The Comparative Susceptibility of *Porphyromonas gingivalis* to the Killing Mechanisms of the Neutrophil

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ABSTRACT

Rebecca Keyser Parsons: The Comparative Susceptibility of *Porphyromonas gingivalis* to the Killing Mechanisms of the Neutrophil (Under the direction of Roland R. Arnold)

Neutrophils are critical in defense against periodontal pathogens such as *Porphyromonas gingivalis*, yet the mechanisms employed by neutrophils to kill this species are unclear. Using a dual-color fluorescence assay, we investigated phagocytosis and killing of *P. gingivalis* by neutrophils from normally healthy human donors *vs.* subjects with either chronic granulomatous disease (CGD) or myeloperoxidase (MPO)-deficiency. *P. gingivalis* was not killed in the absence of phagocytosis, and neutrophil phagocytosis required both complement and high titered specific antibody. Intraphagosomal killing of *P. gingivalis* required a functional NADPH oxidase, which required the availability of oxygen, as there was no killing by CGD neutrophils or by normal neutrophils when NADPH oxidase was inhibited either with anaerobiosis or diphenyl iodonium. Furthermore, MPO was not required for neutrophil-mediated killing of this pathogen, as MPO-deficient neutrophils were fully competent in killing *P. gingivalis*, and MPO inhibition with 4-aminobenzoic acid hydrazide did not block neutrophil killing of *P. gingivalis*. The relative susceptibility of *P. gingivalis* to the reactive oxygen species associated with NADPH oxidase-dependent killing was also investigated. H_2O_2, but not superoxide, was effective in killing *P. gingivalis*, as killing by the xanthine-xanthine oxidase system was blocked by catalase, but not superoxide dismutase. When delivered as a bolus, H_2O_2 killed *P. gingivalis* in a dose-, time-, and temperature-dependent fashion, while ^{-}OCl killed at a critical concentration, independent

of time or temperature. Compared to other species tested, *P. gingivalis* was remarkably sensitive to H_2O_2. Despite having very different susceptibilities to H_2O_2, all test bacteria showed similar susceptibilities to the MPO-H_2O_2 system and to its product, ^-OCl. When administered to logarithmically-varied bacterial densities, H_2O_2 killing was dependent on concentration, but not target density; while ^-OCl killing was lost with increasing target density. Together, these data support the hypothesis that ^-OCl is contact-lethal. In contrast, the time- and temperature-dependent properties associated with MPO-independent H_2O_2-mediated killing are consistent with the conversion of H_2O_2 to a more toxic product by the bacteria itself. The involvement of hydroxyl radical in this bactericidal activity was suggested by the protection afforded by dimethyl sulfoxide against H_2O_2 killing and azide protection against neutrophil-mediated killing of *P. gingivalis*.

This dissertation is dedicated to:
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And to my sister, Kim Keyser –
for enriching my life with love and family;
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LIST OF ABBREVIATIONS AND SYMBOLS

A	absorbance
A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
Ab	antibody
4-ABH	4-aminobenzoic acid hydrazide
Atm.	atmosphere
BPI	bacterial permeability increasing protein
CAT	catalase
CFU	colony forming unit
CGD	chronic granulomatous disease
CR1	complement receptor 1
CR3	complement receptor 3
DAG	diacylglycerol
DAPI	4’,6-diamidino-2-phenylindole
DHR 123	dihydrorhodamine 123
DMSO	dimethyl sulfoxide
DPI	diphenyl iodonium
FC	final concentration
fMLP	N-formyl-methionyl-leucyl-phenylalanine
F. nucleatum	*Fusobacterium nucleatum*
FV	final volume
G	glucose
GCSF	granulocyte colony stimulating factor
GMCSF	granulocyte monocyte colony stimulating factor
GO	glucose oxidase

G6PD	glucose-6-phosphate dehydrogenase
HBSS	Hanks balanced salt solution
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
IgG	Immunoglobulin G
iNOS	inducible nitric oxide synthase
LAD	leukocyte adhesion deficiency
LF	lactoferrin
LJP	localized juvenile periodontitis
Log	logarithmic
Log_{10}	log base 10
LPS	lipopolysaccharide
MMP	metalloprotease
ΔmOD_{30min}	change in optical density over 30 minutes
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NaN_3	sodium azide
$NO^{\cdot}$	nitric oxide
NOI	neutrophil oxidative index
NT	not tested
$^1O_2$	singlet oxygen
$O_2^{\cdot -}$	superoxide radical
^-OCl	hypochlorite
OD	optical density
$ONOO^{\cdot -}$	peroxynitrite
$OH^{\cdot}$	hydroxyl radical

PBS	phosphate buffered saline
P. gingivalis	*Porphyromonas gingivalis*
phox	phagocyte oxidase
PI	propidium iodide
P. intermedia	*Prevotella intermedia*
PKC	protein kinase C
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
ROS	reactive oxygen species
RT	room temperature
S. aureus	*Staphylococcus aureus*
SOD	superoxide dismutase
S. pyogenes	*Steptococcus pyogenes*
UV	ultraviolet
Vmax	maximal velocity of superoxide production
WC	Wilkins-Chalgren
X	xanthine
XO	xanthine oxidase

CHAPTER 1

INTRODUCTION:

LITERATURE REVIEW AND RATIONALE

Periodontal Health and its Relationship with the Neutrophil

Multiple cell types of the innate and adaptive immune systems together provide an effective arsenal of host defense. Among the white blood cell types important to immunity, however, a granulocytic leukocyte called the polymorphonuclear neutrophilic leukocyte (PMN or neutrophil) is the one invariably associated with the health of the periodontium. The neutrophil is a professional phagocyte indispensible to periodontal defense, supported by the fact that individuals with neutrophil deficiencies, or with specific dysfunctions that impair certain but not all neutrophil functions, are highly susceptible to periodontal diseases (1) (2) (3).

Periodontal Diseases as Infectious and Inflammatory Diseases

The periodontal diseases are a clinically diverse group of infection-driven, chronic inflammatory diseases of the gums (gingiva) and the supporting structures of the teeth (periodontium) (4) (5). Specific pathogens are associated with different forms of periodontal disease, from gingivitis, which is considered reversible if treated; to periodontitis, which if left untreated can result in the formation of deep periodontal pockets, irreversible destruction of the attachment apparatus of the teeth (periodontal ligament and connective tissue), resorption of supporting alveolar bone, and ultimately, tooth loss. While gingivitis can progress to periodontitis in the absence of adequate oral hygiene, periodontitis is not an inevitable progression from gingivitis. Additionally, epidemiological surveys have estimated that up to 50% of the US adult population has gingivitis affecting a minimum of three teeth (6), and at least 10% of the US adult population (30 million people in the USA alone) suffers from moderate to severe forms of periodontitis (7). Periodontal diseases are therefore among the most common chronic infectious diseases in humans (8).

Periodontitis can generally be characterized by the rate (chronic or aggressive), extent (localized or generalized), and severity (mild, moderate, or severe) at which attachment and bone loss occur, and is attributed as the major cause of tooth loss in adults (5). While the etiology of periodontitis is multifactorial, it is generally understood that the host's immune and inflammatory responses to specific pathogenic flora are responsible for the periodontal tissue destruction that is the hallmark of this disease (9). Impacting quality of life as well as, perhaps, life expectancy, periodontitis has been linked to an increased risk of premature heart disease, stroke, lung infections, abnormal pregnancy outcomes, and septicemia (10). Therefore, periodontitis, which affects a significant portion of the human population, is not just a matter of aesthetics, but can have a holistic impact on health and well-being (11).

The Complex Flora Associated with Periodontal Health and Disease

It is estimated that upward of 700 different bacterial phylotypes and species colonize the oral cavity of humans (12). Colonizing both non-shedding (teeth) and continually-shedding (mucosal epithelium) surfaces, the bacteria in dental plaque colonize in a sequential fashion, forming surface-attached communities called biofilms (5). The shift from periodontal health to periodontal disease is accompanied by a shift in the microflora of dental plaque, due to architectural changes of the gingival crevice, or sulcus. Specifically, alterations in the depth of the gingival crevice (the space between the tooth and wall of the gingival epithelium), oxygen tension (i.e. decreased oxygen when the gingival crevice transitions to a deeper pocket and/or the inflammatory response reduces local oxygen levels), and increased gingival crevicular fluid (a serous exudate that fills the sulcus and bathes the surrounding tissues) flow are the conditions concomitant with and necessary for the microbiological progression which initiates the transition from gingival health to disease (12).

Periodontal health, defined as the absence of gingival pocket depths of 4mm or greater, is associated with the colonization of predominantly Gram-positive, aerobic and facultative anaerobic bacteria such as *Actinomyces* and *Streptococci* spp., localized predominantly at the gingival margin (13) (14). The transition to gingivitis and periodontitis is associated with biofilm development extending beneath the gingival sulcus, with increased percentages of specific Gram-negative, microaerophilic and strictly anaerobic bacteria, such as *Aggregatibacter, Campylobacter, Fusobacterium, Prevotella, Porphyromonas, Selenomonas, Tannerella,* and *Treponema* spp., etc (5) (13) (14). Continued development of the biofilm relies on interactions such as coaggregation with bacteria from the same or different genera (15), and both earlier and later colonizers are believed to mutually benefit each other. It is generally thought that by providing critical attachment sites and by reducing oxygen tensions to levels permissive to the obligate anaerobic growth of these more fastidious organisms, earlier colonizers facilitate the colonization of later colonizers. By stimulating inflammation and tissue damage, the more pathogenic later colonizers are also beneficial to the earlier colonizers and to the community as a whole by supplying critical nutrients, etc (9) (16) (17).

Socransky and Haffajee *et al* (18) (19) (20) categorized the microflora of biofilms associated with gingival health, gingivitis, and periodontitis, and assigned color designations to these bacteria for the convenience of discussion. Specifically, bacteria were divided into clusters based on association (e.g. bacteria which occur together) and sequence of colonization, as well as pathogenicity or disease severity (12). 'Orange complex' bacteria were classified as bacteria appearing together, later in biofilm development, and comprise species such as *Fusobacterium nucleatum* and *Prevotella intermedia.* During continued maturation of the biofilm, 'orange complex' bacteria, which are associated with both gingivitis as well as mild to moderate forms of periodontitis, are thought to condition the subgingival environment for the subsequent

colonization of ‘red complex’ bacteria. ‘Red complex’ bacteria appear with ‘orange complex’ bacteria, but are overt periodontal pathogens, which appear late in biofilm development, and are associated with more severe forms of adult periodontitis. Comprising only three species, these include *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* (12). Individually and in combination, a plethora of virulence factors possessed by the ‘orange’ and ‘red complex’ pathogens elicit the host responses that lead to the inflammation (gingivitis and periodontitis) and tissue destruction (periodontitis) characteristic of periodontal diseases.

The Inflammatory Cell Infiltrate of Periodontal Lesions

Neutrophils are the predominant inflammatory cell type which infiltrates the gingival sulcus and junctional epithelium, in both health and periodontal disease (13). While neutrophils have been shown to make up about 90-98% of the cell infiltrate of periodontal lesions, around 5% of the population is also constituted by cytokine-producing mononuclear cells such as monocytes and tissue macrophages, T and B lymphocytes, and plasma cells (13) (21). While the absolute numbers of each of these cell types greatly increase with disease severity, the approximate ratio of polymorphonuclear (neutrophils) to mononuclear leukocytes has been shown to stay relatively constant as the degree of inflammation increases in periodontal lesions (13) (22) (23) (24). However, as lesions progress, there is a shift in the predominance of certain lymphocytes present. For instance, as early gingivitis develops into more chronic and severe gingivitis, there is a shift from T cell predominance to B cell predominance (13) (25) (26). As early periodontitis develops into more chronic and severe periodontitis, there is a shift towards antibody-secreting plasma cells (13) (21) (26) (27). Consistent with this, while all of these named cell types are presumably involved in defense within periodontal lesions, the neutrophil axis, in concert with

opsonins such as complement and antibodies, is considered the primary defense mechanism operational in the gingival sulcus (28) (29). Additionally, the principal means by which neutrophils handle periodontal pathogens is through phagocytosis and killing, in a process dependent upon granule fusion with the phagosome (3) (28) (30) (31) (32), and both oxygen-dependent and oxygen-independent killing pathways operational in the neutrophil are considered important in preserving periodontal health (3) (33) (34).

Neutrophil Development in the Bone Marrow

In a process called myelopoiesis, neutrophils develop in the bone marrow in two stages: a mitotic or proliferative phase, followed by a maturation phase. During the mitotic phase, which averages 7.5 days in humans (35), pluripotent stem cells are stimulated to become neutrophil precursors under the influence of myeloid-specific growth factors such as granulocyte colony stimulating factor (GCSF), and to a lesser extent, granulocyte monocyte colony stimulating factor (GMCSF) (36). Stem cells undergo a series of divisions and first become myeloblasts, then promyelocytes, and next myelocytes (36). After this stage, neutrophil precursors lose their ability to divide, but continue to differentiate during the maturation phase into metamyelocytes, band cells, and finally into morphologically mature, segmented neutrophils (33). The maturation phase averages 6.5 days in humans (35).

During both the mitotic and maturation phases in the bone marrow, neutrophil precursors are actively undergoing granule biosynthesis, in a process called granulopoiesis (37) (38). Under normal conditions, the mature neutrophil, which by now has a dense, granular appearance of cytoplasm, is released from the bone marrow into the blood stream two days after maturation is completed, as a terminally differentiated, highly specialized, effector cell. As a fully differentiated and mature granulocyte, the

neutrophil participates in very little *de novo* protein synthesis, which is consistent with the observation that mature neutrophils contain very few mitochondria and a poorly developed endoplasmic reticulum (2). Additionally, neutrophils are considered anaerobic cells, as they contain a rich glycogen supply in their cytoplasm and obtain their energy solely from glycolysis (39).

In a normal, healthy human adult, neutrophils account for 43-65% (normal reference range) of the total circulating white blood cells in peripheral blood. Additionally, after leaving the bone marrow, neutrophils have a short circulating lifespan which averages about 8 hours (13) (36) (40). When stimulated, circulating neutrophils are directed into tissues, where they may remain active for between two and six days, functioning as professional phagocytes (36). If unstimulated, neutrophils will become senescent and undergo apoptosis, and are cleared by the liver and spleen. Studies have demonstrated that the average daily output of neutrophils into the circulation of a 150 lb person is approximately 1×10^{11}, which has the potential to rise by a factor of at least five, during a bacterial infection (35) (41). While the bone marrow contains a reserve pool of between 20 and 50 times the number of neutrophils in the circulation (36), in order to accommodate the increased need for neutrophils during a severe bacterial infection, morphologically immature neutrophils may also be seen in the circulation during illness (35).

The Hierarchy of Granulopoiesis and Degranulation

The cytoplasm of a mature neutrophil is densely populated with granules, which contain not only an arsenal of antimicrobial and potentially cytotoxic substances for the destruction of microbial invaders, but also an important reservoir of membrane receptors, essential for neutrophil functions such as chemotaxis (the directed migration of neutrophils toward a site of infection in tissues) and phagocytosis (the engulfment of

bacteria into intracellular, membrane-enclosed compartments called phagosomes), etc (3) (42). During granulopoiesis, granule proteins are synthesized sequentially, but as a continuum, in the presence of specific transcription factors which are turned on and off, at distinct stages of neutrophil development (37) (38) (43). In general, granule packaging is believed to occur not by sorting, but by the fusion of different transport vesicles which bud off the Golgi at approximately the same time (42) (44) (45). Therefore, granule packaging occurs continually as proteins are synthesized, and granule proteins that are synthesized at the same time will localize to the same granules. Highly heterogeneous, granules have traditionally been categorized into subsets, based on the order of biosynthesis of their characteristic protein contents (37) (38) (43). As such, granules are categorized into primary (or azurophilic), secondary (or specific), and tertiary (or gelatinase) granule subsets, named for their order of appearance during neutrophil development.

While all granule subsets share some proteins such as lysozyme, which is synthesized continually; other proteins, which are synthesized transiently and express minimal overlap, serve as specific markers of particular subsets. For example, 'azurophilic' or 'primary' granules are synthesized and packaged first during granulopoiesis, in myeloblasts and promyelocytes. Named for their affinity for the basic dye azure A due to their peroxidase-positive content (46), azurophilic granules are defined based on the presence of a specific protein/ enzyme, which serves as the marker for this subset, called myeloperoxidase. While myeloperoxidase is found only in azurophilic granules, azurophilic granules are also divided into defensin-poor (synthesized early and are small) and defensin-rich (synthesized later and are larger and dense) granules. Defensin-rich granules appear late in promyelocytes and continue in myelocytes. 'Specific' or 'secondary' granules, which are twice as populous but half the size of azurophilic granules (47), are synthesized and packaged second during

granulopoiesis, in myelocytes and metamyelocytes, and are peroxidase-negative. Specific granules are defined based on the presence of the proteins, vitamin B_{12}-binding protein, and in particular, lactoferrin, which serves as the main marker for this subset. 'Gelatinase' or 'tertiary' granules are synthesized and packaged third during granulopoiesis, in band cells and segmented polymorphonuclear neutrophils, and are defined based on their high content of a proteolytic enzyme called gelatinase. Finally, while considered to be a different organelle than granules, 'secretory vesicles' are purely exocytotic storage vesicles, rich in receptors, and are synthesized and packaged last during granulopoiesis, in segmented neutrophils (37) (38) (42) (43) (44) (45).

All granule subsets and secretory vesicles share the feature that they are membrane-bound organelles, which contain both a phospholipid bilayer membrane, rich in receptors, and an intragranular matrix. With the exception of secretory vesicles, the intragranular matrices of granules are rich in antimicrobial proteins, enzymes, and proteases, which serve as important mechanisms of defense, in both oxygen-dependent and oxygen-independent killing pathways of the neutrophil. Both the synthesis and mobilization of neutrophil granules and secretory vesicles, which are highly regulated events, are essential components of the neutrophil-mediated immune response. For instance, from initial activation and recruitment, to phagocytosis, and the destruction and degradation of microorganisms, degranulation of both granules and secretory vesicles is essential to executing every function of the neutrophil (42).

Additionally, granules and secretory vesicles are mobilized (or degranulated) in the opposite order in which they were synthesized. Therefore, while secretory vesicles are synthesized last, they are mobilized first upon neutrophil activation; and while azurophilic vesicles are synthesized first, they are generally mobilized last during intracellular killing and digestion. In particular, the defensin-rich granules are generally considered to be the last to fuse with the phagosome and the least likely to undergo

exocytosis. Furthermore, while secretory vesicles are purely exocytotic vesicles which fuse only with the neutrophil exterior (plasma membrane); portions of gelatinase and specific granules, in contrast, undergo exocytosis, as well as intracellular fusion with the phagosome. In contrast to the larger specific granules, the smaller gelatinase granules are not as rich in antimicrobial substances, and more readily undergo exocytosis, where they serve primarily to supply membrane receptors and matrix-degrading enzymes during neutrophil extravasation and tissue migration. Only azurophilic granules undergo very limited exocytosis, and are therefore primarily considered lysosomal granules, contributing predominantly to the killing and digestion of phagocytosed organisms (42) (45).

It is also important to note that the act of granule fusion with the plasma membrane serves not only to deliver intragranular matrix contents to the outside environment of the neutrophil, but also serves to incorporate granule membrane components into the plasma membrane and on the exterior neutrophil surface. Similarly, the act of granule fusion with the phagosome membrane serves not only to deliver intragranular matrix contents to the interior environment of the phagosome, but also results in incorporation of granule membrane components into the phagosome membrane and on the interior phagosome surface.

Neutrophil Activation, Diapedesis, and Chemotaxis

The neutrophil response to an infectious insult is a multi-step process, involving neutrophil activation, vascular adhesion and diapedesis, tissue migration through chemotaxis, phagocytosis, and finally, microbicidal activity (3). The initial response is characterized by the activation, and directional recruitment of neutrophils from peripheral blood to sites of infection that involves a process called chemotaxis. Primary contact with stimulated vascular endothelium, occurring especially in the post-capillary venules

adjacent to sites of infection (48), activates neutrophils and initiates a series of exocytotic events by secretory vesicles, which are the first and most rapidly mobilized subcellular constituents. By fusion with the plasma membrane, the membrane contents of secretory vesicles get incorporated into the plasma membrane of the neutrophil, which furnishes the neutrophil's exterior with vital adhesion molecules and receptors, which serve to initiate the earliest functions of the neutrophil (45). Essential to these earliest activities, secretory vesicles supply the neutrophil membrane with β_2-integrins CD11b/CD18 (Mac-1, CR3), the complement receptor 1 (CR1), receptors for formylated bacterial peptides (formylmethionyl-leucyl-phenylalanine (fMLP) receptors), the lipopolysaccharide (LPS)/lipoteichoic acid binding receptor CD14, the FcγIII receptor CD16, and the metalloprotease leukolysin, etc (42) (45). Surface-surface interactions between selectin ligands on activated endothelial cells and integrins on activated neutrophils then mediate the process of neutrophil rolling, tethering, and eventual adhesion to the endothelium. Neutrophils then extravasate between the junctions of endothelial cells into tissues, in a process known as diapedesis. Three important metalloproteases (MMPs) have been identified in neutrophils which facilitate this process by degrading extracellular matrix components, such as collagen, fibronectin, proteoglycan, and laminin. These include gelatinase (or MMP-9), which is a matrix component predominantly found in gelatinase granules; collagenase (MMP-8), which is a matrix component in specific granules; and leukolysin (MMP-25), which is a matrix component in both specific and gelatinase granules, as well as a membrane component of secretory vesicles (42). Therefore, degranulation of secretory vesicles, followed by portions of gelatinase and then specific granules, is concomitant with and necessary for the migration of neutrophils from the vascular basement membrane into infected tissues. Interestingly, the multilobular nucleus of the neutrophil, which contributes to the extreme flexibility of the cell, is another essential feature allowing not only the migration of

neutrophils between the narrow spaces of endothelial cell junctions, and from the bone marrow sinuses into the circulation (3) (35), but also serves to help minimize capillary and tissue damage as neutrophils cross through these structures.

In the periodontium, neutrophils similarly enter the gingival crevice by diapedesis between the endothelial cells of gingival blood vessels and capillaries, and migration through the tissues of the junctional epithelium, by following chemotactic substances which form a gradient (13). Neutrophils can be driven toward bacteria by several types of chemotactic gradients, including microbial products such as LPS and fMLP, complement cleavage products such as C5a, antigen-antibody complexes, pro-inflammatory cytokines such as interleukin-8, and lipid mediators such as leukotriene B4– all of which are found in gingival crevicular fluid during periodontal infections (49).

Neutrophil Phagocytosis and Phagolysosomal Fusion

Phagocytosis is the process by which a microbe is engulfed by a phagocyte (e.g. a neutrophil) into an intracellular and physically sequestered, plasma membrane-lined enclosed compartment, called a phagosome. In a process known as opsonization, phagocytosis is greatly facilitated if microbes are first coated with opsonic proteins. The most important opsonins in serum are the complement fragments, C3b and C3bi, and immunoglobulin G (IgG), which bind to and cross-link with the complement (CR1 and CR3) and Fc receptors on neutrophils (31). Antibody is not always required for complement activation, and phagocytosis may also occur via alternative pathways of complement activation independent of antibody (50), as well as by non-specific opsonins which may also be present in serum (3). Receptor-ligand interactions between the phagocyte and the opsonin-coated microbe typically initiate the process of phagocytosis, where the cell membrane invaginates to form a phagosome (51). Silverstein (52) observed that when a neutrophil comes in contact with a bacterium, the cytoplasm of the

neutrophil first extends out into long, thin pseudopodia, which then spread over and flow around the surface of the targeted bacterium, tightly encompassing it, in a zipper-like fashion. In formation of the phagosome, Zakhireh *et al* (35) also observed that after encasement, the phagosome buds off from the cell periphery and moves centripetally away into the cell's interior. Stossel (53) identified that pseudopodia formation, important for both neutrophil locomotion and phagocytosis, is made possible due to contractile proteins, similar to those of skeletal muscles, such as actin, myosin, actomyosin, and actin-binding proteins. Specifically, the polymerization and rearrangements of these contractile networks, located within the neutrophil cytoskeleton just beneath the plasma membrane, permit the neutrophil motility and formation of pseudopodia, required for phagocytosis (30) (35) (53).

Just after phagocytosis (1-15 min), microbicidal activity begins with the activation of both oxygen-dependent and oxygen-independent killing pathways, which are mediated predominantly by specific and azurophilic granules, which fuse with the phagosome, in a process called phagolysosomal fusion. With very little extracellular medium taken up during phagocytosis (47) (54), the phagosome is designed to be a very narrow space in which a large amount of degranulation occurs, close to the bacterial surface, for microbial killing and digestion (50) (55). A complex series of events are responsible for these processes. In brief, degranulation is activated by a surge of intracellular calcium, followed by the activation of protein tyrosine kinases, which phosphorylate specific phospholipases (e.g. phospholipase C). These in turn catalyze the generation of specific 'messengers' such as inositol triphosphate and diacylglycerol (DAG), which release additional calcium from intracellular stores and activate protein kinase C (PKC), respectively (3) (42). PKC is particularly important for its role in activating the NADPH oxidase, a phagosomal (90-95%) and plasma membrane (5-10%)

-associated enzyme complex (56), that is dormant in resting neutrophils, but rapidly assembled when activated.

Oxygen-Dependent Killing Pathways

As proposed by Klebanoff (57) (58), neutrophils have two major bactericidal mechanisms which can be divided broadly into oxygen-dependent and oxygen-independent killing pathways. While the principal oxygen-dependent pathway of the neutrophil is mediated by the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase enzyme, oxygen-dependent killing may also be divided into myeloperoxidase (MPO)-dependent and MPO-independent mechanisms. However, while only shown to occur under special circumstances, another oxygen-dependent pathway of the neutrophil is one mediated by an enzyme called inducible nitric oxide synthase (iNOS). Believed to be the major oxygen-dependent killing pathway in human macrophages (59), iNOS is not a principal oxygen-dependent killing pathway in human neutrophils. Since iNOS has not been identified in any granule subset, its generation is believed to require *de novo* protein synthesis – which is minimal in the neutrophil as a fully differentiated granulocyte. However, Webb *et al* (60) have shown that iNOS can be induced in human neutrophils in the presence of specific cytokines, including interleukin-1α, tumor necrosis factor-α, and interferon-γ, as well as additional stimuli from adhesion to a surface. Only under these circumstances, and in the presence of oxygen and L-arginine (O_2 + L-arginine → $NO^{\cdot}$ + L-citrulline), is iNOS capable of generating nitric oxide ($NO^{\cdot}$), a reactive nitrogen species which was discovered for its role in the control of vascular tone (59).

The principal oxygen-dependent killing pathway of the neutrophil is mediated by the multi-component NADPH oxidase system, which is responsible for the production of a respiratory burst (named for the large amounts of oxygen that are rapidly consumed by

activated neutrophils). Dependent on the fusion of specific (or secondary) granules with the phagosome, the components of the NADPH oxidase (which are referred to by their molecular weight (kDa) and have been given the designation *phox* for *ph*agocyte *ox*idase (61)), are delivered both to the phagosome membrane and to the phagosome interior or cytosol. These include two membrane-associated components, $gp91^{phox}$ and $p22^{phox}$, which together comprise the cytochrome b_{558}; as well as three cytosolic components, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$. In order to activate the NADPH oxidase, all five NADPH oxidase subunits ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) with the cytosolic GTPase Rac2 (62) (63) must come together and assemble on the interior surface of the phagosome membrane to form the NADPH oxidase complex, a process dependent on phosphorylation of cytosolic subunits by PKC (64). Glucose-6-phosphate dehydrogenase (G6PD) generates NADPH, which provides the NADPH oxidase complex with the electron to reduce oxygen to superoxide anion: $NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\cdot -} H^+$ (30) (56) (62) (64) (65) (66) (67) (68). The genetic deficiencies in phagocytes of either $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, or $p67^{phox}$, or mutations in Rac2 or G6PD, provide the molecular bases for all cases of chronic granulomatous disease (CGD) in humans (69) (70). Neutrophils from subjects with CGD are consequently unable to produce superoxide or any downstream reactive oxygen species, and are considered respiratory burst-deficient (61) (66) (69) (70) (71) (72) (73) (74) (75). In normal, NADPH oxidase-functional neutrophils, superoxide, generated through the respiratory burst, subsequently dismutates to hydrogen peroxide ($O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2$); and from these intermediates, other more reactive oxidants are generated, either by reacting with bacterial iron or with enzyme systems such as MPO, or by the products of these systems reacting with $O_2^{\cdot -}$ and/or H_2O_2, or with each other (47) (63) (76) (77) (78) (79) (80) (81) (82) (83). It is of interest to note that hydrogen peroxide is believed to form in

the phagosome through the collision or interaction of two superoxide molecules, since there is no evidence of SOD in the phagosome (84).

The presence of oxygen and a functional NADPH oxidase are essential in making numerous reactive oxygen species (ROS) within the phagosome, including superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl or H^+ ^-OCl), and hydroxyl radical ($OH^{\cdot}$) (64) (76) (79) (81). Both $O_2^{\cdot-}$ and H_2O_2 are generally regarded as relatively nontoxic and inert (or only mildly oxidative) (79) (85) (86) (87), though their roles in killing bacteria are of particular interest due to the abundance of superoxide dismutases (SOD), catalases, and peroxidases in bacteria ($O_2^{\cdot-}$ and H_2O_2 scavenging enzymes); since the oxygen consumed in the respiratory burst is first converted to $O_2^{\cdot-}$ and then to H_2O_2; since both are generated in close proximity to ingested bacteria; and due to the severe clinical consequences of their absence in CGD (34) (76) (77) (81) (88) (89). Additionally, considerable evidence suggests that $O_2^{\cdot-}$ and/or H_2O_2 play direct (47) or indirect roles in killing ingested bacteria by participating as substrates in almost every known killing system in the neutrophil (76) (81) (90). Furthermore, impaired bactericidal activity in CGD neutrophils, which are incapable of producing their own $O_2^{\cdot-}$ and H_2O_2, is restored in the laboratory by the addition of a H_2O_2-generating system (91) (92) (93) (94) (95) (96). It is also important to note that while $O_2^{\cdot-}$ has limited membrane permeability due its charge; H_2O_2, in contrast, is highly membrane permeable because it is small and uncharged (79). It is likely that this membrane permeability property permits H_2O_2 to gain access to the interior of the cell, where it is converted to more reactive species in direct proximity to vulnerable targets.

The principal enzyme of the neutrophil known to consume H_2O_2 in the phagosome is heme-based MPO, which catalyzes the oxidation of chloride by H_2O_2 to generate the potent oxidant, HOCl or H^+ ^-OCl, the weak acid form of the active ingredient found in household bleach, Na^+ ^-OCl. Dependent on the fusion of

azurophilic (or primary) granules with the phagosome, MPO (an intragranular matrix component defining azurophilic granules and the most abundant protein/enzyme in neutrophils), is delivered to the phagosome, in close proximity to bacteria, where it is proposed to react with H_2O_2, already present (by order of degranulation, H_2O_2 would be present in the phagosome before MPO), converting H_2O_2 to H^+ ^-OCl: MPO + Cl^- + H_2O_2 → H^+ ^-OCl + H_2O. Necessary chloride has been shown to be provided via channels which pump chloride into the phagosome from the outside of the cell (79) (97), where it is present in biological fluids at concentrations higher than required (84); as well as supplied by fluids taken up upon phagocytosis (47). MPO, a strongly cationic 146 kDa hemoprotein (98), has also been shown to coat the surfaces of some negatively-charged bacteria (99), which would initiate HOCl generation even closer to a bacterial surface (84); though most MPO is believed to react with H_2O_2 unbound to bacteria (47). HOCl, which is a small, uncharged, non-radical, highly permeable to bacterial membranes (79), is believed to exert the majority of its antimicrobial activity after entering the cell. Due to its membrane permeability (79), its rapid reactivity (short-lived), and its ability to chlorinate or otherwise modify a wide range of microbial targets, MPO-generated HOCl is regarded as one of the most potent microbicidal and cytotoxic mechanisms of the neutrophil (47) (63) (84) (100) (101) (102). In particular, HOCl has been shown to be highly proficient at disrupting intracellular structures and metabolic processes in bacteria (50) (103) (104) (105); e.g. by cross-linking enzymes, lipids, proteins, and DNA (106); by interfering with DNA and protein synthesis (107); by fragmenting proteins (108); and chlorinating amines to chloramines (104) (109), a secondary more stable product proposed to enhance the toxicity of HOCl in the phagosome (68) (101) (110).

It is also well-supported that in a reductive cellular environment (which is found in a metabolizing cell), H_2O_2 is capable of reacting with bacterial-associated iron and mediating a Fenton reaction, where ferrous iron (Fe^{2+}) reduces H_2O_2 to the most reactive

(i.e. non-specific and diffusion-limited) radical known, hydroxyl radical ($OH^{\cdot}$): $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H_2O + OH^{\cdot}$ (78) (81) (82) (83) (85) (88) (111) (112) (113) (114) (115). Because $OH^{\cdot}$ reacts so quickly with essentially the first molecule it meets (very short range of action), it would likely need to be formed in the immediate vicinity of a crucial target in order to become lethal (116). Upon diffusion inside the cell, H_2O_2 is proposed to react with bacterial iron (e.g. cell-associated iron or hemes, Fe-S clusters, etc) (78) (83) (85) (115) (117) to generate $OH^{\cdot}$ close to DNA; and has been shown to cause DNA modifications and strand breaks in a variety of bacteria (79) (118). Though extremely reactive with most bacterial targets, $OH^{\cdot}$ is especially adept at causing lipid peroxidations (79), which have the potential to propagate rampantly throughout a cell in a domino-like fashion or chain reaction (78).

Due to the instability of these partially reduced oxygen species (e.g. $O_2^{\cdot -}$ and $OH^{\cdot}$), their membrane permeabilities (e.g. H_2O_2 and HOCl), and their rapid reactivity (e.g. H_2O_2, HOCl, and $OH^{\cdot}$) with a wide range of bacterial targets (e.g. bacterial-associated iron, enzymes, proteins, lipids, cell walls, cell membranes, RNA, and DNA etc), ROS pose a significant threat to microorganisms, especially when generated within the phagosome in close proximity to a bacterium, or within a bacterium itself (69) (76) (77) (79).

Oxygen-Independent Killing Pathways

Neutrophils are also proficient at killing most bacteria in the absence of oxygen. Oxygen-independent killing mechanisms, by definition, do not require the presence of oxygen in order to function, and seem to act in both primary, combinatorial, and reserve capacities (35). For instance, many organisms are susceptible to more than one antimicrobial pathway, and so when one is absent, as in MPO-deficiency, it may be handled less effectively, but adequately, by another system, such as by an oxygen-

independent killing mechanism, which may serve as a type of back-up or compensatory mechanism (58). Dozens of antimicrobial substances, which constitute the oxygen-independent killing pathways of the neutrophil, have been isolated from the intragranular matrices of the three granule subsets. Some of the most well-known examples include enzymes such as lysozyme, β-glucuronidase, and phospholipases; proteins such as defensins, lactoferrin, azurocidin, and bacterial permeability increasing protein; and the serine proteases, proteinase-3, elastase, and cathepsin G (42) (45) (61). It is of interest to note that most of the oxygen-independent antimicrobial substances found in neutrophils are arginine- and/or lysine- rich (significance to be discussed). A few of the best-characterized oxygen-independent killing pathways of the neutrophil have been selected for discussion below.

Defensins are small (~3.5 kDa), arginine-rich cationic peptides, which are stored in a subset of the azurophilic granules – the defensin-rich dense granules. Estimated to make up at least 5% of the total protein content of neutrophils, defensins are an important killing mechanism of the neutrophil, and display antimicrobial activities against a broad range of both Gram-positive and Gram-negative bacteria, as well as fungi, enveloped viruses, and protozoa (42) (119) (120). Defensins appear to exert their antimicrobial effects by forming ion-permeable, hydrophobic channels or pores through the lipid bilayer of membranes (121) (122). This increases the membrane permeability of microorganisms, which in turn enhances their susceptibility to a wide range of other microbicidal substances, which are now capable of penetrating.

Bacterial permeability increasing protein (BPI) is a larger (~50 kDa), lysine-rich protein, which is stored in azurophilic granules. Highly cationic, BPI is especially proficient at killing Gram-negative bacteria, at nanomolar concentrations (123). By binding to the outer membranes of negatively-charged LPS residues, BPI appears to exert its antimicrobial effects by disrupting the integrity of bacterial cell membranes (e.g.

inducing lipid rearrangements) (63). This in turn increases the membrane permeability of Gram-negative bacteria, and inhibits their growth (124) (125). Further, by attaching to bacterial LPS, BPI has been shown to function also as an opsonin, facilitating phagocytosis by neutrophils (126).

Serprocidins, such as proteinase-3, elastase, and cathepsin G, are serine proteases (~25-29 kDa) with microbicidal activities (42). Serprocidins are arginine-rich polypeptides, which are stored in azurophilic granules. As cationic and hydrolytic proteins, serprocidins damage and degrade bacteria, but are best known for their proteolytic activities against a wide range of extracellular matrix components, such as elastin, fibronectin, laminin, type IV collagen, and vitronectin; as well as for their roles in activating other antimicrobial enzymes and proteins which get delivered to the phagosome (42) (127). Proteinase-3, for example, has been shown to play an important role in cleaving and activating the specific granule peptide, human cathelicidin protein-18 (hCAP-18), into the antimicrobial peptide cathelicidin LL-37 (128).

Lysozyme, an arginine- and lysine-rich cationic antimicrobial peptide (14 kDa), was originally discovered by Sir Alexander Fleming and has come to be one of the most completely understood enzymes of the neutrophil (35). Lysozyme is present in all three granule subsets, but has peak concentrations in specific granules (129). Lysozyme is an endoacetyl muramidase, specific for cleaving the β-(1,4)-N-acetyl-muramyl-N-acetylglucosamine linkages within the peptidoglycan layer of bacterial cells walls (130). Because the peptidoglycan layer of Gram-negative bacteria is protected by LPS, Gram-negative bacteria are not usually susceptible to lysozyme (131). Additionally, while long known for its ability to lyse certain bacteria, it appears that many pathogens, especially Gram-negative bacteria, are resistant to the lytic action of lysozyme unless they are damaged (e.g. by defensins or by BPI) or opsonized first (e.g. with antibody and complement) (35) (132).

Lactoferrin (LF), a major constituent of specific granules, is an arginine- and lysine- rich, non-heme iron-binding glycoprotein (78 kDa), which has been shown to demonstrate both bacteriostatic and bactericidal activities against a broad range of Gram-positive and Gram-negative bacteria (133) (134) (135) (136). A member of the transferrin family of iron-binding proteins, LF was originally demonstrated to display bacteriostatic activities by binding and sequestering environmental iron, which leads to bacterial nutritional deprivation (137). In addition to its competitive iron-binding capacity, LF has also been shown to cause irreversible membrane damage and cell lysis by binding to bacterial cell membranes via an N-terminal cationic and amphipathic α-helical region called lactoferricin (138). Ambruso and Johnson (139), however, demonstrated that LF is also capable of participating in hydroxyl radical ($OH^{\cdot}$) generation in human neutrophils. Kalmar et al (140) demonstrated that the periodontal pathogen *Aggregatibacter actinomycetemcomitans* was highly susceptible to killing by LF *in vitro* (i.e. apoLF, which is an iron- and anion-free form), by a mechanism consistent with $OH^{\cdot}$ generation. While LF was not capable of killing the periodontal pathogen *Porphyromonas gingivalis* by a similar mechanism, Wei (141), however, discovered that purified apoLF does inhibit the expression of one of *P. gingivalis'* most important virulence factors – the arginine-specific gingipains, a discrete protease of *P. gingivalis*, which specifically cleaves after arginine amino acids (more discussion on these later). Human LF has also been shown to bind and remove the hemoglobin receptor (HbR) from the cell surface of *P. gingivalis*, inhibiting its growth when supplied with a medium containing hemoglobin as its sole iron source (142). Therefore, LF is proposed to function both as an oxygen-independent, as well as a possible oxygen-dependent killing mechanism of the neutrophil.

It is important to note that the antimicrobial function of neutrophils depends on a collection of oxygen-dependent, as well as oxygen-independent killing mechanisms,

which operate simultaneously and work as a partnership, to kill and degrade the plethora of potential infecting organisms that challenge the human host. Many antimicrobial mechanisms therefore exist, and some even overlap, to ensure an effective host defense. While the majority of microorganisms are susceptible to multiple killing systems; killing of other microorganisms may depend on a select mechanism for killing. Due to an intrinsic resistance or due to the possession of defense mechanisms, a microbe may not be effectively handled when one antimicrobial pathway is absent or defective (such as in CGD or MPO-deficiency), if it is its critical killing mechanism and if there is no other killing system which compensates effectively. This situation may result in an infection, which progresses to pathology (disease). Interestingly, neutrophil defects in oxygen-dependent and oxygen-independent killing pathways – as well as quantitative defects, such as neutropenias – can be considered 'experiments in nature', helping to elucidate the host defense mechanisms which are essential for the killing of specific microorganisms. It is the study of human diseases which provide insights into normal host defense that were not likely to have been made otherwise.

Protective and Destructive Roles of Neutrophils in the Periodontium

The best evidence of the importance of neutrophils in the defense of the periodontium is the consequence of neutropenia, which is defined as an absolute neutrophil count of less than 1,500 neutrophils per microliter of blood. In addition, individuals with other specific defects that result in neutrophil dysfunction, such as leukocyte adhesion deficiency (LAD) (adherence, phagocytosis and chemotaxis defects), Papillon-Lefévre syndrome (chemotaxis and phagocytic defects), Chédiak-Higashi syndrome (chemotaxis and phagolysosomal fusion defects), specific granule deficiency (chemotaxis and microbicidal defects due to the absence of secondary, tertiary, and defensin-rich granules), as well as systemic diseases which reduce

neutrophil function (such as diabetes and Down's syndrome), also suffer from rapidly destructive forms of adult periodontitis, which manifest in children with these defects shortly after the teeth erupt (1) (2) (13) (33) (65) (143) (144) (145) (146) (147) (148). Interestingly, individuals with cyclic neutropenia, which is characterized by the cyclic disappearance of neutrophils from the blood and bone marrow at intervals of every few weeks (on average, lasts 3 to 6 days every three weeks), are usually asymptomatic and appear healthy without obvious infections, with the exception of symptoms consistent with the initiation of periodontitis, lasting for the duration of the neutropenia (33). Once neutrophil counts recover in these patients, and after potential infection sources are removed, symptoms typically reverse with periodontal health restored (33). It is important to note that in the absence of the neutrophil, as in subjects with chronic neutropenia or LAD for instance, there is an increased susceptibility to not only periodontal pathogens, but to specific other pathogens as well, in particular, *Staphylococcus aureus* and certain Streptococcus species such as *Streptococococcus pyogenes*. Importantly, all of these neutrophil disorders associated with periodontitis demonstrate that the ability to recruit properly functioning neutrophils in sufficient number to subgingival infection sites, followed by phagocytosis and killing by phagolysosomal fusion, is absolutely crucial in the protection against periodontal disease initiation and in limiting disease progression.

Although neutrophils play an important protective role in the periodontium; neutrophils, when present in chronically elevated levels, are also implicated as the main cell type to contribute to periodontal disease progression, due to the release of antimicrobial substances, reactive oxygen species, and matrix-degrading enzymes into tissues ('double-edge sword' theory) (149) (150) (151) (152) (153). Host tissue damage most likely occurs unintentionally by 'frustrated' neutrophils, unable to clear phagocytosis-resistant bacteria, or when confronted by high concentrations of bacteria

that they are unable to handle (154). In an attempt to control infection via extracellular killing of bacteria, neutrophils release more of their granule contents extracellularly than usual (which in these special circumstances include azurophilic granules). The deployment of an overzealous host response to infection is therefore as potentially damaging to periodontal tissues, as when there is a breakdown in neutrophil defense mechanisms (28) (152).

While neutrophils play both protective and destructive roles in periodontitis, and while the protective and destructive functions of neutrophils are not mutually exclusive in periodontitis; the focus of this dissertation is on the protective role of the neutrophil in periodontal defense – as neutrophils are the one cell type, the protective activities of which, are invariably associated with the health of the periodontium. Despite the importance of neutrophils in the periodontium, however, relatively few studies have examined which killing mechanisms employed by the neutrophil are the most important to the suppression of periodontal pathogens. While it is well-known that the main method by which neutrophils handle potential periodontal pathogens is through phagocytosis and killing (28) (30) (31) (50), the specific bactericidal mechanism(s) that neutrophils use against overt periodontal pathogens have yet to be defined. The objective of this dissertation project was therefore to elucidate the mechanism(s) used by neutrophils to kill *Porphyromonas gingivalis*, the periodontal pathogen most associated with the etiology of both chronic and aggressive forms of severe adult periodontitis (4) (16) (18) (19) (155) (156). Understanding how neutrophils kill *P. gingivalis* may offer clues about how normally healthy people are resistant to periodontitis; and likewise, offer clues about the underlying bases for susceptibility to periodontitis in others.

Discrete Defects in the Principal Oxygen-Dependent Killing Pathway

As previously discussed, the principal oxygen-dependent killing pathway of the neutrophil is mediated by the NADPH oxidase, which can then be sub-divided into MPO-independent and MPO-dependent killing mechanisms. In order to investigate the importance of the NADPH oxidase and MPO in neutrophil-mediated killing of *P. gingivalis*, we examined killing of *P. gingivalis* by neutrophils from normally healthy human donors as controls that had no definable neutrophil defects, deficiencies, or dysfunctions *vs.* from subjects with chronic granulomatous disease (CGD) and from myeloperoxidase (MPO)-deficiency. Both CGD and MPO-deficiency represent congenital defects in discrete components of oxygen-dependent killing. The consequences of these deficiencies are discussed below.

