Biofilms in Obstructive Lung Disease

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Internal Medicine Grand Rounds
UT Southwestern Medical Center
June 24, 2004

"Tell me your friends and I'll tell you who you are"
- Assyrian Proverb

"It is better to be alone than in bad company"
- Ralph Waldo Emerson

This is to acknowledge that Jonathan C. Weissler M.D. has disclosed financial interests or relationships with commercial concerns related directly or indirectly to this program. Specifically Dr. Weissler has received honoraria or consulting fees from Abbott Labs, Pfizer, and Aventis who manufacture antimicrobials which are mentioned in this protocol. Dr. Weissler will be discussing off-label uses of macrolide antibiotics in his presentation.

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Introduction

In contrast to many other body compartments the respiratory tract is chronically exposed to a variety of microbes including bacteria, mycobacteria and fungi which are disposed to produce either colonization or chronic infection. In particular a variety of obstructive lung diseases including cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease are characterized by colonization with well defined species of bacteria. Although the role of bacteria in producing symptomatic exacerbations of these diseases has been appreciated for many years most attention has focused on therapies against specific microbes recovered from patients with these diseases as defined by in vitro response to anti-microbial agents. Recently the concept of bacteria operating as communities on mucosal surfaces rather than as single (planktonic) organisms has gained widespread attention. The coalescence of multiple bacteria, either of the same species or with other pathogens, into a **biofilm** capable of adhering to epithelial cells, indwelling catheters, or heart valves is now well recognized as an important clinical event(16, 34, 71). Experience with several forms of obstructive lung disease suggests that biofilms may be important contributors to the clinical spectrum of these diseases which is often characterized by chronic cough, mucus production, microbial colonization, and progressive airways obstruction.

What's a biofilm ?

Bacteria may exist in both a free floating (planktonic) state or may adhere in communities called biofilms. Bacteria within a biofilm excrete sugar polymers called exopolysaccharides to form a three dimensional matrix which is adherent to either a mucosal or synthetic surface in vivo (13). Biofilms are also frequently found *ex vivo* in bodies of water and are a major problem in water treatment facilities or reservoirs(8, 71). Biofilms have been found in fossils from 3 billion years ago suggesting that they were an early adaptation in the development of bacteria(46).

Figure 1 Microstructure of a biofilm showing mushroom like towers and aqueous channels for water flow (17)

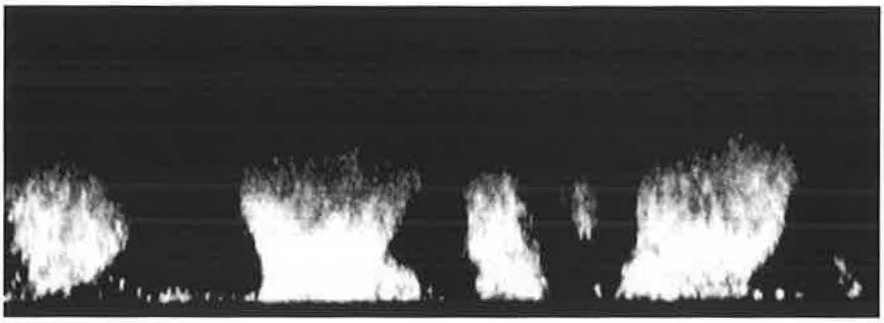


Figure 2: Mushroom-like towers growing in a pseudomonas aeruginosa biofilm (43)

Many bacteria associated with obstructive lung disease including *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus,* and *Haemophillus influenzae* are capable of producing biofilms. In general biofilms are produced as a response to a particular microenvironment and involve a complex series of inter-cellular communications (see below). Analyzed on a genomic level only 1% of PA genes showed differential expression in a biofilm state; about 0.5% of genes were activated while 0.5% of genes were repressed(99). Some of the genes relate to antibiotic resistance while others relate to the biofilm itself. One type of biofilm called a pellicle, occurs at an air-liquid interface. Recently a group of seven adjacent genes, designated *pel* genes, have been identified in a

PA strain (PA14). These genes produce a cellulase- sensitive extracellular matrix composed primarily of glucose. *Pel* mutants were unable to produce the exopoysaccharide and could not form pellicles or a solid surface-associated biofilm(37). Although alginate is the predominate exopolysaccharide produced by mucoid strains of PA nonmucoid strains of PA such as PA14 produce biofilms without alginate(104).

In contrast *staphylococcus epidermidis* species primarily produce a slime substance known as PIA (polysaccharide intercellular adhesin). PIA is composed of *B*-1,6-linked *N*-acetylglucosamines with partly deacetylated residues(42). Production of PIA is controlled by a number of genes comprising the *ica* operon. Mutations in the *ica* operon lead to organisms incapable of forming biofilms. This system is complex however as biofilm-negative mutants have been isolated in whom PIA production is unaffected; hence a number of other proteins are likely necessary for forming biofilms in *staphylococcal* species. In *staphylococcus aureus* the genetic control of biofilm formation is also multifactoral, as will be discussed below. Some strains of *S. aureus* produce a PIA-like substance.

Biofilms are often characterized as one of a number of **virulence factors** that are produced by bacteria. Although virulence in this context pertains to the ability to establish infection from a clinical standpoint this term may be confusing. Bacteria forming biofilms, particularly in the respiratory tract, can be invasive but this is relatively uncommon. Indeed one of the primary characteristics of a biofilm is to promote an environment on a mucosal surface which allows for continued local propagation of a bacterial species.

