



The role of oxygen and the interaction of human neutrophils with viable planktonic and biofilm
Pseudomonas aeruginosa
by Justin Brock Bleazard

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

Human neutrophils are uniquely capable of generating reactive oxidants when exposed to a variety of serum-opsonized particles or soluble inflammatory stimulants. Neutrophils striking ability to pass high-energy electrons to molecular oxygen (O_2) to form the superoxide anion (CF) is central for host antimicrobial defense. This pathway, though effective against bacterial and fungal pathogens, also generates free radicals associated with chronic and acute inflammatory damage of normal neighboring cells. However, bacterial pathogens that employ a biofilm mode of growth present additional obstacles to professional phagocytes such as neutrophils. The biofilm structure, the role of oxygen, and possibly the release of chemoattractants form biological and chemical barriers that may retard the penetration of phagocytes and inhibit host cellular defense mechanisms.

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The results of this study indicate that human neutrophils show a decreased antimicrobial efficacy towards *Pseudomonas aeruginosa* biofilms when compared to their planktonic counterpart. This efficacy was increased by the autologous serum opsonization of bacteria, which agrees with the concept that relevant components of complement are crucial peptide mediators of inflammation. Also, biofilms exposed to 10% normal human serum displayed an immediate marked increase in oxygen consumption that rivaled that of human neutrophils. These results suggest that a dynamic competition between neutrophils and bacteria exists, providing biofilm bacteria with an increased resistance to cellular antimicrobials. Microscopy results indicated both neutrophil plasma membrane stimulation and neutrophil penetration into the biofilm occurred. Further understanding of resistance mechanisms utilized by such bacteria will aid in the treatment of biofilm infection and inflammation.

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PSEUDOMONAS AERUGINOSA

by

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In

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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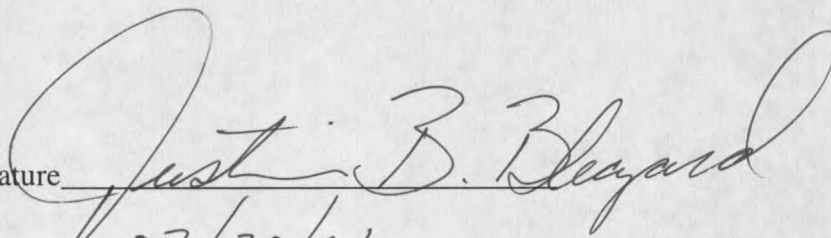

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Abstract

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CHAPTER 1

INTRODUCTION

Role of Human Neutrophils in the Immune System

Human neutrophils or polymorphonuclear leukocytes (PMNs) are professional phagocytes and the immune system's first cellular defense against invading pathogens. They are an essential component of the acute inflammatory response. Neutrophil migration to sites of infection and injury is mediated by numerous chemoattractants including those derived from living and dead bacteria. This process is vital for host defense and tissue repair (Miettinen *et al.*, 1998). When appropriately stimulated, neutrophils produce superoxide anion (O_2^-). The superoxide anion is an important precursor for the production of a variety of toxic oxygen species including hydrogen peroxide, hypochlorous acid, and hydroxyl radical; all at the expense of cytoplasmic NADPH. With these defensive mechanisms, neutrophils can destroy microorganisms and malignant cells but also induce injury to adjacent normal tissues. The enzyme system responsible for the production of superoxide is the NADPH oxidase, a multicomponent complex consisting of flavocytochrome b_{558} , p40, p47, p67 and the small G proteins, Rac2 and Rap1a.

Baldrige and Gerard first discovered the extra respiratory activity of PMNs, "respiratory burst", in 1933 while studying canine neutrophils. Their electrode studies indicated that neutrophils consume large quantities of dissolved molecular oxygen. This seminal discovery was later shown to be central to the

host defense against bacterial and fungal invaders. The essential role played by the NADPH oxidase is unequivocally demonstrated by individuals with Chronic Granulomatous Disease (CGD). These patients lack a functional flavocytochrome b558 or functional assembly proteins and do not efficiently kill biological pathogens such as bacteria. A complete description of (CGD) is found the following section entitled: NADPH Oxidase and the Role of Oxygen.

In their resting state, neutrophils circulate through the vascular system as freely floating cells and by rolling along the vascular endothelium. At sites of injury, infection, or inflammation they strongly adhere to the endothelium and migrate through the vascular layer into the surrounding tissue. As they approach stimulant sources such as chemokines, cytokines, complement components, and chemoattractants, their entire cellular machinery is primed for the release of a potent defensive arsenal. Their ability to generate superoxide is contained by holding the NADPH oxidase in a disassembled configuration. Once neutrophils reach the infection site, they mobilize the NADPH oxidase system and release superoxide radicals into the local environment in order to kill and clear biological pathogens.

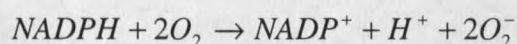
NADPH Oxidase and the Role of Oxygen

The NADPH oxidase reduces molecular oxygen with high energy electrons to produce the oxygen radical superoxide anion. The central redox component of the oxidase is the flavocytochrome b, a heterodimeric, integral membrane glycoprotein composed of 91-kDa (gp91^{phox}) and 22-kDa (p22^{phox})

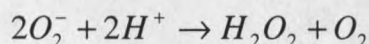
subunits. Two low potential, non-covalently bound hemes are responsible for the transfer of high-energy electrons from NADPH to molecular oxygen.

Flavocytochrome b_{558} possesses NADPH, FAD and possibly O_2 binding sites and is the enzymatic center of the oxidase. The other oxidase components regulate its function. Superoxide is then metabolized further to generate an array of toxic oxygen species responsible for the neutrophil mediated killing of invading pathogens and collateral host tissue damage (Segal & Meshulam, 1979); (Jesaitis *et al.*, 1986); (Parkos *et al.*, 1987); (Burritt *et al.*, 1995). The regulation of this redox component is at the core of controlling microbicidal and inflammatory damage.

The flavocytochrome b requires both NADPH and dissolved molecular oxygen in order to produce toxic oxygen species as described in the following chemical reaction:



(Curnutte & Babior, 1974). The superoxide molecule (O_2^-) spontaneously dismutates at neutral pH or in the presence of superoxide dismutase to form hydrogen peroxide and oxygen as shown in the following reaction (Curnutte & Babior, 1974).



The superoxide anion itself is relatively inactive in hydrophilic environments. However, it is the subsequent toxic oxygen species produced from superoxide side-reactions and production of hydrogen peroxide that delivers the chemical

assault to pathogens. Superoxide is a strong base when confined to hydrophobic environments such as the transmembrane region of cells. Within the transmembrane domain, superoxide reaction with lipids is kinetically favorable at pH values between 7 and 10. Thus, the generation and reaction of superoxide with plasma membranes aids in the killing of microbial invaders. However, the environment and mechanisms with which superoxide reacts in hydrophobic regions makes the direct killing of bacteria by superoxide both difficult to analyze and quantify.

Table 1. Toxic Oxygen Species Produced From The Superoxide Anion

<u>Component</u>	<u>Species</u>
Amines	RNHCl
Dismutation, Superoxide Dismutase	H ₂ O ₂
Hyperhalous Acids	HOCl, HOBr
Hydroxyl Radical	OH [•]
Lipids	Lipid
Peroxides	H ₂ O ₂
Nitric Oxide	ONOOH
Hydroperoxyl Radical	HO ₂ [•]

A complete description of each of the above reactions can be found in, Chapter 47 "Oxygen Metabolites from Phagocytes", by Seymour J. Klebanoff Inflammation: Basic Principles and Clinical Correlates (1999).

Neutrophil influx into extremely anaerobic environments will diminish the cell's ability to form superoxide. The published K_m value, half-saturation coefficient, for intact human neutrophils is 25μM oxygen (Light *et al.*, 1981). Thus, oxygen concentrations below this binding affinity will reduce the rates at which neutrophils clear bacteria and yeast. Pathogenic organisms that are more efficient at metabolizing oxygen will compete with neutrophils for ambient oxygen. Obligate anaerobes and facultative anaerobes can exploit the lack of

oxygen as a way to evade a primary host defense weapon of the phagocytic cells. Additionally, it would seem clear that the flavocytochrome b requires adequate oxygen concentrations to thermodynamically promote superoxide production and clearing of bacterial and fungal populations.

The importance of human neutrophil's NADPH oxidase and "respiratory burst" has been linked to the genetic syndrome called chronic granulomatous disease (CGD). A variety of mutations in the component genes of the NADPH

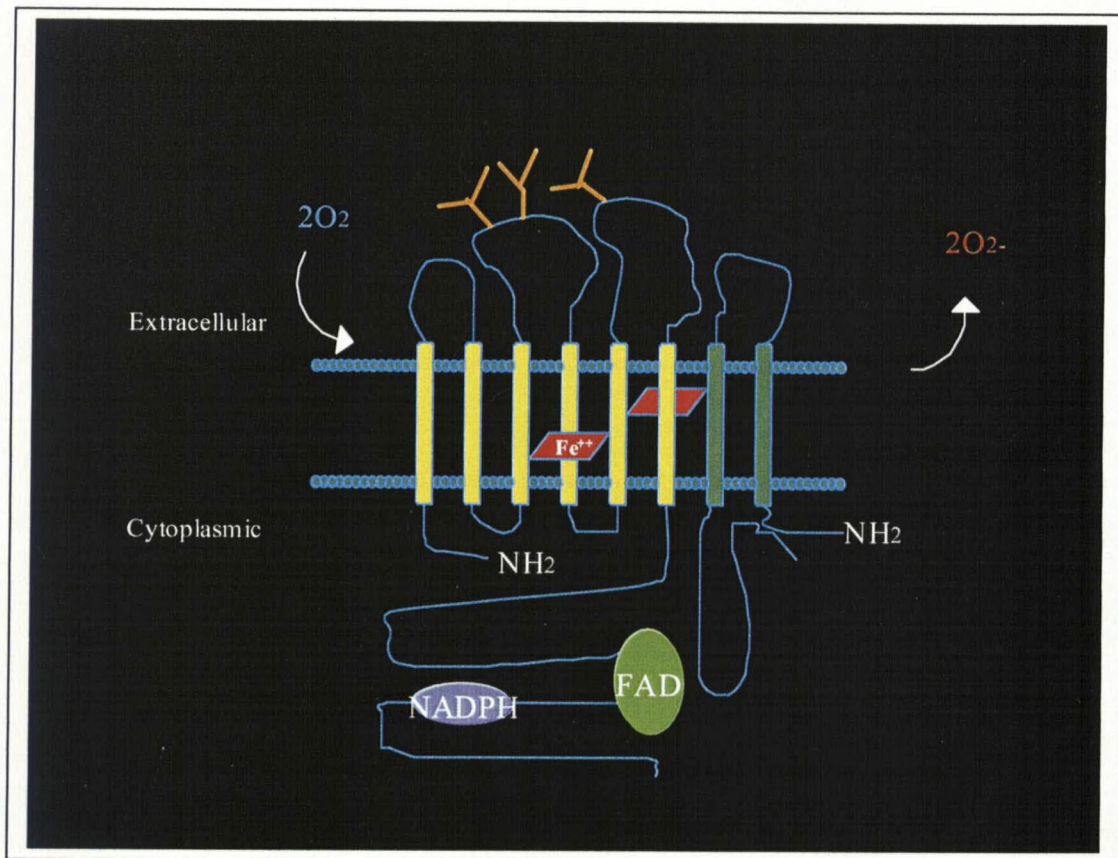


Figure 1. Putative model of the transmembrane protein Flavocytochrome b₅₅₈. Yellow bars indicate transmembrane domains of gp91^(phox), green bars indicate transmembrane domains of p22^(phox), copper branches represent heterogeneous glycosylation sites, blue lines form the extracellular and cytoplasmic loops, red rhombi portray iron hemes and purple and green ovals the NADPH and FAD binding domains respectively.

oxidase complex render the system ineffective or absent. Individuals who carry the CGD genetic disorder have phagocytes that lack the ability to produce or regulate superoxide production. These individuals cannot mount an effective host defense response toward catalase-positive bacteria and yeast such as *Pseudomonas* and *Candida*, respectively. Chronic bacterial and yeast infections call for drastic antimicrobial chemotherapy and often prevent patients with CGD from reaching adulthood. The genetic trait is estimated to effect 1:250,000 (Shatwell & Segal, 1996; Kuijpers *et al.*, 1999).

Normal individuals with a functioning NADPH oxidase system require only 5% of normal superoxide generating levels to maintain their health. It is this enzyme system that monitors superoxide production. The lack of or a deficiency in flavocytochrome b is a directly correlates with an individual's bacterial killing efficiency.

Pseudomonas aeruginosa: A Bacterial Pathogen of Cystic Fibrosis

Pseudomonas aeruginosa is a motile, rod-shaped, gram-negative and environmentally adaptable bacterium. A microbial opportunist, *P. aeruginosa* colonizes a diverse number of tissues within the human host, none more recognized than the pulmonary epithelium. In cystic fibrosis (CF) patient, pulmonary colonization with *P. aeruginosa* exceeds that of all other pathogens and increases drastically through childhood to reach 80-90% of CF adults (Hutchison & Govan, 1999). Individuals with CF fortunate enough to escape the detrimental colonization of *P. aeruginosa* have life expectancies twice that of

infected patients. Chronic bronchiopulmonary infections due to *P. aeruginosa* are the leading cause of morbidity and mortality in CF patients and lead to episodes of pulmonary exacerbation (Hutchison & Govan, 1999).

P. aeruginosa is found in two phenotypes, mucoid and non-mucoid, based on the presence or absence of the encapsulating polysaccharide alginate, respectively. Mucoid *P. aeruginosa* isolated from the sputum of CF patients will rapidly revert to the non-mucoid phenotype after subcultureing. The mucoid strain, once removed from the patient's lung, no longer requires alginate production and its ability to revert to a non-mucoid strain is indicative of *P. aeruginosa*'s adaptive capabilities.

P. aeruginosa will also attach, colonize, and grow on a variety of surfaces such as the pulmonary epithelium and the epidermis of the urinary canal. This sessile form of growth is known as a biofilm and is a mechanism for the increased antimicrobial resistance of microorganisms.

Studies by (Mathee *et al.*, 1999) have indicated that *P. aeruginosa* biofilms exposed to hydrogen peroxide (HP) in vitro may be a stimulatory pathway for alginate production. Mucoid strains of *P. aeruginosa* isolated from CF sputum that had reverted to non-mucoid were treated with 3% HP for 6hrs. Upon completion of HP treatment, the strain became mucoid again. This system may model the effect seen in CF patient's lungs colonized by non-mucoid *P. aeruginosa* followed by infiltration of HP producing polymorphonuclear leukocytes.

Researchers have also suggested the hypertonic environment of CF patient's lungs contributes to the production of alginate. This condition is caused by a dysfunctional locus encoding the cystic fibrosis transmembrane regulator (CFTR) protein, which balances chloride ions across the cell membrane. In either case, CF lungs display a reduced ability to clear bacterial infections. It is believed that the formation of biofilms in combination with alginate production are key mechanisms in resisting opsonophagocytosis and the direct killing of bacteria by phagocytes.

