Biology of *Pseudomonas aeruginosa* in relation to pulmonary infection in cystic fibrosis

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Mearns[1] showed that *Staphylococcus aureus* is usually the initial bacterial pathogen in the lungs of cystic fibrosis (CF) children with respiratory disease, but with increasing age *Haemophilus influenzae* and *Pseudomonas aeruginosa* begin to predominate. *P. aeruginosa* in particular has been associated with the progressive chronic respiratory infection so often seen in CF, but the biological basis of the significant interactions between the microbe and host remains obscure. In the last two decades much research has been directed towards the numerous putative pathogenic factors produced by *P. aeruginosa* and their role in promoting the spread and survival of the organism in the CF lung. As yet, no single factor of the parasite or host has been identified that serves to explain comprehensively the poor prognosis for patients colonized with this organism. The following brief review attempts to summarize our current knowledge of the microbiology of pseudomonas and the relevance of its products to the pathogenesis of infection in CF.

General

P. aeruginosa is the type species of a large genus of strictly aerobic gram-negative rods which is widely distributed in nature as saprophytes or pathogens of plants, insects and animals. *P. aeruginosa* is extremely versatile biochemically, and can grow in environments as diverse as jet fuel and distilled water. It attacks carbohydrates oxidatively, and the majority of strains produce both a yellow fluorescent pigment and a blue pigment, pyocyanin, yielding the characteristic green colouration on agar culture.

In hospitals, *P. aeruginosa* survives well in moist environments such as sinks, respirators, humidifiers etc., and is occasionally found on the hands of medical personnel. Faecal carriage by healthy adults is low (3-12%), but this increases dramatically in hospitalized patients[2]. Infections in the community are rare and are usually limited to 'soggy dermatitis' of interdigital spaces, but recently an increase in cases of folliculitis associated with bathing in contaminated whirlpools, Jacuzzis, etc., has been observed.

When compared with established bacterial pathogens, *P. aeruginosa* must be considered to be only weakly pathogenic and, in the absence of debility in the host, virtually non-virulent. Nevertheless, the species produces a wide array of potential virulence factors (Table 1), which contribute to its pathogenicity in the compromised patient.

Table 1. Virulence factors of Pseudomonas aeruginosa *relevant to infection in cystic fibrosis*

Factor	*Function*
Mucoid polysaccharide	Adherence to epithelia, penetration barrier for phagocytes, antibiotics, etc.
Fimbriae	Adherence to buccal epithelia
Proteases	Local necrosis, splitting of IgG, degradation of fibronectin
Exotoxin A	Systemic toxicity; role in CF obscure
Pyocyanin	Inhibits ciliary function
Lipopolysaccharide/ endotoxin	Unknown - possibly toxic but does not, apparently, lead to toxaemia and shock

Adherence properties

Once colonization of the CF lung occurs, *P. aeruginosa* is seldom, if ever, eradicated despite intensive specific antibiotic therapy. It has been suggested that the CF lung offers a unique habitat for the organism, as it adheres more efficiently to respiratory mucins than other gram-negative bacteria[3]. There are two main schools of thought on the adherence of *P. aeruginosa* to mucosal surfaces. Costerton *et al.*[4] proposed that the mucoid exopolysaccharide (MEP), produced by most CF isolates, forms what they termed a 'glycocalyx' or loose capsule. This is composed of highly organized linear strands of polysaccharide radiating outwards from the cell surface and mediates attachment to respiratory epithelium. MEP is often referred to as alginate because of its chemical similarity to a polysaccharide normally found in seaweed algae. Bacterial microcolonies are entrapped in this layer and protected from attack by host defences.

Although MEP-producing strains (mucoid strains) probably adhere by this mechanism, non-mucoid strains require a different mode of attachment. Baker and Marcus[5] proposed that in CF the oropharynx is colonized by non-mucoid *P. aeruginosa* which adhere to buccal cells with the aid of hair-like projections called fimbriae[6]. Mucoid variants which arise during the infection are aspirated into the lungs and these cells adhere via fibres of MEP. The adherent bacteria then embed themselves in the MEP matrix and elaborate locally various exoproducts which results in necrosis of the lung tissue.