Advantages of Biofilms

There are several potential advantages to bacteria related to formation of biofilms. First the three dimensional structure of the biofilm allows bacteria to be concentrated where nutrients are most plentiful and the presence of water channels within the biofilm enhances elimination of waste material (Figure 1). In conditions of high nutrient states a smooth and compact biofilm may form. In contrast when nutrients are less plentiful mushroom-like colonies form towers of biofilms (Figure 2)(12). Indeed some forms of innate host immunity appear to selectively target the nutitional milieu conducive to biofilm formation. Lactoferrin, a normal constituent of respiratory secretions, chelates iron and causes PA to "keep moving" across the eptihelial surface rather than depositing and setting up a biofilm(85). Within the biofilm cooperation between different species of bacteria may result in metabolites from one species serving as nutrients for a different species(97).

A second advantage is that bacteria contained within biofilms may be less conspicuous to both innate and humoral immune mechanisms. Antigens necessary for inducing humoral immunity may be hidden within the biofilm and ligands necessary for optimal opsonization may be repressed(70). Although the biofim itself induces inflammation the bacteria within the film are usually protected from the effects of the host response.

Another important advantage of a biofilm is that organisms within are less susceptible to anti-microbial therapy. Although early investigation focused on the physical barrier to the antibiotic imparted by the exopolysaccharide resistance in biofilms is clearly more complex. One of the best clinical examples of a biofilm infection is that which occurs in

endocarditis, where sheets of bacteria are adherent to a cardiac valve. It has long been appreciated that some antibiotics, such as second or third generation cephalosporins, with good in vitro activity against bacteria such as methicillin sensitive *Staphylococcus aureus* are inadequate as therapy against endocarditis. Although poor penetration of these drugs is often cited as the explanation for their clinical failure studies in animal models of endocarditis or other infections(2, 19, 25, 90) clearly demonstrate that these drugs penetrate into the biofilm. However a number of potential mechanisms for resistance occur within the biofilm.

Many planktonic colonies of bacteria causing biofilms, such as PA, contain efflux pumps capable of pumping antimicrobial compounds out of the bacteria or biofilm. While these pumps are present within the biofilm their role in effecting antimicrobial resistance is uncertain. Expression of genes for two multidrug (MDR) efflux pumps (MexAB-OprM and MexCD-OprJ) was analyzed in PA forming biofilms. While both were expressed their expression was not greater than in planktonic PA and was down regulated over time in the developing biofilm. Furthermore using a series of MDR mutants none of the four characterized efflux pumps in PA appeared to be responsible for resistance in a biofilm(22). Similar data have been reported in *Candida albicans* biofilms, which are highly resistant to fluconazole. Using mutants lacking one or more of the drug efflux pumps (Cdr1p, Cdr2p, and Mdr1p) found in *C. albicans* it was apparent that while these pumps contributed to drug resistance in the first 6 hours of biofilm formation they were not necessary for high level resistance at 12 or 48 hours(67). More recent data has identified a gene in PA, *ndvB*, which encodes for the synthesis of periplasmic glucans. Organisms which lack this gene are still capable of forming biofilms however they do not develop high level resistance to multiple antibiotics. The periplasmic glucans(glucose polymers) physically interact with tobramycin and may function by sequestering antimicrobial compounds in locations which are harmless to the bacteria(62). Another important mechanism by which antimicrobial resistance may occur is that the densely packed structure of a biofilm is ideal for conjugal transfer of plasmids(40, 66), some of which promote resistance to antimicrobial therapy. In addition antimicrobial therapy may have unintended effects on gene expression within biofilms. Imipenem, a commonly utilized agent against PA, causes an increase in biofilm volume in part due to over-expression of alginate when subinhibitory concentrations of the antibiotic are present in the biofilm(4).

Finally the biofilm constantly undergoes renewal and this provides a mechanism of spreading new colonization along a mucosal surface. Voids develop within PA biofilms and cells are dispersed from within these voids. Integral to this process is something akin to apoptosis of the biofilm. A repeatable process of cell death is observed simultaneously with dispersal of new viable microcolonies of PA, and may be produced by a prophage within the genome of PA(98).

How do bacteria know they are not alone in the infinite universe?

There are several mechanisms by which bacteria communicate to signal the presence of a critical mass which results in the generation of a biofilm. Chief amongst these mechanisms is a series of signals referred to as **quorum sensing (QS).** Through quorum sensing bacteria are capable of communicating with other organisms of the same species or with completely different microorganisms. The molecules secreted by bacteria in this setting are also referred to as **autoinducers** and ultimately result in target-gene expression in nearby bacteria. While the amount of quorum sensing molecules released in general parallels the number of bacteria present these systems are under regulation by a variety of factors. Quorum sensing appears to be so important that multiple systems have been described, likely suggesting that different environments or neighbors elicit different signals(33).