Myeloperoxidase-Deficiency

When Klebanoff (157) demonstrated that MPO (a major constituent of neutrophils, highly conserved in all mammals) contributed to the microbicidal activity of leukocytes, a generation of scientists was stimulated to dissect the specific role of this protein in host defense. As a result of numerous elegant studies, the antimicrobial action of MPO has since been attributed to its unique capacity to produce HOCl within the phagosome (47) (63) (79) (84) (100) (101) (102). Since much of the oxygen consumed by an activated neutrophil is converted to H_2O_2 from $O_2^{\cdot -}$ (158) (159), and since most of the H_2O_2 generated in the neutrophil is reported to be converted to HOCl by MPO (47) (63) (79) (105) (160), MPO is a prime candidate as the dominant oxygen-dependent killing mechanism of the neutrophil. Additionally, MPO-generated HOCl (also the active ingredient in household bleach) has repeatedly been shown to display potent microbicidal activities against a broad range of microorganisms, by chlorinating or otherwise modifying a wide range of microbial targets, including enzymes, proteins,

lipids, cell walls, membranes, RNA, and DNA, etc (47) (63) (79) (84) (100) (101) (102) (161) (162) (163).

The real importance of MPO in host defense, however, is somewhat of an enigma. Despite the abundance of MPO in the neutrophil, the evidence of robust HOCl production in the phagosome (47) (63), and its perceived importance for its potent antimicrobial potential; MPO-deficiency represents the most common form of neutrophil dysfunction in humans, with an estimated incidence of 1 in 2,000 to 4,000 individuals in the USA and Europe (164) (165). Yet, these subjects are remarkably healthy and do not experience an increased frequency of infection (166) (167). In fact, MPO-deficiency is generally considered innocuous and was recently removed as a primary immune deficiency disease (168) (169). However, in *ex vivo* conditions, neutrophils from subjects with MPO-deficiency experience a significantly impaired (i.e. delayed) ability to kill certain targets, such as *S. aureus, Escherichia coli*, *Serratia* spp., and *Candida albicans*, etc (84) (170) (171) (172) (173) (174) (175). Despite this, the only clinical complication reported in these patients is a predisposition to infections with *Candida* spp. in MPO-deficient subjects who also happen to be diabetic (69) (176). Yet even the frequency of *Candida* infections is very low in diabetic MPO-deficient subjects (reported in less than 5%) (167), raising doubts of the importance of MPO in host defense (177) (178) (179). MPO does, however, appear crucial when the host is challenged with high doses of infecting agents, as recently suggested by the studies of Aratani (180). With all things taken together, however, while the significance of MPO has been questioned due to its apparent dispensability in humans, the prevailing current view remains that MPO plays an important role in killing and digestion within the phagosome and that MPO-sufficiency in neutrophils contributes to optimal host defense.

Chronic Granulomatous Disease

CGD is a rare (estimated incidence of 1 in 200,000 to 250,000 individuals) (181), heterogeneous group of neutrophil disorders with genetically distinct forms, all of which are characterized by a deficient respiratory burst capacity in stimulated neutrophils (61). While caused by a wide variety of unique mutations (200 or more) in the components of the NADPH oxidase (e.g. substitutions, deletions, insertions, and splices) (73) (74), CGD is divided into an X-linked form (i.e. $gp91^{phox}$ deficient), which is male-dominated, with females as carriers; as well as autosomal recessive forms (e.g. $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ deficient), which affect both males and females. Autosomal recessive forms are clinically less severe, and are usually diagnosed later in childhood, but are less common than the X-linked form of CGD, which comprises the majority (approximately two-thirds) of CGD cases (61) (74). The $p47^{phox}$ form comprises the second most common form of CGD (approximately one-fourth of cases), and is the least severe autosomal recessive form (33). In contrast, subjects with the X-linked form suffer the most severe clinical courses, have more frequent infections than subjects with the autosomal recessive forms, experience an earlier onset (are usually diagnosed within the first year of life), and have the highest mortality rate in children (65) (68) (74) (182). However, a few CGD patients (usually the $p47^{phox}$ form) go undiagnosed until adulthood, due to less severe infections and longer intervals between infections (168) (183). It is hypothesized that the CGD patients that go undiagnosed until older ages may have compensatory mechanisms in place (e.g. upregulated oxidases, such as *duox,* xanthine oxidase, or glucose oxidase, though upregulated iNOS is associated with interferon-γ treatment) (61) (184) (185); or have 'variant' forms of CGD, producing minute levels of $O_2^{\cdot-}$, which suffice (61) (72) (186) (187) (188). Interestingly, some carriers of X-linked CGD with only 5-10% of NADPH oxidase-functional neutrophils are asymptomatic.

In contrast to MPO-deficiency, the vast majority of subjects with CGD present early in life with severe, life-threatening, and recurrent opportunistic infections, as well as uncontrolled inflammation associated with granuloma formation (74). Additionally, in contrast to MPO-deficiency, the microorganisms that are poorly killed by CGD neutrophils *ex vivo* are typically the pathogens responsible for the characteristic infections in CGD (79) (170) (189) (190) (191) (192). In North America, the majority of infections in CGD are due to only five microorganisms, which include *S. aureus, Burkholderia cepacia, Serratia marcescens, Norcardia*, and *Aspergillus* (72), in tissues that serve as natural barriers to the environment (skin, lungs, gastrointestinal tract, and the lymph nodes downstream from these organs; with liver, spleen, bone, and brain affected as infections spread) (68) (75).

While the lack of correlation between MPO-deficiency and morbidity from infection seems to imply that MPO is not important to host defense; the significant morbidity associated with infections secondary to CGD, in contrast, appears as a dramatic demonstration of the importance of the NADPH oxidase for normal host defense (63). However, another explanation (in addition to potential compensatory mechanisms operating in MPO-deficiency) is that CGD affects a wider spectrum of cells (eosinophils, monocytes, and neutrophils) than MPO-deficiency (monocytes and neutrophils) (79); and perhaps more importantly, affects the oxidative killing pathway higher up (NADPH oxidase), than the more distal effects of MPO-deficiency (63). For instance, in CGD, both MPO-catalyzed and MPO-independent oxidative killing mechanisms are absent (57), which likely explains its greater severity. This translates to the inability of phagocytes to generate $O_2^{\cdot -}$ and every downstream ROS dependent on $O_2^{\cdot -}$ generation, including H_2O_2, HOCl, $OH^{\cdot}$, $ONOO^{\cdot -}$, $^1O_2$ etc; compared to MPO-deficiency, where presumably only HOCl is absent. As a result, the isolated pathogens in CGD are microorganisms believed to be resistant to the oxygen-independent killing

mechanisms of the neutrophil, and are typically catalase-positive, capable of eliminating their own metabolically-generated H_2O_2 (68). Likewise, the microorganisms that are killed normally in CGD are believed to be killed either because they are catalase-negative, and thus able to supply a source of H_2O_2 for their own demise (bacterial catalase is believed to deprive phagocytes from using bacterial-generated H_2O_2 for killing), or because non-oxidative killing mechanisms are effective (68) (193) (194).

Interestingly, while subjects with neutropenia have an increased susceptibility to both staphylococcal and streptococcal infections (195); subjects with CGD show intact bactericidal activity against streptococcal species, but not to certain staphylococcal species such as *S. aureus* (170) (196). This suggests that neutrophil function is important to both Staphylococcus and Streptococcus, but that NADPH oxidase activity is essential to the killing of certain staphylococcal species such as *S. aureus*, but not to streptococcal species. The significant predisposition to severe periodontitis in subjects with quantitative (neutropenias) as well as qualitative neutrophil disorders (adherence, chemotaxis, phagocytosis, and microbicidal defects) also suggests that neutrophil function is essential to the defense against periodontal pathogens such as *P. gingivalis*. In the next three chapters, we examine the conditions, components, and mechanisms essential for neutrophil-mediated killing of this pathogen; and investigate how the susceptibility of *P. gingivalis* (catalase-negative) to the killing mechanisms of the neutrophil compares to the catalase-positive *S. aureus* (a pathogen in both CGD and neutropenia), to the catalase-negative *S. pyogenes* (a pathogen in neutropenia but not in CGD), as well as to other catalase-positive and catalase-negative species associated with periodontitis.

Bacterial Mechanisms of Resistance to Neutrophil-Mediated Killing

In the course of their evolution, bacterial pathogens have developed strategies to resist neutrophil-mediated killing as well as other components of the immune system. Termed virulence factors or virulence traits, because they enhance their survival and pathogenicity (ability to establish an infection and cause disease) in the face of an immune response, these include (but in no means are limited to) the possession of antioxidant defenses, such as superoxide dismutases (SOD), catalases, and peroxidases ($O_2^{\cdot-}$ and H_2O_2 scavenging enzymes); protective outer cell structures such as capsule, LPS, S layers, and protoporphyrin; specialized proteases, which inactivate and dysregulate components of the immune system; and toxins such as endotoxins and leukotoxin, etc (13) (31) (197).

Some of the most important strategies of a pathogen may therefore involve host defense evasion; e.g. inactivating chemotactic factors, resisting serum-mediated killing, resisting phagocytosis, resisting neutrophil-mediated killing (oxygen-independent and/or oxygen-dependent killing mechanisms), provoking inflammation while avoiding host recognition, and altering host immune responses. *P. gingivalis* possesses all of these strategies, which undoubtedly contribute to its pathogenicity.

Porphyromonas gingivalis

Among the major human periodontal pathogens, *Porphyromonas gingivalis* is the oral pathogen most associated with the etiology of both chronic and aggressive forms of severe adult periodontitis (4) (16) (18) (19) (155) (156). *P. gingivalis* is characterized as a Gram-negative, strictly anaerobic, and highly proteolytic bacterium, which obtains its energy by proteolysis of peptides and amino acids, and not sugars (is assacharolytic). *P. gingivalis* possesses an array of virulence factors that contribute to its pathogenic potential. These include numerous proteases, antioxidant defenses, and a variety of

outer cell structures such as capsule, LPS, fimbriae, vesicles, and protoporphyrin, etc (4) (12) (13) (198) (199) (200) (201) (202). A few of *P. gingivalis'* most important virulence characteristics and strategies used to resist neutrophil-mediated killing have been selected for discussion.

Proteases, Capsule, and Antioxidant Defenses

P. gingivalis is a highly proteolytic bacterium, and produces and releases large quantities of a variety of proteases, which have all been implicated in the pathogenesis of periodontitis (203). In fact, the proteolytic activities of *P. gingivalis* alone are implicated in provoking every hallmark feature of periodontitis, including neutrophil accumulation at infected sites, increased gingival crevicular flow, bleeding on probing, formation of the periodontal pocket, destruction of periodontal ligament, and bone resorption; as well as most of the observed deregulated inflammatory and immune features of this disease (4) (9) (12) (28) (200) (201) (204) (205) (206) (207) (208). Some of the proteases identified in *P. gingivalis* include collagenases, hemagglutinins, hemolysins, dipeptidyl peptidases, prolyl tripeptidyl peptidases, serine endopeptidases, periodontains, gingipains, and broad-spectrum proteases (9) (28) (201) (209).

Of the proteases identified in *P. gingivalis*, however, three gingipains, encoded by the genes *rgp*A, *rgp*B, and *kgp*, are generally considered the major virulence factors of this periodontal pathogen, and are believed to be the main tools used by *P. gingivalis* in host defense evasion (201) (204) (210) (211). Belonging to the cysteine and serine catalytic class of peptidases, the gingipains demonstrate 'trypsin-like' activities, though the arginine gingipains (HRgpA and RgpB) specifically cleave after arginine residues (the carboxy-terminal side peptide bond of arginine); whereas, the lysine gingipains (Kgp) specifically cleave after lysine residues (the carboxy-terminal side peptide bond of lysine) (4) (212).

Many of the oxygen-independent killing mechanisms of the neutrophil are highly cationic and rich in arginine and/or lysine residues, and would therefore seem susceptible to digestion by the arginine and lysine gingipains of *P. gingivalis.* Interestingly, this seems to occur as culture supernatants and whole cells of *P. gingivalis* have been shown to digest and inactivate a multitude of antimicrobial peptides, proteins, and enzymes found in neutrophil granules, including lactoferrin, the cathelicidin LL-37, β-defensins, cathepsin G, elastase, and bacterial-permeability increasing factor (209) (213) (214) (215) (216) (217) (218) (219) (220) (221).

Prior studies have suggested that several strains of *P. gingivalis* exhibit remarkable resistance to neutrophil phagocytosis due in part to the production of an extracellular polysaccharide capsule, and to the proteolytic degradation of serum opsonins and their receptors (199) (201) (202) (221) (222) (223) (224) (225) (226) (227). The addition of high titered antibodies (IgG) to *P. gingivalis* can facilitate phagocytosis, however, not only by specificity for the capsule, but through retardation of the proteolytic destruction of the opsonins C3b, C3bi, and IgG, as well as the complement (CR1 and CR3) and Fc receptors on the neutrophil (32) (228) (229) (230). This is speculated to occur by gingipain-specific antibodies at least temporarily inhibiting (masking) the discrete proteases of *P. gingivalis*, permitting increased surface deposition of opsonic complement and C3-IgG complexes (50) (228) (231), so that opsonization and neutrophil phagocytosis of *P. gingivalis* may occur.

Like its mechanism of resistance to phagocytosis (228), the resistance of *P. gingivalis* to the bactericidal activity of serum has been attributed to both capsule and to the proteolytic degradation of complement, which together prevent the membrane attack complex from assembling on and penetrating the bacterial surface (227) (232) (233). *P. gingivalis* is not only resistant to the bactericidal activity of complement components in serum, but also to antibody.

While *P. gingivalis* does not make catalase or peroxidase enzymes to degrade H_2O_2 (234) (235), it does make a black-pigmenting, protoporphyrin layer from hemoglobin (236) (237), as well as rubrerythrin (238), Dps (DNA binding protein from starved cells) (239), an alkyl hydroperoxide reductase (AhpFC) (240) (241), and OxyR (242), which have all been shown to potentially protect *P. gingivalis* against H_2O_2. *P. gingivalis* also makes superoxide dismutases (MnSOD and FeSOD) that potentially protect *P. gingivalis* against $O_2^{\cdot-}$ (234) (243) (244) (245).

Co-opting the Host Inflammatory Response

Besides contributing to host defense evasion, the numerous virulence factors of *P. gingivalis* contribute to its ability to evoke an aggressive inflammatory response, which undoubtedly serves to its benefit. Afterall, it would seem likely that a successful pathogen such as *P. gingivalis* would avoid provoking mechanisms of host defense, unless the net outcome offered some survival advantage (17). It is hypothesized that in early stages of infection, *P. gingivalis* may evade, inactivate, and dysregulate mechanisms of host defense (e.g. pro-inflammatory cytokines, host receptors and adhesion molecules, opsonins, etc), in order to facilitate its establishment (4) (28) (201) (221) (246) (247); while at later stages of infection, *P. gingivalis* may stimulate inflammation (e.g. neutrophil recruitment) in order to meet its nutritional and environmental needs (4) (17) (28). Increased gingival crevicular fluid (serous exudate) and flow (stimulated by *P. gingivalis* via proteolytic activation of the kallikrein/kinin pathways, which release bradykinin and increase vascular permeability/ edema), as well as tissue destruction and degradation of host proteins (by proteases of both neutrophil and *P. gingivalis* origin), provide the assacharolytic *P. gingivalis* with a source of peptides and amino acids for growth and nutrition (4) (28) (205) (206) (207). Bleeding gums (stimulated by *P. gingivalis* via proteolytic dysregulation of the coagulation and

fibrinolysis pathways), followed by hemolysis of red blood cells by hemagglutinins, hemolysins, and gingipains of *P. gingivalis* provide this hemolytic bacterium with its two essential growth requirements, hemin (which provides both nutritional iron and protoporphyrin for black pigmentation, when derived from hemoglobin) and Vitamin K (4) (9) (200) (205) (206) (237) (248); while stimulation of the respiratory burst helps to reduce the subgingival environment by the consumption of oxygen, for the anaerobic growth of this fastidious organism. The ability to resist several features of the immune system (including neutrophil phagocytosis, serum-mediated killing, arginine- and lysine-rich antimicrobial peptides, and pro-inflammatory cytokines, etc) undoubtedly enable *P. gingivalis* to co-opt the host inflammatory response for its survival advantage.

Overall Objectives, Hypothesis, and Study Design

While exceptionally resistant to phagocytosis (32) (224) (225) (226) (227) (228) (249) (250) (251), previous studies in our laboratory have shown that normal neutrophils are readily capable of killing *P. gingivalis*, once phagocytized (32). The overall objective of this study was therefore to elucidate the specific bactericidal mechanism(s) employed by neutrophils to kill phagocytized *P. gingivalis*. Our overall hypothesis was that oxygen-dependent killing mechanisms are important to the killing of *P. gingivalis*, because so many of the oxygen-independent killing mechanisms of the neutrophil appear susceptible to the proteolytic activities of *P. gingivalis* (209) (213) (214) (215) (216) (217) (218) (219). A second objective was to investigate how the susceptibility of *P. gingivalis* to the killing mechanisms of the neutrophil compares relative to selected other pathogens, in order to gauge whether its sensitivity is specific to *P. gingivalis*, or generalizable to other relevant pathogens. The pathogens that were selected for comparison included the catalase-negative, anaerobic periodontal pathogens, *Fusobacterium nucleatum* and *Prevotella intermedia*; the catalase-positive, aerotolerant periodontal pathogen *Aggregatibacter actinomycetemcomitans*; the catalase-positive *Staphylococcus aureus*, and the catalase-negative *Streptococcus pyogenes* – two aerotolerant pathogens responsible for pathology in neutropenia as well as oral infections in humans.

In order to begin to understand the mechanisms required to kill this species, we used a dual-color fluorescence assay to investigate under what conditions the human neutrophil kills *P. gingivalis*. Differential interference contrast optics and the fluorescent dyes, DAPI (4',6-diamidino-2-phenylindole) and PI (propidium iodide), were used to visualize the phagocytic and microbicidal activities of neutrophils under epi-illumination UV microscopy. Bacterial killing was detected by permeability of PI, which enters the bacteria immediately upon loss of membrane integrity or death, at which point they

fluoresce red; whereas, live bacteria exclude PI and fluoresce blue, due to the intercalation of DAPI with DNA. This approach allows direct visualization of both phagocytosis and killing, enabling a clear and straightforward interpretation: the bacteria are either within the confines of the neutrophil plasma membrane or not, and are either alive (blue) or dead (red). In contrast to the neutrophil lysis and plating methods reviewed by Decleva *et al* (170), which commonly overestimate killing (i.e. suggests normal killing when it is not) due to incomplete neutrophil lysis (fewer bacteria are released and plated), and due to the clumping and lysis of the bacteria themselves (clumped bacteria will appear as only 1 CFU); our approach using DAPI/PI affects neither the viability of *P. gingivalis* nor of neutrophils, directly measures (without over- or under-estimation) both the phagocytic and microbicidal activities of neutrophils, and can be preserved on a slide indefinitely for later review. Killing assays to neutrophil products were also performed neutrophil-free, in phosphate buffered saline.

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CHAPTER 2

Human Neutrophil Killing of *Porphyromonas gingivalis* Requires Phagocytosis, Oxygen, and NADPH Oxidase, but Not Myeloperoxidase

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Nonstandard abbreviations used: 4-aminobenzoic acid hydrazide (4-ABH), chronic granulomatous disease (CGD), diphenyl iodonium (DPI), final concentration (FC), glucose-6-phosphate dehydrogenase (G6PD), localized juvenile periodontitis (LJP), myeloperoxidase (MPO), neutrophil oxidative index (NOI), propidium iodide (PI), Wilkins-Chalgren (WC).

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ABSTRACT

Neutrophils are indispensible in the defense against periodontal pathogens such as *Porphyromonas gingivalis.* Yet, relatively few studies have examined the mechanisms essential to killing this species. Using dual color-fluorescence microscopy, we investigated killing of opsonized *P.gingivalis* by neutrophils from normally healthy human donors *vs.* from subjects with chronic granulomatous disease (CGD) or myeloperoxidase-deficiency. Strikingly, no killing of *P.gingivalis* occurred by CGD neutrophils or by normal neutrophils treated with anaerobiosis or the NADPH oxidase inhibitor, diphenyl iodonium (DPI). Myeloperoxidase-deficient neutrophils killed *P.gingivalis* normally, while normal and myeloperoxidase-deficient neutrophils treated with the classic myeloperoxidase inhibitor, sodium azide, ablated their ability to kill *P.gingivalis*. Treatment of normal neutrophils with a more specific myeloperoxidase inhibitor, 4-aminobenzoic acid hydrazide (4-ABH), permitted significant, though delayed, killing of *P.gingivalis*. In contrast, normal neutrophils treated with anaerobiosis or DPI were capable of near normal killing of *Aggregatibacter actinomycetemcomitans*, and reduced, but significant, killing of *Fusobacterium nucleatum*; while 4-ABH reduced killing of *F.nucleatum* and *A.actinomycetemcomitans*, similar to *P.gingivalis*. Taken together, these studies show that neutrophil killing of *P.gingivalis* requires phagocytosis, oxygen, and a functional NADPH oxidase, but not myeloperoxidase, and suggest that *P.gingivalis*, in contrast to select other periodontopathogens, is remarkably completely resistant to oxygen-independent mechanisms of neutrophil killing.

INTRODUCTION

The periodontal diseases are a clinically diverse group of infection-driven, chronic inflammatory diseases of the gums (gingiva) and the supporting structures of the teeth (periodontium) (1) (2). Periodontitis is a destructive inflammatory disease of the periodontium, and is among the most common chronic infectious diseases in humans (3) (4). It has recently been estimated that up to 30 million people in the USA alone (10% of the US population) suffer from moderate to severe periodontitis (5), which is characterized by the formation of deep periodontal pockets, irreversible destruction of the attachment apparatus of the teeth (periodontal ligament and connective tissue), and resorption of supporting alveolar bone, and is the major cause of tooth loss in adults (1) (2) (6). Impacting quality of life as well as, perhaps, life expectancy, periodontitis has been linked to an increased risk of premature heart disease, stroke, lung infections, preterm low birth weight pregnancy outcomes, and septicemia (7). Therefore, periodontal disease is not just a matter of aesthetics, but can have a holistic impact on health and well-being.

It is well-known that neutrophils are indispensable in the defense against periodontal diseases and play an integral role in the maintenance of periodontal health. Individuals with quantitative (neutropenia) and qualitative (adherence, chemotaxis, phagocytosis, degranulation) neutrophil defects, as well as systemic diseases which reduce neutrophil function (such as diabetes and Downs syndrome), have all been found to carry a significant increased risk for developing severe periodontitis (8) (9) (10) (11) (12) (13) (14) (15) (16). Neutrophils are essential as the front line of defense in the periodontium against potential periodontal pathogens, but despite their importance, relatively few studies have examined the mechanisms employed by neutrophils to kill these species. While it is known that the main method by which neutrophils handle

periodontal pathogens is through phagocytosis and killing (11) (17) (18) (19) (20), the specific bactericidal mechanism(s) that neutrophils use against subgingival infections with particular organisms have yet to be defined. The objective of this study was therefore to elucidate the mechanism(s) used by neutrophils to kill the periodontal pathogen *Porphyromonas gingivalis*, the oral pathogen most associated with severe forms of periodontitis in adults (1) (21) (22) (23) (24).

P. gingivalis is a Gram-negative, assacharolytic, strictly anaerobic, and highly proteolytic bacterium, capable of hemagglutination, hemolysis, and destruction of host tissues (25) (26). It possesses an array of virulence factors such as lipopolysaccharide, fimbriae, vesicles, broad-spectrum and discrete proteases, extracellular polysaccharide (capsule), and protoporphyrin, which contribute to its ability to evade the host defenses, provoke inflammation, and successfully establish persistent infections within subgingival pockets (1) (8) (27) (28) (29). Its success as a periodontal pathogen has been linked with its exceptional resistance to serum-mediated, extracellular killing and to phagocytosis (20) (30) (31) (32) (33) (34) (35). However, once phagocytized, normal neutrophils are capable of killing *P. gingivalis* (20). No studies to date have defined the specific mechanism(s) used by neutrophils to kill *P. gingivalis*, once phagocytized.

The mechanisms employed by neutrophils to kill bacteria are oxygen-dependent and oxygen-independent, and both are considered important in preserving periodontal health (11) (13) (36). After phagocytosis, both the oxygen-dependent and oxygen-independent killing mechanisms are dependent on cytoplasmic granule fusion with the phagosome (17) (37). The granule content believed responsible for oxygen-independent killing pathways include enzymes such as lysozyme, β-glucuronidase, and phospholipases; proteins such as defensins, lactoferrin, azurocidin, and bacterial permeability increasing protein; and the serine proteases, elastase and cathepsins (37) (38) (39). The principal oxygen-dependent killing pathway of neutrophils is mediated by

the multi-component NADPH (nicotinamide adenine dinucleotide phosphate) oxidase system, which is responsible for the production of a respiratory burst (named for the rapid consumption of oxygen on activation). It is also mediated by granules (secondary or specific granules), which deliver the subunits of the NADPH oxidase to the phagosome. In this process, the NADPH oxidase subunits ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) with the GTPase Rac2 assemble on the inside of the phagosome membrane to form the NADPH oxidase complex. Glucose-6-phosphate dehydrogenase (G6PD) generates NADPH, which provides the NADPH oxidase with the electron to reduce oxygen to superoxide anion: $NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\cdot -} + H^+$ (12) (17) (40) (41) (42) (43) (44) (45). The genetic deficiency in phagocytes of either $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, or $p67^{phox}$, or mutations in Rac2 or G6PD, provides the molecular basis for all cases of chronic granulomatous disease (CGD) in humans, and neutrophils from subjects with CGD are consequently unable to produce superoxide or any downstream reactive oxygen species, and are considered respiratory burst-deficient (38) (41) (46) (47) (48) (49) (50) (51) (52). However, in normal, NADPH oxidase-functional neutrophils, superoxide, generated in the respiratory burst, subsequently dismutates to hydrogen peroxide ($O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2$), and from these intermediates, other more reactive oxidants are generated, either by reacting with iron or with enzyme systems, or by the products of these systems reacting with $O_2^{\cdot -}$ and/or H_2O_2, or with each other (53) (54) (55) (56) (57) (58). The best described enzyme known to consume H_2O_2 in the phagosome is the heme-based myeloperoxidase (MPO) system, which is a chloride- and H_2O_2-dependent mechanism of generating the strong oxidant, weak acid, hypochlorous acid ($MPO + Cl^- + H_2O_2 \rightarrow HOCl$), and is regarded as one of the most potent microbicidal and cytotoxic mechanisms of neutrophils (53) (59) (60) (61).

In this study, a dual color-fluorescence assay was used to investigate the conditions that influence the ability of human neutrophils to phagocytize and kill *P.*

gingivalis. The importance of oxygen in neutrophil killing of *P. gingivalis* was examined by comparing the influence of aerobic *vs.* anaerobic atmospheres on neutrophil killing of antibody- and complement- opsonized *P. gingivalis*. In order to investigate the importance of the NADPH oxidase, *P. gingivalis* killing by neutrophils from subjects with CGD and by normal neutrophils treated with the classic NADPH oxidase inhibitor, diphenyl iodonium (DPI), was determined. The role of the MPO system in killing was examined by comparing *P. gingivalis* killing by neutrophils from subjects with MPO-deficiency and by normal neutrophils treated with the MPO inhibitors, sodium azide and 4-aminobenzoic acid hydrazide (4-ABH), *vs*. normal neutrophils that had not been treated.

RESULTS

Requirement of Antibody and Complement, but not Oxygen, for Neutrophil Phagocytosis of *P. gingivalis*.

As in previous studies, neutrophil phagocytosis of *P. gingivalis* strain A7436 was highly dependent on both high titered specific antibody and a complement source (Supplemental Table A.1). In the presence of both hyperimmune rabbit antiserum to *P. gingivalis* and human complement, there was a rapid involvement of neutrophils in phagocytosis at the initial time point, which progressed to effectively 100% of neutrophils involved in phagocytosis by two hours. In contrast, in the presence of complement only (without antibody), a maximum of only 10% of neutrophils were defined as phagocytic, and less than 1% were phagocytic in the absence of both antibody and complement (data not shown), through the two hour test period. Additionally, phagocytosis was independent of oxygen, as there were no differences in the rate or extent of neutrophil phagocytosis under aerobic *vs.* anaerobic atmosphere.

Requirement of Phagocytosis and Oxygen for Neutrophil Killing of *P. gingivalis*.

Neutrophil killing of *P. gingivalis* was tested in the presence of antibody and complement, under aerobic *vs.* anaerobic atmosphere (Table 2.1). Despite substantial phagocytosis, there was no evidence of neutrophil killing of *P. gingivalis* at initial time. Under aerobic conditions, the number of phagocytic neutrophils which killed three or more bacteria exceeded 75% by one hour and 90% by two hours. In contrast, anaerobiosis completely eliminated killing of *P. gingivalis,* despite normal phagocytosis. There was no evidence of extracellular killing through two hours (data not shown). These data indicate an absolute dependence on phagocytosis and oxygen for normally

competent neutrophils to kill *P. gingivalis*, and a total resistance of *P. gingivalis* to oxygen-independent killing mechanisms.

Requirement of a Functional NADPH Oxidase for Neutrophil Killing of *P. gingivalis*.

CGD and DPI-Treated Neutrophils. The NADPH oxidase is the principal oxygen-dependent killing pathway of the neutrophil. In order to determine the contribution of this enzyme system in neutrophil killing of *P. gingivalis*, neutrophils from subjects with CGD *vs.* neutrophils from normally healthy donors that were either untreated or treated with DPI were examined using dual color-fluorescence microscopy. Neutrophils from five CGD subjects were included in these studies and their inability to respond with a respiratory burst was confirmed by two different methods. Refer to Supplemental Table A.2 and Methods text in Appendix for detailed descriptions of CGD subjects.

Flow Cytometry. The respiratory burst capacities of neutrophils from the five CGD subjects, compared to normally competent neutrophils from healthy donors (controls), were assessed by flow cytometry (DHR123 method) (Supplemental Table A.3). In PMA-stimulated neutrophil controls, a significant increase in green fluorescence was detected by flow cytometry suggesting normal respiratory burst capacity (control neutrophil oxidative index (NOI) scores ranged from 167-344). In contrast, neutrophils from each of the five CGD subjects were incapable of respiratory burst as detected by negligible fluorescence following PMA-stimulation (CGD NOI scores ranged from 1-6).

Cytochrome C Reduction. The respiratory burst capacities of neutrophils from four of the CGD subjects were also assessed for SOD-inhibitable, cytochrome *c* reduction capacity in response to either PMA or fMLP/cytochalasin b (Supplemental

Table A.3). Consistent with total respiratory burst-deficiency, neutrophils from the CGD subjects tested were completely incapable of superoxide production as determined by an absence of cytochrome *c* reduction, in contrast to the robust responses of controls.

Phagocytosis and Killing of P. gingivalis by CGD Neutrophils. Compared to competent neutrophil controls, CGD neutrophils under aerobic atmosphere were severely compromised in their ability to kill *P. gingivalis*, despite normal phagocytosis (Table 2.2). These data are consistent with the inability of neutrophils to kill *P. gingivalis* under anaerobiosis (Table 2.1), and together indicate that a functional NADPH oxidase is essential for neutrophil killing of *P. gingivalis*.

***Phagocytosis and Killing of P. gingivalis by DPI-Treated Neutrophils*.** DPI treatment of neutrophils inhibits NADPH oxidase-dependent superoxide production (respiratory burst) mimicking the CGD condition (62). DPI at the concentration used in these studies (20μM final concentration (FC)) completely blocked SOD-inhibitable reduction of cytochrome *c* (data not shown), suggesting complete inhibition of respiratory burst superoxide production with DPI. Phagocytosis and killing of opsonized *P. gingivalis* by normally competent neutrophils either untreated (control), DMSO-treated (DPI control), or DPI-treated were tested under aerobic atmosphere (Table 2.3). Untreated neutrophil controls were capable of robust phagocytosis and a clear progression of killing of *P. gingivalis* through three hours. In contrast, treatment of neutrophils with DPI ablated their ability to kill *P. gingivalis* without influencing phagocytosis. Importantly, the DMSO control was capable of normal phagocytosis and killing, comparable to untreated controls – a necessary confirmation of DPI's effect, as DMSO (albeit at much higher concentrations) is a known scavenger of hydroxyl radicals ($OH^{\cdot}$) (63). We therefore hypothesize that the mechanism used by neutrophils to kill *P. gingivalis* is both oxygen- and NADPH oxidase-dependent, and further, because the respiratory burst requires both oxygen and a functional NADPH oxidase, it must be the

products (reactive oxygen species) generated by the respiratory burst that are effective in killing *P. gingivalis*. Which reactive oxygen species most effective in killing *P. gingivalis* was the subject of subsequent studies.

MPO is Unnecessary for Neutrophil Killing of *P. gingivalis*.

MPO-Deficient, Azide- and 4-ABH-Treated Neutrophils. MPO is the most abundant enzyme in neutrophils, and has frequently been described as the dominant bactericidal mechanism of neutrophils (53) (57) (61) (64). In order to determine the contribution of this enzyme system in neutrophil killing of *P. gingivalis*, neutrophils from subjects with MPO-deficiency *vs.* from normally healthy donors that were either untreated or treated with the MPO inhibitors, sodium azide or 4-ABH, were examined. Neutrophils from five subjects with complete or selective deficiencies in MPO were included in these studies and were members of one family. Refer to Methods text in Appendix for detailed descriptions of MPO-deficient subjects.

Transmission Electron Microscopy of Neutrophils from MPO-Deficient Subjects. The granule contents of neutrophils from members of the test family and from gender- and race-matched normal donors (controls) were assessed for MPO by histochemical staining and for lactoferrin by immunogold labeling (Supplemental Figure A.1). Compared to MPO-sufficient controls with 100% of neutrophils showing normal distributions of peroxidase-positive granules (Supplemental Figure A.1A), none of the neutrophils examined from MPO-DS (sister) (Supplemental Figure A.1B, right panel), MPO-DB (brother) or MPO-DF (father) (not shown) contained peroxidase-positive granules, consistent with complete MPO-deficiency. MPO-PDM (mother) (Supplemental Figure A.1B, left panel) showed normal distributions of peroxidase-positive granules in 90% of neutrophils and 10% having no demonstrable peroxidase-positive granules. MPO-PDG (maternal grandmother) (Supplemental Figure A.1C) had

peroxidase staining profiles consistent with partial MPO-deficiency, with approximately 50% of neutrophils having no demonstrable peroxidase-positive granules (right panel) and the remainder appearing normal for peroxidase staining (left panel). All neutrophils from MPO-DS, MPO-DB, MPO-DF, MPO-PDM, and MPO-PDG contained lactoferrin-positive granules (arrows, Supplemental Figure A.1C and data not shown) and a general distribution of granules that were comparable in density to controls.

Phagocytosis and Killing of P. gingivalis by MPO-Deficient Neutrophils. Phagocytosis and killing of *P. gingivalis* under aerobic atmosphere by MPO-deficient neutrophils was indistinguishable from that of normal controls (Table 2.4), indicating that MPO is not required for neutrophil-mediated killing of *P. gingivalis*.

***Influence of Sodium Azide on Neutrophil Killing of P. gingivalis*.** To further explore the potential importance of MPO in neutrophil killing of *P. gingivalis*, neutrophils were treated with the classic MPO inhibitor, sodium azide. In accordance with the literature, we confirmed that azide at the concentration used in these studies (20μM FC) completely abolished MPO enzymatic activity (Supplemental Figure A.2). Additionally, azide treatment did not influence neutrophil phagocytosis of *P. gingivalis* (data not shown), nor did it affect the viability of *P. gingivalis,* as determined by recoverable CFU and propidium iodide (PI) permeability (data not shown). Neutrophil killing of *P. gingivalis* was tested under aerobic atmosphere, with untreated *vs.* azide-treated normal and MPO-deficient neutrophils (Table 2.5). Interestingly, the addition of azide blocked the killing of *P. gingivalis* by both MPO-sufficient and MPO-deficient neutrophils. These data are consistent with the probability that azide is blocking a mechanism – other than MPO – which is essential to the killing of *P. gingivalis*.

***Influence of 4-ABH on Neutrophil Killing of P. gingivalis*.** While azide is indeed a potent MPO inhibitor, it also inhibits other potentially relevant metalloenzymes that contain heme groups (e.g. peroxidases, catalase, superoxide dismutase,

cytochrome oxidases, etc) (65) (66) (67), and is reported to function as a scavenger of hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) (68), and as a potent inhibitor of redox reactions (69) and reactions involving the transition of metals (70). 4-ABH, on the other hand, is reported to be a mechanism-based specific inhibitor of MPO, with no effects on other neutrophil enzymes (71) (72) (73) (74). We confirmed that 4-ABH at the concentration used in these studies (4mM FC) completely abolished MPO enzymatic activity (Supplemental Figure A.2), and had no direct effect on the recovery (CFU) or PI permeability of *P. gingivalis* (data not shown). Unlike the complete ablation of killing observed with azide, the presence of 4-ABH permitted significant, but delayed, killing of *P. gingivalis* by normally competent neutrophils (Table 2.6). While MPO may contribute to the killing of *P. gingivalis*, these data further support that MPO is not a required component for neutrophil killing of this pathogen.

Comparison of the Mechanisms Employed by Neutrophils to Kill *P. gingivalis vs. F. nucleatum* and *A. actinomycetemcomitans*.

DAPI/PI Fluorescence Microscopy of Selected Periodontopathogens. Data from the preceding studies suggest that *P. gingivalis* is completely resistant to neutrophil-mediated non-oxidative mechanisms of killing. Neutrophil killing of *P. gingivalis* was determined to be absolutely dependent on a functional NADPH oxidase, which requires the availability of oxygen; but neutrophil killing of *P. gingivalis* could occur in the absence of functional MPO. The purpose of the next series of experiments was to determine if these observations were unique for *P. gingivalis*.

The micrographs shown in Supplemental Figure A.3 are representative of the data obtained with *P. gingivalis vs. F. nucleatum* and *A. actinomycetemcomitans.* *P. gingivalis* (Supplemental Figures A.3A-C) and *A. actinomycetemcomitans* (not shown) are similar in appearance microscopically, while the fusiform *F. nucleatum*

(Supplemental Figures A.3D-F) is distinguished by its long, spindle-shaped rod morphology and unique knot-like staining of its chromatin. Supplemental Figure A.3A shows four neutrophils at 0 time defined as involved in phagocytosis of *P. gingivalis* (three or more bacteria per neutrophil), with none defined as involved in killing due to the absence of red bacteria (all bacteria were blue). Note: Supplemental Figures A.3B-C and E-F show the same microscopic field; excitation wavelengths permit visualization of both DAPI and PI fluorescence in B and E, whereas narrow band excitation excludes DAPI fluorescence in C and F. Supplemental Figures A.3B-C show four phagocytic neutrophils at 60 min, three of which were involved in killing (three or more red bacteria per neutrophil). Supplemental Figure A.3D shows four neutrophils at 0 time defined as involved in phagocytosis of *F. nucleatum* (three or more bacteria per neutrophil), with none defined as involved in killing (all bacteria were blue), and two neutrophils (arrows) that did not meet the definition of phagocytic. Supplemental Figures A.3E-F show five phagocytic neutrophils at 60 min involved in killing, with the exception of one neutrophil (arrow) that would not have been counted (neutrophil was dead as determined by PI staining of the nucleus).

Influence of Anaerobiosis on Neutrophil Killing, Comparing Select Periodontopathogens. Neutrophil phagocytosis and killing of opsonized *P. gingivalis vs.* opsonized *F. nucleatum* and *A. actinomycetemcomitans* were tested using normally competent neutrophils, under aerobic *vs.* anaerobic atmosphere (Table 2.7). Phagocytosis of *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans* was independent of oxygen, as there were no differences in % phagocytosis under aerobic *vs.* anaerobic conditions at 0 or 90 min. Additionally, there was no evidence of extracellular or serum-mediated killing of *P. gingivalis, F. nucleatum,* or *A. actinomycetemcomitans* through 90 min (data not shown). Despite substantial phagocytosis, there was no evidence of neutrophil killing of any of the test bacteria at

initial time. By 90 min, in the presence of oxygen, however, there was a significant involvement of phagocytic neutrophils in the killing of all three test bacteria. In contrast to the complete ablation of killing that occurs with *P. gingivalis* under anaerobiosis at 90 min, there was a greatly reduced, though significant, % of neutrophils involved in killing *F. nucleatum*; and a reduced, but near normal, % of neutrophils involved in killing *A. actinomycetemcomitans*. These data suggest that *P. gingivalis* is unique among the periodontopathogens tested in its total resistance to neutrophil-mediated non-oxidative mechanisms of killing.

Influence of DPI on Neutrophil Killing, Comparing Select Periodontopathogens. Neutrophil phagocytosis and killing of opsonized *P. gingivalis vs.* opsonized *F. nucleatum* and *A. actinomycetemcomitans* were tested under aerobic atmosphere, using untreated (control) *vs.* DPI-treated normally competent neutrophils (Table 2.8). Untreated neutrophil controls were capable of robust phagocytosis and killing of *P. gingivalis, F. nucleatum,* and *A. actinomycetemcomitans*, with a clear progression through 180 min. Consistent with the anaerobiosis data, in contrast to the complete ablation of killing that occurs with *P. gingivalis* with DPI-treatment of neutrophils over 180 min, there was a greatly reduced, though significant, % of neutrophils involved in killing *F. nucleatum*; and a reduced, but near normal, % of neutrophils involved in killing *A. actinomycetemcomitans*. Importantly, the DMSO control was capable of normal phagocytosis and killing of all three test bacteria, comparable to untreated controls (data not shown). These data are consistent with *P. gingivalis* having a unique relationship with neutrophils. While *F. nucleatum* and *A. actinomycetemcomitans* are susceptible to killing by oxygen-independent, as well as DPI-insensitive mechanisms; *P. gingivalis*, in striking contrast, is completely resistant to killing when DPI-sensitive mechanisms are inhibited.

Influence of 4-ABH on Neutrophil Killing, Comparing Select Periodontopathogens. Neutrophil phagocytosis and killing of opsonized *P. gingivalis vs.* opsonized *F. nucleatum* and *A. actinomycetemcomitans* were tested under aerobic atmosphere, using untreated (control) *vs.* 4-ABH-treated normally competent neutrophils (Figure 2.1). Addition of 4-ABH had no influence on the rate or extent of neutrophil phagocytosis of *P. gingivalis, F. nucleatum,* or *A. actinomycetemcomitans,* but did significantly retard the rate of neutrophil killing of all three test bacteria, compared to untreated controls. For *P. gingivalis* and *F. nucleatum*, the effect of 4-ABH was significant at all time points between 30 min and three hours. The 4-ABH effect on *A. actinomycetemcomitans* only reached significance at the 30 min time point, consistent with its greater susceptibility to non-oxidative killing mechanisms compared with *F. nucleatum* and *P. gingivalis*. These data suggest that MPO or some other yet-to-be-identified 4-ABH-sensitive mechanism contributes to the killing of all three periodontopathogens, but there are highly effective MPO-independent mechanisms utilized by the neutrophil for the killing of all three species.

DISCUSSION

Previous studies in this laboratory using DAPI/PI have shown that *P. gingivalis* is not killed in the absence of phagocytosis, though once phagocytized, neutrophils are readily capable of killing *P. gingivalis* (20). Additionally, prior studies have suggested that several strains of *P. gingivalis* including A7436 exhibit remarkable resistance to neutrophil phagocytosis due in part to the production of a capsule and to the proteolytic degradation of serum opsonins and their receptors (26) (29) (31) (32) (75) (76) (77) (78) (79). High titered antibodies (IgG) to *P. gingivalis* can facilitate phagocytosis not only by specificity for the capsule, but through retardation of the proteolytic destruction of the opsonins C3b, C3bi, and IgG, as well as the complement (CR1 and CR3) and Fc receptors on the neutrophil (20) (35) (80) (81). This is speculated to occur by gingipain-specific antibodies at least temporarily inhibiting (masking) the discrete proteases of *P. gingivalis*, permitting increased surface deposition of opsonic complement and C3-IgG complexes (35) (82) (83). The current study provides further confirmation that neutrophil phagocytosis of *P. gingivalis* strain A7436 requires both complement and high titered specific antibodies, as there was only a small subset of neutrophils capable of phagocytosis in the presence of complement only (Supplemental Table A.1), and absolutely no phagocytosis in the absence of both of these opsonins (data not shown). Furthermore, there was no evidence of extracellular killing under any test condition (data not shown).

In addition to phagocytosis, neutrophil killing of *P. gingivalis* A7436 absolutely requires a functional NADPH oxidase, which is dependent on the availability of oxygen, as there was no killing of *P. gingivalis* by normal neutrophils under anaerobic atmosphere (Table 2.1); by neutrophils from subjects with CGD (Table 2.2); or by normal neutrophils treated with the NADPH oxidase inhibitor, DPI (Table 2.3). In contrast,

normal neutrophils were capable of reduced, though significant, killing of *F. nucleatum*, and of near normal killing of *A. actinomycetemcomitans*, under anaerobiosis (Table 2.7) and in the presence of DPI (Table 2.8). Because even a small amount of contaminating oxygen proved sufficient to allow neutrophil killing of strain A7436 (unpublished observations), all reagents were thoroughly pre-reduced before use in anaerobic experiments, and neutrophil preparations required a minimum of one hour in the anaerobic chamber prior to the addition of opsonized bacteria. These findings extend those of Odell *et al* (84), who previously reported an oxygen requirement for neutrophil-mediated killing of *P. gingivalis* strain W83 (an encapsulated, disseminating strain, like strain A7436).

These data further suggest that *P. gingivalis*, in contrast to select other periodontopathogens, is remarkably resistant to the oxygen-independent killing mechanisms of the neutrophil, which by definition should still be operational under anaerobiosis, in CGD, and in the presence of DPI. Like its mechanism of resistance to phagocytosis (35), the resistance of *P. gingivalis* to the bactericidal activity of serum has been attributed to both the production of capsule and to the proteolytic degradation of complement, which together prevent the membrane attack complex from assembling on and penetrating the bacterial surface (30) (85). Additionally, the resistance of *P. gingivalis* to the non-oxidative killing mechanisms of the neutrophil may also be attributed to the robust proteolytic activities of *P. gingivalis*, since culture supernatants and whole cells of *P. gingivalis* are able to digest and inactivate a multitude of antimicrobial proteins, enzymes, and peptides, including lactoferrin, the cathelicidin LL-37, β-defensins, cathepsin G, elastase, and bacterial-permeability increasing factor (84) (86) (87) (88) (89) (90) (91) (92). Interestingly, Bachrach *et al* (93) suggest that the resistance of *P. gingivalis* strain ATCC 33277 (a non-encapsulated, non-disseminating strain) to direct killing by select antimicrobial peptides, including the neutrophil peptide

LL-37, can be attributed more to the low affinity of these antimicrobial peptides, rather than to the proteolytic activities of *P. gingivalis*.