Pseudomonas aeruginosa Biofilm Mode of Growth

We have selected *Pseudomonas aeruginosa* as a model microorganism because it is an opportunistic pathogen that is often referred to when analyzing a biofilm mode of growth. It is also well known that chronic *P. aeruginosa* infections of airways or infections resulting from the use of catheters or other invasive medical devices are some of the most difficult to treat. Eradication of these bacteria using anti-*Pseudomonas* agents is rarely achieved. In most cases a short-term transient improvement in health occurs followed by a recurrence of symptoms (Kobayashi, 1995). Researchers have illustrated that the increased virulence displayed by *P. aeruginosa* is due in part, to a biofilm mode of growth (Hoiby *et al.*, 2001). Here mass transport of anti-*Pseudomonas* agents is limited by a reaction-diffusion mechanism. However, this mechanism only provides a limited description of the increased virulence of bacteria growing as a biofilm. A more general description of *P. aeruginosa*'s increased virulence must take into account reaction-diffusion with other supporting mechanisms, for example;

phenotypic changes, heterogeneity within the biofilm, and resistance to phagocytosis.

A number of investigators including (Liu *et al.*, 1998), (Elkins *et al.*, 1999), and (Stewart *et al.*, 2000) have shown that biofilm resistance to antimicrobials is much greater than the resistance of planktonic organisms. It is this recalcitrance to a broad spectrum of antimicrobials that makes biofilm control difficult. The retarded penetration of HP into biofilms formed of *P. aeruginosa* serves as a model for describing the mechanism of reaction-diffusion. *P. aeruginosa* resists HP penetration by limiting the diffusion and transport of HP. Diffusion coefficients for HP in biofilms are approximately 80-90% of the diffusion coefficient in water (Stewart, 1998) (Cochran *et al.*, 2000). The diffusional-transport of HP, however, is retarded not only by the formation of a biofilm structure but also by the reaction with catalase A and B. Transport of antimicrobial agents such as HP or HOCl is hindered from the bulk fluid to the substratum because these substances oxidize the polymeric matrix of the biofilm. However, if ample time is allowed, a steady state value within the biofilm eventually arises based on Fickian diffusion.

Where:

$$(1) \quad J_{Az} = -D_{AB} \frac{dC_A}{dz}$$

Thus, a chemical reaction between the biocide and the polymeric matrix takes place within the biofilm and consumes the HP at a rate far greater than the flux by the differential concentration between the liquid-biofilm interface and the

substratum. This oxidation reaction of the polymeric matrix, assuming first order kinetics, is defined below as:

$$(2) \quad r_A = -k_A C_A$$

However, during the dismutation of superoxide to hydrogen peroxide, oxygen is produced. Additionally, a significant fraction of the hydrogen peroxide may react with catalase to produce more oxygen. These reactions result in a cyclic process of oxygen consumption and hydrogen peroxide production. Therefore a combination of reactions is needed to define the dynamic course of hydrogen peroxide transport into the biofilm. Assuming first order kinetics for each reaction (3) dismutation and (4) catalase will result in an overall oxygen consumption reaction defined as (S):

$$(3) \quad r_B = -k_B C_B$$

$$(4) \quad r_C = -k_C C_C$$

$$(5) \quad S = \sum r_A + r_B + r_C + \dots + r_n$$

Combining equation (1) and (5) yields a generic reaction-diffusion equation that is expressed below as the change of concentration versus time with flux designated by (J_{Az}).

$$(6) \quad \frac{dC}{dt} = \frac{d}{dz}(J_{Az}) - S$$

Support for the above equation has been provided by biological assays and microelectrode measurements of HP penetration and killing of wild-type and catalase mutant *P. aeruginosa* biofilms by (Liu *et al.*, 1998), (Elkins *et al.*, 1999) and (Stewart *et al.*, 2000). Each has indicated *P. aeruginosa*'s dependence on catalase for survival. (Elkins *et al.*, 1999) directly compared *katA* and *katB* catalase mutants to wild-type cells. The researchers showed that planktonic and biofilm *katA* mutants were susceptible to HP doses of 50mM while *katB* mutants and wild-type cells were not affected by the treatment. These experiments suggest that KatA catalase is the dominant form and is responsible for the protection of *P. aeruginosa* biofilms against HP. These results combined with those of (Ma & Eaton, 1992) also show that the aggregated or biofilm growth of single-celled organisms is a successful defense against antimicrobial treatments such as HP.

Biofilms structural heterogeneity is an additional factor in influencing bacterial virulence (Wimpenny *et al.*, 2000; Xu *et al.*, 1998). The employment of confocal scanning laser microscopy (CSLM) has shown that biofilms contain a complex structure containing channels, pores, microcolonies and interstitial voids (Yang *et al.*, 2000). However, biofilms are often described and modeled as flat slabs divided between the bulk fluid and substratum. Local growth conditions, microorganisms, flow velocity, biofilm porosity and density all affect the local intrabiofilm mass transport. For example, distinguishing between the water in the biofilm matrix and the bulk fluid of the biofilm generates intrinsic problems when studying local mass transport phenomena (Lewandowski, 1994).

The use of cathodically polarized microelectrodes, however, has recently allowed the measurement of local effective diffusivity coefficients in biofilms (Beyenal & Lewandowski, 2001). This examination shows that the limiting current of the microelectrode is proportional to the flux of the analyte under study and is mathematically related to Fick's law. Such an approach has tied the consumption of HP or other analytes in biofilms to the reaction-diffusion mechanism by analytically displaying the chemical species profiles through the biofilm (Rasmussen & Lewandowski, 1998; Beyenal & Lewandowski, 2001).

A collaboration of groups at the Department of Clinical Microbiology, Copenhagen, Denmark and the University of Calgary, Alberta, Canada permitted the first analysis of the response of human polymorphonuclear leukocytes (PMNs) to *P. aeruginosa* biofilms (Jensen *et al.*, 1990). Their experiments suggest that PMNs respond to attached bacteria differently than their planktonic counterpart. Chemiluminescence assays were used to examine the respiratory burst of human neutrophils when interacting with planktonic and resuspended biofilm *P. aeruginosa*. The neutrophil's superoxide responses were slower and lower in magnitude for resuspended biofilms than for planktonic cultures. A recent report by (Costerton *et al.*, 1999) states that neutrophils can clear planktonic bacteria but only undergo frustrated phagocytosis when faced with sessile communities of bacteria. In such cases, the ability of neutrophils to ingest large foreign particles is ultimately limited by the size of the phagosome. Particles that are too great in size induce a physical response where the neutrophils are unable to close the "zipper" of the phagosome and seal in its contents. This process is defined as

frustrated phagocytosis. Frustrated phagocytosis causes the neutrophil to release its hydrolytic enzymes and superoxide into the local environment in an attempt to kill bacteria or fungal invaders.

Hypothesis

The hypotheses for the research presented in this thesis are as follows. (1) Planktonic human neutrophils will consume dissolved molecular oxygen when challenged with a variety of soluble stimuli or bacteria in an unopsonized or opsonized state. (2) Planktonic human neutrophils will release superoxide during their respiratory burst that will dismutate into measurable quantities of hydrogen peroxide. (3) Neutrophils exposed to *P. aeruginosa* biofilms will be stimulated into a respiratory burst that is less than planktonic neutrophils. (4) Neutrophils in contact with a *P. aeruginosa* biofilm will undergo frustrated phagocytosis.

Objectives

The objectives of this thesis research were to: (1) characterize the nature of biochemical interactions of PMNs with planktonic *P. aeruginosa*; (2), quantify oxygen consumption rates during neutrophil respiratory burst and subsequent production of hydrogen peroxide in *P. aeruginosa* biofilms; (3) morphologically determine whether PMNs undergo frustrated phagocytosis and are thus prevented from undergoing chemotaxis and effective superoxide production in biofilms.

CHAPTER 2

MATERIALS AND METHODS

Reagents/Materials

Sodium chloride, citric acid, modified Hank's balanced salt solution (HBSS), phorbol-12-myristate-13-acetate, superoxide dismutase, copper chloride dihydrate, cobalt chloride hexahydrate, calcium chloride, 25% glutaraldehyde, sucrose, and zinc chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Gelatin, D-glucose, ammonium sulfate, ammonium molybdate, magnesium sulfate heptahydrate, sodium phosphate, potassium phosphate, potassium cyanide, iron chloride, hydrochloric acid, and 50ml plastic centrifuge tubes were purchased from Fisher Scientific (Tustin, CA). Yeast extract was purchased from Difco Co. (Franklin Lakes, NJ). LB agar, LB broth and hydrogen peroxide (30%) was purchased from EM Sciences (Aurora, CO). Aseptic vacuum-bottle filters 0.2 μm with 44mm necks were purchased from Millipore (Bedford, MA). In-line and syringe filters 0.2 μm were purchased from Corning Inc., (New York, NY). Platinum wire TC grade (99.99%) and silver wire (99.99%) with 0.002 in. and 0.004 in. diameters respectively were purchased from California Fine Wire Company, (Grover Beach, CA). Silver chloride solution saturated with potassium chloride was purchased from Orion (Beverly, MA).

Microorganisms:

Pseudomonas aeruginosa strain PAO1, used in these studies, was provided as a kind gift from Dr. Michael Franklin, Department of Microbiology, Montana State University–Bozeman. Information on *Pseudomonas aeruginosa* strain PAO1 can be found at www.pseudomonas.com.

Isothermal Flat Plate Open-Conduit Bioreactor (IFPOCB)

Pure culture *P. aeruginosa* biofilms were grown isothermally in a Flat Plate Open-Conduit Bioreactor (IFPBOC). The reactor is made of ¼ in. polycarbonate with glass microscope slide inserts serving as the biofilm substratum. It has a total working volume of 350 ml, including the recycle loop and heat exchanger. The fresh media feed flow rate is 0.6ml/min. This yields a retention time (R_t) equal to 7 hrs. A laminar flow velocity profile, Reynolds number (N_{Re}) 50–200, maintained within the reactor is controlled by the recycle loop's flow velocity. The reactor recycle ratio was set at 300 for the duration of the experiment. Two mixing chambers at each end of the reactor combined with the high recycle ratio provide a uniform substrate concentration across the flat plate of the reactor. A small air pump is used to provide aseptic oxygen to the reactor through an inline 0.2µm filter. A glass tube within a tube heat exchanger placed in the recycle loop controls the reactor's temperature at a constant 37°C. This temperature is achieved with a parallel co-current flow pattern in the heat exchanger with water bath reservoir set at 40°C.

IFPOCB was washed twice daily for 20 minutes with sterile water to remove excess cell debris and planktonic microorganisms. Prior to experiments, the IFPOCB was rinsed with sterile water and equilibrated in HBSS at 37°C.

Isolation of Human Neutrophils and Human Serum

Human neutrophils were purified from fresh whole citrated blood using gelatin sedimentation as previously described by (Parkos *et al.*, 1987). Isolated cells were stored on wet ice, (0°C), in modified Hank's balanced salt solution at approximately 10^7 - 10^8 cells/ml until needed. For use in experiments, cells were resuspended and allocated to microfuge tubes and warmed by water-bath to 37°C.

Human serum was prepared by allowing fresh whole blood to clot at room temperature in sterile glass tubes. After clot retraction the serum was pulled from the clot tube placed in sterile microfuge tubes and spun at a relative centrifugal force (RCF) of 5000g for 2 minutes to remove any excess cells and clot debris. The supernatant was transferred via pipette to sterile microfuge tubes and stored at 4 °C.

Measurement of Dissolved Oxygen

Dissolved oxygen measurements were performed with a modified Clarks type electrode with guard cathode as described by (Jorgensen & Revsbech, 1988). The cathode was polarized with a constant potential of -0.80 V. Dissolved oxygen electrodes were calibrated with air-saturated and nitrogen-saturated HBSS.

Measurement of Hydrogen Peroxide

Hydrogen peroxide measurements were performed with a glass encased platinum wire anode against a silver/silver chloride reference electrode from Fischer Scientific, (Tustin, CA) as described by (Rasmussen & Lewandowski, 1998). The anode was polarized with a constant potential of +0.80V. Hydrogen peroxide electrode was calibrated using standardized 30% hydrogen peroxide solutions diluted into hanks balanced salt solution. A picoammeter/DC voltage source 4140B Hewlett Packard, was used to supply the proper potentials under immersion. A micromanipulator, from World Precision Instruments (Sarasota, FL), in conjunction with a computer controller and stepper motor both from Oriel, (Stratford, CT) was used to position microelectrodes. The measurements were directed to a computer-based data acquisition system which monitored the current and the position of the microelectrode. An inverted microscope made by Olympus (Tokyo, Japan), was used to locate microelectrode tips during biofilm measurements. Autoclave-resistant tubing, #13, #14, #16 neoprene, pump heads, 1-100rpm and 6-600rpm motors from Cole-Parmer, (Vernon Hills, IL) were used to generate a 5ml/min flow in an open-channel reactor for measurements.

Planktonic and Biofilm Viabilities

Planktonic viabilities of PAO1 in the presence of human neutrophils were determined in the following manner. Overnight cultures of PAO1 were grown in LB broth at 37°C in a water bath shaker. The cells were harvested and pelleted at RCF of 7500 g in a Sorval centrifuge at 4°C for 10 minutes. The pellet was

resuspended in cold filter-sterilized HBSS, mixed with a vortex stirrer for 1 min and repelleted at RCF of 7500 g and 4°C for 10 minutes. The PAO1 pellet was resuspended in cold HBSS divided into two samples, diluted to an optical density of 0.6 absorbance units, and blanked against HBSS, in a 96-well plate reader by (Molecular Devices, Palo Alto, CA). The samples were serially diluted in sterile plastic tubes, containing filter sterilized HBSS, to a total volume of 900 µl. The control and test samples were incubated in a water bath shaker at 37°C. The controls received 100µl additional HBSS and the test samples received 100 µl of a suspension of fresh human neutrophils (PMN) in the same buffer to give a final concentration of 10^7 cell/mL. After 30 minutes of incubation at 37°C in the water bath shaker, 10µl samples were drop-plated on LB agar of both the control and test vials. Cultures were grown overnight in an air convection incubator at 37°C for up to 24 hours the plates were counted visually for viable cell colonies.

Biofilm viabilities in the presence of human neutrophils were determined as follows. PAO1 biofilms were grown on (25mm x 75mm x 1mm) microscope slides in an IFPOCB as mentioned above. After 5-7 days of growth the reactor was rinsed with 2L of sterile water followed by 1L of filter-sterilized HBSS. The microscope slides containing the attached biofilm were removed from the reactor and placed in rectangular polycarbonate boxes. Control biofilms were exposed to 6 ml HBSS for 30 minutes while biofilms treated with neutrophils were exposed to 6ml of 10^8 PMN/ml for 30 minutes. Serum-opsonized biofilms were exposed to 3 ml of 10% normal autologous human serum for 30 minutes followed by 3ml of

2×10^7 PMN/ml and a second incubation for 30 minutes. Thirty-minute incubations were performed at 37°C in an air-convection incubator. The microscope slides were scraped then placed into 50ml plastic centrifuge tubes along with the 6ml of liquid volume. The centrifuge tubes were mixed by vortex for 10 minutes to create a uniform suspension of cells. Upon completion of resuspension, 100µL aliquots were removed and serially diluted in filter sterilized HBSS. Four to six 10µL aliquots of each dilution were drop plated on LB agar and incubated at 37°C overnight, and viable colonies visually counted 24 hours later.