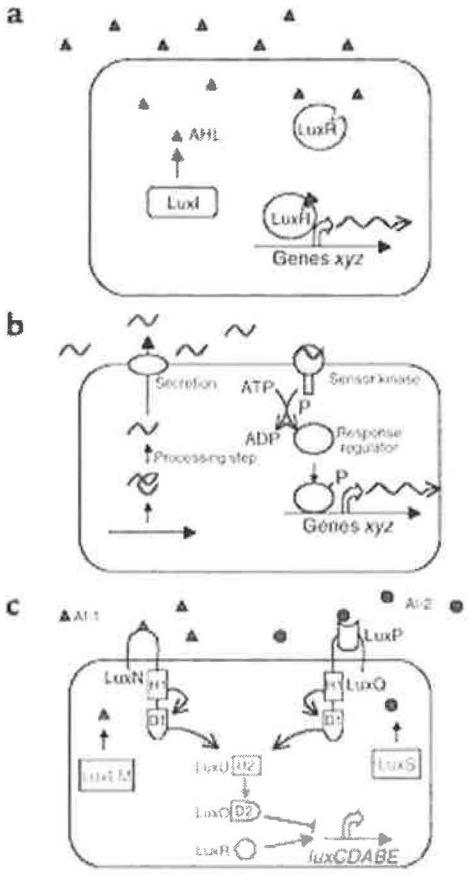


Figure 3. Three different quorum sensing circuits in bacteria. (A) Gram negatives produce AHLs (triangles) which freely diffuse across the cell membrane and bind LuxR-type proteins. (B) Gram positives synthesize oligopeptides (wavy lines) that are actively secreted, interact with a membrane bound sensor kinase and ultimately result in phosphorylation of a response regulator which acts as a transcription factor. (C) A hybrid of the two systems occurs in *Vibrio harveyi* where an AHL called AI-1 and a furanosyl borate diester called AI-2 are produced by different Lux systems than in (A). In the absence of autoinducers different Lux proteins are capable of acting as sensor kinases and cause phosphorylation of LuxO, which acts as a response regulator (33)

Different QS mechanisms exist in gram-negative and gram-positive bacteria (Figure 3). Gram negatives predominantly rely on a system producing *N*-acyl-homoserine lactones (AHL) which interact with regulator proteins of the LuxR family(15) (see below). The AHLs freely diffuse from the cytoplasm to the extracellular milieu and then into the cytoplasm of a different bacteria. The AHL then binds to a Lux-R type protein which becomes capable of acting as a transcription factor by binding to chromosomal DNA. Other systems have been described in *Pseudomonas* including the *Pseudomonas* quinolone signal (PQS) which is capable of interacting with products of the AHL system and more recently the VqsR (virulence and quorum-sensing regulator) which interacts with the LuxR regulator (54). Although AHL-LuxR pairings are relatively specific for certain species of bacteria, which limits cross-talk between species, the AHL system can also be utilized under certain conditions for inter-species communication(59).

Gram positive organisms, such as *staphylococcal* species, synthesize oligopeptides which are modified at specific amino acids and must be actively secreted. These peptides interact with surface bound kinases (sensor kinases) to phosphorylate specific proteins, called response regulators which then act as transcription factors(110). Some gram negative bacteria such as certain *Vibrio* species utilize a hybrid of the AHL/sensor kinase system to form autoinducers specifically for communicating with different bacterial species(60). In addition recent data has demonstrated that some strains of *Escherichia coli* utilize a sensor kinase pathway (RcsC) during the generation of biofilms(35). However to date there are no gram positive organisms that have been found to utilize the AHL system. Because QS mechanisms are thought crucial to the formation of biofilms and offer opportunities for novel therapies it is important to concentrate on QS in specific bacteria important in obstructive lung disease.

Quorum Sensing and Biofilms in *Pseudomonas aeruginosa*

PA is a common bacteria in the respiratory tract of patients with cystic fibrosis, diffuse panbronchiolitis and bronchiectasis. There are two AHL systems, utilizing different AHLs and LuxR protiens, which have been well characterized in PA(88). The *las* system consists of the LasI synthase protein which produces the AHL *N*-(3-oxodododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL) which interacts with the LasR transcriptional regulator. Only the multimeric form of LasR can bind DNA and serve as a transcription factor. Importantly changes in nutrients, oxygen concentration, or other environmental conditions appear to have a marked influence on the genes regulated by QS pathways(95).

The second AHL system in PA is the *Rh1* pathway. The Rh1 synthase produces the AHL *N*-butanoyl-L-homoserine lactone (C4-HSL) which interacts with the Rh1R transcriptional regulator. The *las* system exerts a control on the *Rh1* system, in part owing to the slower diffusion of 3O-C-12-HSL out of the target cells cytoplasm. Indeed a specific efflux pump is involved in clearance of the *las* AHL from the target cell(74).

Experimental models have clearly demonstrated the importance of QS in both acute and chronic lung infection with PA(73, 80, 87, 105). Mutants lacking functional las/Rh1 were less virulent, produced less inflammation, less biofilm, and were more easily cleared by the host (Figure 4).

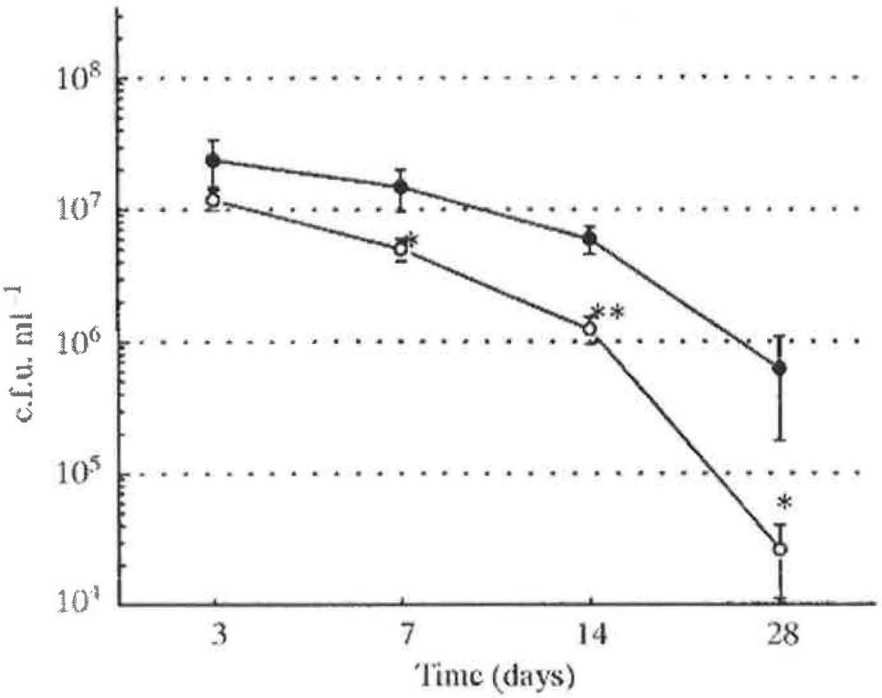


Figure 4. PA mutants lacking functional las/Rh1 (open circles) were cleared more efficiently than wild type strain PAO1 (closed circles) from a rat model of chronic PA lung infection(105)