Because A7436 appeared completely resistant to the oxygen-independent killing mechanisms of the neutrophil, we hypothesize that the success of *P. gingivalis* as a periodontal pathogen may not only be linked with its exceptional resistance to phagocytosis and to extracellular killing, but to its requirement for oxygen (i.e. a respiratory burst) in order to be killed. Despite a large number of neutrophils; reduced oxygen conditions, which prevail in the periodontal pocket (94), likely explain the ability of this strict anaerobe to resist killing (reduces the ability of neutrophils to kill this pathogen), as well as to persist and multiply in active periodontitis. Believed to parallel the anoxic conditions in severe periodontitis, *P. gingivalis* is not killed by neutrophils when atmospheric oxygen levels are reduced due to the inability of these neutrophils to mount a respiratory burst (95). It is further hypothesized that *P. gingivalis* may even benefit from the presence of neutrophils, which would make major contributions to the reduction of oxygen at sites of inflammation, while contributing to the tissue destruction necessary to provide this fastidious, assacharolytic bacterium with a source of peptides and amino acids.

Localized in the azurophilic (primary) granules, MPO is the most abundant enzyme in neutrophils (96) (97), and is proposed to play a central role in oxygen-dependent killing in the phagosome by catalyzing the generation of hypochlorous acid (HOCl) from H_2O_2 ($MPO + Cl^- + H_2O_2 \rightarrow HOCl$) in close proximity to bacteria (59) (64). HOCl is regarded as one of the most potent microbicidal mechanisms of neutrophils because of its reactivity and ability to chlorinate or otherwise modify a wide range of bacterial targets, including enzymes, proteins, lipids, cell walls, membranes, RNA, and DNA (53) (57) (60) (61) (96) (98) (99) (100) (101). Furthermore, since much of the oxygen consumed by an activated neutrophil is converted to H_2O_2 from $O_2^{\cdot -}$ (102) (103),

and since most of the H_2O_2 generated in the neutrophil is reported to be converted to HOCl by MPO (53) (57) (61) (104) (105), MPO is a prime candidate as the dominant oxygen-dependent killing mechanism of the neutrophil. Despite its abundance and perceived importance, however, MPO-deficiency represents the most common form of neutrophil dysfunction in humans, with an incidence of 1 in 2,000 to 4,000 individuals (106), and these subjects do not experience an increased frequency of infections (including periodontitis) and are remarkably healthy (107) (108). In fact, MPO-deficiency is generally considered innocuous and was recently removed as a primary immune deficiency disease (109) (110). Despite the impaired ability of their phagocytes to kill certain targets, such as *Staphylococcus aureus* and *Candida albicans* (59) (111) (112), the only clinical complication reported in these patients is a predisposition to infections with *Candida* spp. in MPO-deficient subjects who also happen to be diabetic (51) (113). However, even the frequency of *Candida* infections is very low (reported in less than 5% of MPO-deficient subjects) (108), raising doubts as to the importance of MPO in host defense (114) (115) (116).

In initial experiments, MPO was found to be dispensable in the killing of A7436, as subjects with MPO-deficiency (partial and complete) were fully capable of killing strain A7436, comparable to MPO-sufficient controls (Table 2.4). The killing of A7436 was next examined by neutrophils treated with the MPO inhibitors, sodium azide and 4-ABH. Neutrophils from normally healthy donors and from MPO-deficient subjects treated with the classic MPO inhibitor, sodium azide, were not capable of killing *P. gingivalis* strain A7436 (Table 2.5), while normal neutrophils treated with the more specific MPO inhibitor, 4-ABH, were still capable of effective, though delayed, killing of *P. gingivalis* (Table 2.6). Comparable to *P. gingivalis*, normal neutrophils treated with 4-ABH were also delayed in their ability to kill *F. nucleatum* and *A. actinomycetemcomitans* (Figure 2.1).

Important to the interpretation of the azide data, however, is the fact that while azide is a highly potent inhibitor of MPO activity (by directly attaching to the coordinate position of the iron in the heme moiety) (117), azide is not specific for MPO and is reported to also inhibit the activity of other enzymes in the neutrophil (65) (66) (67) (118), without affecting respiratory burst or granule release (71) (119). Interestingly, azide is also reported to function as a hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) scavenger (68), as well as a potent inhibitor of redox reactions (69) and reactions involving the transition of metals (70). Therefore, azide may have protected A7436 by functioning as a radical scavenger or by preventing toxicity by H_2O_2. For instance, by binding and bridging transition metals (70), azide may have prevented H_2O_2 from participating in a Fenton Reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H_2O + OH^{\cdot}$), in the absence of competition with MPO for H_2O_2. It is also conceivable that azide may have protected A7436 by shutting it down, since azide has been used to inhibit the metabolism of *P. gingivalis* (120) (121) (122) (123). This is consistent with the concept presented by Imlay *et al* (124), who suggested that active bacterial metabolism is required for killing by H_2O_2. In contrast to azide, 4-ABH is a more specific inhibitor of MPO, with no effects on other neutrophil enzymes (71) (72) (73) (74), so the influence of 4-ABH on neutrophil killing of A7436 is believed to more directly reflect the contribution of MPO in killing. Taken together, the results indicate that while MPO may contribute to the oxidative killing of *P. gingivalis*, this enzyme is not necessary for neutrophil-mediated killing of this pathogen. Furthermore, these data suggest that the main mechanism that normal and MPO-deficient neutrophils use to kill A7436 is an MPO-independent, but azide-sensitive mechanism. Additionally, because neither azide nor 4-ABH affects the respiratory burst or degranulation (71) (119), *P. gingivalis* A7436 is likely killed by a mechanism subsequent to the initial generation of superoxide by the NADPH oxidase.

It is important to note that the MPO-deficient family reported in this study was identified in the course of clinical evaluation of the children, for recurrent pulmonary infections and asthma. It seems unlikely that the MPO-deficiency was the predisposing factor for their clinical symptoms, especially considering that the father (MPO-DF) proved to be MPO-deficient and reported a normal health history. It would be difficult to explain the symptoms of the daughter (MPO-DS) or son (MPO-DB) based on MPO-deficiency, and instead are likely a reflection of a humoral immune deficiency, as their immunoglobulin values were low, they were non-responsive to pneumococcal vaccine, and symptoms responded to gamma globulin therapy (Appendix). Neither the father (MPO-DF) nor the children (MPO-DS and MPO-DB) had detectable MPO in their neutrophils, but were capable of normal killing of *P. gingivalis*, at a level and kinetic that were not discernibly different from controls, as were the neutrophils from the partially-deficient grandmother (MPO-PDG) and mother (MPO-PDM) (Table 2.4).

MPO-deficiency was clearly demonstrated by histochemical intracellular staining of neutrophils visualized by electron microscopy, which provides the additional benefit of assessing functional MPO (i.e. peroxidase activity) within neutrophils (125). This method also detected functionally normal eosinophils in the MPO-deficient subjects, which confirms the findings of Mauch *et al* (109), that MPO-deficient subjects retain functional eosinophil peroxidase. Additionally, none of these MPO-deficient subjects had discernible deficiencies in lactoferrin-containing granules, and the numbers and distribution of non-MPO granules appeared normal (Supplemental Figures A.1B-C).

One possible explanation of the 4-ABH data (reduced kinetics of killing of A7436), in light of the MPO-deficient subject data (normal killing of A7436), is that MPO-deficient subjects may have compensatory killing mechanisms in place in the absence of MPO. For instance, augmented inducible nitric oxide synthase (iNOS) expression and increased NO production observed in MPO-null mice during bacterial sepsis is proposed

to compensate for the lack of HOCl in host defense, in the absence of MPO (126). Another possibility is that H_2O_2 may build up in the absence of MPO to kill directly or through participation in $OH^{\cdot}$ generation (57) (59) (127). This is consistent with the findings of Winterbourn (128) that suggested that $OH^{\cdot}$ generation is more favorable in MPO-deficient neutrophils due to the absence of competition with MPO for H_2O_2. In addition, the neutrophils of MPO-deficient subjects consume more oxygen in the respiratory burst (65) (129), and have extended superoxide and hydrogen peroxide production, compared to MPO-competent neutrophils (64) (130). It has been suggested that the MPO system may inactivate the respiratory burst, and consequently, the absence of MPO may permit a sustained burst (57) (65). Furthermore, it is also possible that increased phagocytosis (131), degranulation (132), and/or enhanced non-oxidative killing in the absence of MPO (99) (133) may compensate in MPO-deficiency. Alternative or upregulated MPO-independent killing mechanisms such as these may therefore account for greater (normal) killing of *P. gingivalis* by MPO-deficient neutrophils, compared to neutrophils treated with 4-ABH, and may explain the low (normal) morbidity in subjects with MPO-deficiency.

In contrast to MPO-deficiency, subjects with CGD present early in life with severe, life-threatening and recurrent opportunistic infections, as well as uncontrolled inflammation associated with granuloma formation. In addition, the microorganisms that are poorly killed by CGD neutrophils *ex vivo*, as well as by neutrophils in anaerobic conditions and in the presence of DPI, are typically the pathogens responsible for the characteristic infections in CGD (57) (62) (111) (134) (135) (136). The significant morbidity associated with infections secondary to CGD may appear as a dramatic demonstration of the importance of the NADPH oxidase for normal host defense, while the lack of correlation between MPO-deficiency and morbidity from infection may imply that MPO is not important to host defense (53). However, another explanation (in

addition to potential compensatory mechanisms operating in MPO-deficiency) is that CGD affects a wider spectrum of cells (eosinophils, monocytes, and neutrophils) than MPO-deficiency (monocytes and neutrophils) (57), and perhaps more importantly, affects the oxidative killing pathway higher up (NADPH oxidase), than MPO-deficiency where the defect is more distal (53). For instance, in CGD, both MPO-catalyzed and MPO-independent oxidative killing mechanisms are absent (137), which likely explains its greater severity. This translates to the inability of phagocytes to generate $O_2^{\cdot-}$ and every downstream ROS dependent on $O_2^{\cdot-}$ generation, including H_2O_2, HOCl, $OH^{\cdot}$, $ONOO^{\cdot-}$, $^1O_2$ etc, compared to MPO-deficiency, where presumably only HOCl is absent. As a result, the isolated pathogens in CGD are organisms believed to be resistant to the oxygen-independent killing mechanisms of the neutrophil, and are typically catalase-positive organisms, capable of destroying their own metabolically-generated H_2O_2 (45). Likewise, the organisms that are killed normally in CGD are believed to be killed either because they are catalase-negative, and thus able to supply a source of H_2O_2 for their own demise (bacterial catalase is believed to deprive phagocytes from using bacterial-generated H_2O_2 for killing), or because non-oxidative killing mechanisms are effective (45) (138) (139).

In North America, the majority of infections in CGD are due to only five microorganisms, which include *S. aureus, Burkholderia cepacia, Serratia marcescens, Norcardia*, and *Aspergillus* (47), in tissues that serve as natural barriers to the environment (skin, lungs, gastrointestinal tract, and the lymph nodes downstream from these organs, with liver, spleen, bone, and brain affected as infections spread) (45) (50). In contrast, subjects with CGD are neither abnormally predisposed to periodontal infections (140) (141) (142) (143) nor to anaerobic bacterial infections in general (141) (144). Curiously, while CGD and DPI-treated neutrophils are incapable of killing both the catalase-positive *S. aureus* (111) and the catalase-negative *P. gingivalis* (Tables 2.2

and 2.3), *S. aureus*, but not *P. gingivalis*, is a definitive pathogen in CGD. Furthermore, because subjects with CGD are characteristically susceptible to *Staphylococcus* infections, we ideally wanted to include *S. aureus* in our neutrophil killing studies along with *P. gingivalis.* However, in preliminary experiments, it was discovered that sub-optimal PI concentrations killed *S. aureus* (as detected by immediate PI uptake and logarithmic reductions in CFU/mL by plating) (Supplemental Table A.4), so it was not possible to include this pathogen in our phagocytosis and killing studies using DAPI/PI.

The observation that catalase-positive, but not catalase-negative microorganisms are infecting agents in CGD, and that subjects with CGD are capable of normal killing of catalase-negative microorganisms (e.g. *Streptococcus* species), but not catalase-positive organisms (e.g. *Staphylococcus* species) (145), led to the hypothesis that CGD neutrophils are compensated in part by H_2O_2, generated by catalase-negative bacteria, which serves as a substrate for the MPO system after phagocytosis (138) (146). This concept has been challenged in mouse models of CGD, which have shown catalase-negative mutants of *Aspergillus* and *S. aureus* to be equally virulent to their catalase-positive counterparts (147) (148), and due to the finding in this model that the H_2O_2 produced by catalase-negative organisms was insufficient to cause significant iodination, a marker for chlorination (147). However, these conclusions are confounded by the fact that CGD mice displayed aberrant inflammatory responses even to sterile hyphae (149), and developed fatal infections with normally avirulent auxotrophs (150).

It therefore appears that the traditionally held theory remains, but that the level of H_2O_2 production may be more important than catalase production, in terms of susceptibility and resistance to killing by CGD neutrophils (59). The recent work of Saito *et al* (151), which showed that H_2O_2-producing strains of *Streptococcus pyogenes* are rapidly killed by CGD neutrophils, whereas H_2O_2-non-producing strains of *S. pyogenes*, which are killed by normal neutrophils, are resistant to killing by CGD

neutrophils; as well as studies with other mutant strains of H_2O_2-producing, catalase-negative organisms (139) (152) (153) (154) also support this concept. Therefore, it is not inconceivable that *P. gingivalis* is not killed by CGD neutrophils because it does not generate H_2O_2. While *P. gingivalis* does not make catalase or peroxidase enzymes to degrade H_2O_2 (155) (156), it does make a protoporphyrin layer from hemoglobin (157) (158), as well as rubrerythrin (159), Dps (DNA binding protein from starved cells) (160), an alkyl hydroperoxide reductase (AhpFC) (161) (162), and OxyR (163) which have all been shown to potentially protect *P. gingivalis* against H_2O_2. It is possible that a strict anaerobic metabolism may safeguard *P. gingivalis,* as it is presumed to not be metabolically active in the presence of oxygen.

The question still remains as to why subjects with CGD, whose neutrophils are incapable of killing *P. gingivalis*, rarely present with periodontal or other anaerobic infections. It is possible that the answer lies in the absence of the respiratory burst itself. For instance, the inability to reduce the microenvironment (i.e. rid oxygen) in CGD, despite its inability to kill, may prevent this strict anaerobe from growing and thus establishing an infection in healthy, aerated periodontal tissues. It is also well-supported that ROS from the respiratory burst are the major contributors to the severe periodontal tissue destruction associated with aggressive periodontitis (141), so the absence of a respiratory burst in CGD may explain the absence of characteristic periodontal tissue damage. This is consistent with the findings of Gronert *et al* (164), which associated locally inflamed sites in subjects with localized aggressive periodontitis with excessive superoxide production. Additionally, this concept is consistent with the findings of Mydel *et al* (165), which found that the host respiratory burst paradoxically improved the survival of *P. gingivalis* in the mouse subcutaneous chamber model by enhancing local and systemic inflammation. Conversely, mice without a respiratory burst survived and were resistant to *P. gingivalis* infection, while mice with an intact

NADPH oxidase succumbed to *P. gingivalis* challenge. Excessive neutrophil-generated inflammation in wild type mice may not only have provided reduced oxygen conditions, but a source of nutrients for the growth of this anaerobic and assacharolytic bacterium, and was shown to be completely responsible for the morbidity and mortality associated with *P. gingivalis* infection. Another explanation which may partially account for the lack of predisposition to periodontitis in subjects with CGD is long-term antibiotic use after diagnosis with CGD. This seems a less likely explanation, however, as patients with other neutrophil disorders appear highly predisposed to developing severe periodontitis, despite aggressive antibiotic regimens (166).

In conclusion, we know that *P. gingivalis* requires phagocytosis and the production of a Respiratory Burst in order to be killed by neutrophils, but which NADPH oxidase-dependent product(s) most important in neutrophil-mediated killing of *P. gingivalis* remained unclear in these studies. While MPO does not appear to be a required component for neutrophil killing of this pathogen; it does, however, appear to play a role by participating in the kinetics of killing *P. gingivalis*. In an attempt to determine which ROS or oxidative mechanism(s) of the neutrophil are most effective in killing *P. gingivalis*, the susceptibility of *P. gingivalis* to MPO-dependent and MPO-independent H_2O_2-mediated killing, in comparison to selected other organisms, was the subject of subsequent studies.

MATERIALS AND METHODS

Bacterial Growth and Storage:

Strain CDC A7436 is a clinical isolate of *P. gingivalis* that was chosen for its expression of multiple virulence traits considered representative of this periodontopathic species. *P. gingivalis* A7436 was originally isolated from a patient with aggressive periodontitis, and was characterized by the late V.R. Dowell (167), and by this laboratory (20) (33) (34) (35) (82), and others (168) (169) (170). Its virulence characteristics have been studied in a variety of animal models (33) (171) (172) (173). A7436 is encapsulated and exhibits a remarkable resistance to phagocytosis (20) (33) (35) (82). In the mouse subcutaneous chamber model, A7436 is capable of spreading and dissemination, producing secondary lesions away from the injection site, septicemia, and death (33) (34) (170). Due to the hydrophilic nature of its extra-cellular polysaccharide layer (capsule), strain A7436 does not auto-aggregate, clump, or associate with neutrophils (75) (81) (174). It is a Gram-negative short rod, β-hemolytic, and readily forms black-pigmentation on blood-based agars.

P. gingivalis was grown at 36^{o}C in a flexible film anaerobic chamber (Coy Laboratory Products Inc, Ann Arbor, MI) in an atmosphere of 85% N_2 – 10% H_2 – 5% CO_2, in 9mL volumes of Wilkins-Chalgren (WC) Anaerobe Broth Medium (Oxoid Ltd, Basingstroke, Hampshire, England) in glass test tubes, without shaking. This broth medium contains tryptone, gelatin peptone, yeast extract, glucose, sodium chloride, L-arginine, sodium pyruvate, menadione, and haemin. For the sake of consistency and to minimize mutation, bacteria were freshly grown from minimally passaged, aliquoted frozen stocks each week before use in the phagocytosis and killing assays. Briefly, 100µL aliquots of *P. gingivalis* (grown in WC to mid-exponential phase or to ~0.5 optical density ($A_{\lambda 660nm}$)) were stored in 1mL glass vials containing 0.5mL sterilized skim milk

(Sigma, St. Louis, MO), at -80^{o}C until use. Individual frozen aliquots were thawed at room temperature and recovered by inoculation into 9mL volumes of non-reduced WC. From this, 3-5 drops (using a sterile plastic transfer pipette) were inoculated into fresh 9mL volumes of non-reduced WC, and from this, 1mL was inoculated into a second 9mL volume of non-reduced WC to make a 10-fold dilution, and placed into the anaerobic incubator for growth. These broth cultures took approximately 60 hr to initially recover from frozen stock in WC. Upon initial visible growth, broth cultures were serially diluted (1 drop and 10-fold from that) into fresh 9mL volumes of reduced WC. Bacteria were harvested from overnight cultures (~18 hr) using the dilution that best fit early- to mid-exponential phase with an $A_{\lambda 660nm}$ of 0.2-0.6. The starting inoculum assured that the test bacteria used in all experiments had undergone a minimum of five doublings. Individual frozen aliquots were also checked for purity by streaking for isolation on both aerobic and anaerobic blood agar plates. An absence of growth on aerobic blood agar plates (TSA agar with 5% sheep blood (PML Microbiologicals, Mississauga, ON, Canada), incubated aerobically at 36^{o}C) and a morphologically pure culture on anaerobic blood agar plates (reducible Brucella blood agar (Anaerobe Systems, Morgan Hill, CA), incubated anaerobically at 36^{o}C) assured purity and was confirmed by Gram stain (agar and broth cultures) and distinct odor.

F. nucleatum strain 1594 and *A. actinomycetemcomitans* strain CDC A7154 (leukotoxin producing, serotype b clinical isolate) were grown and stored in an identical manner to *P. gingivalis*.

Bacterial Labeling with DAPI

One 9mL overnight culture (~18 hr) in early- to mid-exponential phase ($A_{\lambda 660nm}$ of 0.2-0.6) was divided into six 1.7mL polypropylene centrifuge tubes (VWR International, West Chester, PA), and centrifuged for four min at 10,000xg. Supernatants were

discarded, and bacteria were concentrated into one tube by resuspending pellets serially in one 1mL volume (q.s. to 1.5mL) of HBSS (Hanks Balanced Salt Solution, GIBCO, Grand Island, NY) without metals (calcium or magnesium) or phenol red, followed by DAPI (4', 6-diamidino-2-phenylindole, Sigma Chemical Co, St. Louis, MO) labeling (45µL of 7.5µg/mL) for ten min at room temperature, without motion in the dark. This was followed by three washes (four min at 10,000xg) in 1.5mL of fresh HBSS without metals. After the fourth and final spin, the pellet was resuspended in 0.75mL of HBSS with calcium and magnesium (with metals), and adjusted with HBSS with metals to a final $A_{\lambda 660nm}$ =0.2 (approximately 10^8 CFU/mL), using a spectrophotometer (Beckman, Fullerton, CA).

Patient Selection

***CGD Subjects*:** Five CGD subjects included in this study were genetically typed (Supplemental Table A.2), and are characterized in detail in the Supplemental Methods text in the Appendix. Briefly, subjects were confirmed to have no respiratory burst function, as determined by an absence of cytochrome *c* reduction (inability to produce superoxide) and by flow cytometry (inability to produce hydrogen peroxide) (Supplemental Table A.3).

***MPO-Deficient Subjects*:** Five MPO-deficient subjects included in this study were members of one family identified with MPO-deficiency by histochemical staining of granule content (Supplemental Figure A.1), and are characterized in detail in the Supplemental Methods text.

Neutrophil Separation

Standard venipuncture was used to collect approximately 20-30mL of peripheral blood in Vacutainer® collection tubes containing heparin (86 USP units) (BD, Franklin Lakes, NJ), from consenting healthy donors and from patients with CGD or from MPO-

deficiency (or from minors with parental consent). A discontinuous one-step Histopaque (Histopaque, density 1.077, Sigma-Aldrich, St. Louis, MO) and Mono-poly (Mono-poly resolving medium, density 1.114, ICN Biomedicals Inc, Aurora, OH) gradient system described previously (175) was used for the isolation of peripheral blood neutrophils from whole blood. Briefly, Mono-poly (on bottom), Histopaque, and blood (on top) were carefully layered with a 3:1:5 ratio (respectively) in a sterile, 50mL polypropylene conical centrifuge tube. A single centrifugation at 500xg for 40 min at room temperature resulted in the separation of whole blood leukocytes into two distinct leukocyte bands (mononuclear layer and neutrophil layer) above an erythrocyte sediment. The neutrophil layer was carefully collected with a sterile plastic Pasteur pipette, and washed twice with 45mL of HBSS without metals in a sterile, 50mL polypropylene tube, using centrifugation at 400xg for 10 min at room temperature. After the second wash, neutrophils were collected by gently resuspending the pellet in 1mL of HBSS without metals, followed by counting neutrophils with a hemacytometer. Viability (>99%) was assessed by PI exclusion, and purity (>98%) was checked by differential microscopy of Wright-stained cytospin slides. Neutrophils were adjusted to $2x10^7$ cells/mL with HBSS without metals.

Generation of Rabbit Antiserum

High-titered antisera to *P. gingivalis* strain A7436 were produced in New Zealand White female rabbits based on a procedure described by Cutler (82). Aliquots were stored at –80^{o}C until use, and were thawed on ice at time of assay, and used only once.

Human Complement

Normal human serum from a single healthy adult male donor (AB positive) was used as a complement source. This serum had no detectable antibodies to *P.*

gingivalis A7436, as determined by ELISA and by Western blots of outer membrane proteins or of purified lipopolysaccharide. This serum was not opsonic for A7436 with normal human neutrophils at any concentration tested including undiluted, nor was this serum bactericidal for A7436, *F. nucleatum* strain 1594, or *A. actinomycetemcomitans* strain A7154. Unlike with *P. gingivalis* and *A. actinomycetemcomitans*, the opsonic conditions for *F. nucleatum* do not require the addition of an antibody source (just active complement). Aliquots were stored at –80^{o}C until use, and were thawed on ice at time of assay, and used only once.

Localized Juvenile Periodontitis (LJP) Serum

Previous studies in this laboratory have characterized the phagocytosis and killing requirements for *A. actinomycetemcomitans* (176). In addition to active complement, LJP serum (serotype b reactive) is required as an antibody source to not only neutralize *A. actinomycetemcomitans* leukotoxin but to optimize phagocytosis. Sera obtained from LJP subjects were pooled and frozen in aliquots at -80^{o}C until use. Aliquots were thawed on ice at time of assay, and used only once.

Phagocytosis and Killing Assay

The dual-color fluorescence phagocytosis and killing assay using DAPI/PI first described by Kalmar *et al* (176) and modified by Cutler *et al* (20) (82) was used to assess neutrophil phagocytosis and killing of *P. gingivalis*, as well as *F. nucleatum* and *A. actinomycetemcomitans* in select experiments. DAPI is a vital stain, which binds to A/T-rich regions of double-stranded DNA, and forms a highly fluorescent complex (blue color) when bacteria are alive (177). PI is a red fluorescing viability dye, which is excluded by the cytoplasmic membranes of live bacteria and enters the bacteria upon loss of membrane potential, associated with death (178). Using the combination of

DAPI and PI allows monitoring of phagocytosis and killing of bacteria, discrimination of intra- and extra-cellular bacterial killing, and determination of phagocyte death. Two independent observers, each calibrated in the assay, viewed coded slides without the bias of knowing the nature of the samples. At least 100 neutrophils were counted by each observer and averaged to obtain values for % phagocytosis and killing. The final estimated ratio of bacteria to neutrophils was ~20:1.

Phagocytosis was expressed as the percentage of total neutrophils involved in phagocytosis, defined as having three or more bacteria (alive or dead) clearly contained within the neutrophil, confirmed using differential interference contrast optics. Killing was expressed as the percentage of phagocytic neutrophils involved in killing, defined as containing three or more red or dead bacteria. (For examples of data collection, see Supplemental Figure A.3).

For aerobic assays, all reagents were equilibrated to room temperature in ambient atmosphere for at least one hr, and phagocytosis and killing assays were performed in a 37^{o}C Fisher Isotemp dry bath (Fisher Scientific Company, Fairlawn, NJ), without rocking. For anaerobic assays, all reagents were equilibrated to ambient temperature in the anaerobic chamber (atmosphere of 5% CO_2/10% H_2/85% N_2) for at least one hr, and phagocytosis and killing assays were performed in a 37^{o}C incubator inside the anaerobic chamber, without rocking. Efforts were made to fully reduce the reagents used for anaerobic experiments, including neutrophil preparations, because any amount of oxygen introduced into the chamber (e.g. through ice or by acclimation for less than one hr) proved sufficient to allow neutrophil killing of *P. gingivalis*. It also was necessary to refresh the catalyst prior to each experiment. Anaerobiosis of the chamber was assured with methylene blue indicators.

In summary, DAPI-labeled *P. gingivalis* (100µL at $A_{\lambda 660nm}$ =0.2) were opsonized (and gently mixed) with human complement (12.5µL) and hyperimmune rabbit antiserum

(12.5µL), in a 1.7mL polypropylene centrifuge tube (VWR International) for 10 min at 37^{o}C, with rocking. DAPI-labeled *A. actinomycetemcomitans* and *F. nucleatum* were opsonized with the same source of human complement (12.5µL) as *P. gingivalis*, but with either 12.5µL of LJP serum for *A. actinomycetemcomitans*, or with 12.5µL of heat-inactivated (at 56^{o}C for 30 min just prior to use) human complement for *F. nucleatum*. After opsonization, PI (10µL of 7.5µg/mL) and purified neutrophils (125µL at 2x10^{7} cells/mL) were added and gently resuspended, immediately initiating the start of the experiment (time 0). In selected experiments, the classic NADPH oxidase inhibitor, DPI (Sigma; 20µM FC, in less than 0.3mM FC DMSO); the classic MPO inhibitor, sodium azide (Sigma; 20µM FC); or the more specific MPO inhibitor, 4-ABH (Sigma; 4mM FC), was added to neutrophil suspensions (pre-incubated together for one min prior to the addition of PI and opsonized bacteria), in order to examine DPI-, azide-, and 4-ABH-inhibitable killing of test bacteria. At the prescribed intervals, 30µL aliquots were removed (after gently resuspending) and cytospun (Shandon Inc., Sewickley, PA) for 3 min at 850rpm onto pre-cleaned microscope slides (VWR International). Once dried (≤1 min after spin finished), glass cover slips (VWR) were mounted with cyanoacrylate (Krazy Glue™), and slides were examined by epi-illumination UV microscopy with oil immersion (63x) on a Zeiss Axioskop to access phagocytosis and killing by neutrophils. Approximate fluorescence excitation/ emission maxima for DAPI and PI, when bound to DNA, were 358/461 and 535/617, respectively.

Statistical Analysis

As appropriate, the data are expressed as means and standard deviations. Differences in means between normally distributed data sets were tested for significance using the paired Student's t test with confidence set at $p<0.05$.

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Table 2.1. Influence of Anaerobiosis on Neutrophil Killing of Opsonized *P. gingivalis*

	Aerobic Atmosphere		Anaerobic Atmosphere	
Time	**% Phagocytosis**	**% Killing**	**% Phagocytosis**	**% Killing**
0 hr	71 (5)	1 (1)	72 (6)	1 (1)
1 hr	97 (5)	76 (14)	96 (4)	*1 (1)
2 hr	98 (3)	92 (5)	98 (3)	*1 (2)

Results are expressed as the % of total neutrophils involved in phagocytosis (three or more alive or dead bacteria per neutrophil), and the % of phagocytic neutrophils involved in killing (three or more dead bacteria per neutrophil). Experiments were performed at 37^{o}C under aerobic *vs*. anaerobic atmosphere, using normally competent neutrophils from healthy donors. Phagocytosis and killing (%) values are the means from three or more independent experiments, and values in parentheses are the standard deviations of those means. Asterisks indicate that the difference, compared to aerobic controls, is statistically significant ($p<0.05$).

Table 2.2. CGD *vs.* Normal Neutrophil Killing of Opsonized *P. gingivalis* at 1 hr

Subject	**% Phagocytosis**	**% Killing**
CGD 1	97	3
CGD 2	98	2
CGD 3	75	7
CGD 4	90	2
CGD 5	95	0
Controls	96 (2)	73 (12)

Experiments were performed under aerobic atmosphere. Control values are the means from normally competent neutrophils from five different healthy donors, and values in parentheses are the standard deviations of those means. Values for CGD Subjects 1-5 represent data obtained from a single sample.

Table 2.3. Influence of DPI on Neutrophil Killing of Opsonized *P. gingivalis*

	Control		DMSO Control		DPI-Treated	
Time	% Phagocytosis	% Killing	% Phagocytosis	% Killing	% Phagocytosis	% Killing
0 hr	71 (5)	1 (1)	70 (6)	1 (1)	68 (1)	1 (1)
1 hr	97 (5)	76 (14)	97 (5)	80 (6)	97 (5)	*1 (1)
2 hr	98 (3)	92 (5)	99 (4)	87 (9)	99 (2)	*1 (2)
3 hr	100 (1)	98 (3)	100 (1)	97 (3)	99 (1)	*1 (1)

Experiments were performed under aerobic atmosphere using normally competent neutrophils. Neutrophils were either untreated (control) or treated with DPI (20µM FC in less than 0.3mM final FC DMSO). Phagocytosis and killing (%) values are the means from three or more independent experiments, and values in parentheses are the standard deviations of those means. Asterisks indicate that the difference, compared to untreated controls, is statistically significant ($p<0.05$).

Table 2.4. Normal *vs.* MPO-Deficient Neutrophil Killing of Opsonized *P. gingivalis*

	40 min		60 min	
Subject	% Phagocytosis	% Killing	% Phagocytosis	% Killing
Controls	95 (2)	59 (18)	97 (5)	76 (14)
MPO-DS	93	61	99	85
MPO-DB	96	67	98	78
MPO-DF	93	44	97	63
MPO-PDM	96	41	99	66
MPO-PDG	95	68	93	88

Experiments were performed under aerobic atmosphere. Control values are the means from normally competent neutrophils from five different healthy donors, and values in parentheses are the standard deviations of those means. MPO-sufficient controls showed normal distributions of peroxidase-positive granules in 100% of neutrophils. MPO-DS and MPO-DB were siblings whose neutrophils were devoid of MPO-positive granules, as were those of the father, MPO-DF. MPO-PDM was the mother of MPO-DS and MPO-DB, and showed normal distributions of peroxidase-positive granules in 90% of neutrophils, with 10% having no demonstrable peroxidase-positive granules. MPO-PDG was the maternal grandmother with 50% of neutrophils devoid of MPO-positive granules. Values for MPO-deficient subjects represent data obtained from a single sample.

Table 2.5. Influence of Sodium Azide (NaN_3) on Normal *vs.* MPO-Deficient Neutrophil Killing of Opsonized *P. gingivalis*

	40 min		60 min	
	% Killing		% Killing	
Subject	**- NaN_3**	**+ NaN_3**	**- NaN_3**	**+ NaN_3**
Controls	59 (18)	*5 (5)	76 (14)	*7 (8)
MPO-DB	67	7	78	11
MPO-DF	44	3	63	9
MPO-PDG	68	3	88	8
MPO-PDM	41	0	66	6

Experiments were performed under aerobic atmosphere. Neutrophils were either untreated (- NaN_3) or treated with 20µM FC NaN_3 (+ NaN_3). Control values are the means from normally competent neutrophils from four different healthy donors, and values in parentheses are the standard deviations of those means. Asterisks indicate that the difference, compared to untreated controls, is statistically significant ($p<0.05$). Values for MPO-deficient subjects represent data obtained from a single sample.

Table 2.6. Influence of 4-ABH on Neutrophil Killing of Opsonized *P. gingivalis*

	Control		4-ABH Treated	
Time	**% Phagocytosis**	**% Killing**	**% Phagocytosis**	**% Killing**
0 min	71 (5)	1 (3)	72 (5)	1 (1)
30 min	87 (5)	75 (5)	85 (6)	*43 (4)
1 hr	97 (5)	87 (6)	97 (4)	*61 (5)
2 hr	98 (3)	94 (5)	98 (3)	*76 (4)
3 hr	100 (2)	98 (2)	99 (2)	*80 (5)

Experiments were performed under aerobic conditions using normally competent neutrophils. Neutrophils were either untreated (control) or treated with 4-ABH (4mM FC). Phagocytosis and killing (%) values are the means from three or more independent experiments, and values in parentheses are the standard deviations of those means. Asterisks indicate a significant difference from untreated controls ($p<0.05$).

Table 2.7. Influence of Anaerobiosis on Neutrophil Killing of Opsonized *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans*

Time	Atm.	*P. gingivalis*		*F. nucleatum*		*A. actinomycetem-comitans*	
		% Phagocytosis	**% Killing**	**% Phagocytosis**	**% Killing**	**% Phagocytosis**	**% Killing**
0 min	**Aerobic**	71 (4)	1 (1)	70 (6)	1 (1)	80 (8)	2 (1)
	Anaerobic	72 (6)	1 (1)	72 (8)	1 (1)	81 (9)	1 (1)
90 min	**Aerobic**	94 (5)	81 (9)	80 (6)	82 (8)	98 (1)	90 (5)
	Anaerobic	95 (4)	*1 (1)	81 (7)	*10 (1)	99 (1)	*70 (5)

Experiments were performed under aerobic *vs*. anaerobic atmosphere, using normally competent neutrophils. Phagocytosis and killing (%) values are the means from three or more independent experiments, and values in parentheses are the standard deviations of those means. All bacteria were tested at the same time using aliquots of the same neutrophil populations. Asterisks indicate a significant difference compared to aerobic atmosphere. The % killing values of *F. nucleatum* and *A. actinomycetemcomitans* at 90 min under anaerobiosis were significantly different from that of *P. gingivalis* and from each other ($p<0.05$).

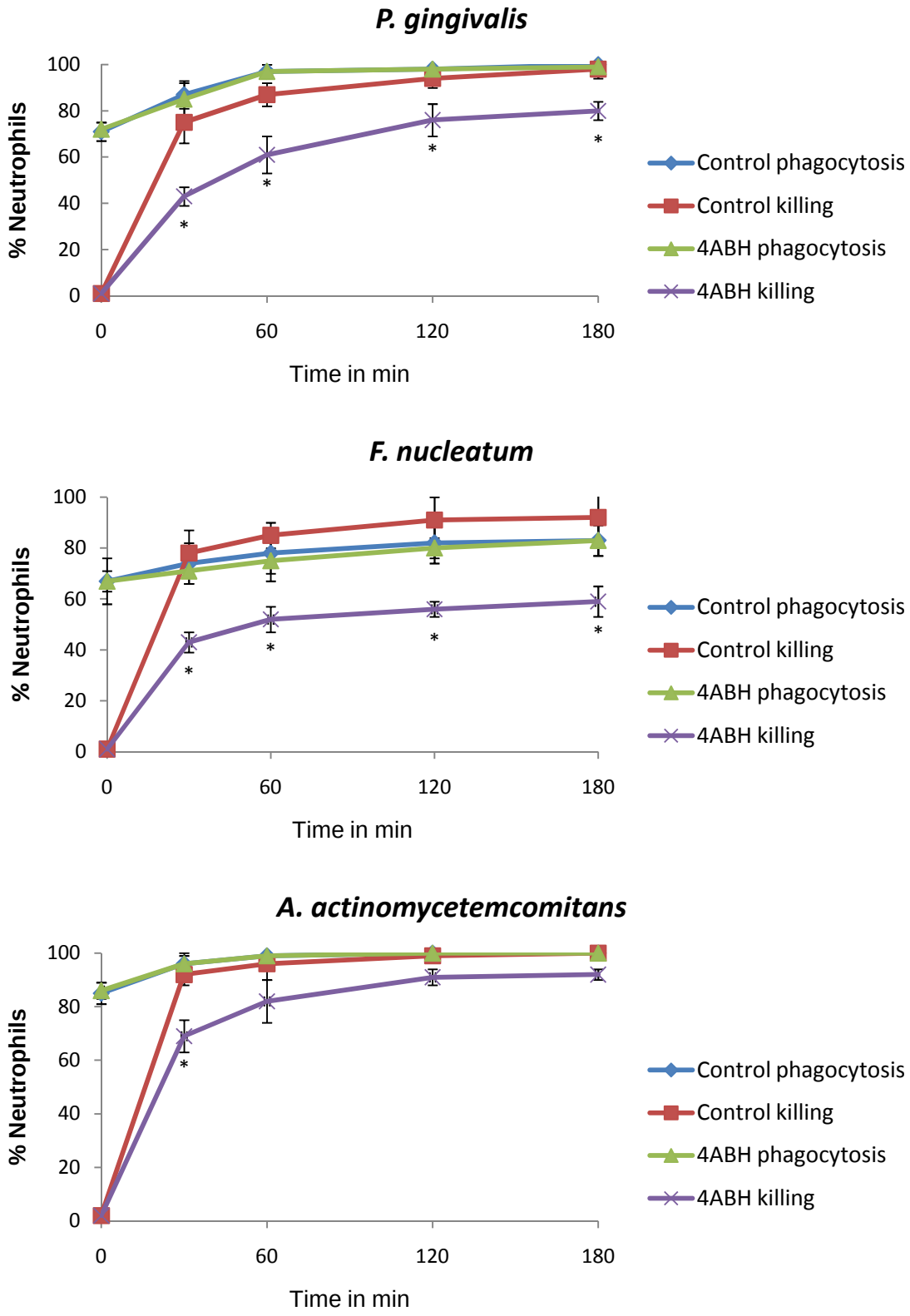
Table 2.8. Influence of DPI on Neutrophil Killing of Opsonized *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans*

Control	***P. gingivalis***		***F. nucleatum***		***A. actinomycetem-comitans***	
Time (min)	**% Phagocytosis**	**% Killing**	**% Phagocytosis**	**% Killing**	**% Phagocytosis**	**% Killing**
0	72 (4)	1 (1)	67 (9)	1 (1)	85 (4)	2 (1)
30	92 (6)	77 (9)	74 (8)	78 (9)	96 (3)	92 (4)
60	97 (3)	85 (5)	78 (8)	85 (5)	99 (1)	96 (6)
120	99 (1)	91 (4)	82 (8)	91 (9)	100 (1)	99 (1)
180	100 (1)	92 (4)	83 (6)	92 (9)	100 (1)	100 (1)

DPI-Treated	***P. gingivalis***		***F. nucleatum***		***A. actinomycetem-comitans***	
Time (min)	**% Phagocytosis**	**% Killing**	**% Phagocytosis**	**% Killing**	**% Phagocytosis**	**% Killing**
0	70 (5)	1 (1)	65 (5)	1 (1)	85 (4)	2 (1)
60	95 (3)	*1 (1)	76 (7)	*13 (4)	98 (2)	*81 (5)
120	98 (1)	*1 (1)	81 (5)	*16 (3)	100 (1)	*90 (4)
180	99 (1)	*1 (1)	82 (6)	*19 (3)	100 (1)	*91 (3)

Experiments were performed under aerobic atmosphere using normally competent neutrophils. Neutrophils were either untreated (control) or treated with 20µM FC DPI. Phagocytosis and killing (%) values are the means from three or more independent experiments, and values in parentheses are the standard deviations of those means. All bacteria were tested at the same time using aliquots of the same neutrophil populations. Asterisks indicate a significant difference from the results obtained from untreated controls at the same time point. The % killing values of *F. nucleatum* and *A. actinomycetemcomitans* with DPI-treated neutrophils were significantly different from that of *P. gingivalis* and from each other at the same time points ($p<0.05$).

Figure 2.1. Influence of 4-ABH on Neutrophil Killing of Opsonized *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans*



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CHAPTER 3

The Relative Susceptibilities of *Porphyromonas gingivalis* to the Reactive Oxygen Species Associated With Neutrophil-Mediated Killing

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Running Title: Relative Susceptibilities of *P. gingivalis* to Principal ROS

ABSTRACT

Porphyromonas gingivalis is an overt periodontal pathogen associated with the etiology of chronic and aggressive forms of adult periodontitis. It was previously shown that human neutrophils require a functional NADPH oxidase to kill *P. gingivalis*. This report examines the relative susceptibilities of *P. gingivalis* to the principal reactive oxygen species associated with NADPH oxidase-dependent, neutrophil-mediated killing. *P. gingivalis* was killed by both the xanthine- xanthine oxidase and glucose- glucose oxidase systems. Killing by the xanthine- xanthine oxidase system was blocked by catalase, but not by superoxide dismutase. When delivered as a bolus, H_2O_2 killed *P. gingivalis* in a time- and dose- dependent fashion. Compared to other strictly anaerobic periodontal pathogens, all strains of *P. gingivalis* independent of capsule or virulence type were consistently more susceptible to low concentrations of H_2O_2. In contrast, aerotolerant pathogens independent of catalase production were highly resistant to H_2O_2. The addition of myeloperoxidase protected *P. gingivalis* and *Fusobacterium nucleatum* against bactericidal concentrations of H_2O_2, but enhanced killing of all test bacteria including *P. gingivalis* and *F. nucleatum* at concentrations between 1 and 10µM H_2O_2, with an optimum at 5µM. The MPO enzymatic inhibitor 4-aminobenzoic acid hydrazide completely abolished this activity. There was no killing by the myeloperoxidase system when H_2O_2 concentrations exceeded 10µM. These data suggest that *P. gingivalis* and possibly other H_2O_2-sensitive bacteria may be killed in the phagosome by H_2O_2 alone, and that phagosomal concentrations of H_2O_2 must be tightly regulated for MPO to function as a bactericidal constituent.

INTRODUCTION

Porphyromonas gingivalis is an overt periodontal pathogen associated with the etiology of chronic and aggressive forms of adult periodontitis (1) (2) (3) (4) (5). *P. gingivalis* is characterized as a strictly anaerobic, Gram-negative short rod, which is β-hemolytic and readily forms black-pigmentation on blood-based agars. Several studies have shown that different isolates of *P. gingivalis* express discrete virulence traits in animal models that may reflect distinct clinical presentations (6) (7) (8) (9) (10) (11) (12). One virulence type in animal infection models is represented by strains including A7436, W83, and W50, which are encapsulated, highly resistant to phagocytosis, hydrophilic and non-clumping, and demonstrate spreading and dissemination from sequestered infection sites. Another virulence type is represented by strains including 381, ATCC 33277, and HG405, which, in contrast, are non-encapsulated, more readily phagocytosed, hydrophobic and auto-aggregating, and demonstrate local persistent inflammation that can progress to abscess formation, but does not disseminate or spread from sequestered infection sites.

Previous studies have suggested that several strains of *P. gingivalis* including A7436 exhibit remarkable resistance to neutrophil phagocytosis due in part to the production of a capsule, but also due to the proteolytic degradation of serum opsonins and their receptors (13) (14) (15) (16) (17) (18) (19) (20) (21) (22). However, phagocytosis-resistant strains of *P. gingivalis* including A7436 are readily phagocytized in the presence of serum containing complement and high titered specific antibody (23) (24). Additionally, *P. gingivalis* is not killed in the absence of phagocytosis, though *P. gingivalis* is readily killed by normal neutrophils once phagocytized (23) (24).

It was previously reported in this laboratory that in addition to phagocytosis, neutrophil killing of *P. gingivalis* A7436 absolutely requires a functional NADPH oxidase,

which is dependent on the availability of oxygen, as there was no killing of *P. gingivalis* by neutrophils from subjects with chronic granulomatous disease (CGD); by normally competent neutrophils treated with the NADPH oxidase inhibitor, diphenyleneiodonium (DPI); or by normally competent neutrophils under anaerobic atmosphere (24). In all three cases, the NADPH oxidase was inhibited, superoxide was not generated, and there was no killing of *P. gingivalis*, despite normal phagocytosis, even after several hours. In contrast, competent neutrophils treated with either anaerobiosis or DPI were capable of near normal killing of *Aggregatibacter actinomycetemcomitans*; and significant, though substantially reduced, killing of *Fusobacterium nucleatum*. Compared to these periodontal pathogens, *P. gingivalis* was remarkably resistant to the non-oxidative killing mechanisms of the neutrophil, which by definition, should still be operational under anaerobiosis, in the presence of DPI, and in CGD; and was instead completely dependent on an NADPH oxidase- dependent mechanism for killing.

