



Interaction between human neutrophils and *Pseudomonas aeruginosa* biofilm : morphological and biochemical characterization
by Maiko Sasaki Papke

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:

Pseudomonas aeruginosa is an organism which forms biofilms in the environment as well as on the tissues of immunocompromised patients, such as cystic fibrosis (CF) patients. To better understand the interaction between the host immune system and *P. aeruginosa* biofilm, morphology, phagocytic capability, and degranulation profile of the neutrophils in association with *P. aeruginosa* biofilm were characterized. Neutrophils were found to remain rounded in association with the biofilm using fluorescence microscopy, confocal laser scanning microscopy, and scanning electron microscopy. They were also immobile when exposed to the biofilm, and settled on the surface of the bio film without displaying evidence of pseudopodia formation. Planktonic bacteria were released into the medium from the biofilm when exposed to serum or neutrophils, obscuring the view of the neutrophils.

The rounded and unpolarized morphology of neutrophils suggested that they were inactive in mounting the assault. However, myeloperoxidase and lactoferrin, granule components released in the extracellular medium assayed as indication of degranulation, were found to be up to 40% and up to 80% of maximal release respectively when neutrophils were exposed to the *P. aeruginosa* biofilms. Maximal release levels of myeloperoxidase and lactoferrin were established by addition of formylmethionylleucylphenylalanine and dihydro cytochalasin B as stimulants of neutrophils. Furthermore, transmission electron microscopy revealed that the ventral side of neutrophils facing the biofilm was actively involved in phagocytosis of the bacteria despite the fact that they appeared inactive from the dorsal side, not facing the *P. aeruginosa* biofilm. In summary, neutrophils are unpolarized and immobilized on the dorsal side, but are capable of phagocytosis and partial degranulation.

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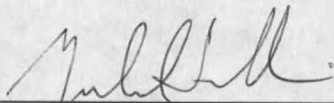
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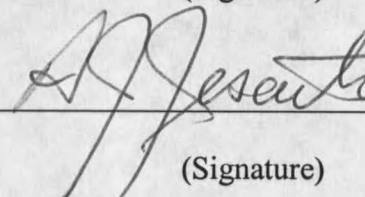
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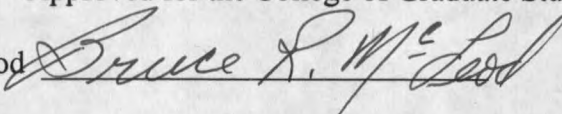
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ABSTRACT

Pseudomonas aeruginosa is an organism which forms biofilms in the environment as well as on the tissues of immunocompromised patients, such as cystic fibrosis (CF) patients. To better understand the interaction between the host immune system and *P. aeruginosa* biofilm, morphology, phagocytic capability, and degranulation profile of the neutrophils in association with *P. aeruginosa* biofilm were characterized. Neutrophils were found to remain rounded in association with the biofilm using fluorescence microscopy, confocal laser scanning microscopy, and scanning electron microscopy. They were also immobile when exposed to the biofilm, and settled on the surface of the biofilm without displaying evidence of pseudopodia formation. Planktonic bacteria were released into the medium from the biofilm when exposed to serum or neutrophils, obscuring the view of the neutrophils.

The rounded and unpolarized morphology of neutrophils suggested that they were inactive in mounting the assault. However, myeloperoxidase and lactoferrin, granule components released in the extracellular medium assayed as indication of degranulation, were found to be up to 40% and up to 80% of maximal release respectively when neutrophils were exposed to the *P. aeruginosa* biofilms. Maximal release levels of myeloperoxidase and lactoferrin were established by addition of formylmethionylleucylphenylalanine and dihydro cytochalasin B as stimulants of neutrophils. Furthermore, transmission electron microscopy revealed that the ventral side of neutrophils facing the biofilm was actively involved in phagocytosis of the bacteria despite the fact that they appeared inactive from the dorsal side, not facing the *P. aeruginosa* biofilm. In summary, neutrophils are unpolarized and immobilized on the dorsal side, but are capable of phagocytosis and partial degranulation.

CHAPTER 1

INTRODUCTION

Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessively inherited disorder caused by a defect on a 230 kb-gene on chromosome 7, coding for a 1480 amino acid-long cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al.*, 1989; Ratjen and Doring, 2003). There are approximately 30,000 CF patients in the United States, and 1 in 31 (1 in 28 Caucasians) carries a defective allele (<http://www.cff.org>). The CFTR is a 175 kDa glycoprotein, and is a 12-transmembrane chloride ion channel. The two membrane-spanning regions are separated by a nucleotide binding domain and a regulatory domain, and the C-terminal nucleotide binding domain flanks the second transmembrane domain (Stern, 1997; Bradbury, 1999; Schwiebert *et al.*, 1999; Sheppard and Welsh 1999; Zielenski, 2000). There have been over 800 defects found to date (Zielenski, 2000), but a phenylalanine deletion at position 508 ($\Delta 508$) is the most commonly found mutation in CF, comprising over 70 % of all the existing mutations, and hence also the most well studied (Tummler and Kiewitz 1999; Zielenski, 2000). This mutation is located in the first nucleotide binding domain, and leads to abnormal processing and trafficking of CFTR to the apical surfaces of the epithelial cells (Zielenski, 2000). The rest of the mutations comprises the remaining 30 % of all the CF-causing genetic defects, but the frequency of each mutation is very low (Tummler and Kiewitz 1999; Zielenski, 2000).

Due to the malfunction of CFTR as a chloride channel, osmolarity in the lung microenvironment is altered, leading to dehydration. Dehydration and subsequent thickening of mucous layer, which protects the lung epithelial cells, lead to aberrant mucociliary clearance of the particulate matters and bacteria from the lung (Pilewski and Frizzell, 1999). Furthermore, the increased concentration of chloride ions has been implicated in lowered bactericidal ability of the lung epithelial cells and effectiveness of bactericidal properties of defensin, a granular content of neutrophils, increasing neutrophil IL-8 production, accelerating the neutrophil apoptosis and lysis, and its ability of bacterial killing. Thus, higher chloride concentration aids in higher neutrophil influx into the lung environment through increased release of IL-8 and other chemoattractants and reduces the ability to kill the invading bacteria, sustaining the microbial presence in the CF lungs. (Smith *et al.*, 1996; Goldman *et al.*, 1997; Bals *et al.*, 1998; Tager *et al.*, 1998). The damage of the lung tissues in CF are most frequently seen in the airway walls, submucosa, and the structures supporting them. The first sign of CF pathogenesis is seen as a dilation of submucosal gland ducts, which can be observed as early as 4 months after birth (Sturgess *et al.*, 1982). The epithelial cell lining damage and the loss of cilia from the epithelial cells can also be observed. This loss compromises the ability of mucociliary clearance, as well as increased viscosity of the airway surface fluid resulting from the increased chloride concentration (Bedrossian *et al.*, 1976). Although the CF patients experience the first infection and/or colonization with either *H. influenzae* or *S. aureus*, they can be eradicated relatively easily with administration of antibiotics. However, other predominant infectious agent, *P. aeruginosa*, is more difficult to treat due to its multi-drug resistance (Alonso, *et al.*, 1999; Di Martino *et al.*, 2000; Westbrook-

Wadman *et al.*, 1999). The infection of *P. aeruginosa* can be seen in one third of the patients as early as 3 years of age (Rosenfeld *et al.*, 1999), and the colonization can be seen as early as 10 years of age (Baltimore *et al.*, 1989; Pilewski and Frizzel, 1999). Although the lungs of the CF newborns are virtually indistinguishable from those of the healthy newborns, the proinflammatory cytokines can be detected before their first year of life, indicating the inflammation in the lungs due to the bacterial colonization (Armstrong *et al.*, 1997; Khan *et al.*, 1995). However, even before the onset of bacterial colonization, these cytokines can be detected, though at lower level. This implies the inherently higher influx of neutrophils in CF patients (Khan *et al.*, 1995).

Although the relationship between CFTR defect and the CF pathogenesis has not yet been clearly demonstrated, there are a number of defects seen in the epithelial cells carrying two CF alleles. These defects include decreased glutathione transport (Gao *et al.*, 1999), altered cytokine expression (Bonfield *et al.*, 1999; Francoeur and Denis *et al.*, 1995; Schwiebert *et al.*, 1999), and altered terminal glycosylation of surface glycoconjugate molecules (Scanlin *et al.*, 1999). Glutathione (GSH) is a scavenger of free radicals produced by lipid peroxidation upon neutrophil degranulation. Thus, it serves as a quenching agent to minimize the damage of the epithelial cells during inflammation (Gao *et al.*, 1999). CF cells have been observed to secrete decreased amount of anti-inflammatory interleukin-10 (IL-10) and increased expression of chemoattractant interleukin-8 (IL-8) and interleukin-6 (IL-6). IL-10 is constitutively expressed by epithelial cells of healthy subjects as well as B- and T-lymphocytes (Bonfield *et al.*, 1995). IL-10 functions as an anti-inflammatory mediator by inducing the

expression of I κ B. I κ B, in turn, inhibits the activation of NF- κ B by preventing its nuclear translocation. NF- κ B, when activated, function as a transcriptional regulator of proinflammatory cytokines such as IL-6 and IL-8. IL-10 expression is induced by tumor necrosis factor- α (TNF- α) and lipolysaccharides (LPS) (Moore *et al.*, 2001; Bonfield *et al.*, 1995).

In addition to the IL-10 production by epithelial cells, macrophages in the lungs are shown to secrete IL-10. However, in CF lungs, lack of IL-10 production by epithelial cells may suffice to create an imbalance of IL-10 availability in the lung microenvironment. Thus, the anti-inflammatory responses are overwhelmed by pro-inflammatory responses elicited by overproduction of IL-6 and IL-8. The imbalance of the pro-inflammatory cytokines in the lung microenvironment leads to increased neutrophil influx, as well as enhanced LPS release from bacterial infection.

Neutrophil influx and subsequent activation lead to secretion of more pro-inflammatory cytokines, thereby inducing a snowball effect of more neutrophils in the lungs. Removal of neutrophils as well as bacteria becomes difficult due to the dysfunction of mucociliary clearance mechanism. Mucociliary clearance is hindered partly due to the fact that the viscosity of the lung airway fluid are increased. Furthermore, proteases, either host or bacterial in origin, levels are increased in CF lungs and this might interfere further with the clearance of apoptotic neutrophils by macrophages (Chmiel *et al.*, 2002). Thus, not only the increased influx of neutrophils, it is also the lack of clearance of neutrophils that exacerbate the lung damage. Furthermore, apoptotic process of neutrophils appear to be somewhat different. According to Dacheux

et al., the mode of neutrophil death is not apoptotic, but rather oncotic. The main difference of the two processes are the lack of fragmentation of DNA induced by caspases (Dacheux *et al.*, 2000). Whichever the mode of neutrophil death, the fact remains that the unfragmented DNA is present in the airway surface fluid in large quantity, contributing to the increased viscosity of the fluid.

Furthermore, presence of elastase, both neutrophil- and *P. aeruginosa*-derived, may also enhance the detachment of epithelial cells from the basal membrane, producing ulcers. Elastase is also known as an inducer of mucous secretion by the epithelial cells, further increasing the viscosity of the airway surface fluid (Sommerhoff *et al.*, 1990; Smallman *et al.*, 1984). Elastase impairs ciliary function, stimulates mucus production and hypersecretion, and induces mucus cell hypertrophy and hyperplasia (Stockley, 1994). Elastase also increases the respiratory epithelial expression of MUC5AC mRNA, which encodes respiratory mucin, by stabilizing it (Fischer and Voynow, 2000; Voynow *et al.*, 1999). Elastases are also found to induce the production and secretion of neutrophil chemoattractants such as IL-8 by epithelial cells and activating complement 5 by releasing C5a (McElvaney *et al.*, 1992; DiMango, E., 1995). The other host-derived molecules that may contribute to the deterioration of the lung environment are defensins. Defensins are a non-enzymatic cytotoxins found in the azurophilic granule of neutrophils. They insert into the bacterial cell wall to lyse the organism. However, they have been known to cause havoc if it is secreted extracellularly. Defensins induce the release of the neutrophil chemoattractant chemokine interleukin-8 from respiratory epithelial cells (Hiemstra *et al.*, 1998), and increase the proliferation rate of epithelial cells (Aarbiou *et*

al., 2002). Since *P. aeruginosa* is known to bind to epithelial cell mucin components (Ramphal, R., 2002) and repairing epithelial cells (de Bentzmann *et al.*, 1996a; de Bentzmann *et al.*, 1996b), action of defensin may induce more adhesion of bacteria to the epithelial cell layer. Thus, these host-derived molecules designed to combat against the infection often exacerbate the damage experienced by the host tissues.

Pseudomonas aeruginosa

As suggested above, the most problematic bacterial agent in CF pathogenesis is *P. aeruginosa*. It is a gram negative, aerobic, rod-shaped, respiratory, and motile organism, belonging to the bacterial family *Pseudomonadaceae*. It is ubiquitous in the environment, but its most common habitats are soil and water. Its optimal growth temperature is 37 °C, but can tolerate up to 42 °C, and it can also use more than 30 organic compounds for growth. Furthermore, although growth of *P. aeruginosa* is strictly respiratory, it is able to use NO₃ as an electron acceptor.

The ability of *Pseudomonas* bacteria to tolerate various growth conditions makes them a successful microorganism in many environments as well as a successful and important opportunistic human pathogen (Campa *et al.*, 1993). However, association of *P. aeruginosa* with healthy human subject is very uncommon. Only 2 % of the healthy human fecal samples collected outside of the hospitals are found to contain *P. aeruginosa* (Lanyi *et al.*, 1966). Furthermore, its requirement for moisture for growth limits the colonization on human skin (McBride *et al.*, 1975; Doring, 1991). Despite the fact that *P. aeruginosa* are rarely associated with healthy human subjects, they can cause serious infection to immuno-compromised patients suffering from CF, cancer, neutropenia, or

severe burns. *P. aeruginosa* is also known to cause urinary tract infections, respiratory system infections, dermatitis, osteomyelitis, pneumonia, otitis, endocarditis, and bacteremia (Campa, Ed.).

Colonization of these bacteria often involves formation of biofilms, which makes eradication rather difficult (Hoiby and Olling, 1977). Biofilm formation is often accompanied by phenotypic changes from non-mucoidy to mucoidy (Lam *et al.*, 1977). If surgically implanted devices become colonized with these bacteria, the only course of action may be to exchange the device (Shirtliff *et al.*, 2002). Eradication of this microorganism is problematic due to its growth mode during infection and its nature. *Pseudomonas* bacteria are inherently resistant to many antibiotics. In biofilms are more resistant to antibiotics and other bactericidal agents. The high intrinsic resistance of *P. aeruginosa* is thought to come from the lower outer-membrane permeability of this species, coupled with secondary resistance mechanisms such as an inducible cephalosporinase or antibiotic efflux pumps, which take advantage of low, the unusual outer-membrane permeability (Hancock, 1998). Costerton *et al.* has also suggested that the mucoid phenotype of *P. aeruginosa*, producing massive amount of exopolysaccharide alginate, which serves as a barrier from the host defense systems (Costerton, 1987).