Given the importance of the QS system in PA (and other bacteria) it is not surprising that innate immunity exists against the AHL system in plants, bacterial species, and mammals(48). The seaweed *Delisea pulchra* remains free of slime from biofilms despite existing in water with high counts of bacteria. Coating the surface of this seaweed are molecules called **furanones**, which share the same structure as the AHLs and compete for binding on LuxR proteins(63). A soil species of *Bacillus* produces an enzyme, coded by the *aiiA* gene, which hydrolyzes the lactone ring of AHLs (24) thus preventing the formation of biofilms by other organisms. Finally differentiated human respiratory epithelial cells inactivate the AHL products of the *las* system but not the *Rh1* system (14), suggesting that innate immunity limits the colonization of the respiratory tract by PA under normal circumstances.

The production of QS inhibitors by biotechnology companies is an area of burgeoning interest(50). Naturally occuring furanones have little effect on quorum sensing in PA(51).

In contrast synthetic furanones have been developed which are capable of inhibiting QS in PA leading to dysfunctional biofilms, increased susceptibility to antimicrobials, and inhibition of virulence factors including antibiotic efflux pumps(52, 53). In a mouse model of PA pulmonary infection the administration of furanones for 3 days resulted in a significant reduction in bacterial load(106). Unfortunately the toxicity of halogenated furanones, which have the greatest effect on the AHL system, appears to be significant.

At present the only commonly utilized agents with activity against the AHL system are the macrolide antibiotics. These drugs contain either a 14-(erythromycin/clarithromycin), 15-(azithromycin), or 16- member (josamycin) lactone ring. All macrolides inhibit protein synthesis; the 14- and 15- member macrolides appear to inhibit AHL synthesis in PA(93). Macrolides appear to affect both the *las* and *Rh1* pathways and decrease biofilm formation by some strains of PA(32). Whether the beneficial properties of macrolides in some patients with cystic fibrosis and chronic colonization with PA relates to the effect on QS mechanisms is unclear (see below). Nevertheless response to macrolides in patients with obstructive lung disease, as will be discussed in a latter section, may be a marker for identifying biofilm-related diseases.

Quorum Sensing and Biofilms in *Staphylococcal* species

Staphylococcal species are frequently found colonizing the repiratory tract in patients with bronchiectasis and diffuse panbronchiolitis. The *S. aureus* quorum sensing signal system is encoded by the accessory gene regulator (*agr*) locus and provides a different perspective on the role of QS systems in biofilm formation compared to the situation observed in *Pseudomonas*(109). The *agr* system is important in infections such as osteomyelitis and endocarditis but the relationship to biofilm formation is complex. Environmental factors, the site of infection, and the strain of S. aureus studied appear to impact biofilm formation. Other regulatory elements such as staphylococcal accessory regulator (SarA) are also involved in regulating production of the *agr* derived products RNAII and RNAIII, which serve as the effector molecules of the locus. Many *agr* mutants are still able to form biofilms and *agr* expressing strains form biofilms only under specific conditions. In addition the *ica* operon which is responsible for biofilm formation in *S. epidermidis* is expressed in virtually all *S. aureus* strains(18) which do not produce biofilms.

Coagulase negative *staphylococcal* species (CoNS) such as *S. epidermidis* frequently form biofilms(70). Interestingly vancomycin resistant CoNS appear to be at a selective disadvantage in terms of forming biofilms(45), which may explain the relative paucity of such isolates in clinical medicine. Consistent with this observation vancomycin by itself does not appear to affect biofilm formation by CoNS. However macrolide antibiotics and rifampin (which also affects ribosomal function and protein synthesis) appear to be beneficial in treating biofilms formed by CoNS when utilized with vancomycin(75).

Quorum Sensing and Biofilms in *Haemophilus influenzae*

Nontypeable *haemophilus influenzae* (NHI) is a gram-negative bacteria and a common colonizer of the upper and lower respiratory tract. Invasive infection from NHI is