Not surprisingly, the pathogens responsible for the characteristic infections in CGD are killed poorly by CGD neutrophils *ex vivo*, as well as by neutrophils under anaerobic conditions and in the presence of DPI (25) (26) (27) (28) (29) (30) (31). Additionally, the common pathogens in CGD are catalase-positive organisms resistant to the non-oxidative killing mechanisms of the neutrophil (32). Conversely, the organisms that are killed normally in CGD are believed to be killed either because non-oxidative killing mechanisms are effective; or because they are catalase-negative, and consequently contribute the missing H_2O_2 to the phagosome environment through their own metabolism (32) (33) (34). Predictably, the neutrophils of subjects with CGD show intact bactericidal activity against catalase-negative organisms such as streptococcal species (31), but not against certain catalase-positive organisms such as *Staphylococcus aureus* (27) (31). Therefore, subjects with CGD show an increased susceptibility to *S. aureus*, but not to streptococcal infections. However, while DPI-

treated, anaerobic, and CGD neutrophils are incapable of killing both the catalase-positive *S. aureus* (27) (29) and the catalase-negative *P. gingivalis* (24); *S. aureus*, but not *P. gingivalis*, is a recognized pathogen in CGD. In fact, subjects with CGD are neither abnormally predisposed to periodontal infections (35) (36) (37) (38), nor to anaerobic bacterial infections in general (36) (39). In contrast, subjects with neutropenia have an increased susceptibility to both *S. aureus* and to certain streptococcal species such as *Streptococcus pyogenes* (40), as well as to a variety of species associated with periodontitis (35) (41) (42) (43), including *F. nucleatum, Prevotella intermedia*, and *P. gingivalis*. Periodontitis is a mixed flora infectious and inflammatory disease, the pathology of which is associated with a relatively small group of specific periodontal pathogens.

While *P. gingivalis* does not make catalase or peroxidase enzymes to degrade H_2O_2 (44) (45), it does make a protoporphyrin layer from hemoglobin, which accounts for its ability to form black-pigmentation on blood-based agars (46) (47), as well as rubrerythrin (48), Dps (DNA binding protein from starved cells) (49), an alkyl hydroperoxide reductase (AhpFC) (50) (51), and OxyR (52), which have all been shown to potentially protect *P. gingivalis* against H_2O_2. *P. gingivalis* also makes superoxide dismutases (MnSOD and FeSOD) (44) (53) (54) (55), which potentially protect *P. gingivalis* against $O_2^{\cdot-}$.

In addition to capsule, which contributes to its resistance to both neutrophil phagocytosis and to serum-mediated killing (22) (56) (57); *P. gingivalis* produces a variety of proteases (e.g. gingipains), which have been shown to digest and inactivate a multitude of antimicrobial peptides, proteins, and enzymes found in neutrophil granules, when combined with culture supernatants or whole cells of *P. gingivalis* (18) (58) (59) (60) (61) (62) (63) (64) (65) (66). While many of these non-oxidative killing mechanisms of the neutrophil are highly cationic and rich in arginine and lysine residues, and would

therefore seem susceptible to digestion by the arginine and lysine gingipains of *P. gingivalis*, its mechanism of resistance to the non-oxidative killing mechanisms of the neutrophils remains unknown. Interestingly, Bachrach *et al* (67) suggested that the resistance of *P. gingivalis* strain ATCC 33277 (a non-encapsulated, non-disseminating strain) to direct killing by select antimicrobial peptides, including the neutrophil peptide LL-37, can be attributed more to the low affinity of these antimicrobial peptides, rather than to the proteolytic activities of *P. gingivalis*.

The principal oxygen-dependent killing pathway of the neutrophil is mediated by the multi-component NADPH oxidase system, which consumes large amounts of oxygen from which it directly generates superoxide anion: $NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\cdot -} + H^+$ (32) (68) (69) (70) (71). Dependent on the fusion of specific (or secondary) granules with the phagosome, the components of the NADPH oxidase are delivered both to the phagosome membrane and to the cytosol. These include two membrane-associated components, $gp91^{phox}$ and $p22^{phox}$, which together comprise the cytochrome b_{558}; as well as three cytosolic components, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$. In order to activate the NADPH oxidase, all five NADPH oxidase subunits ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) with the cytosolic GTPase Rac2 (72) (73) must come together and assemble on the interior surface of the phagosome membrane to form the NADPH oxidase complex. Superoxide subsequently dismutates to hydrogen peroxide: $O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2$, and from these intermediates, other more reactive oxidants are generated, either by reacting with enzyme systems such as myeloperoxidase (MPO) or with bacterial iron, or by the products of these systems reacting with $O_2^{\cdot -}$ and/or H_2O_2, or with each other (25) (73) (74) (75) (76) (77) (78) (79) (80) (81). It is important to note that the oxidative killing mechanisms of the neutrophil do not operate alone, as there are multiple antimicrobial protein constituents and enzymes which also get delivered upon gelatinase, specific, and then azurophilic granule fusion with the phagosome.

While both superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are generally regarded as relatively nontoxic and inert (or only mildly oxidative) (82) (83) (84) (85), their roles in killing microbial pathogens are of particular interest due to the abundant production of enzymes dedicated to the neutralization of these two species, including superoxide dismutases (SOD), catalases, and peroxidases ($O_2^{\cdot-}$ and H_2O_2 scavenging enzymes) in bacteria. Furthermore, the neutrophil responds to phagocytic targets by consuming oxygen in the respiratory burst, which is first converted to $O_2^{\cdot-}$ then to H_2O_2, in close proximity to ingested bacteria. The importance of these species to phagocyte-mediated clearance of potential pathogens is most evident in the severe clinical consequences of their absence in CGD (74) (75) (76) (86) (87) (88). Additionally, considerable evidence suggests that $O_2^{\cdot-}$ and/or H_2O_2 play direct or indirect roles in killing ingested microorganisms by participating as substrates in almost every known killing system in the neutrophil (74) (75) (79) (89). In addition, impaired bactericidal activity in CGD or DPI-treated neutrophils, which are incapable of producing their own $O_2^{\cdot-}$ and H_2O_2, is restored by the addition of a H_2O_2-generating system (90) (91) (92) (93) (94) (95). It was therefore valuable to assess the potential contributions of both superoxide and hydrogen peroxide in the killing of *P. gingivalis* in this study.

The principal enzyme constituent of the neutrophil antimicrobial armament is heme-based MPO, which utilizes H_2O_2 to generate the strong oxidant, weak acid, hypochlorite ($H^+ \, ^-OCl$ or HOCl), also known as bleach: $MPO + Cl^- + H_2O_2 \rightarrow H^+ \, ^-OCl$ (96) (97). Localized in the azurophilic (primary) granules, MPO is the most abundant enzyme in neutrophils (98) (99), and is delivered to the phagosome in high concentration and in close proximity to microbial targets, following the initiation of the respiratory burst by specific granule fusion with the phagosome (73) (79) (100). MPO-generated ^-OCl displays potent microbicidal activities against a broad range of microorganisms, by chlorinating or otherwise modifying a wide range of microbial targets, including enzymes,

proteins, lipids, cell walls, membranes, RNA, and DNA, etc (73) (79) (96) (97) (98) (99) (101) (100) (102) (103). Since much of the oxygen consumed by an activated neutrophil is converted to H_2O_2 from $O_2^{\cdot-}$ (104) (105), and since most of the H_2O_2 generated in the neutrophil is reported to be converted to the bactericidal ^{-}OCl by MPO (25) (73) (79) (106) (107), MPO is proposed to play a central role in oxygen-dependent killing in the phagosome. However, the true contribution of MPO in bactericidal function within the neutrophil has been questioned as MPO-deficient subjects are remarkably healthy and do not experience an increased frequency of infection (108) (109). Yet, in *ex vivo* conditions, neutrophils from subjects with MPO-deficiency demonstrate an impaired ability to kill certain targets, such as *S. aureus* (27) (110) (111) (112) (113). This led to the question as to whether MPO-deficient neutrophils were capable of killing *P. gingivalis.*

While sodium azide totally blocked the killing of phagocytized *P. gingivalis* by MPO-competent neutrophils, neutrophils from subjects with MPO-deficiency were not compromised in their ability to kill *P. gingivalis* (24). Furthermore, in contrast to the total inhibition of killing found with azide, competent neutrophils were capable of highly efficient phagocytic killing of *P. gingivalis* in the presence of the more specific inhibitor, 4-aminobenzoic acid hydrazide (4-ABH) (24). Neither azide nor 4-ABH affects the respiratory burst of stimulated neutrophils (114) (115). These data therefore suggest that the killing of phagocytized *P. gingivalis* is dependent on the reduced oxygen species of a functioning NADPH oxidase system. Further, this killing occurs independent of MPO, but by an azide-sensitive mechanism.

As suggested from preceding studies, *P. gingivalis* A7436 is completely resistant to the non-oxidative killing mechanisms of the neutrophil, and is instead completely dependent on a functional NADPH oxidase for killing (24). However, which reactive oxygen species (ROS) or NADPH oxidase-dependent product(s) central to neutrophil-

mediated killing of *P. gingivalis* remained unclear. The objective of the current study was therefore to examine the susceptibility of *P. gingivalis* to the principal NADPH oxidase-dependent products (ROS) of the neutrophil, in an attempt to determine which oxidative mechanism(s) or ROS are most important to killing *P. gingivalis.* To begin to address this, the killing potentials of the two initial products of the NADPH oxidase, superoxide and hydrogen peroxide, were examined in a cell-free system of killing and in the absence of other neutrophil products such as MPO. The xanthine (X) – xanthine oxidase (XO) system was chosen for the enzymatic generation of both exogenous superoxide and hydrogen peroxide. The individual contributions of these ROS were distinguished by the inclusion of superoxide dismutase (SOD) and/or catalase, and by the glucose (G) – glucose oxidase (GO) system for the generation of hydrogen peroxide free of superoxide. As MPO is a principal constituent of the phagosome environment, the *in vitro* influences of MPO activity on the interactions of H_2O_2 with *P. gingivalis* were also examined.

The distinct effects of anaerobiosis and DPI-treatment on neutrophil-mediated killing of *P. gingivalis* and *F. nucleatum vs*. that of *A. actinomycetemcomitans*, as well as the subtle differences in the ability of neutrophils to kill these three bacteria with 4-ABH-treatment (24), suggested there may be insights to be gained by comparing their relative susceptibilities to the NADPH oxidase-dependent ROS. Likewise, an expansion to other logical pathogens for comparison with *P. gingivalis* was included, in order to create a hierarchical ranking of bacterial susceptibilities to the principal NADPH oxidase-dependent ROS.

S. aureus is a catalase-positive, aerotolerant, Gram-positive bacterium, and is the prototypic pathogen in CGD and an important pathogen in neutropenia. Conversely, *S. pyogenes* is also an aerotolerant, Gram-positive bacterium, but is catalase-negative, and is an important pathogen in neutropenia but is not considered a

pathogen in CGD. These observations suggest that, like *P. gingivalis*, the neutrophil is important to host defense against both of these pathogens, but that the mechanisms employed by the neutrophil to kill each are distinct.

The catalase-negative, anaerobic, Gram-negative, and non-pigmenting *F. nucleatum* was included in the previous studies (24), and was found to be readily phagocytized and killed by neutrophils. Both DPI and anaerobiosis dramatically reduced, but did not eliminate, the killing of this species (24). *P. intermedia,* like *F. nucleatum*, is considered an important member of the mixed anaerobic population that constitutes a periodontopathic flora of the subgingival biofilm (116) (117) (118). *P. intermedia* is closely related to *P. gingivalis* evolutionarily and, like *P. gingivalis*, makes a black-pigmenting, protoporphyrin layer on blood-based media (119), and is a catalase-negative, Gram-negative, strict anaerobe.

A. actinomycetemcomitans is also a Gram-negative periodontal pathogen but, unlike the others, is catalase-positive and capable of growth in the presence of oxygen (120) (121). It is an etiologic agent in certain forms of aggressive periodontitis, where it can be isolated in high numbers in relatively pure form from active disease sites (122). It makes a carbohydrate capsule and produces a leukotoxin that is potent against human neutrophils (123) (124) (125) (126) (127) (128) (129). Susceptibility to *A. actinomycetemcomitans* has been associated with discrete neutrophil dysfunctions (126) (130) (131). In contrast to *P. gingivalis*, *A. actinomycetemcomitans* is readily killed by normally competent neutrophils treated with DPI and by normally competent neutrophils under anaerobic atmosphere (24).

MATERIALS AND METHODS

Bacterial Growth and Storage:

Strain CDC A7436 is a clinical isolate of *P. gingivalis* that was chosen for its expression of multiple virulence traits considered representative of this periodontopathic species. *P. gingivalis* A7436 was originally isolated from a patient with aggressive periodontitis, and was characterized by the late V.R. Dowell (132), and by this laboratory (8) (23) (133) (134) (135), and others (9) (136) (137). Its virulence characteristics have been studied in a variety of animal models (133) (138) (139) (140). A7436 is encapsulated and exhibits a remarkable resistance to phagocytosis (23) (133) (134) (135). In the mouse subcutaneous chamber model, A7436 is capable of spreading and dissemination, producing secondary lesions away from the injection site, septicemia, and death (8) (9) (133). Due to the hydrophilic nature of its extra-cellular polysaccharide layer (capsule), strain A7436 does not auto-aggregate, clump, or associate with neutrophils (11) (13) (141). It is a Gram-negative short rod, β-hemolytic, and readily forms black-pigmentation on blood-based agars.

P. gingivalis was grown at 36^{o}C in a flexible film anaerobic chamber (Coy Laboratory Products Inc, Ann Arbor, MI) in an atmosphere of 85% N_2 – 10% H_2 – 5% CO_2, in 9mL volumes of Wilkins-Chalgren (WC) Anaerobe Broth Medium (Oxoid Ltd, Basingstroke, Hampshire, England) in glass test tubes, without shaking. This broth medium contains tryptone, gelatin peptone, yeast extract, glucose, sodium chloride, L-arginine, sodium pyruvate, menadione, and haemin. For the sake of consistency and to minimize mutation, bacteria were freshly grown from minimally passaged, aliquoted frozen stocks each week before use in the killing assays. Briefly, 100µL aliquots of *P. gingivalis* (grown in WC to mid-exponential phase or to ~0.5 optical density (OD) at $A_{\lambda 660nm}$) were stored in 1mL glass vials containing 0.5mL sterilized skim milk (Sigma, St.

Louis, MO), at -80^{o}C until use. Individual frozen aliquots were thawed at room temperature (RT) and recovered by inoculation into 9mL volumes of non-reduced WC. From this, 3-5 drops (using a sterile plastic transfer pipette) were inoculated into fresh 9mL volumes of non-reduced WC, and from this, 1mL was inoculated into a second 9mL volume of non-reduced WC to make a 10-fold dilution, and placed into the anaerobic incubator for growth. These broth cultures took approximately 60 hr to initially recover from frozen stock in WC. Upon initial visible growth, broth cultures were serially diluted (1 drop and 10-fold from that) into fresh 9mL volumes of reduced WC. Bacteria were harvested from overnight cultures (~18 hr) using the dilution that best fit early- to mid-exponential phase with an $A_{\lambda 660nm}$ of 0.2-0.6. The starting inoculum assured that the test bacteria used in all experiments had undergone a minimum of five doublings. Individual frozen aliquots were also checked for purity by streaking for isolation on both aerobic and anaerobic blood agar plates. An absence of growth on aerobic blood agar plates (TSA agar with 5% sheep blood (PML Microbiologicals, Mississauga, ON, Canada), incubated aerobically at 36^{o}C) and a morphologically pure culture on anaerobic blood agar plates (reducible Brucella blood agar (Anaerobe Systems, Morgan Hill, CA), incubated anaerobically at 36^{o}C) assured purity and was confirmed by Gram stain (agar and broth cultures) and distinct odor.

Prevotella intermedia ATCC 25611, *Fusobacterium nucleatum* 1594, and *Aggregatibacter actinomycetemcomitans* CDC A7154 (leukotoxin-producing, serotype b clinical isolate), as well as *P. gingivalis* strains HG405, FDC 381, and ATCC 33277 used in select experiments, were grown and stored in an identical manner to *P. gingivalis* strain CDC A7436. *Staphylococcus aureus* ATCC 25923 and *Streptococcus pyogenes* ATCC 19615 were grown in WC broth at 36^{o}C in ambient atmosphere.

Cell-free Killing Assays

All killing assays were performed *in vitro*, in cell-free, aerobic conditions, in 1X sterile filtered phosphate buffered saline (PBS) (0.0067 PO_4) without calcium or magnesium (HyClone Laboratories, Thermo Scientific, Logan, Utah). All reagents were freshly prepared just prior to the start of experiments, and were equilibrated to RT in ambient atmosphere before use. Bacteria were prepared last (after reagents) to maximize their viability.

Briefly, bacteria were harvested from ~18 hr overnight cultures (grown either anaerobically or aerobically in WC, depending on species; see above), using the broth culture that best fit early- to mid-exponential phase with an OD of 0.2-0.6 at $A_{\lambda 660nm}$ (see above). All bacteria were adjusted in aerobic conditions with non-reduced WC to a final $A_{\lambda 660nm}$ = 0.1, using a spectrophotometer (Beckman, Fullerton, CA). Bacteria were then serially diluted in PBS (non-reduced) by performing three 10-fold dilutions, and then diluted by one-half after the addition of reagents in experiments. Done this way, the final bacterial density in experiments was consistently between 4.5-5.2 $\log_{10}$ CFU/mL. All killing assays were set up at RT in 1mL final volumes (FV) in 1.7mL polypropylene centrifuge tubes (VWR International, West Chester, PA). Following set-up and initial plating (0 time), tubes were placed in a 37^{o}C Fisher isotemp dry bath (Fisher Scientific Company, Fairlawn, NJ) without shaking, until subsequent time points.

At each time point, samples were briefly vortexed (<1 s) and spiral plated (Spiral Systems, Bethesda, MD) to the surface of non-reduced WC agar (Oxoid Ltd), for growth and bacterial enumeration. The spiral plating system delivers 49µL of liquid sample in an Archimedes spiral pattern onto the surface of a rotating agar plate. The dispensing stylus deposits logarithmically decreasing volumes of sample as it moves from the center of the plate outwards, and because of this unique plating action, samples that normally require several serial dilutions prior to plating can be plated directly without

dilution. After the growth of countable colonies, plates were enumerated by an automatic colony counter with computer software (ProtoCOL, Synbiosis USA, Frederick, MD) specifically programmed for determining spiral plated counts. Results were represented as recoverable $\log_{10}$ CFU/mL. Logarithmic reductions in recoverable CFU/mL were considered significant; i.e. ≥1 log reduction which equated to a ≥90% reduction in CFU/mL was considered significant, while <1 log reduction was not considered significant. The lower limit of detection in samples enumerated by the spiral plate method was 1.31 $\log_{10}$ CFU/mL. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments using overlapping concentrations of test reagents and progressive refinement of the dilutions around the effective concentrations.

After plating, agar surfaces were briefly allowed to dry in ambient air (dried within 5 min of plating), and were quickly placed into the anaerobic chamber incubator (for *P. gingivalis*, *P. intermedia*, *F. nucleatum*, and *A. actinomycetemcomitans*) or aerobic incubator (for *S. aureus* and *S. pyogenes*) for growth. Plates were incubated at 36^{o}C, and recoverable CFU were countable after 4-6 days for *P. gingivalis* and *P. intermedia*; after 2-3 days for *F. nucleatum*, *A. actinomycetemcomitans*, and *S. pyogenes*; and after 1 day for *S. aureus*. It is noteworthy that various conditions (e.g. low humidity or mild oxygen contamination) impacted the recovery time of CFU of the strict anaerobes such as *P. gingivalis* in experiments. Inhibitory concentrations of hydrogen peroxide with any bacterial species often resulted in recoverable CFU that were smaller or took longer to grow than controls.

Xanthine (X) – Xanthine Oxidase (XO) Killing Assays

The xanthine (X) – xanthine oxidase (XO) system was used to enzymatically generate exogenous superoxide and/or hydrogen peroxide in a cell-free system of killing

(Figure 3.1). Xanthine is a catabolic product of purine nucleotide metabolism, and generates both superoxide and hydrogen peroxide when used as the substrate for XO (by definition, 1U of XO will convert 1µmole of xanthine to uric acid/min at pH 7.5 at 25^{o}C). Since urate is a byproduct of the X-XO system, it remained present in the assay +/- superoxide dismutase (SOD) and catalase, but was not found to contribute to either protection or enhancement of killing. Xanthine (Sigma-Aldrich, St. Louis, MO) was freshly prepared as a 100mM stock in 1M NaOH, and then serially diluted in PBS and added to experiments at a final concentration (FC) of 100µM. XO (Sigma, from buttermilk) was freshly prepared as a 1U/mL stock in PBS, and then diluted in PBS and added to experiments at a FC of 15mU/mL. SOD (Boehringer Mannheim, Mannheim, Germany, from bovine erythrocytes) was used to eliminate superoxide in the X-XO system, by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and oxygen. SOD was freshly prepared as a 1mg/mL stock in PBS, and added to experiments at a FC of 0.1mg/mL. Catalase (Sigma, from bovine liver) was used to eliminate hydrogen peroxide in the X-XO system, by converting hydrogen peroxide to oxygen and water (by definition, 1U of catalase decomposes 1µmole H_2O_2/min at pH 7 at 25^{o}C). Catalase was freshly prepared as a 5,000U/mL stock in PBS, and then diluted in PBS and added to experiments at a FC of 125mU/mL.

Preliminary experiments that titered xanthine and XO led to the selection of 100µM xanthine and 15mU/mL XO as optimal for use in these experiments. 125mU/mL catalase and 0.1mg/mL SOD were also chosen as optimal in preliminary assays. Reagents were added quickly with consistent timing to reaction tubes, with brief vortexing (<1 s) after each addition. In order to set up the X-XO system in favor of radical generation (or inhibition of discrete ROS) before challenge with *P. gingivalis*, the following order of addition was used: 1st: 100µL of XO, 2nd: 100µL of catalase (or 100µL of PBS), 3rd: 100µL of SOD (or 100µL of PBS), 4th: 100µL of xanthine, 5th: 600µL of

bacteria (final bacterial density per tube approximately 4.5 $\log_{10}$ CFU/mL in 1mL FV). After all reagents were combined, vortexing following the addition of bacteria started the experiment (0 time). All samples were spiral plated.

Cytochrome *C* Reduction/ Oxidation Assay

The ability of SOD and catalase to eliminate superoxide and hydrogen peroxide (respectively) in the X-XO system was confirmed by cytochrome *c* oxidation in the presence of SOD, and cytochrome *c* reduction in the presence of catalase (data not shown), using a microtiter spectrophotometric assay, based on the SOD-inhibitable reduction of cytochrome *c* used to measure neutrophil oxidative burst function (superoxide production) upon stimulation with PMA (132) (142). Briefly, cytochrome *c* reduction/ oxidation were assessed by measuring the change in OD at $A_{\lambda 550nm}$ every 10 s for 30 min in a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA) at RT. Agitation was applied immediately prior to each reading using the auto-mixing feature of the instrument. The microplate reader was connected to a PC with Softmax software (Molecular Devices) allowing Vmax estimates, OD change measurement, and curve fittings. Cytochrome *c* (Sigma), SOD, catalase, xanthine, and XO were freshly made using PBS (see above methods), and test wells contained a FC of 0.11μM cytochrome *c*, 125mU/mL catalase or 0.1mg/mL SOD, 100μM xanthine, and 15mU/mL XO, in a FV of 100μL. A positive slope correlated with cytochrome *c* reduction by superoxide in the presence of X-XO + CAT, while a negative slope correlated with cytochrome *c* oxidation by hydrogen peroxide in the presence of X-XO + SOD. An absence of cytochrome *c* reduction or oxidation, as demonstrated by a Vmax of zero, and a negligible OD change over 30 min, was observed in samples with X-XO + SOD and CAT.

Glucose (G) – Glucose Oxidase (GO) Killing Assays

The glucose (G) – glucose oxidase (GO) system was used to enzymatically generate exogenous hydrogen peroxide in a cell-free system of killing (Figure 3.2). When β-D-glucose is used as the substrate for GO, GO catalyzes the oxidation of β-D-glucose to D-gluconolactone and hydrogen peroxide (by definition, 1U of GO will oxidize 1μM of β-D-glucose to D-gluconolactone and hydrogen peroxide/min at pH 5.1 at 35^{o}C). GO (Sigma, from *Aspergillus niger*, type VII) was freshly prepared as a 1,000U/mL stock in PBS, and then titered in experiments using PBS so that the FC of GO started at 1U/mL, and decreased 10-fold from this. β-D-glucose (EM Science, Gibbstown, NJ) was freshly prepared in PBS and added to experiments at a FC of 160mM, which allowed glucose to be in excess and GO to be the limiting reagent upon titration.

Reagents were added quickly with consistent timing to reaction tubes, with brief vortexing (<1 s) after each addition. In order to set up the G-GO system in favor of hydrogen peroxide generation before challenge with *P. gingivalis*, the following order of addition was used: 1st: 250μL of GO (or 250μL of PBS), 2nd: 250μL of glucose (or 250μL of PBS), 3rd: 500μL of bacteria (final bacterial density per tube approximately 4.5 $\log_{10}$ CFU/mL in 1mL FV). After all reagents were combined, vortexing following the addition of bacteria started the experiment (0 time). All samples were spiral plated.

Hydrogen Peroxide (H_2O_2) Killing Assays

H_2O_2 was delivered as a bolus (non-enzymatic delivery of H_2O_2) to *P. gingivalis*, in order to determine the killing kinetics of *P. gingivalis* with H_2O_2 over time (Figure 3.3); and to *P. intermedia*, *F. nucleatum*, *A. actinomycetemcomitans*, *S. aureus*, and to *S. pyogenes* (Figure 3.4), in order to determine how the susceptibility of *P. gingivalis* to H_2O_2 compares with these selected bacteria. Dilutions of H_2O_2 were freshly prepared in PBS before each experiment from a 30% w/w stock solution (Sigma), and molar

concentrations (c) were determined using the Beer's Law formula, A = cbε, where A was the OD of the H_2O_2 dilution in PBS at $A_{\lambda 230nm}$ in a quartz cuvette, ε was an 81 $cm^{-1}M^{-1}$ extinction coefficient, and b equaled 1. Known concentrations of H_2O_2 were then titered in PBS and are reported as approximate FC. 500µL of bacteria (final bacterial density approximately 5.0 $\log_{10}$ CFU/mL) were added to 500µL of hydrogen peroxide (or 500µL of PBS), and samples were spiral plated (see above) at specific time points in order to discern killing curves. Initial experiments tested the killing activity of ten-fold dilutions of H_2O_2. Subsequent experiments progressed from five-fold and then two-fold dilutions around the effective concentration of the preceding experiment to refine the titration.

Myeloperoxidase (MPO) – Hydrogen Peroxide (H_2O_2) Killing Assays

The MPO-H_2O_2 system was used to enzymatically generate exogenous hypochlorous acid (HOCl or bleach) in a cell-free system of killing (Figures 3.5 and 3.6). Human neutrophil MPO (Athens Research and Technology Inc, Athens, GA) was prepared in 50mM sodium acetate pH 6 + 100mM NaCl as a 20,000mU/mL stock, and frozen as 30µL aliquots at -20°C until use. MPO was thawed on ice and dilutions were prepared in PBS just before experiments. Dilutions of H_2O_2 were also freshly prepared in PBS from a 30% w/w H_2O_2 stock solution (Sigma), and molar concentrations were determined spectrophotometrically using the Beer's Law formula (see above). The source of chloride in the MPO-H_2O_2 system of generating HOCl ($Cl^- + H_2O_2 + H^+ + MPO \rightarrow HOCl + H_2O$) was derived from PBS (HyClone Laboratories, Thermo Scientific), which contained 0.15M NaCl. This concentration provided an excess of chloride, and as a result, chloride was not a rate-limiting reagent in the MPO-H_2O_2 system. In order to set up the MPO- H_2O_2 system in favor of HOCl generation before challenge with bacteria, MPO and H_2O_2 were incubated together for exactly 20 s before the addition of bacteria. Hence, reagents were added in the following order, with brief vortexing (<1 s) after each

addition: 1st: 250µL of MPO (or 250µL of PBS), 2nd: 250µL of H_2O_2 (or 250µL of PBS), 3rd: 500µL of bacteria (final bacterial density approximately 4.5 - 5.0 $\log_{10}$ CFU/mL). After all reagents were combined, vortexing following the addition of bacteria started the experiment (0 time). All samples were spiral plated.

In 4-aminobenzoic acid hydrazide (4-ABH) studies (Figure 3.6), the effect of 4-ABH (an inhibitor of MPO enzymatic activity) on bacterial killing by the MPO- H_2O_2 system was examined. 4-ABH (Sigma) was prepared as a 100mM stock in 10mM HCl, and then diluted in PBS and added at a FC of 4mM (chosen as an optimal concentration in preliminary experiments to inhibit MPO peroxidatic activity). In order to favor the activity of 4-ABH, MPO and 4-ABH were incubated together for exactly 10 s before the addition of H_2O_2. Next, in order to set up the MPO- H_2O_2 system in favor of HOCl generation before bacterial challenge, H_2O_2 plus MPO +/- 4-ABH were incubated together for an additional 20 s before the addition of bacteria. Hence, reagents were added in the following order, with brief vortexing (<1 s) after each addition: 1st: 225µL of MPO (or 225µL of PBS), 2nd: 25µL of 4-ABH (or 25µL of PBS), 3rd: 250µL of H_2O_2 (or 250µL of PBS), 3rd: 500µL of bacteria (final bacterial density approximately 4.5 - 5.0 $\log_{10}$ CFU/mL). After all reagents were combined, vortexing following the addition of bacteria started the experiment (0 time). All samples were spiral plated.

RESULTS

Catalase, but not SOD, blocks X-XO Killing of *P. gingivalis*

The X-XO system was used to enzymatically generate both exogenous superoxide and hydrogen peroxide in a cell-free system of killing (Figure 3.1). Catalase (CAT) and/or superoxide dismutase (SOD) were used with this system, in order to determine the susceptibility of *P. gingivalis* to these individual ROS. The X-XO system is proposed to resemble the NADPH oxidase in the phagosome, by generating superoxide first, which subsequently dismutates to hydrogen peroxide (143) (144). Additionally analogous, the X-XO system enzymatically generates these species in a continual or sustained fashion (provided there is enough substrate), as opposed to delivering them all at once to bacteria, as a bolus. Non-recoverable, logarithmic reductions in CFU/mL were interpreted as killing.

In order to examine the effect of both superoxide and hydrogen peroxide generation on *P. gingivalis*, A7436 was challenged with X (100µM FC) and XO (15mU/mL FC), and killing was compared to controls, which contained no X or XO. Significant killing did not occur in the control (no X or XO) over the two hour test period. However, X-XO killed *P. gingivalis* in a time-dependent manner: Killing did not occur at 0 time, though significant killing had occurred by one hour (>2 log reductions or >99% reduction in $\log_{10}$ CFU/mL), with additional and complete killing (no detectable CFU) by two hours. Sensitivity to X-XO suggested that *P. gingivalis* is susceptible to any or all of the components generated by this system (superoxide, hydrogen peroxide, and urate). However, the addition of SOD + CAT to X-XO (which eliminated both superoxide and hydrogen peroxide, but not urate) resulted in complete protection from killing, comparable to controls. Additionally, no killing occurred in controls with SOD alone (without X or XO) or CAT alone (without X or XO), or in controls with SOD + CAT alone

(without X or XO). These data support that the killing which occurred in the X-XO system is due to the ROS generated by the X-XO system: superoxide and/or hydrogen peroxide, but not urate.

In order to discern the susceptibility of *P. gingivalis* to superoxide individually, A7436 was challenged with X-XO plus CAT. Under these conditions, superoxide was generated as determined by cytochrome *c* reduction, and there was no discernible killing of A7436. In contrast, when challenged with X-XO plus SOD, A7436 killing was equivalent to that of X-XO alone. Under these conditions, hydrogen peroxide was generated as determined by cytochrome *c* oxidation. These data indicate that hydrogen peroxide alone can kill A7436, and that superoxide under the conditions of these experiments has no effect on *P. gingivalis* A7436.

P. gingivalis **is Killed by G-GO**

The G-GO system was next used to enzymatically generate exogenous hydrogen peroxide in a cell-free system of killing (Figure 3.2). The G-GO system also generates hydrogen peroxide in a sustained fashion (145), but unlike X-XO and the NADPH oxidase, the G-GO system does not generate superoxide first, but directly generates hydrogen peroxide. GO was titered in the presence of excess glucose (FC of 160mM), which varied the rate of hydrogen peroxide generation.

The G-GO system killed *P. gingivalis* A7436 in a time- and rate- dependent manner. There was no evidence of killing at the initial plating (0 time) with any of the GO concentrations tested. There was no killing through two hours with 100µU/mL GO (0.0001U/mL GO), but a ten-fold increase in GO (0.001U/mL GO) resulted in killing by one hour and total killing by two hours. Higher concentrations of GO (≥0.01U/mL GO) resulted in total killing by one hour. This GO-dependent killing of *P. gingivalis* A7436 was completely blocked by the addition of 125mU/mL catalase (data not shown), which

supports that the killing which occurred in the G-GO system is due to hydrogen peroxide alone.

Killing of *P. gingivalis* by H_2O_2 Requires Time

Data from the X-XO (Figure 3.1) and G-GO systems (Figure 3.2) suggest that *P. gingivalis* is susceptible to hydrogen peroxide without exogenous co-factors, and that superoxide has no influence on A7436 under these conditions. Furthermore, the effect of GO titration suggests there is a threshold concentration of H_2O_2 necessary to kill *P. gingivalis*. The fact that there was no detectable reduction in recoverable CFU/mL at the initial time point, regardless of the concentration of GO tested, also suggests a time-dependent kinetic of killing by H_2O_2. In order to validate the H_2O_2-sensitivity of *P. gingivalis* and to address the killing kinetic of *P. gingivalis* by H_2O_2, the bactericidal activity of H_2O_2 delivered as a bolus (non-enzymatic delivery of H_2O_2) was next examined.

In initial experiments, the bactericidal activity of H_2O_2 on *P. gingivalis* A7436 was titrated from 10mM H_2O_2 using ten-fold dilutions to determine approximate sensitivity (data not shown). Figure 3.3 represents the data from a two-fold titration from 100µM H_2O_2, which was spiral plated at select times through three hours. As suggested in Figure 3.3, H_2O_2, delivered as a bolus, killed *P. gingivalis* in a time-and dose-dependent fashion. There was no reduction in recoverable CFU/mL upon initial plating, despite concentrations of H_2O_2 as high as 10mM (data not shown). This suggests that the process of plating neutralizes any additional bactericidal activity of H_2O_2 that may have occurred on the plate, and that the killing events occur in the reaction volume only. Additionally, there was a concentration-dependent, logarithmic killing progression over the three hour test period with all H_2O_2 concentrations tested, including 6.25µM H_2O_2.

P. gingivalis is Remarkably Susceptible to H_2O_2, compared with Select Pathogens

These data indicate that low, micromolar concentrations of H_2O_2 are capable of killing *P. gingivalis* in the absence of exogenous co-factors. The next series of experiments were designed to determine if this H_2O_2-sensitivity is unique to this strain of *P. gingivalis* and/or to this species. In Figure 3.4, the sensitivity of *P. gingivalis* to H_2O_2 was compared with other periodontal pathogens, including *P. intermedia*, *F. nucleatum*, and *A. actinomycetemcomitans*, as well as with other important human pathogens associated with neutrophil dysfunctions, including *S. aureus* and *S. pyogenes*. Four *P. gingivalis* strains (A7436, HG405, 381, and 33277) were also included. The killing of all test bacteria by H_2O_2 was a time-dependent event (data not shown). At 60 min, there were logarithmic reductions in recoverable CFU/mL of all four *P. gingivalis* strains with 20µM H_2O_2 (Figures 3.4A and B). At 60 min, *P. intermedia* required 100µM H_2O_2 (Figure 3.4B), while *F. nucleatum* required >300µM H_2O_2 (Figure 3.4A), in order to achieve logarithmic reductions. In contrast, the three species that are capable of growing in an aerobic environment, *A. actinomycetemcomitans, S. aureus,* and *S. pyogenes,* required >1,250µM, >2,500µM, and >5,000µM H_2O_2, respectively, to achieve logarithmic reductions at 60 min (Figures 3.4A and B).

Influence of MPO on the Bactericidal Activity of H_2O_2 with *P. gingivalis* and *F. nucleatum*

In initial experiments to determine the influence of MPO on the bactericidal activity of H_2O_2, H_2O_2 was titrated using two-fold dilutions from 1,600µM, in the presence of 2mU/mL MPO with both *P. gingivalis* A7436 (data not shown) and *F. nucleatum* (Figure 3.5A). This concentration of MPO totally protected both bacteria against the bactericidal activity of even the highest concentrations of H_2O_2. In subsequent experiments, H_2O_2 was titrated from 40µM with increased concentrations of MPO

(Figures 3.5B and C). In Figure 3.5B, again the bactericidal activity of H_2O_2 against *P. gingivalis* was eliminated by the addition of MPO. However, at sub-lethal concentrations of H_2O_2, there was a dose-dependent killing of *P. gingivalis* in the presence of 12mU/mL MPO (Figure 3.5B). This killing activity peaked at 5µM H_2O_2, lessened at 10µM H_2O_2, and was lost with greater H_2O_2 concentrations (Figure 3.5B). Over the same range of H_2O_2 concentrations, the MPO system resulted in a strikingly similar pattern of killing with *F. nucleatum* (Figure 3.5C), despite its logarithmically greater resistance to MPO-independent killing by H_2O_2.

Relative Sensitivities of the different target bacteria to the MPO System

These data are consistent with a bactericidal mechanism involving the generation of HOCl by the peroxidatic activity of MPO, which occurs over a discrete range of H_2O_2 concentrations from 0.6µM to 10µM, with an optimum at 5µM H_2O_2. Also, this bactericidal activity and/or the peroxidatic generation of HOCl by MPO appears to be a time-dependent event (Figures 3.5B and C). Higher concentrations of H_2O_2 apparently interfere with this peroxidatic activity, and the presence of MPO limits the activity and/or availability of bactericidal concentrations of H_2O_2 (Figure 3.5).

The next series of experiments (Figure 3.6) was designed to compare the relative patterns of sensitivities to MPO-dependent killing of H_2O_2-resistant species compared to that of the more H_2O_2-sensitive species. Each of the six target bacteria were treated with a titration of H_2O_2 through 5µM, in the presence of MPO ranging in concentration from 0 to 24mU/mL. As can be seen, there was a variable requirement of the MPO concentration required to enhance killing by H_2O_2, depending on the target bacterium. The two catalase-producing species (Figures 3.6D and E) required the highest concentration of MPO (12-24mU/mL) to achieve logarithmic killing by the system; whereas *F. nucleatum* (Figure 3.6C) showed logarithmic killing with the lowest

concentration/ activity of MPO (3-6mU/mL). Surprisingly, all of the test bacteria were sensitive to the same optimum concentration of H_2O_2, provided there was sufficient MPO. All killing activity was inhibited by 4-ABH (Figure 3.6), which is a potent, mechanism-based inhibitor of MPO peroxidatic activity (146). The ablation of killing with 4-ABH supports that the killing in this system is due to the peroxidatic generation of HOCl by MPO. Neither sub-optimal nor optimal MPO concentrations alone, or 4-ABH (at 4mM FC) alone, demonstrated any bactericidal activity with any of the species tested (Figure 3.6).

DISCUSSION

Since human neutrophils require a functional NADPH oxidase in order to kill phagocytosed *P. gingivalis* (24), it was important to examine the roles of the principal products of the NADPH oxidase ($O_2^{\cdot-}$, H_2O_2, and MPO-generated ^{-}OCl) in this killing process. In preliminary experiments, XO was titrated in the presence of excess xanthine in PBS to screen for any bactericidal activity against *P. gingivalis* and to determine a concentration that yielded a definable killing kinetic and was capable of SOD-inhibitable reduction and catalase-inhibitable oxidation of cytochrome *c* (data not shown). The XO enzyme system was found capable of potent killing of *P. gingivalis* with an activity yielded by 15mU/mL in a reaction mixture containing 100μM xanthine. This combination resulted in significant, but not total, killing at one hour (Figure 3.1). This activity would be estimated to generate approximately 30nmoles/min of $O_2^{\cdot-}/H_2O_2$, for a total of 1.8μmoles over the initial one hour treatment period. These data suggested that either, both, or the combination of $O_2^{\cdot-}$ and H_2O_2, without the possible co-factors of the phagosome environment, was capable of killing *P. gingivalis*. Catalase, but not SOD, was capable of total protection of *P. gingivalis* against this bactericidal activity, suggesting that H_2O_2 was solely responsible for killing (Figure 3.1).

The catalase-inhibitable bactericidal activity of the GO system provided further evidence for the direct bactericidal activity of H_2O_2 against *P. gingivalis* in the absence of $O_2^{\cdot-}$ (Figure 3.2). The titration of GO in the presence of excess glucose in PBS yielded total killing by one hour with an activity of 0.01U/mL; while an activity of 0.001U/mL GO yielded a less than one log reduction in recoverable CFU/mL by one hour, but total killing after two hours of treatment. This is consistent with a bactericidal rate of H_2O_2 generation of between 10nmoles and 100nmoles/min, strikingly similar to the 30nmoles/min estimated with XO. These data clearly implicate H_2O_2 as the sole active

agent in both enzyme systems. The absence of demonstrable bactericidal activity by $O_2^{\cdot-}$ with the XO system would be consistent with its lack of diffusibility across a membrane (79) (82). In support of inaccessibility to lethal targets, unpublished studies in this laboratory found that *P. gingivalis* was killed by paraquat, a toxic agent thought to act via the endogenous generation of $O_2^{\cdot-}$ within a cell (147). *P. gingivalis* sensitivity to H_2O_2 but not to $O_2^{\cdot-}$ is also consistent with *P. gingivalis* possessing SOD (MnSOD and FeSOD), but not catalase or peroxidase enzymes (45). It is also possible that the rate of exogenous $O_2^{\cdot-}$ generated under the conditions employed was insufficient for killing.

Treatment of *P. gingivalis* with a single bolus of discrete concentrations of H_2O_2, as opposed to the enzymatic generation of uniformly increasing concentrations, permitted discrimination of the rate of H_2O_2 killing, from that of the enzyme generating systems (Figure 3.3). From these studies, it is clear that there is a dose-dependent progression of killing by H_2O_2. There was no evidence of killing at the initial time point with any of the concentrations tested, including much higher levels (data not shown). With the highest treatment doses, logarithmic killing was evident within 30 min; and with the lower concentrations, killing continued through three hours. These data collectively suggest that the effects of a bolus of H_2O_2 are continuous through three hours and are not neutralized in the reaction volume. In contrast, the conditions associated with spiral plating do appear to block the progression of killing on a plate, permitting colony formation with ultimately lethal concentrations of H_2O_2, possibly through repair of damage. There was evidence of delayed appearance (requiring longer incubation times) and smaller colonies with the intermediate doses (data not shown), which may reflect such repair activities.

Interestingly, to achieve the same level of killing in the same period of time (60 min) as that with the enzyme- generated systems, a bolus of H_2O_2 at a concentration between 25 and 50µmoles/mL was required (Figure 3.3) *vs*. the 30nmoles estimated to

be generated in the first minute and the 1,800nmoles generated in the first hour in the XO system (Figure 3.1). This would suggest that a sustained rate of H_2O_2 generation, as would be found in the phagosome, would be highly efficient at killing.

The level of susceptibility of strain A7436 was apparently reflective of a species characteristic, as the other strains of *P. gingivalis* tested were similarly sensitive to direct killing by H_2O_2 (Figure 3.4B), despite differences in virulence properties such as capsule production. Furthermore, this level of susceptibility of *P. gingivalis* to the direct bactericidal effects of H_2O_2 would be well within the range of the H_2O_2 concentrations (2μM) modeled by Winterbourn *et al* (79), sustained in the phagosome environment post- delivery of MPO. As MPO is considered to be the primary consumer of H_2O_2 in the phagosome (25) (73) (79) (106) (107), the concentrations of NADPH oxidase products that the phagocytized bacterium would be exposed to before delivery of MPO would be in great excess of bactericidal concentrations.

Like *P. gingivalis*, *P. intermedia* is also a strict anaerobe, which synthesizes an extracellular protoporphyrin layer from the heme constituent of hemoglobin that results in black pigmentation of colonies grown on blood-based media (119). Because of their many similarities, these two bacteria were originally classified as subspecies of the same species, *Bacteroides melaninogenicus* (148). It was, therefore, not surprising that its sensitivity to the bactericidal activity of H_2O_2 proved similar to that of the *P. gingivalis* strains (Figure 3.4B). *P. intermedia* was not included for comparison with *P. gingivalis* in the neutrophil killing studies (24) that lead to the current studies, as it was not readily phagocytized. It is not known whether it would show a similar dependence on NADPH oxidase activity.

In contrast, *F. nucleatum* was readily phagocytized and killed by human neutrophils, without a requirement of an exogenous antibody source for opsonization (24). Its neutrophil-associated killing was significantly depressed when the activity of

the NADPH oxidase was inhibited, either through oxygen limitation (anaerobiosis) or by chemical (DPI) inhibition (24), indicating an importance of the respiratory burst in neutrophil killing of *F. nucleatum*. Unlike the complete ablation of killing with *P. gingivalis*, there remained a significant level of killing of *F. nucleatum* under these conditions, suggesting a sensitivity to oxygen-independent mechanisms, or an increased sensitivity to low levels of residual ROS. In the current studies, however, *F. nucleatum* was found to be relatively more resistant to direct killing by H_2O_2 (Figures 3.4A and B), which may be consistent with its reported ability to grow in the presence of low levels of oxygen (up to 6%) and its apparently less fastidious requirements for establishing infection (149).

The neutrophil-mediated killing of *A. actinomycetemcomitans*, unlike that of *P. gingivalis* and *F. nucleatum*, was relatively insensitive to inhibition of NADPH oxidase activity (24), supporting this bacterium's sensitivity to oxygen-independent killing mechanisms, as previously reported by others (150) (151) (152). Unlike the strict anaerobes included in this study, *A. actinomycetemcomitans* characteristically produces catalase and can grow in the presence of atmospheric oxygen (120) (121). It was also predictably more resistant to the direct bactericidal activity of H_2O_2, than was *F. nucleatum* (Figure 3.4A).

