Evaluation of *Pseudomonas aeruginosa* Exotoxin A and Elastase as Virulence Factors in Acute Lung Infection

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Acute *Pseudomonas aeruginosa* pneumonia was established in guinea pigs by intratracheal instillation of bacteria. Challenge strains included PAO-1, a strain known to produce exotoxin A, alkaline protease, and elastase, and several PAO-1 mutants deficient in either biologically active exotoxin A or elastase production. Survival, intrapulmonary killing of bacteria, and blood cultures were compared among the groups. Strains of *P. aeruginosa* deficient in active elastase production appeared to be less virulent than the parent strain and were more easily cleared from the lung. Opposite results were obtained for the exotoxin A-deficient mutants. These data suggest that elastase, but not exotoxin A, was an important virulence factor during acute pneumonia due to *P. aeruginosa*.

Infections with *Pseudomonas aeruginosa* have become an important clinical problem, and pneumonia caused by this organism is particularly difficult to eradicate by conventional antibiotic therapy (18). This has stimulated much interest in elucidating the mechanism of pathogenicity for this organism, including study of several extracellular secretory products as possible virulence factors. Although studies in animal models have suggested important pathogenic roles for *P. aeruginosa* exoproducts, including exotoxin A (11, 20, 21) and proteases (4, 9, 13, 21), there is little information about their specific roles during acute *P. aeruginosa* pneumonia. Accordingly, we used a guinea pig model of acute *P. aeruginosa* pneumonia to evaluate directly the effect of in vivo exotoxin A or elastase production on the pathogenesis of this infection. For these studies, a parent strain of *P. aeruginosa*, PAO-1, known to produce both exotoxin A and elastase, was compared for virulence with PAO-1 mutants deficient in the secretion of active exotoxin A or elastase.

**MATERIALS AND METHODS**

**Animals.** Hartley strain guinea pigs weighing 350 to 400 g were obtained from Charles River Breeding Laboratories, Bar Harbor, Maine, and fed water and standard laboratory chow (Ralston Purina, St. Louis, Mo.).

*P. aeruginosa* infection. The parent and mutant strains used in this study have been described and characterized previously. The parental strain, PAO-1, produces normal elastase, alkaline protease, and exotoxin A (5). The PAO-1 mutants include strain T-1, an exotoxin A-deficient mutant (Tox−) (11); strain E-64, which produced biologically inactive elastase (Elast−) (12); and PR-1, which produces a nontoxic but immunologically cross-reactive exotoxin A protein (CRM) (3). Bacteria were grown overnight in trypticase soy broth (TSB; Gibco Laboratories, Grand Island, N.Y.) at 37°C in a shaking water bath. For selected studies, strains PAO-1 and T-1 were grown in a deferrated dialyze of TSB with 1% glycerol and 0.05 M monosodium glutamate (1). The bacteria were then washed and standardized to the desired concentration by spectrophotometric and standard pour plate dilution methods, as described previously (2). The method for producing experimental acute *P. aeruginosa* pneumonia in guinea pigs has been described previously (14, 15). Briefly, intratracheal bacterial instillations were performed on anesthetized guinea pigs with 0.5-ml portions of bacteria suspended in isotonic saline.

To ensure the stability of the mutant strains after in vivo passage, we verified the phenotype of each after organisms were isolated from the lungs of selected animals with pneumonia. This involved assaying the strains for exotoxin A and elastase production in broth cultures, as previously described (11). No change in production capacity for exoproducts by any of the four strains was found after passage in guinea pig lungs. A wide range of challenge doses was used to determine the 50% lethal dose (LD50) for each of the four infection groups by the method of Reed and Muench (17). Selected groups of animals were sacrificed at various times to compare rates of intrapulmonary killing among the four strains (2). Equivalent inocula (0.8 × 10⁵ colony-forming units [CFU]) were employed for each strain in these experiments. A sample of blood was obtained from each animal by direct heart puncture immediately after death and cultured in TSB, and growth was then assessed on blood agar plates.
TABLE 1. Survival and bacteriological correlates for experimental P. aeruginosa pneumonia with parent and mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>(LD_\text{so}(\times 10^9))</th>
<th>CFU ((\times 10^7)) per ml of lung homogenate (mean ± SEM)*</th>
<th>Blood cultures (no. positive/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO-1 (parent)</td>
<td>2.80</td>
<td>292 ± 127</td>
<td>3/24</td>
</tr>
<tr>
<td>T-1 (Tox&quot;)</td>
<td>0.33</td>
<td>7,424 ± 3,507</td>
<td>14/24*</td>
</tr>
<tr>
<td>PR-1 (CRM)</td>
<td>0.73</td>
<td>383 ± 120</td>
<td>9/20*</td>
</tr>
<tr>
<td>E-64 (Elast&quot;)</td>
<td>5.10</td>
<td>53 ± 18</td>
<td>3/20</td>
</tr>
</tbody>
</table>