P. aeruginosa Toxins and Their Contribution to CF Pathogenesis

P. aeruginosa produces wide variety of toxins and polysaccharides that affect the function of surface airway epithelial cells and neutrophils. The list of the toxins include but not limited to: 1) leukocidin, 2) neutrophil inhibitor, 3) elastase, 4) exotoxin

A, 5) pyocyanin, and 6) exoenzyme S. Leukocytin is a 27 kDa protein that affects neutrophils' motility, impairs bactericidal capacity and phagocytosis. It is also known to lyse neutrophils at higher concentration (Scharmann *et al.*, 1976; Baltch *et al.*, 1985; Kluftinger *et al.*, 1989). Neutrophil inhibitor is a 65 kDa protein that interferes with neutrophils' chemotactic and phagocytic abilities (Nonoyama *et al.*, 1979). Elastase digests complement components and immunoglobulin, thus interfering with the host innate immune system (Schultz and Miller, 1974). Exotoxin A inhibits NAD-dependent protein synthesis, thus and is toxic to the neutrophils (Iglewski and Kabat 1975).

Pyocyanin is a redox, phenazine pigment that partly gives the organism its characteristic color. However, it is also known as a toxin that disrupts epithelial cell layers, slows the ciliary beat frequency of epithelial cells (Kanthakumar *et al.*, 1993), and induces apoptosis of neutrophils (Usher *et al.*, 2002). Furthermore, pyocyanin also increases the expression of IL-8 level of the surface airway epithelial cells. Thus, pyocyanin also is implicated in increased infiltration of neutrophils in the lung microenvironment (Denning *et al.*, 1998). Finally, exoenzyme S is a bifunctional cytotoxin that can act as an ADP-ribosyltransferase and GTPase-activating protein, but its mode of cytotoxicity has not yet been well defined (Krall *et al.*, 2002).

The first step in infection is adhesion and colonization. Adhesins are required for this step, and because of their function, they are also considered as virulence factors. *P. aeruginosa* is thought to have many adhesins, including exopolysaccharide alginate (Doig *et al.*, 1987), exoenzyme S (Baker *et al.*, 1991), LPS (Pier *et al.*, 1997; Pier, 2000), flagella (Feldman *et al.*, 1998; Ramphal *et al.*, 1996), and pili (Darzins and Russell, 1997).

Although they probably all play roles in adhesion in varying degrees, pili are considered to be the major adhesin involved in the initial adhesion before colonization. The next stage of the infection is local invasion, which leads to the third stage, dissemination and systemic disease.

P. aeruginosa in Biofilm Mode of Growth

P. aeruginosa as well as many other bacteria that thrive in their environment form biofilms. The formation of biofilms and the mode of growth of bacteria within biofilms have been extensively studied especially on clinically significant bacterial species such as *P. aeruginosa* and *S. aureus*. The biofilm is a very dynamic and complex system, and there are three models available today to describe such structures.

Wimpenny and Colsanti developed a modeling system on biofilm structures. Their models are: 1) heterogeneous mosaic model, 2) dense confluent model, and 3) penetrated water channel model. The first two models do not include water channels within the biofilm organization. However, the first model contains the pillars and substructures that are sparsely distributed in the place of the biofilm, whereas the second model describes more or less the confluent growth of bacteria forming a biofilm. The third model is also similar to the first model, this so called heterogeneous mosaic model, in that the biofilm contains substructures resembling pillars. However, the notable difference lies in the inclusion of water channel among such substructures (Wimpenny and Colasanti, 1997). Although in their work, they describe three distinct models, they mention that they are not mutually exclusive. Given a specific biofilm system, each model may be used to describe it, but certain characteristics of other models may be evident in a smaller scale.

P. aeruginosa biofilm studied has been shown to resemble the third model, penetrated water channel model, where the mushroom-like pillar structures loom from the attachment surface, fuse at the top, entrapping the water channel within the biofilm (Costerton *et al.*, 1999).

The matrix of biofilm contains a very complex array of molecules. It is composed of secreted bacterial polymers, nutrients, and environmental and cellular debris, although the bulk of the biofilm matrix is composed of glycocalyx, also known as exopolysaccharides (Sutherland, 2001). Exopolysaccharides (EPS) provides the framework of biofilm in which bacterial cells and other components of the biofilm are embedded (Danese *et al.*, 2000). The composition of the biofilm may vary dramatically depending on its growth condition as well, further illustrating the dynamic nature of biofilm (Kolenbrander *et al.*, 1993).

During the biofilm mode of growth, *P. aeruginosa* exhibits altered gene expression. The differential gene expression during biofilm mode of growth as opposed to planktonic growth condition may be as low as 1 % (0.5 % upregulated and 0.5 % down regulated) of total genome (Whiteley *et al.*, 2001). However, in *P. putida*, the differentially expressed proteins may account for 50 % of total proteome (Sauer *et al.*, 2002). The differentially expressed genes encompass many aspects of bacterial existence. Some of structural, metabolic, translational, and motility-related proteins were found to be either upregulated or downregulated. Whiteley *et al.* has found that all the motility-related proteins that were found to be altered in their expression levels were downregulated (Whiteley *et al.*, 2001). Furthermore, some gene products that give the

bacteria more resistance to antibiotics directly or indirectly also were found to be either down- or up-regulated (Xu *et al.*, 2000).

In order to achieve the differential gene expression during the biofilm mode of growth, individual bacterial cells need to communicate with each other. The cell-to-cell communication is accomplished using two simple but elegant communication systems; hierarchical yet intertwined. The first is the LasI/LasR system, and the secondary system is the RhII/RhlR system. In the LasI/LasR system, LasI induces the production of signaling molecule, homoserine lactone (HSL). It is secreted to the extracellular matrix via efflux pump. As the concentration of extracellular HSL is increased, the intracellular concentration also increases through equilibrating mechanism. Once the critical level of intracellular HSL concentration is achieved, it then activates LasR transcriptional activator. This will induce the transcription of various gene products such as serine protease and endotoxinA. The second system also involves diffusible HSL homolog. Upon reaching the threshold concentration, it binds and activates the RhlR transcriptional regulator, which, in turn regulates the pyocyanin and lectins. In the early stages of the biofilm growth, there is an inhibitory interaction between RsaL and lasI. RsaL binds to the regulatory region of *lasI* operon, inhibiting the activation of the operon. As the biofilm matures, the threshold concentration of LasI competitively removes the inhibitor of RsaL, thereby initiating the signaling cascade (Shiriff *et al.*, 2002). Interesting point to note is that the Rhl system is inhibited by an alternative sigma factor RpoS (Whiteley *et al.*, 2000). This finding is significant since in biofilm mode of growth, RpoS was found to be downregulated (Whiteley *et al.*, 2001).

Biofilm formation confers advantages to the bacteria. Bacteria forming biofilms are much more resistant to antibiotics compared to planktonic growing cells of the same isolate. The antibiotics to which they are resistant include aminoglycosides, beta-lactam antibiotics, fluoroquinolones (Anwer and Costerton, 1990; Giwercman *et al.*, 1992). β -lactamase, which cleaves the β -lactam antibiotics, is found to be increased during the course of antibiotic treatment (Giwercman *et al.*, 1992). The resistance to antibiotics may be attributed to several different factors involved in biofilm formation. It has been known that the slow growing cells are more resistant to antibiotics due to the slower metabolic rate and subsequent uptake of the antibiotic molecules. Furthermore, due to the physical proximity to the liquid phase of the bacterial cells surrounded by EPS matrix may also account for the slower growth rate and less exposure to the antibiotics (Brown *et al.*, 1990; Hoiby and Koch, 1990).

Not only does biofilm formation gives bacteria antibiotic resistance, it also provides resistance to other host-derived molecules such as lactoferrin. Lactoferrin is a neutrophil granular protein that binds the free ferric ions from the environment. Since iron is found as components of many bacterial regulatory enzymes and proteases, lactoferrin is toxic to the bacteria at higher concentration (Hassett *et al.*, 1996; Singh *et al.*, 2002). Lactoferrin has been also suggested to enhance the bactericidal activity of antibiotics (Ellison, 1994) and to inhibit the formation of mature biofilm at sublethal concentration (Singh *et al.*, 2002). Although planktonic and young biofilm forming bacteria are susceptible to lactoferrin-mediated killing, mature biofilms are not affected by lactoferrin (Singh *et al.*, 2002).

P. aeruginosa growing in biofilms are also found to reduce oxidative burst of neutrophil up to 25 % in comparison with the planktonic bacteria and reduce the extent of complement cascade activation (Jensen *et al.*, 1990; Jensen *et al.*, 1992). Complement activation is mediated mainly by LPS, and reduction of complement activation may be explained by the enhanced expression level of exopolysaccharides (Whiteley *et al.*, 2001).

Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes (PMN), are a class of granulocyte that represent 50 to 60% of the total circulating leukocytes. The normal concentration of neutrophils in circulation is about 4.4×10^6 cells/ml. They are approximately 10 μm in diameter. The nuclei of neutrophils are multi-lobed and chromatin-dense (Williams, 4th Ed.). They were believed to be terminally differentiated cells that do not have active metabolism, but the recent findings indicate that mature neutrophils undergo transcriptional and translational activities such as IL-8, IL-1, and TNF- α (Witko-Sarsat *et al.*, 2003) as well as an active modulation of numerous genes after bacterial challenge (Kobayashi *et al.*, 2002).

They originate as hematopoietic stem cells in bone marrow differentiating through the myeloblast differentiation pathway. It takes about 2 weeks for them to originate and mature within the bone marrow before entering the circulation. Once the neutrophils mature and enter circulation, they may be present in a marginal pool without activation for 4 to 10 hours. When there is no inflammation found, they are often found in a resting state within “physiological regional granulocyte pool” (Peters, 1998), mainly narrow pulmonary capillaries. When they are in the resting stage prior to activation,

active adhesion processes are not involved (Doyle *et al.*, 1997; Mizgerd *et al.*, 1998). However, once activated and entered the tissues, they survive from one to two days. Senescent neutrophils are believed to undergo apoptosis, and apoptotic neutrophils are removed by tissue macrophages (Kobayashi *et al.*, 2002).

Once the neutrophils enter the circulation, they are sequestered to the site of inflammation. The local endothelial cells expresses E- and P-selectins induced by inflammation mediators released by damaged tissues, such as IL-1, TNF- α , histamine, and LPS, which are recognized by the neutrophil counterreceptors. Due to the low flow and inflammation and dilation of the capillaries, neutrophils adhere to the endothelial wall transiently to start the tethering and rolling. During migration, neutrophils may encounter chemoattractant gradient. Chemoattractant gradients serve as a beacon and a priming agent to activate the neutrophil integrin, CD11b/CD18, which are required for firm adhesion and spreading prior to extravasation into the tissues. Chemoattractants may include bacterially derived formylated peptides, and host derived factors such as IL-8 and C5a (Witko-Sarsat *et al.*, 2000).

Neutrophils are also one of the professional phagocytes, and are the first to be recruited to the site of infection or injury. Their targets include bacteria, fungi, protozoa, viruses, virally infected cells and tumor cells, and they remove those agents mainly through phagocytosis upon opsonization. Neutrophils are summoned to the site of inflammation and or infection through chemoattractant gradients. Neutrophil chemoattractants include IL-8 and complement fragment C5a. Upon target recognition, neutrophils initiate ingestion through membrane extension. Upon ingestion,

enveloping the target cells or particulate matters, a formation of phagolysosome ensues. Fusion of lysosome to phagosome releases the lysosomal content, creating a toxic environment within the phagolysosome. At this time, azurophilic granules and specific granules also fuse with the phagosomal membrane making the phagolysosome which introduce their contents including various serine proteases and lactoferrin.

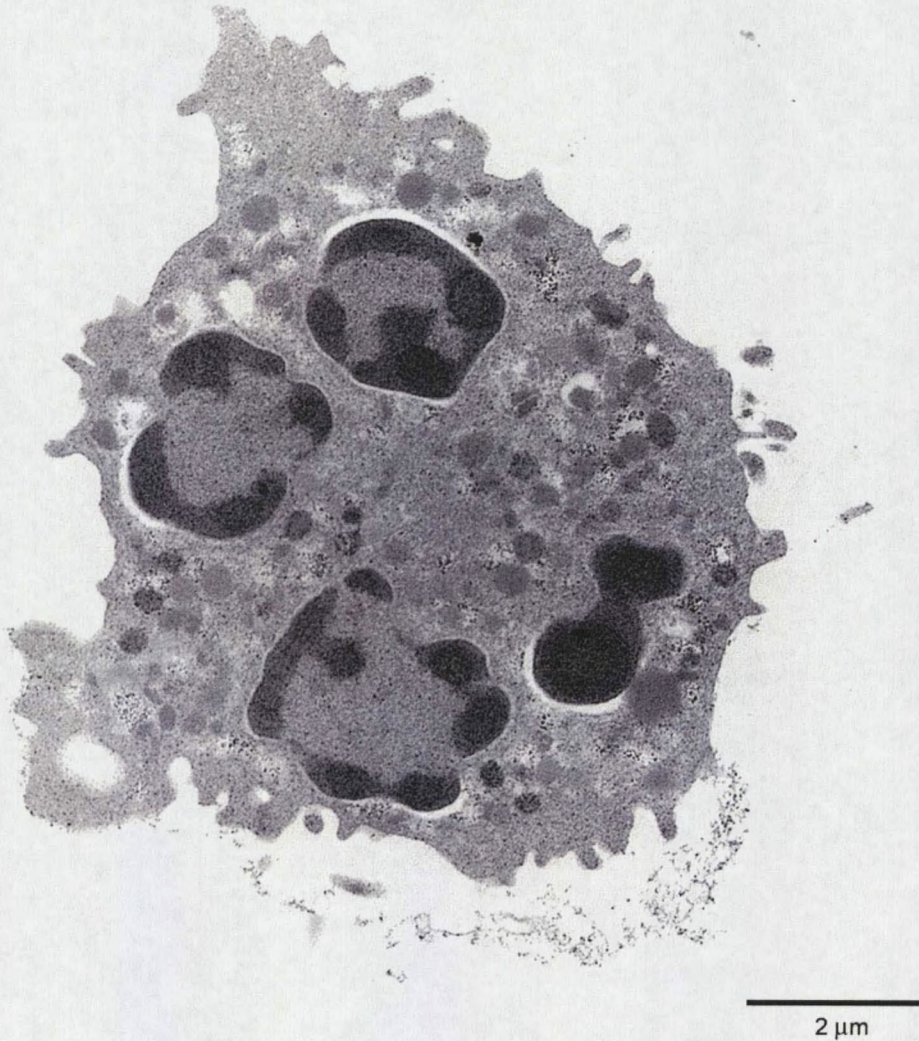
Reactive oxygen species synthesized *de novo* during the respiratory burst, in which consumption of oxygen by the neutrophil increases dramatically, also take part in creating the cytotoxic environment for the engulfed bacteria. Although the reactive oxygen species usually remain within the cells, if the target is too large for the neutrophils to engulf, neutrophil undergoes a process called frustrated phagocytosis in which reactive oxygen species are secreted to the extracellular environment. The presence of reactive oxygen species in extracellular environment may be beneficial in killing the organisms that are too large to be phagocytosed. However, these molecules also damage the host cells and overwhelm the host's neutralizing mechanism if present in large quantity. Much lung damage in CF is due to the excessive inflammation and subsequent release of reactive oxygen species and other compounds released from neutrophils.

