Characterization of *Pseudomonas cepacia* Isolates from Patients with Cystic Fibrosis

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*Pseudomonas cepacia* infections which follow a fulminant course and which include septicemia are being reported with increasing frequency from cystic fibrosis patients. Forty-eight *P. cepacia* isolates from cystic fibrosis patients were screened for production of potential virulence factors. A majority of strains tested produced protease and lipase. Eleven strains harbored plasmids of approximate molecular weights in the range $50 \times 10^6$ to $100 \times 10^6$. Twenty-two strains produced a smooth lipopolysaccharide. Studies are presently under way to determine the role of these potential virulence factors in the pathogenesis of *P. cepacia* disease.

Chronic pulmonary infection is the most significant cause of morbidity and mortality in patients with cystic fibrosis (CF) (19). It is estimated that from 40 to 90% of CF patients have chronic respiratory tract colonization with *Pseudomonas aeruginosa* (10). Poor clinical status is associated with increased numbers of this organism in the sputum (A. S. Hanissian, G. Templeton, R. W. Chandler, and H. Robinson, Abstr. Cystic Fibrosis Club Annu. Meet. 1974). Although other species of *Pseudomonas* are less frequently isolated from CF patients (14), in recent years *Pseudomonas cepacia* has been isolated with increasing frequency (A. Isles, H. Levison, C. Newth, M. Carey, and P. Fleming, American Pediatric Society, Annu. Meet. Cystic Fibrosis Club Abstr. 1982). There is growing evidence that this organism may be associated with poorer pulmonary function (C. Prober, personal communication); in some cases, infection with *P. cepacia* is associated with rapid progression to respiratory failure and death (11).

In contrast to the large amount of information on *P. aeuginosa* virulence factors, a paucity of published information exists concerning the virulence factors of *P. cepacia*. The present study was undertaken to determine the existence of potential virulence factors of *P. cepacia*. With this information one might then be able to define the pathogenesis of *P. cepacia* lung infections in CF patients.

A total of 48 *P. cepacia* strains isolated from the sputum of CF patients were used in this study. Twenty-five strains were obtained from C. L. Prober, The Hospital for Sick Children, Toronto, Ontario; 20 strains were from J. D. Klinger, Rainbow Babies' and Children's Hospital, Cleveland, Ohio; and 3 strains were from Foothills Hospital, Calgary, Alberta. Strains were stored in sterile skimmed milk (10% [wt/vol]) at $-70^\circ$C.

Protease (15), elastase (12), lipase (3), gelatinase (13), and hemolysin (12) production were determined by plate assays. Positive reactions were defined as zones of activity of more than 2 mm around a 15-mm inoculum after incubation at 37°C for 48 h.

Culture supernatants for assay of toxin activity were obtained after overnight growth of each of the 48 strains in tryptic soy broth dialyzed deferrated with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) and supplemented with 1% glycerol and 0.05 M monosodium glutamate (TSBDC) (12). For exoenzyme S production, the above medium was supplemented with 10 mM nitrotriacetic acid (1). Toxin A production was identified by a significant increase in ADP ribose (ADPR) transferase activity in TSBDC culture supernatants after preincubation with 4 M urea–1% dithiothreitol (16). Partially purified elongation factor two extracted from wheat germ was used as substrate (2).

Plasmid DNA was isolated essentially by the method of Maniatis et al. (9), except that bacteria were grown overnight on L-agar plates and all volumes were increased 10-fold.

DNA was subjected to electrophoresis in a horizontal 0.7% (wt/vol) agarose gel in Tris-borate buffer (89 mM Tris, 2.5 mM EDTA, 89 mM boric acid, pH 8.0) at a constant 75 V. Gels were stained with ethidium bromide (1 $\mu$g/ml) and photographed.

Chinese hamster ovary cells (CHO cells), mouse (L-929) fibroblasts, and HeLa cells obtained from the American Type Culture Collection (Rockville, Md.) were grown to monolayers at 37°C under 5% CO₂ in RPMI 1640 medium (Flow Laboratories, Mississauga, Ontario) supplemented with 10% fetal calf serum and 50 $\mu$g of gentamicin per ml. Cells were resuspended in fresh growth medium to a final density of $2 \times 10^5$ cells per ml, and 100 $\mu$l was dispensed into each well of a 96-well microtiter plate. After incubation for 24 h at 37°C, 100 $\mu$l of fresh growth medium was added to each well followed by 100 $\mu$l of serial twofold dilutions of bacterial culture supernatant diluted in tissue culture medium. Plates were incubated for 72 h at 37°C under 5% CO₂. Cytotoxicity was scored microscopically, and the data were expressed as the reciprocal of the highest dilution of culture supernatant which gave rise to cytotoxicity. Supernatants from *P. aeruginosa* strains PAO and PAO-PR1 (20) were used as positive and negative controls, respectively.