Scanning Confocal Laser Microscopy (SCLM)

Monoclonal antibody 7D5, courtesy of Dr. Michio Nakamura from the Institute of Tropical Medicine Nagasaki, Japan, was labeled with Alexa Fluor 488 or Alexa Fluor 568 dye (Molecular Probes). Both Alexa Fluor 488 and 568 dye (1mg) was solubilized in 0.1mL of DMSO. 25 µL of dye was added to 2 mg of 7D5 antibody in 9:1 parts 1X PBS to 1M sodium bicarbonate. The mixture was incubated at room temp for 1 hr and mixed by gentle inversion every 10 minutes in the absence of light. A BioRad size exclusion column (10D-G) was used to separate the labeled antibody from the free dye. The liquid-mobile phase used to elute the protein was PBS. The labeling efficiency was determined using BCA protein assay and with the fluorochrome's absorbance spectrum (see Molecular Probes Handbook).

Scanning Electron Microscopy (SEM)

Biofilms were grown on atomically flat silicon slides for seven days as described above. The slides were removed from the reactor and immersed in HBSS at 37°C. Neutrophils at 1×10^7 cells/mL were added to the surface of the biofilm and incubated at 37°C in an air convection incubator for 30 minutes. Serum labeled biofilms were exposed to 10% normal autologous human serum prior to addition of neutrophils at the identical concentration. Control biofilms were also placed in HBSS for 30 minutes or 10% normal human serum in HBSS prior to fixing. All samples were fixed overnight at room temperature using 0.1X PBS solutions containing 2% gluteraldehyde and 8% sucrose. The biofilm samples were rinsed 2 times with PBS for 15 minutes. This rinse was followed by a series of dehydration washes in 50, 70, 90 and 100% ethanol for 10 minutes each. Once in 100% ethanol, the samples were transferred to brass containers for critical point drying with ethanol and CO₂. Following critical point drying the samples were mounted on SEM stages with double stick carbon tape and sputter-coated with 20nm of Au-Pd in the presence of Argon. The samples were then stored in desiccant at room temp until examined with a JEOL 6100 scanning electron microscope with RUNCO digital imaging system.

CHAPTER 3

RESULTS

Growth Curve Analysis for *Pseudomonas aeruginosa* strain PAO1

The growth of *Pseudomonas aeruginosa* strain PAO1 was measured in order to determine the time necessary to reach exponential growth phase in the microorganism's life cycle. The cell density was determined by optical density at 600nm at specific times. All suspensions were grown in LB broth. Figure 2 depicts an average of three separate measurements versus time.

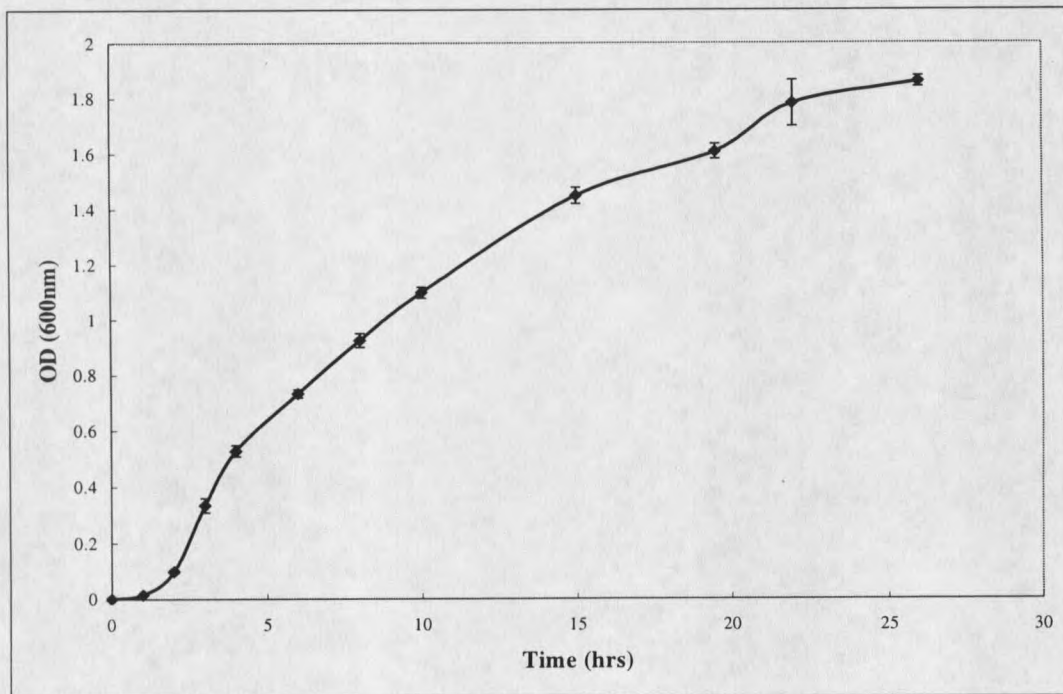


Figure 2. *Pseudomonas aeruginosa* growth curve at 37°C showing the dependence of optical density of the cell suspension versus time. Since OD is an exponential function of cell density, it is clear that the exponential growth phase was achieved after 3-5 hours and maintained for up to 25 hours. All subsequent experiments employed *P. aeruginosa* in the initial period of exponential growth including inoculation of reactors. Absorbances were corrected to an optical pathlength of 1cm.

Stimulation of Planktonic Human Neutrophils

Human neutrophils have long been observed to consume O_2 after stimulation by serum-opsonized particles and soluble stimulants such as PMA (Segal & Coade, 1978; Segal & Meshulam, 1979; Parkos *et al.*, 1987) (Vasilij & Pick, 1993) and (Vasilij & Pick, 1994). To demonstrate the sensitivity of the microelectrode system, determine the maximum oxygen consumption rate of neutrophils, and the lowest steady state oxygen concentration produced by neutrophils in suspension, this study used three modes of stimulants. These modes included PMA ($1\mu M$) as the soluble stimulant as well as unopsonized and normal autologous serum-opsonized viable *P. aeruginosa* both at 10^7 cfu/mL. PMA stocks 5mg/mL were prepared in DMSO, aliquoted and stored at $-20^\circ C$ until use. PMA was used as a positive control stimulant, since it yields the maximum respiratory burst in neutrophils without degranulation of the primary granules. PAO1 samples were prepared from fresh overnight cultures and used at an optical density of 0.6 (OD). The reactor vessel was maintained at $37^\circ C$ with a water bath and isolated from ambient O_2 by purging with N_2 over the measurement chamber. The plastic cuvette containing the sample was stirred with a magnetic stir bar to keep the suspension uniform and maintain a time averaged maximum mass transport to the electrode's membrane surface.

To evaluate the rate of oxygen consumption for each stimulant used, the curves such as those shown in Figure 3, were plotted and the slope (dC/dt) was numerically determined using a linear best-fit function in Microsoft Excel. The maximal rate of oxygen consumption was then calculated and compared by

normalizing to the number of neutrophils yielding a (nmol O₂/(10⁷PMN)/min basis.

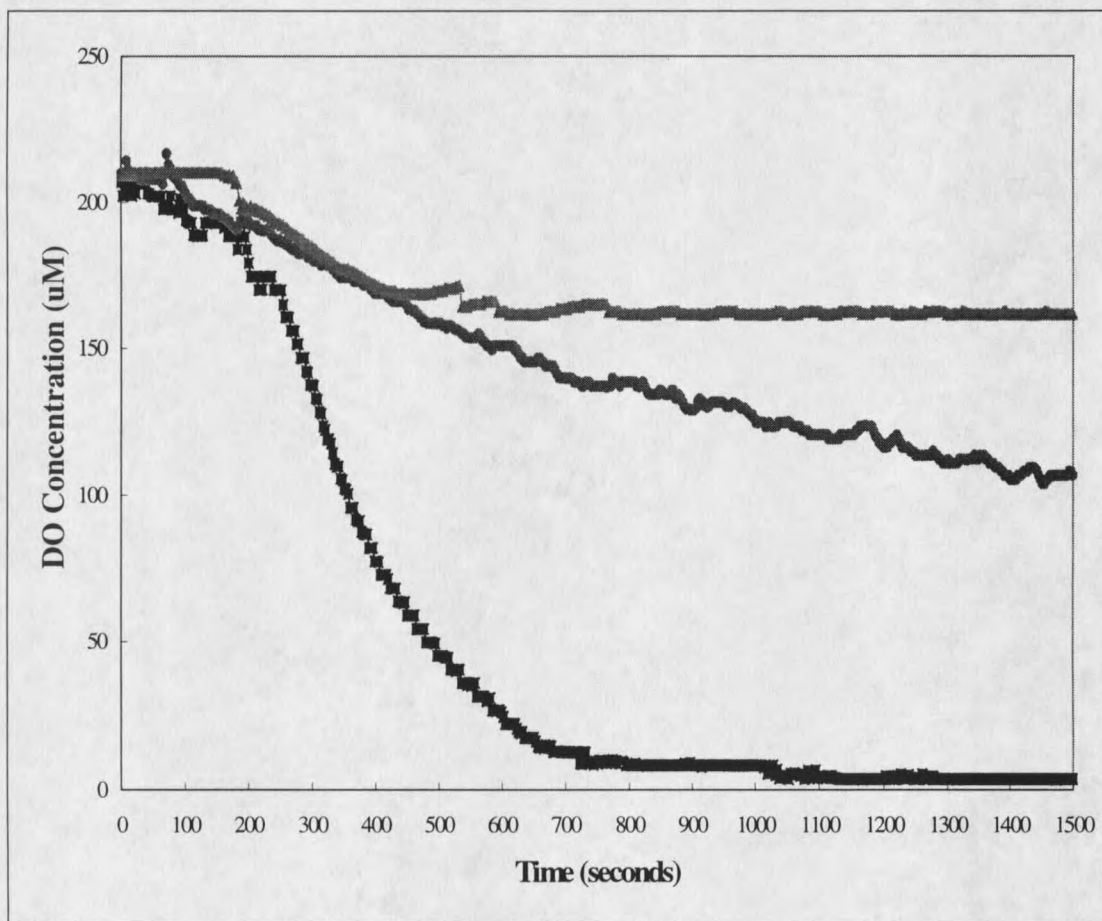


Figure 3. Dissolved oxygen concentration as a function of time in suspension of neutrophils stimulated by: PMA (■), 10% autologous human serum opsonized PAO1 (●), and PAO1 (▲)

Table 2. Maximum Oxygen Consumption Rates for Human Neutrophils

Stimulant	Rate(nmol O ₂ /(10 ⁷ PMN)/min
PMA	100.3 +/- 8.4
10% Serum-opsonized PAO1	57.7 +/- 2.4
PAO1	32.6 +/- 5.0
No Stimulant	2.1 +/- 1.2

The rates of oxygen consumption obtained for PMA stimulated neutrophils agrees with the literature value reported by (Light *et al.*, 1981), of 114.8 +/- 17.4. The maximum oxygen consumption rates for neutrophils suspended with autologous serum-opsonized PAO1 and unopsonized PAO1 are similar to those found for IgG labeled latex beads by (Segal & Jones, 1979) and to those observed for serum labeled zymosan particles by (Curnutte *et al.*, 1975). As Table 2 indicates, the oxygen consumption rates achieved for each type of stimulant differ considerably.

Table 3 indicates the minimum average steady state oxygen concentrations reached by the neutrophils for each stimulant after 30 minutes of observation.

Table 3. Minimum Oxygen Values Obtained for Suspended Human Neutrophils

Stimulant	Oxygen Concentration (μM)
PMA	5.9 +/- 2.3
10% Serum-opsonized PAO1	106.0 +/- 3.8
PAO1	155.0 +/- 5.6

Hydrogen Peroxide Production by Stimulated Planktonic Human Neutrophils

To determine the subsequent hydrogen peroxide concentration produced from the dismutation of superoxide by stimulated human neutrophils in suspension, an amperometric glass encased platinum electrode was employed. Measurements were taken using a similar experimental setup as described for O₂ (see materials and methods) and are shown in Figure 4. To accelerate superoxide

anion conversion at neutral pH to hydrogen peroxide, superoxide dismutase (SOD) was included in the measuring buffer for the soluble stimulant PMA. This process at its maximum converted 30% of the dissolved molecular oxygen to hydrogen peroxide.

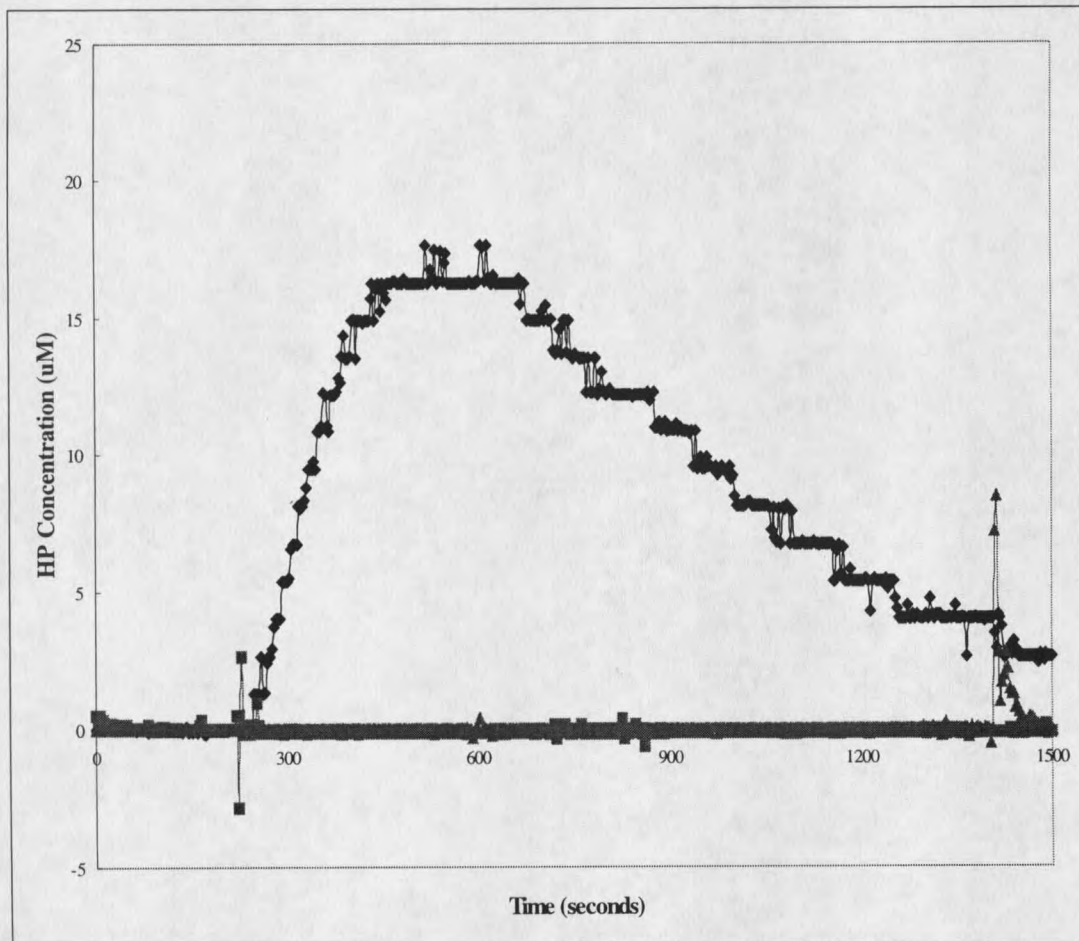


Figure 4. Hydrogen peroxide production by suspended human neutrophils stimulated with PMA (◆), PAO1 (■), and 10% autologous serum opsonized PAO1 (▲). Hydrogen peroxide spike during serum opsonized profile is addition of hydrogen peroxide to confirm functioning microelectrode.