Neutrophils contain three different granules. They are, in order of maturation, 1) azurophilic or primary granules, 2) specific or secondary granules, and 3) tertiary granules. Azurophilic granules consist of approximately 20% of all granular population in neutrophils. They are about 0.5 μm in diameter. They are electron dense and appear quite dark and oval in shape (Murphy, 1976). Azurophilic granules contain

myeloperoxidase, serine proteases such as neutrophil elastase (NE) (Fischer and Voynow, 2000), defensins (Lehrer and Ganz, 2002), and bactericidal permeability increasing proteins (BPI) (Calafat *et al.*, 2000). Myeloperoxidase is a heme-containing enzyme that catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions (Hampton, M.B., 1998). Defensins are 3-kD proteins that disrupt bacterial membranes (Lehrer and Ganz, 2002). Bactericidal/permeability-increasing protein is a 55 kDa protein that is primarily associated with the azurophilic granule membrane. Upon activation of neutrophils, fusion of azurophilic granules, release their contents into the phagosome, and binds to lipopolysaccharides. Specific or secondary granules are defined as peroxidase-negative granules, and are more electron lucent than azurophilic granules. They are about 0.2 μm in diameter, and oval in shape. They contain lactoferrin and lysozyme (Murphy, 1976) and other important host defensive proteins. The tertiary granules are also defined as peroxidase negative granules, and they contain gelatinase (Borregaard *et al.*, 1993).

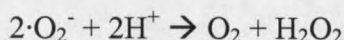
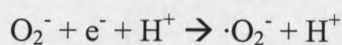
Although azurophilic granules are considered to be the granules containing the most potent bactericidal components, other compounds synthesized *de novo* by neutrophils contribute greatly to their bactericidal activities. Neutrophil undergo respiratory burst, a process in which the oxidative metabolism is activated. There are at least two types of free radicals, produced by neutrophils. They are the reactive oxygen

Figure 1. Transmission electron micrograph of human neutrophil. Oval, electron dense granules are azurophilic. The light colored, round granules are specific granules. The small specs visible especially around the peripheral regions are glycogen granules.

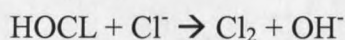
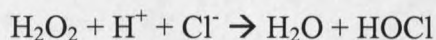


intermediates (ROI) and reactive nitrogen intermediates. The reactive oxygen species is produced by NADPH oxidase, which is an electron transport chain found in the wall of the endocytic vacuole of professional phagocytes and in B and T lymphocytes. NADPH oxidase in resting neutrophils is dissociated and inactive, but upon activation, the components are recruited to the luminal side of the phagosome, assemble, and start the

production of reactive oxygen species. In the process of generation of ROI, NADPH is used as an electron donor to reduce molecular oxygen to superoxide anion, which then is chemically dismutated by action of superoxide dismutase to hydrogen peroxide as shown below. Although hydrogen peroxide is not considered to be highly toxic to bacteria, it is known to induce a mutation in *P. aeruginosa mucA22*, which is involved in switching to the mucoidy phenotype (Mathee *et al.*, 1999).



Superoxide ion can serve both as a one-electron reductant or a one-electron oxidant, though its biological activity is relatively low due to its mild reductant/oxidant nature. However, since it is capable of permeating the membranes through anion channel, it can be converted to other ROI such as hydrogen peroxide and hydroxyl radical ($\cdot\text{OH}$) distal to where it originates. Hydrogen peroxide is a bacteriostatic agent rather than a bactericidal agent. It is an effective bactericidal agent only in a higher dosage of 500 μM (Hyslop *et al.*, 1995). Hydrogen peroxide serves as a substrate of myeloperoxidase, component in azurophilic granules, to generate hypochlorous acid, which is in turn metabolized further to hypochlorite and chlorine as shown below.



Hypochlorous acid is the most powerful substances involved in microbicidal and cytotoxic reactions. They are membrane permeable, and are known to crosslink proteins and oxidize iron containing molecules (Hampton *et al.*, 1998). Other reactive oxygen species include hydroxyl radical whose cytotoxic activity has not yet been fully determined due to its transient nature. However, it seems that the hydroxyl radical may be important in generating hypochlorous acid in the presence of chloride ions (Czapski *et al.*, 1992).

Although pathogenesis of CF has not yet been clearly demonstrated, numerous factors from both the host and the bacteria are significant in this fatal genetic disorder. Much of fatality occurs due to the tissue damage incurred by the hyperactive host defense mechanism, rather than the damage incurred from the colonization of *P. aeruginosa* (Chmiel *et al.*, 2002). The interaction between neutrophils and *P. aeruginosa* has been extensively studied. However, most of the studies employed the planktonic *P. aeruginosa*. Since *P. aeruginosa* forms biofilm within the CF lungs, it is more pertinent to study the interaction between neutrophils and bacteria growing in biofilm. However, such studies have been limited to date. In the work described in this thesis, the interaction between *P. aeruginosa* biofilm and neutrophils has been examined.

CHAPTER 2

MATERIALS AND METHODS

Serum Preparation

Thirty milliliters (30 mL) of healthy donor's blood was drawn and placed in a glass Corex® tube, and let stand for 1 hour at room temperature. The clot was centrifuged at 12,000 rpm in the Beckman model J6-B centrifuge with bucket rotor, TY JS 3.0 (Beckman Coulter, Fullerton, CA), for 10 minutes. Serum was carefully aspirated, aliquoted and kept at 4°C or -20 °C until used.

PMN Preparation

Fifty milliliters (50 mL) of healthy human donors' blood was drawn and centrifuged at 14,000 rpm for 20 minutes in the Beckman bucket rotor centrifuge at room temperature. The serum and the buffy coat layers were carefully aspirated. Red blood cell layer was mixed gently with 50 mL 3.7 % gelatin/1% sodium chloride (NaCl)/phosphate buffered saline (PBS) solution, and incubated at 37 °C for 30 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes at room temperature. The supernatant was removed, and the pellet was lysed in 10mL Lysis buffer over ice by gentle mixing. The solution was centrifuged at 12,000 rpm at 4 °C, and the pellet was resuspended in 5 mL Dulbeccos' Phosphate Buffered Saline (DPBS). The final concentration of polymorphonuclear leucocytes (PMN) was adjusted to 1×10^7 cells/mL. The cells were kept on ice until used.

Pseudomonas aeruginosa Biofilm Preparation

P. aeruginosa strains PAO1 and PAO1 containing plasmid, pMF230 (stock number 1325) were used in this study. PAO1-GFP (Nivens *et al.*, 2001) constitutively expresses green fluorescent protein (GFP), *gfp-mut2*. PAO1 was maintained on L agar (10g tryptone, 5 g yeast extract, 5 g NaCl, and 7.5 g Bacto Agar per liter (Difco Co., Franklin Lakes, NJ)). PAO1-GFP was maintained on L agar containing 300 µg/mL carbenicillin (Anatrace, Inc., Maumee, OH).

For development of biofilms used in granule release assays, wild type strain, PAO1 and GFP-expressing *P. aeruginosa* strain, 1325 were grown overnight. 1 mL overnight culture was inoculated into the 100 mL PIA broth overnight. 200 µL of the culture was placed in the 96-well plate (Phenix Research Products, Hayward, CA) and grown for 3 days at 37 °C. The broth was changed every 8 hours to ensure the healthy biofilm formation.

Scanning electron microscopy analyses were performed on *P. aeruginosa* biofilm grown in a Flat Plate Open-Conduit Bioreactor for 7 days at 37 °C. The reactor vessel is made of 0.25 in. polycarbonate, and has a working volume of 350 mL inclusive of the recycle loop and the heat exchanger. A laminar flow was maintained by velocity of the recycle loop, and the recycle ratio was set at 300. The vessel was connected to an air pump equipped with 0.2 µm filter to supply fresh, aseptic oxygen to the reactor system.

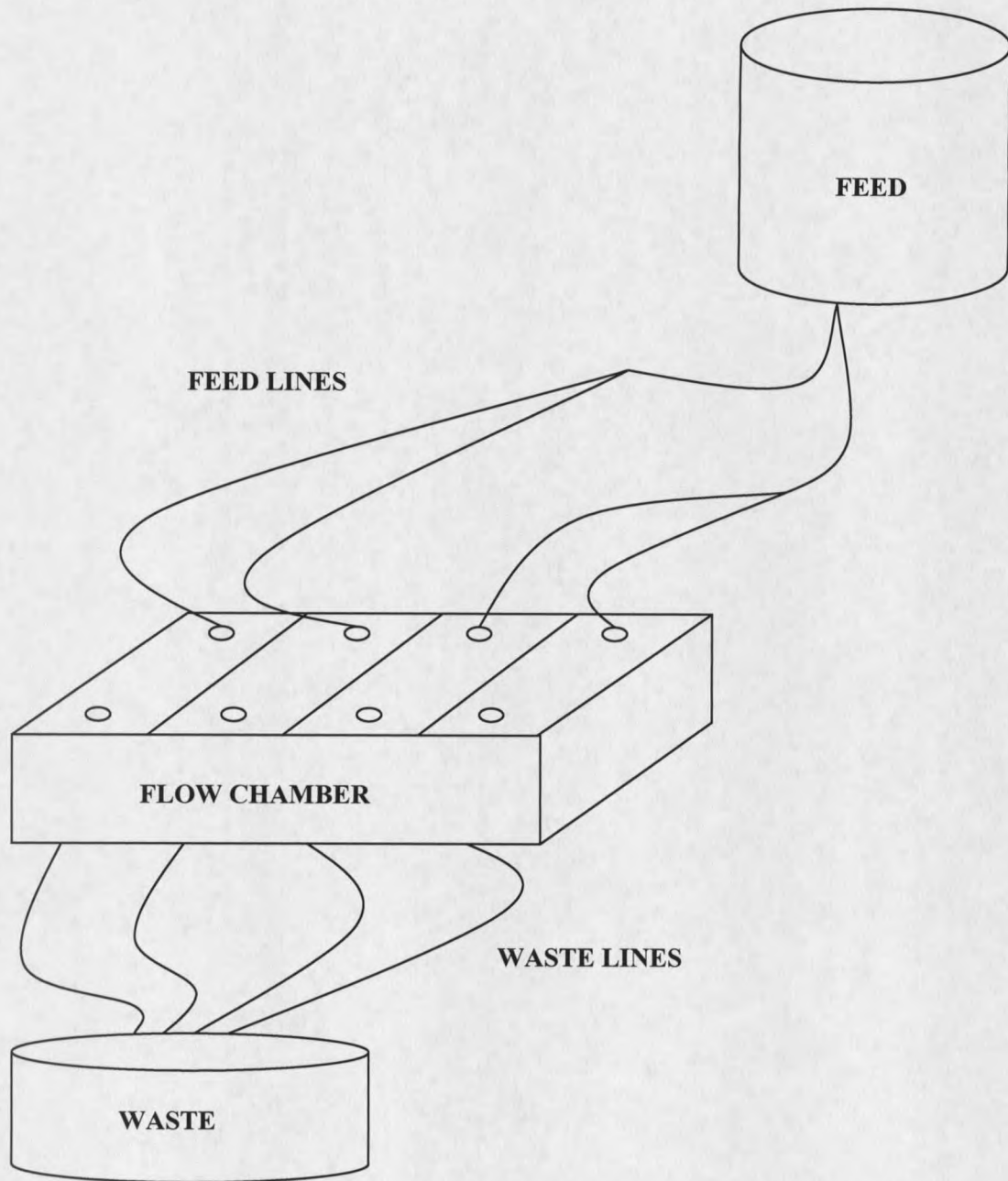
For Fluorescent Microscopy, Confocal Scanning Laser Microscopy, and Transmission Electron Microscopy

Biofilm used for fluorescence microscopy, confocal scanning laser microscopy, and transmission electron microscopy was grown in a drip flow system (Fig. 2) was used to form biofilm of PAO1, FRD1, and PAO1-GFP. Polycarbonate membranes (Osmonics Laboratory Products, MN) were placed in the vessel and autoclaved. Bacterial cultures were started in 10X Biofilm medium (0.09 mM sodium glutamate, 0.5 mM glycerol, 0.02 mM MgSO_4 , 0.15 mM NaH_2PO_4 , 0.34 mM K_2HPO_4 , and 145 mM NaCl per liter, pH 7.0) until the cell density reached approximately 10^7 cells/mL. The culture was then diluted ten fold in saline as an inoculum. The 5 mL was inoculated into the each chamber of the vessel, covering the membranes. After the bacteria were allowed to settle and attach to the membrane for 20 minutes, the excess fluid was drained, and the flow of 1X biofilm media was started. The bacteria were grown for 3 days.

Drip Flow Reactor

A drip flow reactor is made of $\frac{1}{4}$ in. plexiglass walls with four chambers to receive one slide per chamber. Each chamber is equipped with 0.125 (1/8) in. plexi glass lid, secured by hex nuts. The lid also is equipped with an opening to accommodate the feed line. At the front, bottom part of the chamber, attachment to accommodate the waste line is located. The inlet and outlet spouts are located opposite from each other to allow the drainage of the fluid to allow the maximal attachment and growth of bacteria. Feed lines are threaded through 1-100 rpm pump with corresponding number of pump heads for each feed line. The flow rate was adjusted to 1.2 mL/min.

Figure 2. Diagram of drip flow reactor used for growing *P. aeruginosa* biofilm.



Myeloperoxidase Assay

The myeloperoxidase assay was conducted in 96-well plates in which biofilms were grown. The supernatant was removed from the biofilm for myeloperoxidase detection, and the media of the biofilm was changed once, one hour before the assay. In experiments investigating the interaction between neutrophils and opsonized biofilm, biofilm was pretreated with 10 % autologous serum in 190 μL HBSS or 200 μL HBSS at room temperature for 30 minutes. The biofilm was rinsed three times gently with HBSS immediately before the experiment, and adjusted amount of HBSS to the final volume of 200 μL was added to each well. In experiments to establish the maximal myeloperoxidase release, 1 μM formyl-Met-Leu-Phe (fMLF) and 10 μM dihydrocytochalasin B (dhCB) were added to the wells. Human neutrophils were gently overlayed at the final concentration of 4×10^5 cells/well, and the mixture was incubated for 45 minutes at 25 °C or 4 °C. The plate was shaken briefly to ensure the mixing of the myeloperoxidase released by the PMN and centrifuged in a Beckman J6B centrifuge equipped with Js3.0 rotor and ELISA plate adaptors at 1000 rpm for 10 minutes at room temperature. Immediately after the centrifugation, 50 μL -70 μL of supernatant was removed from the top gently and placed in the well of a clean 96-well Costar E2 plate. The aliquot was maintained on ice until the myeloperoxidase assay was conducted. 50 μL substrate containing 1M sodium citrate, pH 4.2, 3.3 % hydrogen peroxide (H_2O_2), and 1 mM 2,2'-azino-bis(3ethylbenzthioziline-6-sulfonic acid) diammonium salt (ABTS) and 50 μL 1M sodium citrate, pH 4.2, were added. The mixture was incubated at room temperature for 1 hour to allow the color to develop. Color development was linear with

time. The myeloperoxidase release was assayed colorimetrically, read at 405 nm using Spectra Max 250 (Molecular Devices, Sunnyvale, CA) (Parkos *et al.*, 1985).

β -Glucuronidase Assay

β -glucuronidase detection was conducted in the same assay fluids as prepared in the myeloperoxidase assay. The supernatant was collected upon centrifugation and added to the solution containing 0.2 M acetate buffer, pH4.5, and 0.06 M phenolphthalein mono- β -D-Glucosiduronic acid (PGA) in final concentration of 20 %. The mixture was incubated at 37.5 °C for 16 hours, and the reaction was quenched with 5 X volume of .02 M Glycine with 0.2 % sodium lauryl sulfate (SDS) to the total volume of 200 μ L. The glucuronidase released was assayed colorimetrically, read at 540 nm using Spectra Max 250. The amount was quantitatively calculated against the standard curve containing 2 – 20 μ g of PGA.