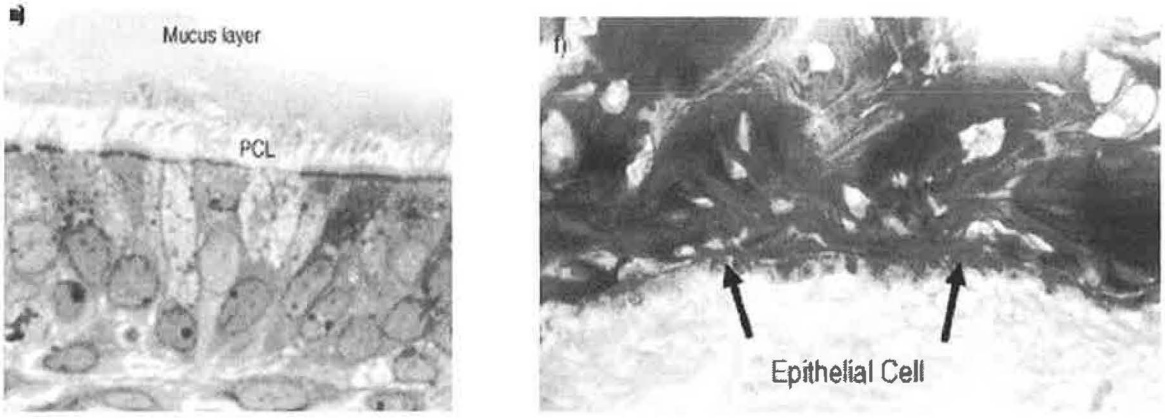
decidedly rare despite its' ubiquitous presence in the lower respiratory tract of patients with chronic obstructive pulmonary disease (COPD). The detection of products of the AHL species in NHI infection *in vivo* has not been rigorously studied. It is clear that biofilm formation occurs in some strains of NHI(26) as a result of the up-regulation of virulence factors. In particular the production of a group of proteases referred to as **autotransporter** proteins appears to be an important end-product of QS in NHI(49) and *Moraxella catarrhalis*, another chronic colonizer of the lower respiratory tract in COPD. One such autotransporter, *hap*, mediates bacterial adherence to extracellular matrix and the formation of microcolonies leading to biofilm formation. However many *hap* expressing strains of NHI do not form biofilms *in vitro*, though the relevance of *in vitro* conditions to biofilm formation *in vivo* is very much in question. More recently attention has focused on lipooligosaccharides (LOS) expressed on the cell surface of NHI. LOS in biofilms formed by NHI must be sialyted; in the absence of sialic acids biofilm formation was significantly reduced(91).

Biofilms in Specific Forms of Obstructive Lung Disease

Cystic Fibrosis

There is uniform agreement that chronic colonization of the lower respiratory tract with mucoid and non-mucoid strains of PA in patients with cystic fibrosis (CF) is due to the formation of biofilms(11, 77, 108). Although patients are also infected with other microbes such as *S. aureus, H. influenzae*, and atypical mycobacteria PA ultimately infects in excess of 80% of CF patients(21). Colonization with PA occurs early in life and often predates clinical symptoms. However the transformation to mucoid strains, particularly *P. cepacia* (also referred to as *Burkholderia cepacia*), is associated with a progressive decline in pulmonary function. While CF patients with normal flora have a normal FEV_1, those with PA have 65% and those with *P. cepacia* 55% of the predicted value(61). Indeed this organism is considered impossible to eradicate, even following double-lung transplantation(89), and is viewed as an absolute contraindication to lung transplantation in CF.

The pathogenesis of CF is complex but the airway lesion appears to be a direct result of abnormalities in the amount of fluid on the epithelial surface(11). In particular mutations in the cystic fibrosis transmembrane regulator protein (CFTR) result in depletion of the fluid layer just above the epithelial cell. Loss of the periciliary liquid (PCL) compartment in turn results in cilial dysfunction and an inability to clear bacteria (Figure 5).



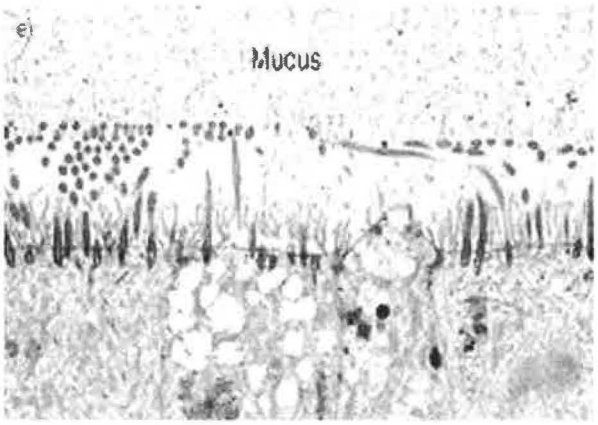


Figure 5. Light micrograph of 6 week old human bronchial air-liquid interface culture revealing distinct mucus and PCL layer(above left); in contrast in freshly isolated CF airway mucus is directly adherent to the epithelium in the absence of the PCL(above right); low power electron micrograph (left) of CF airway culture showing "bent over" cilia and thickened mucus adhering to the glycocalyx coating ciliary shafts(11)

Coincident with the loss of PCL there is an increase in epithelial oxygen consumption which results in the development of hypoxic conditions within the thickened mucus layer on the epithelial surface. PA which deposit on this surface penetrate into the hypoxic zone and produce increased amounts of alginate, which further exacerbates the local hypoxia(102). The evidence that QS mechanisms are operative in CF is extensive. Biologically active components of the AHL system have been detected in the sputa of CF patients and expression of the *las* system has also been identified(29, 86). Moreover AHLs were detected in CF patients colonized with PA but not *S. aureus*((64). Important interactions have been described between the AHL system in PA and *P.cepacia* leading to coordinated expression of virulence factors(59). More recently the Pseudomonas quinolone signal (PQS) has been found to be actively secreted by PA isolated from patients with CF early in the course of the disease(44).

A major component of the biofilm in CF airways is DNA. Clinical trials have demonstrated that aerosolized DNA-ase improved pulmonary function and reduced exacerbation rate(38). Traditionally the DNA has been viewed as part of the detritus of dead bacteria and inflammatory cells. However recent studies suggest that DNA is actively secreted by PA and that this results in stabilization of the biofilm as well as enhanced gene transfer between bacteria(40, 66).