The relative susceptibility of *P. gingivalis* to H_2O_2 was also compared to that of the catalase-positive, aerotolerant *S. aureus*, a pathogen in CGD and in neutropenia; and to that of the catalase-negative, aerotolerant *S. pyogenes*, a pathogen in neutropenia but not in CGD. Unfortunately, neither of these species could be included in the comparative studies of neutrophil phagocytosis and killing (24), as they were highly sensitive to the fluorescent dyes used to visualize these cell processes (*S. pyogenes* was killed by DAPI, and *S. aureus* was killed by PI) (Table A.4 and described in text of Appendix). Other studies, however, have demonstrated the importance of a

functioning respiratory burst for optimal killing of *S. aureus* by human neutrophils (26) (27) (28) (29) (32) (33). It has been suggested that the differences in susceptibility of CGD patients to these two species relate to the inability of *S. pyogenes* and other non-catalase- producing bacteria to clear bacterially-generated H_2O_2 in the phagosome (33) (153). However, both of these species were relatively resistant to direct killing by H_2O_2 (Figures 3.4A and B). Moreover, the catalase-negative *S. pyogenes* proved even more resistant than the catalase-positive *S. aureus* (Figure 3.4B), challenging this traditionally held theory. The greater resistance of streptococcal species than *S. aureus* to H_2O_2 has previously been ascribed to its abundant possession of cytosolic peroxidases (154).

MPO is the major protein constituent of azurophilic (or primary) granules and is the most abundant enzyme in neutrophils (98) (99). The hierarchy of granule fusion would dictate that the components of the NADPH oxidase system would already be assembled and functioning on the phagosome membrane, by the time MPO is delivered (25) (73). Based on the results of a variety of studies, MPO is considered the major consumer of H_2O_2 in the phagosome as modeled in (79).

MPO is a heme-containing oxidoreductase that undergoes a series of redox reactions that involve interconversions of the relevant intermediates: ferric and ferrous peroxidase, compound I, compound II, and compound III (155). Depending on substrate availability, MPO can path through the halogenation cycle and/or the peroxidase cycle, and/or can act as a pseudo-catalase (155). The antimicrobial activity of MPO, however, has been attributed to the peroxidatic oxidation of chloride to the potent bactericidal agent, $H^+ {}^-OCl$, within the phagosome (73) (79) (82) (96) (100) (102) (103). Of the mammalian peroxidases, only MPO is capable of oxidizing chloride at a reasonable rate (156). Despite the attractiveness of the antimicrobial properties of this product, however, the actual contributions of MPO to the bactericidal activities in the phagosome environment are unclear and may depend on the ratios of substrate

availability (79). Kettle and Winterbourne (157) suggested that MPO possesses true catalase activity, the extent of which is so important, compared to other peroxidases, that MPO should be considered as a catalase/peroxidase.

The current studies were therefore designed to first examine the possibility that MPO provides protection against the direct bactericidal activity of H_2O_2. H_2O_2 was titrated to cover a range of concentrations to include levels that far exceeded that which is necessary for minimal bactericidal activity, with relatively low concentrations of MPO in order to minimize protein effect. MPO clearly afforded protection against the direct bactericidal activity of H_2O_2 for both *F. nucleatum* and *P. gingivalis* (Figures 3.5A and B). The extremely low concentration of protein required for this level of activity and the relatively high levels of H_2O_2 that MPO protected against, would suggest that this inhibition is due to the enzymatic properties of MPO, rather than to competitive inactivation or to a protein effect.

During the course of titration of H_2O_2, there were suggestions of killing with low levels of H_2O_2 in the presence of MPO, as was evident with *F. nucleatum* treated with 12.5μM H_2O_2, in the presence of 2mU/mL MPO, in Figure 3.5A. This MPO-dependent killing appeared to be more consistent with higher concentrations of MPO. The titrations of H_2O_2 and MPO were therefore refined to yield a clearer picture of this bactericidal activity of the MPO system. The chloride was assumed to be in substrate excess, at the 150mM concentration in PBS. Both *P. gingivalis* and *F. nucleatum* were killed optimally when treated with a concentration of H_2O_2 of 5μM, and killing was diminished at 10μM and lost at 20μM (Figures 3.5B and C).

There was a requirement for higher concentrations of MPO with *P. gingivalis* than with *F. nucleatum* (Figures 3.5B and C, and Figures 3.6A and C). This MPO- and H_2O_2-dependent killing extended to all of the other target bacteria, provided that MPO was present in sufficient quantity (Figures 3.6B, D, E and F). The surprising observation

was that 5μM H_2O_2 proved optimal for the killing of all target bacteria, which suggested that an optimally lethal concentration of what is presumed to be HOCl was achieved at this concentration, and that all of the species were equally susceptible to this product, independent of their relative susceptibilities to MPO-independent H_2O_2.

The requirement for different concentrations of MPO is interesting from several perspectives. *P. gingivalis* is highly resistant to the non-oxidative mechanisms of neutrophil killing, presumably through proteolytic inactivation of the bactericidal proteins and peptides of granules (18) (58) (59) (60) (61) (62) (63) (64) (65) (66). These granule constituents are rich in arginines and/or lysines, which are evidently the targets of the gingipains that define this pathogen (1). MPO is also especially rich in arginines, and its structure includes critical lysines (158). It therefore would not have been a surprise if *P. gingivalis* was highly resistant to any MPO-dependent activities. This could have been an explanation for the apparent independence of MPO for neutrophil-mediated killing of this pathogen (24). While *P. gingivalis* required more MPO than did *F. nucleatum* to demonstrate MPO-dependent killing; the ability to produce catalase (Figures 3.6D and E) or some other H_2O_2 competitor, appears to offer a more likely explanation for the species-dependent requirements for higher concentrations of MPO for activity. While cytosolic peroxidases may afford better protection against the toxicity of MPO-independent H_2O_2 (154), which diffuses into the cell; surface-associated catalase may afford better protection against the MPO-dependent effects of H_2O_2. Competition with MPO for H_2O_2 by an extracellular protoporphyrin is also suggested by the similarity of MPO requirements for *P. gingivalis* (46) (47) and *P. intermedia* (119) *vs.* that of the non-pigmented *F. nucleatum*.

If antagonistic interactions occur in the phagosome between MPO and bactericidal concentrations of H_2O_2, it is unclear what role MPO plays in the phagosome. The data in this study suggest that *P. gingivalis* and possibly other H_2O_2-sensitive

bacteria may be directly killed in the phagosome by low micromolar concentrations of H_2O_2, which are suggested to remain steady state in the phagosome after MPO delivery (79), and that phagosomal concentrations of H_2O_2 must be tightly regulated in order for MPO to function as a bactericidal constituent. As proposed by Reeves *et al* (159), it is possible that MPO has an alternative role in the phagosome such as H_2O_2 scavenging, which may serve to dampen the toxicity of the neutrophil against high concentrations of H_2O_2 and by cytotoxic enzymes. This is consistent with the observation that the majority of chlorinated proteins in the phagosome are of host origin (160).

Killing by H_2O_2 could be a likely mechanism of killing *P. gingivalis* within the phagosome, before MPO is delivered, due to its unusually high susceptibility to H_2O_2. H_2O_2 is hypothesized to kill by diffusion inside the bacterial cell, where it reacts with bacterial-associated iron, mediating a Fenton reaction, where ferrous iron (Fe^{2+}) reduces H_2O_2 to the most reactive (i.e. non-specific and diffusion-limited) radical known, hydroxyl radical ($OH^{\cdot}$) (75) (77) (80) (81) (83) (87) (161) (162) (163) (164) (165) (166). This scenario appears consistent with the fact that H_2O_2-mediated killing of *P. gingivalis* does not occur instantly, but that *P. gingivalis* and H_2O_2 (generated enzymatically at a sufficient rate or delivered as a bolus at a sufficient dose) need time together in order for H_2O_2 to become lethal – a mechanism consistent with requiring time in order for H_2O_2 to enter the bacteria, find and react with bacterial-associated iron, generate something more toxic (e.g. $OH^{\cdot}$), and cause a lethal, irreparable hit (e.g. damage to DNA).

It is therefore suggested from these studies that in order to kill *P. gingivalis*, MPO is an unnecessary co-factor, because H_2O_2 at a minimal dose is a highly effective mechanism of killing *P. gingivalis*. The fact that the specific granules are delivered to the phagosome before the primary granules assures that the NADPH oxidase system is delivering ROS to the bacterial target for a period of time in the absence of MPO (25) (167). This would presumably allow at least transient MPO-independent H_2O_2 activity

in the phagosome at levels that would exceed bactericidal concentrations. The differential susceptibility to very low doses of H_2O_2, compounded with the observation that the MPO-H_2O_2 system works only within a narrow range, and that MPO activity is highly susceptible to neutralization, makes H_2O_2 a highly desirable candidate as the effector mechanism of killing *P. gingivalis*.

Figure 3.1. Catalase, but not Superoxide Dismutase, blocks Xanthine – Xanthine Oxidase Killing of *P. gingivalis*. Results are expressed as recoverable $\log_{10}$ CFU/mL. Samples were spiral plated (limit of detection: 1.31 $\log_{10}$ CFU/mL), and logarithmic differences in recoverable CFU/mL were considered significant. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments. Experiments were performed at 37°C.

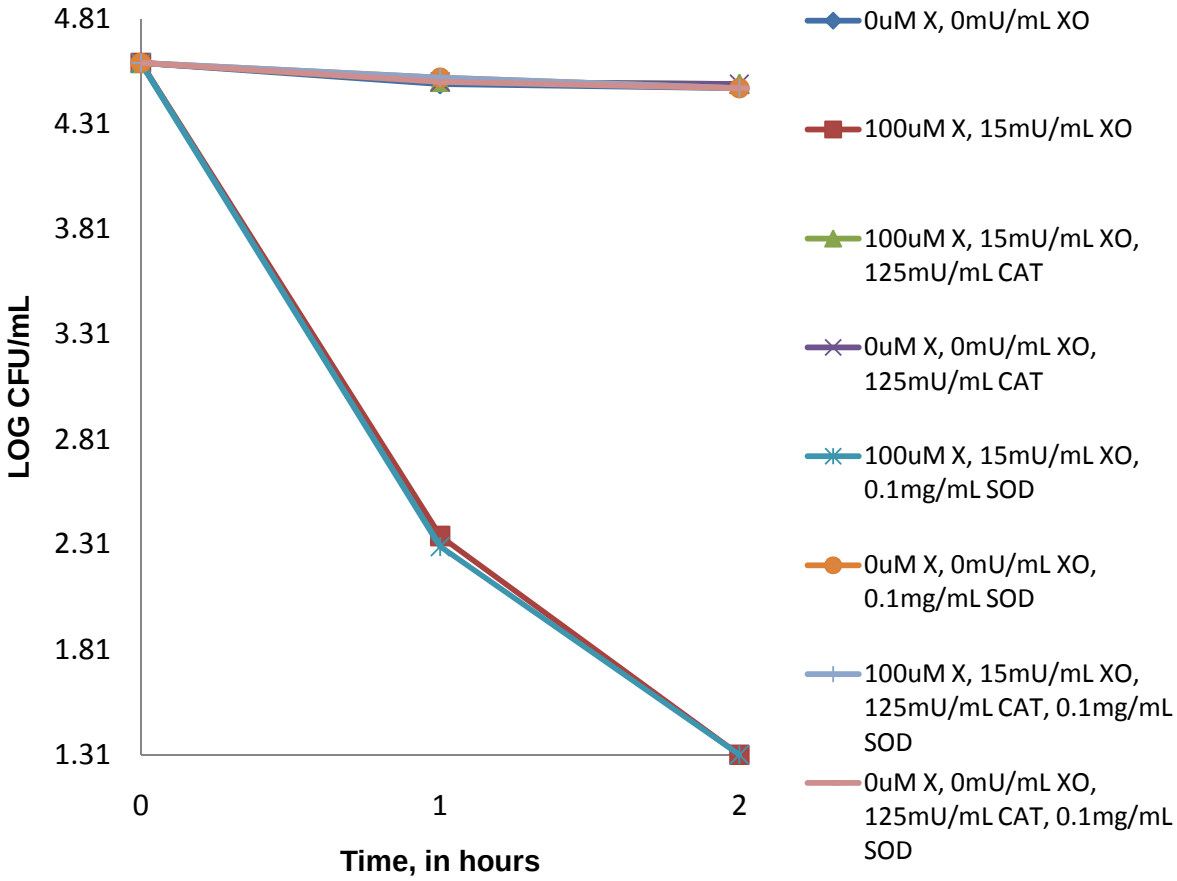


Figure 3.2. Titration of Glucose Oxidase- Dependent Killing of *P. gingivalis*. System contained 160mM FC glucose as enzyme substrate. Results are expressed as recoverable $\log_{10}$ CFU/mL. Samples were spiral plated (limit of detection: 1.31 $\log_{10}$ CFU/mL), and logarithmic differences in recoverable CFU/mL were considered significant. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments. Experiments were performed at 37°C.

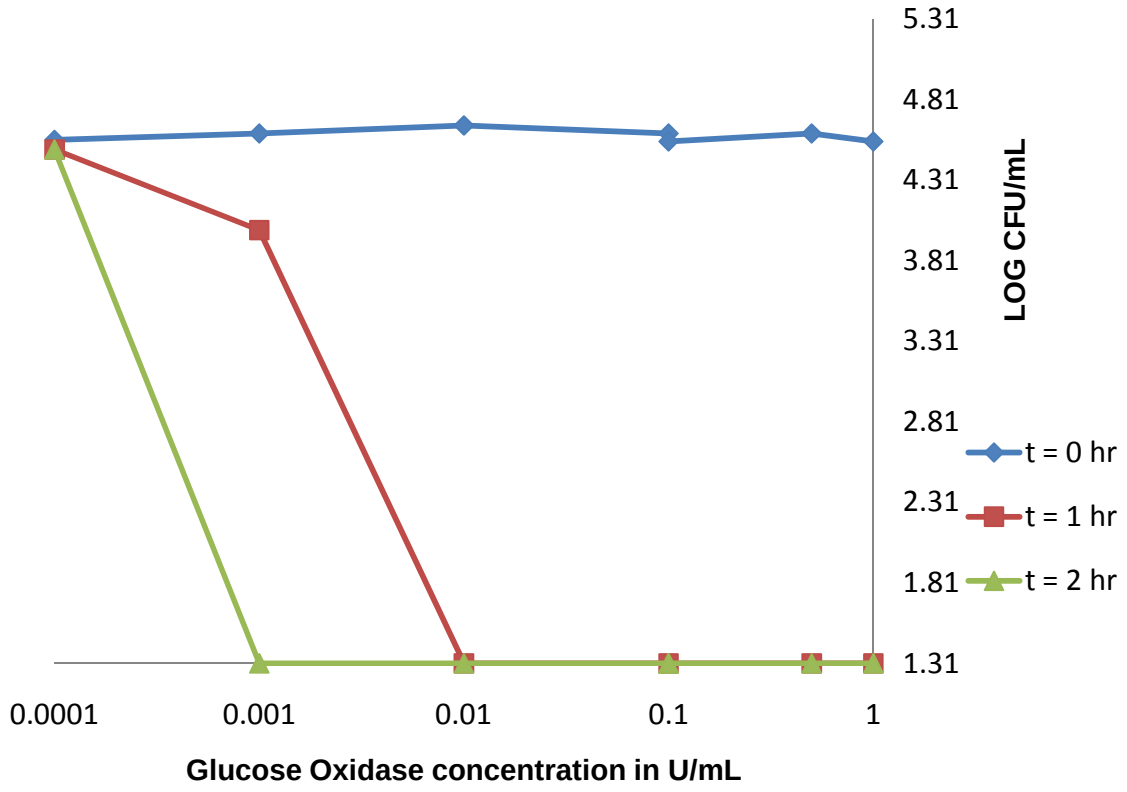


Figure 3.3. Kinetics of *P. gingivalis* Killing by Hydrogen Peroxide. Results are expressed as recoverable $\log_{10}$ CFU/mL. Samples were spiral plated (limit of detection: 1.31 $\log_{10}$ CFU/mL), and logarithmic differences in recoverable CFU/mL were considered significant. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments. Experiments were performed at 37°C.

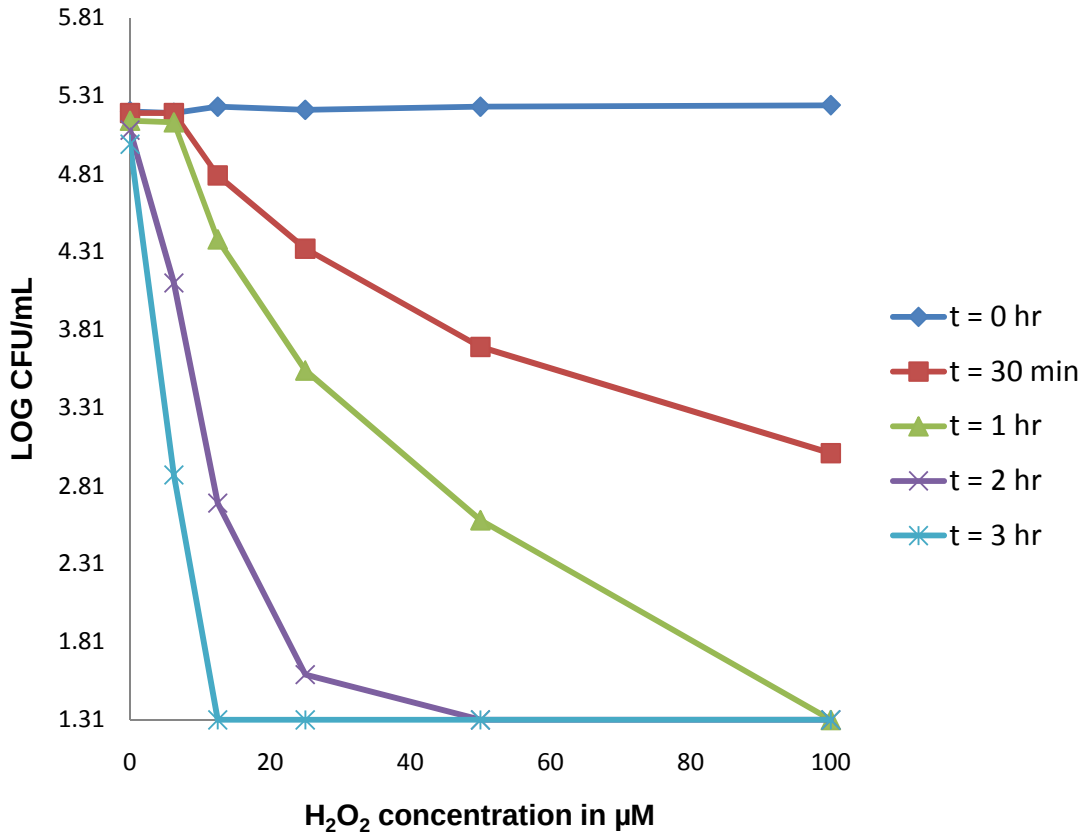
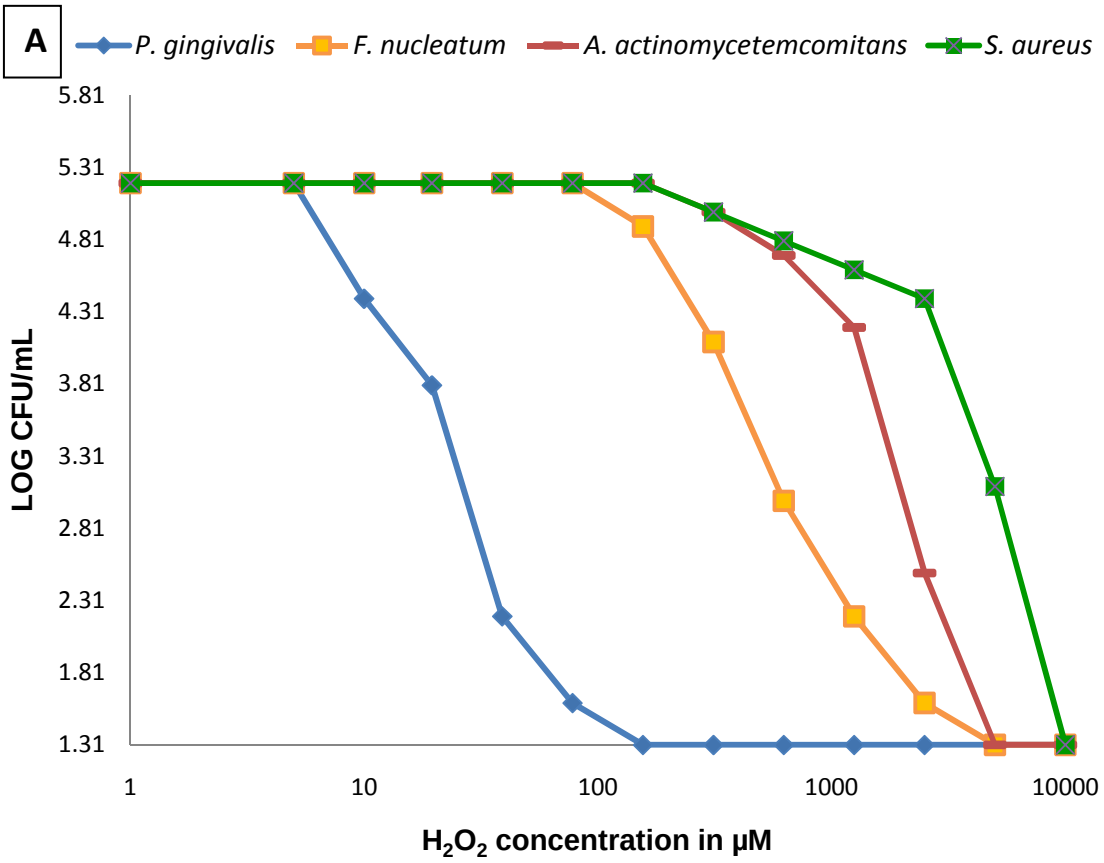


Figure 3.4. Titration of H_2O_2-Mediated Bactericidal Activity against *P. gingivalis*, *P. intermedia, F. nucleatum*, *A. actinomycetemcomitans*, *S. aureus* and *S. pyogenes*. H_2O_2 was delivered as a bolus to bacterial targets in PBS, in order to compare susceptibilities to H_2O_2 at 60 min. Results are expressed as recoverable $\log_{10}$ CFU/mL. Samples were spiral plated (limit of detection: 1.31 LOG CFU/mL), and logarithmic reductions (≥1 log reductions) in recoverable CFU/mL were considered significant. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments. Experiments were performed at 37°C.



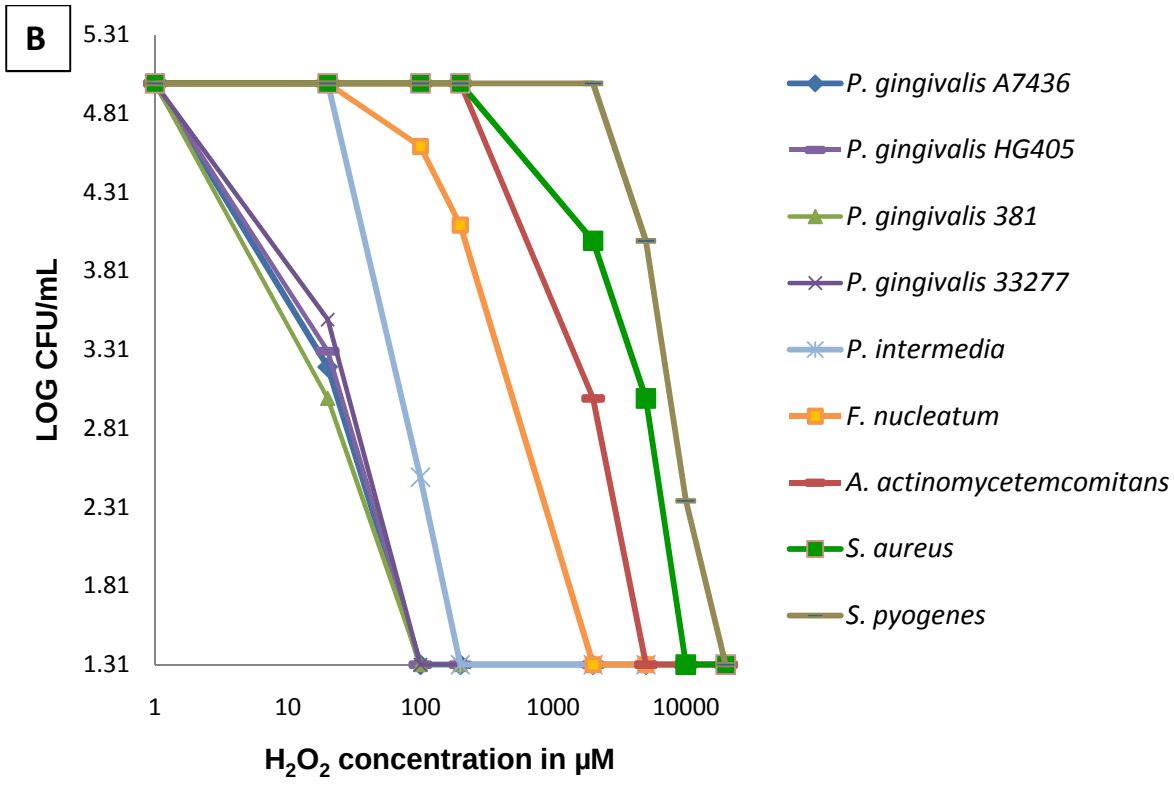
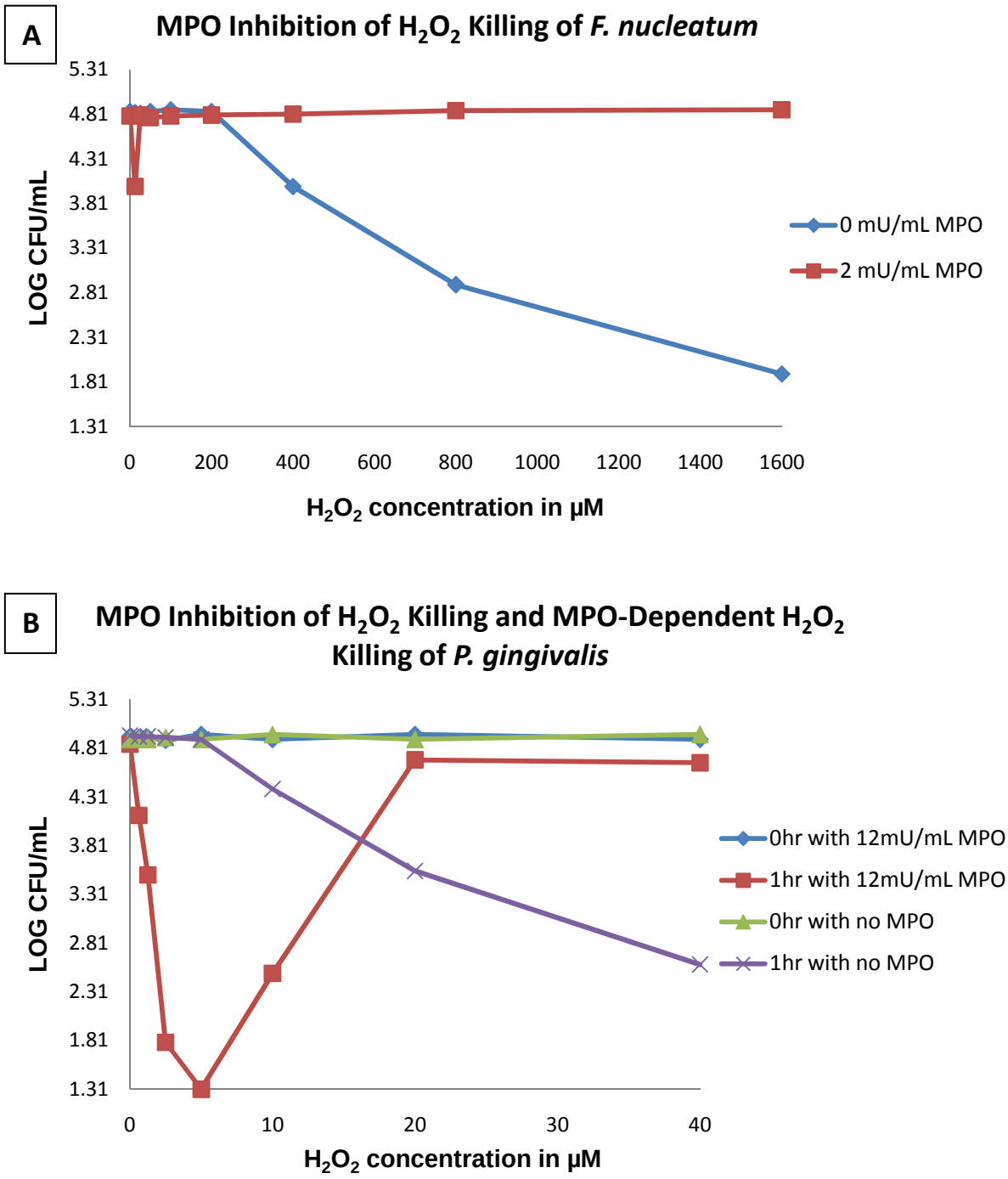

B
5.31
4.81
4.31
3.81
3.31
2.81
2.31
1.81
1.31
LOG CFU/mL
1
10
100
1000
10000
H2O2 concentration in µM
P. gingivalis A7436
P. gingivalis HG405
P. gingivalis 381
P. gingivalis 33277
P. intermedia
F. nucleatum
A. actinomycetemcomitans
S. aureus
S. pyogenes

Figure 3.5. MPO Inhibition of H_2O_2 Killing and MPO-Dependent H_2O_2 Killing of *F. nucleatum* and *P. gingivalis*. H_2O_2 was titrated in the presence of varying concentrations of MPO as indicated. Results are expressed as recoverable $\log_{10}$ CFU/mL after incubation in PBS at 37°C for 60 min. Samples were spiral plated (limit of detection: 1.31 $\log_{10}$ CFU/mL), and logarithmic differences in recoverable CFU/mL were considered significant. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments.



C

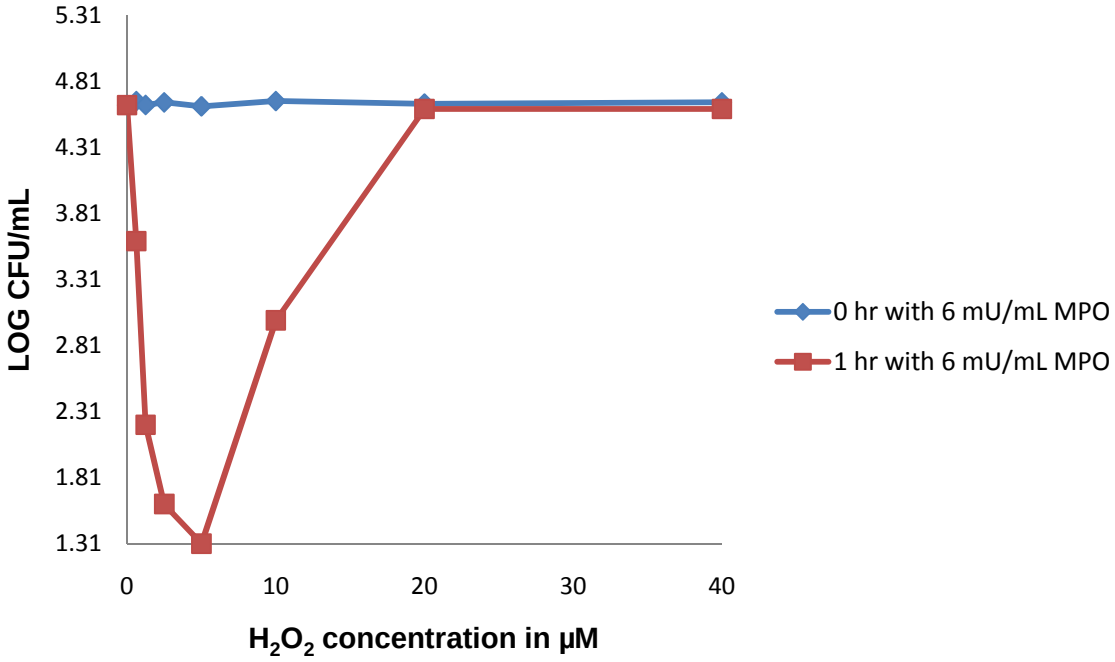
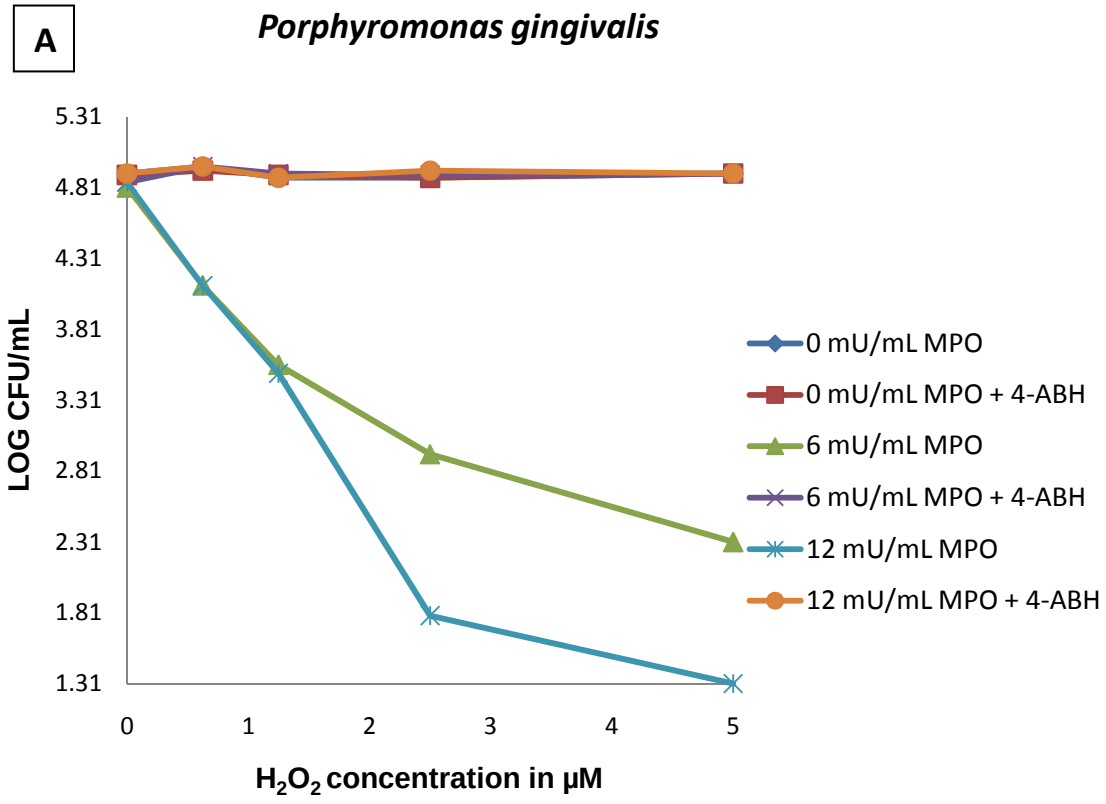
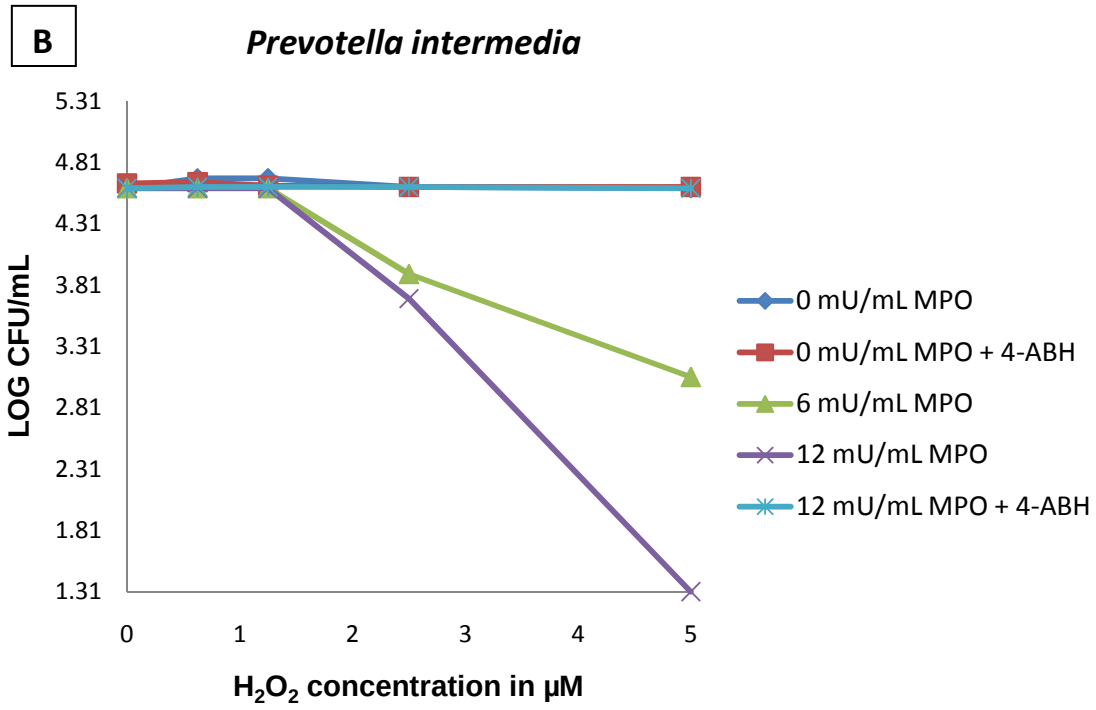
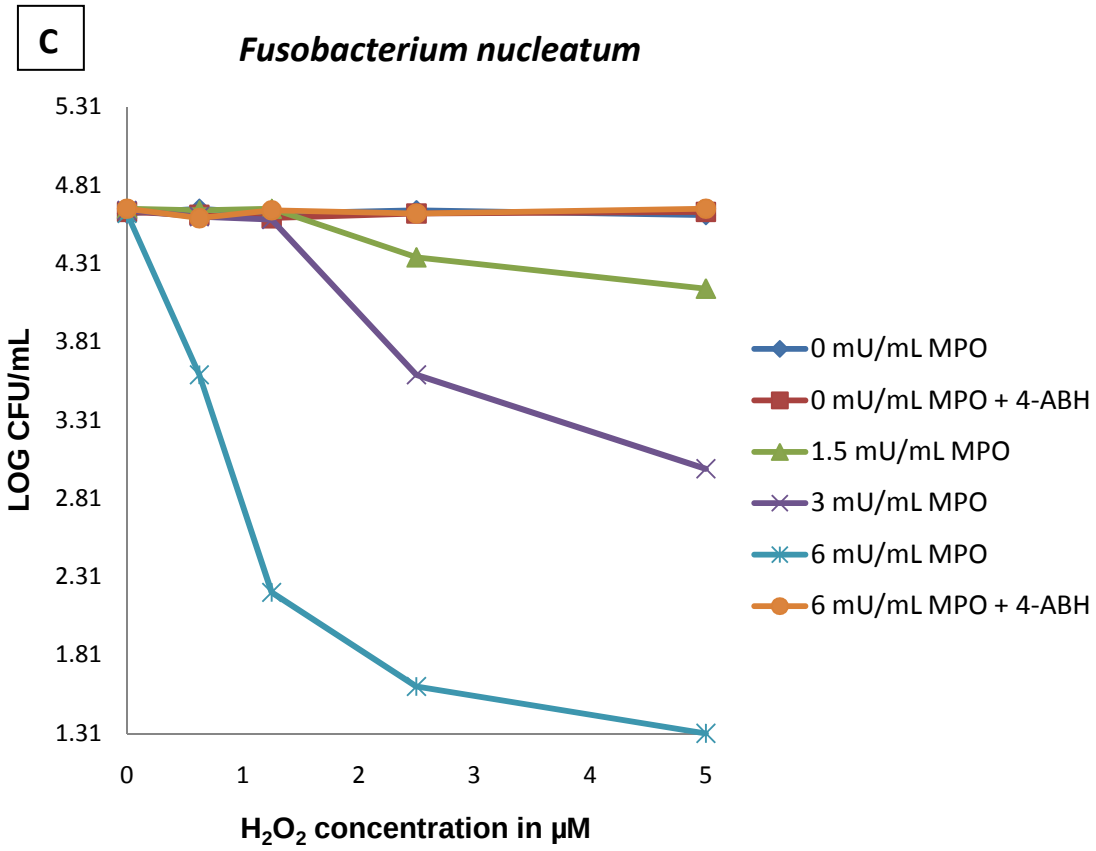

MPO-Dependent H_2O_2 Killing of *F. nucleatum*
LOG CFU/mL
5.31
4.81
4.31
3.81
3.31
2.81
2.31
1.81
1.31
0
10
20
30
40
H_2O_2 concentration in µM
0 hr with 6 mU/mL MPO
1 hr with 6 mU/mL MPO

Figure 3.6. 4-ABH-Inhibitable Killing of Various Bacterial Species by the MPO System. H_2O_2 was titrated from 0-5µM in the presence of varying concentrations of MPO as indicated. Target bacteria include *P. gingivalis* A7436 (A); *P. intermedia* (B); *F. nucleatum* (C); *A. actinomycetemcomitans* (D); *S. aureus* (E); and *S. pyogenes* (F). The final concentration of 4-ABH in the reaction mixtures was 4mM where indicated. Results are expressed as recoverable $\log_{10}$ CFU/mL after incubation in PBS at 37°C for 60 min. Samples were spiral plated (limit of detection: 1.31 $\log_{10}$ CFU/mL), and logarithmic differences in recoverable CFU/mL were considered significant. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments.

