Production of an extracellular toxic complex by various strains of *Pseudomonas cepacia*

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Summary. Six isolates of *Pseudomonas cepacia*, representing various serotypes of the organism and possessing similar degrees of virulence in mice, were examined for their production of an extracellular toxic complex (ETC) *in vitro*. This compound is lethal for mice and produces extensive lung pathology in rats; it is composed of a surface carbohydrate antigen, lipopolysaccharide and protein. All six isolates produced the ETC. The LD₅₀ values for the six ETC preparations ranged from 395 µg for strain 61g to 1750 µg for strain 90ee. Only two of the six ETC preparations contained ketodeoxyoctonate detectable by the methods used, and these two were the most toxic. Rabbit antiserum to the ETC of a serotype D strain could significantly protect mice only against serotype D strains. Examination of the various phases of growth of *P. cepacia* showed that there was extracellular release of the ETC beginning in the early logarithmic phase and continuing through the late stationary phase. The presence of the ETC in the supernatant fluids was due to release of this material rather than to cell lysis. In addition, at least one strain of *P. cepacia* was shown to produce an alginic acid-like compound.

Introduction

The importance of *Pseudomonas cepacia* as an opportunist pathogen in hospitalised patients and other compromised individuals is becoming increasingly evident (Goldmann and Klinger, 1986). Data from cystic fibrosis (CF) centres across the USA demonstrate that CF patients are at especially high risk of colonisation and infection (Corey *et al.*, 1984; Isles *et al.*, 1984; Thomassen *et al.*, 1985, 1986) and are currently the largest patient population infected with *P. cepacia*. This is particularly distressing because of the cases of bacteraemia and fulminant necrotising pneumonia reported in this population (Isles *et al.*, 1984; Thomassen *et al.*, 1985). Because of the organism's resistance to a wide range of antimicrobial compounds, treatment of *P. cepacia* infections in CF patients is particularly difficult (Goldmann and Klinger, 1986).

We have recently described the production of an extracellular toxic complex (ETC) that appeared to be responsible for the lethality and the extensive lung pathology produced by *P. cepacia* in animals (Straus *et al.*, 1988). The ETC consisted of a surface

carbohydrate antigen (CSA), lipopolysaccharide (LPS) and protein. Saponification studies demonstrated that the toxicity of the ETC was most probably associated with the LPS. We also showed that this material enhanced the virulence of *P. cepacia* when co-injected into mice (Straus *et al.*, 1988). Because the ETC from only one strain of *P. cepacia* was employed in the above study, we have examined additional strains for the production of this compound.

Materials and methods

Bacteria, bacterial virulence and media

The *P. cepacia* strains employed in these studies were K19–2 (serotype A), K30–6 (serotype A), Pc 61g (serotype D), 710m (serotypes A, D, E), 90ee (serotypes A, B, C, D, E), Pc99bb (serotype B), 5530pk (serotype C), Pc 527i (serotype D) and K63–2 (serotype E). The serotyping and origin of these strains were described by McKevitt *et al.*, 1987). The storage of the organisms and the media employed have been previously described (Anwar *et al.*, 1983b); LD₅₀ was determined as previously described (Straus *et al.*, 1988) and calculated by the method of Reed and Muench (1938).

Purification, quantitation and chemical characterisation of the ETC from various strains of P. cepacia

Purification and quantitation of the ETC were done as previously described (Straus *et al.*, 1988). The purified ETC was assayed for protein, ketodeoxyoctonate (KDO) and hexose by methods previously described (Straus *et al.*, 1988). LPS was determined by the Limulus amoebocyte lysate (LAL) assay (Sullivan *et al.*, 1976). Uronic acid content was measured by the procedure of Blumenkrantz and Asboe-Hansen (1973), with glucuronic acid (sodium salt; Sigma Chemical Co., St Louis, MO, USA) as the standard. KDO assays on the ETC were also run after exposure of the ETC to sweet potato acid phosphatase (25 units; Sigma) as described by Uehara *et al.* (1974) to determine if there were phosphate esters at the 4th or 5th carbon atoms of KDO.

