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CFTR AND PSEUDOMONAS INFECTIONS IN CYSTIC FIBROSIS

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TABLE OF CONTENTS

1. ABSTRACT

Pseudomonas aeruginosa is a significant threat to human health as it is frequently recalcitrant to conventional antibacterial therapy. This ubiquitous gram-negative bacterium is notorious for its nutritional and ecological flexibility and its resistance to both antibiotic treatments and sanitary measures. These properties contribute to its prominence as a leading source of opportunistic nosocomial (hospital acquired) and a less appreciated, but significant cause of community acquired infections. *P. aeruginosa* remains a considerable problem for patients with burns, neutropenic individuals, and cystic fibrosis patients (CF). In this review, we will address the current issues in *P. aeruginosa* infections in CF. A major emphasis will be placed on the factors predisposing CF patients to colonization with *P. aeruginosa*.

2. *P. AERUGINOSA* AND RESPIRATORY COMPLICATIONS IN CYSTIC FIBROSIS

The major cause of high morbidity and mortality in CF remain the chronic respiratory infections with *P. aeruginosa* (1). CF is the most common lethal genetic disorder among Caucasians in the United States and worldwide. Presently, approximately 30,000 children, adolescents, and adults in the United States are affected by this disease, as reported on the Cystic Fibrosis Foundation Web site (http://www.cff.org). The median age of survival is 32.3 years of age (2). A variety of symptoms, the most common ones being salty-tasting sweat and skin, persistent cough, wheezing, and failure to thrive (due to intestinal defects, malnutrition and anorexia) characterize the disease. The usual complications in the respiratory tract are: hemoptysis (blood in the sputum); pneumothorax (collapsed lung); atelectasis (air resorbtion leaving the lobe or segment airless) caused by complete mucus plugging; dilated bronchioles and bronchi and weakened bronchioles and bronchi walls; fibrosis (scar tissue); and low oxygen levels. Respiratory failure in CF is usually at the end of a long process where frequently there is no longer enough healthy lung tissue left to eliminate CO_2 (3). It is widely believed that the respiratory sequelae in CF and progressive deterioration of respiratory function are the result of persistent bacterial colonization (culminating with chronic *P. aeruginosa* infections) and chronic inflammation. According to a comprehensive report by Fitzsimmons (4), incidence of *P. aeruginosa* infection among CF patients in the age group over 26 years exceeds 80%. The relationship between *P. aeruginosa* and CF has been the subject of numerous studies but the reasons for this unusually firm association are not completely understood. In subsequent sections, some of the underlying causes and possible explanations for the predilection for bacterial infections in CF, in particular the persistent infections with *P. aeruginosa*, are discussed. However, it is essential to remember that *P. aeruginosa* is not the only pathogen in CF, and that its prevalence in CF is not based solely on predisposing genetic and physiological factors. The association is also based on the unique recalcitrance of this organism to antibiotic treatments, its evasion of immune mechanisms possibly related to the biofilm mode of growth (5) and the selection of mucoid variants over time in the CF lung (1).

3. BIOFILM FORMATION

In the environment, *P. aeruginosa* preferentially exists as a biofilm, but the types of the exopolysaccharides synthesized in ecological sites are not currently known (6). Biofilm development involves microcolony formation and these have been observed in the lungs of CF patients (5) and have been seen in transmission electron micrographs of the sputum (7). A recent study supports the notion that *P. aeruginosa* grows as a biofilm in the CF lung since the ratio of two homoserine lactones in patient sputum (N-(3-oxododecanoyl)-L-HSL and N-butyryl-L-HSL) resembles the *in vitro* ratio seen in laboratory grown biofilms (7). Biofilms have been regarded as a protective mode of growth because the organisms are inaccessible to phagocytic cells (8) and possibly antibiotics (7). Biofilms may also confer antibiotic resistance properties either by forming a polyanionic shield (9) or providing an environment that favors slow metabolism and growth of the bacteria relative to the free swimming planktonic form (10). Recent genetic studies of biofilm development (11) have uncovered a potential link between biofilm formation and quorum sensing systems which affect expression of other virulence factors (elastase, exotoxin A, alkaline protease) in *P. aeruginosa*.