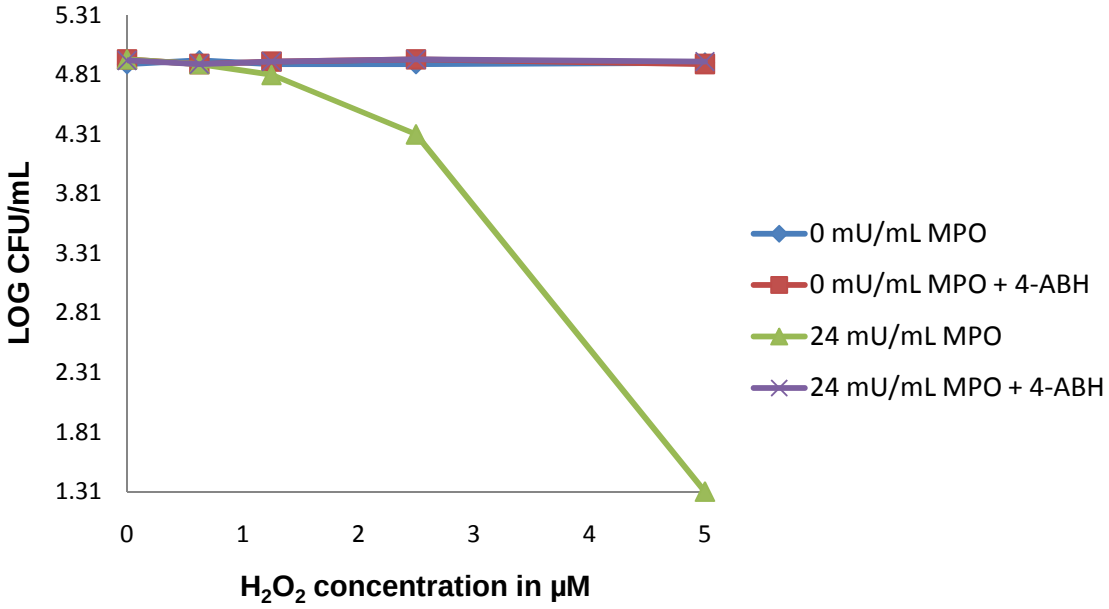



B
Prevotella intermedia
5.31
4.81
4.31
3.81
3.31
2.81
2.31
1.81
1.31
LOG CFU/mL
0
1
2
3
4
5
H2O2 concentration in µM
0 mU/mL MPO
0 mU/mL MPO + 4-ABH
6 mU/mL MPO
12 mU/mL MPO
12 mU/mL MPO + 4-ABH


C
Fusobacterium nucleatum
5.31
4.81
4.31
3.81
3.31
2.81
2.31
1.81
1.31
LOG CFU/mL
0
1
2
3
4
5
H2O2 concentration in µM
0 mU/mL MPO
0 mU/mL MPO + 4-ABH
1.5 mU/mL MPO
3 mU/mL MPO
6 mU/mL MPO
6 mU/mL MPO + 4-ABH

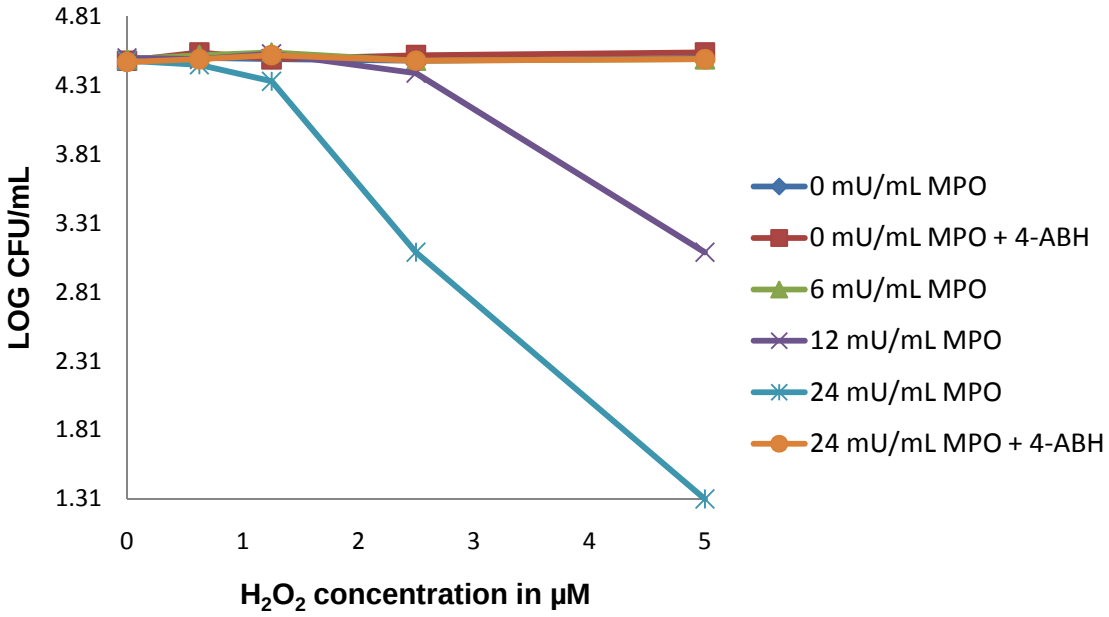
D

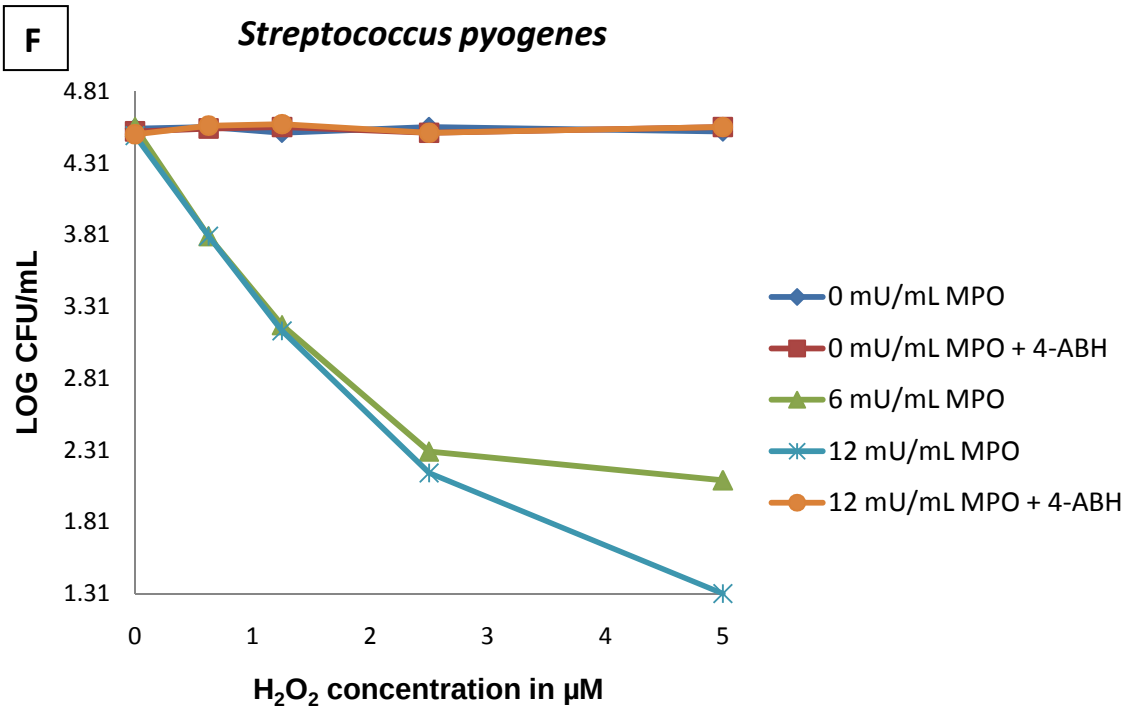
Aggregatibacter actinomycetemcomitans



E

Staphylococcus aureus




F
Streptococcus pyogenes
4.81
4.31
3.81
3.31
2.81
2.31
1.81
1.31
LOG CFU/mL
0
1
2
3
4
5
H2O2 concentration in µM
0 mU/mL MPO
0 mU/mL MPO + 4-ABH
6 mU/mL MPO
12 mU/mL MPO
12 mU/mL MPO + 4-ABH

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CHAPTER 4

Differential Mechanisms of Killing by Hydrogen Peroxide *vs.* Bleach

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Running Title: Differential Killing by H_2O_2 *vs.* Hypochlorite

ABSTRACT

Previous studies designed to determine the NADPH oxidase-dependent, neutrophil-mediated mechanisms of killing *Porphyromonas gingivalis*, an overt periodontopathogen, demonstrated both MPO-dependent and MPO-independent bactericidal activity with H_2O_2, effective against *P. gingivalis* and all other bacterial species tested. While *P. gingivalis* proved exceptionally sensitive to MPO-independent H_2O_2 bactericidal activity, all test species were susceptible to the same concentrations of H_2O_2 in the presence of optimal MPO activity. The aim of the current study was to investigate the differential mechanisms of killing by H_2O_2 *vs.* that of the presumed bactericidal product of MPO, hypochlorite (bleach). While the bactericidal activity of H_2O_2 was time- and concentration-dependent, bleach killed immediately without temporal progression. Despite having very different susceptibilities to H_2O_2, all test bacteria showed identical susceptibilities to bleach. The bactericidal activity of H_2O_2 was also temperature-dependent, with intermediate killing at room temperature and no killing on ice. In striking contrast, the bactericidal activity of bleach was temperature-independent. When the bacterial target numbers were varied relative to fixed concentrations of H_2O_2, the highest bacterial densities were killed exponentially better than the lower bacterial densities. In contrast, the bactericidal activity of bleach was lost as the bacterial target densities increased. Dimethyl sulfoxide competitively inhibited the bactericidal activity of H_2O_2 with all test species, suggesting a role for hydroxyl radical ($OH^{\cdot}$). These data are consistent with a mechanism that requires bacterial conversion of H_2O_2 to the more reactive $OH^{\cdot}$, proximal to a susceptible vital target, in contrast to the direct bactericidal activity of the MPO product, hypochlorite.

INTRODUCTION

The principal oxygen-dependent killing pathway of the neutrophil is mediated by the multi-component NADPH oxidase system, which is responsible for the production of a respiratory burst, where large amounts of oxygen are consumed in the enzymatic generation of superoxide anion: $NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\cdot -} + H^+$ (1) (2) (3) (4) (5). Once generated, superoxide subsequently dismutates to hydrogen peroxide ($O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2$), and from these intermediates, which are generally considered relatively nontoxic and inert (or only mildly oxidative) (6) (7) (8), other more reactive oxidants are generated, either by reacting with enzyme systems or with bacterial iron, or by the products of these systems reacting with $O_2^{\cdot -}$ and/or H_2O_2, or with each other (6) (9) (10) (11) (12) (13) (14) (15) (16) (17).

The best described enzyme known to consume H_2O_2 in the phagosome is the heme-based myeloperoxidase (MPO) system, which is a H_2O_2-dependent mechanism of generating the strong oxidant, weak acid, hypochlorous acid (HOCl): $MPO + Cl^- + H_2O_2 \rightarrow HOCl$. Due to its reactivity and ability to chlorinate or otherwise modify a wide range of bacterial targets, MPO-generated HOCl is regarded as one of the most potent microbicidal and cytotoxic mechanisms of the neutrophil (6) (11) (15) (18) (19) (20) (21). It is also well-supported that in a reductive cellular environment, as found in a metabolizing cell, the toxicity of H_2O_2 can be enhanced by reacting with bacterial-associated iron and mediating a Fenton reaction, where ferrous iron (Fe^{2+}) reduces H_2O_2 to the most reactive (i.e. non-specific and diffusion-limited) radical known, hydroxyl radical ($OH^{\cdot}$): $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H_2O + OH^{\cdot}$ (7) (10) (13) (16) (17) (22) (23) (24) (25) (26) (27). H_2O_2 is membrane permeable, and upon diffusion inside the cell, is believed to react with bacterial iron to generate $OH^{\cdot}$ close to DNA. Like the membrane-permeable HOCl, $OH^{\cdot}$ is capable of powerful oxidative damage, which if not repaired,

leads to lethality (6) (28). The instability of these partially reduced oxygen species, their membrane permeabilities, and their rapid reactivity with a wide range of bacterial targets (e.g. cell- associated iron or hemes, Fe-S clusters, enzymes, proteins, lipids, cell walls, membranes, RNA, and DNA etc) pose a significant threat to microorganisms, and when generated within the phagosome in close proximity to bacteria, are important for microbial killing and digestion (6) (9) (12) (29) (30).

From preceding studies, the MPO-independent bactericidal activity of H_2O_2, effective against *Porphyromonas gingivalis* and all other species tested, was demonstrated to be both time- and dose- dependent (31). A significant range of susceptibilities to H_2O_2 was demonstrated among the various species tested. Of all the species tested, all strains of the periodontal pathogen *P. gingivalis* were consistently more susceptible to low concentrations of H_2O_2, and experienced logarithmic killing with ≥20μM H_2O_2 at one hour. In comparison, other anaerobic periodontal pathogens, *Prevotella intermedia* and *Fusobacterium nucleatum*, required approximately 5- and 15-fold, respectively, higher concentrations of H_2O_2 to be killed logarithmically at one hour. In contrast, the aerotolerant pathogens *Aggregatibacter actinomycetemcomitans, Staphylococcus aureus,* and *Streptococcus pyogenes* required approximately 100-, 150-, and 250- fold, respectively, higher concentrations of H_2O_2 to be killed logarithmically at one hour. Interestingly, however, while there were major differences in the susceptibilities of these bacteria to MPO-independent H_2O_2, there were no differences in the susceptibilities of these bacteria to the H_2O_2 required for MPO-dependent killing. As long as there was optimum MPO activity available (to presumably exceed the ability of the catalase- producing bacteria to scavenge exogenous H_2O_2), there was no difference in the amount of H_2O_2 required to kill with MPO. This seemed to suggest that these test bacteria were not different in their

susceptibility to the product of the MPO-H_2O_2 system (HOCl), despite having very different susceptibilities to H_2O_2.

The objective of the current study was therefore to investigate the differential killing mechanisms of H_2O_2 *vs.* bleach, in an attempt to better understand how these oxidative killing mechanisms may operate in the neutrophil with various bacteria. To begin to address this, select bacteria were challenged with either a bolus of H_2O_2 or sodium hypochlorite (Na^+ OCl^-, which is the salt form of HOCl generated by the MPO-H_2O_2 system in the neutrophil), in order to compare the killing kinetics of H_2O_2 *vs.* bleach. The effects of varying temperature and bacterial density on H_2O_2- *vs.* bleach-mediated killing were also assessed. Finally, the ability of the hydroxyl radical scavenger, dimethyl sulfoxide (DMSO), to protect against H_2O_2-mediated killing of these bacteria was examined.

MATERIALS AND METHODS

Bacterial Growth and Storage:

Strain A7436 was an original clinical isolate of *P. gingivalis*, and was chosen for its expression of multiple virulence traits considered representative of this periodontopathic species. *P. gingivalis* A7436 was originally isolated from a patient with aggressive periodontitis, and was characterized by the late V.R. Dowell (32), and by this laboratory (33) (34) (35) (36) (37), and others (38) (39) (40). Its virulence characteristics have been studied in a variety of animal models (34) (41) (42) (43). A7436 is encapsulated and exhibits a remarkable resistance to phagocytosis (33) (34) (36) (37). In the mouse subcutaneous chamber model, A7436 is capable of spreading and dissemination, producing secondary lesions away from the injection site, septicemia, and death (34) (35) (40). Due to the hydrophilic nature of its extra-cellular polysaccharide layer (capsule), strain A7436 does not auto-aggregate, clump, or associate with neutrophils (44) (45) (46). It is a Gram-negative short rod, β-hemolytic, and readily forms black-pigmentation on blood-based agars.

P. gingivalis was grown at 36^{o}C in a flexible film anaerobic chamber (Coy Laboratory Products Inc, Ann Arbor, MI) in an atmosphere of 85% N_2 – 10% H_2 – 5% CO_2, in 9mL volumes of Wilkins-Chalgren (WC) Anaerobe Broth Medium (Oxoid Ltd, Basingstroke, Hampshire, England) in glass test tubes, without shaking. For the sake of consistency and to minimize mutation, bacteria were freshly grown from minimally passaged, aliquoted frozen stocks each week before use in the killing assays. Briefly, 100µL aliquots of *P. gingivalis* (grown in WC to mid-exponential phase or to ~0.5 optical density (OD) at $A_{\lambda 660nm}$) were stored in 1mL glass vials containing 0.5mL sterilized skim milk (Sigma, St. Louis, MO), at -80^{o}C until use. Individual frozen aliquots were thawed at room temperature (RT) and recovered by inoculation into 9mL volumes of non-

reduced WC. From this, 3-5 drops (using a sterile plastic transfer pipette) were inoculated into fresh 9mL volumes of non-reduced WC, and from this, 1mL was inoculated into a second 9mL volume of non-reduced WC to make a 10-fold dilution, and placed into the anaerobic incubator for growth. These broth cultures took approximately 60 hr to initially recover from frozen stock in WC. Upon initial visible growth, broth cultures were serially diluted (1 drop and 10-fold from that) into fresh 9mL volumes of reduced WC. Bacteria were harvested from overnight cultures (~18 hr) using the dilution that best fit early- to mid-exponential phase with an $A_{\lambda 660nm}$ of 0.2-0.6. The starting inoculum assured that the test bacteria used in all experiments had undergone a minimum of five doublings. Individual frozen aliquots were also checked for purity by streaking for isolation on both aerobic and anaerobic blood agar plates. An absence of growth on aerobic blood agar plates (TSA agar with 5% sheep blood (PML Microbiologicals, Mississauga, ON, Canada), incubated aerobically at 36^{o}C) and a morphologically pure culture on anaerobic blood agar plates (reducible Brucella blood agar (Anaerobe Systems, Morgan Hill, CA), incubated anaerobically at 36^{o}C) assured purity and was confirmed by Gram stain (agar and broth cultures) and distinct odor.

The pathogens that were selected for comparison with the catalase-negative, strictly anaerobic periodontal pathogen, *P. gingivalis*, include the catalase-negative, microaerophilic periodontal pathogen, *Fusobacterium nucleatum*; the catalase-positive, aerotolerant periodontal pathogen, *Aggregatibacter actinomycetemcomitans*; the catalase-positive, aerotolerant pathogen, *Staphylococcus aureus*; and the catalase-negative, but peroxidase-positive, aerotolerant pathogen, *Streptococcus pyogenes* (both *S. aureus* and *S. pyogenes* are pathogens in subjects with neutrophil dysfunctions). *Fusobacterium nucleatum* strain 1594, and *Aggregatibacter actinomycetemcomitans* strain A7154 (leukotoxin-producing, serotype b clinical isolate) were grown and stored in an identical manner to *P. gingivalis* strain A7436. *Staphylococcus aureus* strain 25923

and *Streptococcus pyogenes* strain 19615 were also grown in WC broth at 36^{o}C, but in ambient atmosphere.

Cell-Free Killing Assays

All killing assays were performed *in vitro*, in cell-free, aerobic conditions, in 1X sterile filtered phosphate buffered saline (PBS) (0.0067 PO_4) without calcium or magnesium (HyClone Laboratories, Thermo Scientific, Logan, Utah). All reagents were freshly prepared just prior to the start of experiments, and were equilibrated to RT (unless otherwise stated) in ambient atmosphere before use. Bacteria were prepared last (after reagents) to maximize their viability.

Briefly, bacteria were harvested from ~18 hr overnight cultures (grown in WC at 36^{o}C, either anaerobically or aerobically, depending on species; see above), using the broth culture that best fit early- to mid-exponential phase with an OD of 0.2-0.6 at $A_{\lambda 660nm}$ (see above). All bacteria were adjusted in aerobic conditions with non-reduced WC to a final $A_{\lambda 660nm}$ = 0.1, using a spectrophotometer (Beckman, Fullerton, CA). Bacteria were then serially diluted in PBS (non-reduced) by performing three 10-fold dilutions, and then diluted by one-half after the addition of reagents in experiments. Done this way, the final bacterial densities in experiments was consistently between 4.2-5.2 $\log_{10}$ CFU/mL. All killing assays were set up at RT (unless otherwise stated) in 1mL final volumes (FV) in 1.7mL polypropylene centrifuge tubes (VWR International, West Chester, PA). Following set-up and initial plating (0 time), tubes were placed in a 37^{o}C Fisher isotemp dry bath (Fisher Scientific Company, Fairlawn, NJ) (unless otherwise stated) without shaking, until subsequent time points.

At each time point, samples were briefly vortexed (<1 s) and either spiral plated (Spiral Systems, Bethesda, MD) or plated by delivering 10µL of sample (spot assayed) to the surface of non-reduced WC agar (Oxoid Ltd), for growth and bacterial

enumeration. In some experiments, detection of logarithmic differences in recoverable CFU was sufficient, so samples plated by the spot assay method (10µL of sample delivered as a localized drop or spot to the surface of an agar plate) was more practical because it allowed dozens of samples to be compared side by side on the same plate, capturing more data on one plate and conserving materials and time, with the additional benefit that it allowed easy detection of logarithmic differences in recoverable CFU between samples. Logarithmic reductions in bacterial densities per spot were estimated by comparison to calibration standard controls, which were serially diluted 10-fold. Results in these experiments were approximated to the nearest log and were reported as either % recoverable $\log_{10}$ CFU/mL (Table 4.1) or recoverable $\log_{10}$ CFU/mL (Table 4.3). All other data reported (Figures 4.1 and 4.2 and Table 4.2) were quantitated by spiral plating and were reported as recoverable $\log_{10}$ CFU/mL. The densities of the bacterial suspensions permitted direct spiral plating without dilution. After the growth of countable colonies, plates were enumerated by an automatic colony counter with computer software (ProtoCOL, Synbiosis USA, Frederick, MD) specifically programmed for determining spiral plated counts. Logarithmic reductions in recoverable CFU/mL were considered significant. The lower limit of detection in samples enumerated by the spot assay method was 2.0 $\log_{10}$ CFU/mL, and the limit of detection in samples enumerated by the spiral plate method was 1.31 $\log_{10}$ CFU/mL. Samples plated both ways were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments using overlapping concentrations of test reagents and progressive refinement of the dilutions around the effective concentrations.

After plating, agar surfaces were briefly allowed to dry in ambient air (dried within 5 min of plating), and were quickly placed into the anaerobic chamber incubator (for *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans*) or aerobic incubator (for *S.*

aureus and *S. pyogenes*) for growth. Plates were incubated at 36°C, and recoverable CFU were countable after 4-6 days for *P. gingivalis*; after 2-3 days for *F. nucleatum*, *A. actinomycetemcomitans*, and *S. pyogenes*; and after 1 day for *S. aureus*. Inhibitory concentrations of hydrogen peroxide or bleach with any bacterial species often resulted in recoverable CFU that were smaller or took longer to grow than controls.

Hydrogen Peroxide (H_2O_2) Killing Assays

Dilutions of H_2O_2 were freshly prepared in PBS before each experiment from a 30% w/w stock solution (Sigma), and molar concentrations (c) were determined using the Beer's Law formula, $A = cb\varepsilon$, where A was the OD of the H_2O_2 dilution in PBS at $A_{\lambda 230nm}$ in a quartz cuvette, ε was an 81 $cm^{-1}M^{-1}$ extinction coefficient, and b equaled 1. Known concentrations of H_2O_2 were then titrated in PBS. 500µL of bacteria were added to 500µL of H_2O_2 (or 500µL of PBS), and samples were either spot or spiral plated (see above) over time in order to discern killing kinetics.

In temperature studies (Figure 4.1), the effect of varying temperature on H_2O_2-mediated killing of *P. gingivalis* was examined. Small volumes of *P. gingivalis* (~3.5mL) and the various H_2O_2 dilutions (~1mL each), separately contained in 5mL polypropylene culture tubes (VWR International), were either pre-incubated in an ice water bath (1°C), in a water bath at RT, or in a water bath maintained at 37°C, for at least 15 min for equilibration to these temperatures. After 15 min of equilibration and just prior to being combined (the start of the experiment), bacteria and H_2O_2 were briefly vortexed. Empty reaction tubes (1.7mL polypropylene centrifuge tubes, VWR) were also pre-equilibrated in the various temperature baths, and experiments were completely set-up and performed in the various water baths in which they were equilibrated (ice, RT, 37°C). For instance, bacteria and H_2O_2 that had been chilled on ice for 15 min were combined on ice in reaction tubes that had also been chilled on ice. 500µL of bacteria (final

bacterial density approximately 5.0 $\log_{10}$ CFU/mL) were added to 500µL of H_2O_2 (titrated using 2-fold dilutions from 100µM, final concentration (FC)) (or 500µL of PBS) in the three test temperature conditions. Samples were incubated in their respective temperature baths for the duration of the experiment, with the exception of brief vortexing and spiral plating at initial time and at 60 min.

In bacterial density studies (Table 4.3), the effect of varying bacterial density on H_2O_2-mediated killing of *P. gingivalis* and *S. aureus* was examined. *P. gingivalis* and *S. aureus* were titrated 10-fold, so that final bacterial densities varied logarithmically from 8.0 – 3.0 $\log_{10}$ CFU/mL after the addition of hydrogen peroxide (FC listed in µM). Samples were spot assayed (see above), in order to discern logarithmic differences in recoverable CFU/mL between samples. Since the limit of detection was 2.0 $\log_{10}$ CFU/mL, a maximum of one log reduction could be detected in samples containing a bacterial density of 10^3 CFU/mL, and a maximum of six log reductions could be detected in samples containing a bacterial density of 10^8 CFU/mL. Note: Results denoted as <2 recoverable $\log_{10}$ CFU/mL indicates no recoverable CFU on replicate plating. Results denoted as 8 recoverable $\log_{10}$ CFU/mL, for instance, in samples with a starting inoculum of 10^8 CFU/mL, indicate no logarithmic reductions detected, and so on.

In the dimethyl sulfoxide (DMSO) studies (Figure 4.2), the effect of DMSO on H_2O_2-mediated killing was examined with select bacteria. DMSO (Pierce, Rockford, IL) was diluted in PBS and titrated using 10-fold dilutions from 100mM. H_2O_2 was also titrated in PBS (FC listed in µM). Reagents were added quickly with consistent timing to reaction tubes, with brief vortexing (<1 s) after each addition. In order to favor the activity of DMSO, DMSO was present with the bacteria before challenge with H_2O_2. Hence, reagents were added in the following order: 1^{st}: 250µL of DMSO (or 250µL of PBS), 2^{nd}: 500µL of bacteria, 3^{rd}: 250µL of H_2O_2 (or 250µL of PBS). After all reagents

were combined, vortexing following the addition of H_2O_2 started the experiment (0 time), and all samples were spiral plated at 0 and 60 min.

Bleach Killing Assays

Dilutions of sodium hypochlorite (bleach) were freshly prepared in PBS before each experiment from a stock solution of 6.15% vol/vol Na^+ OCl^- (Clorox Bleach, Oakland, CA) that was opened for the first time. Sodium hypochlorite concentrations were titrated using either 2- or 10-fold dilutions, starting from different initial concentrations based on the progression of the outcome of experiments, and are expressed as % FC, vol/vol. Each repetition included overlapping concentrations.

In temperature studies (Table 4.1), the effects of varying temperature on sodium hypochlorite-mediated killing of *P. gingivalis* were examined. Small volumes of *P. gingivalis* (~4mL) and the various dilutions of bleach (~0.5mL each), separately contained in 5mL polypropylene culture tubes (VWR International), were either pre-incubated in an ice water bath (1^{o}C) or in a water bath maintained at 37^{o}C, for at least 15 min for equilibration to these temperatures. After 15 min of equilibration and just prior to being combined (the start of the experiment), bacteria and the bleach dilutions were briefly vortexed. Empty reaction tubes (1.7mL polypropylene centrifuge tubes, VWR) were also pre-equilibrated in the temperature baths, and experiments were completely set-up and performed in the water bath in which they were equilibrated (ice or 37^{o}C). 500µL of bacteria (final bacterial density 5.0 $\log_{10}$ CFU/mL) were added to 500µL of bleach (or 500µL of PBS). Samples were incubated in either ice or at 37^{o}C for the duration of the experiment, with the exception of brief vortexing and plating (spot method) at 0, 1, and 2 hr.

In Table 2, the killing kinetics of select bacteria with sodium hypochlorite were compared at 37^{o}C. 500µL of bacteria (starting inoculums between 4.4 – 4.8 $\log_{10}$

CFU/mL, final bacterial densities) were added to 500µL of bleach (or 500µL of PBS). Samples were incubated at 37^{o}C in a Fisher isotemp dry bath for the duration of the experiment, with the exception of brief vortexing and spiral plating at 0 and 60 min.

In bacterial density studies (Table 4.3), the effects of varying bacterial density on sodium hypochlorite-mediated killing of *P. gingivalis* and *S. aureus* were examined. *P. gingivalis* and *S. aureus* were titrated using 10-fold dilutions, so that final bacterial densities varied logarithmically from 8.0 – 3.0 $\log_{10}$ CFU/mL after the addition of bleach. Bleach was titrated using 10-fold dilutions from 1% v/v. Samples were spot assayed (see above), in order to discern logarithmic differences in recoverable CFU/mL between samples. Since the limit of detection was 2.0 $\log_{10}$ CFU/mL, a maximum of one log reduction could be detected in samples containing a bacterial density of 10^3 CFU/mL, and a maximum of six log reductions could be detected in samples containing a bacterial density of 10^8 CFU/mL. Note: Results denoted as <2 recoverable $\log_{10}$ CFU/mL indicates no recoverable CFU on replicate plating. Results denoted as 8 recoverable $\log_{10}$ CFU/mL, for instance, in samples with a starting inoculum of 10^8 CFU/mL indicates no logarithmic reductions detected, and so on.

RESULTS

H_2O_2-Mediated Killing is Temperature- Dependent

The effects of varying temperature on H_2O_2-mediated killing of *P. gingivalis* were examined after 60 min of incubation (Figure 4.1). As was seen in previous studies (31), concentrations of H_2O_2 in excess of 12.5μM resulted in exponential killing of *P. gingivalis* at 60 min and at 37°C. There was a significant reduction in the effectiveness of H_2O_2 at room temperature (100μM was required for logarithmic reduction), and killing was totally eliminated when the reaction mixture was maintained on ice. This protection on ice was evident for *P. gingivalis* even with H_2O_2 concentrations 100-times higher than the minimal bactericidal concentration (data not shown). Ice also protected *S. aureus* from bactericidal concentrations of H_2O_2 (data not shown).

Sodium Hypochlorite-Mediated Killing is Time- and Temperature- Independent

As can be seen in Table 4.1, the bactericidal activity of sodium hypochlorite (bleach) was dose-dependent and was evident upon initial plating, suggesting that killing with bleach resulted on contact. Unlike the activity of H_2O_2, there was no temporal progression of killing with bleach. Furthermore, there was no influence of temperature, as treatment on ice resulted in killing activity equivalent to that at 37°C. Likewise, the killing of *S. aureus* with bleach was equivalent at 37°C and on ice (data not shown).

Previous studies have indicated that there is a range of susceptibilities among the various species tested to MPO-independent H_2O_2 bactericidal activity; however, *P. gingivalis* is consistently the most sensitive species to H_2O_2 in the absence of MPO (31). In contrast, these same species were susceptible to the same H_2O_2 concentrations in the MPO system (31). To extend this comparison, the susceptibilities of these species to bleach, as well as the killing kinetics with bleach, were determined in comparison to

that of *P. gingivalis* (Table 4.2). Consistent with the bactericidal activity in the MPO system (31), the susceptibilities of *F. nucleatum, S. aureus,* and *S. pyogenes* to bleach were strikingly similar to that found with *P. gingivalis* (Table 4.2). Furthermore, with all bactericidal concentrations of bleach, there were immediate logarithmic reductions in recoverable CFU/mL with each of the target bacteria, and very little evidence of killing progression over time in the presence of bleach. Identical susceptibilities to bleach were also observed with other tested bacteria, including *P. intermedia* and *A. actinomycetemcomitans* (data not shown).

The Effects of Bacterial Density on H_2O_2- *vs.* Sodium Hypochlorite-Mediated Killing

In the next series of experiments (Table 4.3), the densities of *P. gingivalis* and *S. aureus* were varied relative to titrated concentrations of either H_2O_2 or bleach, in order to determine the influence of varying target to bactericidal agent ratios, on outcome. Again there was no evidence of bacterial killing at the initial time point with H_2O_2, despite its relative increase in concentration to target ratio. This means that not only did the bacteria survive initial contact, but that there was no continued effect after plating. These experiments were done as spot assays so there were none of the dilution effects characteristic of spiral plating. Additionally, the non-lethal concentrations of 6.25µM H_2O_2 and 600µM H_2O_2 for 10^5 CFU/mL of *P. gingivalis* and *S. aureus*, respectively, were insufficient doses to kill any bacterial density, after 60 min of treatment. As in previous experiments (31), treatment of 10^5 CFU/mL of *P. gingivalis* or *S. aureus* with either 12.5µM or 2000µM H_2O_2, respectively, resulted in one logarithmic reduction in recoverable CFU/mL by 60 min (Table 4.3). Furthermore, these concentrations resulted in one logarithmic reduction despite increasing the target bacterial density 1,000-fold, or

decreasing the target bacterial density 100-fold. Two-fold increases in H_2O_2 generally resulted in logarithmic increases in killing regardless of bacterial density.

In contrast to H_2O_2, bleach killed *P. gingivalis* and *S. aureus* at initial time (0 min), when challenged with a sufficient (lethal) dose (≥0.001% Na^+ OCl^- v/v), and showed only a hint of killing progression over time with certain concentrations of bleach, unlike H_2O_2 (Table 4.3). Additionally, in contrast to H_2O_2, bleach (at 0.001% and 0.01% Na^+ OCl^- v/v) killed the lower bacterial densities of *P. gingivalis* and *S. aureus* better than the higher bacterial densities, at 0 and 60 min. In fact, 0.001% Na^+ OCl^- v/v killed the lower densities of *P. gingivalis* and *S. aureus* (≤10^5 CFU/mL) completely at 60 min, while there was no evidence of killing at the higher bacterial densities (≥10^6 CFU/mL) with this same concentration of bleach. Similarly, 0.01% Na^+ OCl^- v/v killed the lower densities of *P. gingivalis* and *S. aureus* (≤10^7 CFU/mL) at 60 min, while the highest bacterial density (10^8 CFU/mL) of *P. gingivalis* and *S. aureus* was not killed at all. With the next logarithmically higher bleach concentrations (0.1% and 1% Na^+ OCl^- v/v), however, every bacterial density of *P. gingivalis* and *S. aureus* was killed completely at 0 and 60 min.

Effect of DMSO on H_2O_2-Mediated Killing

The killing kinetics, temperature requirements, and the effects of bacterial density on the bactericidal activities of H_2O_2 were consistent with a requirement for bacterial conversion of H_2O_2 to a lethal product. The possible nature of this product was examined with the hydroxyl radical scavenger, DMSO (47), as a competitor of H_2O_2-mediated killing of *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *S. aureus* (Figure 4.2). DMSO was titrated in 10-fold increments, starting from 100mM FC. The outcome measure was recovery of $\log_{10}$ CFU/mL by spiral plating. Logarithmic

protection from killing (≥1 log increases in recoverable CFU/mL) in the presence of DMSO was considered significant.

As was previously determined, there was a broad range of susceptibilities to H_2O_2 for these selected bacterial species and the titrated concentrations were varied accordingly to allow the expression of bactericidal activity against each species (Figure 4.2). There was dose-dependent protection with DMSO against the bactericidal activities of H_2O_2 with all four species. With all four species, there was significant protection with 100mM DMSO and no effect with 0.1mM DMSO. The protective effects of DMSO were most evident with the most H_2O_2-resistant species tested, *S. aureus*. These data support hydroxyl radical as the effector mechanism of H_2O_2-mediated killing of *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *S. aureus*.

DISCUSSION

In previous studies, it was determined that the overt periodontopathogen, *P. gingivalis*, was remarkably resistant to killing by oxygen-independent mechanisms operating in the phagolysosome of a neutrophil, as there was no evidence of killing by normally competent neutrophils equilibrated in an anaerobic environment (48). This was in striking contrast to the highly efficient, though reduced, phagolysosome killing of the aerotolerant periodontopathogen, *A. actinomycetemcomitans*, under anaerobic conditions. Neutrophil-dependent killing of a second anaerobic periodontal bacterium, *F. nucleatum*, was also dramatically reduced in the absence of oxygen, but unlike with *P. gingivalis*, there was evidence of significant, though markedly delayed, killing, suggesting that this species shows some degree of sensitivity to oxygen-independent killing. Chemical (DPI) inhibition of the NADPH oxidase of the neutrophil also totally ablated killing of *P. gingivalis*, consistent with the inability of the NADPH oxidase-dysfunctional neutrophils of chronic granulomatous disease (CGD) patients to kill this pathogen (48). In contrast, DPI minimally reduced neutrophil killing of *A. actinomycetemcomitans*, and had substantial, but not total, inhibitory effects on the killing of *F. nucleatum* (48). These data were consistent with the relative sensitivities of these three pathogens to MPO-independent H_2O_2-mediated killing (31).

Myeloperoxidase-deficient neutrophils demonstrated efficient killing of *P. gingivalis* that was indistinguishable from that of MPO-sufficient control neutrophils (48). In contrast, selective inhibition of MPO with 4-ABH significantly delayed the killing progression of *P. gingivalis* by otherwise competent neutrophils in atmospheric oxygen (48). Subsequent studies determined that H_2O_2 was capable of MPO-independent killing that was actually totally blocked in the presence of MPO (31). The species, *P. gingivalis*, proved to be exceptionally sensitive to H_2O_2, compared to a range of

resistances for other species (31). In contrast, an MPO-dependent killing was demonstrable with sub-cidal concentrations of H_2O_2. All species, independent of their relative differences in sensitivities to H_2O_2, were killed by the same optimal concentration of H_2O_2 in the MPO-dependent system (31). These studies do not resolve what is actually happening in the phagosome and how this might differ from one target species to another.

The current studies clearly indicate that the principal purported bactericidal product of the MPO system, hypochlorite, is capable of potent killing of a range of target species. Surprisingly, these species were all killed by the same concentration of hypochlorite, if adjusted to the same number of target colony forming units (Table 4.2). This activity was energy-independent (no temperature effect) and the killing event was initiated on contact, as there was no significant progression of killing beyond the initial plating (time 0) (Table 4.1). These data suggest that a critical concentration of hypochlorite, generated in proximity to any of the target species, would result in an immediate lethal hit. If hypochlorite is responsible for the lethal outcome in the MPO-dependent killing of the previous studies (31), then the time required to kill must relate to the enzymatic kinetics necessary to reach critical lethal concentrations.

Our previous data would also suggest that hypochlorite is only generated by MPO across a very limited range of H_2O_2 concentrations in the presence of non-limiting concentrations of chloride (31). The maximum concentration of hypochlorite achievable at optimal H_2O_2 concentrations would be at 5μM (31). The 1% (v/v) treatment solution of the bleach reagent used in these experiments is estimated to contain ~70μM NaOCl. Dilutions to 0.001% (~70nM) were effective at killing 10^5 CFU/mL of target bacteria, but ten-fold more (~700nM) was required to affect killing of 10^6 CFU/mL and to give the first evidence of a temporal requirement with 10^7 CFU/mL, with both *P. gingivalis* and *S. aureus* (Table 4.3). Micromolar concentrations of hypochlorite were required to kill 10^8

CFU/mL. It is likely that at concentrations of <0.001%, the bleach is effectively neutralized by the reaction environment, as there was no evidence of killing with 0.0001% bleach, even with a 100-fold reduction in target bacteria density (Table 4.3). It is therefore estimated that a maximum of 90% of the bleach would be available for interaction with the bacteria in the 0.001% treatment mixture.

From this it can be estimated that a maximum of 60pmoles of reactive hypochlorite would be available in a one mL volume, resulting in a ratio of 10^6 molecules of NaOCl/ CFU of target bacteria. The best estimate for the minimum lethal number of molecules of hypochlorite would best be derived from the 0.01% treatment of 10^7 CFU/mL, as the delay in evident killing was apparent at this ratio of agent to target (Table 4.3). This would suggest that treatment conditions containing a minimum of 10^5 molecules of hypochlorite per target CFU would be required to achieve a lethal hit. The Winterbourn model (15) of ROS reactions in the neutrophil phagosome estimates that HOCl would be generated at the rate of 134mM/min in the phagosome volume. This would result in concentrations orders of magnitude greater than that necessary to affect a lethal hit based on the observations in the current study. This excess quantity may be necessary to overcome the competition from the protein- rich environment of the phagosome.

An assumption of the Winterbourn model (15) is that $O_2^{\cdot-}$ and H_2O_2 are generated initially in the presence of optimal concentrations of MPO. In this model (15), H_2O_2 is consumed by MPO as rapidly as it is derived from the dismutation of $O_2^{\cdot-}$ to yield a steady state concentration of H_2O_2 in the low micromolar range. In contrast to this model, however, the NADPH oxidase is assembled in active form on the phagosome membrane surface, primarily from fusion with the secretory specific granule, prior to the delivery of MPO-containing lysosomal primary granules (1) (2) (3) (4) (5) (11). This would result in the generation of 5.2mM/sec of $O_2^{\cdot-}$ and dismutation to H_2O_2 in immediate

proximity and in high concentration to the bacterial target, at least initially in the absence of the influence of MPO. It therefore is of importance to consider the nature of the interactions of H_2O_2 that lead to the lethal events previously described (15).

Unlike hypochlorite, exposure of target bacteria to lethal concentrations of MPO-independent H_2O_2 resulted in a progressive loss of recoverable CFU, with no killing evident on initial contact, even at concentrations in great excess of that necessary to totally kill (Table 4.3) (31). Reducing the treatment concentrations of H_2O_2 lengthened the time it took to observe killing (31). It is important to note that in order to achieve logarithmic reductions in CFU of *P. gingivalis* by 60 min, a concentration of ~20μM H_2O_2 was required; while even 6.25μM was bactericidal if the treatment was allowed to continue to 180 min (31). All concentrations killed better the longer treatment was continued. This is in striking contrast to the all-or-none activity of NaOCl (Tables 4.1-3).

Further insight into the action of H_2O_2 can be gained from examining the influence of altering the target densities of the bacteria, relative to the treatment concentrations of H_2O_2 (Table 4.3). Consistent with all previous observations with *P. gingivalis* (31), there was a logarithmic reduction in the population density of 10^5 CFU/mL, after 60 min of treatment with 12.5μM, and no detectable reduction at 6.25μM (Table 4.3). Unlike with bleach, this concentration (12.5μM) also affected logarithmic reductions with each of the higher densities including 10^8 CFU/mL. Conversely, this concentration resulted in logarithmic, but not total, killing of the lower densities of bacteria, and these reductions in bacterial density did not permit lower concentrations of H_2O_2 to kill. Each two-fold increase in H_2O_2 concentration resulted in a logarithmic increase in killing of *P. gingivalis* through 100μM (Table 4.3). As previously determined (31), the concentration of H_2O_2 required to affect logarithmic reductions in *S. aureus* were exponentially higher, but the killing properties were affected in the same way by altering target density as with *P. gingivalis* (Table 4.3). In contrast, 0.001% NaOCl

resulted in a three log reduction in the 10^5 CFU/mL suspensions of both *P. gingivalis* and *S. aureus* at 60 min, but failed to kill ≥10^6 CFU/mL, while totally killing ≤10^5 CFU/mL (Table 4.3). Increasing the concentration of NaOCl directly increased the number of bacteria that were killed. Despite the increased resistance of *S. aureus* to H_2O_2, the susceptible target densities as CFU/mL for NaOCl were identical for *P. gingivalis* and *S. aureus* (Tables 4.3).

These data are consistent with the hypothesis that bleach is directly and immediately toxic; whereas, H_2O_2 requires conversion to something else in order to kill. The fact that the bactericidal activity was concentration-dependent, but independent of target density, is consistent with each target bacterium acting on the H_2O_2 in its immediate vicinity, and converting it to a more reactive lethal product. H_2O_2 is freely diffusible and relatively non-reactive (6) (7) (8). In the presence of exogenous H_2O_2, a bacterium would likely achieve a critical bactericidal concentration of H_2O_2 within the periplasmic membrane that is then converted to more reactive ROS in proximity to vital targets such as DNA. This reaction is likely energy-dependent and possibly dependent on the metabolism of the target bacteria (17). This would explain the temperature-dependence for H_2O_2 toxicity and the kinetics of killing (Figure 4.1). If the lethal hit is delivered to the interior of each target, then this would explain the varied concentration requirements of the treatment environment, but the lack of influence by changing bacterial density (Table 4.3). Bacteria that are able to reduce the environmental concentrations of H_2O_2 (e.g. the catalase- producers such as *S. aureus* and *A. actinomycetemcomitans*) would require greater concentrations to raise the internal H_2O_2 to lethal levels. Likewise, bacteria that are capable of converting H_2O_2 post-diffusion to more innocuous products (e.g. the cytosolic peroxidases of streptococci) would also require higher exogenous concentrations.

It is clear that H_2O_2 in the presence of a suitable transition metal can be converted to the highly reactive and potentially toxic product, $OH^{\cdot}$, through the Fenton reaction (7) (10) (13) (16) (17) (22) (23) (24) (25) (26) (27), though there is controversy regarding whether this ROS plays any role in the bactericidal activity in the phagosome due to competition of H_2O_2 with MPO (49). In our previous studies with human neutrophils, we reported that sodium azide totally inhibited phagosome killing of *P. gingivalis*, not only in normally competent neutrophils, but also with MPO-deficient neutrophils (48). These observations, plus the lack of similar effect using 4-ABH treatment, suggested that *P. gingivalis* was being killed by an azide-inhibitable, MPO-independent mechanism (48). In addition to a number of other properties, azide is reported to scavenge $OH^{\cdot}$ (50). In an attempt to determine if $OH^{\cdot}$ was contributing to the toxicity of MPO-independent H_2O_2 bactericidal activity, the highly specific $OH^{\cdot}$ scavenger (47), DMSO, was examined to determine its ability to inhibit H_2O_2-mediated killing of the various target bacteria (Figure 4.2). These data indicate that killing of each of the bacteria, regardless of their H_2O_2 requirements for killing, could be significantly reduced with DMSO. Given the reactive nature of $OH^{\cdot}$ (6) (7) (10) (13) (16) (17) (22) (23) (24) (25) (26) (27), and the presumed proximity to the susceptible target that this ROS must be generated, any protective effect by DMSO is highly suggestive of the involvement of $OH^{\cdot}$ in killing.

These studies suggest that if bacteria are exposed to levels of H_2O_2 sufficient to reach critical lethal concentrations inside the cell, then this could be an effective vehicle for delivery of $OH^{\cdot}$. In previous studies, it was reported that the presence of low concentrations of MPO would subvert this bactericidal activity (31). Inhibition by MPO assumes that it has access to H_2O_2 on the outside of the cell before it has had a chance to diffuse to the target sites. Once within the bacterial membrane, MPO would be ineffective at neutralizing the bactericidal progression of H_2O_2. During the process of

encountering an opsonized target, there is a hierarchical delivery of discrete granule subsets through fusion with the plasma membrane and the developing phagosome (1) (2) (3) (4) (5) (11). This results in an ordered delivery of the granule defining constituents contained in both the milieu of the matrix and in the granule membrane. This process results in prescribed progressive exposure of the phagocytized target to a discretely changing environment.

In particular consideration for the studies reported here, the first granules and subcellular membrane vesicles shown to be delivered are those containing the membrane constituent components of NADPH oxidase, followed quickly by the mobilization of the cytosolic constituents, resulting in an active enzyme complex that rapidly consumes oxygen to generate $O_2^{\cdot -}$ at a rate estimated to approach 5.2mM/sec, delivering both $O_2^{\cdot -}$ and H_2O_2 to the surface of the bacteria in the initial absence of MPO. This high level of NADPH oxidase products in the small volume of the phagosome could quickly result in critical concentrations of H_2O_2 within the interior of the bacteria that may even be facilitated by the production of superoxide dismutase, but not catalase or peroxidase by the bacteria, as would be the case with *P. gingivalis*. This could even be true for the mM concentrations of H_2O_2 necessary to kill the more resistant target bacteria. The MPO would then be delivered by the later arriving azurophilic granules (11) that presumably would subvert the NADPH oxidase products away from H_2O_2 to mM concentrations of HOCl, and a steady state of $O_2^{\cdot -}$ and low levels of H_2O_2 that would preserve the peroxidatic activity of the enzyme. This suggests that both MPO-independent and MPO-dependent mechanisms of H_2O_2-mediated killing may be progressively functioning in the phagosome.

Figure 4.1. Effects of Temperature on Hydrogen Peroxide-Mediated Killing of *P. gingivalis* at 60 min. Results are expressed as recoverable $\log_{10}$ CFU/mL. Samples were spiral plated (limit of detection: 1.31 $\log_{10}$ CFU/mL), and logarithmic differences in recoverable CFU/mL were considered significant. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three independent experiments. Experiments were performed in water baths either with ice, at room temperature (RT), or at 37°C. Bacteria and H_2O_2 were equilibrated for at least 15 minutes on ice, at RT, or at 37°C before the start of these experiments. Asterisks indicate significant differences from the 37°C treatment values.

