MONTANA STATE UNIVERSITY

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
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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 dihidro 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.

INTERACTION BETWEEN HUMAN NEUTROPHILS AND *PSEUDOMONAS*

AERUGINOSA BIOFILM: MORPHOLOGICAL AND BIOCHEMICAL

CHARACTERIZATION

by

Maiko Sasaki Papke

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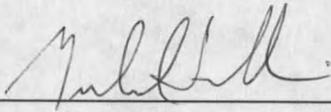Bozeman, Montana

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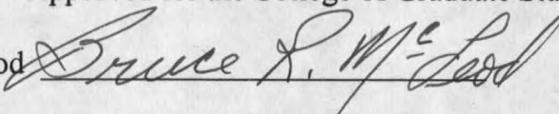
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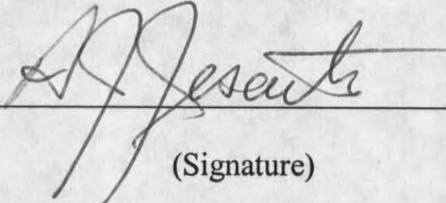
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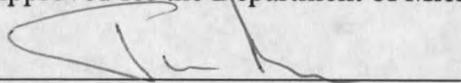
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ABSTRACT

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.

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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 transmembreane 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 (Δ508) 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; Westbrock-

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). Glutahione (GSH) is a scanvenger 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 inteleukin-10 (IL-10) and increased expression of chemoattractant inteleukin-8 (IL-8) and interleukin-6 (IL-6). IL-10 is consititutively 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 IκB. IκB, in turn, inhibits the activation of NF-κB by preventing its nuclear translocation. NF-κB, 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 inbalance of IL-10 availability in the lung microenvironment. Thus, the anti-inflammatory responses are overwhelmed by pro-inflammtory 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 (Sommerfoff *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.

<center>*Pseudomonas aeruginosa*</center>

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 NO_3 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. aerugionosa* 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 exopolyssaccharide 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. Leukocydin 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 interfereing 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 cytotoxity 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 (Wimpennyand 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; hierchical yet intertwined. The first is the LsasI/LasR system, and the secondary system is the RhlI/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 equilibriating 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 estages of the biofilm growth, there is an inhibitory interaction between RsaL and lasl. RsaL binds to the regulatory region of *lasl* operon, inhibiting the activation of the operon. As the biofilm matures, the threshold concentration of LasI competitively removes the inhibiton of RsaL, thereby initiating the signaling cascade (Shirtiff *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 $4.4E10^6$ 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. Durings 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 in 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