Pseudomonas aeruginosa in cystic fibrosis: pathogenesis and new treatments

Pseudomonas aeruginosa *is the most problematic organism in patients with cystic fibrosis. This article reviews its pathogenic mechanisms, with particular relevance to the pathophysiology of the cystic fibrosis airway, and recent advances in therapeutic approaches.*

Cystic fibrosis is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR encodes a protein whose primary role is the regulation of chloride secretion and sodium absorption at epithelial surfaces. Over 1800 mutations in CFTR have been described (Cystic Fibrosis Mutation Database, 2011), although the most common mutation (Phe508del: previously termed ΔF508; a deletion of phenylalanine at codon 508) occurs in approximately 70% of patients.

Disruption of the homeostatic balance of sodium, chloride and water at the epithelial surface results in a failure of mucociliary clearance, such that inhaled bacteria are less effectively cleared from the airways. As a consequence, the lungs of a patient with cystic fibrosis eventually become populated by bacteria that would normally be cleared by the immune defences in a healthy individual. This results in a cycle of chronic pulmonary infection and inflammation, with a concomitant decrease in lung function, punctuated by occasional infective exacerbations with symptoms of cough, breathlessness and increased sputum production. These exacerbations are of clinical importance because up to 25% of patients fail to fully recover to their previous baseline lung function (Sanders et al, 2010).

The bacteria isolated from the lungs of patients with cystic fibrosis in infancy are common respiratory pathogens – *Staphylococcus* and *Haemophilus* spp. – similar to those that may be isolated in healthy children with an intercurrent pneumonia. The major pathogen in cystic fibrosis, however, is *Pseudomonas aeruginosa*. *P. aeruginosa* is found in almost 80% of patients by 18 years of age (Geller, 2009), and the organism can be isolated from the lower respiratory tract of infants and young children, even shortly after a diagnosis of cystic fibrosis is made following neonatal screening (Hilliard et al, 2007; Stafler et al, 2011). There is a wide distribution of *Pseudomonas* genotypes isolated from children with cystic fibrosis, strongly supporting the notion that infection mainly arises from environmental sources rather than as a result of cross-infection; patient-to-patient spread has been a problem in some centres, however, and highly transmissible 'epidemic' strains have been identified (Cheng et al, 1996). If initial infection is not successfully eradicated, chronic lower airways infection with *Pseudomonas* results in increased endobronchial inflammation and poorer clinical status (Sagel et al, 2009), and so it is against this bacterium that the majority of new antibiotic therapies in cystic fibrosis are targeted.

In addition to *P. aeruginosa*, the lungs of patients with cystic fibrosis may provide a suitable environment for populations of other microbial species that are not normally found in healthy lungs; *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Burkholderia cepacia complex*, atypical mycobacteria species and *Aspergillus fumigatus* are all opportunistic pathogens. While *B. cepacia complex* infection is associated with a decrease in clinical status, including manifesting as cepacia syndrome (high fevers, necrotizing pneumonia and bacteraemia), the clinical sequelae of infection with bacteria such as *Stenotrophomonas* and *Achromobacter* are less clear, and there is at least some evidence that certain of them may be markers of increased disease severity rather than the cause.

Davies et al (2007) described the mainstays of antibiotic therapy in cystic fibrosis, which are based on the concept of initial prevention of infection with prophylactic oral penicillins, limitation of bacterial load with regular nebulized antibiotics against *Pseudomonas*, and treatment of exacerbations with oral or intravenous antibiotics as appropriate. This review will concentrate on recent developments in the knowledge of *Pseudomonas* behaviour in the lungs of patients with cystic fibrosis, and the emerging targets for antibiotic therapy revealed by these discoveries.

Pseudomonas aeruginosa in the lungs of patients with cystic fibrosis

P. aeruginosa is a common environmental Gram-negative bacillus, found in damp conditions such as around sinks, in drains, or in ventilator tubing and equipment. It

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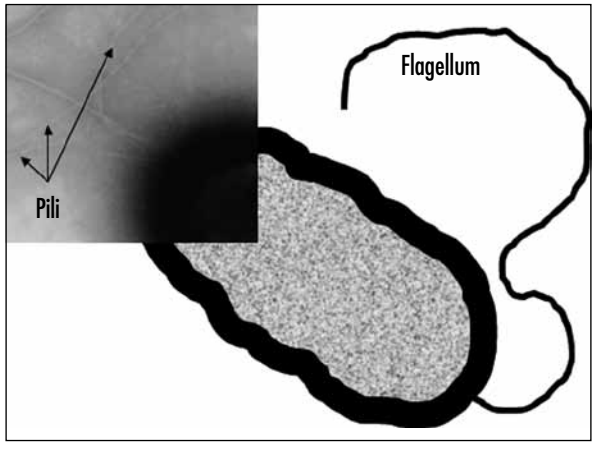
clearly has a particular affinity for the lungs of patients with cystic fibrosis; in part this appears to be the result of a number of intrinsic and adaptive qualities, but it is incompletely understood.