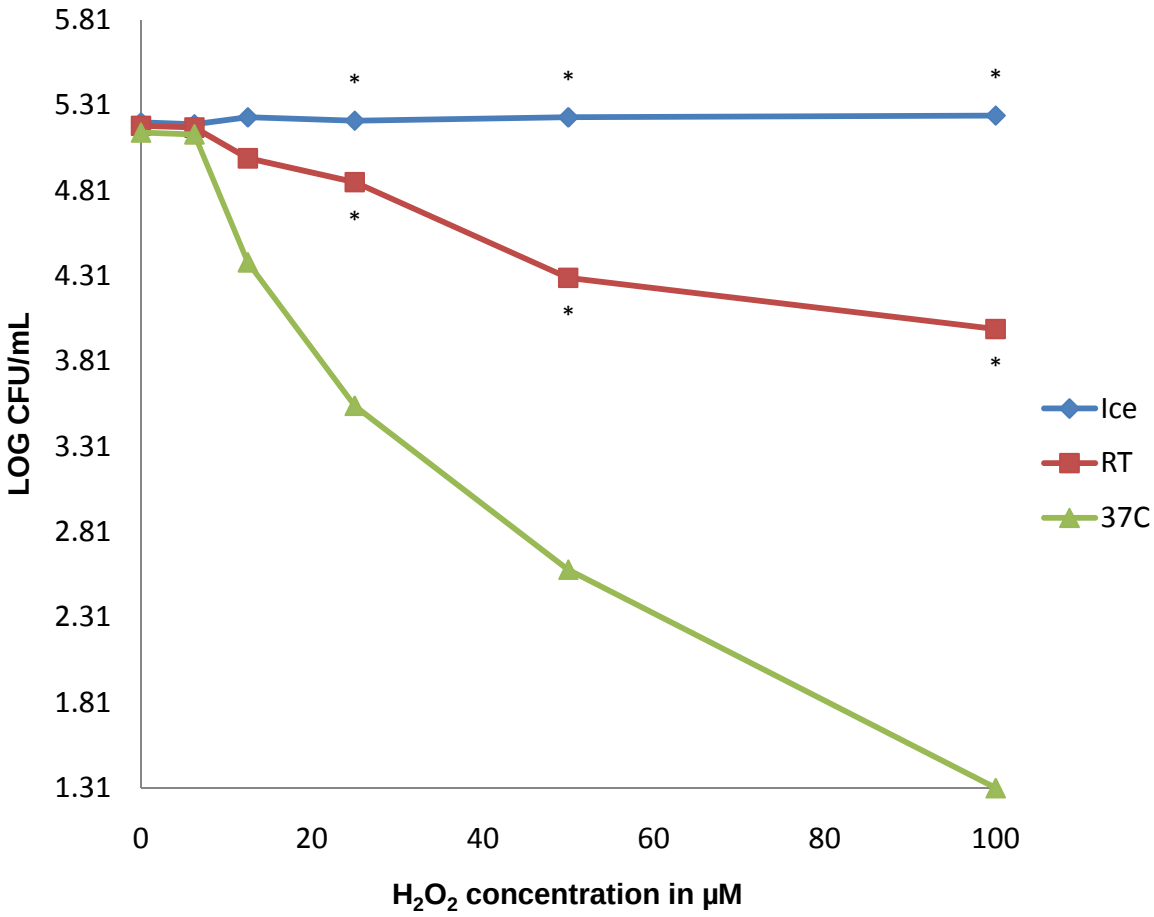


Table 4.1. Effects of Time and Temperature on Sodium Hypochlorite-Mediated Killing of *P. gingivalis.* Sodium hypochlorite (bleach) concentrations are expressed as % final concentrations, vol /vol. Treatment volumes contained a final density of ~10^5 CFU/mL of *P. gingivalis*. Since the limit of detection was 2.0 $\log_{10}$ CFU/mL, a maximum of three log reductions could be detected. Killing was expressed as % recoverable $\log_{10}$ CFU/mL, where 100% recoverable CFU/mL indicates no logarithmic reductions detected (no killing) and <0.1% recoverable CFU/mL indicates there were no recoverable CFU. Samples were plated at least in duplicate. Data are representative of three independent experiments. Experiments were performed in water baths either at 37°C or with ice. Bacteria and H_2O_2 were equilibrated for at least 15 minutes at 37°C or on ice before the start of these experiments.

37°C	**% Recoverable CFU/mL**		
% Bleach	**t = 0 hr**	**t = 1 hr**	**t = 2 hr**
0	100	100	100
10^{-6}	100	100	100
10^{-5}	100	100	100
10^{-4}	100	100	100
10^{-3}	1	<0.1	<0.1
10^{-2}	<0.1	<0.1	<0.1
10^{-1}	<0.1	<0.1	<0.1
1	<0.1	<0.1	<0.1

Ice	**% Recoverable CFU/mL**		
% Bleach	**t = 0 hr**	**t = 1 hr**	**t = 2 hr**
0	100	100	100
10^{-6}	100	100	100
10^{-5}	100	100	100
10^{-4}	100	100	100
10^{-3}	1	<0.1	<0.1
10^{-2}	<0.1	<0.1	<0.1
10^{-1}	<0.1	<0.1	<0.1
1	<0.1	<0.1	<0.1

Table 4.2. Killing Kinetics of *P. gingivalis, F. nucleatum, S. aureus,* and *S. pyogenes* with Sodium Hypochlorite at 37°C at 0 and 60 min. Sodium hypochlorite (bleach) concentrations are expressed as % final concentrations, vol /vol. Samples were spiral plated (limit of detection: 1.31 $\log_{10}$ CFU/mL, where <1.31 indicates no recoverable CFU on replicate plating), and logarithmic reductions in recoverable CFU/mL were considered significant. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three independent experiments.

Recoverable $\log_{10}$ CFU/mL							
	P. gingivalis		*F. nucleatum*		*S. aureus*		
% Bleach	**0 min**	**60 min**	**0 min**	**60 min**	**0 min**	**60 min**	
0	4.64	4.6	4.4	4.16	4.51	4.46	
2×10^{-6}	4.65	4.69	4.38	4.21	4.54	4.45	
2×10^{-5}	4.6	4.62	4.37	4.18	4.52	4.47	
2×10^{-4}	4.68	4.65	4.38	4.2	4.51	4.49	
2×10^{-3}	<1.31	<1.31	<1.31	<1.31	<1.31	<1.31	
2×10^{-2}	<1.31	<1.31	<1.31	<1.31	<1.31	<1.31	
2×10^{-1}	<1.31	<1.31	<1.31	<1.31	<1.31	<1.31	
2×10^{0}	<1.31	<1.31	<1.31	<1.31	<1.31	<1.31	

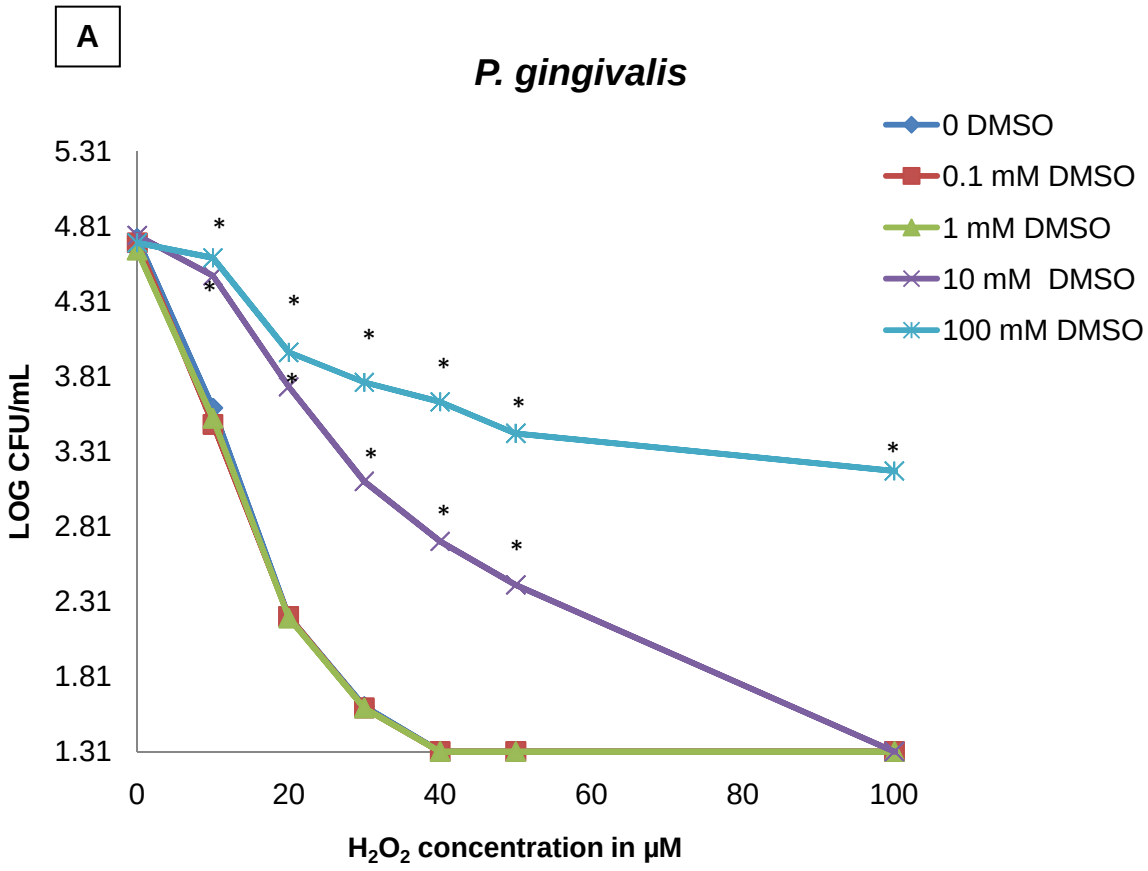
Recoverable $\log_{10}$ CFU/mL							
	P. gingivalis		*S. aureus*		*S. pyogenes*		
% Bleach	**0 min**	**60 min**	**0 min**	**60 min**	**0 min**	**60 min**	
0	4.79	4.7	4.51	4.41	4.47	4.44	
3.13×10^{-4}	4.74	4.75	4.51	4.39	4.48	4.48	
6.25×10^{-4}	4.77	4.68	4.63	4.5	4.53	4.52	
1.25×10^{-3}	2.76	<1.31	2.42	<1.31	2.35	<1.31	
2.5×10^{-3}	<1.31	<1.31	<1.31	<1.31	<1.31	<1.31	
5×10^{-3}	<1.31	<1.31	<1.31	<1.31	<1.31	<1.31	
1×10^{-2}	<1.31	<1.31	<1.31	<1.31	<1.31	<1.31	

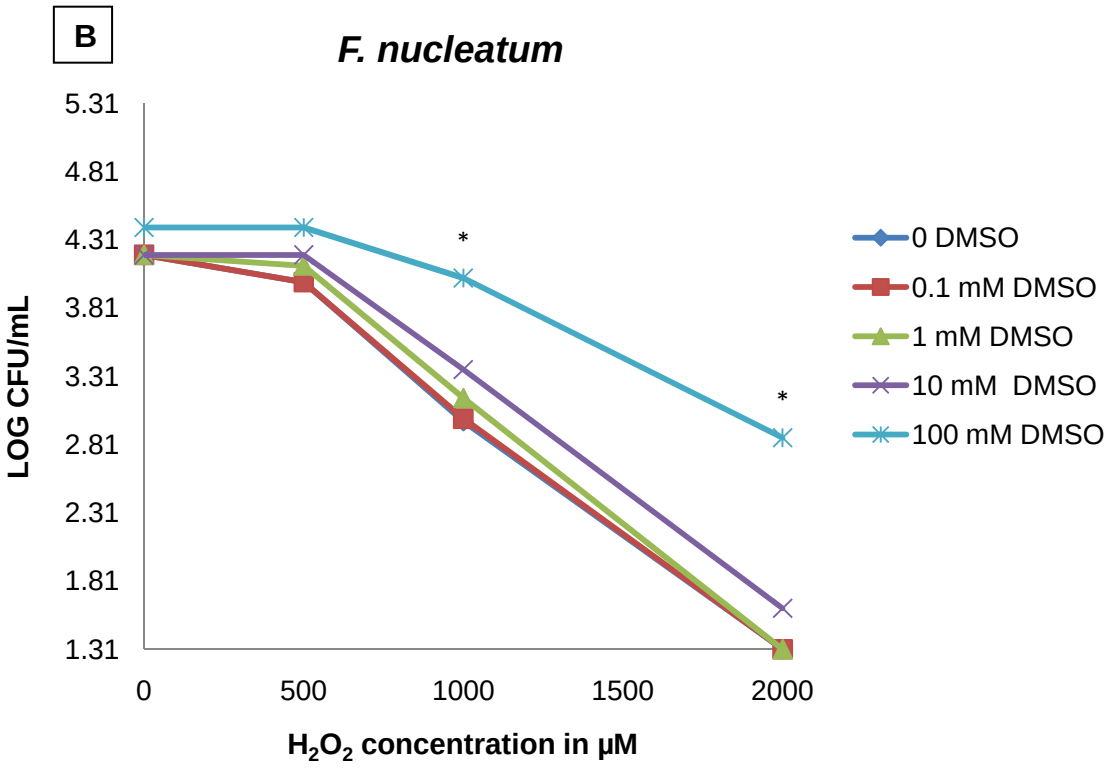
Table 4.3. Effects of Varying Bacterial Density on Hydrogen Peroxide- *vs*. Sodium Hypochlorite- Mediated Killing of *P. gingivalis* and *S. aureus* at 37°C at 0 and 60 min. Results are expressed as recoverable log_{10} CFU/mL. H_2O_2 concentrations are expressed as FC in µM, and sodium hypochlorite (bleach) concentrations are expressed as % FC, vol /vol. *P. gingivalis* and *S. aureus* were titered 10-fold, so that starting inoculums varied logarithmically per sample, from 10^8 – 10^3 log_{10} CFU/mL, final concentration. Limit of detection was 2.0 log_{10} CFU/mL; <2 indicates no recoverable CFU on replicate plating. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three independent experiments.

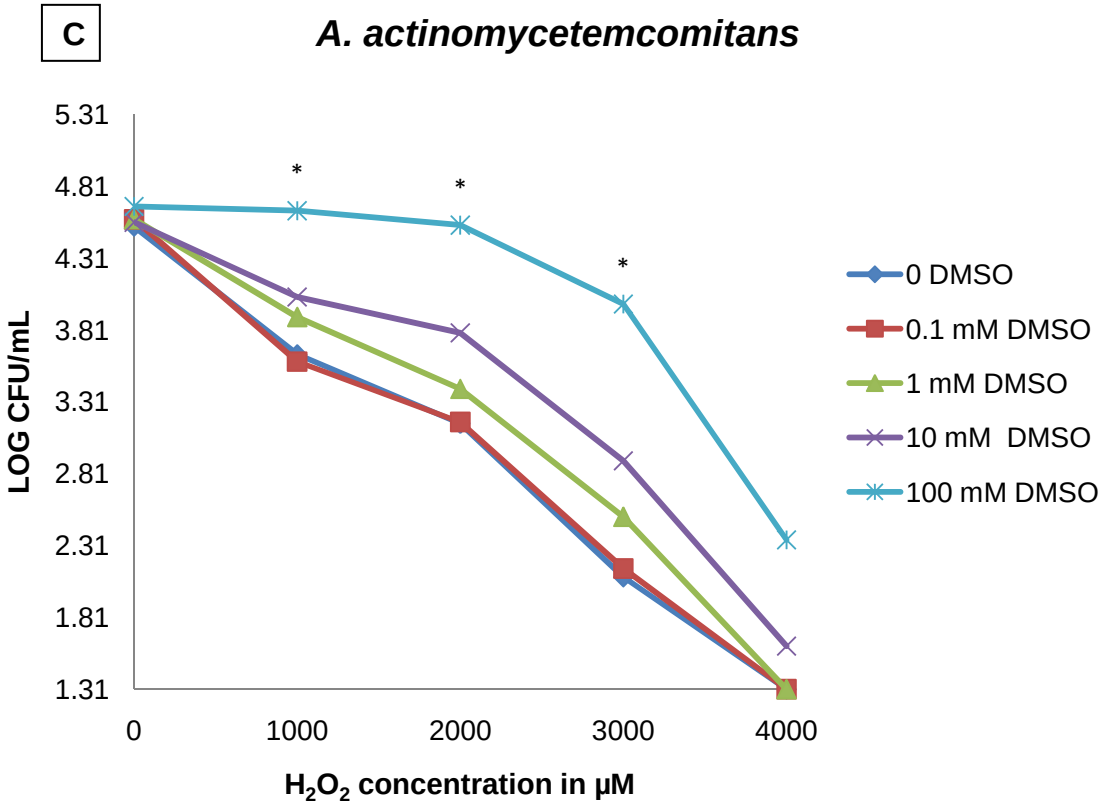
0 min	*P. gingivalis* Recoverable Log_{10} CFU/mL							0 min	*P. gingivalis* Recoverable Log_{10} CFU/mL					
[H_2O_2] in µM	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**		**% Bleach**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
0	3	4	5	6	7	8		**0**	3	4	5	6	7	8
6.25	3	4	5	6	7	8		**10^{-4}**	3	4	5	6	7	8
12.5	3	4	5	6	7	8		**10^{-3}**	<2	<2	3	6	7	8
25	3	4	5	6	7	8		**10^{-2}**	<2	<2	<2	<2	7	8
50	3	4	5	6	7	8		**10^{-1}**	<2	<2	<2	<2	<2	<2
100	3	4	5	6	7	8		**1**	<2	<2	<2	<2	<2	<2
60 min								**60 min**						
[H_2O_2]	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**		**% Bleach**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
0	3	4	5	6	7	8		**0**	3	4	5	6	7	8
6.25	3	4	5	6	7	8		**10^{-4}**	3	4	5	6	7	8
12.5	2	3	4	5	6	7		**10^{-3}**	<2	<2	<2	6	7	8
25	<2	2	3	4	5	6		**10^{-2}**	<2	<2	<2	<2	4	8
50	<2	<2	2	3	4	5		**10^{-1}**	<2	<2	<2	<2	<2	<2
100	<2	<2	<2	2	3	3		**1**	<2	<2	<2	<2	<2	<2

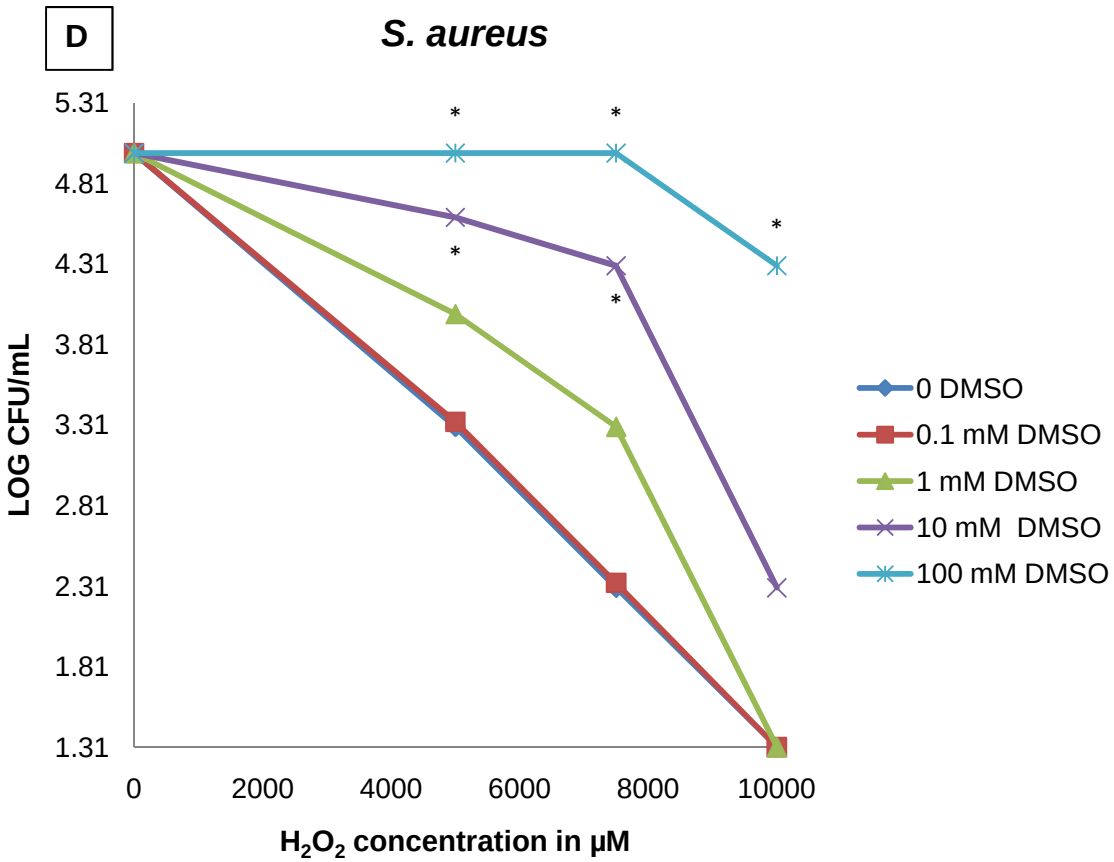
0 min	*S. aureus* Recoverable Log_{10} CFU/mL							0 min	*S. aureus* Recoverable Log_{10} CFU/mL					
[H_2O_2]	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**		**% Bleach**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
								0	3	4	5	6	7	8
0	3	4	5	6	7	8		**10^{-4}**	3	4	5	6	7	8
600	3	4	5	6	7	8		**10^{-3}**	<2	<2	<2	6	7	8
2000	3	4	5	6	7	8		**10^{-2}**	<2	<2	<2	<2	7	8
6000	3	4	5	6	7	8		**10^{-1}**	<2	<2	<2	<2	<2	<2
								1	<2	<2	<2	<2	<2	<2
60 min								**60 min**						
[H_2O_2]	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**		**% Bleach**	**10^3**	**10^4**	**10^5**	**10^6**	**10^7**	**10^8**
								0	3	4	5	6	7	8
0	3	4	5	6	7	8		**10^{-4}**	3	4	5	6	7	8
600	3	4	5	6	7	8		**10^{-3}**	<2	<2	<2	6	7	8
2000	2	3	4	5	6	7		**10^{-2}**	<2	<2	<2	<2	4	8
6000	<2	2	3	4	4	5		**10^{-1}**	<2	<2	<2	<2	<2	<2
								1	<2	<2	<2	<2	<2	<2

Figure 4.2. Effects of DMSO on H_2O_2- Mediated Killing of *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans,* and *S. aureus* at 60 min and 37°C. Results are expressed as recoverable $\log_{10}$ CFU/mL. Samples were spiral plated (limit of detection: 1.31 $\log_{10}$ CFU/mL), and logarithmic protection from killing (≥1 log increases in recoverable CFU/mL compared to killing by H_2O_2 in the absence of DMSO) was considered significant as indicated by asterisks. Samples were plated at least in duplicate, and colony counts were averaged. Data are representative of three or more independent experiments.




B
F. nucleatum
5.31
4.81
4.31
3.81
3.31
2.81
2.31
1.81
1.31
LOG CFU/mL
0
500
1000
1500
2000
H2O2 concentration in µM
*
*
0 DMSO
0.1 mM DMSO
1 mM DMSO
10 mM DMSO
100 mM DMSO


C
A. actinomycetemcomitans
5.31
4.81
4.31
3.81
3.31
2.81
2.31
1.81
1.31
LOG CFU/mL
0
1000
2000
3000
4000
H2O2 concentration in µM
*
*
*
0 DMSO
0.1 mM DMSO
1 mM DMSO
10 mM DMSO
100 mM DMSO


D
S. aureus
5.31
4.81
4.31
3.81
3.31
2.81
2.31
1.81
1.31
LOG CFU/mL
0
2000
4000
6000
8000
10000
H2O2 concentration in µM
0 DMSO
0.1 mM DMSO
1 mM DMSO
10 mM DMSO
100 mM DMSO

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CHAPTER 5

GENERAL DISCUSSION

AFTERWORD

It is well-known that neutrophils are indispensable to periodontal health, supported by the fact that individuals with impaired neutrophil functions have a high prevalence of an early onset of aggressive periodontitis (1) (2) (3) (4) (5) (6) (7) (8) (9) (10). The health of periodontal tissues is therefore highly dependent on proper neutrophil function (e.g. chemotaxis, phagocytosis, bactericidal mechanisms), as periodontitis, which is sometimes mislabeled or generalized by medical professionals (non-dentist) as severe gingivitis, stomatitis, or ulcerative oral lesions around the teeth, is frequently one of the first manifestations of a systemic breakdown in neutrophil function (11) (12). While these periodontal infections may initially appear in these patients as severe gingivitis, they are not simply gingivitis, and the patient should be referred to a periodontist, for identification of specific periodontal pathogens and therapies appropriate to these microorganisms (8) (11). Besides tooth loss, the importance of prompt treatment is implicated in the fact that periodontitis is linked to an increased risk of premature heart disease, stroke, lung infections, preterm low birth weight pregnancy outcomes, and septicemia, as well as to a variety of other chronic health issues (13).

In this laboratory, plaque samples cultured from children and adolescents who present with periodontitis show unusually high levels of specific adult periodontal pathogens, which behave as opportunistic pathogens when there is a breakdown in periodontal defense. While normally healthy children and adolescents are suspected to be exposed to adult periodontal pathogens by parents etc (14) (15) (16), the diagnosis of periodontitis in children and young adults is highly abnormal, and is suggestive of a serious underlying medical illness such as a neutrophil disorder (8) (11) (12). The abnormal presentation of periodontitis in young individuals is also suggestive that the

periodontium is normally resistant to developing periodontal disease as long as the periodontal (i.e. neutrophil) defense mechanisms remain intact.

Despite the importance of neutrophils in the maintenance of periodontal health, relatively few studies have examined the critical killing mechanisms used by neutrophils when periodontal tissues are exposed to particular pathogens. While it is understood that the main method by which neutrophils handle potential periodontal pathogens is through phagocytosis and killing (6) (17) (18) (19) (20), the specific post-phagocytic bactericidal mechanism(s) essential to the defense against specific periodontal pathogens have yet to be defined. While adult periodontitis is a mixed flora infectious and inflammatory disease, pathology is associated with a relatively small group of overt periodontal pathogens (21).

The overall objective of this dissertation was therefore to elucidate the mechanism(s) used by neutrophils to kill *Porphyromonas gingivalis*, an overt periodontal pathogen that is the most associated with the etiology of both chronic and aggressive forms of severe adult periodontitis (14) (22) (23) (24) (25). This dissertation project, which focused on defining the conditions and components essential for neutrophil-mediated killing of this pathogen, as well as understanding the relative susceptibility of this species to these killing mechanisms compared to other relevant pathogens, have undoubtedly offered valuable insights into how normally healthy people are resistant to periodontitis and likewise how others are susceptible to periodontitis; as well as offered clues about therapies appropriate to this pathogen.

Several studies have shown that different isolates of *P. gingivalis* express discrete virulence traits in animal models, which may reflect distinct clinical presentations (26) (27) (28) (29) (30) (31) (32). Strain A7436 is a clinical isolate of *P. gingivalis* that was chosen for its expression of multiple virulence traits considered representative of this periodontopathic species, which include capsule and resistance to phagocytosis (27)

(33) (34) (35). *P. gingivalis* A7436 was originally isolated from a patient with aggressive periodontitis, and has been characterized by the late V.R. Dowell (36), and by this laboratory (27) (28) (34) (35) (37), and others (29) (33) (38). In the mouse subcutaneous chamber model, A7436 is remarkably resistant to neutrophil phagocytosis and is capable of spreading and dissemination, leading to septicemia and death of the mouse (27) (28) (29).

OVERALL SUMMARY, DISCUSSION, AND CONCLUSIONS

It is widely held that the primary defense mechanism operative in the periodontal pocket involves the teamwork of neutrophils, antibody, and complement (19). The importance of these components in periodontal defense seem to be supported by the prolific serous and cellular infiltrate in diseased pockets, which is dominated by antibody-secreting plasma cells and neutrophils, which comprise at least 95% of the leukocyte influx (39) (40). Previous studies have shown that *P. gingivalis* has a striking ability to recruit neutrophils into infected sites, as well as to induce a significant serum and local antibody response, which is predominantly IgG (27) (41) (42) (43). While subjects with adult periodontitis have been shown to have elevated IgG antibody titers to many *P. gingivalis* antigens (41) (42) (43), serum samples isolated from many of these subjects, however, lack opsonic capability toward specific strains of *P. gingivalis* such as A7436 (20), which suggests that the antibody response is not always protective in periodontitis. In the face of a large number of neutrophils, as well as antibody and complement, *P. gingivalis* has the potential to multiply and thrive in active periodontitis (44), as well as in the mouse subcutaneous chamber model, where certain strains including A7436 are remarkably adept at evading phagocytosis (27). Low avidity IgG responses have been attributed to a lack of sufficient antibody specificity to opsonize and phagocytose infecting strains (20) (45), and have even been suggested to function as an immune decoy to divert the attentions of the immune response (19). Some *P. gingivalis* infections appear unable to be resolved (refractory), and these studies elucidate many of the reasons why.

The main objective of chapter 2 was to examine what is necessary in order for *P. gingivalis* to be killed by neutrophils, since the periodontium is resistant to infections with periodontal pathogens such as *P. gingivalis*, when the protective functions of neutrophils

are intact. After neutrophil chemotaxis to an infection site, the first step in defense against *P. gingivalis* is phagocytosis. In order to begin to define the specific components essential for neutrophil-mediated suppression of this pathogen, a dual-color fluorescence assay was used to investigate under what conditions the human neutrophil phagocytizes and then kills *P. gingivalis* A7436. Differential interference contrast optics and the fluorescent dyes DAPI/PI were used to visualize the phagocytic and microbicidal activities, respectively, of neutrophils under epi-illumination UV microscopy. This approach allows direct visualization of both phagocytosis and killing, enabling a clear and straightforward interpretation: the bacteria are either within the confines of the neutrophil plasma membrane or not, and are either alive (blue) or dead (red). Figure A.3 in the appendix accompanying chapter 2 presents examples of how the data in chapter 2 were obtained.

Preliminary studies in this laboratory using DAPI/PI have shown that *P. gingivalis* is not killed in the absence of phagocytosis, though normally competent neutrophils are capable of killing *P. gingivalis*, once phagocytized (20). Previous studies have also suggested that several strains of *P. gingivalis* including A7436 exhibit remarkable resistance to neutrophil phagocytosis due in part to the production of a capsule, and to the proteolytic degradation of serum opsonins and their receptors (26) (46) (47) (48) (49) (50) (51) (52) (53) (54). However, phagocytosis-resistant strains of *P. gingivalis* including A7436 are readily phagocytized in optimum conditions, which include the presence of serum containing both complement and hyperimmune antiserum specific for *P. gingivalis* (37). It has been shown that the addition of high titered specific antibody (IgG) to *P. gingivalis* can facilitate phagocytosis not only by specificity for capsule, but also through reducing and/or slowing the proteolytic destruction of the opsonins C3b, C3bi, and IgG, as well as the complement (CR1 and CR3) and Fc receptors on the neutrophil (20) (34) (55), enabling a window of time for phagocytosis to take place. This

is speculated to occur by gingipain-specific antibodies binding to and temporarily inhibiting (masking) the discrete proteases of *P. gingivalis*, thereby enhancing opsonization by increasing surface deposition of opsonic complement and C3-IgG complexes (34) (45) (56).

In chapter 2, we confirmed that neutrophil phagocytosis of *P. gingivalis* A7436 requires opsonization with both complement and high titered specific antibody, as there was only a small subset (10%) of neutrophils capable of phagocytosis of A7436 in the presence of complement but not specific antibody (Table A.1), and essentially no neutrophils capable of phagocytosis of A7436 in the absence of both of these opsonins (data not shown), through the two hour test period. This was in striking contrast to neutrophils in the presence of both complement and high titered specific antibody to *P. gingivalis*, which resulted in a rapid and significant involvement of neutrophils in phagocytosis of A7436 at initial sampling (>70% at 0 time) that progressed to effectively 100% of neutrophils involved in phagocytosis by two hours (Table A.1). It is possible that the small subset (10%) of neutrophils able to phagocytize *P. gingivalis* A7436 in the presence of complement but not specific antibody (Table A.1) was somehow different from the neutrophils (90%) unable to phagocytize A7436 in the absence of specific antibody. Perhaps this small subset expressed different or a greater numbers of receptors for phagocytosis, or may simply reflect the percentage of neutrophils able to phagocytize *P. gingivalis* through the alternative pathway (34) (45), before complement and/or its receptors are degraded by *P. gingivalis*. Likewise, 90% may reflect the percentage of neutrophils unable to phagocytize *P. gingivalis* in time. This is in agreement with the concept proposed by Cutler *et al* (34) (45), suggesting that most of the opsonic activity of antibody for *P. gingivalis* A7436 occurs via antibody-dependent alternative pathway opsonization, though contributions are also made by the classical

pathway. Additionally, phagocytosis was shown to occur independent of the availability of oxygen (Table A.1).

From previous studies using electron microscopy, *P. gingivalis* A7436 is singly taken up into individual phagosomes (unpublished observations) for killing. No studies to date clearly define the specific mechanism(s) used by neutrophils to kill *P. gingivalis*, once phagocytized. The significant findings of chapter 2 were that in addition to phagocytosis, neutrophil killing of *P. gingivalis* A7436 absolutely requires a functional NADPH oxidase, which is absolutely dependent on the availability of oxygen. There was essentially no killing of phagocytized *P. gingivalis* by normally competent neutrophils in anaerobic atmosphere (Table 2.1); by neutrophils from subjects with chronic granulomatous disease (CGD) in aerobic atmosphere (Table 2.2); or by normally competent neutrophils in aerobic atmosphere in the presence of the NADPH oxidase inhibitor, diphenyleneiodonium (DPI) (Table 2.3). In all three cases, the NADPH oxidase was inhibited, superoxide was not generated (confirmed in Table A.3 and data not shown), and there was no killing of *P. gingivalis* A7436, despite normal phagocytosis, even after several hours. This was in striking contrast to NADPH oxidase-functioning neutrophils from normally healthy donors in the presence of oxygen (Table A.3), where there was a significant involvement of phagocytic neutrophils in killing by one hour (>75%) that progressed to nearly 100% involved in killing by three hours (Table 2.3).

Because even a small amount of oxygen introduced into the anaerobic chamber was enough oxygen to allow neutrophil killing of A7436 (unpublished observations), efforts were required to sufficiently reduce all reagents before use in anaerobic experiments, which required at least one hour. Preliminary experiments attempted shorter and longer reduction times, though reducing reagents for less than one hour left enough oxygen to allow neutrophil killing of A7436, while going much longer started to

compromise the viability of neutrophils due to their short half-lives, and showed more apoptosis at later time points. This provided further confirmation that normally competent neutrophils are capable of killing A7436 if even small amounts of oxygen are available.

In order to determine whether the oxygen and NADPH oxidase requirements for killing *P. gingivalis* may be unique or common to other species associated with periodontitis, neutrophil killing of *P. gingivalis* was compared with that of *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum*, under aerobic *vs.* anaerobic atmosphere, and in the presence and absence of DPI. Untreated normal neutrophils under aerobic atmosphere were capable of rapid cell-association and killing of all three periodontal pathogens, with progressions of each through 180 min (Table 2.8). In contrast to *P. gingivalis*, normal neutrophils under anaerobiosis (Table 2.7) or DPI (Table 2.8) were capable of near normal killing of *A. actinomycetemcomitans*; and substantial, though significantly reduced, killing of *F. nucleatum*. Compared to these species associated with periodontitis, *P. gingivalis* A7436 was remarkably, totally resistant to the non-oxidative killing mechanisms of the neutrophil, which by definition, should still be operational under anaerobiosis, in the presence of DPI, and in CGD; and was instead completely dependent on an NADPH oxidase-dependent mechanism for killing. In contrast, *A. actinomycetemcomitans* was highly susceptible to non-oxidative killing mechanisms, as well as to DPI-insensitive mechanisms; while *F. nucleatum* was relatively more sensitive to non-oxidative killing mechanisms, as well as to DPI-insensitive mechanisms, than was *P. gingivalis*, which was totally resistant to both.

There was absolutely no evidence of oxygen-independent killing of A7436, including that of serum-mediated, extracellular killing in the absence of phagocytosis. Like its mechanism of resistance to phagocytosis (34), the resistance of *P. gingivalis* to the bactericidal activity of serum is also attributed to both the production of capsule and

to the proteolytic degradation of complement in serum, which together prevent the membrane attack complex from assembling on and penetrating its surface (54) (57) (58).

In addition to capsule, *P. gingivalis* produces and releases large quantities of broad-spectrum, as well as specific and discrete proteases, which undoubtedly contribute to its pathogenicity and ability to evade many of the host defenses. Of the many proteases identified in *P. gingivalis*, the three gingipains, encoded by the genes *rgp*A, *rgp*B, and *kgp*, are generally considered the major virulence factors of this periodontal pathogen (46) (59), and are reported to be responsible for at least 85% of its overall proteolytic activity (60) (61). Demonstrating 'trypsin-like' activities, the arginine gingipains (HRgpA and RgpB) specifically cleave after arginine residues; whereas, the lysine gingipains (Kgp) specifically cleave after lysine residues (22) (62). Both cell-associated and released, the gingipains are hypothesized to at least partially account for the remarkable resistance of *P. gingivalis* to the non-oxidative killing mechanisms of the neutrophil. Afterall, since many of the proteins and peptides contributing to the non-oxidative killing mechanisms of the neutrophil are highly cationic and rich in arginine and lysine residues, they seem highly susceptible to digestion by the arginine and lysine gingipains of *P. gingivalis*. Consistent with this, the robust proteolytic activities of *P. gingivalis* both in culture supernatants and by whole cells of *P. gingivalis* have been shown to digest and inactivate a multitude of oxygen-independent, antimicrobial peptides, proteins, and enzymes found in neutrophil granules, including lactoferrin, the cathelicidin LL-37, β-defensins, cathepsin G, elastase, bacterial-permeability increasing factor, etc (50) (63) (64) (65) (66) (67) (68) (69) (70) (71). Interestingly, Bachrach *et al* (72) suggested that the resistance of *P. gingivalis* strain ATCC 33277 (a non-encapsulated, non-disseminating strain) to direct killing by select antimicrobial peptides, including the neutrophil peptide LL-37, can be attributed more to the low affinity of these

antimicrobial peptides to *P. gingivalis*, rather than to the proteolytic activities of *P. gingivalis*.

The studies thus far have suggested that once phagocytized, the mechanism used by neutrophils to kill *P. gingivalis* A7436 is entirely phagocyte oxidase (*phox*) activity- dependent. Requiring specific (or secondary) granule fusion with the phagosome, the five NADPH oxidase subunits ($gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$), which are referred to by their molecular weight (kDa) and have been given the designation *phox* for *ph*agocyte *ox*idase (73), are delivered to the phagosome upon activation, at which time they assemble on the interior surface of the phagosome membrane to form the NADPH oxidase complex (74) (75). In what is known as the respiratory burst, large amounts of oxygen are consumed by the NADPH-dependent oxidase complex, in the enzymatic generation of superoxide anion: $NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\cdot -} + H^+$ (17) (76) (77) (78) (79). The genetic deficiency in phagocytes of either $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, or $p67^{phox}$ results in the vast majority of CGD cases (>99%), and neutrophils from subjects with CGD are consequently unable to produce superoxide or any downstream reactive oxygen species (ROS), and are considered respiratory burst-deficient (73) (78) (80) (81) (82) (83) (84) (85) (86). In normal, NADPH oxidase-functional neutrophils, superoxide, the direct product of the respiratory burst, is subsequently dismutated to hydrogen peroxide ($O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2$), and from these two initial products, other more reactive oxidants are generated, either by reacting with iron or with enzyme systems such as myeloperoxidase (MPO), or by the products of these systems reacting with $O_2^{\cdot -}$ and/or H_2O_2, or with each other (75) (87) (88) (89) (90) (91). In CGD, both MPO-catalyzed and MPO-independent oxidative killing mechanisms are absent (92), which explains its severity. This translates to the inability of phagocytes to generate $O_2^{\cdot -}$ and every downstream ROS dependent on $O_2^{\cdot -}$

generation, including H_2O_2, HOCl, $OH^{\cdot}$, $ONOO^{\cdot-}$, $^1O_2$ etc; compared to MPO-deficiency, where presumably only HOCl is absent.

Still unknown at this point, was whether the NADPH oxidase-dependent killing of *P. gingivalis* is dependent or independent of additional contributions by MPO. Heme-based MPO is the most abundant enzyme in neutrophils (93) (94), and is localized in the azurophilic (or primary) granules. By azurophilic granule fusion with the phagosome, MPO is delivered in high concentration and in close proximity to microbial targets, following the initiation of the respiratory burst by specific granule fusion with the phagosome (75) (95) (96). Once present, MPO is proposed to utilize H_2O_2, already available in potentially large concentrations, to generate the strong oxidant, weak acid, hypochlorous acid (H^+ $^-$OCl or HOCl), also known as bleach: MPO + Cl^- + H_2O_2 → H^+ $^-$OCl (97) (98). MPO-generated HOCl displays potent microbicidal activities against a broad range of microorganisms, by chlorinating or otherwise modifying a wide range of microbial targets, including enzymes, proteins, lipids, cell walls, membranes, RNA, and DNA, etc (75) (93) (94) (95) (96) (97) (98) (99) (100) (101). Since most of the oxygen consumed by an activated neutrophil is converted to H_2O_2 from $O_2^{\cdot-}$ (102) (103), and since the vast majority of the H_2O_2 generated in the neutrophil is reported to be converted to HOCl by MPO (76) (91) (95) (104) (105), MPO is believed to play a dominant role in oxygen-dependent killing in the phagosome.

Despite its abundance and perceived importance, however, subjects with MPO-deficiency are remarkably healthy and do not experience an increased frequency of infections (106) (107). In fact, MPO-deficiency is generally considered innocuous and was recently removed as a primary immune deficiency disease (108) (109). While their phagocytes show an impaired ability to kill certain targets *ex vivo*, such as *Staphylococcus aureus* and *Candida albicans* (97) (110) (111), the only clinical complication that has been reported in these patients is a predisposition to infections

with *Candida* spp. in MPO-deficient subjects who also happen to be diabetic (*S. aureus* is not a problem) (85) (112). Interestingly though, even the frequency of *Candida* infections is very low in these subjects (reported in less than 5%) (107), raising doubts about the true importance of MPO in host defense (113) (114) (115). This led to the question as to whether MPO had any importance in neutrophil killing of *P. gingivalis* A7436.

In initial experiments, MPO was found to be completely dispensable in the killing of *P. gingivalis* A7436, because subjects with MPO-deficiency (Figure A.1B) were fully capable of killing A7436, comparable to MPO-sufficient (Figure A.1A) neutrophils from normally healthy donors (Table 2.4). Since *P. gingivalis* was killed normally in the absence of MPO in neutrophils, MPO was not suggested to be a necessary or important component for neutrophil-mediated killing of *P. gingivalis*. For comparison, the killing of *P. gingivalis* was next examined by neutrophils treated with the classic MPO inhibitors, sodium azide and 4-aminobenzoic acid hydrazide (4-ABH).

The addition of azide blocked the killing of *P. gingivalis* A7436 by both MPO-sufficient and MPO-deficient neutrophils, despite normal phagocytosis (Table 2.5). These data are consistent with the probability that azide is blocking a mechanism, other than MPO, which is essential to the killing of *P. gingivalis* by normal and MPO-deficient neutrophils. In comparison, MPO-competent neutrophils treated with the more specific MPO inhibitor, 4-ABH, were capable of effective, though delayed, killing of *P. gingivalis*, despite normal phagocytosis (Table 2.6). For instance, while the number of neutrophils involved in phagocytosis of A7436 did not vary in the presence of 4-ABH, fewer 4-ABH treated phagocytic neutrophils were involved in killing at each time point; and the number of 4-ABH-treated phagocytic neutrophils that killed at two hours was comparable to the number of untreated phagocytic neutrophils that killed at 30 min (Table 2.6).

Similar to *P. gingivalis*, normal neutrophils treated with 4-ABH were also capable of effective, though delayed, killing of both *F. nucleatum* and *A. actinomycetemcomitans*, with no effects on phagocytosis (Figure 2.1); however, the data demonstrate a greater effect of 4-ABH on neutrophil killing of *F. nucleatum* than on neutrophil killing of *P. gingivalis*, and the least effect on neutrophil killing of *A. actinomycetemcomitans*. For *P. gingivalis* and *F. nucleatum*, the effect of 4-ABH was significant at all time points between 30 min and three hours; while the 4-ABH effect on *A. actinomycetemcomitans* only reached significance at the 30 min time point, consistent with its greater susceptibility to non-oxidative and DPI-insensitive killing mechanisms compared with *F. nucleatum* and *P. gingivalis* (Tables 2.7 and 2.8). Additionally, *A. actinomycetemcomitans* was killed equivalently by neutrophils treated with either DPI or 4-ABH, suggesting that 100% of its oxidative killing is attributable to MPO.

Taken together, these data suggest that MPO or some other yet-to-be-identified 4-ABH-sensitive mechanism contributes to the killing of all three periodontopathogens, but that there are highly effective MPO-independent mechanisms utilized by the neutrophil for the killing of all three species. Because neither azide nor 4-ABH affects the respiratory burst or degranulation in stimulated neutrophils (116) (117), these data also suggest that *P. gingivalis* A7436 is killed by a mechanism subsequent to the initial generation of superoxide by the NADPH oxidase. Furthermore, these data suggest that the dominant mechanism that normal and MPO-deficient neutrophils use to kill *P. gingivalis* is an MPO-independent, but an azide-sensitive mechanism.

While sodium azide is a highly potent inhibitor of MPO enzymatic activity (confirmed in Figure A.2), by directly attaching to the coordinate position of the iron in the heme moiety (118), it is unclear what azide was blocking (i.e. what unidentified co-factor) in neutrophil killing of *P. gingivalis*. Important to the interpretation of the azide data is the fact that azide is not a specific inhibitor of MPO and is also reported to inhibit

the activity of other metalloenzymes in the neutrophil and have other effects as well (119) (120) (121) (122). For instance, besides inhibiting other heme-based enzymes, azide is also reported to function as a hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) scavenger (123), as well as a potent inhibitor of redox reactions (124) and reactions involving the transition of metals (125). Therefore, azide may have protected A7436 by functioning as a radical scavenger or by preventing toxicity attributable to H_2O_2. By binding and bridging transition metals (125), azide may have prevented H_2O_2 from participating in a Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H_2O + OH^{\cdot}$), in the absence of competition with MPO for H_2O_2. It is also conceivable that azide may have protected A7436 by shutting down its metabolism, since azide has been used in a number of studies to inhibit the metabolism of *P. gingivalis* (126) (127) (128) (129). This is consistent with the concept presented by Imlay *et al* (130), who suggested that an active bacterial metabolism is required for killing by H_2O_2.

It is also important to note that while 4-ABH is also a highly potent inhibitor of MPO enzymatic activity (confirmed in Figure A.2), and is reported to be a more specific inhibitor of MPO than is sodium azide with no effects on other neutrophil enzymes (116) (131) (132) (133); it is also possible that 4-ABH may have had additional effects on the neutrophil to account for the discrepancy in MPO-deficient *vs.* 4-ABH-treated neutrophil killing of *P. gingivalis*. Another possibility is that MPO-deficient neutrophils may have compensatory or alternative killing mechanisms in place in the absence of MPO, which is not inconsistent with our data that MPO may actually afford protection to bacteria against H_2O_2-mediated killing. In the absence of MPO, H_2O_2 may build up and be more available to kill directly or participate in $OH^{\cdot}$ generation (91) (97) (134). This is consistent with the findings of Winterbourn (135), which suggested that $OH^{\cdot}$ generation is more favorable in MPO-deficient neutrophils due to the absence of competition with MPO for H_2O_2. In addition, attributed to a lack of inactivation of the respiratory burst

due to the absence of MPO (91) (119), MPO-deficient subjects consume more oxygen in the respiratory burst than normal (119) (136), and have extended superoxide and hydrogen peroxide production compared to individuals with MPO-sufficiency (98) (137). Alternative or upregulated MPO-independent killing mechanisms such as these may therefore account for greater (normal) killing of *P. gingivalis* by MPO-deficient neutrophils, compared to neutrophils treated with 4-ABH, and may explain the low (normal) morbidity in subjects with MPO-deficiency.