Fibronectin is a protease-sensitive glycoprotein secreted by mucosal epithelial cells. It serves to protect the respiratory epithelia from bacterial colonization and there is evidence that this coating substance is lost from cells as a consequence of illness or other factors associated, perhaps, with hospitalization. A direct correlation has been shown between loss of fibronectin and an increase in salivary proteases

which degrade fibronectin, and increased colonization by *P. aeruginosa*[7].

Extracellular products of *P. aeruginosa*

Proteases

At least three distinct proteases have been identified in *P. aeruginosa*, and two of them, elastase and alkaline protease, are associated with virulence[8]. Elastase activity of strains gives rise to haemorrhagic skin lesions and, in combination with alkaline protease, to tissue necrosis. Jagger *et al.*[9] found that mucoid *P. aeruginosa* from CF were less proteolytic than the non-mucoid isolates in the same specimen. They proposed that the proteolytic activity of non-mucoid forms - which are more frequent in the early stages of colonization - assisted the establishment of the mucoid variety, presumably by degrading protective proteins of the mucosa. A more simple explanation may be that MEP restricts the passage of enzyme to the epithelial cell surface.

There is evidence, however, to suggest that elastase can interfere with host defences by cleaving IgG molecules into biologically less active subunits[10]. Certainly, the majority of CF patients who are chronically colonized with *P. aeruginosa* have high levels of serum anti-protease antibody[11], but it is unknown whether these antibodies neutralize bacterial proteases in the lung. Furthermore, the proteolytic activity of bronchial secretions in CF is not restricted to *P. aeruginosa* alone, as proteases derived from granulocytes contribute significantly to this activity[12].

Phospholipase

Berka *et al.*[13] showed that all strains of *P. aeruginosa*, from various clinical sources, produced a heat-labile phospholipase (C) which, in conjunction with a glycolipid, is responsible for the haemolytic activity of the species. Phospholipase C expression was highest in non-mucoid CF isolates, but mucoid strains were poor producers of the enzyme.

We were able to confirm this in our laboratory, but could find no correlation between the production of phospholipase C by CF isolates, and the severity of respiratory infection in patients assessed by the length of colonization, lung function and clinical score (unpublished).

Exotoxin A

This toxin is a proenzyme of molecular weight 66 000-71 000 daltons. Its mode of action is to transfer adenosine diphosphate ribose (ADPR) from nicotinamide adenine dinucleotide (NAD) to elongation factor 2 (EF-2). Transfer of ADPR to EF-2 irreversibly inactivates the latter and stops peptide elongation and protein synthesis in the mammalian cell. The cytotoxicity of exotoxin A is manifest by many pathological changes, including diffuse liver cell necrosis, leucopenia, pulmonary haemorrhage and renal necrosis. The toxin is produced *in vivo* in humans and is immunogenic, and several studies attest to its role in systemic infections with *P. aeruginosa*[14,15].

The role of exotoxin A production in CF is obscure. Antibody to the toxin is readily demonstrable in the serum of CF patients[11] but does not appear to protect the patient from subsequent infection. Exotoxin A is also toxic for human macrophages[16] and this feature may assist the survival of *P. aeruginosa* in the lung.

Pyocyanin

It has been known for many years that the pigment pyocyanin is toxic for both mammalian and some bacterial cells. Pyocyanin oxidizes NAD and releases oxygen-free radicals, in the absence of enzyme action, through the electron transport system. Its role in the pathogenesis of pseudomonas infection has been largely ignored by researchers, but recently Wilson *et al.*[17] showed that pyocyanin and its derivative, 1-hydroxyphenazine, have a marked and profound effect on ciliary function in humans. Using ciliated nasal epithelial cells, they demonstrated that pyocyanin of CF and other isolates caused slowing of ciliary beat and dyskinesia. The effect was reversible and the epithelia remained viable. The implication of this finding for CF patients has yet to be assessed, but the disruption of a primary host defence mechanism by *P. aeruginosa* might influence the establishment, and persistence, of the organism in the respiratory tree. Pyocyanin is certainly produced *in vivo*, as evidenced by the appearance of green sputum in colonized patients and its solubility in chloroform extracts of sputum. Moreover, the bactericidal activity of pyocyanin against many gram-positive and gram-negative bacteria may suppress the normal flora of the host and facilitate colonization by *P. aeruginosa*.