Establishing infection

P. aeruginosa is thought initially to establish infection in the lungs of patients with cystic fibrosis through its ability to adhere to the respiratory epithelium (both secretions and cell surfaces) via pili and a single long flagellum (*Figure 1*). In individuals who do not have cystic fibrosis this is prevented by the presence of a healthy, normal depth of airway surface liquid and mucus lining the respiratory tract, in which the bacteria are trapped and removed by the normal action of the mucociliary escalator. They are then either swallowed or exhaled. Importantly, their brief presence in the healthy airway will not trigger an inflammatory response. In patients with cystic fibrosis the airway's surface liquid layer is reduced in depth, although data suggest that a 'second hit', in the form of a viral infection, may be a requirement for the bacteria to establish a foothold, in addition to the underlying CFTR defect (Tarran et al, 2005). *P. aeruginosa* is not distributed homogenously in the lungs of patients with cystic fibrosis, but instead shows an affinity for the hypoxic environment of the thick mucus plugs that occur in the airway lumen; the motile organism is able to penetrate deep into airway mucus to avoid the initial host immune defence (Worlitzsch et al, 2002).

Mucociliary clearance thus fails to remove the inhaled bacteria, which may adhere directly to the epithelial cell surface membrane; in-vitro studies have demonstrated binding directly to surface glycolipids carrying the GalNAcβ1-4Gal disaccharide, and one of these, asialoGM1, has been reported to be more abundant at the cell surface layer in cystic fibrosis (Saiman and Prince, 1993) and restorable by CFTR correction (Davies et al, 1997). Whether this mechanism is important in vivo is less clear.

Figure 1. Electron micrograph showing adherent pili with schematic representation of long flagellum.



Maintaining infection

Once infection has been established, *P. aeruginosa* switches its focus from binding to the cell surface to avoiding the host's immune response. The cycle of host inflammatory responses via neutrophil oxidative bursts, superoxide anion generation and the actions of inflammatory cytokines is thought to be key in the progressive destruction of lung tissue and subsequent bronchiectasis that are the radiological and histological hallmarks of this disease.

This cat-and-mouse battle with the immune system provides a fascinating insight into possible future targets for anti-pseudomonal therapies (*Table 1*). It is hypothesized that the trigger for these responses is the relatively hypoxic, anaerobic environment of the thick mucus of the respiratory surface of the lungs of the patient with cystic fibrosis (Hassett et al, 2010). *P. aeruginosa* has the ability rapidly to alter its phenotype as a result of the hypermutability of its DNA-RNA transcription properties. Not only can it switch genes off and on rapidly, but it also commonly undergoes a mutation that switches off its DNA mismatch-repair capabilities, resulting in common DNA transcription errors going through to cell division in an unedited state. This results in a greater than 20-fold increase in the spontaneous mutation rate and generates numerous in-vivo mutants which may carry selective advantages in their microenvironment.

First, *P. aeruginosa* looks to protect itself from immune recognition. The pili and flagellae of *P. aeruginosa* are strongly immunogenic, and once *P. aeruginosa* has established itself in the lung it decreases expression of these structures (Murray et al, 2007). Second, possibly to provide a physical barrier to antigen–antibody binding, *P. aeruginosa* has the ability to convert to a mucoid phenotype and secrete large quantities of alginate exopolysaccharides.