Possibly independent of the "natural" biofilm mode of growth, presumed to take place during initial infections, is the second phase of infection in which mucoid (exopolysaccharide alginate overproducing) mutants of *P. aeruginosa* are selected in the CF lung (1). The mucoid mutants of *P aeruginosa* produce copious amounts of exopolysaccharide independent of the adherence to the substrate (*i.e.* even when grown as planktonic forms) or other conditions normally required for biofilm formation. Thus, mucoid *P. aeruginosa* mostly likely exist in CF in a different type of biofilm than biofilms seen in various ecological niches with wild type, non-mucoid organisms. It is important to note that serious clinical deterioration (12, 13) usually coincides with conversion to mucoidy (*e.g.* selection of *muc* mutants which overproduce alginate) which occurs at various times upon the initial colonization with nonmucoid strains (13-16) It is not known whether the relatively high densities of mucoid *P. aeruginosa* (as sometimes seen in the lumen of the respiratory tract in CF) and its conventional toxins or some as yet unidentified products, expressed only by mucoid mutants, play a role in increased inflammation and lung damages associated with the emergence of mucoid strains.

4. ESTABLISHMENT OF CHRONIC INFECTIONS IN CF AND CONVERSION TO MUCOIDY IN *P. AERUGINOSA*

With age, *P. aeruginosa* becomes the predominant colonizer in the lungs of more than 80% of CF patients (4). The establishment of chronic *P. aeruginosa* infection is associated with a poor prognosis for CF patients (14) and the infection is biphasic. The first phase (0-5.5 years) consists of intermittent infection of *P. aeruginosa* in the lungs and seroreactivity to no more than two *P. aeruginosa* antigens, but this phase does not cause significant decline in pulmonary function (12,15). The next stage is characterized by a chronic and permanent infection, which coincides with the conversion of *P. aeruginosa* to mucoid phenotype (12, 13). Throughout this phase, *P. aeruginosa* can be continuously isolated for at least 6 months, seroreactivity to *P. aeruginosa* antigens is increased, and the presence of the same strain can be documented for years by fingerprinting techniques (1, 13, 15).

Mucoid isolates produce exopolysaccharide alginate, a liner polymer of (1→4)-linked β-D-mannuronic acid and its C-5 epimer α-L-guluronic acid. These isolates are primarily found in CF lungs, but can be isolated during chronic urinary tract infections (17, 18). Alginate is a highly hydrophilic non-toxic compound that forms a capsule-like matrix in the presence of calcium. All aspects of the expression of the alginate system have been extensively reviewed (1). Inactivating point mutations in the gene *mucA*, negative regulator of the alternative sigma factor AlgU, cause the switch from nonmucoid to mucoid form in 80% of all mucoid strains (1, 17, 19-21). When AlgU is no longer inhibited by *mucA*, transcription of the alginate biosynthetic genes is no longer repressed and alginate is overproduced causing mucoid phenotype.

P. aeruginosa persistence in the CF lung depends on alginate production (17). Studies carried out *in vitro*, supporting the role of alginate in pathogenesis, have been extensively reviewed (1). Alginate may prevent uptake by phagocytic cells via effective opsonization or may play a role in adhesion and antibiotic resistance in the context of a biofilm. Aside from the presumed selective pressure favoring the emergence of a *mucA* mutation in the CF lung, experimental evidence for an *in vivo* role of alginate was missing until Boucher *et al.* demonstrated that non-mucoid *P. aeruginosa* are cleared less efficiently from the murine lung (17). More recent directions (Firoved and Deretic, unpublished) are focusing on understanding what other *P. aeruginosa* genes are upregulated in *mucA* mutant *P. aeruginosa*, as the genes could contribute to increased inflammation and clinical deterioration associated with conversion to mucoidy (12, 13).