Mucoid *v.* non-mucoid strains in CF

There has been considerable discussion in the literature of the significance of the mucoid phenotype in CF. This form is found in 60-90% of chronically colonized CF patients, but is rare (<1%) in other conditions, with the exception of chronic infection in bronchiectasis, and persistent colonization or infection of the urinary tract with *P. aeruginosa*.

The mucoid character is chromosomally encoded, and can be readily selected from non-mucoid cultures by bacteriophages[18] and antibiotics in sublethal concentrations[19]. There is little doubt that mucoid cultures arise *in vivo* from parent non-mucoid forms, but the mechanism of selection is unknown. Bacteriophages have been demonstrated in CF sputa[20], and phages isolated from mucoid strains were better able to convert non-mucoid to mucoid strains than phages from non-mucoid CF isolates[21].

Pier and others[22] purified the MEP substance of mucoid CF cultures and showed that it was antigenic. CF patients colonized with mucoid *P. aeruginosa* have significantly raised titres of circulating antibody to MEP in comparison to controls, but the antibody does not appear to influence colonization with mucoid or non-mucoid *P. aeruginosa*.

Mucoid strains of *P. aeruginosa* are not homogeneous, even within the same specimen, and 100-fold differences in the susceptibility of mucoid colonies in single sputum samples to β-lactam antibiotics has been reported[23]. This heterogeneity is also reflected in the varieties of colonial appearance of mucoid strains so often seen on primary agar culture.

The published data on the comparative antibiotic sensitivity of mucoid and non-mucoid strains are contradictory[24,25], but in the author's experience resistance to antibiotics occurs much less often in CF isolates than in those from other clinical sources. Indeed, about 20% of CF isolates are hypersensitive to β-lactam antibiotics, and a minimum inhibitory concentration (MIC) of 5 mg per litre or less of carbenicillin is not unusual[26]. Hypersensitivity of

strains extends to other antibiotics, including trimethoprim and nalidixic acid, but it has not been observed with aminoglycosides. Despite the apparent sensitivity of CF isolates, it is truly perplexing when variants with obvious increased sensitivity to an antibiotic continue to be isolated during active therapy with that antibiotic.

The increased permeability to cell wall agents results in the suppression of the growth of about one-fifth of all strains of *P. aeruginosa* for CF on selective media[27]. Many laboratories use commercial agars containing the agents Irgasan (ICI), nalidixic acid or cetrimide at concentrations inhibitory to these isolates, and in order to obtain the maximal rate of isolation of pseudomonas from patients, the inclusion of a non-selective medium is recommended.

The diffusion of positively charged antibiotics, e.g. gentamicin, through the negatively charged MEP may be retarded and result in the decreased sensitivity of these strains to antimicrobials[28]. *In vivo*, penetration of antibiotics into sputum containing MEP may, therefore, be considerably reduced, and the resulting suboptimal concentration of antibiotic delivered to the bacterial cell surface may then select mucoid variants.

Surface antigen variation of *P. aeruginosa* in CF

The dominant antigenic determinants or epitopes on the cell surface of *P. aeruginosa* are contained in the lipopolysaccharide (LPS) component of the cell wall. LPS confers somatic antigen (0) specificity, and 17 0-antigens have been identified in the species[29]. 0-specificity lies in the repeating sugar units of the side chain of LPS, and this is covalently linked to a conserved core polysaccharide which, in turn, is bound to lipid A embedded deeper in the outer membrane. Loss of 0-side chain by a strain exposes lipid A, which is capable of activating complement, and the cell becomes sensitive to the bactericidal action of normal human serum and loses its specific 0 serotype. LPS composed of the three regions is often referred to as 'smooth', and LPS deficient in 0-side chains and/or core components as 'rough'.

Classically, strains of *P. aeruginosa* from a variety of clinical sources possess smooth LPS and are serum resistant but, in contrast, about 80% of CF isolates have rough LPS and are serum-sensitive[30,31].

Penketh *et al.*[30], in a study of 49 adult CF patients who had respiratory infections with *P. aeruginosa*, showed that a specific 0 serotype was detected in only 32% of 109 isolates from these patients. Strains were classified by whether they exhibited serotype specificity and their sensitivity to serum. They found that patients who had recently been colonized generally harboured strains which had a specific 0 serotype antigen and were resistant to serum; those patients who had been infected for many years had isolates which were invariably defective in these properties. A striking feature of the study was that patients who harboured strains which appeared to be transitional forms between smooth and rough LPS chemotypes were often the most ill by clinical score, were hospitalized longer and contributed more to the overall mortality.