This percent was calculated by integrating the hydrogen peroxide profile versus time and comparing this value to the amount of oxygen consumed by the same individuals neutrophils in suspension. Since the measurement apparatus has a constant volume, the two measurements could be compared directly. PMA stimulated neutrophils produced a maximum concentration of 16-25uM H_2O_2 . The hydrogen peroxide is consumed by intracellular neutrophil reactions including glutathione peroxidase/glutathione system, catalase, and peroxidase all present in normal functioning neutrophils after 30 minutes of stimulation (Hallett, 1989). Neither PAO1 stimulated neutrophils nor 10% autologous serum opsonized PAO1 stimulated neutrophils produced detectable concentrations of hydrogen peroxide. The lower limit of hydrogen peroxide detection for this system is approximately 100nM.

Dissolved Oxygen Consumption Induced by Neutrophilic

Interaction with PAO1 Biofilms

Aerobic and facultative microbes consume O_2 whether growing as suspended cells or attached to a surface. To compare their oxygen consumption to their planktonic counterparts a microelectrode, (see materials and methods) held in place by a motorized micromanipulator, was used to measure the spatial dissolved oxygen concentration gradients through PAO1 biofilms exposed to human neutrophils. A constant dissolved oxygen concentration at the biofilm bulk fluid interface was set by bubbling air into the reactor feed line. Baseline measurements were taken through the biofilm to determine the inherent

respiration rate of the biofilm. By allowing neutrophils to settle onto fixed and living biofilm surfaces, oxygen consumption due to neutrophil insult could be measured by monitoring the dissolved oxygen gradients and hence consumption through the biofilm.

The oxygen concentration dependence on depth above and below the biofilm interface is shown in Figure 5. Each curve represents the O_2 gradient of biofilm alone and then after several 5-minute intervals after allowing neutrophils settling on the unopsonized biofilm. Clearly, addition of neutrophils increases O_2 flux and decreases O_2 concentration above and below the calculated biofilm interface.

To better approximate a more physiologically relevant state, biofilms were also exposed to 10% autologous normal human serum for 30 minutes prior to introduction of neutrophils. Baseline profiles of dissolved oxygen were measured prior to opsonization and neutrophil induction. The oxygen profiles for these situations are shown in Figure 6. Unchanging dissolved oxygen profiles of the untreated biofilm indicate that the biofilm has reached a steady-state level for oxygen consumption. Interestingly, the application of normal human serum to the biofilm increases its respiration rate of the biofilm. This result is clearly evident from the decrease in oxygen concentration at the biofilm liquid interface from $80\mu M$ to $50\mu M$. 10% normal human serum increased the respiration rate of the biofilm by a factor of 4. The measurement was made by comparing the flux generated by the biofilm before and after exposure with 10% serum. The increased rate approaches the consumption rate of stimulated human neutrophils

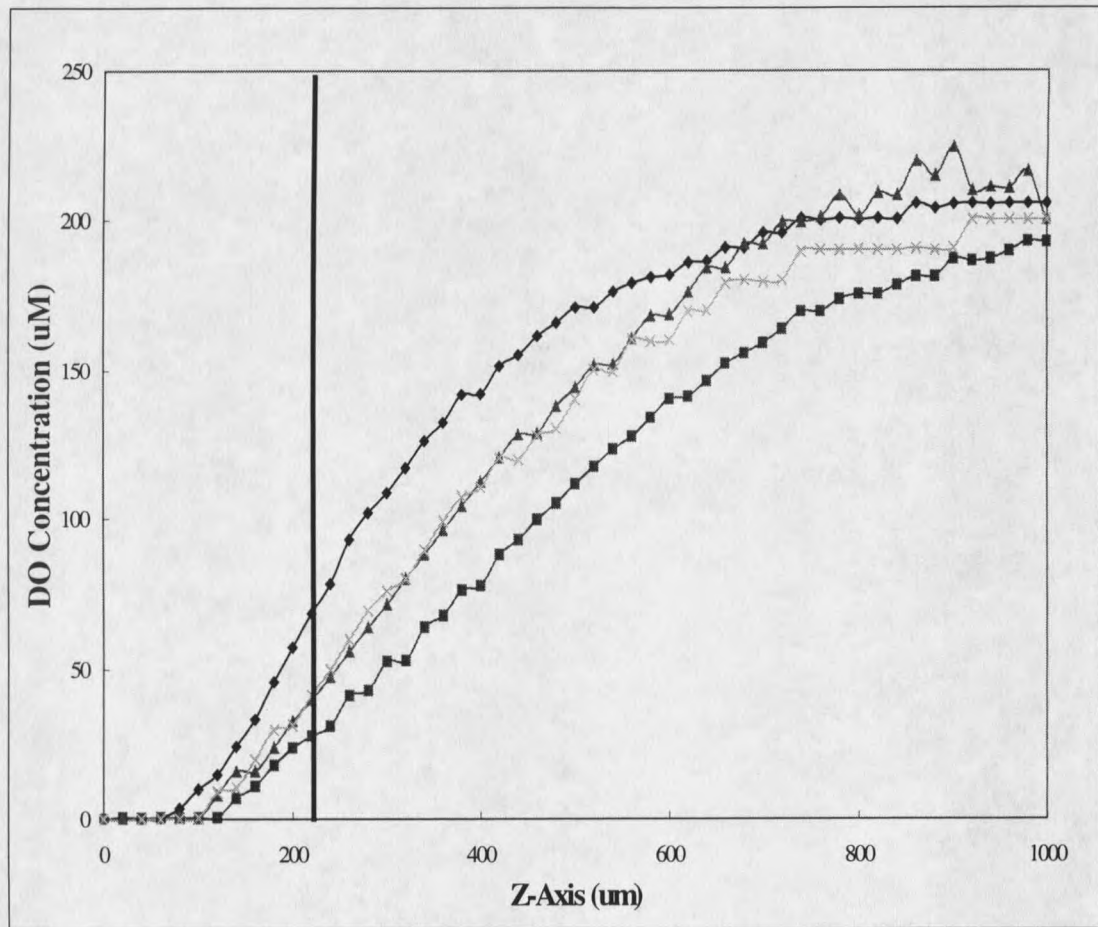


Figure 5. Dissolved oxygen (DO) gradients through a pure culture PAO1 biofilm and their dependence on neutrophil addition. Z-axis identifies the position of microelectrode above the substratum in microns. The biofilm substratum is identified at (0 μ m). The black bar at 230 μ m denotes the liquid-biofilm interface, and positions above 230 μ m the bulk fluid. The normal respiring biofilm is represented by (◆). Additional curves represent profiles at five minute intervals after the addition of 10^8 neutrophils 5min (■), 10min (▲), and 15min(×).

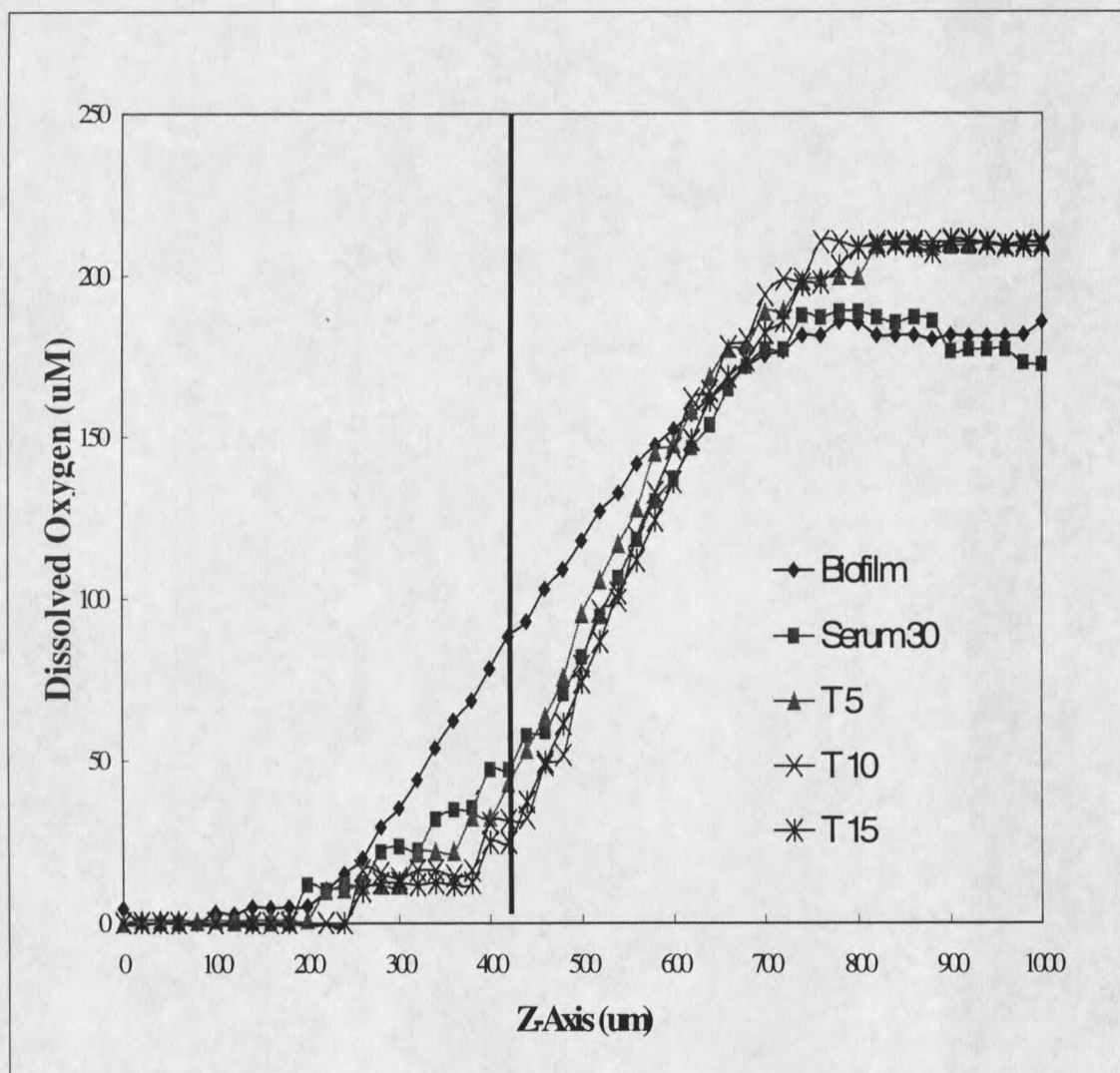


Figure 6. Profile of dissolved oxygen across the biofilm system along the Z-axis. The effect of both serum opsonization and neutrophil settling on dissolved oxygen gradients through a pure culture PAO1 biofilm. Zero denotes the biofilms substratum, (black bar at 420 μ m) the liquid-biofilm interface, and positions greater than 420 μ m the bulk fluid. Normal respiring biofilm at time zero is represented by ($\diamond$). Biofilm after 30 minutes of exposure to 10% normal human serum ($\blacksquare$), additional curves represent time profiles at five minute intervals after the addition of 10^8 neutrophils 5min ($\blacktriangle$), 10min($\times$), and 15min($\circ$).

on unopsonized biofilms. Upon the addition of neutrophils an even greater decrease in oxygen concentration at the biofilm liquid interface occurs, positively identifying additional oxygen consumption due to neutrophil addition. The effect is enhanced when settled on opsonized biofilms rather than unopsonized biofilms.

Since the diffusion coefficient for dissolved oxygen is known ($9 \cdot 10^{-6} \text{ cm}^2 \text{ sec}^{-1}$) for the bulk solution, the flux of oxygen to the biofilm surface may be calculated by extrapolating the numerically determined slope (dC/dz) for each location and time profile (Villaverde *et al.*, 2001). The slopes were bound between the surface of the biofilm, indicated by a small inflection in the curve, and the bulk solution. The region between the liquid-biofilm interface and the bulk solution is a linear function. All slopes calculated between these points had R^2 -values of 0.98 or greater in order to be used. The differential oxygen change between the bulk fluid and the substratum determines the changes taking place in the biofilm. Figure 7 shows that the oxygen flux towards the surface of the unopsonized biofilm increased by 436%. In repeated experiments this increase ranged from 235% to 440%. The variability of these measurements is in large part due to the differences among individual blood donors. Repeated experiments using the same individual's serum and neutrophils yielded consistent and reproducible results.

When the biofilms were exposed to 10% normal human serum, the calculated fluxes differed considerably from those observed in the unopsonized counterparts. Figure 8 shows that the serum-opsonized biofilms increased their

oxygen flux at least 2.5 fold during the 30 minutes of exposure to the serum. The

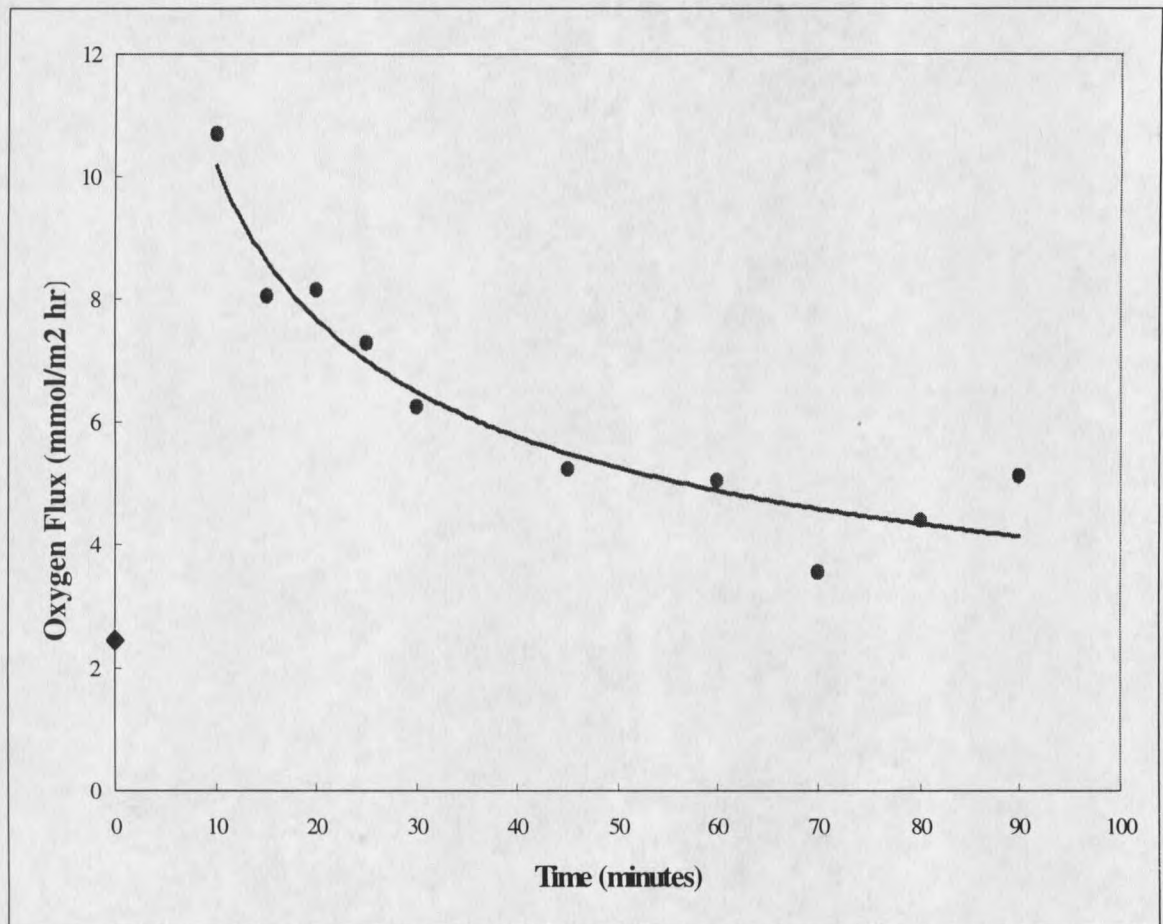


Figure 7. Oxygen flux to unopsonized viable PAO1 biofilm before and after addition of human neutrophils. (◆) Represents the oxygen flux to the biofilm prior to induction of human neutrophils with error representative of the size of the symbol. (●) Represents the oxygen flux to the biofilm after settling of neutrophils. Flux value at 5 minutes had R^2 -value less than 0.98 and was not used. This measurement was typical of a single patient's neutrophils to the surface of a biofilm and was repeated more than ($n=3$).

marked increase of oxygen flux to the biofilm reaches approximately 80% of the values observed when neutrophils were exposed to the opsonized biofilm. This increase represents a 20% change in oxygen flux to the biofilm. Obviously the

opsonization of the biofilm effects the total oxygen consumption of the biofilm and the neutrophils. This value depended significantly on donors used for neutrophil and autologous serum preparation and probably represents normal population variants.