5. CFTR AND THE GENETIC BASIS OF CYSTIC FIBROSIS

CF is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a protein that functions as an apical membrane chloride channel (22). Various CFTR mutations affect the processing, intracellular localization, and function of this protein. Mutations in CFTR also have pleiotropic effects on the function of other ion transport processes (e.g. amiloride-sensitive sodium channel, the outwardly rectifying chloride channel, the Na/H+ exchanger, and bicarbonate conductance) (23). In addition, genetic modifiers within and outside of the CFTR locus could provide an explanation for the observation that CFTR genotype does not always correlate with disease severity in both mice and humans (24).

The most common mutant *CFTR* allele, deltaF508 probably arose in an individual over 50,000 years ago and this individual's progeny now number between 20-50 million offspring (25). Although the deltaF508 mutation is the most common alteration (accounting for 70% of the cases), there are more than 950 CF-causing alleles of *CFTR* described thus far (24). At least two theories have been proposed to explain the emergence of CF. Individuals with one mutant CFTR allele have been suggested to be more resistant to cholera toxin (26) and to *Salmonella* infections (27). However, this issue is still considered controversial and unresolved.

6. UNDERSIALYLATION OF EPITHELIAL GLYCOCONJUGATES AND INCREASED ADHERENCE OF *P. AERUGINOSA* TO CF RESPIRATORY EPITHELIUM

Several studies have suggested that glycoproteins and glycolipids on the apical membranes of CF cells are undersiaylated and that expression of deltaF508 in heterologous cells results in decreased sialylation of glycoconjugates on the plasma membrane (28). One hypothesis to explain the altered sialylation is that CFTR could be playing a role in facilitating acidification of the *trans*-Golgi network (TGN), by providing negatively charged chloride and maintaining charge neutrality as protons are pumped into the lumen of these organelles (29). Like most enzymes, TGN sialyltransferases have a pH optimum and if the loss of *CFTR* results in an increased compartmental pH in the TGN then this could cause a reduction in the sialylation of glycoconjugates. However, several studie**s** have failed to detect reduced acidification of TGN (30). Despite the publication of several analyses regarding pH in CF cells, there is presently no consensus on this issue. *CFTR* may also be involved in membrane trafficking as it associates with endosomes (31) and appears to play a role in endosome fusion (32). In this model, CFTR is a putative regulator of vesicular trafficking and could affect the time that membrane proteins spend in relevant compartments, such as TGN, where they get exposed to sialyl transferases. Regardless of the exact mechanism, the increased levels of asialoganglioside 1 (aGM1), and potentially undersialylated glycoproteins (33), on CF epithelial cells promote adherence and colonization with *P. aeruginosa* (34, 35). *P. aeruginosa* pilin and whole cells (34, 35) bind better to aGM1 than to the sialylated ganglioside GM1 (36). Despite some controversy regarding *P. aeruginosa* adhesion to aGM1, the issue of TGN function remains critical to understanding infections in CF.

7. REDUCED UPTAKE OF *P. AERUGINOSA* BY CF RESPIRATORY EPITHELIAL CELLS

In a recent, thought provoking model (37), *P. aeruginosa* is perceived as binding to the first extracellular loop of CFTR, after which it is taken up by the epithelial cells, and eliminated from the respiratory tract via cell desquamation. In this scenario, deltaF508 CFTR, which is not properly folded and remains trapped in the ER, never traffics to the apical plasma membrane thus eliminating the receptor for *P. aeruginosa* uptake by epithelial cells. If one assumes that uptake of the bacteria is part of a clearance mechanism, then the absence of CFTR in the plasma membrane could result in inefficient elimination of *P. aeruginosa*. According to some views, this proposal is controversial due, among other issues, to the preferential patterns of *P. aeruginosa* uptake *in vitro* via the basolaterlal plasma membrane domain while CFTR is mostly present in the apical membrane. More studies are needed to prove or refute this model.