Determination of the rate of production of ETC and percent lysis of P. cepacia at various points in the growth curve

Cultures of strain Pc 61g were grown in 1 L of Anwar's medium (Anwar *et al.*, 1983b) for 72 h. Samples (200 ml) were removed at optical-density (OD_{540}) values of 0.1 (lag phase), 0.3 (early log phase), 0.6 (late log phase), 1.0 (early stationary phase), and 1.3 (late stationary phase). The samples were immediately chilled on ice, and centrifuged at 17 700 *g* for 30 min at 4°C. The supernates were dialysed with stirring at 4°C against 8 L of deionised water for 3 days (with daily changes of water), and lyophilised. Samples were suspended in 5 ml of distilled water and assayed for ETC by radial immunodiffusion as previously described (Straus *et al.*, 1988). To determine the percentage lysis of *P. cepacia* at various points in the growth curve, we used the procedure described by Straus (1987).

LD50 determinations and animal protection studies

The LD50 of each of the ETC preparations was determined as previously described (Straus *et al.*, 1988) and the value was calculated by the method of Reed and Muench (1938). Antiserum to the purified ETC of strain Pc 61g was prepared in rabbits as previously described (Straus *et al.*, 1988) and serum was then referred to as immune rabbit serum (IRS). The following protocol was used to determine if anti-Pc 61g ETC antibody would protect experimental animals against homologous and heterologous strains of *P. cepacia*. Strains K19-2 (serotype A), Pc 99bb (serotype B), 5530 pk (serotype C), Pc 527i (serotype D), K63-2 (serotype E) and Pc 61g (serotype D) were grown to the stationary phase (OD_{540} = 1.0) in Anwar's defined media (Anwar *et al.*, 1983b). For every 5 ml of washed *P. cepacia* cells in a beaker, 1.0 ml of IRS was added. The same amount of washed bacteria was added to a beaker containing 1.0 ml of normal rabbit serum (NRS) which served as a control. The beakers

were incubated with stirring for 1 h at room temperature (RT). Then approximately 10 LD50 of *P. cepacia* of each serotype (exposed to either IRS or NRS) was injected intraperitoneally (i.p.) into Swiss-Webster mice. Deaths were recorded in both groups for 5 days.

Analysis of CSA for alginic-acid

Purification and quantitation of the CSA of strain Pc 61g was done as previously described (Straus *et al.*, 1988). CSA was analysed for hexuronic acid by the gas chromatographic method of Vadas *et al.* (1981). The method was verified by the analysis of alginic acid (Sigma: Catalogue No. A-2033). Gas chromatography was performed on a Perkin Elmer gas chromatograph, Model 3300, equipped with a flame ionisation detector. Acetyl groups were determined as the ferric hydroxamate by the method of McComb and McCready (1957).

Isolation and characterisation of LPS from P. cepacia strain 61g

P. cepacia strain 61g was grown for 24 h in Anwar's medium as previously described (Straus *et al.*, 1988) and 10 L were centrifuged at 17 700 *g* for 60 min. The LPS was purified from this cell pellet by the procedure of Staub (1967) and further purified by ultracentrifugation (Chedid *et al.*, 1963). Endotoxic activity of the LPS was measured by the LAL assay (Sullivan *et al.*, 1976). The LD50 value of the purified LPS preparation was determined in the same manner as that employed to determine the toxicity of the ETC preparations. The purified LPS from strain Pc 61g was also examined for the presence of KDO as previously described (Straus *et al.*, 1988).

Results

Production of ETC by various strains of P. cepacia

Table I shows the production and LD50 values in mice of ETC from six *P. cepacia* isolates. All six isolates produced ETC with LD50 values ranging from 395 µg for strain Pc 61g to 1750 µg for strain Pc 99bb. However, all were relatively avirulent when injected i.p. into mice; the LD50 values for the six strains ranged from 2.18×10^8 cfu to 9.56×10^8 cfu. Furthermore, there was a wide range of variation in ETC production by these strains. For example, we were able to obtain 70 mg of purified ETC from 10 L of strain 710m, whereas we obtained only 3 mg from 10 L of strain K30-6.

