesions and pulmonary haemorrhage. Exo-enzyme S, like ETA, is a ADP-ribosyl transferase produced by P. aeruginosa. Production of exo-enzyme S is fairly common among clinical isolates of P. aeruginosa, especially in strains from burn infections and bacteraemias. Exo-enzyme S is also known as an important virulence factor in a burned mouse model and a chronic rat lung infection model.

The role of these exo-enzymes in the development of endogenous P. aeruginosa bacteraemia is still unclear. Because animals injected directly with a large amount of gram-negative bacteria die of endotoxic shock within a short period, the assessment of other virulence factors in conventional systemic infection models may be difficult. In the infection model used in the present study, each step involved in the production of systemic
bacteraemia mimics the clinical pathophysiology of primary bacteraemia originating in the intestinal microflora.

In the present study, there was little difference in mortality between mice given strain PA01 or its elastase-deficient mutant, PAO-E64. These data suggest that elastase is not an essential factor in endogenous _P. aeruginosa_ bacteraemia in mice. When exo-enzyme S-producing strains and their mutants were evaluated, strain 388 exhibited low virulence in contrast to 388E, a mutant producing exo-enzyme S. The data suggest that exo-enzyme S, like ETA, is an important factor in _P. aeruginosa_ bacteraemia and lethality for mice. It is also possible that the ability to produce both ETA and exo-enzyme S is essential for lethality in this model, since DG1-ExS5, which produces ETA but not exo-enzyme S, had a relatively low virulence.

With the alkaline protease mutants of strain PAKS-1, mortality was significantly lower than in mice given the parent strain. These results suggest that alkaline protease is also an important factor in bacteraemia.

In conclusion, we speculate that exo-enzyme S and alkaline protease, but not elastase, affect one or more of the steps involved in colonisation of the mouse intestine by _P. aeruginosa_, in its subsequent invasion of the blood stream, and in its resistance to phagocytosis by Kupffer cells.

References