Should antibodies or antibiotic molecules penetrate this barrier, *P. aeruginosa* carries a second line of active defenses. It is capable of secreting protease enzymes, which hydrolyse antibody, complement proteins and inflammatory cytokines such as interleukin-8. *P. aeruginosa* also has a multitude of defence mechanisms against

Table 1. *Pseudomonas aeruginosa* in the lung of the patient with cystic fibrosis

Adheres to respiratory epithelium
Penetrates hypoxic mucus plugs
Generates in-vivo mutants
Decreases expression of external structures
Secretes protease enzymes
Efflux pumps and beta-lactamases
Forms a biofilm via quorum sensing
Exopolysaccharide (alginate) secretion

antibiotics. The thin, Gram-negative cell wall allows antibiotics to penetrate the microbe, which then responds with efflux pumps and beta-lactamases. Selection pressure towards resistance is driven by the ability of colonies of bacteria to form complex groups, in which many individual organisms may be exposed to sub-lethal doses of antibiotics and begin to develop resistance to them.

Properties of biofilms

As *P. aeruginosa* infection becomes well established, the bacteria show the ability to develop into more complex population organizations in which they act as a group to provide collective protection. This is achieved by the organization of a biofilm. A biofilm is an aggregation of micro-organisms within which bacteria are metabolically inactive and relatively resistant to antibiotics (Sintim et al, 2010). The formation of biofilms by bacterial pathogens has long been recognized in nature (rivers and plants), on artificial in-vivo devices such as prosthetic heart valves, and in areas of the body where immune penetration is known to be limited, such as infected bone (osteomyelitis). Biofilm production is not unique to *P. aeruginosa* – many other common human pathogens also form such structures. The biofilm itself consists of the population of bacteria, trapped endogenous mucins, DNA breakdown products from damaged leucocytes and an exopolysaccharide matrix or 'sticky extracellular slime' (Nadell et al, 2008) which is either adhered to the cell surface membrane, or more commonly a 'bacterial raft', free-floating in the respiratory mucus.

The trigger for biofilm formation is unclear, but is considered to be intimately related to the ability of bacteria to detect the concentration of their population in their immediate environment; a phenomenon described as 'quorum sensing'. *P. aeruginosa* secrete molecules called acyl homoserine lactones that can freely diffuse across bacterial cell membranes throughout their colony and are sensed by neighbouring bacteria. Once the colony is of sufficient size the acyl homoserine lactone reaches a concentration which leads to gene expression in adjacent bacteria, resulting in production of exopolysaccharides which then act to protect the entire group. The triggers for this process are hypothesized to be local micro-environmental hypoxia (Yoon et al, 2002) or exposure to antibiotics. Biofilms are thought to increase in mass until they periodically rupture and shed a bolus of bacteria out into their immediate milieu, and this has been proposed as one mechanism causing the intermittent exacerbations that typify the clinical course of cystic fibrosis.

Designing drug therapies targeting *P. aeruginosa* is therefore complicated by three main obstacles:

1. The bacteria are uniquely suited to invading the lungs of patients with cystic fibrosis, using extrinsic structures which are quickly shed once infection is established, rendering them relatively free from immune surveillance
2. They are able to excrete exopolysaccharides that impair antibiotic penetration and facilitate the development of resistance as small quantities of antimicrobials persist in the biofilm and are available to drive selection pressure
3. Finally, it is worth recognizing that this process is not homogenous throughout the lung, and that non-mucoid wild-type *P. aeruginosa* species may be continually reinfecting areas of well-aerated, relatively aerobic lung parenchyma immediately adjacent to a mature biofilm colony in an anaerobic mucus plug.

This complexity means that there is unlikely to be a single chemotherapeutic solution to this complex picture (*Table 2*).

New approaches to antibiotic development

Penetrating the biofilm

To overcome the physical barrier that the biofilm confers it is important to be able to deliver effective concentrations of antibiotic molecules directly to the site of the biofilm, the airway lumen. Inhaled (nebulized) antibiotics including the aminoglycoside tobramycin and the polymixin colomycin are mainstays of current therapy for cystic fibrosis (Geller, 2009). Delivering antibiotics via the inhalational route, while time consuming for patients and carers, avoids high systemic levels of drugs with well-recognized oto- and nephrotoxic side effects. Inhalational preparations of further classes of antibiotics such as levofloxacin (a quinolone) are currently in development, and aztreonam (a monobactam) is now licensed for prescription (Retsch-Bogart et al, 2009; Sabet et al, 2009).