The compositions of the various ETC preparations are also shown in table I. One of the most toxic ETC (that from strain 710m with an LD50 value of 440 µg) had the highest percentage of LPS (41.8%). The least toxic ETC (that from strain Pc

Table I. Production and LD50 values in mice of purified ETC from six isolates of *P. cepacia*

Isolate	LD50* (cfu)	ETC produced (mg/10 L)†	Presence of KDO‡	ETC LD50 (µg)*	Percentage composition of ETC§			
					LPS	Hexose	Protein	Uronic acid
K19-2	2.41×10^8	27.0	—	1000	29.1	58.2	11.6	0.9
K30-6	4.85×10^8	3.0	—	1000	27.7	33.3	36.3	2.2
Pc 61g	2.89×10^8	20.5	+	395	29.7	61.9	1.9	6.3
710 m	5.20×10^8	70.0	+	440	41.8	46.8	8.3	2.9
90ee	9.56×10^8	11.2	—	1500	27.1	29.8	38.0	4.8
Pc 99bb	2.18×10^8	14.9	—	1750	22.7	49.1	23.6	4.0

* Determined after i.p. injection by the method of Reed and Muench (1938).

† Purified as previously described (Straus *et al.*, 1988).

‡ Determined by the method of Osborn (1963); the assay can detect the presence of ≥ 0.5 µg of KDO/200 µl.

§ Chemical analyses were performed as described in *Materials and methods*.

99bb with an LD50 value of 1750 µg) had the lowest percentage of LPS (22.7%). It was also quite interesting to note that the two most toxic ETC preparations (those from strains Pc 61g and 710m) possessed detectable levels of KDO whereas the other four ETC preparations, with LD50 values of ≥ 1000 µg, did not appear to possess KDO. Pre-incubation of the six ETC preparations with 25 units of sweet potato acid phosphatase had no effect on the amount of KDO found in each preparation.

Production of an alginic acid-like material by strain Pc 61g and an examination of the toxicity of its LPS

To determine if strains of *P. cepacia* taken from cases of CF produce alginic acid, the carbohydrate surface antigen (CSA; pool II from DEAE-Sepharcel; Straus *et al.*, 1988) was analysed for its composition by gas chromatography as well as for acetylation. It consisted of approximately $72 \pm 3\%$ guluronic acid and $11 \pm 4\%$ mannuronic acid. No other carbohydrates were present. The material was $1.75 \pm 0.04\%$ acetylated.

The purified LPS from strain Pc 61g was found to be toxic in mice and possessed an LD50 in mice (when injected i.p.) of 1000 µg. This purified LPS preparation also possessed KDO, because it gave a pink colour with maximal absorption at 549 nm in the assay described by Osborn (1963).

*Ability of antibodies to ETC to passively protect experimental animals against homologous and heterologous *P. cepacia* challenge*

Approximately 10^9 cfu of at least one of the various serotypes of *P. cepacia* were incubated with IRS and NRS to determine if antibody to the ETC

of one strain could protect experimental animals against homologous or heterologous challenge, or both. As can be seen in table II, rabbit antiserum to purified ETC from strain Pc 61g significantly protected experimental animals only against serotype D organisms. IRS also appeared to offer some protection against strain K63-2, which is a serotype E strain. However, strain K63-2 appeared to be less virulent than the other strains tested, and the difference in mortality was not statistically significant. Antiserum to the ETC of strain Pc 61g did

Table II. Ability of rabbit antibodies to ETC to protect passively against homologous and heterologous *P. cepacia* challenge

Source of serum	Bacterial challenge strain*	Serotype	Number of mice dead/ number of mice challenged (%)
IRS	K19-2	A	5/5 (100)
NRS	K19-2	A	5/5 (100)
IRS	99bb	B	5/5 (100)
NRS	99bb	B	5/5 (100)
IRS	5530pk	C	5/5 (100)
NRS	5530 pk	C	5/5 (100)
IRS	61g	D	1/10 (10)†
NRS	61g	D	10/10 (100)
IRS	527i	D	4/10 (40)†
NRS	527i	D	10/10 (100)
IRS	K63-2	E	6/16 (37.5)
NRS	K63-2	E	11/15 (73.3)

IRS=immune serum from rabbits immunised with purified ETC from strain Pc 61g (serotype D) as described in the *Materials and methods*. NRS=Normal rabbit serum.