Strains of *P. aeruginosa* with altered surface antigens may be selected for *in vivo* by the readily demonstrable antibody response to 0-specific smooth LPS in CF patients[32], or by exposure to sublethal concentrations of cell-wall antibiotics, or both. Preliminary results of a study of the antibody response of CF patients to the rough LPS of their variant strains indicates that these forms may not be recognized as readily as typical strains. It seems reasonable, therefore, to suggest that antigenic variation of the initial infection strain might contribute to its survival in CF.

Antigenic variation can be defined as a process by which an organism gains a selective advantage by periodically changing its antigenic profile to avoid elimination by the host's immune system. This type of variation has been well documented in diseases caused by some trypanosomes and plasmodia, and a situation analagous to *P. aeruginosa* in CF occurs in repeated asymptomatic bacterial infection of the urinary tract due to *Escherichia coli* in schoolgirls[33].

The loss of 0-specific components of LPS results in a decrease in virulence; Cryz *et al.*[34] showed that, for burned mice, an LPS-defective variant was 1000-fold less virulent than its parent. The continued survival of these organisms is, therefore, paradoxical in that they are avirulent, sensitive to serum, hypersensitive to antibiotics, and yet contribute to mortality in CF patients.

The frequency of serum-sensitive *P. aeruginosa* in CF serves to explain why extension of organisms from the diseased lung to the blood is rare. Furthermore, most CF patients' serum is bactericidal for their autologous strain of pseudomonas, although a minority produce a serum factor, possibly IgG, which blocks the bactericidal activity of their serum[35].

Vaccines

In experimental animals, vaccination with a variety of pseudomonas products has been shown to enhance resistance to infection with *P. aeruginosa*[36]. LPS vaccines have been developed for the treatment of burns, in particular, and the reported results are encouraging. However, as CF patients generally possess a highly competent systemic humoral immunity towards most pseudomonas antigens, the benefit of vaccination as a treatment for established pseudomonas infections is questionable. The injection of vaccine in patients with pre-existing circulating antibody may allow the formation of circulating antigen-antibody complexes, which may give rise to tissue damage. The prospective use of a vaccine to prevent colonization showed that it failed to reduce the rate of colonization of CF with *P. aeruginosa*[37]. Indeed, patients who received a polyvalent vaccine experienced more rapid clinical deterioration than those who remained uncolonized and, strangely, 65% of isolates of *P. aeruginosa* from vaccinated children were mucoid compared with 74% of non-mucoid isolates from the control group.

Pseudomonas cepacia

Recently there have been reports of the frequent isolation of *P. cepacia* from CF patients, and colonization rates of up to 20% have been cited[38]. Traditionally a cause of onion-rot, *P. cepacia* is of interest to medical bacteriologists principally because of its apparent predilection for contaminating hospital pharmacy products and its ability to thrive in disinfectants such as chlorhexidine[39]. *P. cepacia* is constitutively resistant to colistin and many other antibiotics, and this feature has been utilized for its isolation from mixed cultures. Its pathogenic role in

CF is unclear, but some researchers consider its presence to be unwelcome and associated with increased morbidity[40].

Antibiotics and *P. aeruginosa*

P. aeruginosa is resistant to many antibiotics at concentrations that can be achieved *in vivo*. In respiratory infections in CF there is the added difficulty of achieving bactericidal concentrations because the organisms are embedded in mucus and alginate, and the passage of antibiotics to the bacterial surface is severely restricted. Table 2 lists the major antibiotics which have proved to be clinically useful in the treatment of *P. aeruginosa* infections in CF.