In Figure 8 neutrophils were added to the opsonized biofilm surface at 30 minutes. The elevated oxygen flux after 50-90 minutes of neutrophil exposure in both biofilm cases was interesting as is seen in the right most curve. Clearly, a rapid decline was observed down to levels observed only with unopsonized biofilms.

To test the physiological significance of this O₂ flux behavior, a *P. aeruginosa* biofilm was grown for seven days and fixed in 4% paraformaldehyde in PBS, pH 7.4 for 2 hrs. After careful rinsing and reequilibration in HBSS, dissolved oxygen profiles of the biofilm were measured. Figure 9 shows that fixation eliminated biofilm respiration since the dissolved oxygen concentration was saturated at 210μM through the biofilm. Neutrophils were introduced onto this biofilm and oxygen gradients determined at five-minute intervals. The oxygen concentration profiles showed changes that clearly resulted from the respiratory burst reducing the dissolved oxygen concentration at the surface of the biofilm to 50μM. The neutrophils gradually lost their ability to maintain this oxygen flux. Figure 10 shows that no steady state oxygen concentration had been achieved at the surface of the biofilm. After 15 minutes the cells apparently slowed in their superoxide production as the oxygen concentration above and in the biofilm rose.

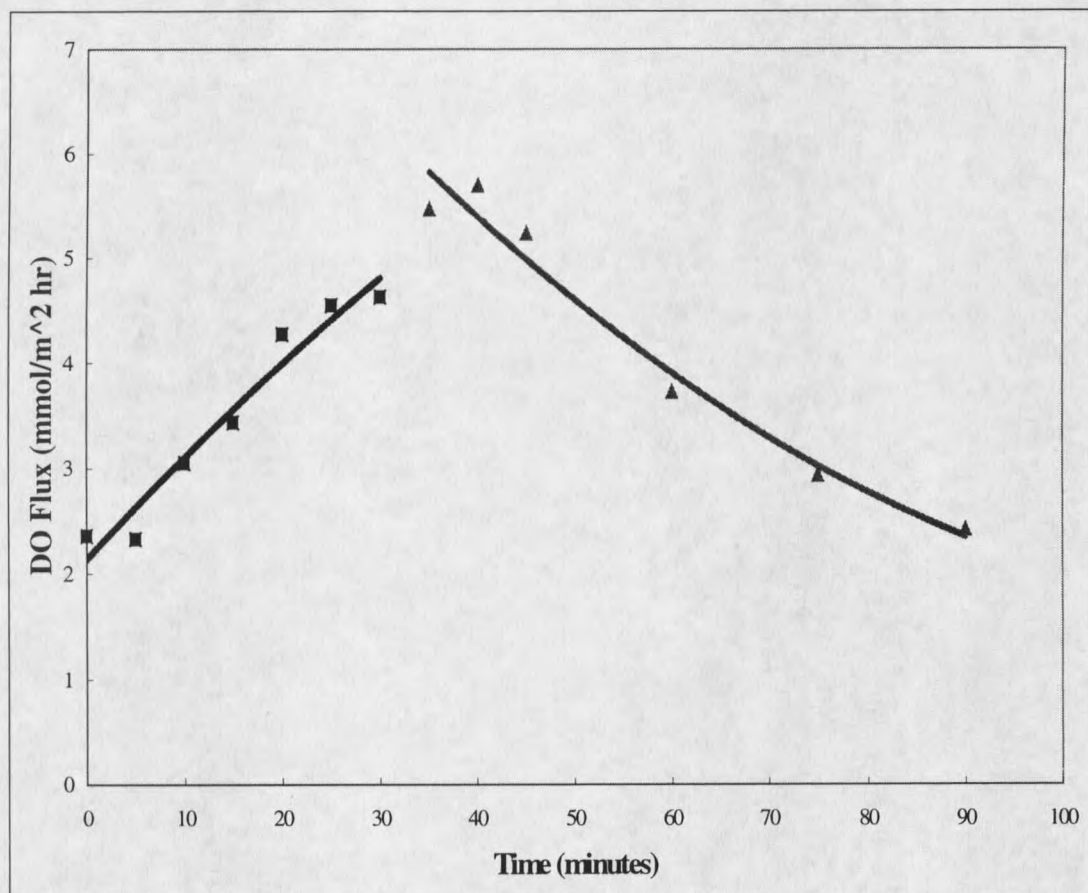


Figure 8. Serum and neutrophil-induced changes in oxygen flux through PAO1 biofilm. Rapid increase in biofilm oxygen consumption during exposure to 10% normal human serum indicated by (■). Biofilms oxygen flux is approximately 2.5 fold greater after exposure to serum. Initial increase of oxygen flux from neutrophil exposure and gradual decrease to the normal oxygen flux of the biofilm indicated by (▲).

However, even after 60 minutes of neutrophil exposure the dissolved oxygen concentration had not returned to a steady state concentration of 210 μ M. These results clearly show the contribution of neutrophils to oxygen consumption and allow calculation of the individual contribution of biofilm and blood cells to oxygen consumption in the unopsonized system.

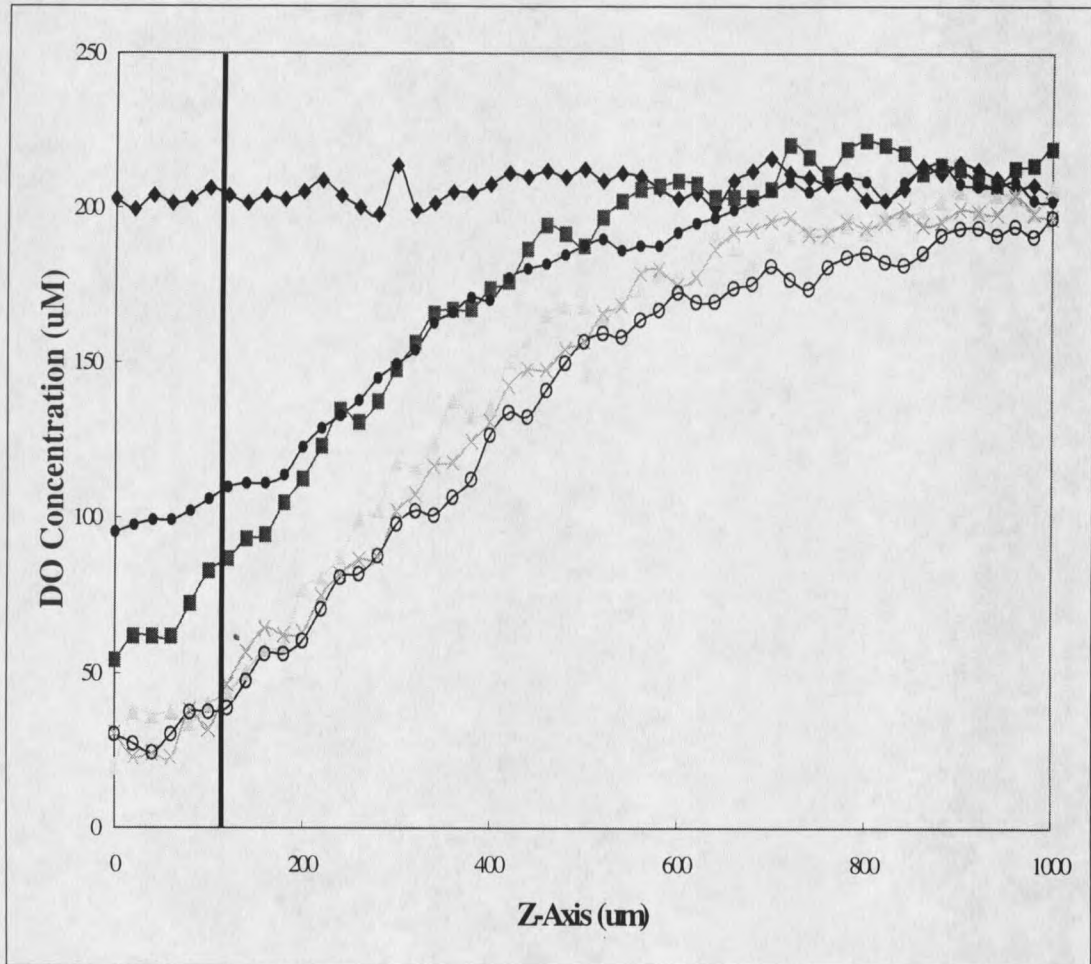


Figure 9. Profiles of dissolve oxygen concentration across a fixed biofilm system. Oxygen profiles of 4% paraformaldehyde fixed biofilms prior to and during phagocytic neutrophil response. Position (0 μ m) is substratum, position (160 μ m) is calculated biofilm liquid interface and positions (160 μ m-1000 μ m) indicate the bulk fluid. Oxygen profiles were taken at time zero ($\blacklozenge$) and at five minute intervals following settling of neutrophils. Time displayed in figure after neutrophil introduction are 5min ($\blacksquare$), 10min ($\blacktriangle$), 15min ($\times$), 30min ($\bigcirc$) and 60min ($\bullet$).

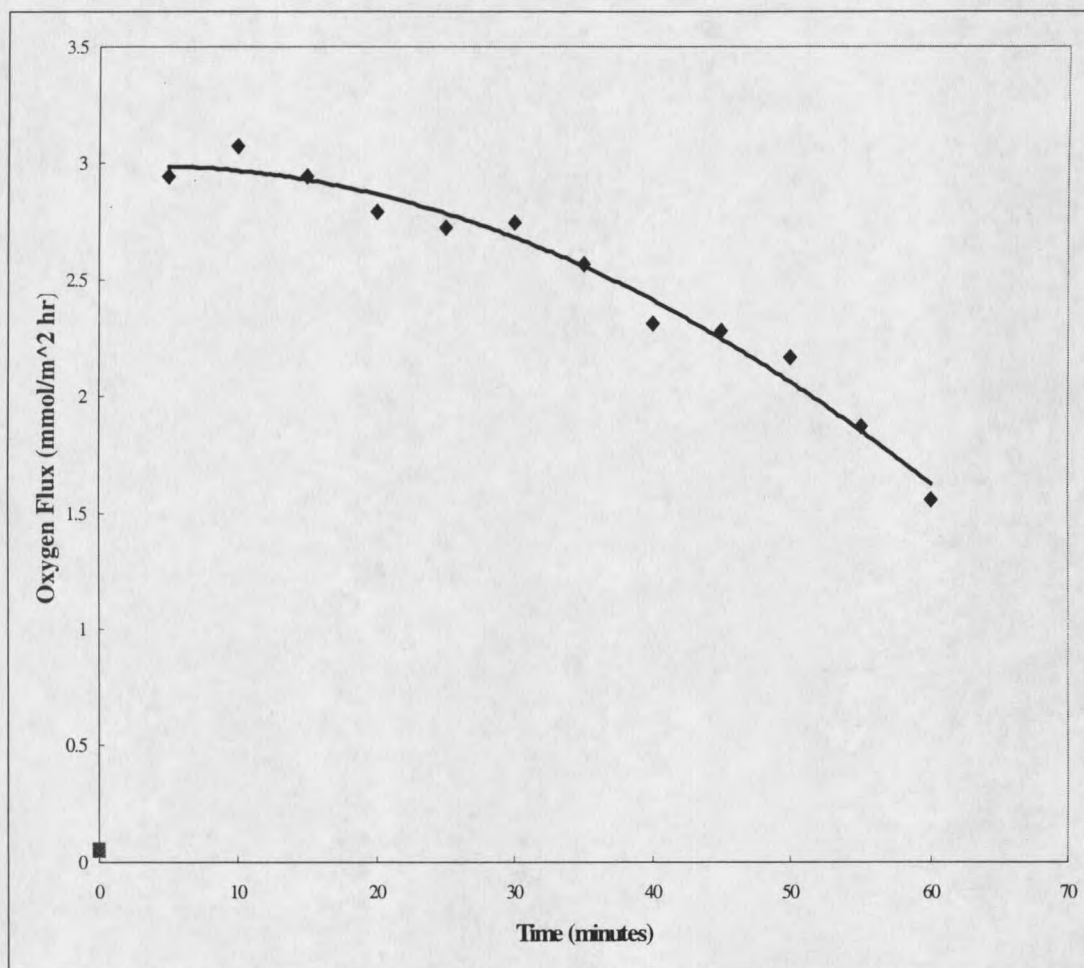


Figure 10. Oxygen flux towards 4% paraformaldehyde fixed biofilm before and after introduction of neutrophils. Baseline flux towards biofilm is represented by (■) with size of symbol representative of standard deviation in measurement. Increase in oxygen flux caused by respiratory burst of neutrophils is indicated by (◆).

Hydrogen Peroxide Gradients in PAO1 Biofilms

In planktonic environments, no extracellular H₂O₂ production from neutrophils could be detected as indicated in Figure 4. To determine if free H₂O₂ could be detected in the presence of biofilms, hydrogen peroxide gradients

induced by human neutrophils stimulated by both 10% autologous serum-opsonized PAO1 and unopsonized PAO1 biofilms were measured using a glass encased platinum electrode. Micromolar concentrations of hydrogen peroxide could be measured near the surface and penetrating the biofilm as Figures 11 and 12 indicate. In repeated experiments surface concentrations of 0.5 μM to 1.4 μM were obtained after 5 to 10 minutes of neutrophils settling, presumably from the spontaneous or enzymatic dismutation of superoxide during the neutrophils respiratory burst. Hydrogen peroxide at the biofilm surface could be measured for approximately 30 minutes before falling below detectable limits. Since neutrophils are responsible for consuming O_2 to levels of approximately 50 μM when stimulated by the biofilm (Figure 5) but only produce 0.5 to 1.4 μM H_2O_2 at the surface of the biofilm, then hydrogen peroxide is being consumed by other reactions and/or superoxide is being converted to species other than hydrogen peroxide. These results suggest that a maximum of only 2-6% of the superoxide generated is being converted to measurable levels of H_2O_2 in the spontaneous dismutation reaction. Opsonizing the biofilm surface with normal human serum did not affect the hydrogen peroxide concentrations at the biofilm surface or substratum.