* Dose c. 1×10^9 cfu (10 LD50).

† Significantly different from control by Fisher's Exact Test ($p < 0.005$).

Table III. Production of ETC by strain Pc 61g during different phases of growth in Anwar's medium

Growth phase	Total cells/ 200 ml	Total ETC (mg)/ 200-ml supernate	ETC produced/ cell (ng)	Total supernate DNA (μ g)	Total DNA (supernate plus pellet) (μ g)	Percentage cell lysis (total supernate DNA/ total DNA)
Lag	7.5×10^6	ND	0	ND	< 10	0
Early log	1.14×10^8	3.0	26.3	ND	200	0
Late log	6.92×10^8	3.5	5.06	ND	925	0
Early stationary	9.51×10^8	8.0	8.42	ND	980	0
Late stationary	1.09×10^9	10.0	9.17	37.5	1237	3.0

ND = Not detected.

not protect the mice against challenge with the other serotypes.

Production of ETC at various points in the growth curve of strain Pc 61g

Table III shows the production of ETC by strain Pc 61g during different phases of the growth curve. This table shows that, beginning in early log and continuing to late stationary phase, there is a continuous release of ETC. A determination of the total DNA found in the supernate allowed for an examination of the relationship between cell lysis and the extracellular release of the ETC. The only detectable cell lysis (3.0%) occurred in the late stationary phase.

Discussion

We have recently described the production of an ETC by a strain of *P. cepacia* (Pc 61g) which appeared to be responsible for the lethality and the extensive lung necrosis associated with pneumonia produced by this organism in an animal model (Straus *et al.*, 1988). In this report, we have shown that a further five strains of *P. cepacia* also produce ETC (table I). The amount of ETC produced ranged from 3 mg/10-L culture of strain K30-6 to 70 mg/10-L culture of strain 710m. The significance of this finding is unclear because the LD₅₀ values for all the *P. cepacia* strains we examined, after i.p. injection, were $> 2.1 \times 10^8$ cfu for out-bred white mice. This is in agreement with the work of Jonsson (1970) who reported that the *P. cepacia* isolates he examined were not pathogenic for mice or guinea-pigs except in "extremely large doses".

The toxicity (LD₅₀ values) of the ETC preparations also varied widely (from 395 μ g for strain Pc 61g to 1750 μ g for strain Pc 99bb). The most toxic ETC preparations, those from strains Pc 61g and

710m, contained the highest concentrations of LPS. When the LPS from one strain of *P. cepacia* (Pc 61g) was isolated and purified, it was found to possess KDO and to be toxic in mice. This confirms that the LPS portion of the ETC confers toxicity on the complex. It should also be noted that the most toxic ETC preparations also possessed KDO, whereas the least toxic ETC preparations apparently did not. Previously, we have been able to detect extracellular KDO in approximately 20% of the *P. cepacia* strains that we examined (Straus *et al.*, 1988). Manniello *et al.* (1979) were the first to report that there was no detectable KDO in biologically active LPS preparations extracted from the strains of *P. cepacia*, as determined by the procedure of Osborn (1963). This conclusion was supported by Anwar *et al.* (1983a) in their studies on the outer membrane of one strain of *P. cepacia*. In this study, we treated the ETC with sweet potato acid phosphatase (Uehara *et al.*, 1974) before examination for KDO by the procedure described by Osborn (1963). This enzyme has been shown to hydrolyse phosphate esters of secondary alcohols, such as those found in the 4-phosphate and 5-phosphate esters of KDO. Treatment of all ETC preparations with sweet potato acid phosphatase had no significant effect on the amount of KDO detected in each preparation, indicating that no phosphate esters of secondary alcohols were present. We have examined a further 25 strains and have found that approximately 25% of them produce a KDO-possessing ETC (Straus *et al.*, unpublished observations) that is detectable by the methods we are currently employing. However, it should be pointed out that several authors have reported finding LPS lacking KDO in other bacteria, which were later shown by more stringent methods to contain KDO (Rietschel *et al.*, 1988). We are now attempting to detect KDO in the strains that we consider to be KDO-lacking by

more sophisticated technology such as gas chromatography.