One of the limitations of antibiotic molecules with respect to penetrating biofilms is that they often exist as charged particles when in liquid suspension, and therefore are less able to penetrate into the hydrophobic slime that makes up the biofilm. In a novel attempt to overcome this, a liposomal preparation of amikacin (an aminoglycoside) has been developed (Meers et al, 2008). In this inhalational preparation, the amikacin is packaged into liposomes 0.3 μm in diameter with an outer layer made up of the surfactant constituents dipalmitoyl phosphatidylcholine and cholesterol. These liposomes

Table 2. Anti-pseudomonal strategies

Nebulized antibiotics
Liposomal preparations
Prodrug antibiotics
Antibiotic-metabolite degrading compounds
Quorum-sense molecular targets
Nitrite anion oxidative stress
Lytic phage
Antibodies (monoclonal, IgY)

are then able to penetrate the biofilm and provide a prolonged delivery of aminoglycoside directly to the bacteria inside. It is proposed that liposomes are ruptured in situ by lytic defence molecules that are secreted by the *P. aeruginosa* called rhamnolipids, a so-called 'Trojan horse' method of drug delivery. In addition to amikacin, tobramycin has also now been developed as a liposomal preparation (Alipour et al, 2009) and has a fourfold increase in bactericidal activity compared to the freely distributed drug.

Innovative approaches to resistance

The above example of using bacterial self-defense molecules to release an antibiotic from its delivery system is conceptually similar to a familiar strategy in pharmacokinetics: the use of prodrugs. Prodrugs are commonly used in medicine, an example being the administration of diamorphine, which relies on first-pass metabolism in the liver and conversion to the active metabolite morphine. When bacteria first encounter antibiotic molecules they rely on influx and efflux pumps to internalize, metabolize and remove metabolite remnants. These metabolites may persist in structures such as biofilms for prolonged periods of time, allowing bacteria which have not been killed by the initial antibiotic to have a sustained phase of exposure to near-identical, non-lethal molecules and thus build resistance mechanisms. An important future strategy for antibiotic delivery is to design pro-antibiotics which would be taken up by bacteria and activated by them once the molecule is inside the cytoplasm (Devasahayam et al, 2010).

The problem of persistent inactive metabolites can also be overcome by packaging the antibiotic with an enzyme that will actively degrade these metabolites, reducing the ability of nearby bacteria to become acquainted with them. Tarkkanen et al (2008) looked at the feasibility of administering a penicillin antibiotic intravenously with a concurrent oral dose of a recombinant beta-lactamase and demonstrated a decrease in ampicillin-induced changes in intestinal microflora.

New antibiotic targets

Given the recent increase in our understanding of how biofilms make antibiotic delivery so haphazard, it makes sense to see if we can prevent the formation of the biofilm in the first instance. As already discussed, quorum sensing is dependent on interbacterial communication using sense molecules such as acyl homoserine lactone, so efforts are being made to prevent acyl homoserine lactone production, blunt its bioavailability or block its receptor interaction. Antibodies directed against acyl homoserine lactone and acyl homoserine lactone-degrading enzymes have both been demonstrated to inhibit the activities of *P. aeruginosa* during in-vitro studies (Willcox et al, 2008), and pre-infection immunization with an acyl homoserine lactone–protein conjugate decreases mortality in mice subsequently exposed to *P. aeruginosa* (Miyairi et al, 2006).

Our understanding of how acyl homoserine lactone signalling is triggered is at an early stage, but increasing evidence is implicating cyclic diguanylmonophosphate in both transcription of virulence factor genes and regulation of molecules involved in biofilm production (Hassett et al, 2010). Specific phosphodiesterase enzymes that degrade cyclic diguanylmonophosphate may therefore have the potential to disrupt quorum sensing and biofilm production (Sintim et al, 2010). These findings could provide cystic fibrosis clinicians with a number of exciting possibilities for therapies, both in the window before *P. aeruginosa* infection is established, and then in disrupting quorum sensing and subsequent biofilm production once *P. aeruginosa* has been cultured from the airway.

New antimicrobial approaches

To persist in a biofilm, *P. aeruginosa* has to develop the ability to metabolize under anaerobic conditions without creating toxic metabolites that will kill both itself and its neighbours, and it does this by transcribing and activating nitric oxide reductase. Bacteria that do not have the ability to reduce nitrite ions will die under anaerobic conditions (Yoon et al, 2002) as a result of overproduction of endogenous nitric oxide. A novel approach against *P. aeruginosa* when it is in biofilm is therefore to flood it with nitrite ions, overcoming the nitric oxide reductase and causing cell death. Major et al (2010) demonstrated that an acidified preparation of sodium nitrite ($NaNO_2$) kills not only the mucoid form of *P. aeruginosa*, but also *Staphylococcus aureus* and *B. cepacia complex* organisms via the generation of NO_2- ions.