Figure 11

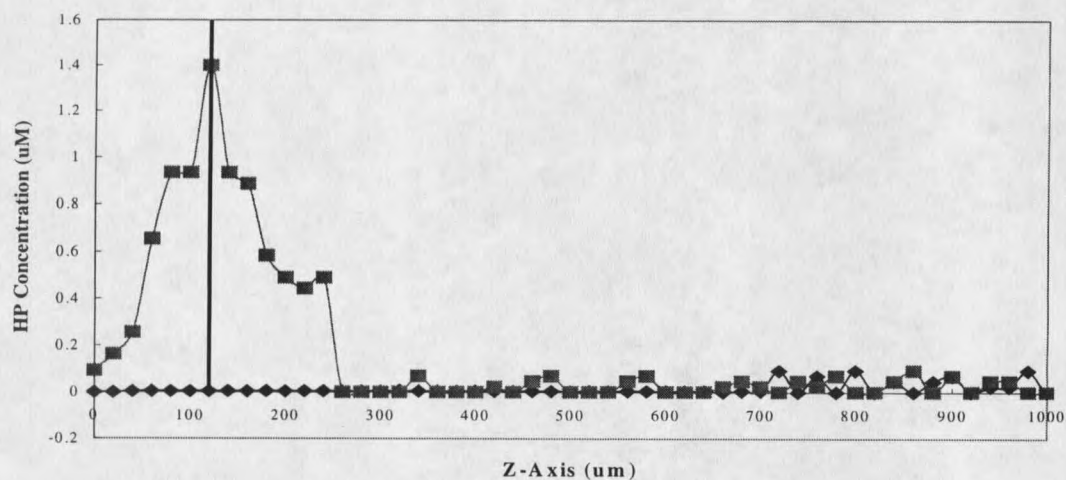


Figure 12

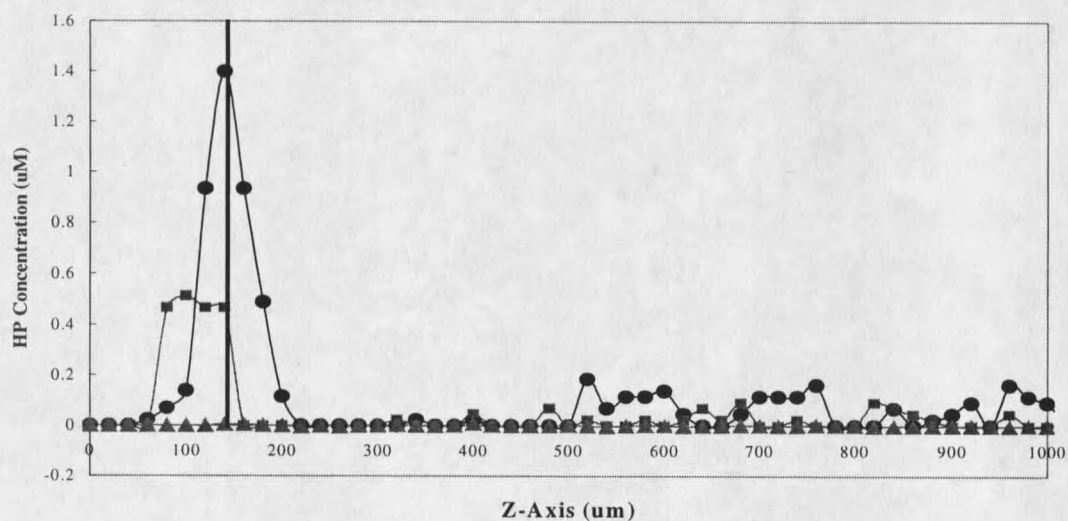


Figure 11. Microelectrode analysis of hydrogen peroxide through the *P. aeruginosa* biofilm system. Hydrogen peroxide profile through a PAO1 Biofilm. HP profile of untreated biofilm is represented by (◆), HP production after settling of neutrophils is represented by (■). Figure 12. Additional hydrogen peroxide profiles of neutrophils on PAO1 biofilm, time represented are 15min (●), 25min (■), and 30min (▲).

Planktonic and Biofilm Viabilities

Clearly, biofilm formation prohibits significant oxygen consumption and oxidant production by neutrophils. To determine the effect of the neutrophils on biofilms, bacterial viability was examined. Planktonic and biofilm *P. aeruginosa* bacterial cells were exposed to human neutrophils or opsonized with 10% autologous serum followed by human neutrophils. Cell cultures were put into suspension, diluted and drop-plated on LB agar. Plates were counted for viable colony forming units after 24 hours of growth and compared. Statistically, the viable CFU did not vary between the suspended and surface attached organisms as indicated in Figure 13. The standard deviations between the samples were large enough to encompass any differences. However, if the reduced CFUs normalized to a per neutrophil basis are compared, a marked difference is observed. Figure 14 shows the effect of exposure of both planktonic and biofilm bacteria on killing rate by neutrophils alone and by neutrophils after prior opsonization. The result suggests that planktonic bacteria are 17 to 29 times more likely to be killed by neutrophils than their biofilm counterpart. Thus the formation of the biofilm structure resist both phagocytosis and opsonophagocytosis.

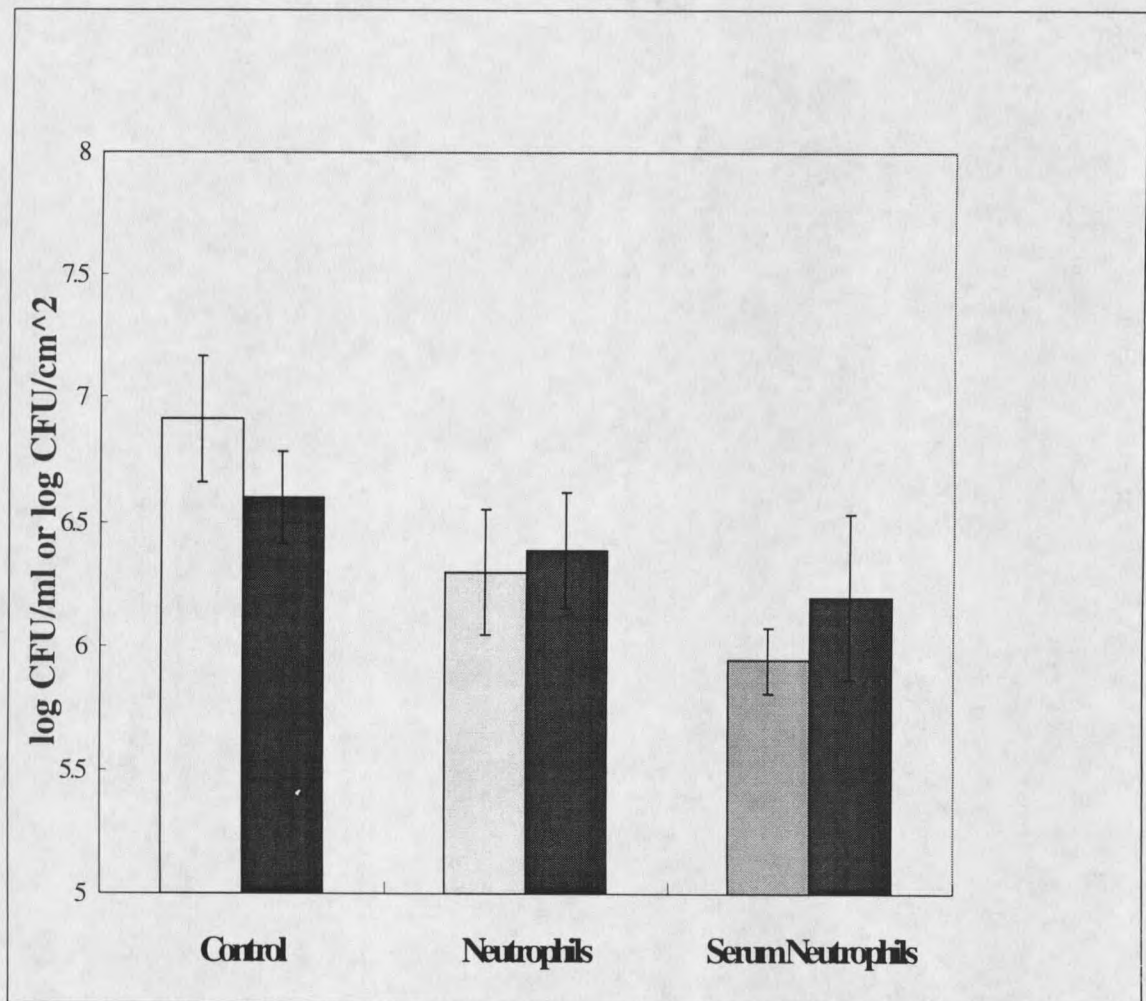


Figure 13. Viable colony forming unit counts of *P. aerug.* challenged with human neutrophils. Viable cell counts of planktonic (grayed) and biofilm (solid) *P. aerug.* bacterial cells were exposed to human neutrophils, or 10% autologous human serum followed by human neutrophils and compared to untreated microorganisms. Neutroph reduced planktonic cell counts by approximately 0.5 log units where opsonization neutrophil introduction reduced viable cell counts by 1 log. Biofilm cultures were not affected as greatly by neutrophils or the combination of serum opsonization and cell cultures experienced log reductions of approximately 0.3 and 0.6, respectively.

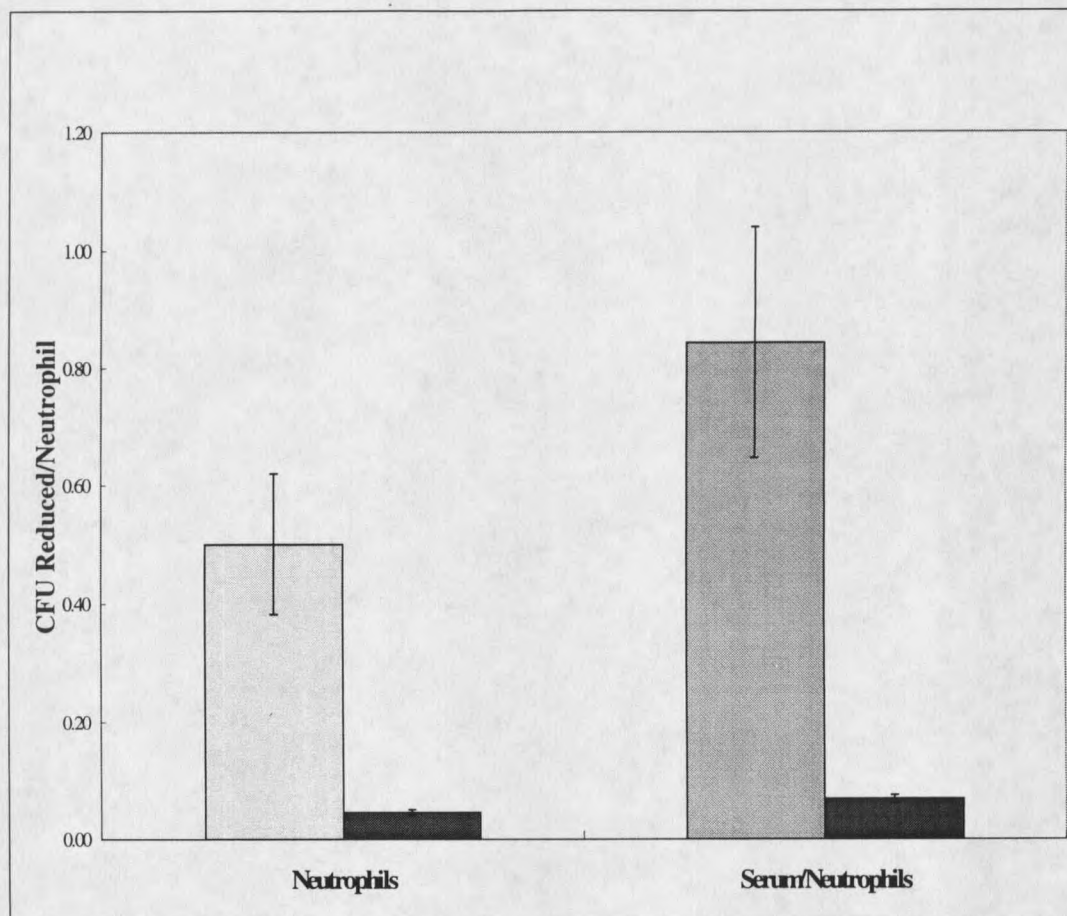


Figure 14. Viability normalized to a per neutrophil basis. Viability data in Figure 13 were normalized to the number of neutrophils used in each treatment. Planktonic (shaded) bacteria were treated with 10^7 PMN. Biofilm (solid) bacteria were treated with 10^8 PMN/ml (see Materials and Methods). The results suggest neutrophils have greater killing efficacy for planktonic *P. aeruginosa* when compared to biofilm cells. When neutrophils were exposed to unopsonized planktonic and biofilm *P. aeruginosa* the killing ratio of planktonic cell is approximately 17 times that of biofilm cells. When neutrophils were exposed to 10% autologous serum opsonized bacteria the killing efficiency rose for both, however, increasing the relative killing ratio to approximately 29:1.

Scanning Electron Microscopy

The effect of neutrophil mediated killing *Pseudomonas aeruginosa* is clearly reduced by biofilm formation. To demonstrate that neutrophils were in

fact intact, functional and pervasive in this experiment, a scanning electron microscopy (SEM) analysis was carried out on *P. aeruginosa* biofilms before and during exposure to 10% human serum and neutrophils.

Figures 15 –19 are full frame typical SEM images of: (15) *P. aeruginosa* biofilm, (16) *P. aeruginosa* biofilm after 30 minutes of incubation with 10% normal human serum, (17) human neutrophils on *P. aeruginosa* biofilm, (18) human neutrophils on *P. aeruginosa* biofilm after 30 minute incubation in 10% autologous normal human serum. Selected frames, Figure (19), show human neutrophils carrying out phagocytosis of PAO1 bacterium.

The surface of the biofilm did not appear to change when exposed to 10% human serum when compared to the control biofilm surface. Also, no distinguishable difference could be found between neutrophils interacting on biofilms or opsonized biofilms. Interestingly, the majority of neutrophils were round in morphology but did show a degree of morphologic activation in membrane structure. In fact very little evidence of a polarized morphology or phagocytosis was observed, suggesting a “paralysis” of sorts in phagocytosis. Comparing SEM images of biofilm shows that 1 in 160 are actively involved in phagocytosis.

Neutrophils along the biofilm surface, however, do show ruffled plasma membranes, a known characteristic of activation. Neutrophils were also found embedded in the biofilm or along the heterogeneous channels or pores of the biofilm and appear to be rounded. To determine whether the phagocytic granulocytes appeared to employ the heterogeneity of the biofilm as a means to migrate into the internal structure of the biofilm. Further studies using transmission electrons microscopy and confocal fluorescence microscopy will be necessary.