Lactoferrin Assay

Lactoferrin detection procedures were conducted in the same assay fluids as prepared in the myeloperoxidase assay. The supernatant was collected upon centrifugation of the neutrophils interacting with the biofilm. Lactoferrin was quantitatively detected using western blot. Human milk lactoferrin (Sigma, St. Louis, MO), Cappel rabbit anti-human lactoferrin, ICN Biomedicals, Inc., Irvine, CA), and peroxidase-conjugated or alkaline phosphatase-conjugated goat anti-rabiit (H + L) for chemiluminescence or colorimetric immunoblot assays (Bio-Rad Laboratories, Hecules, CA) were used as standards, primary antibody, and secondary antibody respectively. The

primary antibody was diluted to 1:8000 for chemiluminescence and 1:1000 for conventional analysis. Western blots were analyzed according to the Pierce specifications using Pierce chemiluminescence assay kit (Pierce, Rockford, IL) or BCIP/NBT Phosphatase Substrate System (KPL, Gaithersburg, MD). Immunoblots were scanned to create a TIFF file, which was analyzed by ImageQuant Molecular dynamics, Sunnyvale, CA) software densitometrically to quantify the amount of lactoferrin in the supernatant collected.

Fluorescence Microscopy

Isolated and purified neutrophils were stained with 9.6 nM BODIPYTM FL SSE (Molecular Probes, Eugene, OR) for 5 minutes, and rinsed twice in DPBS. The biofilm of PAO1-GFP grown over the polycarbonate membranes in a drip-flow reactor as described above were gently rinsed twice in DPBS. The neutrophils were layered over the biofilm and allowed to settle for 1 hour before the microscopy. The membranes were rinsed in DPBS once gently as not to disturb the interaction, and fixed for 30 minutes in 3.7 % paraformaldehyde (PFA) at room temperature. The membranes were placed over the slides to be viewed under 100X magnification using Nikon Eclipse E600 fluorescence microscope, equipped with SpotTM digital camera system.

Scanning Confocal Laser Microscopy

P. aeruginosa biofilm was grown in the drip flow reactor over glass coverslips for 72 hours as described above. The biofilm-covered coverslips were removed from the vessel, moved to a 30mm-petri plates containing HBSS, rinsed once, and covered with 15

mL HBSS. Neutrophils stained with 16 μ M BODIPY FL SSE as described above in the fluorescence microscopy section. 50 μ L neutrophil suspension was placed over the biofilm. The interaction of bacteria and neutrophils were observed using Leica TCS-SP confocal microscope (Leica Microsystems Inc., Heidelberg) with 63X water immersion objective. GFP was excited using 488 nm line of argon laser, and the emission was collected at 500 to 550 nm to visualize the *P. aeruginosa* biofilm. BODIPY FL was excited with 568 line of krypton laser, and emission was collected at 590 to 700 nm. Appropriate neutrophil density over the biofilm was chosen for observation and photographed. The images were process using the shadow projection function of Imaris 3.0 (Bitplane Inc., St. Paul, MN) and Adobe Photoshop (Adobe Systems, San Jose, CA).

Scanning Electron Microscopy

P. aeruginosa biofilm was grown over silicon slides for 7 days in isothermal flat plate open-conduit bioreactor as described above. The slides were removed from the growth vessel and covered with HBSS at 37 °C. 10 % autologous serum was added to the suspension to study the interaction between the opsonized *P. aeruginosa* biofilms and the neutrophils; HBSS was substituted for the autologous serum for the control experiments. 1×10^7 cells/mL neutrophils were gently added to the surface of the biofilm, and the slides were incubated in an air convection incubator at 37 °C for 30 minutes. Samples were fixed in 2 % gluteraldehyde/8 % sucrose in 0.1X PBS. The samples were then reinsed twice, 15 minutes each, in PBS. After samples were rinsed twice in PBS, 15 minutes/rinse, they were dehydrated in successively higher concentration of ethanol, 50, 70, 90, and 100 %, 10 minutes/dehydration step. The samples were then processed with

ethanol and carbon dioxide in the brass vessel for critical point drying, then immersed in 100 % ethanol once. The samples were prepared for SEM by sputter-coating them with 20 nm Gold-Palladium in the presence of argon. JEOL 6100 scanning electron microscope (JEOL USA, Inc., peabody, MA) equipped with RUNCO digital imaging system (Runco International, Union city, CA) was used to capture and analyze the image data.

Transmission Electron Microscopy

The bacteria grown over a polycarbonate membrane in a drip flow reactor described above was removed from the vessel and rinsed twice with DPBS gently as not to disrupt the biofilm. The membranes were placed in 3cm-petri dishes containing 2 mL DPBS. 50 μ L of PMN solution at 2×10^7 cells/mL was layered over the membrane gently. PMN was allowed to settle for 1 hour before fixation. The cells were fixed in 2 % glutaldehyde (Electron Microscopy Scientifics, Fort Washington, PA) solution in DPBS overnight at 4 °C. The membranes were rinsed three times in 0.1 M phosphate buffered saline (PSBS), pH7.2, 10 minutes per rinse. The membrane was further fixed in 2 % osmium tetroxide (OsO_4) for four hours. The membranes were rinsed twice, 10 minutes each in PSBS. The samples were successively dehydrated as follows: twice, 10 minutes each in 50% ethanol (EtOH), 70 % EtOH overnight, 95 % EtOH for 10 minutes, thrice in 100 %, 10 minutes each, twice, 10 minutes each in 100 % propylene oxide (PO). The samples were then embedded in the Spurr's medium (23.6% vinylcyclohexane dioxide, 14.2 % DER 736 resin, 61.3 % Nonenyl succinic anhydride modified, and 0.9 % 2-Dimethylaminoethanol) in a successively higher concentration as follows: 2:1/PO:Spurr's

for 30 minutes, 1:2/PO:Spurr's for 1 hour, and 100 % Spurr's medium overnight. The embedding procedures were performed at room temperature with vigorous shaking. The samples were cut into approximately 1mm squares, placed into the capsules with Spurr's medium, and baked overnight at 70 °C. The samples were sectioned into 70 nm slices using a RMC MTXL ultra-microtome (Boeckler Instruments, Inc., Tucson, AZ). The prepared sections were examined using a Hitachi H-7100 electron microscope (Hitachi High Technologies America, Pleasanton, CA) equipped with an AMT digital camera system coupled with a 1K CCD camera (Advanced Microscopy Techniques, Inc., Danvers, MA). Sectioning and imaging were performed at Electron Microscopy facility at University of Montana, Missoula, MT.

Fluorescence Microscopy

Neutrophils were stained with 9.6 nM BODIPY FL SSE™ for 5 minutes, rinsed twice in DPBS. The biofilm of PAO1-GFP grown over the polycarbonate membranes were gently rinsed twice in DPBS. The neutrophils were layered gently over the biofilm and allowed to settle for 1 hour before the microscopy. The membranes were placed over the slides to be viewed under 100X magnification using Nikon Eclipse E600 fluorescence microscope, equipped with Spot™ digital camera system. The dye did not have any adverse effects on neutrophil motility or function (data not shown).

CHAPTER 3

RESULTS

Attachment and Spreading of Neutrophils on Inanimate Glass or Plastic Surface

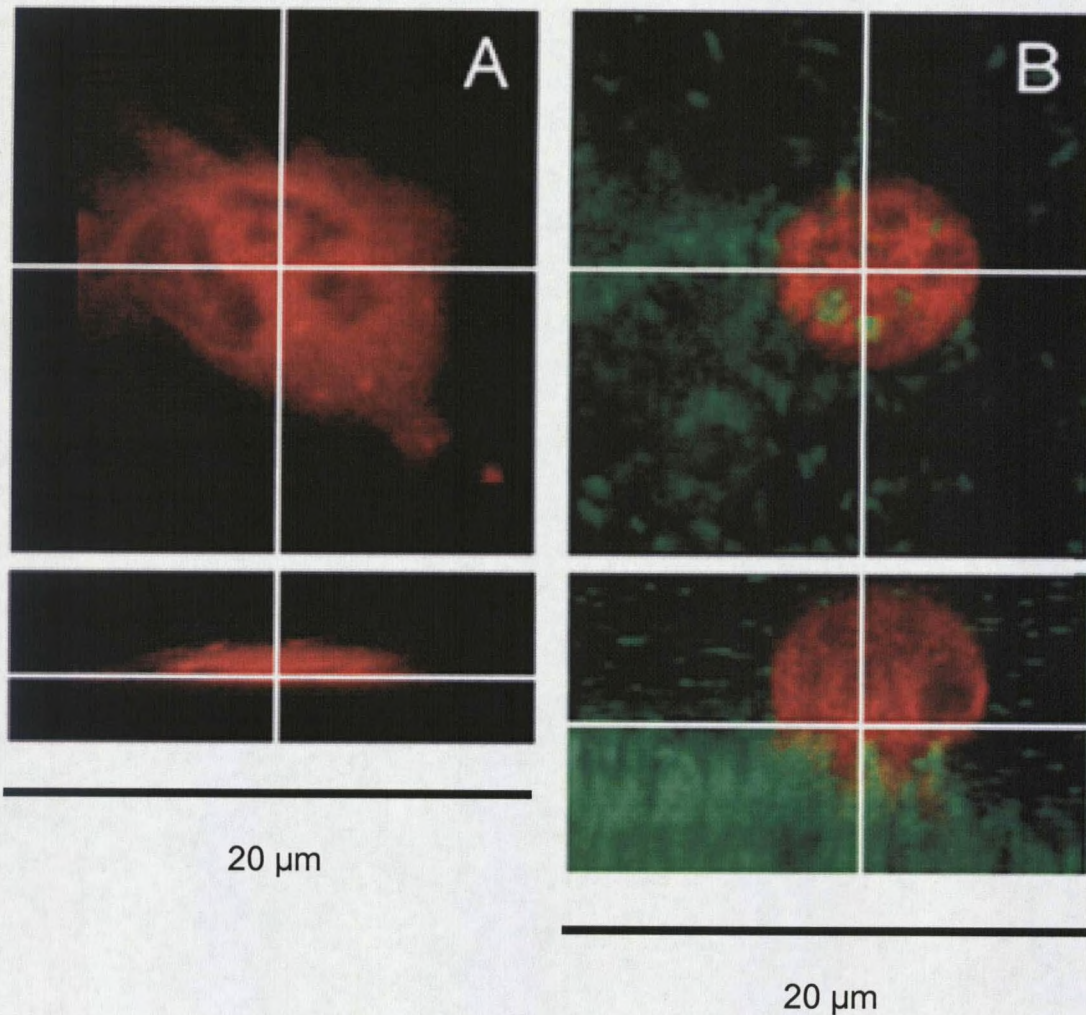
To evaluate the interaction between neutrophils and *P. aeruginosa* biofilm coated surfaces, the morphology of neutrophils attaching to an inanimate surface, such as glass and polycarbonate membranes, was established. Human neutrophils were isolated and purified from peripheral blood of healthy subjects as described in the Materials and Methods section. Fig. 3 shows neutrophils and an eosinophil adhering to a polycarbonate membrane visualized using transmission electron microscopy (TEM). When neutrophils were settled on a polycarbonate membrane without biofilm for an hour, they adhere to the membrane firmly. The unattached surface appears somewhat rounded, but the overall morphology appears flattened. Neutrophils stained with BODIPY FL SSE (Molecular Probes) adhering to a glass slide were imaged using scanning confocal laser microscopy (SCLM) performed by Deb Berglund (Fig. 4 A) and fluorescence microscopy (Fig. 5 A). The advantage of SCLM is that it allows to view the object under consideration from all directions, although the resolution is lower than that of TEM. Fluorescence microscopy, on the other hand, lower resolution between TEM and SCLM, and does not allow the side views. The appearance of neutrophils imaged by both SCLM and fluorescence microscopy confirms that the neutrophils assume similar morphology when they are settled onto either the glass or polycarbonate surfaces. Furthermore, neutrophils stained with cell-permeable BODIPY do not appear to be impaired in either viability or functions such as cytoskeleton remodeling, as they settle and adhere normally to the untreated

surfaces. Then neutrophil membranes also show modest ruffling without evidence of polarization in any specific direction as expected (Fig. 3 and 4 A). Despite the fact that the resolution is relatively low, Fig. 5 A and b show that untreated neutrophils are quite flat. Furthermore, the images shown also indicate that the neutrophils may be focally adhered to the glass surface. There are bright tight spots seen in the generally dark interior of the neutrophils, indicating focal adhesion. In Fig. 5 B, neutrophils were treated with 10 μ M cytochalasin B which inhibits the actin polymerization. With the cytoskeletal remodeling capacity crippled, neutrophils settled over the glass slide remained fully rounded. The apparent diameter of the treated neutrophils is approximately half of the non-treated neutrophils on average. Furthermore, treated neutrophils also do not appear to have a staining pattern suggesting the focal adhesion seen in non-treated neutrophils (Fig. 5 A and B).

Figure 3. Transmission electron micrograph of neutrophils and an eosinophil attaching to the polycarbonate membrane. Cells are rounded but tightly adherent to the membrane. Moderate membrane ruffling of neutrophils can be seen.



Figure 4. SCLM images of neutrophils from the dorsal (top) and transverse (bottom) views. A. A neutrophil is tightly adherent to the glass slide, spreading. B. Neutrophil interacting with *P. aeruginosa* biofilm. Neutrophil is rounded without evidence of membrane ruffling. It sits atop the biofilm, and individual bacterial cells can be seen adhering to the neutrophil. Experiments were conducted and analyzed by Deb Burglund.