One important set of observations in CF is that the bacterial species present once a biofilm is established is remarkably constant(89). In a study of 48 patients followed longitudinally over two years the strain of PA isolated during 94% of exacerbations was genetically identical to that present during times of clinical stability (1). This is strongly suggestive that the unique ecological niche of the biofilm provides a selective advantage for the strain of bacteria responsible for it's production. **Stability of bacterial flora may thus be an important component in identifying biofilm related obstructive lung diseases.**

In contrast the role of macrolides in CF as a "disrupter" of biofilms is less clear(76). Although initial pilot studies reported an 11% improvement in the FEV_1 of children with CF treated for >3 months with azithromycin, a 15 month randomized, placebo-controlled crossover trial showed only a 5% increase in lung function(28),(Figure 6).

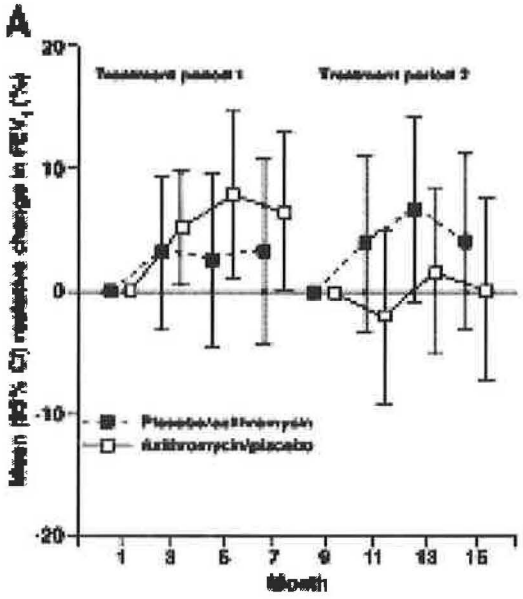


Figure 6: CF patients treated with azithromycin demonstrated significant improvements in FEV_1 in a crossover trial(28).

A large trial sponsored by the Cystic Fibrosis Foundation, which has not been formally published, showed a statistically significant benefit in improving lung function over a 5 month period in patients on azithromycin though the absolute increase in FEV_1 was only 6%. However it was noted that a subset of patients had a much more significant (>15%) improvement in FEV_1.

The mechanisms by which macrolide antibiotics might impact lung function in CF is diverse. In addition to decreasing synthesis of AHLs, mucus release from epithelial cells is inhibited by some macrolides(84) and a variety of pro-inflammatory cytokines(20, 23) may also be down regulated. The antibacterial properties of macrolides likely also play a role in clinically benefitting some patients with CF. While the anaerobic milieu of CF biofilms renders most antibiotics less effective against PA the antibacterial effect of macrolides under these conditions is increased logarithmically(22). The failure of macrolides to dramatically improve function in the majority of patients with CF may also be mutifactoral, given the marked and irreversible anatomic changes which occur with bronchiectasis, and the continued dysfunction of the CFTR in these patients.

Diffuse Panbronchiolitis

Diffuse panbronchiolitis (DPB) is a rare disorder leading to chronic airflow obstruction. Although more prevalent in the Far East, DPB is now well recognized in North America(36, 69). The syndrome is characterized by a history of long standing sinusitis, isolation of either PA or *S.aureus* from the respiratory tract, chronic cough and

mucus production, and severe airflow obstruction. Lung volume studies typically display an increase in residual volume due to the marked air trapping caused by small airways obstruction. High resolution chest CT scans usually disclose evidence of "bronchiole-ectasis" with centrilobular nodules and dilated bronchioles distally. If open biopsy is performed a pattern of inflammation with lymphocytes, plasma cells and foamy macrophages is found centered on the bronchiole. In most tertiary centers the diagnosis of DPB is usually suspected on clinical, radiographic, and microbiologic criteria.

Pseudomonas strains isolated from DPB patients often have mucoid properties similar to that observed in CF(103, 107). Patients with DPB have usually been misdiagnosed for years with asthma or some other form of obstructive disease, and have often received trials of antimicrobial therapy. Usually these patients have also received intensive forms of anti-inflammatory therapy with corticosteroids or other agents without demonstrable effect. In contrast use of macrolide antibiotics is associated with a marked, and prompt, improvement in symptoms and pulmonary function(36, 41, 58, 79). In addition a highly significant improvement in survival was reported in DPB patients treated with erythromycin(58), (Figure 7). Similar results have also been reported with clarithromycin(55).

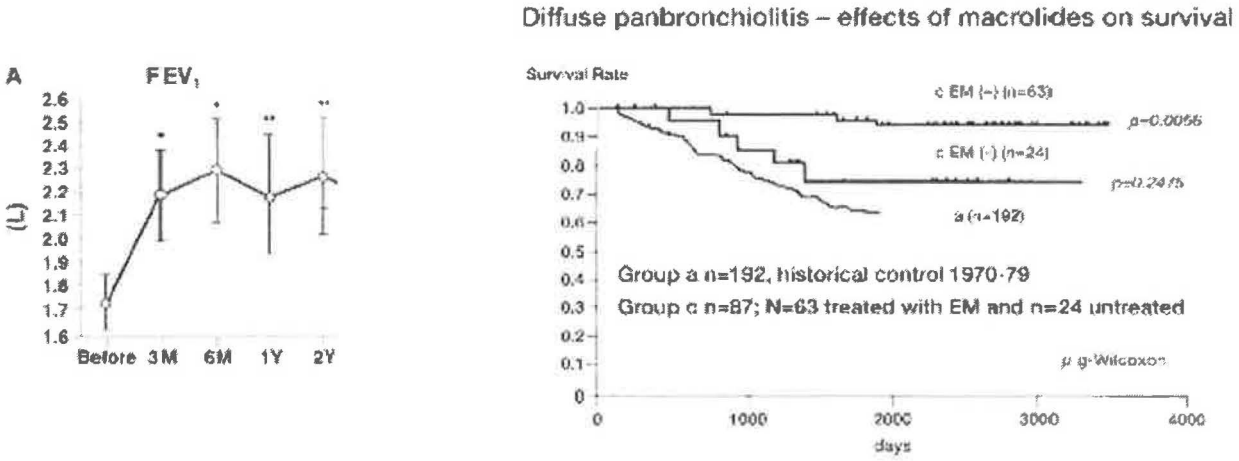


Figure 7: Improvement in FEV1 (left) and survival (right) in Japanese patients with DPB treated with erythromycin (EM+) compared to those not treated (EM-) or historical controls (a)(58, 79).