Figure 15. *Pseudomonas aeruginosa* biofilm.

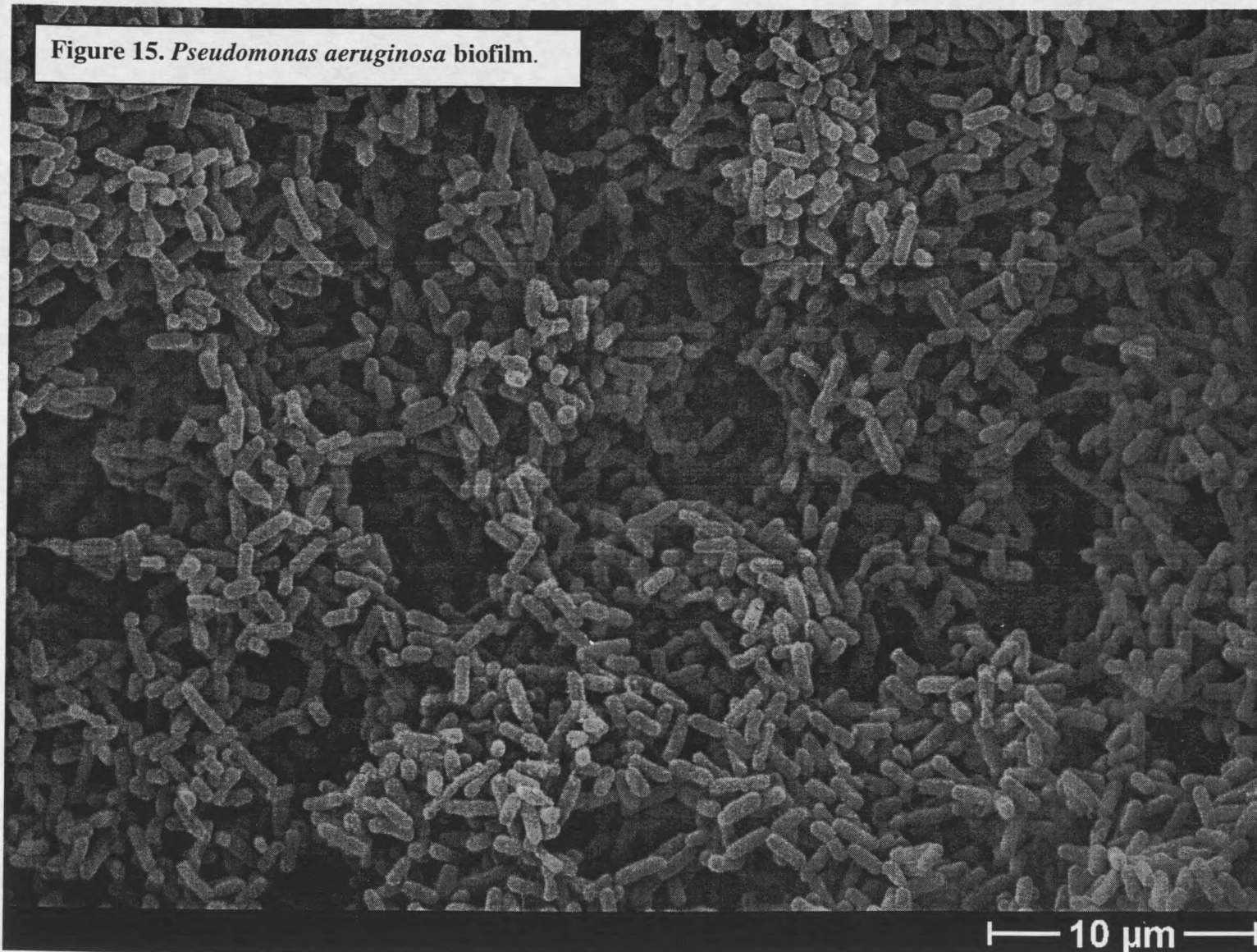
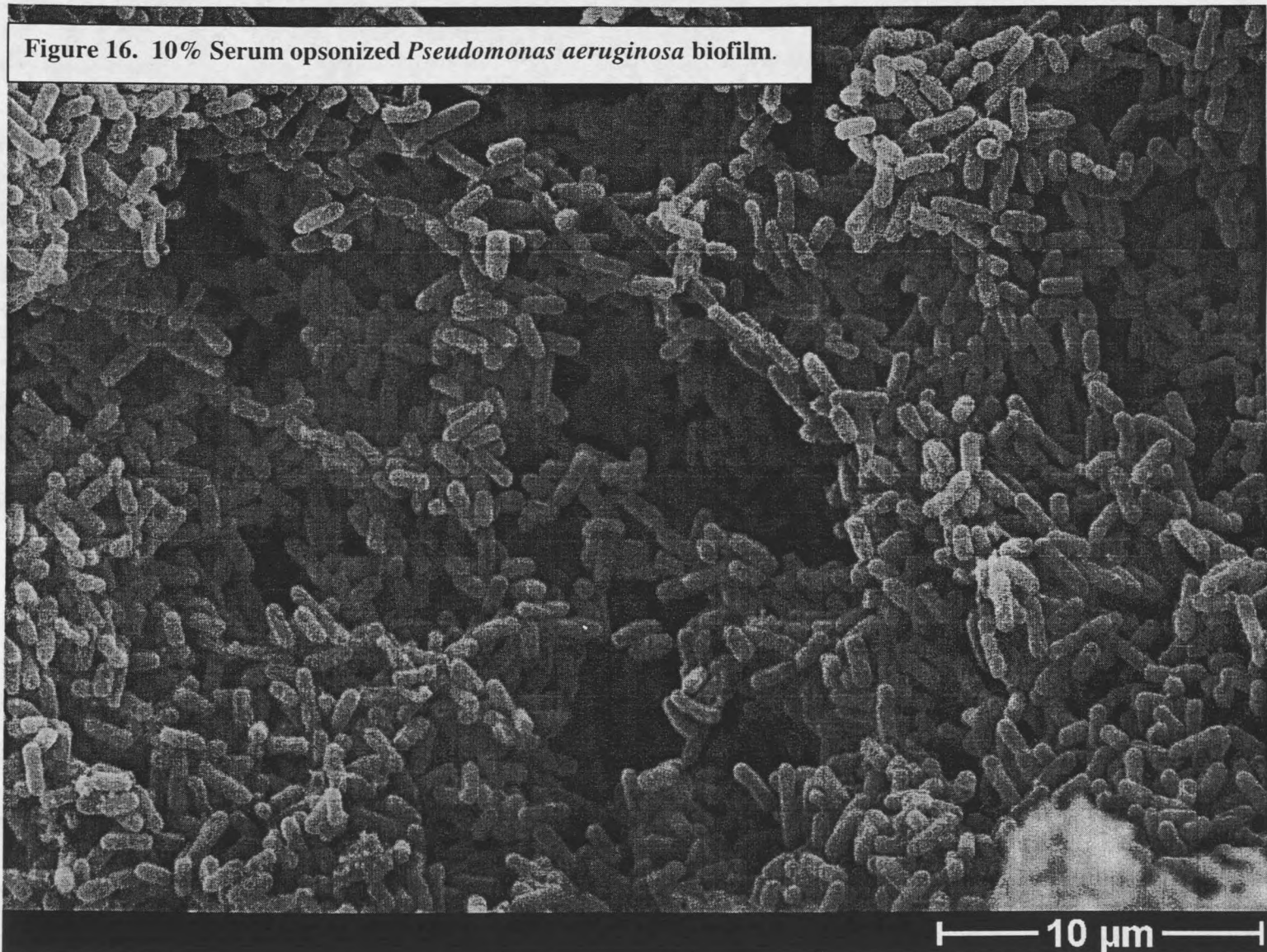


Figure 16. 10% Serum opsonized *Pseudomonas aeruginosa* biofilm.



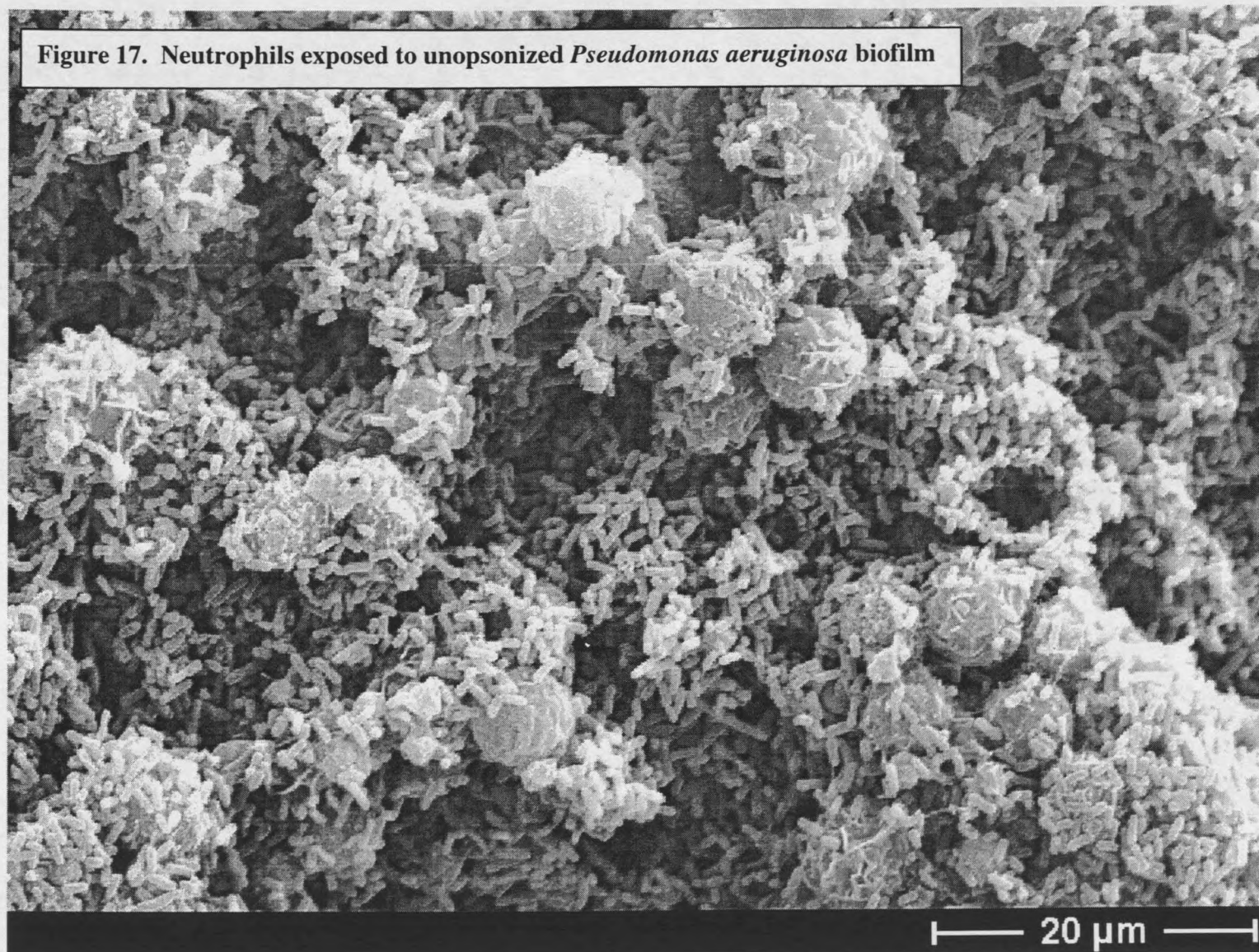


Figure 18. Neutrophils exposed to 10 % serum opsonized *Pseudomonas aeruginosa* biofilm

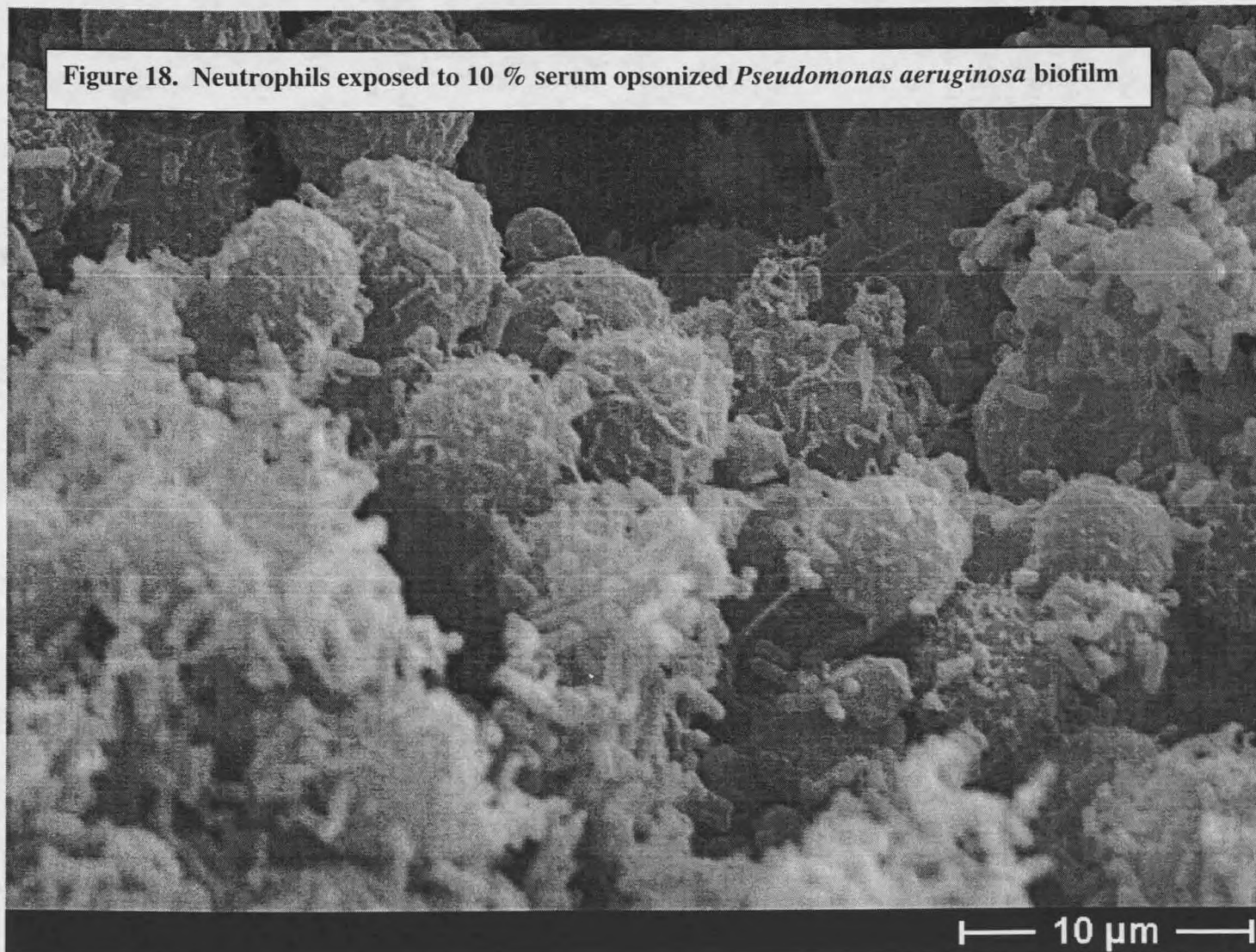
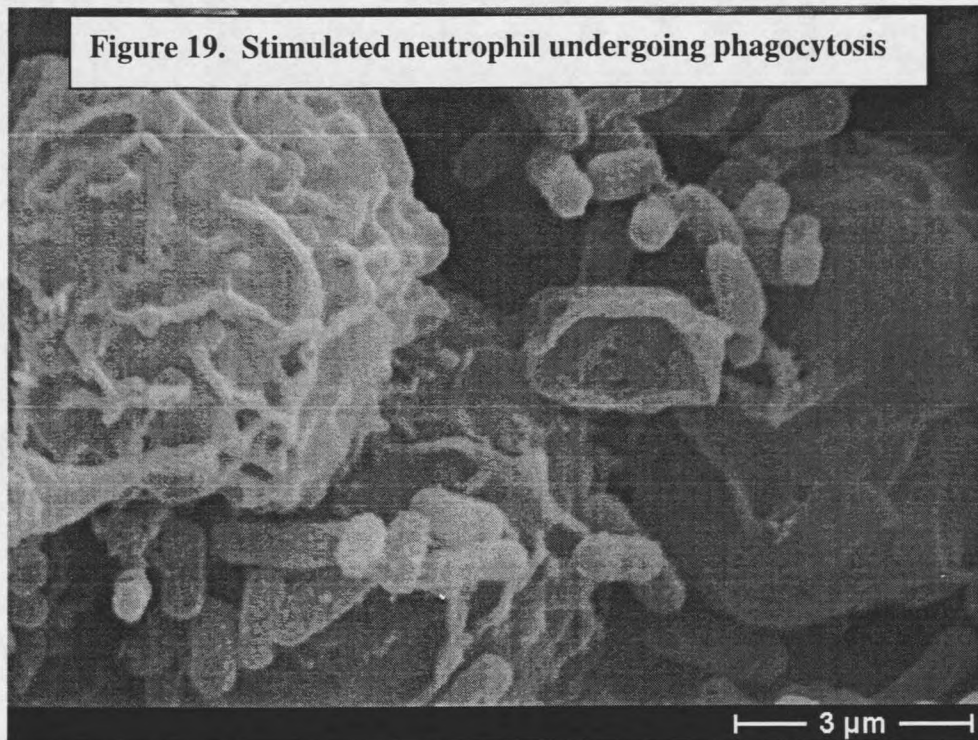