* Values are for groups of eight animals sacrificed 12 h after infection.

** Greater than parent strain (\(P < 0.02\), Student’s \(t\) test)

† Different than parent strain (\(P < 0.01\), chi square analysis, Yate’s correction).

‡ Less than strain PR-1 (\(P < 0.01\)).

§ Different than parent strain (\(P < 0.05\)).

The lungs were next removed and quantitatively cultured as previously described (2).

RESULTS

Table 1 shows the \(LD_\text{so}\) for the parent and each mutant strain of P. aeruginosa, the mean number of CFU per milliliter of lung homogenate cultured at 12 h, and the cumulative blood culture findings. The \(LD_\text{so}\) of the parental strain (\(2.8 \times 10^9\)) was approximately 10-fold greater than that of the T-1 (Tox") mutant strain, with the PR-1 (CRM) mutant strain being intermediate between these. The E-64 (Elast") mutant strain was associated with the highest \(LD_\text{so}\), almost twice the parental value. The number of organisms cultured from the lungs of infected animals paralleled these \(LD_\text{so}\) values. Strain E-64-infected animals had the lowest numbers of bacteria, followed by parental strain-infected animals; the groups infected with strains PR-1 and T-1 showed the least effective intrapulmonary killing of the organism. The mean CFU value for the T-1 group includes only those animals that survived until the time of sacrifice. Four T-1-infected animals died within 12 h.

To ensure that each study group received pulmonary inocula of equivalent size, we sacrificed several animals (two to three) in each study group immediately after lung challenge and quantitatively cultured the lungs. The numbers of CFU in the lungs were similar among the groups immediately after challenge (mean values were PAO-1, \(3.86 \times 10^5\); T-1, \(1.74 \times 10^5\); PR-1, \(2.70 \times 10^5\); and E-64, \(5.27 \times 10^5\)). These values also showed that by 12 h after lung challenge (Table 1) bacterial counts had increased in the T-1-infected group (\(1.6 \log_{10}\), remained constant in the parental and PR-1 groups, and decreased in the E-64 group (\(1 \log_{10}\)).

Evidence that normal in vitro production of exotoxin A is inhibited by media containing high levels of iron (1) suggested the possibility that our standard TSB did not support proper exotoxin A generation by the parent strain. This in vitro effect may or may not be important after in vivo infection is established. To evaluate this potential artifact, we carried out further challenges with strains PAO-1 and T-1, each grown in deferrated TSB (1). Of four animals infected with \(10^8\) CFU of strain PAO-1, only one died; death occurred 70 h after infection. Of four animals infected with \(10^8\) CFU of strain T-1, all died within 24 h of infection. Thus, it did not appear that the paradoxically higher \(LD_\text{so}\) of strain PAO-1 compared with strain T-1 could be explained by an artifact related to high iron content in the culture medium.

To explore the possibility that the presence of exotoxin A was actually protective during acute pneumonia, we challenged guinea pigs with \(3 \times 10^7\) CFU of the exotoxin A-deficient strain T-1 or with a mixture of \(3 \times 10^7\) CFU each of strains T-1 and the toxin A-producing parent PAO-1. Another group was challenged with \(3 \times 10^7\) CFU of PAO-1 alone. Mortality rates were five of eight for T-1 alone, three of eight for T-1 plus PAO-1, and zero of eight for PAO-1 alone. Other groups were challenged with T-1 alone or a mixture of T-1 plus PAO-1, as above, and were sacrificed 6 h after infection. The mean numbers of viable bacteria (± standard error of the mean) in the lung homogenates were 47.3 (± 14.8) \(\times 10^7\) \((n = 4)\) for T-1 and 80.5 (± 6.8) \(\times 10^7\) \((n = 4)\) for the mixture of T-1 plus PAO-1. In vitro assays determined that approximately 50% of the organisms retrieved from the mixed infection were exotoxin A-deficient organisms (21). Since the group receiving the mixed infection (total challenge inoculum, \(6 \times 10^7\) CFU) had roughly twice the number of organisms in their lungs as did the T-1 group (challenge inoculum \(3 \times 10^7\) CFU), there was little evidence for superior early bacterial killing with the mixed infection.