There has recently been a resurgence of interest in antibacterial therapies that use bacteriophages, viruses that specifically target bacteria. These were first identified in 1917 and used to treat a variety of infectious diseases until the emergence of antibiotics in the early 1940s. Their use continued in the countries of the Eastern bloc during the second half of the 20th century. As more and more pathogens emerge that are resistant to the current model of antibiotic therapies, 'phage' are receiving more interest. Carmody et al (2010) have demonstrated the ability of phage to decrease inflammation and bacterial load in a murine *B. cepacia* infection model. A lytic phage against *P. aeruginosa* has been identified and has demonstrated promising results against *P. aeruginosa* in a mouse lung model (Morello et al, 2011). Lytic phage are therefore perhaps promising new candidates for antipseudomonal therapy in cystic fibrosis and related disorders involving chronic *Pseudomonas* infection.

There are two monoclonal antibody-based antipseudomonal immunotherapeutic agents currently in development. Fab 1A8, a human-derived monoclonal antibody against the *Pseudomonas* type 3 exotoxin secretion system, has been demonstrated in a mouse model to decrease mortality in the face of a lethal dose of *P. aeruginosa* (Baer et al, 2009), and panobacumab, a human monoclonal anti-lipopolysaccharide IgM targeted against

Pseudomonas serotype O11, is reported to increase survival in adults with nosocomial *Pseudomonas* pneumonia in the intensive care setting (Lu et al, 2011). Immunoglobulin from chicken egg yolks (IgY) has been shown in an early phase case-control study to delay *Pseudomonas* colonization in cystic fibrosis patients (Kollberg et al, 2003), and a phase III trial is anticipated.

Unknown unknowns

Our current understanding of the micromilieu of the lungs of patients with cystic fibrosis has been challenged over the past decade by two main findings; the discovery of the unique properties of *P. aeruginosa* once it genoconverts to an anaerobic environment, and the realization that many more species of bacteria may be found in the lungs of patients with cystic fibrosis as novel microbiological tools increase in sensitivity. Degand et al (2008) used matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify non-fermenting Gram-negative bacteria in the lungs of patients with cystic fibrosis and isolated 549 strains of bacteria from just over 100 patients. As well as the common bacteria (400 strains of *P. aeruginosa* and 54 strains of *A. xylosoxidans* were isolated), species such as *Ralstonia*, *Bordatella*, *Inquilinus* and *Cupriavidus* were identified.

Establishing the pathogenicity, virulence or indeed clinical significance of organisms as yet unheralded in the mainstream cystic fibrosis literature represents a major challenge to our future understanding of the microbiological strategies required in this illness; should we eradicate *P. aeruginosa* and remove a major competitor from the cystic fibrosis airway allowing further Gram-negative species to thrive? And what is the complex interplay between neighbouring biofilms comprised of different species of bacteria? If we impair quorum sensing for one population then will a competitor simply emerge with as yet unknown clinical consequences?

Conclusions

The expansion in our knowledge about the environment of *P. aeruginosa* in the lungs of patients with cystic fibrosis and the physical barriers it creates provides a number of targets for emerging antibiotic therapies, be they simply new inhalational antibiotics or novel drug delivery mechanisms such as liposomes. As we unravel the complex molecular interplay both on the intracytoplasmic and intermicrobial level that determines quorum sensing and biofilm behaviour, new targets for modification and attenuation of these processes are unveiled, and novel drug therapies such as anti-acyl homoserine lactone strategies may be designed. Finally, approaches ranging from simple oxidative stress by nitrite salts through to species-specific lytic phage and targeted monoclonal antibodies may provide those caring for children and adults with cystic fibrosis with a new range of treatments for the future. **BJHM**

KEY POINTS

- *Pseudomonas aeruginosa* is able to make structural and genetic adaptations once inside the lung of a person with cystic fibrosis in order to establish infection.
- Once infection is established, the bacteria form a biofilm to protect the colony from the immune response.
- Novel antipseudomonal therapies can be targeted at the quorum sense molecules that control biofilm formation, and packaged in molecules designed for biofilm penetration.
- Lytic phage and monoclonal antibody may be the 'next wave' of antipseudomonal therapies.

Conflict of interest: none.

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