Figure 19. Stimulated neutrophil undergoing phagocytosis



CHAPTER 4

DISCUSSION

The purpose of the experiments described in this thesis was to evaluate the potential of *Pseudomonas aeruginosa* biofilms to serve as a defensive structure against oxidative insult by the innate immune system of humans. These studies have clearly shown that *P. aeruginosa* biofilms provide extraordinary protection from oxidative attack by human inflammatory cells. These findings are in agreement with and provide mechanistic support for other studies showing the resistance of biofilms to antimicrobial treatment (Brown & Gilbert, 1993), (Gilbert *et al.*, 1997) and (Costerton *et al.*, 1995). The previous studies infer that the mechanism of reduced biofilm susceptibility results from a failure of antimicrobial compounds to penetrate the biofilm. Relatively few studies have examined the effect of human-derived antimicrobials on biofilms.

Different investigators have proposed hypotheses explaining how medically-relevant biofilms might escape human immune surveillance and oxidative attack. However, few hypotheses have actually been tested thus suggesting that a wealth of information might be derived from analyses such as the one described herein. Of particular importance, is the establishment of a paradigm for future evaluation of therapeutic strategies to enhance oxidative clearing of biofilm infection.

Our studies clearly show that human neutrophils have a decreased antimicrobial efficacy when presented with bacteria attached to a surface as a biofilm. Neutrophils of normal healthy adults cleared planktonic bacteria more efficiently than biofilm bacteria *in vitro*. The neutrophil mediated killing of planktonic bacterial cells has been examined in the past correlating the number of bacterial cells ingested with superoxide production (Segal & Coade, 1978). Other investigators have examined the number and types of bacteria taken up by neutrophils. In these varied studies sampling time, growth mediums, bacteria types, bacteria to phagocyte ratios, phagocyte purification and donor differences make the data difficult to compare. For example, Yasuda tested the viability of *E. coli* when treated with human neutrophils. Samples were taken on 1-hour intervals without the constant addition of neutrophils. The work described in this thesis as well as that performed by {Hampton & Winterbourn 1999 86 /id} indicates that the respiratory burst of neutrophils is greatest between the first 5-10 minutes and approaches zero after 30 minutes. Thus the impact of oxidative killing is greatest within the first 10 minutes and later sampling confuses the importance of early and late neutrophilic events.

In 1992, Jensen and coworkers performed the first known comparison of the oxidative burst of neutrophils challenged by planktonic and resuspended biofilm *Pseudomonas aeruginosa* (Jensen *et al.*, 1992). Their chemiluminescent analysis shows that neutrophils produce greater quantities of superoxide after exposure to planktonic bacteria rather than resuspended biofilm bacteria. Our microelectrode measurements have oxygen metabolism in neutrophils systems

interacting with planktonic bacteria and with biofilm bacteria. These measurements profile the z-axis of the biofilm not just flocculant aggregates of the biofilm. We have quantitatively expressed these results as rates of oxygen consumption by these neutrophil systems in Tables 2 and 4. The rate of oxygen consumption for neutrophils stimulated by planktonic *P. aeruginosa* system is 150% of that found for the neutrophil/biofilm system. The serum opsonization of *P. aeruginosa* increased the oxygen consumption rate for the neutrophil/planktonic bacterial system by 2 fold. Serum opsonization also increased the oxygen consumption rate for the neutrophil/biofilm by 2 fold. Therefore, the system contains stimulated by serum-opsonized planktonic PAO1 consumed 132% more oxygen than the corresponding PAO1 biofilm system. Comparing the two cases of neutrophils stimulated by unopsonized and opsonized PAO1 suggests that serum opsonization amplifies the killing response of human neutrophils as expected. Paraformaldehyde treated controls indicated that this increase is at least in part caused by the neutrophils oxidative bursts. Tables 2 and 4 also show that neutrophil/biofilm systems consume oxygen at lower rates than neutrophils exposed to planktonic bacteria.

Serum opsonization in planktonic settings has been found to effect bacterial growth rates. Serum exposure precedes interaction of invasive *Pseudomonas aeruginosa* infections with neutrophils. Therefore, neutrophil association with *Pseudomonas aeruginosa* biofilms exposed to human serum was assessed. The results, as shown in Figure 8, clearly indicate that *P. aeruginosa* biofilms when exposed to human serum increase their metabolic activity by

196%. In 1986, Britigan analyzed the effects of 10% serum on planktonic *Escherichia coli*, *Staphylococcus aureus*, and *Neisseria gonorrhoeae* and found that basal respiration rates increased by 157%, 199% and 356% respectively. Their work also described the competition that these microorganisms have when competing against human neutrophils for oxygen. Britigan reported that *E. coli*, *S. aureus*, and *N. gonorrhoeae* lower the respiratory burst of human neutrophils by 52.7%, 88.6% and 91.7% respectively when analyzed with luminol. The results of this study and Britigan's work support the fact that there is a dynamic competition between neutrophils and bacteria for molecular oxygen *in vitro*. The work also indicates that bacteria have the ability to increase their metabolic rates in response to stimulatory factor(s) in order to compromise the local neutrophil function.

Since, *P. aeruginosa* is a complement sensitive bacteria species, viability measurements were assessed when neutrophils were challenged with both unopsonized and 10% serum-opsonized planktonic and biofilm PAO1. Our results agree with traditional observation that the addition of normal human serum increases the antimicrobial efficacy of human neutrophils. It also confirms the work showing that the complement system is activated by both planktonic and biofilm *P. aeruginosa* (Jensen *et al.*, 1990). In later studies, {Jensen, Kharazmi, et al. 1992 119 /id}, these investigators noted that the lipid A part of lipopolysaccharide (LPS) activates the classical pathway of complement while the saccharide portion stimulates the alternative pathway. Their arguments suggested that *Pseudomonas* products such as alginate could mask the surface of the

biofilm, protecting it from complement activation and support the observation by Pedersen and coworkers that alginate itself does not stimulate either complement pathway (Pedersen *et al.*, 1990).

Biofilms are known to resist chemical challenge by failure of the antimicrobial compound to penetrate the biofilm due to a reaction-diffusion interaction. This concept has been put forth by several researchers in the microbiology literature including (Costerton *et al.*, 1987), (Nichols, 1989) (Nichols *et al.*, 1989). However, living cells such as human neutrophils do not react in the same manner as chemicals. Instead the phagocytes actively carry the ability to produce antimicrobial compounds into the biofilm. Once inside the biofilm they could release oxidative compounds as antimicrobials. Thus, host cellular defense does not strictly obey a reaction diffusion model for antimicrobial release. However, the neutrophil's superoxide production is limited by a reaction-diffusion model where the bacteria consume oxygen at a rate great enough that the biofilm is not fully penetrated with oxygen. Oxygen then becomes the limiting substrate in the production of neutrophil-induced antimicrobials. Therefore, as the oxygen concentration approaches zero the superoxide production follows.

Pseudomonas aeruginosa biofilms contain an additional defense mechanism in the form of catalase. Catalase positive bacteria resist the antimicrobial production of hydrogen peroxide by phagocytes. Therefore, a cyclic process involving the consumption of oxygen, the subsequent production of

hydrogen peroxide, and the catalytic action of catalase produces a dynamic effect at the biofilms surface that is difficult to quantify.

As stated in the introduction, neutrophils are the first line of cellular immune defense against bacterial pathogens such as *P. aeruginosa*. The neutrophil defensive arsenal, however, requires intimate contact in most cases in order to achieve effective concentration of antimicrobials. The contact is achieved by direct migration leading ultimately to contact and phagocytosis. Other investigators have made conclusions about the interactions between phagocytes and biofilms based on microscopy data of neutrophils exposed to charged or foreign surfaces exhibiting frustrated phagocytosis (Costerton *et al.*, 1999). Our results employing SEM to analyze the biofilm surface in contact with settling neutrophils suggests a different picture. The difference appears not only in phagocytic process but apparently in the morphology and locomotion of the inflammatory cells. Normally within five minutes of being stimulated in suspension isotropically or while adherent with in the presence of chemoattractant gradients, neutrophils display a polarized morphology which in the latter case is in the direction of the chemotactic gradient. Our results show that neutrophils coating and penetrating the biofilm display a lack of polarization almost as if the cells have become "paralyzed" or desensitized. One explanation might be neutrophils coming in contact with the biofilm surface are "over" stimulated by high concentrations of bacterially produced stimuli. If the concentration of stimulant were sufficiently high to saturate chemotactic receptors neutrophils would not be able to recognize a chemical gradient becoming immobilized.

Although plausible, this explanation is incompatible with observations made on isolated neutrophils exposed to saturating chemoattractant concentrations. First, in a high isotropic concentration of fMetLeuPhe, neutrophils still assume a polarized stimulated morphology. Second, on surfaces in high concentrations of chemotactic gradients neutrophils still demonstrate active and rapid chemokinesis and a stimulated polarized morphology. Thus biofilms opsonized or otherwise appear to have special properties, perhaps inhibitors that are actively released or prevent active phagocytosis. Such properties might derive from bacteria in contact or interacting with a surface or possibly a bacterial product that is released during normal metabolism. In any case biofilms appear to be structures that obtain significant protective and defensive mechanisms against opsonophagocytosis.

Attempts were made to describe both quantitatively and qualitatively the presence of neutrophils in the biofilm with SCLM. However, these results are still inconclusive and were not included in this thesis.

CHAPTER 5

CONCLUSIONS

The major findings of this study are as follows. The antimicrobial capacity of human neutrophils against *P. aeruginosa* decreases when the bacteria attach to a surface. The neutrophils exhibited an increase in the antimicrobial efficacy when both planktonic and biofilm *P. aeruginosa* were exposed to 10% normal human serum prior to human neutrophils, which agrees with the concept that relevant components of complement C3a, C4a, and C5a are crucial peptide mediators of inflammation.

Phorbol-12-myristate-13-acetate (PMA) stimulation of planktonic human neutrophils caused the maximum consumption rates of oxygen, (100nmol/10⁷PMN/min), and the resulting maximum concentration of hydrogen peroxide in the presence of superoxide dismutase (16-20uM).

Dissolved oxygen consumption rates with systems of human neutrophils and planktonic *P. aeruginosa* differed from those exhibited by neutrophil/biofilm systems. Neutrophils exposed to planktonic bacteria induced oxygen consumption rates 163% (PAO1), to 288% (serum-opsonized PAO1) greater than oxygen consumption rates observed with neutrophils on biofilms.

Low levels of hydrogen peroxide appear to be generated near the surface and throughout the biofilm upon neutrophil activation suggesting significant neutrophils penetration. Hydrogen peroxide production could be measured for 25-30 minutes after exposure of neutrophils to the biofilm surface. Of the total

dissolved oxygen that was consumed only 6% could be measured as hydrogen peroxide. This measurement may vary greatly due to the dynamic cycling of oxygen found at the biofilms interface.

Oxygen concentration profiles of biofilms directly exposed to neutrophils indicated an increase in the oxygen flux towards the biofilm surface, demonstrating the "respiratory burst" of human neutrophils. However, oxygen profiles produced by biofilms incubated with 10% normal human serum produced entirely different patterns. The serum acted as a preliminary stimulant to the biofilm. A marked increase in the flux to the biofilm surface could be measured and ranged from 150 to 220%. The increased oxygen consumption by the biofilm was donor dependent. The microbial oxygen consumption of the biofilm thus competed with that of neutrophils.

The results of this study provide insight into the nature of the physical stimulation and phagocytosis of surface attached bacteria by neutrophils. Human neutrophils in contact with the biofilm surface do not undergo frustrated phagocytosis as previously believed. Instead, a paralysis in both chemokinesis and phagocytosis appears to occur. Only 1 in 160 neutrophils found on the biofilm surface was actively involved in frustrated phagocytosis. Thus an unknown biochemical product of *Pseudomonas aeruginosa* biofilms may act on neutrophils to inactivate or freeze the phagocytosis process. Neutrophils were found imbedded in the biofilm and employed the biofilm porosity to access the deeper layers of the biofilm. This may be the reason for low levels of hydrogen peroxide measured at the biofilms substratum and suggests that there might be

population of neutrophils unaffected by the inhibitory properties observed in the morphologic studies. The significance of these measurements is that they may provide insights into the countermeasures that pathogens in biofilms employ to evade cellular host defense mechanisms.

In summary, the results in this thesis support the well-known fact that microbial biofilms are difficult to eradicate. Then show that the local oxygen concentration fully governs the neutrophil superoxide production when exposed to biofilms and protects the biofilms interior structure. They also suggest that a dynamic mechanism between neutrophils and microbial biofilms exists in the cycling of local oxygen again contributing to biofilm protection from these inflammatory cells. Evidence to support frustrated phagocytosis by neutrophils in biofilms was not observed. Instead neutrophils, at least a significant population of neutrophils were unable to penetrate biofilms or be morphologically stimulated upon contact with biofilms.

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