The etiology of DPB is unknown. However the dramatic response to macrolides may provide the clearest example of the effectiveness of these drugs on biofilms in clinical pulmonary medicine. In contrast to CF there is little reason to believe that

macrolides exert their effect through either anti-inflammatory or anti-bacterial machanisms in DPB. Patients have usually failed to respond to other anti-inflammatory medications or antimicrobials prior to the institution of macrolides. **The rapid and sustained improvement in pulmonary function following the administration of macrolides may thus serve as a marker for identifying other biofilm related lung diseases.**

Other "Bronchiolar" Diseases

The experience with DPB and CF has lead to speculation that other forms of obstructive lung disease are secondary to biofilms. A number of diseases thought to primarily be the result of small airways or bronchiolar obstruction(81) have been identified. In general these pateints may exhibit a disproportional reduction in "midflows" (the FEF_{25-75}) and evidence of air trapping with increased residual volumes. One such bronchiolar disease is bronchiolitis obliterans (BO) which is commonly seen following lung transplantation. In contrast to DPB or CF there is little to suggest that quorum sensing/biofilms are found routinely in bacteria colonizing the lower respiratory tract of transplant patients. Nevertheless macrolides have been demonstrated to improve airflow in patients with a clinical diagnosis of post-transplant BO. Five of six patients with BO demonstrated a significant improvement in FEV_1 (17% improvement, absolute increase of 0.5 L) following continuous therapy with azithromycin(39).

These observations have lead to empiric trials of macrolides in patients with either known or suspected bronchiolar diseases. Two such patients are described below.

Patient 1

A 56 year old woman with a chronic unremitting cough for 8 years, severe airways obstruction, and chest CT scan demonstrating nodular densities failed to improve on bronchodilators and inhaled/systemic corticosteroids. Because of her abnormal chest CT she underwent an open lung biopsy. Specimens reviewed at the Mayo Clinic were consistent with constrictive bronchiolitits (considered identical to BO).

The patients FEV_1 one year post operatively ranged between 0.98 and 1.10 L. She was empirically started on clarithromycin and treated for 9 months. There was no improvement in symptoms or FEV_1 and the macrolide was discontinued.

Patient 2

A 57 year old non-smoking man with a 3 year history of progressive dyspnea, wheezing, and productive cough. Despite high doses of inhaled corticosteroids and bronchodilators the patient had unremitting symptoms. He was treated with oral antibiotics 3-4 times per year during this period and noted that he did not respond to amoxicillin or fluoroquinolones but did improve following therapy with azithromycin.

Upon presentation his FEV_1 was 1.49 L (43% predicted), FVC 2.9 L (68% predicted) and his FEF_{25-75} was 12% predicted. Lung volumes revealed his total lung capacity to be 96% predicted, his residual volume was 131% predicted. Chest x-ray was unremarkable. He was continued on his other medications and started on azithromycin. Upon returning 3 weeks later he reported a marked improvement in symptoms within 48 hours of starting the macrolide; repeat spirometry revealed an FEV_1 of 2.11 L (61% predicted), FVC 3.72 L (87% predicted), and his FEF_{25-75} remained 15% predicted.

He did well and ultimately stopped his macrolide, though he continued his inhaled corticosteroids and bronchodilators, after 6 months because he felt well. He returned 6 months later with worsening symptoms for 3 months. Spirometry revealed an FEV_1 of 1.44 L (40% predicted). He was restarted on a macrolide and again noted marked clinical improvement within a few days. He returned 6 weeks later and spirometry now revealed an FEV_1 of 2.05 L (56% predicted).

The patients described above illustrate the spectrum of response to macrolides for presumed bronchiolar disease. Given the relative lack of serious side effects to therapy with macrolides a trial in patients with refractory/severe airways obstruction is reasonable. When patients do respond it is often within a week of initiation and is usually unequivocal. Whether the mechanism by which patients respond pertains to disruption of biofilms or is a direct effect on epithelial cell/goblet cell mucus secretion is unclear.

Bronchiectasis

Bronchiectasis is an obstructive lung disease characterized by chronic cough, usually productive of muco-purulent sputa, chronic bacterial colonization, and radiographic evidence of dilated/ectatic bronchi(6). Although numerous syndromes of bronchiectasis have been described, many with unique underlying mechanisms for producing the disease, the bacterial species isolated are quite similar across the spectrum of the disease (30). PA, NHI, and *staphylococcal* species are frequently isolated from sputa. Colonization with mucoid strains of PA is associated with a worse prognosis and quantitatively worse lung function (31). Numerous trials with multiple different antimicrobial therapies have demonstrated a benefit, which is usually not related to the *in vitro* sensitivities of the bacteria isolated. Indeed sterilization of sputa is rarely achieved, even when therapy is tailored towards the antimicrobial sensitivities of the organisms(7, 78). Detailed molecular typing of bacteria in most types of bronchiectasis has not been performed making it difficult to judge whether the bacterial flora remains constant. A study of low dose erythromycin in non-CF patients with bronchiectasis, many of whom were colonized with PA, revealed a significant improvement in lung function and sputum volume in patients receiving the macrolide compared to placebo though sputum pathogens were not altered(94).