Table 2. Major antibiotics active against Pseudomonas aeruginosa

Antibiotic	MIC_{50}● *(mg/l)*	MIC_{90}● *(mg/l)*
Amikacin	4	32
Azlocillin	16	64
Carbenicillin	64	128
Cefsulodin	4	16
Ceftazidime	2	8
Ciprofloxacin	0.25	1
Gentamicin	2	8
Piperacillin	16	64
Ticarcillin	32	128
Tobramycin	1	4

●Minimum inhibitory concentration for 50% and 90% of clinical isolates (values taken from published literature)

There appears to be no obvious choice of a particular drug or combination of drugs, judging by the large number of reported trials which, unfortunately, often differ markedly in experimental design, thus making simple comparison difficult[41-43]. Good to excellent clinical improvement of patients with pulmonary infections due to *P. aeruginosa* have been obtained with aminoglycosides and a variety of semi-synthetic penicillins or new cephalosporins, either alone or in combination, but few comparative trials have been undertaken. Nevertheless, it has emerged that a combination of ticarcillin and tobramycin is superior to carbenicillin and gentamicin[43]. Other therapeutic trials reported include piperacillin and tobramycin[44], cefsulodin[45], ceftazidime[46,47], and amikacin[48].

The potential importance of altered pharmacokinetics of aminoglycosides and cephalosporins in the treatment of CF should be recognized. The increased extracellular volume associated with malnutrition, and the elevated renal clearance of these drugs in CF patients, has led to the conclusion that doses of aminoglycosides, in particular, should be larger than those used in the treatment of other patients[49].

Bacteriologic improvement in CF following antibiotics is often short-lived, and in acute exacerbations there is often only a small decrease in the viable count of pseudomonas in the sputum; sometimes clinical improvement occurs without a decrease in the numbers of the organisms[50].

There have been two controlled studies of the use of aerosolized antibiotics in the treatment of respiratory infection in CF. In the first, inhaled cephaloridine was compared with oral cloxacillin in a 6-month trial[51]. The carriage rates of staphylococci and pseudomonas remained the same throughout the trial and the authors concluded that aerosolized anti-staphylococcal drugs did not give any marked benefit to the patient. In contrast, the study carried out at the Brompton Hospital, London (double-blind randomized crossover study of young adults)[52] utilized aerosolized carbenicillin and gentamicin for the treatment of pseudomonas infection and obtained encouraging results in terms of clinical improvement. Emergence of resistance in the bacterial population was not found. The theoretical risk of selecting resistant forms by this method of treatment is probably acceptable because alternative antibiotics, highly active against resistant strains (e.g. amikacin), are available.

A later study of 9 patients[53] concluded that prophylactic inhalation of antibiotics may lead to a significant improvement in the lifestyle of CF patients who present with moderate to moderately severe lung disease, and are chronically infected with *P. aeruginosa*.

There are currently a number of fluorinated carboxyquinolones, namely enoxacin, ofloxacin, perfloxacin and ciprofloxacin, which have activity against *P. aeruginosa*. Ciprofloxacin has recently entered clinical trials, and detailed data are eagerly awaited. It is highly active *in vitro* and its major benefit over other similarly potent anti-pseudomonals is its oral route of administration.

Clinical impressions of ciprofloxacin, albeit anecdotal, seem to be favourable, and although resistance may emerge during prolonged treatment[54], it ceases to be a problem when the drug is withdrawn. As with other antibiotics, complete eradication of *P. aeruginosa* from the lung is not achieved. Nevertheless, the oral route, combined with its proven activity and apparent lack of side effects, augurs well for the use of ciprofloxacin in the treatment of CF patients with exacerbations of pseudomonas pulmonary infection.

Future prospects

Our knowledge of the biology of *P. aeruginosa* has been helpful in identifying factors which, to a greater or lesser degree, contribute to the severity of lung infection in CF. Nevertheless, recurrent and chronic pulmonary infection is still the major cause of morbidity and mortality in CF[55]. The pathogenesis and virulence of *P. aeruginosa* in CF is obviously multifactorial, and much of the data on the relevance of these factors is contradictory and highlights the complexity and heterogeneity of the species in CF. The basic defect in CF patients has yet to be identified, and this may prove to contribute significantly to the association of pseudomonas and CF. Longitudinal studies are needed of the acquisition of *P. aeruginosa* (and *P. cepacia*) by patients, the colonial, antigenic and antibiotic-sensitive variants which arise during infection, and the immune response to these forms both systemically and in lung secretions. Viruses may play an important role in predisposing infants to bacterial infection, and the contribution of other factors such as sputum biochemistry, and perhaps even diet, probably require further investigation.

Finally, the rather impressive status of *P. aeruginosa* as a 'harbinger of death' for CF patients[56] has been greatly reduced by medical and scientific advances, and there is every reason to be optimistic that

further research will show a way for it to be relegated to a lower and more manageable league of micro-organisms in CF.

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