Lipopolysaccharide (LPS) isolation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and silver staining of gels were carried out as described previously by Hitchcock and Brown (6).

The bacterial strains used in this study were screened for the production of protease, elastase, hemolysin, gelatinase, and lipase. Results are shown in Table 1. None of the 48 *P. cepacia* strains examined produced detectable levels of ADP ribose transferase activity as compared with controls, nor was there any evidence of cytotoxicity at dilutions greater...
than 1:8 on any of the three cell lines examined. The highest dilution of culture filtrate at which a positive effect was observed was 1:8.

Since many studies have shown that the genetic determinants for certain virulence factors are carried on plasmids, we screened all strains for the presence of plasmid DNA. Eleven strains were found to harbor plasmids; of these 11 strains, 7 carried plasmids which had an approximate molecular weight of $60 \times 10^6$ as compared with the Salmonella typhimurium cryptic plasmid. Two other strains carried larger plasmids of an approximate molecular weight of $90 \times 10^6$. One strain, P. cepacia 5530pk, carried two plasmids which had approximate molecular weights of $100 \times 10^6$ and $50 \times 10^6$, respectively. An additional strain, P. cepacia 701J, carried a single plasmid which had a molecular weight of ca. $50 \times 10^6$. We have not as yet assigned a phenotypic function to any of these plasmids.

LPSs from proteinase K-treated whole cell lysates of all P. cepacia strains used in this study were subjected to electrophoresis in polyacrylamide gels and visualized by the silver stain. Twenty-two strains possessed smooth LPS, whereas 26 strains possessed rough LPS. The silver-stained profiles of the LPSs of one rough and two smooth P. cepacia strains are shown in Fig. 1. Both smooth strains showed heavily stained bands distributed throughout the lane; these bands are absent in the two rough strains shown. Similar staining patterns for rough and smooth strains were obtained with LPSs from the remaining 44 strains.

The majority of P. cepacia strains tested produced protease and lipase. Most of the proteolytic strains were active against casein and gelatin but not against elastin. Whether a role exists for protease and lipase in the pathogenesis of P. cepacia lung infections is undetermined.

None of the P. cepacia strains used in this study produced toxin A, exoenzyme S, or other detectable extracellular products capable of producing a cytotoxic effect in vitro with the three cell lines employed. Testing of additional cell lines could be employed which might demonstrate P. cepacia supernatant cytotoxic activity; however, the likelihood of obtaining positive results seems doubtful.

P. cepacia strains isolated from CF patients have been shown to be resistant to a variety of antibiotics (14), and it has been suggested (L. L. Mackenzie and P. H. Gilligan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C34, p. 317) that the ability of P. cepacia strains to infect the respiratory tract of CF patients may be due to its antibiotic resistance. Plasmids have previously been isolated from P. cepacia (4, 18), including resistance plasmids (18). In this study, we isolated plasmids from 11 of 48 P. cepacia strains. However, we found that plasmid isolation resulted in variable agaro agar electrophoresis patterns. Williams et al. (18) have suggested that difficulty in isolation of plasmid DNA from P. cepacia is due to the presence of nucleases active in the presence of detergents, one of which, sodium dodecyl sulphate, was used in this study. The possibility exists, therefore, that plasmids are present in some apparently plasmidless strains but were not isolated by the procedure used.

The LPS of gram-negative bacteria is considered to be an important virulence factor in the pathogenesis of disease due to these organisms (8). In lung infections, Hudson et al. (7) noted that aerosols of endotoxin caused the accumulation of granulocytes in airways, and Weinbaum et al. (17) reported that polymorphonuclear leukocyte proteases are capable of disrupting pulmonary structure. Thus, it would appear that continued presence of a granulocytic chemotaxant may indirectly cause lung damage by the persistent inflammatory response.

Studies are currently under way in our laboratory to determine the role of P. cepacia protease, lipase, plasmids, and LPS in disease due to this organism.

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