Interaction between Neutrophils and *P. aeruginosa* Biofilm

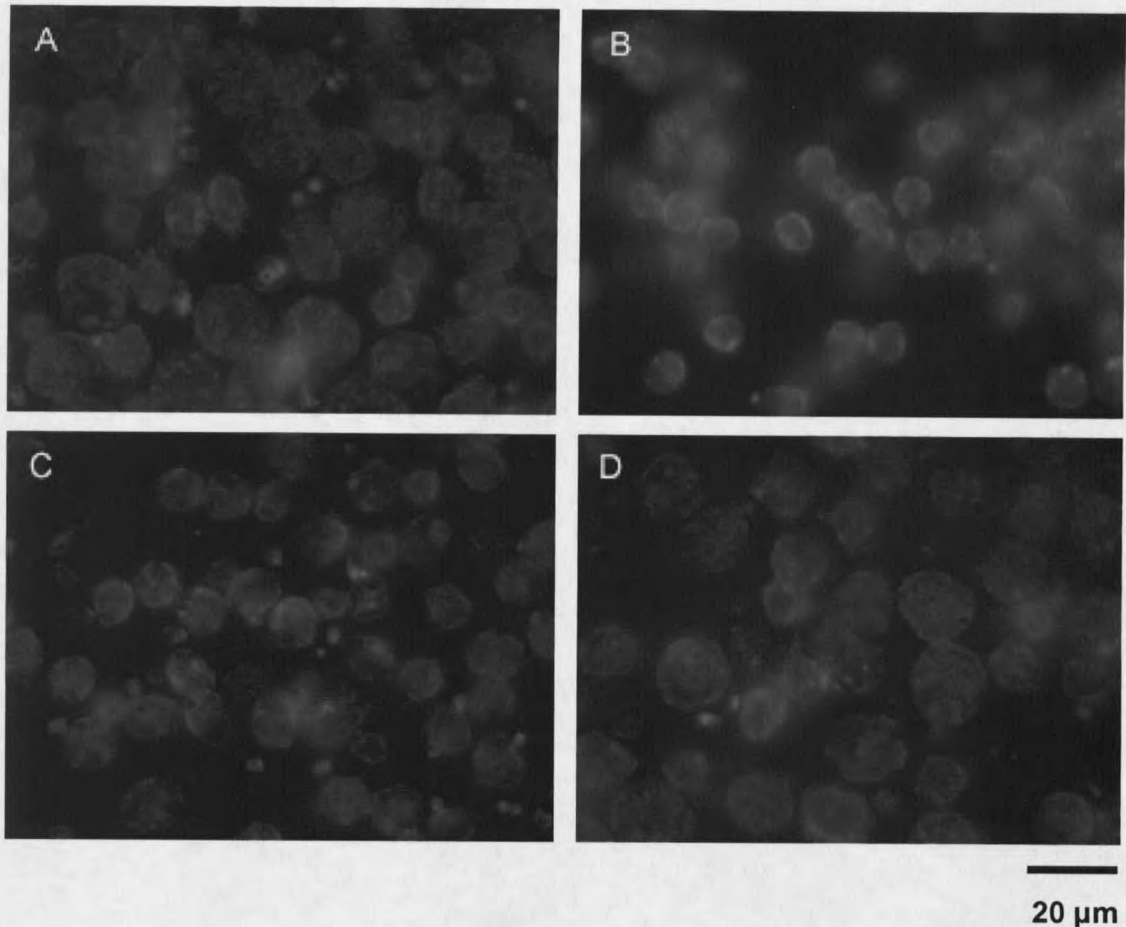
As established in the earlier section, neutrophils settling on the inanimate surfaces spread and adhere to the substrata. However, upon introduction of neutrophils to the *P. aeruginosa* biofilm grown in various conditions, neutrophils exhibit a significantly

different morphology. Fig. 4 B shows a neutrophil settled onto the 3-day old biofilm grown in the drip flow reactor. The neutrophil appears spherical from the dorsal view, and the diameter of the neutrophil also is approximately half of the flattened, adherent neutrophil shown in Fig. 4a. Similar observations were made by fluorescence microscopy (Fig. 6) with *P. aeruginosa* biofilm. To investigate the behavior of neutrophils upon introduction to the biofilm, neutrophils were given the following treatments. In Fig. 6 A, B, and C, untreated, control neutrophils were allowed to settle over the biofilm. In Fig. 6 D, E, and F, 10% autologous serum was added to the biofilm prior to addition of neutrophils, allowed to settle over the biofilm to simulate in an environment approximately the inflammatory state. In Fig. 6 G, H, and I, 10 μ M dhCB and 1 μ M fMLF were added as neutrophils were layered over the biofilm. fMLF, in the presence of dihydrocytochalasin B, is a potent secretagogue of neutrophils of bacterial origin that induces the nearly maximal degranulation of neutrophils. In the last set of three, 10 % autologous serum, dhCB, and fMLF were added to simulate the neutrophil undergoing degranulation in the presence of opsonized bacteria (Fig. 6 J, K, and L). The right column (Fig. 6 A, D, G, and J) represents the images of neutrophils alone. The middle column (Fig. 6 B, E, H, and K) represents the images of the biofilm alone. The left column represents the overlayed images of both the neutrophils and biofilm. The same field of view and depth of the samples were photographed for all the images shown. Biofilm and neutrophil images were photographed Fig. 6 A, B, and C represent non-treated neutrophils interacting with the biofilm. The baseline behavior of neutrophils in the above mentioned conditions in the absence of bacteria was established by layering the neutrophils on the glass slide as shown in Fig. 5. Non-treated neutrophils and neutrophils

treated with fMLF and dhCB (Fig. 6 A-C and G-I) showed relatively rounded morphology in accordance with the results from the SCLM study. Although the membranes of neutrophils in the presence of opsonized bacteria appeared somewhat more ruffled, it may be due to the background green fluorescence from the surrounding bacteria in addition to the low resolution. Evidence of directional polarization was not seen.

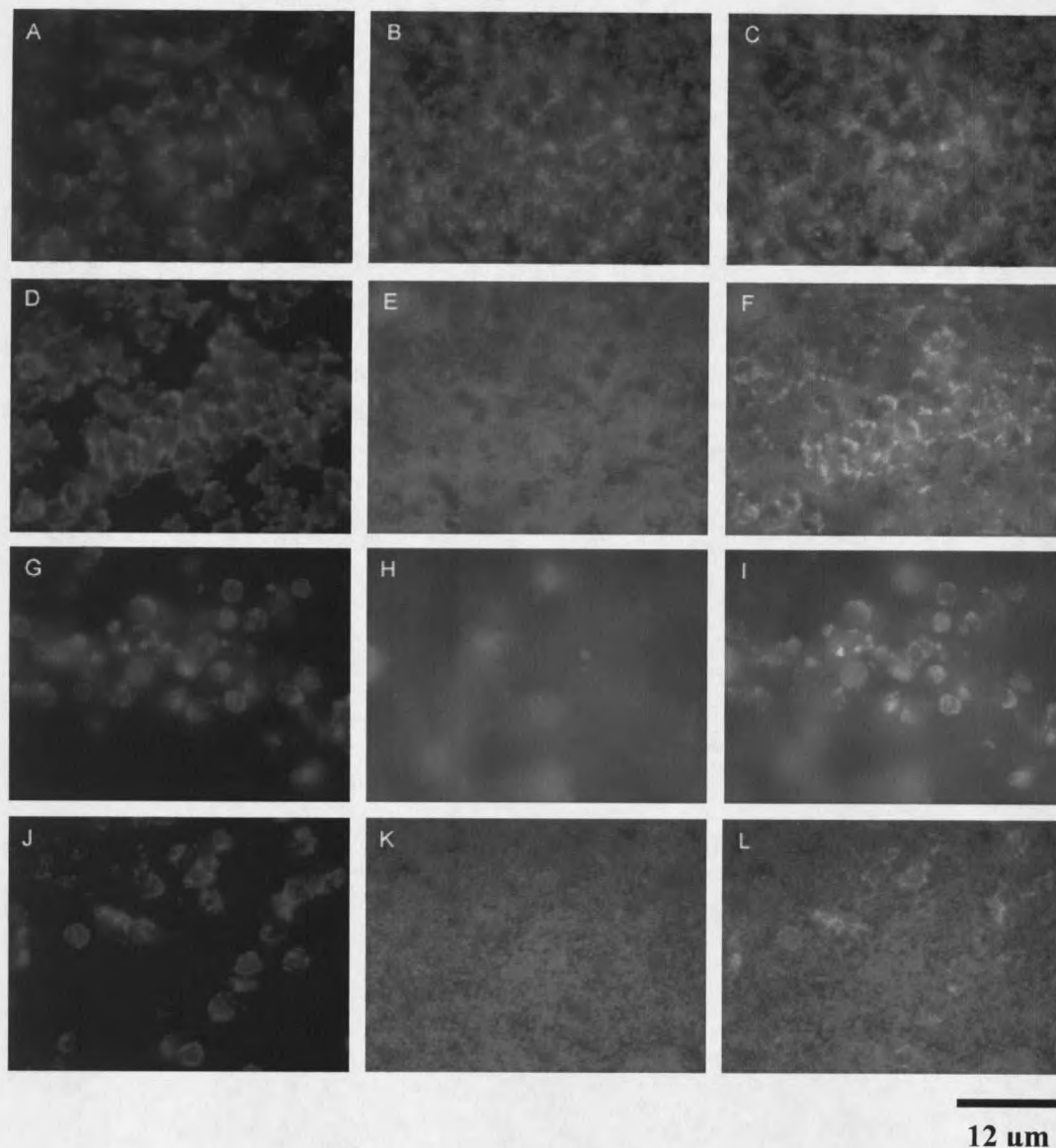
Along with the observation that the neutrophils remain relatively rounded, another curious phenomenon was noted. All the neutrophils observed over the biofilm appeared to be sitting atop the biofilm rather than invading it. Fig. 4 B clearly demonstrates an example of a neutrophil interacting with the biofilm without invading it. This observation confirmed in Fig. 6 G, H, and I, where the locations of neutrophils and the biofilm were so starkly different that they could not be photographed together effectively (Fig. 6 I). Interestingly, the “ventral”, or bacteria-facing side of the neutrophil appears to have membrane protrusions extending toward the neutrophil-biofilm interface. Furthermore, individual bacterial cells appear to be surrounded by the pseudopodia extended by the neutrophils at the neutrophil-biofilm interface. This suggests that neutrophils are still capable of cytoskeletal remodeling and possibly phagocytosis, despite the fact that they are immobile and remain rounded on the dorsal side (Fig. 4 B). However, the resolution was insufficient to make firm conclusions.

Figure 5. Neutrophils stained with 10 nM BODIPY FL SSE. Neutrophils were stained and allowed to settle over glass slides. After 1 hour incubation at room temperature, neutrophils were fixed in 3.7 % PFA for 30 minutes. A. Neutrophils in HBSS. B. Neutrophils treated with 10 μ M dhCB and 1 μ M fMLF. C. Neutrophils treated with 10 % autologous serum. D. Neutrophils treated with serum, dhCB, and fMLF.



To confirm the rounded morphology with membrane protrusions on the ventral or bacteria adjacent side of neutrophils, interaction between neutrophils and *P. aeruginosa* biofilm was studied using SEM, performed by Justin Bleazard (Jesaitis *et al.*, 2003). Biofilms were grown in the isothermal channel bioreactor under continuous flow as described in the Materials and Methods section.

Figure 6. Fluorescence micrographs of neutrophils interacting with *P. aeruginosa* biofilm, constitutively expressing GFP. Neutrophils and biofilm were imaged separately at the same field and the depth. A-C. Images of neutrophils, biofilm, and overlay of both in HBSS. D-F. Images of neutrophils, biofilm, and an overlay of both in 10 % autologous serum. G-I. Images of neutrophils, biofilm, and overlay of both in 10 μ M dhCB and 1 μ M fMLF. J-L. Images of neutrophils, biofilm, and overlay of both in serum, dhCB, and fMLF.



Neutrophils were added to the biofilm when appropriate, and incubated for 30 minutes.

Biofilm with or without neutrophils were then fixed in glutaraldehyde for SEM sample preparation.

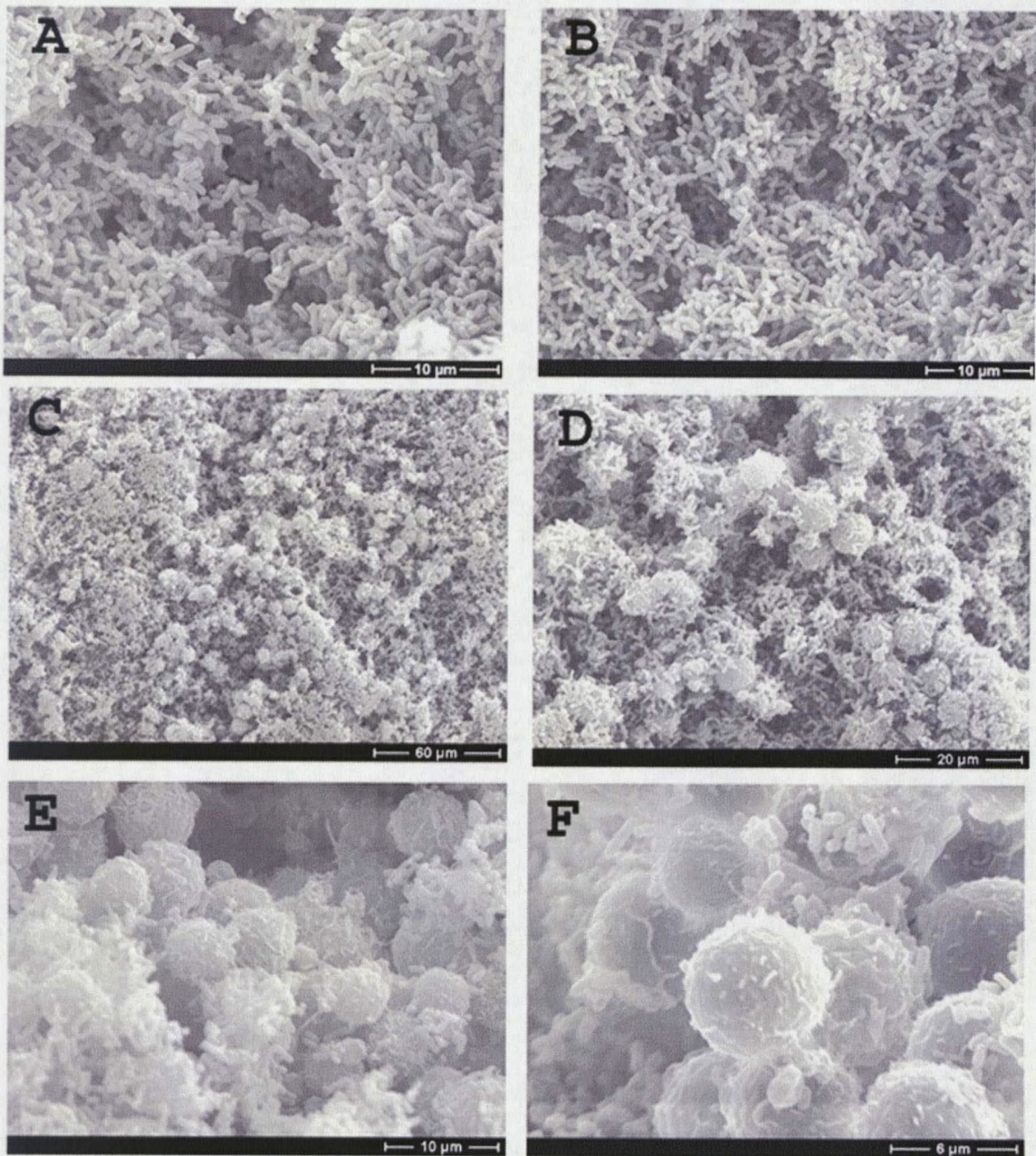
Fig. 7 A and B represent the *P. aeruginosa* biofilm alone without addition of neutrophils in the presence and absence of 10 % autologous serum respectively. *P. aeruginosa* formed dense biofilm with complex structures including troughs and channels, resembling the dense confluent model described by Wimpenny and Colasanti. (Wimpenny and Colasanti, 1997). However, since SEM does not allow the sectional view of the biofilm, so it is difficult to positively correlate with the particular model developed by Wimpenny and Colasanti. Opsonization with serum does not seem to affect the ultrastructure of biofilm after 30 minutes of exposure at the resolution shown here. Fig. 7 C and E show the interaction of neutrophils with non-opsonized *P. aeruginosa* biofilm, Fig. 7 E shown at higher resolution. Fig. 7 D and F shows the neutrophils with opsonized biofilm, Fig. 7 F shown at higher resolution. Although the neutrophils appeared to have moderately ruffled membranes when introduced to the biofilm using fluorescence microscopy (Fig. 6 D and J), Fig. 7 E and F clearly demonstrated that there was no significant difference in the appearance of neutrophils interacting with the non-opsonized or opsonized biofilm. All the visible neutrophils were spherical with minor membrane ruffling, much less than what Fig. 6 D and J might have suggested. There was no evidence of cytoskeletal remodeling, polarization or frustrated phagocytosis. Thus, the morphology suggested that the neutrophils did not appear activated in spite of an abundant presence of bacterial chemoattractants (Fig. 7 C, D, and E) or complement

components resulting from incubation of the biofilm with 10 % autologous serum (Fig. 7 D and F).