DISCUSSION

P. aeruginosa produces a variety of extracellular products which possess potent biological activity in mammalian tissues (8). The heat-labile protein exotoxin A (7) and two major proteolytic enzymes, elastase and alkaline protease (10), have been studied to determine their role as virulence factors in Pseudomonas infection (4, 9, 11, 13, 16, 20–22). Several studies suggest that P. aeruginosa proteases may be involved in the pathogenesis of acute hemor-
rhagic pneumonia. In one study, intranasal instillation of Pseudomonas elastase in mice resulted in intrapulmonary hemorrhage (9). More recently, a mixture of Pseudomonas proteases was isolated from culture filtrates and directly instilled into the trachea of rabbits (4). Again, hemorrhagic lung lesions resembling those seen with acute P. aeruginosa pneumonia were created. In addition, indirect evidence that protease and elastase contribute to the virulence of P. aeruginosa was provided by successful immunization studies which used Formalin-inactivated protease vaccine in a mink model of Pseudomonas pneumonia (6). To date, however, no in vivo evaluation of acute pulmonary infection comparing protease-producing and nonproducing strains of Pseudomonas has been described. Even less information exists for defining the role of in vivo exotoxin A production in acute P. aeruginosa pneumonia.

The present studies clearly showed that the potential for local exotoxin A production was not necessary for P. aeruginosa to cause serious acute infection of the lungs. In fact, pneumonia caused by a strain deficient in exotoxin A production (T-1) and, to a lesser extent, a strain deficient in production of biologically active exotoxin A (PR-1) paradoxically resulted in lower LD50's and decreased intrapulmonary killing of bacteria compared with infection caused by the parent strain. Recent studies (21) which used identical challenge strains of P. aeruginosa in a rat model of chronic lung infection also revealed that intrapulmonary killing of strain PAO-1 was superior to that of strains T-1 and PR-1 during the early period of infection (3 days), but not after longer periods of infection. One explanation for these unsuspected findings could be that these strains were not truly isogenic mutants. Considering the rather extensive characterization of these strains (3, 5, 11, 12), however, this seems unlikely. Another possibility is that intrapulmonary production of exotoxin A in infected lung tissue paradoxically served to stimulate certain local factors for early pulmonary host defense, such as phagocyte recruitment or complement production with bactercidal activity. To explore whether exotoxin A stimulates early components of pulmonary host defense, we performed mixing experiments. Animals were challenged with the toxin-deficient strain (T-1) or with a mixture of T-1 and the toxin-producing parent strain (PAO-1). Even though animals receiving the mixture were infected with twice the number of bacteria, they experienced lower mortality than the group receiving T-1 alone. These data and those shown in Table 1 support the provocative notion that exotoxin A may be useful in early pulmonary defenses against infection.

In contrast to the findings with exotoxin-deficient strains, infections with the elastase-deficient mutant, strain E-64, resulted in a higher LD50 and increased intrapulmonary killing. Although indirect evidence exists that elastase plays a virulence role in acute P. aeruginosa pneumonia (6), this model offers a direct methodology for establishing this fact. Past studies with an infected corneal ulcer model have shown a less important role for local elastase production (11). Thus, it appears that the tissue virulence of elastase may vary with organ site, being important for elastase-rich organs, such as the lung (19), and not as important for certain nonpulmonary sites (11).

The present study emphasizes the value of in vivo studies in assessing the significance of in vitro phenomena. Furthermore, it appears that there is an organ-specific component involved in P. aeruginosa virulence factors and that a variety of experimental models of infection should be employed to explore fully bacterial pathogenicity.

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