8. ALTERED AIRWAY SURFACE LIQUID AND IMPAIRED *P. AERUGINOSA* KILLING

The composition of airway surface liquid (ASL) is believed to be altered in CF lungs relative to non-CF lungs. The alteration may inactivate some endogenous anti-microbials (e.g. defensins) thus promoting infection and lung pathology. Recent work suggests that CF nasal secretions are active against some organisms, but less active against *P. aeruginosa* than normal secretions (38). ASL composition has ramifications beyond CF as nasal secretions from non-CF donors have been found to contain several antimicrobial compounds capable of decreasing bacterial load in the nasal passages, but *S. aureus* nasal non-CF carriers were found to have less microbiologically active nasal fluids (39).

The electrolyte content of the ASL is predicted to be different (e.g. 170 mM chloride in CF vs. the normal 85 mM (40)) because the mutations in CFTR create a defective chloride channel that is unable to transport chloride properly. Based on the premise that salt concentration is increased in CF airway fluids, as observed in CF sweat secretions, several reports (41-43) have indicated that there may be a link between the electrolyte imbalance and infections in CF. According to other reports (44, 45), the volume, but not the electrolyte composition of the airway surface fluid, appears to differ between normal and CF respiratory epithelia. Thus, the proposals that increased salt in CF lung fluid interferes with the action of antimicrobial peptides awaits the resolution of this controversy.

9. REDUCED INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) AND NITRIC OXIDE (NO) PRODUCTION IN CF AND *P. AERUGINOSA* COLONIZATION

Although results have varied, nasal NO production in CF patients is reduced relative to non-CF control patients and orally exhaled NO output in CF patients is lower in comparison to patients having other inflammatory conditions (46). iNOS is constitutively expressed in normal airway epithelial cells (47, 48) and its induction, likely in neutrophils, can be detected in submucosal inflammatory cells (49). In contrast, both CF human airway epithelial cells and the *CFTR*-mutant transgenic murine airway epithelial cells are deficient in iNOS expression (49, 50), although the iNOS gene is intact. Reduced NO levels have been linked to the hyperabsorption of sodium in CF (50). Guanylate cyclase

(cGMP) is stimulated by NO, and, as a result, likely downregulates sodium absorption mediated by the epithelial sodium channel ENaC. In CF respiratory epithelia, a reduction in NO production results in diminished cGMP and contributes to increased sodium absorption, which is already altered because of disrupted interactions between CFTR and ENaC (51). As a result, the ASL is absorbed more in CF epithelia, contributing to altered mucus hydration and impaired mucocilliary clearance.

Nitric oxide has been implicated as bactericidal and bacteristatic agent (52) and the reduced NO output of CF patients has been implicated in susceptibility to *P. aeruginosa* (50). In the latter study, *P. aeruginosa* was cleared less efficiently by lungs of normal mice treated with S-methylisothiourea, an inhibitor of iNOS, suggesting the involvement of NO in bacterial clearance (50). *In vitro*, *P. aeruginosa* was sensitive to an NO-releasing compound, sodium nitroprusside, and the anti-bacterial activity of this compound was abolished by the addition of the NO scavenger N-methyl-D-glucamine dithiocarbamate (50). Furthermore, iNOS knockout animals display a measurable, albeit not dramatically reduced, drop in clearance of *P. aeruginosa* in an aerosol infection model (53).

10. INFLAMMATION IN CF

Polymorphonuclear leukocytes dominate the chronic inflammation and respiratory tissue destruction that are hallmarks of CF (54). The anti-inflammatory drug ibuprofen slows inflammation-induced deterioration of lung function (55). Bronchoalveolar lavage (BAL) fluid of CF patients shows elevated levels of pro-inflammatory cytokines TNF-alpha, IL-1 and IL-8 and reduced amounts of the anti-inflammatory cytokine IL-10 (56). IL-10 plays a critical role in preventing excessive damage to host tissues in an aerosol infection model. Repeated exposure to *P. aeruginosa* aerosols resulted in increased lung pathology in IL-10 knockout mice relative to control animals (57). In addition, BAL fluids of children aged between 3 months to 7 years with CF and minimal or no clinical lung disease displayed increased levels of neutrophils, IL-8 and TNF-alpha suggesting that spontaneous inflammation exists in CF patients prior to microorganism colonization (54, 57). However, it is difficult to prove that the individuals enroled in that study have not been exposed to any infectious agents.