Non-tuberculous mycobacteria (NTM) are frequently isolated from patients with bronchiectasis and in some individuals, such as those with "nodular bronchiectasis", are likely responsible for the majority of symptoms. NTM routinely exist as biofilms in the natural environment(47). The most important NTM in bronchiectasis is *M. avium*

complex (MAC). MAC in biofilms are less susceptible to antimicrobials and detergents; the formation of a biofilm for MAC and other NTM is largely dependent on the environmental condition studied and the nutrients available (5, 12). This point bears special emphasis as it is now clear that mycobacterial species express different genes *in vivo* than under culture conditions and also alter gene expression depending on the immune status of the host(92). The beneficial effect of azithromycin and clarithromycin on patients with MAC-associated bronchiectasis however appears predominantly due to the anti-microbial properties of these drugs and is frequently associated with sterilization of sputa following treatment(9, 96).

Asthma and COPD

There is little data to suggest that chronic infection with bacteria or other biofilm forming microorganisms is central to the pathogenesis of asthma. Indeed utilization of empiric antimicrobial therapy is not recommended for exacerbations of asthma. Although some studies have demonstrated the presence of *Chlamydia* or *mycoplasma* in airway biopsies of asthmatics this is not a uniform finding(27, 56, 57). Studies in asthmatics treated with macrolides empirically have largely yielded unimpressive results in terms of improving pulmonary function, even in those individuals with evidence of *mycoplasmal* or *Chlamydial* infection(10, 57).

In contrast patients with COPD are often infected with bacteria both during times of clinical stability and exacerbation, and therapy with antimicrobials is an important component of treating acute exacerbations of COPD(3, 72, 100). Indeed a direct correlation between the amount of bacteria present in the lower respiratory tract and the rate of decline in pulmonary function in stable COPD patients has been identified (Figure 8)(101). Moreover colonization with a **new** strain of bacteria was associated with an accelerated decline in FEV_1 (Figure 9). Similarly acute exacerbations in COPD, in contrast to those observed in CF are often associated with a new strain of bacteria(82). Although some studies suggest that a subset of COPD patients may be persistently colonized with the same strain of *Haemophilus* (68) others suggest that acquisition of a new strain is associated with an increased risk of exacerbation (83). In toto this would suggest that the "ecology" of the lower airway in patients with COPD is open to colonization with new organisms, a characteristic that makes a biofilm mediated process unlikely.

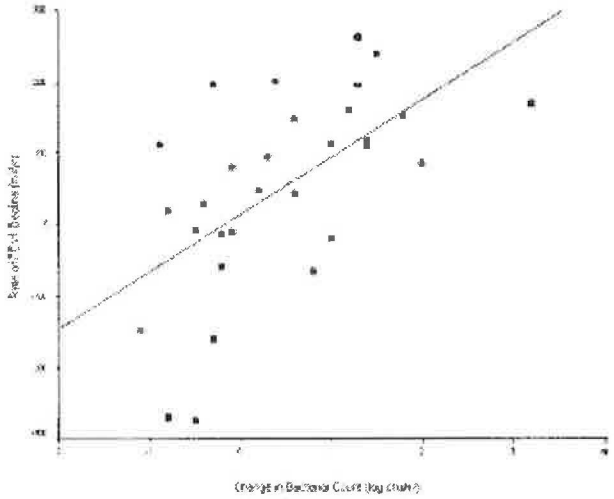


Figure 8 : Increase in bacterial load (log cfu) is associated with an accelerated decline in FEV_1 in patients with stable COPD(101).

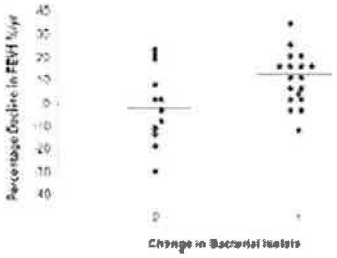


Figure 9: Change in bacterial flora to one or more new isolates of bacteria is associated with an accelerated decline in FEV_1(101)

Similarly there is little data that macrolides are superior in treating acute exacerbations of COPD(65) to other antimicrobials. Short term trials of empiric macrolide therapy for stable COPD have been reported in abstract form to have little impact on airway function or exercise tolerance.

Conclusion

Bacterial communtities forming biofilms in large and small airways are likely to play an important role in producing symptoms in a variety of obstructive lung diseases. Although the evidence for biofilm production is greatest in CF and DPB where mucoid and non-mucoid strains of PA are found, it is likely that biofilms occur in other obstructive diseases characterized by chronic bacterial colonization. The study of biofilms is important yet difficult as there may be gross differences in bacterial behavior in organisms *in vivo* compared to that evidenced by *in vitro* assays. The disconnect between antimicrobial efficacy against planktonic bacteria and those forming biofilms should temper therapeutic recommendations based on *in vitro* sensitivities alone in these diseases. While the evidence that macrolide antibiotics alter biofilm formation is most convincing in diseases involving colonization with PA, there may be other explanations for the efficacy of macrolides in CF, DPB, and other bronchiolar diseases. Nevertheless development of novel agents capable of disrupting biofilms formed by bacteria will likely occur in the near future; these agents may prove to be valuable markers for investigating lung diseases where biofilms play a prominent role.

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