Neutrophils, when added to the *P. aeruginosa* biofilm, lined the troughs and channels of biofilm structures, and as a result, the biofilm appeared more level with filled channels (Fig. 7 C). *P. aeruginosa* often adhered to the surfaces of neutrophils, quite extensively at times as shown in Fig. 7 C and D. Presence of serum components did not appear to affect either the ability of neutrophils to fill the channels or bacterial adherence to neutrophils (Fig. 7 C through F).

Since SEM does not show the sectional view, it is difficult to ascertain whether or not the neutrophils undergo morphological alteration at the neutrophil-biofilm interface. Although the neutrophils do not appear to be involved in phagocytosis as indicated by the adherence of bacterial cell to the dorsal side of neutrophils. However, since structural and physiological differences do exist between *P. aeruginosa* growing in biofilm or planktonic modes of growth (Costerton *et al.*, 1999), lack of evidences of cytoskeletal remodeling of neutrophils associating with individual *P. aeruginosa* cells does not necessarily describe what takes place at the biofilm interface. Another interpretation of the observation that neutrophils fill the biofilm channels is that they may be infiltrating or burrowing in the biofilm as seen in the left bottom corner of Fig. 7 D. However, SCLM (Fig. 4 B) and fluorescence microscopy (Fig. 6 I) images suggest that neutrophils are immobilized on the biofilm and are not burrowing into the biofilm matrix. Unfortunately the low resolution obscures accurate view of neutrophils infiltrating the biofilm.

Figure 7. Scanning electron micrograph of *P. aeruginosa* biofilm and neutrophils. A. Image of biofilm in HBSS. B. Image of biofilm in HBSS with 10 % autologous serum. C and E. Images of Biofilm and neutrophils in HBSS. D and F. Images of biofilm and neutrophils in 10 % autologous serum. Samples were processed and imaged by Justin Bleazard.

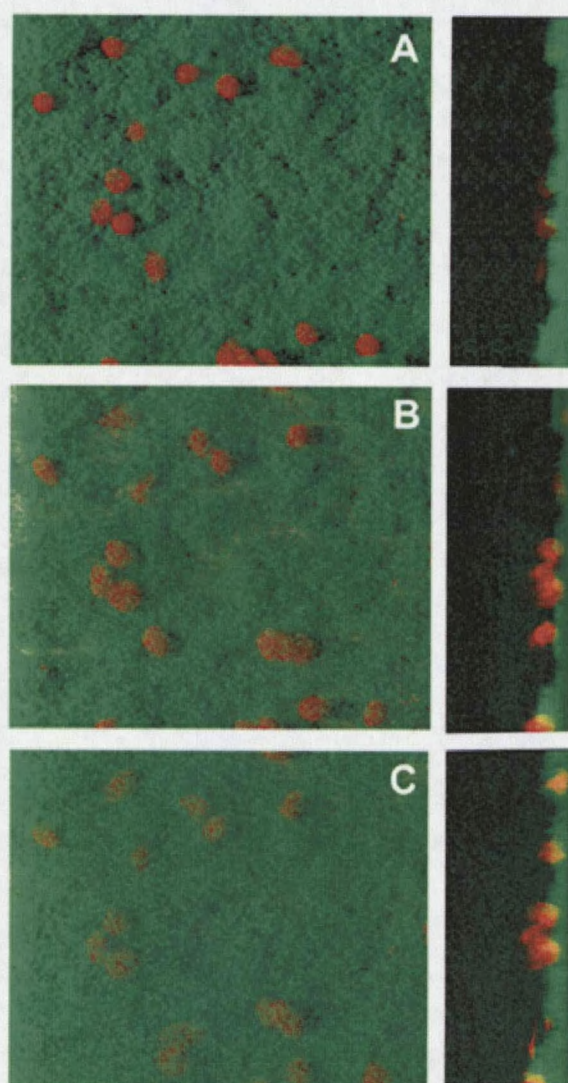


Apparent Inability of Neutrophils to Penetrate Biofilm Structure

To investigate further whether or not neutrophils burrow into the biofilm, BODIPY-stained neutrophils were imaged using SCLM in a time-course. As established in the previous section, BODIPY does not appear to hinder a neutrophil's ability to remodel its cytoskeleton (Fig. 4 A and Fig. 5 A). In Fig. 8, neutrophils were imaged at 5- (Fig. 8 A), 15- (Fig. 8 B), and 60- (Fig. 8C) minute time points in the presence of 10 % autologous serum, assayed and photographed by Deb Berglund. The left column represents the "dorsal" view whereas the right column represents the transverse view. At 5-minute time point, most neutrophils appeared to be loosely associated with the biofilm as indicated by the absence of planktonic bacteria obstructing the view. One of the neutrophils in Fig. 8 A (top left quadrant) appeared somewhat buried within the biofilm, and a few neutrophils had seemingly adherent bacteria. After 15 minutes, most neutrophils appeared to be half buried or partially absconded by the biofilm (Fig. 8 B) and later after 60 minutes completely buried or obscured (Fig. 8 C). Transverse views show that the neutrophils do not burrow but become obscured. The series of transverse views shown in Fig. 8 clearly demonstrate that bacteria somehow dislodge themselves from the biofilm structure to form a cloud above the neutrophils, increasingly obscuring the view of the leukocytes from the top as time passed. Furthermore, the transverse views also demonstrate that the position of neutrophils in relationship to the biofilm does not change over time. The depth at lateral position of the neutrophils on biofilm appear to be approximately the same; the slight differences seen among the three transverse panels do not account for the seeming burrowing of neutrophils into the biofilm seen in the dorsal

views and are probably due to inaccuracies in choosing transverse sections. Furthermore, the observation that neutrophils do not seem to move laterally also supports the idea of motile cells in this dynamic system is *P. aeruginosa*, and not neutrophils.

Figure 8. Confocal scanning laser micrographs of neutrophils appearing to burrow into the biofilm over a course of 60 minutes. A. 5 minute time point. B. 15 minute time point. C. 60 minute time point. The experiments were performed and analyzed by Deb Burglund.



This “blizzard” or bacterial cloud formation effect of bacteria leaving the biofilm, progressively obscuring the view, is not solely due to the presence of neutrophils in the system. 10 % autologous serum alone also causes the individual cells to leave the biofilm structures into the surrounding medium. To rule out neutrophils as a sole cause of *P. aeruginosa* cells leaving the biofilm, time course experiment was conducted with or without serum in the absence of neutrophils. Fig. 9 shows the effect of saline alone or 10 % serum without neutrophils in the system. The images were captured at 20, 30, 45, 60, 75, and 90 minutes. When serum is absent, “blizzard” effect can be seen but at much retarded rate (Fig. 9 A through F). However, when serum is present, considerable number of *P. aeruginosa* cells can be seen in the medium at 15-minute time point (Fig. 9 G). The number of individual cells in the medium increases dramatically over the 90-minute period in the presence of serum (Fig. 9 G through L). Although the density of planktonic bacteria at 70- and 90-minute time point does not seem to differ drastically, the increasing trend can still be seen.

To investigate the contribution of neutrophils to this “blizzard” effect, *P. aeruginosa* biofilm was compared to conditions in which there were: a) neutrophil alone in the absence of serum (Fig. 10 A and B), b) neutrophils and 10 % autologous serum (fig. 10 C and D), and c) 0.2 % sodium azide pretreatment of the biofilm (NaN_3) to inhibit bacterial respiration in the absence of serum (Fig. 10 D and E). The upper panel images (Fig. A, C, and E) were photographed at 15-minutes time point whereas the lower panel images (Fig. B, D, and F) were photographed at 45-minute time point. In the absence or presence of serum with viable biofilm, neutrophils do elicit the “blizzard” effect (Fig. A

through D). However, NaN_3 abolishes this phenomenon, suggesting that viable *P. aeruginosa* cells are responding to both serum and neutrophils somewhat additively as stimuli (Fig. 10 E and F). Interestingly, the neutrophils appear much more active and motile on the NaN_3 -treated biofilm, suggests that active bacteria may be required to cause the inhibition of neutrophil motility.

Figure 9. Serum effect induced by the 10 % autologous serum in a time course. A-F. Biofilm in HBSS successively photographed at 20, 30, 45, 60, 75, and 90 minutes. G-L. Biofilm in 10 % autologous serum at the same time points. The panel G was substituted for the 20 minute time point due to the loss of data. The micrograph was obtained from an experiment conducted under identical condition. Samples were prepared and analyzed by Deb Burglund.

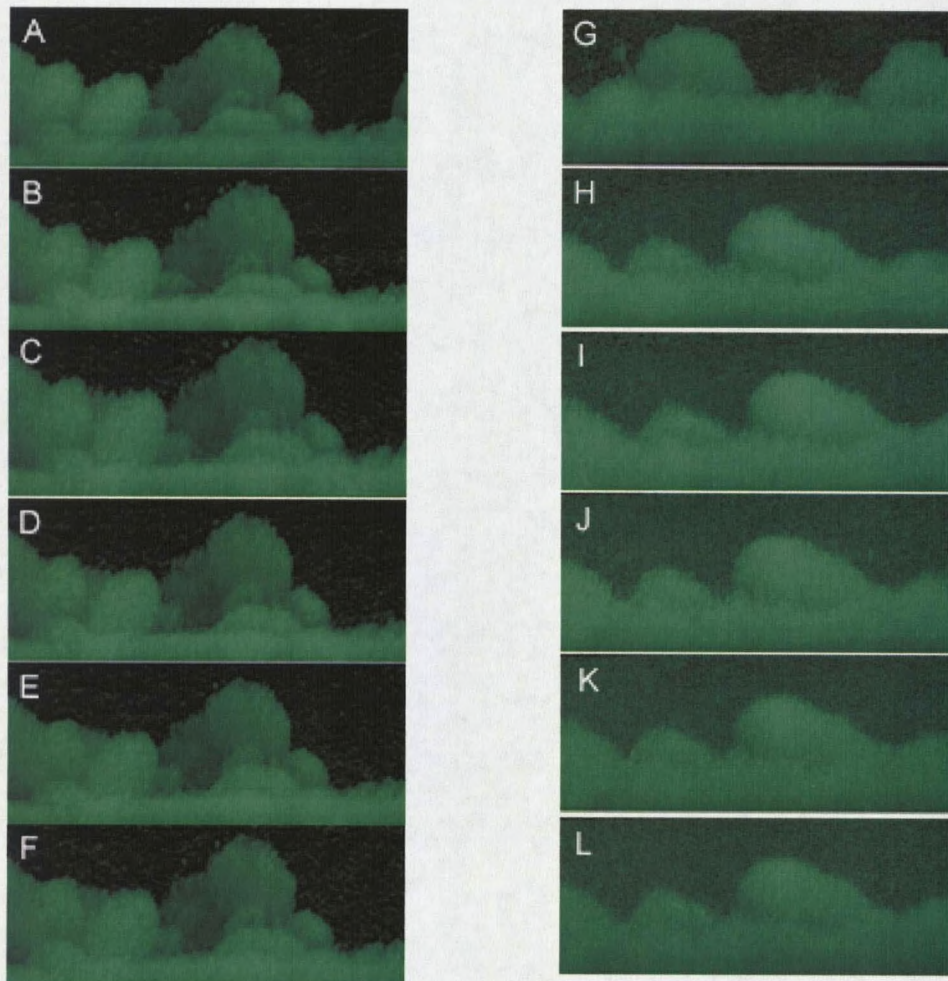
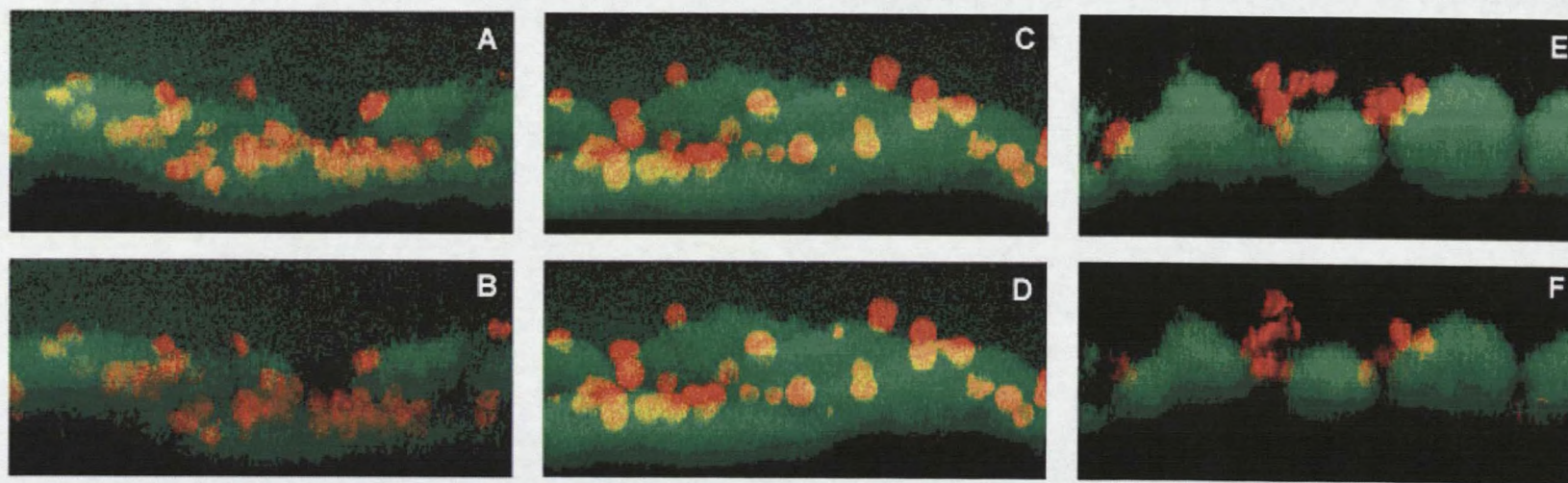


Fig. 10. SCLM showing the “blizzard” effect in the presence of 10 % autologous serum. A, B. Neutrophils and biofilm in HBSS at 15 and 45 minutes. C, D. Biofilm and neutrophils in the presence of serum. E, F. Biofilm treated with sodium azide to stop the respiration of bacteria. Note the absence of planktonic bacteria in the background even after 45 minutes.



155 μ m

Granule Release by Neutrophils in Response to *P. aeruginosa* Biofilm

The main defense mechanism that neutrophils employ to combat the infection with *P. aeruginosa* and other organisms is phagocytosis. Upon formation of phagosome within the neutrophils, neutrophil granules such as azurophilic and specific granules fuse with the phagosome, releasing their contents to the phagosomal lumen. The contents of azurophilic granules, also known as primary granules, include myeloperoxidase, a myriad of hydrolases, and other enzymes including myeloperoxidase and β -glucuronidase, which cleaves the important bacterial carbohydrates. Myeloperoxidase is an enzyme that catalyzes the formation of hypochlorous acid from H_2O_2 and Cl^- . Specific granules on the other hand, contain lactoferrin which functions as a chelator of ferric ions essential for bacterial survival, and have stores of the membrane component of the NADPH oxidase, flavocytochrome b, which forms O_2^- . Neutrophils and macrophages are messy eaters and often release contents of granules into the extracellular medium suggesting that this is a parallel, active process. When the target is too large to be phagocytosed, neutrophils go through frustrated phagocytosis in which the granules fuse with the cell membrane, releasing the contents extracellularly. The targets typically are larger organisms such as fungal agents, but Costerton *et al.* has suggested that the neutrophils also undergo frustrated phagocytosis against *P. aeruginosa* growing in a biofilm (Costerton *et al.*, 1999).