Immunisation of rabbits with the ETC of strain Pc 61g resulted in the production of antibodies that could protect 90% of experimental animals against 10 LD₅₀ doses of the homologous strain (table II). Indeed, this antiserum could protect 60% of experimental animals against 10 LD₅₀ doses of the heterologous *P. cepacia* strain 527i. Both of these strains, however, possess the D serotype, which may explain the protective capability of the serum. In developing the serotyping scheme for *P. cepacia*, McKevitt *et al.* (1987) showed that the LPS determined serotype specificity. Therefore, antibodies against the LPS-containing ETC of strain Pc 61g would be expected to opsonise all serotype D strains, and possibly the serotype E strains, because serum to whole cells of serotype D *P. cepacia* agglutinated serotype E organisms (McKevitt *et al.*, 1987). The data in table II are fairly convincing that antibodies to the LPS of *P. cepacia* can offer protection against experimental challenge. Other studies have shown the protective effect of antibodies to LPS, e.g., in *P. aeruginosa* infections, the most important protective antibodies are directed against cell associated antigens of the organism (Young, 1972). These antibodies do not appear to inactivate any known virulence factor of the bacterium, but rather function as opsonic antibody to increase the efficiency of phagocytosis and resultant killing of the pathogen by professional phagocytes (Young and Armstrong, 1972). McKevitt *et al.* (1987) have shown cross-reactions between type A, which has smooth LPS phenotype, and type B which has a rough phenotype. In that study, the serotype A strain agglutinated in anti-serum prepared against both the serotype A and serotype B strains. The serotype B strain agglutinated only with its homologous serum. The authors concluded from these data that the serotype B strain is a subtype of the serotype A strain, because immunoblotting of the purified LPS preparations of the two strains demonstrated that the antibodies were directed against different determinants. This suggests that the core region of *P. cepacia* LPS may be an important antigenic determinant.

The ETC of strain Pc 61g appears to be released in all phases of growth except the lag phase (table III). ETC is released as a natural product of cell growth and is not due to cell lysis. This is not surprising, because it has been shown that, as other gram-negative bacteria grow *in vitro*, they shed

outer membrane LPS into liquid media (Mackowiak, 1984). These LPS molecules, most commonly found in association with phospholipids and bacterial outer membranes (Rothfield and Pearlman-Kothencz, 1969; Gankema *et al.*, 1980), are thought to be released into the external environment by bacteria in fragments of outer membranes (Russell, 1976). We have previously shown that a similar LPS-containing complex is produced *in vivo* by at least one strain of *P. cepacia* (Straus *et al.*, 1988).

At least one strain of *P. cepacia* (Pc 61g) produces an alginic acid-like compound, the CSA. Polysaccharides produced by mucoid strains of *P. aeruginosa* are heteropolymers composed of D-mannuronosyl and L-guluronosyl moieties and invariably contain acetyl groups (Carlson and Matthews, 1966; Linker and Jones, 1966; Evans and Linker, 1973). Evans and Linker (1973) compared the polysaccharides from mucoid strains of *P. aeruginosa* isolated from CF patients and patients with urinary tract infections for algal alginic acid. They showed that the ratio of L-guluronosyl to D-mannuronosyl moieties and the acetyl content of the polymers differed, depending upon the strain of bacterium examined. In their study, D-mannuronosyl residues were the most abundant and, in certain instances, represented as much as 91% of the polysaccharide. The percentage acetylation of strains in their study ranged from 13.4 to 4.1%. Algal alginate, also composed entirely of D-mannuronosyl and L-guluronosyl residues (Linker and Jones, 1966; Evans and Linker, 1973) was found to be unacetylated. In this study, we have shown that the alginic acid-like compound produced by *P. cepacia* strain 61g is approximately 72% guluronic acid and is 1.75% acetylated. We showed in a previous report (Straus *et al.*, 1988) that active immunisation of mice with this material (the CSA) could protect them against at least one heterologous strain of *P. cepacia*. It is difficult, at this time, to relate this finding to the protection studies with rabbit antibody to the ETC of the same organism, because ETC and CSA certainly represent different compounds in view of their dissimilar elution patterns on DEAE-Sephacel (Straus *et al.*, 1988). The significance of these findings is unclear.

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