The connection between *CFTR* mutations and the hyper-excitability of the immune system is not known. One hypothesis suggests the involvement of NFkB (a transcriptional factor controlling expression of several pro-inflammatory cytokines) (58), since exogenous stimuli appear to activate NFkB in CF epithelial cells above levels observed in normal epithelial cells. However. the presence of serum in other studies may have skewed the results. Current studies suggest that basal levels of NFkB may not differ in CF vs. non-CF tracheo-bronchial tissue (59), so the role of NFkB remains to be resolved.

Another hypothesis for the apparent hyper-reactivity of CF cells is an ER-retention and ER overload response (60) generated by a mutation that blocks processing of CFTR molecules. Intracellular adhesion molecules (ICAM-1) expression could also contribute to the recruitment and accumulation of neutrophils. In addition, T cells from CF patients may be defective in IL-10 production (61), supporting the observation that normal lung cells produce more IL-10 than CF lung cells (56). The imbalance between pro- and anti-inflammatory cytokines in CF has been shown to upregulate NFkB (58) and IL-8, leading to tissue damage, but the intrinsic cause is not yet known.

11. MODELS OF RESPIRATORY INFECTION IN *CFTR* TRANSGENIC MICE

The *CFTR*-knockout mice, while displaying many characteristics of intestinal disease in CF, fail to develop respiratory infections or other signs of overt lung disease. This disappointing limitation of the *CFTR* transgenics, that have been expected to provide a model for infection and inflammation in CF, has been linked to the presence of alternative Cl^- and Na^+ channels in mice that could compensate for the lack of a functional *CFTR* (62). When the mutant $CFTR^{m1UNC}/CFTR^{m1UNC}$ (carrying the S489X nonsense mutation (63)) has been backcrossed repeatedly into a C57BL/6J background, the resulting congenic mice (64) (strain designation C57BL/6J $CFTR^{m1UNC}/CFTR^{m1UNC}$) have developed some features of respiratory disease in CF: e.g. acinar and alveolar hyperinflation has been noted, possibly secondary to a build up of mucus causing ball valve obstruction effect. These effects, seen in the congenic mice, have been attributed to a loss of epithelial response to UTP, an agonist of P_{2U} type purinergic receptor causing IP_3 production and associated release of Ca^{2+} intracellular stores. As manifested by the lack of nasal potential difference response in congenic mice upon stimulation by UTP, it is possible that these phenomena are related to a loss of the BAPTA-inhibitable Ca^{2+}-activated Cl^- channel. The congenic mice also developed detectable fibrosis and early increase in neutrophils which subsided with time, while the modest increase in the number of interstitial macrophages and fibroblasts remained statistically significant with age. However, no spontaneous colonization with the typical CF pathogens, including *P. aeruginosa*, have been observed in congenic animals.

Several studies (65, 66) have been reported using *CFTR* knockout mice carrying the S489X mutation ($CFTR^{m1UNC}$ (63)) and forced *P. aeruginosa* infections via instillation of bacteria-containing agar beads into the respiratory tract. In one of these reports, no differences in *P. aeruginosa* CFU recovered from the lungs have been noted between *CFTR* and normal mice (66), but excessive inflammation was observed in the *CFTR* knockouts. In the other report (65), similar lung pathology was observed in both groups of mice, but the CF mice showed increased *P. aeruginosa* CFU recoverable from the lung. The differences between these two reports can be ascribed to the use of congenic animals in the latter study. Both groups of investigators have nevertheless observed increased mortality among CF mice infected with *P. aeruginosa*

embedded in agar beads (58-82% mortality in CF vs. 12-23% mortality in controls), possibly reflecting excessive inflammatory response in CF transgenics. The excessive inflammatory response has been validated by the reported two-fold increase in MIP-2 and KC, the putative murine functional equivalents of human IL-8. TNF-alpha was also increased in the lungs of infected CF animals relative to colonized controls. However, the overall increase in inflammatory cells in infected *CFTR* animals was relatively modest compared to infected normal controls (65), although, as expected, the percentage of neutrophils in bronchoalveolar lavage fluid went from the very low fraction of less than 5% to over 40% in infected animals.