The morphological study employing the fluorescence microscopy, SCLM, and SEM did not definitively establish neutrophil phagocytosis of *P. aeruginosa*. Granular release assays were conducted to assess the degree to which neutrophils could be

stimulated by biofilm and to determine if in fact leaking of granule material occurs during the phagocytosis process. Fig. 11 shows the myeloperoxidase release by neutrophils under various conditions. The amount released under different conditions was normalized relative to the amount released when neutrophils were maximally stimulated with known secretagogue, 1 μ M fMLF, in the presence of 10 μ M dhCB. When the biofilm was not opsonized, neutrophils released approximately 20 % maximal activity. Upon opsonization, however, the amount released increased to 40 %, nearly doubling the release amount. Serum and fMLF/dhCB had somewhat additive effect. However, the relatively small increase in the amount released suggest that neutrophils were maximally stimulated. To independently confirm the extracellular release of the azurophilic granule content, the amount of β -glucuronidase release, another azurophilic granule enzyme, was also investigated (Fig. 12). Fig. 12 shows the small additive effect of serum and fMLF/dhCB in the presence of biofilm, which confirm the results from the myeloperoxidase assay. However, this assay does not appear to be as sensitive as myeloperoxidase assay, since the percent increase when neutrophils encounter opsonized biofilm is rather small. It is interesting to note here is that biofilm induced higher release of myeloperoxidase when compared to the release in contact with planktonic *P. aeruginosa*.

To examine the release of specific granule proteins, the release of lactoferrin, chelator of ferric ions, was determined. Lactoferrin not only limits the availability of iron required for bacterial metabolism, it is also known to damage the Gram-negative bacterial

cell wall and to inhibit the biofilm formation by inducing the twitching motility (Ellison, 1994; Singh *et al.*, 2002).

Figure 11. Myeloperoxidase release profile of the neutrophils treated with or without addition of serum, fMLF, and or dhCB. The solid black bars represent the percent maximal of myeloperoxidase release by neutrophils associated with the *P. aeruginosa* biofilm. The gray bars represent that of the neutrophils in association with the plastic control surface.

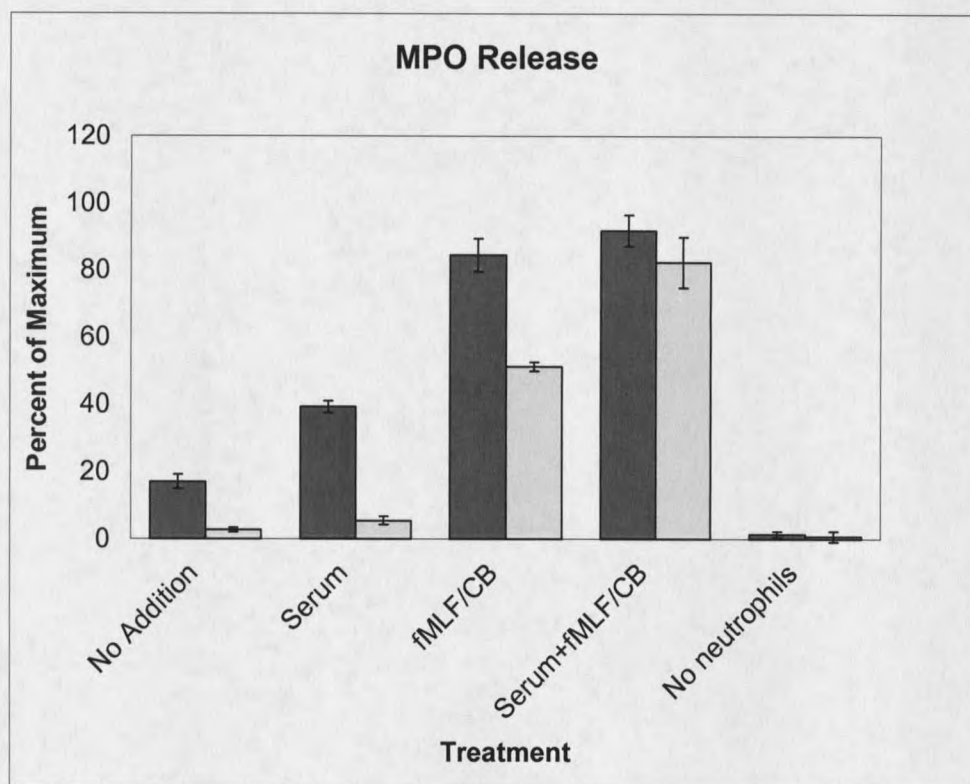


Fig. 13 shows lactoferrin was indeed secreted into the extracellular medium when neutrophils associated with the biofilm. The maximal release occurred when neutrophils were stimulated with the secretagogue, fMLF in the presence of dhCB. The quantitative analysis using Western blot revealed that $0.9 \pm 1.0 \mu\text{g}$ per 4×10^5 neutrophils. Interestingly, the secreted amount of lactoferrin does not seem to

significantly differ from that of plastic in the control plate, however, release of lactoferrin is more easily stimulated in neutrophil due to lower signal transduction requirements (Jaconi *et al.*, 1990).

Figure 12. Beta-glucuronidase release profile of neutrophils in association with *P. aeruginosa* biofilm (blue), plastic control substratum (magenta), and planktonic *P. aeruginosa*. Experiments conducted by Al Jesaitis.

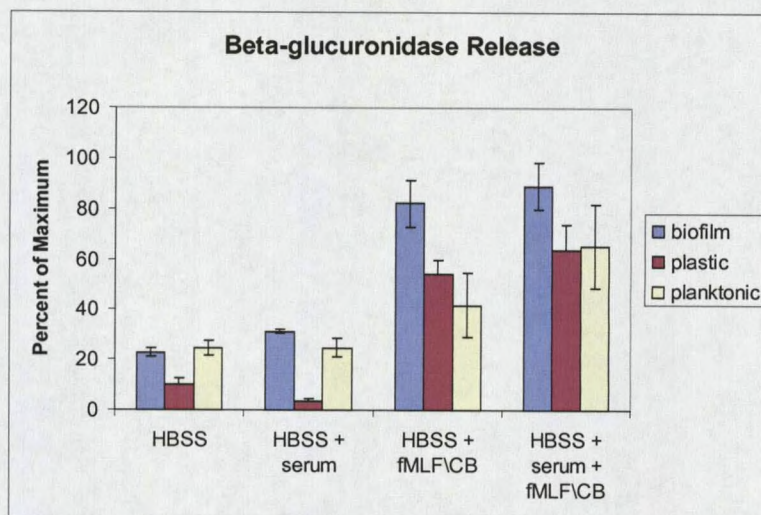
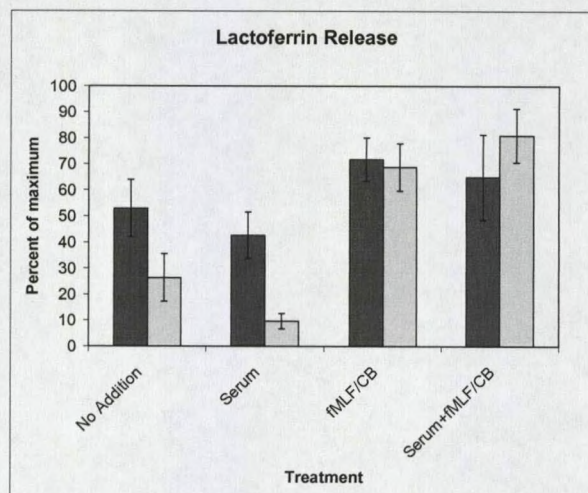


Figure 13. Lactoferrin release profile of neutrophils in association with *P. aeruginosa* biofilm (dark grey) or plastic control (light grey). Experiments conducted by Connie Lord.



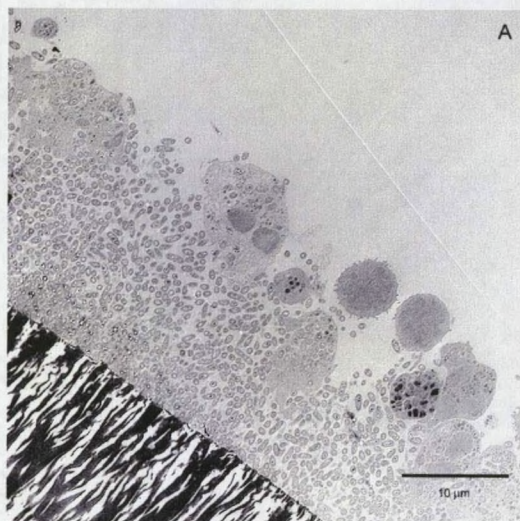
Neutrophil Phagocytosis of *P. aeruginosa* Growing in the Biofilm

Investigation of interaction between neutrophils and *P. aeruginosa* using fluorescence microscopy, SCLM, and SEM has suggested neutrophils appeared to be inhibited in their motility. Enzymatic assays and oxygen consumption measured suggested that neutrophils were functional and sensitive to stimuli (Jesaitis *et al.*, 2003). However, the first and the foremost mechanism of defense, phagocytosis, by inference from morphology could not be conclusively shown. To better assess the neutrophils' phagocytic capability, neutrophils interacting with the biofilm were examined using TEM. Since TEM allows the transverse imaging of the samples with high resolution down to the suborganelle level, it should provide more definitive evidence for or against neutrophil engagement in phagocytosis when in contact with biofilm. *P. aeruginosa* biofilms were grown in a drip flow reactor over polycarbonate membranes as growth substrata at 37 °C for three days. Neutrophils were layered over the biofilm in: 1) HBSS, 2) 10 % autologous serum, 3) dhCB and fMLF, and 4) Serum, dhCB, and fMLF. After 1-hour incubation, the samples were fixed, dehydrated, and embedded in the epoxy resin as described in the Materials and Methods section. Sectioning and microscopy were performed by the technical staff at the TEM facility of University of Montana.

This analysis clearly indicated that neutrophils were engorged with bacteria. With (Fig. 14) or without (Fig. 15) serum, neutrophils remained rounded on the dorsal surfaces, but actively extending the membrane portions into the surfaces of biofilm, interacting with it. Individual *P. aeruginosa* cells were found within the phagolysosome of neutrophils with clearly visible compartmentalization, and more than one bacterial

cells were often present in one phagolysosome (Fig. 15 B). In the presence of dhCB and fMLF with (Fig. 16) or without (Fig. 17) serum, the neutrophils showed no active interaction with the biofilm. Under these conditions, neutrophils did not contain any bacteria-containing phagolysosome, and there was no evidence of extended pseudopod formation at the biofilm interface. Neutrophils appeared relatively spherical overall and effectively degranulated, which was expected due to the action of dhCB, which impairs actin polymerization and promotes full degranulation. However, under no conditions did neutrophils appear to travel deep into the biofilm. Instead, they appear to adhere to the biofilm interface, actively phagocytosing the bacteria with which they come in contact if dhCB is not included in treatment. However, as SCLM results indicated, the distribution of neutrophils vertically and horizontally seems to be rather limited. Thus, despite the rounded dorsal appearance of neutrophils, they are clearly capable of phagocytosing the bacteria at the biofilm interface.

Figure 14. Transmission electron micrograph of neutrophils phagocytosing the bacteria grown in a biofilm. (A) Neutrophils shown with *P. aeruginosa* biofilm. (B) Larger magnification of a neutrophil engorged with *P. aeruginosa*.



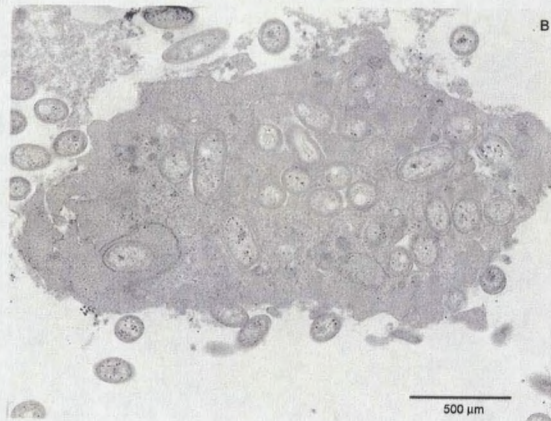


Figure 15. TEM image of neutrophils engorged with *P. aeruginosa* pre-treated with 10% autologous serum. (A) Neutrophils engorged with *P. aeruginosa*. (B) Larger magnification of a neutrophil engaged in phagocytosis.

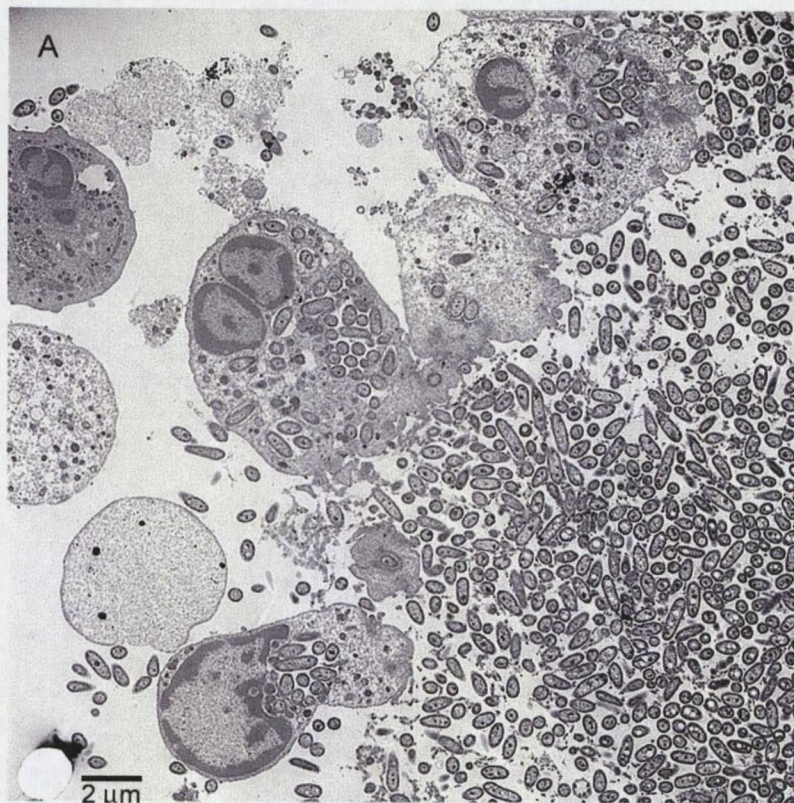




Figure 16. TEM image of neutrophils in association with *P. aeruginosa* biofilm pretreated with fMLF and dhCB. Cytoskeletal control of neutrophils are compromised, and the neutrophils remained rounded.

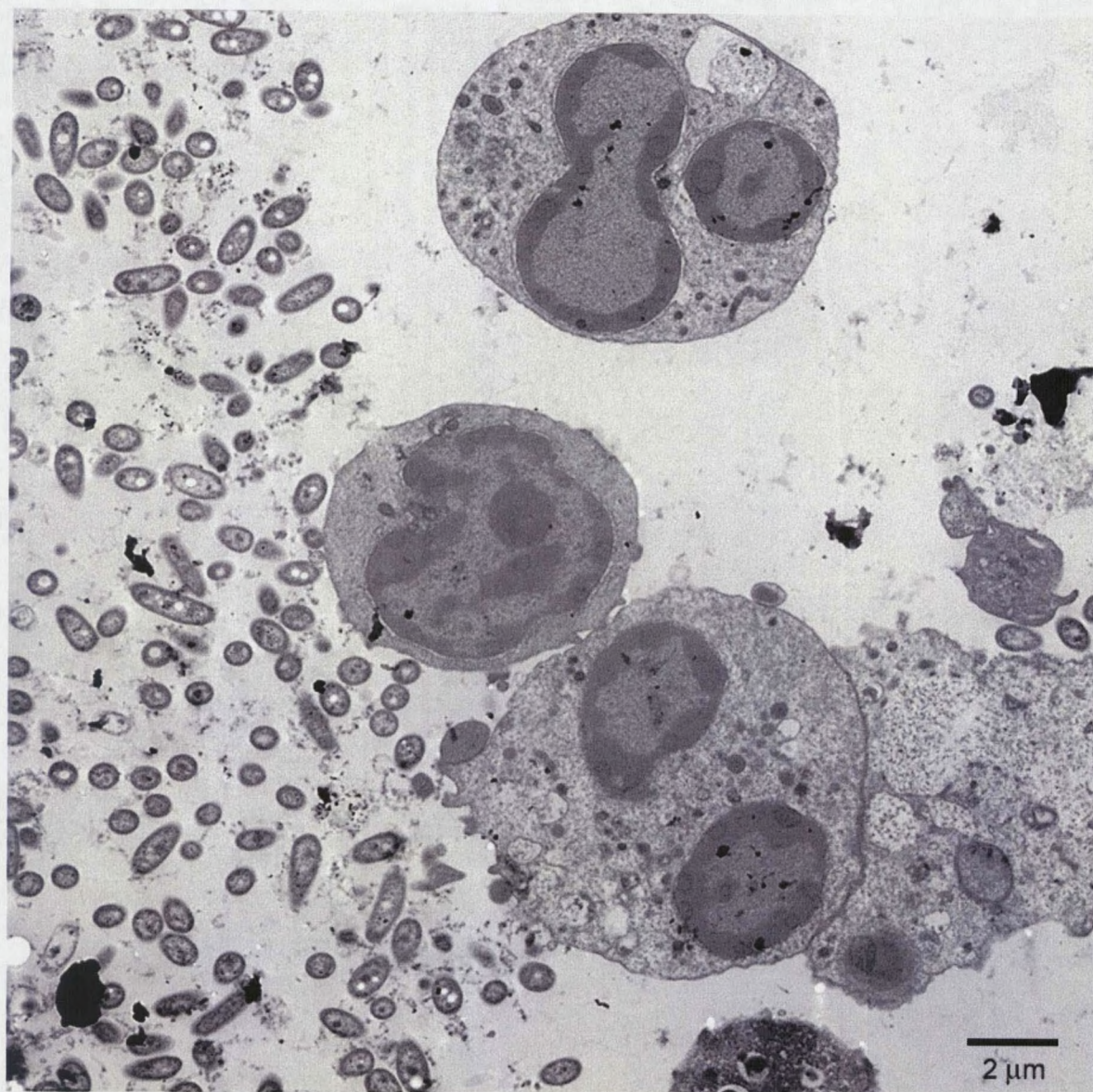
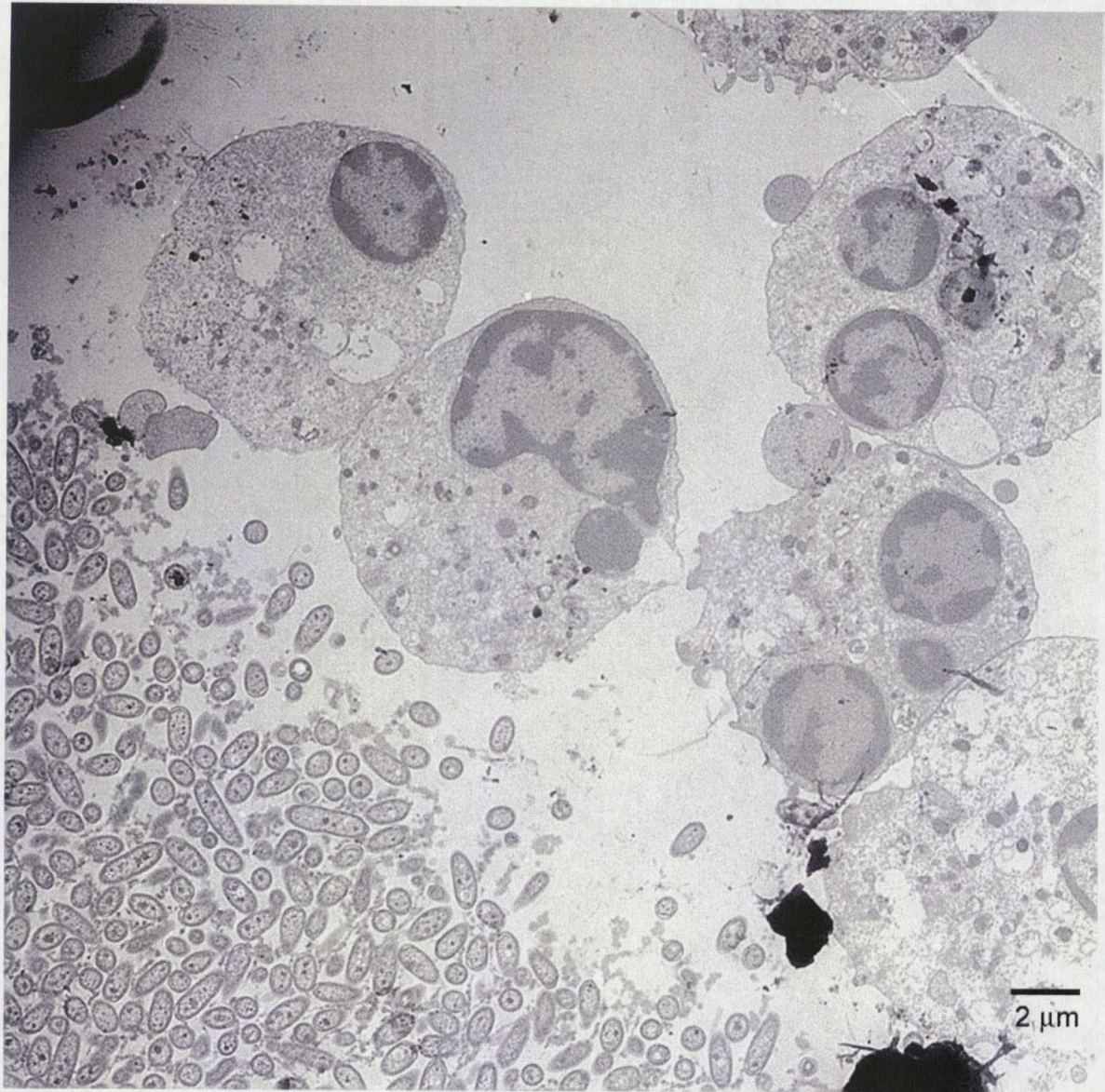


Figure 17. TEM image of neutrophils in association of *P. aeruginosa* biofilm pretreated with 10% autologous serum, fMLF, and dhCB.



CHAPTER 4

DISCUSSION

Many bacteria, pathogenic and non-pathogenic, are known to form biofilms in the environment in which they thrive. Much of what has been known about the pathogenesis of diseases was derived using the planktonic cultures of pathogenic organisms. However, many pathogenic organisms that colonize the human body by forming biofilms appear to be difficult to eradicate under various therapeutic or antibiotic regimens. Recently, the role that biofilms play in human diseases has been under renewed investigation.

In cystic fibrosis, a mutation in CFTR alters the lung environment in such a way that the colonization of opportunistic pathogen, *P. aeruginosa*, is permitted. Although the pathogenic role of defective CFTR in CF have not been clearly identified, it is evident that the lung environment of young CF patients allow an opportunistic pathogen to infect, colonize, and devastate the host immune system. Both host immune system components and bacteria are known to cause the lung damage CF patients suffer, but the damage incurred by the neutrophils influx and subsequent activation appears to be greater than that caused by the bacteria.

Neutrophils represent the first line of defense against the bacterial infection. Upon encounter with potential infectious agents, they engage their phagocytic machinery to remove the threat. Neutrophil granules containing proteases and non-enzymatic bactericidal compounds fuse with the phagosome. Concomittantly, the oxidative burst

produces an array of reactive oxygen species. However, toxic components of neutrophils still leak out into the environment, exacerbating damage seen in CF lungs. The leak might be due to failure to seal the phagolysosome completely, but such leakage also takes place during frustrated phagocytosis. Frustrated phagocytosis is a process in which a neutrophil attempts to engulf targets too large for the cell. Granule contents are released during this process. Though frustrated phagocytosis is effective in combating agents such as parasites or fungi, it also results in damage of the host tissues.

Much of the investigation on interaction between neutrophils and *P. aeruginosa* has been conducted using the planktonic bacteria. However, *P. aeruginosa* forms biofilm in the CF lungs, and the growth and metabolic characteristics of biofilm significantly differ from that of planktonic organisms (Lam *et al.*, 1980; Xu *et al.*, 2000; Saur *et al.*, 2002; Whiteley *et al.*, 2001). That is likely to be one of the probable reasons why *P. aeruginosa* escapes the immune surveillance and is able to colonize the CF lungs. Only recently, the investigation of *P. aeruginosa* biofilms in relationship to CF pathogenesis has intensified.

In our study, both morphological and biochemical characterization of neutrophil-biofilm interaction were attempted, using PAO1 and PAO1-GFP biofilms as model systems. Interaction of neutrophils with *P. aeruginosa* biofilms were imaged with several microscopy methods in an attempt to verify that neutrophils are indeed functional against the biofilms.

Fluorescence microscopy, SCLM, and SEM results all clearly indicated that the neutrophils remained rounded with little or no evidence of directional motility upon

encounter with the *P. aeruginosa* biofilm. As seen in Fig. 1, neutrophil in planktonic *P. aeruginosa* suspension exhibited random pseudopodia formation along with extensive membrane ruffling. Neutrophils also adhered to and spreaded over various substrata including polycarbonate membranes and glass surfaces. As seen in the fluorescence micrograph of Fig. 5, stained but unstimulated neutrophils were able to adhere to the glass surface tightly, forming a flat disc with even distribution of membrane in a radial fashion.

Neutrophils, however, did not display such characteristics of adhesion and spreading when they came in contact with the biofilm. They appeared to settle on the biofilm, remaining round and virtually immobile. *P. aeruginosa* biofilm present a plethora of chemoattractants such as fMLF and inflammatory activators such as LPS. Since neutrophils still retained the ability to adhere to the polycarbonate membrane in the presence of planktonic *P. aeruginosa* as seen in Fig. 3, it can be inferred that the physical and chemical characteristics of biofilm as a whole may contribute to inability of neutrophils to initiate in normal migratory responses.

Due to their morphology, it was originally hypothesized that *P. aeruginosa* biofilms may inhibit neutrophils' cytoskeletal remodeling capability, with an action similar to a fungal toxin, cytochalasin B. In fact, the neutrophil treated with 10 μ M cytochalasin B as seen in Fig. 5 B closely resembles the morphology of neutrophil interacting with biofilm (Fig. 4 B). However, as transmission micrography clearly demonstrated, untreated neutrophils are capable of engulfing bacteria, whereas dhCB-treated neutrophils failed to do so. Furthermore, upon close inspection using TEM,

neutrophils do extend small, local pseudopodia allowing them to envelop bacteria and complete the process of phagocytosis. On the other hand, neutrophils treated with dhCB remain spherical even at the biofilm interface. Other difference to note is that dhCB-treated cells remain rounded and look devoid of granules (Fig. 16), whereas untreated cells appear to be capable of local bacterial engulfment on the surface of the biofilm albeit their shallow depth of reach (Fig. 14).

One speculative explanation for such a limited response may be that bacterial toxins such as exoenzyme S or exoenzyme T are injected into the neutrophil. ExoS and ExoT are *P. aeruginosa* toxins that are delivered to the targets via type III secretion mechanism which requires cell-to-cell contact. ExoS is a bifunctional cytotoxin that can act as an ADP-ribosyltransferase or as a GTPase activating protein (GAP). GAP functions to accelerate the exchange of GTP-bound form of rho family of GTP-binding proteins, thereby inhibiting them (Gumbiner *et al.*, 1996). ExoS is known to alter the localization patterns of Rac and Cdc42, which are involved in formation of lamellipodia and filopodia respectively (Gumbiner *et al.*, 1996), and inhibiting them (Krall *et al.*, 2002). Thus, it is possible that ExoS may be involved in selective or partial inhibition of neutrophil cytoskeletal remodeling capability. Adherence and phagocytosis, however, may be regulated by other GTPases. The effect of these toxins on neutrophils is not well understood. There may also be differential expression pattern of such toxin between *P. aeruginosa* growing in biofilm versus planktonic mode. However, to the author's knowledge, differential expression pattern of ExoS has not been reported to date. Thus, it may be of interest to pursue this possibility in the future.

Leid *et al.* has suggested that neutrophils actively bore into the *Staphylococcus aureus* biofilm, leaving a halo of clearance around the neutrophils (Leid *et al.*, 2002). However, according to our results, neutrophils do not actively migrate into the biofilm surface. As seen in the SCLM time-lapse images, bacteria obscure the neutrophil from the dorsal viewpoint (Fig. 8). This response similar to flight is induced either by addition of neutrophil or serum alone, indicating that host immune component also can induce such a response (Fig. 9). The respiratory inhibitor of bacteria, NaN_3 , abolishes the “blizzard” effect seen after addition of either agent (Fig. 10 E and F), further supporting the notion that only viable bacteria are able to respond to such extracellular stimuli or host digestive pressure.

Singh *et al.* has reported that a specific granule protein, lactoferrin, induces the twitching motility in *P. aeruginosa* biofilm (Singh *et al.*, 2002). Lactoferrin is a ubiquitous iron chelating compound found in various human secretions including tears and milk. Chelation of iron stimulates the bacterial cells to initiate in surface motility via Type IV pili. This appears to be crucial in the initial stage of biofilm formation, as lactoferrin was found to be inhibitory of biofilm formation whereas it did not affect the biofilm to a significant degree once the biofilm matures (Singh *et al.*, 2002).

Despite the fact that neutrophils appear to be immobilized at the surface of the biofilm, we clearly have shown that they are capable of granule release. Furthermore, as the TEM images demonstrated, neutrophils are able to phagocytose bacteria at the biofilm interface. Serum treatment did not appear to significantly alter the neutrophil behavior in this regard, since neutrophils in the absence of 10 % autologous serum

carried out phagocytosis quite effectively as shown in Fig. 13 B. When the cytoskeletal remodeling mechanism was crippled by 10 μ M dhCB, neutrophils were incapable of phagocytosis, indicating that the lack of motility in the presence of biofilm does not indicate that the phagocytosis is inhibited (Fig. 16).. This differential response suggests that the neutrophils may somehow be selectively inhibited by *P. aeruginosa*. This interesting result will need further investigation.

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