In our own studies (53) using aerosol delivery model (57), we have observed peculiar variability among CF knockout mice in their ability to clear *P. aeruginosa*. For example, we noted that $CFTR^{m1UNC}/CFTR^{m1UNC}$ either clear or do not clear *P. aeruginosa* depending upon their nutritional status. Furthermore, the FABP-hCFTR $CFTR^{m1UNC}/CFTR^{m1UNC}$ bitransgenic mice (FABP stands for the Fatty Acids Binding Protein promoter expressed in intestinal epithelial cells), which have their gastrointestinal defect corrected (67), clear *P. aeruginosa* from the lung quite efficiently. In pursuit of the potential relationship between nutrition and pulmonary clearance of *P. aeruginosa* in CF, we have recently shown that malnourished normal mice show reduced capacity to clear *P. aeruginosa*. This defect can be promptly restored by placing the malnourished mice on complete and well balanced diet. In keeping with our observations, the severity of intestinal disease is intensified in back-crossed congenic C57BL/6J $CFTR^{m1UNC}/CFTR^{m1UNC}$ animals vs. $CFTR^{m1UNC}/CFTR^{m1UNC}$ mice, which correlates with signs of pulmonary disease in the congenics (64). In another study (65), congenic C57BL/6J $CFTR^{m1UNC}/CFTR^{m1UNC}$ mice that showed a 10-fold increased burden in *P. aeruginosa* cfu were also 25-30% underweight relative to C57BL/6J controls. Although the authors of that study reported no correlation between animal weight and *P. aeruginosa* clearance, the same animals were used to establish higher *P. aeruginosa* numbers in CF lungs and all *CFTR* transgenic mice, without exceptions, were of lower body weight than any of the normal controls. Based on these considerations and our own observations with malnourished animals, we propose that the unusual susceptibility of the CF lung to bacterial infections, including *P. aeruginosa*, may be secondary to intestinal problems and the generally poor nutritional status of individuals with CF. This proposal is further substantiated by the fact that aggressive nutritional intervention improves the clinical outlook for CF patients (68).

12. *IN VITRO* MODELING AND FUTURE DIRECTIONS

The literature reveals that there are a great variety of methodologies that can be used for studying the cell biology and cellular microbiology of CF. There is a distinct set of questions that need to be addressed, ranging from methodological to strategic points of interest. Primary cells, rather than immortalized tissue culture cells, are likely to resemble most closely what is actually occurring within a patient, but primary cells are more difficult to work with and the heterogeneity of samples could complicate data analysis. When choosing an immortalized cell line, the question to be asked may dictate the cell line. For example, MDCK cells, which do not express CFTR *in vivo*, have been studied extensively, but can conclusions based solely on experimental work with these cell lines be applied to the less extensively studied CF bronchial epithelial cell line IB3-1? Can the basic membrane and protein trafficking experiments performed in the less defined IB3-1 cell line be as informative as those performed in the well-defined MDCK cell line? For microbiological assays, will the growth methods of the microorganism affect the results? Could DNA microarrays provide an avenue for exploring differential virulence factor gene expression *in vivo* and *in vitro*? Will *P. aeruginosa* genomics (http://www.pseudomonas.com) and proteomics provide a decisive advantage in treating patients infected with this pathogen? Are better vaccines, antibiotics, and delivery systems the answer? Can better animal and tissue culture models of CF be developed to faithfully represent infection and inflammation in CF? These types of questions, among others, are the motivating force behind contemporary CF research.

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