The conclusions from chapter 2 are that *P. gingivalis* A7436 is remarkably resistant to the non-oxidative killing mechanisms of the neutrophil, in comparison to other periodontal pathogens tested; and is instead completely dependent on a functional NADPH oxidase, which requires the presence of oxygen, in order to be killed by neutrophils. Additionally, this *phox*-dependent killing is consistent with a mechanism subsequent to the initial generation of superoxide by the NADPH oxidase, though the role and contributions of MPO in this killing remain unclear. Which NADPH oxidase-dependent ROS proved most important in neutrophil-mediated killing of *P. gingivalis* was therefore the subject of chapter 3.

Since the NADPH oxidase is central to killing *P. gingivalis*, it was important to examine the susceptibility of *P. gingivalis* to the three principal products of the NADPH oxidase: $O_2^{\cdot-}$, H_2O_2, and MPO-generated HOCl. To begin to address this, it was first examined whether the killing of *P. gingivalis* can be duplicated in a cell-free system using phosphate buffered saline (PBS); and if so, whether either or both of the two initial products of the NADPH oxidase ($O_2^{\cdot-}$ and H_2O_2) are capable of killing *P. gingivalis* alone and in the absence of other neutrophil co-factors such as MPO. The killing potentials of the two initial products of the NADPH oxidase system, $O_2^{\cdot-}$ and H_2O_2, were examined using the xanthine (X) – xanthine oxidase (XO) system. The X-XO system was chosen because it emulates the NADPH oxidase by enzymatically generating exogenous $O_2^{\cdot-}$,

which subsequently dismutates to H_2O_2. In order to determine the individual contributions of these ROS, superoxide dismutase (SOD) was used to eliminate $O_2^{\cdot -}$ in the X-XO system, and catalase was used to eliminate H_2O_2 in the X-XO system.

P. gingivalis was indeed killed by the X-XO system (Figure 3.1), confirming that *P. gingivalis* can be killed in a cell-free system by $O_2^{\cdot -}$ and/or H_2O_2, in the absence of other neutrophil co-factors such as MPO. Killing of *P. gingivalis* by the X-XO system was completely blocked by catalase, but SOD offered no protection through the two hours tested (Figure 3.1). Under X-XO plus catalase, superoxide was generated as determined by cytochrome *c* reduction (data not shown), and there was no discernible killing of A7436 (Figure 3.1). In contrast, when challenged with X-XO plus SOD, hydrogen peroxide was generated as determined by cytochrome *c* oxidation (data not shown), and A7436 killing was equivalent to that of X-XO alone (Figure 3.1). Because superoxide under the conditions of these experiments had no effect on *P. gingivalis* A7436, the direct product of the NADPH oxidase ($O_2^{\cdot -}$) was not suggested to be bactericidal to *P. gingivalis*. Killing consistent with H_2O_2 was demonstrated to account for 100% of the killing in the X-XO system, which suggested that H_2O_2 but not $O_2^{\cdot -}$ is important to the killing of *P. gingivalis*. *P. gingivalis* sensitivity to H_2O_2 but not to $O_2^{\cdot -}$ is consistent with *P. gingivalis* possessing SOD (MnSOD and FeSOD), but not catalase or peroxidase enzymes (138). Toxicity to H_2O_2 but not to $O_2^{\cdot -}$ is also consistent with the fact that H_2O_2 is membrane-permeable, while $O_2^{\cdot -}$ is not (139).

Since these data indicate that H_2O_2 alone can kill A7436, the sensitivity of *P. gingivalis* to H_2O_2 free of $O_2^{\cdot -}$ was the next center of focus. The glucose (G) – glucose oxidase (GO) system was chosen because it enzymatically generates H_2O_2 directly in a sustained fashion, allowing the susceptibility of *P. gingivalis* to H_2O_2 to be assessed when generated at various rates. *P. gingivalis* was killed by the G-GO system (Figure 3.2), which confirmed the X-XO data that H_2O_2 is indeed effective in killing *P. gingivalis*

when generated at a sufficient rate, which increased with GO concentration. Additionally, this time- and rate- dependent killing of A7436 by the G-GO system was completely blocked by the addition of catalase (data not shown), further implicating a direct toxicity of H_2O_2. Furthermore, the temporal effect of GO titration suggests there is a threshold concentration of H_2O_2 necessary to kill *P. gingivalis*. In order to validate the H_2O_2-sensitivity of *P. gingivalis* and to address the killing kinetic of *P. gingivalis* by H_2O_2, the bactericidal activity of H_2O_2, delivered non-enzymatically as a bolus, was next examined.

Boluses of H_2O_2 were titrated and challenged with *P. gingivalis*, in order to determine the approximate sensitivity of *P. gingivalis* to H_2O_2 in a pure system, and to determine the killing kinetic of *P. gingivalis* with H_2O_2 over time. H_2O_2 delivered as a bolus killed *P. gingivalis* in a time- and dose-dependent fashion (Figure 3.3), which suggested that H_2O_2-mediated killing of *P. gingivalis* does not occur instantly, but that *P. gingivalis* and H_2O_2 (generated enzymatically at a sufficient rate or delivered as a bolus at a sufficient concentration) need time together in order for H_2O_2 to be lethal to *P. gingivalis*. Killing did not occur at initial time with H_2O_2, though significant killing occurred by 30 min, with additional killing occurring at each subsequent time point. Specifically, there was a concentration-dependent, logarithmic killing progression over the three hour test period with all H_2O_2 concentrations tested, including as low as 6.25µM H_2O_2 (Figure 3.3). Furthermore, since there was no reduction in recoverable CFU/mL upon initial plating, despite concentrations of H_2O_2 as high as 10mM (data not shown), the process of plating was suggested to neutralize any additional bactericidal activity of H_2O_2, further suggesting that the killing events occur in the reaction volume only.

These data indicate that low, micromolar concentrations of H_2O_2 are capable of killing *P. gingivalis* in the absence of exogenous co-factors. The purpose of the next series of experiments was to determine how the susceptibility of *P. gingivalis* to H_2O_2

compares on a grand scheme relative to other bacteria with relevant features. Other pathogens, with and without catalase, and with different relationships with oxygen, that are either associated with periodontitis, CGD, and/or neutropenia were selected for comparison.

The relative susceptibility of *P. gingivalis* A7436 (an encapsulated strain of *P. gingivalis*) to H_2O_2 was first compared with other well-studied, non-encapsulated strains of *P. gingivalis*, including ATCC 33277, 381, and HG405, in order to determine if the sensitivity of A7436 to H_2O_2 is a feature shared by the species and is independent of capsule production. It was hypothesized that encapsulated strains like A7436 may show greater resistance to H_2O_2 than non-encapsulated strains due to protection afforded by the extracellular polysaccharide capsular layer. All strains of *P. gingivalis* independent of capsule production or virulence type, however, showed nearly identical sensitivities to H_2O_2 (Figure 3.4B). Therefore, the susceptibility of A7436 to very low concentrations of H_2O_2 (Figures 3.3 and 3.4A-B) does not appear to be a feature unique to strain A7436, but appears to be a unique feature representative of *P. gingivalis* as a species.

The sensitivity of *P. gingivalis* to H_2O_2 was next compared to *Prevotella intermedia*, a catalase-negative, strictly anaerobic periodontal pathogen, similar in ancestry to *P. gingivalis*. Not surprisingly, *P. intermedia* (which like *P. gingivalis*, also requires heme and Vitamin K for growth, readily forms black-pigmentation on blood-based agars due to the production of protoporphyrin, and is a strict anaerobe) was the most similar to *P. gingivalis* in sensitivity to H_2O_2 (Figure 3.4B).

The sensitivity of *P. gingivalis* to H_2O_2 was also compared to *F. nucleatum* and *A. actinomycetemcomitans*, two periodontal pathogens that were consistently more sensitive to neutrophil killing than was *P. gingivalis* (Tables 2.7 and 2.8). *F. nucleatum* is a catalase-negative, periodontal pathogen like is *P. gingivalis*; while *A.*

actinomycetemcomitans, in contrast, is a catalase-positive, periodontal pathogen. Both are capable of growing in microaerophilic conditions, though *A. actinomycetemcomitans* has a greater ability to grow in the presence of oxygen than does *F. nucleatum*, in stark contrast to *P. gingivalis*, which requires anaerobiosis for growth. Based on the anaerobic (Table 2.7) and DPI (Table 2.8) data, it was possible that both *F. nucleatum* and *A. actinomycetemcomitans* were more sensitive to H_2O_2 in the neutrophil than was *P. gingivalis*, if minute levels of oxygen were present in the anaerobic chamber which contributed to H_2O_2 production. This did not seem to be the case, however, as even *F. nucleatum* (which was more dependent on oxygen and a DPI-sensitive mechanism for neutrophil-mediated killing than was *A. actinomycetemcomitans*) was approximately 15- to 25- fold more resistant to H_2O_2 than was *P. gingivalis* (Figures 3.4A and B). The even greater resistance of *A. actinomycetemcomitans* to H_2O_2, in comparison to *F. nucleatum* (Figures 3.4A and B), is likely associated with its ability to produce catalase, and perhaps other antioxidant defense mechanisms, which allow *A. actinomycetemcomitans* to grow in the presence of oxygen.

The relative susceptibility of *P. gingivalis* to H_2O_2 was also compared with the catalase-positive *Staphylococcus aureus*, a pathogen in CGD and in neutropenia, which is capable of growing in the presence of oxygen; and to the catalase-negative *Streptococcus pyogenes*, a pathogen in neutropenia but not in CGD, which is also capable of growing in the presence of oxygen. While the various test bacteria showed a wide range of sensitivities to H_2O_2, *P. gingivalis* was consistently much more susceptible to low concentrations of H_2O_2 than all other test bacteria (Figure 3.4B). The three aerotolerant pathogens (*S. aureus*, *S. pyogenes*, and *A. actinomycetemcomitans*) independent of catalase production were the most resistant to H_2O_2 (Figures 3.4A and B). Interestingly, *S. pyogenes* (catalase-negative) was more

resistant to H_2O_2 than was *S. aureus* (catalase-positive), which is consistent with the findings of Wilson *et al* (140), who attributed greater H_2O_2 resistance not to catalase production, but to the more prolific production of peroxidases in streptococci, compared to *S. aureus*. Additionally, the requirement of time in order for H_2O_2 to kill was common with all bacteria that were tested (data not shown).

Since MPO is a principal constituent of the phagosome environment and is suggested to be the major consumer of H_2O_2 in the phagosome (95), the *in vitro* influences of MPO activity on the interactions of H_2O_2 with *P. gingivalis* were also examined. While *P. gingivalis* was remarkably sensitive to low concentrations of H_2O_2 in the absence of other neutrophil co-factors such as MPO, which suggests that H_2O_2 alone is a highly effective mechanism of killing *P. gingivalis;* the MPO-H_2O_2 system was next examined in order to determine what role MPO plays in the interaction of H_2O_2 with *P. gingivalis*, and specifically, whether MPO has any effect on H_2O_2-mediated killing of *P. gingivalis*. Previous studies suggested that MPO is not essential in the killing of *P. gingivalis* or in the killing of either of the other two bacteria that were tested, *F. nucleatum* and *A. actinomycetemcomitans.* Subjects with MPO-deficiency were fully capable of killing *P. gingivalis* A7436, comparable to MPO-sufficient controls (Table 2.4); while 4-ABH similarly did not block neutrophil-mediated killing of either *P. gingivalis, F. nucleatum*, or *A. actinomycetemcomitans* (Figure 2.1). It was suggested, however, that MPO may play a greater role in the killing of *F. nucleatum* than *P. gingivalis*, and the least role in the killing of *A. actinomycetemcomitans* (Figure 2.1).

F. nucleatum was initially chosen for comparison with *P. gingivalis*, because like *P. gingivalis*, *F. nucleatum* is a catalase-negative, anaerobic periodontal pathogen, but in contrast to *P. gingivalis*, requires approximately 15-fold higher concentrations of H_2O_2 to achieve logarithmic reductions (Figures 3.4A and B). Interestingly, despite both bacteria having very different sensitivities to H_2O_2, the addition of MPO protected both *F.*

nucleatum and *P. gingivalis* against bactericidal concentrations of H_2O_2, and surprisingly, there was no killing of either *P. gingivalis* or *F. nucleatum* in the presence of MPO with concentrations of H_2O_2 exceeding that necessary for MPO-independent bactericidal activity (Figures 3.5A and B). Additionally, while the mechanism of protection by MPO against bactericidal concentrations of H_2O_2 is unknown (e.g. whether MPO scavenges H_2O_2, and whether it is an enzymatic process or not), it has previously been suggested that ≥100µM H_2O_2 inhibits the peroxidatic activity of MPO (141), or transitions it to that of catalatic activity (142). To our knowledge, however, this is the first evidence that MPO interaction with H_2O_2 protects bacteria from killing and/or that MPO neutralizes bactericidal concentrations of H_2O_2. The total block of H_2O_2-mediated killing of *P. gingivalis* and *F. nucleatum* with the addition of MPO is not inconsistent with our earlier data which suggested that MPO is not essential to the killing of either *P. gingivalis* or *F. nucleatum*.

While the addition of MPO offered protection against higher, bactericidal concentrations of H_2O_2; MPO was also shown to enhance the killing of *P. gingivalis* (Figure 3.5B) and *F. nucleatum* (Figure 3.5C), but only within a very narrow range of sub-lethal concentrations of H_2O_2, which gave little or no killing alone. Therefore, while *P. gingivalis* was already remarkably sensitive to very low concentrations of H_2O_2 (10µM or greater H_2O_2 directly kills *P. gingivalis* at one hour); *P. gingivalis* was sensitive to even lower concentrations of H_2O_2 at one hour, in the presence of MPO (Figure 3.5B).

Additionally, while MPO-dependent killing was only evident within a very narrow range of H_2O_2 concentrations, MPO was shown to lower the amount of H_2O_2 required to kill every one of the six test bacteria, including *P. gingivalis* (Figure 3.6A), *P. intermedia* (Figure 3.6B), *F. nucleatum* (Figure 3.6C), *A. actinomycetemcomitans* (Figure 3.6D), *S. aureus* (Figure 3.6E), and *S. pyogenes* (Figure 3.6F), at one hour. The addition of MPO enhanced the killing of all six test bacteria, specifically, at concentrations between 0.6

and 10μM H_2O_2, with an optimum at 5μM. This bactericidal activity was best interpreted to be due to the conversion of H_2O_2 to HOCl, since the MPO peroxidatic inhibitor 4-ABH completely protected all test bacteria against this killing at sub-optimal and optimal MPO concentrations (Figures 3.6A-F).

Interestingly, while all six test bacteria showed major differences in their range of sensitivities to H_2O_2 in terms of the concentrations of H_2O_2 that were required to kill (Figure 3.4B), all six test bacteria surprisingly had comparable susceptibilities to the MPO-H_2O_2 system; and unlike with H_2O_2, there was no differential susceptibility of test bacteria to the product of the MPO-H_2O_2 system (HOCl), as long as there was sufficient MPO available (Figures 3.6A-F). While the addition of MPO enhanced the killing of all test bacteria at an optimum of 5μM H_2O_2, there were two- to four- fold differences between species in the concentration of MPO required to function with 5μM H_2O_2. This was likely a reflection of catalase possession by *S. aureus* and *A. actinomycetemcomitans*, causing these two bacteria to require more MPO (24mU/mL for optimal activity), in order to exceed the ability of their catalase to scavenge H_2O_2; as well as a reflection of some level of H_2O_2 scavenging, by protoporphyrin perhaps, possessed by the two black-pigmenting bacteroides, *P. gingivalis* (143) (144) and *P. intermedia* (145); and due to the possession of peroxidases by *S. pyogenes,* which like catalase, may compete with MPO for H_2O_2 (the later three species required 12mU/mL for optimal activity). The non-black-pigmenting, non-catalase and non-peroxidase -producer, *F. nucleatum*, in contrast, required the least MPO (6mU/mL) activity to function optimally with 5μM H_2O_2 (Figures 3.6C), which suggested that *F. nucleatum* is the least efficient at scavenging H_2O_2 compared to the other species, and is the most sensitive to MPO since it required the least MPO activity for killing. These data are also interesting in the context of the differential effects of 4-ABH on neutrophil-mediated killing of *F. nucleatum* and *A. actinomycetemcomitans* compared to *P. gingivalis* (Figure 2.1), which showed

the greatest reduction in the number of phagocytic neutrophils capable of killing *F. nucleatum* in the presence of 4-ABH, in contrast to *A. actinomycetemcomitans*.

Because it would appear likely that MPO would be neutralized in the phagosome by the copious amounts of H_2O_2 produced there, it is unclear whether MPO plays an important role in killing in the phagosome if antagonistic interactions between MPO and H_2O_2 occur in the phagosome. The data in chapter 3 suggest that *P. gingivalis* and possibly other H_2O_2-sensitive bacteria may be killed in the phagosome by H_2O_2 alone, in the period of time before MPO gets delivered; and that phagosomal concentrations of H_2O_2 must be tightly regulated for MPO to function as a bactericidal constituent, once MPO is delivered. As proposed by Reeves *et al* (115), it is possible that MPO has an alternative role in the phagosome such as H_2O_2 scavenging, which may serve to dampen the toxicity of the neutrophil; not only by "mopping up" excess H_2O_2, but by limiting the activities of cytotoxic and degradative enzymes. This is consistent with the observation that the majority (94%) of chlorinated proteins in the phagosome are of host origin (146). This may also occur outside the 'frustrated' neutrophil, which releases MPO to its exterior, into the gingival crevice.

Our data suggest that killing by H_2O_2 would be an effective mechanism of killing *P. gingivalis* within the phagosome, especially before MPO gets delivered, and possibly even after MPO gets delivered, if even low micromolar concentrations of H_2O_2 are present and left unscavenged by MPO, due to this bacterium's unusually high susceptibility to low micromolar concentrations of H_2O_2. This is consistent with the neutrophil phagosome model of Winterbourn *et al* (95), which suggested that low micromolar concentrations of H_2O_2 are steady state in the phagosome, despite robust HOCl generation. This sustained low level H_2O_2 concentration could potentially be all that is required to kill *P. gingivalis*.

H_2O_2 is hypothesized to kill by diffusion inside the bacterial cell, where it reacts with bacterial-associated iron, mediating a Fenton reaction, where ferrous iron (Fe^{2+}) reduces H_2O_2 to the most reactive (i.e. non-specific and diffusion-limited) radical known, hydroxyl radical ($OH^{\cdot}$) (89) (130) (147) (148) (149) (150) (151) (152) (153) (154) (155) (156). This scenario appears consistent with the fact that H_2O_2-mediated killing of *P. gingivalis* does not occur instantly, but that *P. gingivalis* and H_2O_2 (generated enzymatically at a sufficient rate or delivered as a bolus at a sufficient concentration) need time together in order for H_2O_2 to become lethal – a mechanism consistent with requiring time in order for H_2O_2 to enter the bacteria, find and react with bacterial-associated iron, generate something more toxic (e.g. $OH^{\cdot}$), and cause a lethal, irreparable hit (e.g. damage to DNA). It is therefore suggested from these studies that in order to kill *P. gingivalis*, MPO is an unnecessary co-factor. Because H_2O_2 alone at very low concentrations is a highly effective mechanism of killing *P. gingivalis*, compounded with the observation that H_2O_2 must be tightly regulated for MPO to function as a bactericidal constituent (i.e. the MPO-H_2O_2 system works only within a very narrow range of H_2O_2 concentrations and MPO is highly susceptible to neutralization by H_2O_2), and perhaps most importantly, due to the order of delivery to the phagosome (i.e. H_2O_2 is present before MPO gets delivered) – makes H_2O_2 a highly desirable candidate as the effector mechanism of killing *P. gingivalis*. For instance, H_2O_2 may have a front-end ability to kill *P. gingivalis*, before MPO even gets delivered.

In conclusion from chapter 3, the MPO-independent bactericidal activity of H_2O_2, effective against *P. gingivalis* and all other species tested, was demonstrated to be both time- and dose- dependent; though a significant range of susceptibilities to H_2O_2 was demonstrated among the various species tested. Of all the species tested, all strains of the strictly anaerobic, catalase- and peroxidase-negative, periodontal pathogen *P. gingivalis* were consistently more susceptible to low concentrations of H_2O_2; and at 60

min, there were nearly identical logarithmic reductions in recoverable CFU/mL of all four *P. gingivalis* strains with 20µM H_2O_2 (Figures 3.4A and B). In comparison, other catalase- and peroxidase-negative, anaerobic periodontal pathogens, *P. intermedia* and *F. nucleatum* required, respectively, 100µM H_2O_2 (Figure 3.4B) and >300µM H_2O_2 (Figure 3.4A) for logarithmic reductions. In contrast, the three species that are capable of growing in an aerobic environment, *A. actinomycetemcomitans, S. aureus,* and *S. pyogenes,* required >1,250µM, >2,500µM, and >5,000µM H_2O_2, respectively, for logarithmic reductions (Figures 3.4A and B). Interestingly, however, while there were major differences in the susceptibilities of these bacteria to MPO-independent H_2O_2, there were no differences in the susceptibilities of these bacteria to the H_2O_2 required for MPO-dependent killing. As long as there was optimum MPO activity available, there was no difference in the amount of H_2O_2 required to kill with MPO. This seemed to suggest that these test bacteria were not different in their susceptibility to the product of the MPO-H_2O_2 system (HOCl), despite having very different susceptibilities to H_2O_2.

The objective of chapter 4 was therefore to investigate the differential killing mechanisms of H_2O_2 *vs.* bleach, in an attempt to better understand how these oxidative killing mechanisms may operate in the neutrophil with various bacteria. To begin to address this, select bacteria that were revealed to have very different susceptibilities to H_2O_2 were challenged with a bolus of sodium hypochlorite (Na^+ ^-OCl, which is the salt form of H^+ ^-OCl generated by the MPO-H_2O_2 system in the neutrophil), in order to compare susceptibilities to H_2O_2 *vs.* bleach. Despite requiring logarithmically different concentrations of H_2O_2 in order to be killed (Figures 3.4A and B), these bacteria showed identical susceptibilities to bleach (Table 4.2). When titered in 10-fold increments, sodium hypochlorite killed all bacteria (shown with *P. gingivalis, F. nucleatum,* and *S. aureus*) completely and instantly with the same lethal dose of 0.002% Na^+ OCl^- v/v (Table 4.2). Identical susceptibilities to bleach were also observed with other tested

bacteria, including *P. intermedia* and *A. actinomycetemcomitans* (data not shown). Upon refinement of the titration using 2-fold dilutions, again sodium hypochlorite killed all the tested bacteria (shown with *P. gingivalis, S. aureus,* and *S. pyogenes*) with the same concentration of between 0.00125 - 0.0025% Na^+ OCl^- v/v (Table 4.2).

In contrast to H_2O_2, which killed all test bacteria with a concentration-dependent, kinetic killing progression over time; bleach killing occurred in an all-or-none fashion, instantly, and with no definable killing progression over time (Tables 4.1 and 4.2). If delivered at a sufficient concentration (≥0.001% Na^+ OCl^- v/v), bleach killed instantly (at 0 time, without much additional killing with 0.001% Na^+ OCl^- v/v after this initial time point) (Table 4.1). Conversely, if challenged with an insufficient dose (≤0.0001% Na^+ OCl^- v/v), bleach did not kill *P. gingivalis* at all, even through two hours. These data suggest that bleach either kills completely and instantly with a sufficient dose – or as quickly as it finds its target, because by one hour, nothing was recovered with 0.001% Na^+ OCl^- v/v, while the dose higher (0.01%) killed everything at 0 time – or kills nothing, even after two hours, if administered too low a dose.

Additionally, because *P. gingivalis* was capable of being killed by a bolus of bleach immediately (at 0 time), this suggests that it takes time for the MPO- H_2O_2 system to generate HOCl; but once available at a sufficient (lethal) concentration, bleach does not require time in order to kill, and kills on contact. This is in contrast with a bolus of H_2O_2, which does not kill on contact, but instead requires time with the bacteria in order to kill. Unlike with bleach, there is a clear killing progression with H_2O_2, with additional killing at each subsequent time point with decreasing H_2O_2 concentrations.

The effects of varying temperature on H_2O_2- *vs.* bleach- mediated killing were also assessed. H_2O_2, delivered as a bolus, killed *P. gingivalis* better at 37^{o}C than at room temperature, and was completely blocked when treatment was placed on ice (1^{o}C) (Figure 4.1). The temperature effects on H_2O_2 killing were not limited to *P. gingivalis*

and was also tested and observed with *S. aureus* (data not shown). In striking contrast to H_2O_2, which showed a lag time for killing that was directly temperature-dependent (37^oC), and a killing progression over time with lower and lower concentrations; bleach required a critical concentration in order to kill, and killing occurred equivalently at 37^oC and on ice, and at 0 and 60 min (i.e. same endpoints over time) (Table 4.1).

The effects of varying bacterial density on H_2O_2- *vs.* bleach- mediated killing were also assessed. When administered to logarithmically-varied bacterial densities, H_2O_2 logarithmically killed every bacterial density with the same H_2O_2 concentration(s); while bleach killed the lower bacterial densities completely, while the higher bacterial densities were not killed at all – consistent with all-or-none killing by bleach, and decreased effectiveness of bleach as the target density was increased (Table 4.3). Whereas H_2O_2 had a clearly titratable effect at 60 min, showing dose-dependent increases in killing at every bacterial density; bleach did not show a clear titration at any bacterial density, but an all-or-none effect at 0 and 60 min. For instance, 10^8 *P. gingivalis* went from no killing (8 $\log_{10}$ CFU/mL) to complete killing (<2 $\log_{10}$ CFU/mL) with 0.1% Na^+ OCl^- v/v. These data are consistent with the hypothesis that bleach is contact-lethal with a critical concentration, whereas H_2O_2 must be converted to something else in order to become lethal.

The possible nature of this product was next examined with the highly specific, hydroxyl radical scavenger, DMSO (157), with H_2O_2-mediated killing of *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *S. aureus* (Figure 4.2). Any protection by DMSO is used as a direct indicator of $OH^{\cdot}$ generation (130) (148) (149) (157) (158). DMSO similarly protected all four species from H_2O_2-mediated killing, in a dose-dependent manner: 100mM DMSO protected best, followed by 10mM DMSO. At a minimum concentration of H_2O_2 which gave 100% killing (no recoverable CFU on replicate plating), 100mM DMSO gave >99% protection of *P. gingivalis*, >95% protection

of *F. nucleatum,* >90% protection of A*. actinomycetemcomitans*, and 99.9% protection of *S. aureus.* These data support hydroxyl radical as the effector mechanism of H_2O_2-mediated killing of *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *S. aureus*.

The killing kinetics, temperature requirement differences, and the effects of bacterial density on the bactericidal activities of H_2O_2 *vs.* bleach, are consistent with the hypothesis that bleach is directly toxic; whereas H_2O_2 requires conversion to something else, likely by the bacteria itself (e.g. intracellular bacterial iron), in order to kill. This time-dependent event is consistent with the conversion of H_2O_2 to the toxic product of $OH^{\cdot}$. Additionally, $OH^{\cdot}$ is so reactive that it has been defined as diffusion-limited (89) (91). Its immediacy of reaction with DNA (130), before DMSO could make contact with $OH^{\cdot}$, could therefore explain why even 100mM DMSO could not protect from H_2O_2-mediated toxicity entirely. In addition, DMSO may not have protected completely because even minute amounts of $OH^{\cdot}$ is highly effective in bacterial killing, as proposed by Repine *et al* (148). However, others have suggested that DMSO may not protect completely because the immediate product of the Fenton reaction is a ferryl radical (FeO^{2+}), rather than a free hydroxyl radical (130) (149).

Due to the requirement of temperature ($37^{o}C$) for killing, and due to the total block of killing with H_2O_2 on ice (Figure 4.1), which is suggested to inhibit bacterial metabolism, as well as energy-dependent activities independent of metabolism (159) (160); these data are also consistent with the hypothesis that conversion of H_2O_2 to $OH^{\cdot}$ requires an active bacterial metabolism or is energy-dependent, and that the bacteria themselves contribute to their own demise. Imlay *et al* (130) (161) also suggested that an active bacterial metabolism is required for killing by H_2O_2. Specifically, an active bacterial metabolism is suggested to be necessary to provide the reductive cellular environment to reduce ferric iron to ferrous iron, the form of which is required for

participation in the Fenton reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H_2O + OH^{\cdot}$ (158) (159) (160) (161) (162) (163). These data also support the hypotheses that azide-treated neutrophils are unable to kill *P. gingivalis* either due to $OH^{\cdot}$ scavenging (123), or by preventing H_2O_2 toxicity by shutting down bacterial metabolism or by inhibiting energy-dependent redox reactions (126) (127) (128) (129), required for $OH^{\cdot}$ production by the Fenton reaction (124) (125).

SPECULATION AND FUTURE STUDIES

Over one hundred years ago, Elie Metchnikoff recognized the importance of phagocytes in host defense against invading microorganisms, but noted that "what substances within the phagocyte that harm and destroy the microbes are quite undecided" (164). One hundred years later, much is known about the principal ROS associated with neutrophil-mediated killing, though knowledge of the killing biochemistry within the phagosome remains limited by the specificity of inhibitors; the inaccessibility of probes, detectors, and scavengers; and the variations which exist between individuals, infecting organisms, and infection-host dynamics which are too complex to study– leaving much open to speculation. While the studies described here have begun to elucidate the components required for neutrophil-mediated killing of *P. gingivalis*, many questions remain unanswered. While studying the antimicrobial activities of the neutrophil in isolation with a pure bacterial culture of *P. gingivalis* in PBS may be an inaccurate representation in light of the polymicrobial and complexity of infection conditions *in vivo;* taking such a reductionist approach is a prerequisite to understanding the specific killing mechanisms that may be of importance to this organism. While understanding how *P. gingivalis* is killed has offered clues about potential treatments for this pathogen, as well as insights into the neutrophil-pathogen interactions that may contribute to the pathogenesis of periodontitis; many things remain unclear, such as how exactly this relates to neutrophils and to the gingival pocket *in situ*, in periodontitis. A few of the speculations and unanswered questions derived from these studies are outlined below.

Because *P. gingivalis* showed an absolute dependence on phagocytosis and a functional NADPH oxidase in order to be killed, and was completely resistant to the oxygen-independent killing mechanisms of the neutrophil when oxygen was unavailable; it is likely that the success of *P. gingivalis* as a periodontal pathogen is not only linked with its exceptional resistance to phagocytosis and to extracellular and serum-mediated killing, but to its specific requirements for high titered specific antibody in order to be phagocytosed, and the availability of oxygen (for the production of a respiratory burst) in order to be killed in the phagosome.

One hypothesis as to why *P. gingivalis* infections can present as refractory in subjects with severe periodontitis is that deep pockets are anoxic. Despite a large number of neutrophils; reduced oxygen conditions, which prevail in the periodontal pocket (165), likely explain the ability of this pathogen to resist killing, as well as to persist and multiply in active periodontitis. In the anoxic conditions of severe periodontitis, *P. gingivalis* would not be killed by gingival crevicular neutrophils due to the inability of these neutrophils to mount a respiratory burst in the absence of oxygen (166). It is further hypothesized that *P. gingivalis* may even benefit from the presence of neutrophils, which would make major contributions by consuming available oxygen in the periodontal pocket (anaerobiosis is required for the growth of this strictly anaerobic bacterium); while contributing to tissue destruction, necessary to provide this fastidious assacharolytic bacterium with a source of peptides and amino acids for growth and nutrition.

It is also hypothesized that the neutrophil may be of more importance in the prevention of the initiation of periodontal disease, when the pockets are shallow and more oxygenated, allowing the function of the NADPH oxidase; than in progressive and severe disease states, where the pockets are deep and anoxic. The massive influx of neutrophils in periodontitis has been shown to form a leukocyte wall that becomes

interposed between plaque and the junctional epithelium and sulcus (3), which likely consumes all available oxygen along the interface into the periodontal pocket, leaving the center of the pocket anaerobic. In conjunction, fibroblasts are known to wall off the area, sequestering infection and inflammation, while limiting the re-introduction of oxygen into the area, as a consequence. Re-introducing oxygen into the pocket via scaling and debridement, and especially periodontal surgery, so that the neutrophil NADPH oxidase can function again, would likely be an excellent treatment for periodontal infections with *P. gingivalis.*

Immunizing with specific virulence determinants of *P. gingivalis*, including both capsule and gingipains, may also facilitate clearance by increasing the subset (10% in these studies) of neutrophils capable of phagocytosis in the absence of specific antibody. In addition, since the majority of the proteins and peptides contributing to the non-oxidative killing mechanisms of the neutrophil are arginine- and lysine- rich, they appear incredibly susceptible to cleavage and inactivation by the arginine and lysine gingipains of *P. gingivalis*. Introducing gingipain-specific antibody or protease inhibitors specific to gingipains into infection sites known to have *P. gingivalis* may also serve as an effective treatment by neutralizing these activities, and may increase the ability of neutrophils to kill this pathogen by non-oxidative mechanisms when oxygen is unavailable.

Another potential treatment of periodontitis would be to target pro-inflammatory pathways, since disease progression is due in large part to the consequences of inflammation. It is hypothesized that neutrophil function is altered in periodontitis in the presence of pro-inflammatory cytokines, the influence of which may enhance phagocytosis at the expense of effective killing. Despite an abundance of neutrophils, *P. gingivalis* flourishes in periodontitis, and it is important to understand why. For example, it would be important to understand why *in situ* neutrophils, under the influence

of *P. gingivalis* and/or pro-inflammatory cytokines do not kill as effectively, and whether they are still capable of delivering granule components for killing; furthermore, if oxygen were reintroduced, would effective killing resume, and why not, if not? For instance, it has been suggested in the studies of Abe *et al* (167) that *P. gingivalis* may be capable of altering or shutting down the oxidative killing mechanisms of the neutrophil, which is an interesting concept that warrants further study. It is also unknown whether *P. gingivalis* and/or pro-inflammatory cytokines are capable of inhibiting neutrophil degranulation after phagocytosis, which can be determined by electron microscopy of phagolysosomal fusion events using histochemical staining of MPO-containing granules and immunogold labeling of lactoferrin-containing granules, as well as cytochrome *c* reduction assays of neutrophils for the determination of respiratory burst capacity.

Furthermore, it is interesting that *P. gingivalis* absolutely requires phagocytosis in order to be killed, which requires the presence of serum containing both complement and high titered specific antibody. It is possible that *P. gingivalis* interferes with the respiratory burst in the absence of antibody, explaining why *P. gingivalis* is not killed on the surface of neutrophils, despite the purported production of superoxide and hydrogen peroxide proximal to a bacterial surface and on the outside of the neutrophil before phagocytosis occurs (an estimated 10% of specific granules are exocytosed while the remainder are delivered to the phagosome after phagocytosis). Potential experiments could be to measure the cytochrome *c* reduction capacity of neutrophils in the presence of *P. gingivalis* and in the presence and absence of antibody and complement, after the addition of cytochalasin B/ PMA *vs.* fMLP, with and without SOD and/or catalase; and how well this correlates with the ability of *P. gingivalis* to be killed on the surface of neutrophils under these conditions. While the use of PMA would allow us to differentiate the effects of the respiratory burst independent of MPO release due to the selective release of specific granules, fMLP would allow us to examine the influence of

both granule populations (specific and azurophilic). Additionally, anaerobiosis and DPI could also be used to differentiate the contributions of oxygen- and NADPH oxidase-dependent killing pathways, respectively, in extracellular neutrophil-mediated killing of *P. gingivalis* in the presence of cytochalasin B/ PMA *vs.* fMLP.

Bacterial virulence has been suggested to be connected with the ability of bacteria to synthesize specific antioxidant enzymes that help to neutralize the oxidative killing mechanisms of the neutrophil. Many bacteria synthesize antioxidant enzymes such as SOD, catalase, and peroxidases, which may contribute to their resistance to killing by neutrophils. SOD is thought to protect *Nocardia asteroides* (168) (169), *Listeria monocytogenes* (170), and *Shigella flexneri* (171) (172) from phagocytic killing; whereas, catalase activity has been suggested to protect *S. aureus* (173) and *Neisseria gonorrhea* (174) from phagocytic killing. While SOD in *P. gingivalis* has not been associated with resistance to neutrophil killing, it has been found to contribute to its remarkable ability to survive during transient exposure to oxygen (175). As suggested by Lynch *et al* (175), initial colonization events may pose the most crucial oxidative stresses to *P. gingivalis*, and given the varying oxygen tension in periodontal pockets, aerotolerance in the absence of growth may be as significant a virulence trait of *P. gingivalis* as any other. SOD in *P. gingivalis* may therefore contribute not only to its ability to successfully colonize, but in protection against oxygen exposure during periodontal procedures (e.g. scaling, planing, and surgery).

While *P. gingivalis* does not make catalase or peroxidase enzymes to degrade H_2O_2 (138) (176), it does make a protoporphyrin layer from hemoglobin, which is proposed to serve as an antioxidant barrier (143) (144), as well as rubrerythrin (177), Dps (DNA binding protein from starved cells) (178), an alkyl hydroperoxide reductase (AhpFC) (179) (180), and OxyR (181), which have all been shown to potentially protect *P. gingivalis* against H_2O_2. *P. gingivalis*, however, is remarkably sensitive to low micromolar concentrations of H_2O_2, so it is apparent that these antioxidant mechanisms against H_2O_2 are not very effective against H_2O_2-mediated killing, at least in the conditions (PBS) tested.

Since H_2O_2-mediated lethality in bacteria is associated with the possession of intrinsic iron (88) (148), and is due to overwhelming DNA damage due to inefficient repair (130) (159) (161); the exceptional susceptibility of *P. gingivalis* to H_2O_2 may be due to several things. For instance, *P. gingivalis* may possess 1- fewer scavenging enzymes or 2- fewer DNA repair mechanisms; 3- a greater number or 4- a greater accessibility of lethal targets; or 4- a greater amount of intrinsic iron (e.g. iron-sulfur clusters) available to generate $OH^{\cdot}$. These are unknown variables which warrant future study. It is also unknown whether heme iron in protoporphyrin is capable of participating in Fenton chemistry, which could account for the relatively greater sensitivity of both *P. gingivalis* and *P. intermedia* to H_2O_2, compared to the other test species that do not make protoporphyrin. It is possible that the iron abundant within the protoporphyrin layer that covers the surfaces of these bacteria provide additional iron sources for the generation of $OH^{\cdot}$, close to vital targets such as DNA.

Preliminary studies in this laboratory, which compared H_2O_2-mediated killing of *P. gingivalis* in PBS *vs.* in various complex media (e.g. in Wilkins-Chalgren broth (WC) as well as in trypticase soy broth (TSB)), suggested that *P. gingivalis* has an advantage over other organisms in WC because of how resistant it became to killing in WC; i.e. *P. gingivalis* required approximately 100-fold more H_2O_2 in WC, in order to achieve equivalent killing in WC and PBS (unpublished observations). WC, which contains haemin, afforded a protection to *P. gingivalis* against H_2O_2-mediated killing not evident in TSB, which does not contain haemin. In contrast to *P. gingivalis*, *S. aureus* showed very similar killing in PBS and WC, suggesting that haemin is what was protecting *P. gingivalis* against H_2O_2-mediated killing in WC. It is therefore possible that *P. gingivalis* in growth-conditions *in situ*, where red blood cells and thus haemin are available, may enhance the resistance of *P. gingivalis* by permitting the up-regulation or accretion of potential defense mechanisms such as protoporphyrin, which is dependent on haemin derived from hemoglobin. As suggested by others (156) (182), it is also possible, though unlikely given the negligible effect on *S. aureus* susceptibility, that the rich medium of WC (which contains buffer salts, proteins, etc) accounts for the reduced bactericidal activity of H_2O_2, by its components scavenging H_2O_2. All of these observations warrant further study.

Experiments could include examining *P. gingivalis* sensitivity to H_2O_2 under the influence of different growth phases (i.e. lag, exponential, and stationary) and examining what defense mechanisms are up-regulated or accreted (e.g. protoporphyrin) at the different growth phases and under the growth conditions of WC *vs.* the non-growth conditions of PBS, to possibly account for its greater sensitivity to H_2O_2 in PBS compared to WC. Additional experiments could examine *P. gingivalis* (and *P. intermedia*) sensitivity to H_2O_2 when protoporphyrin is promoted (e.g. when grown on blood-based medias which allow protoporphyrin to be accreted *vs.* on non blood-based

medias such as WC), in comparison to non-black pigmenting mutants that make little or no protoporphyrin (99) (143) (144) (145); and if there are differences in the effectiveness of heme-derived *vs.* hemoglobin-derived protoporphyrin. For instance, growth on media containing hemoglobin but not heme/haemin is necessary for black-pigmentation (99), a result of modification of protoporphyrin. PBS and TSB could also be enriched with heme/ haemin (*vs.* hemoglobin) in order to confirm the influence of haemin in WC, and to evaluate the potential effects of metabolism, in the increased resistance of *P. gingivalis* in WC compared to PBS.

Despite the protective role of neutrophils in the periodontium, neutrophils are the main cell type associated with the pathogenesis of periodontitis, and are responsible for a significant amount of damage accompanying disease activity. Host tissue damage most likely occurs unintentionally by 'frustrated' neutrophils, unable to clear phagocytosis-resistant bacteria, or when confronted by high concentrations of bacteria which they are unable to handle, such in the absence of oxygen (183). In the presence of pro-inflammatory cytokines, neutrophils become hyper-responsive, and are believed to deliver more of their granule contents extracellularly, which in these special circumstances include MPO. It is highly plausible that the primary strategy of *P. gingivalis* in the periodontium may be to minimize the presence of oxygen, which would minimize the production of H_2O_2, and may do so by utilizing host elements.

If *P. gingivalis* is as sensitive to H_2O_2 in the gingival crevice as it is in PBS, it may even benefit from the presence of MPO, which may function as a H_2O_2 scavenger or a catalase in high concentrations of H_2O_2 (115) (142) (and Figure 3.5B). By consuming H_2O_2, the catalase-like activity of MPO in the gingival crevice would convert H_2O_2 to oxygen and water, thereby protecting H_2O_2-sensitive bacteria such as *P. gingivalis* against H_2O_2. In the presence of red blood cells in the gingival crevice due to bleeding gums, hemoglobin would not only be available to provide haemin to *P. gingivalis*, but could also serve to "mop up" excess oxygen from these catalatic activities of both catalase and MPO. It is also likely that extra-phagosomal H_2O_2 either released by neutrophils into the gingival pocket or provided as a clinical treatment, would not be effective in periodontitis due to the abundance of catalase from red blood cells, which would readily rid H_2O_2 in periodontal tissues.

While it is hypothesized from our studies that MPO protects enzymatically (e.g. functions as a catalase) in the presence of high H_2O_2 concentrations, this also warrants confirmation, which can be achieved with 4-ABH under these conditions; the alternative

possibility, though unlikely given the negligible protein contribution, was that it was a protein effect.

In future studies, it would be of upmost importance to better understand the function of MPO in the context of what is actually happening in the phagosome. For instance, at a pH of 7.4, MPO was highly effective at blocking killing by ≥10μM H_2O_2 (Figure 3.5), and thus it remains unclear whether MPO can play an important role in killing in the phagosome, if antagonistic interactions between MPO and H_2O_2 occur in the phagosome, as they were shown to occur in these studies. Furthermore, it would be important to better understand what shifts the function of MPO (e.g. whether pH is involved or if purely dependent on substrate availability such as H_2O_2 concentration) – from being bactericidal, to host protective, and potentially protective of bacteria, depending on H_2O_2 concentration.

In future studies, it would also be important to understand the influence of order of addition. For instance, in these studies, MPO was combined with H_2O_2 for several seconds before bacteria were added, which was designed to favor HOCl generation. It would be interesting to determine whether exposure of bacteria first with H_2O_2, at various concentrations and for various lengths of time, followed by the addition of MPO, would have a different effect. For instance, the H_2O_2 to MPO ratios required for killing may be different if the order of addition is altered, due to additional H_2O_2 scavenging opportunities by bacteria, if exposed to H_2O_2 for a period of time before MPO. Additionally, the pre-treatment of bacteria with H_2O_2, followed by the addition of MPO, would presumably also be comparable to the order of addition which occurs within the phagosome, and may allow H_2O_2 to enter the bacteria and initiate MPO-independent killing events before MPO gets delivered. In these circumstances, the subsequent addition of MPO may not show as much protection against H_2O_2-mediated killing as previously seen, or may show no protection at all, if H_2O_2 has already entered the cell at

the site of delivery, and has already reached lethal concentrations within the bacterial cell. A lethal, intracellular build-up of H_2O_2 may likely occur before MPO is delivered via an azurophilic granule, which would fuse at a separate and distinct site of the phagosome.

H_2O_2, which is generated proximal to a bacterial surface and is rapidly membrane- diffusible, would also be less available to react with MPO when it is delivered later, at a separate site of the phagosome, for the subsequent generation of HOCl. In contrast, the Winterbourn model (95) assumes that all of the H_2O_2 in the phagosome will be consumed immediately by MPO, and that H_2O_2 is unavailable for purposes other than the generation of bleach. In context of this model and our own, it would be extremely important to better understand the timing of azurophilic granule fusion with the phagosome and the timing of MPO release, subsequent to the initiation of the respiratory burst, as previous studies in this laboratory using electron microscopy have suggested that H_2O_2 generation subsequent to specific granule fusion with the phagosome is initiated tens of minutes before MPO-containing azurophilic granules are seen to be delivered (unpublished observations). Additionally, it would be important to take into account the effect of antioxidant defenses of bacteria into this model, as suggested in Figures 3.4 and 3.6 and in the discussions of chapters 3 and 4.

In addition, in future studies, it would also be important to understand the influence of pH on killing. The studies here employed a pH of 7.4, which is the presumed pH when MPO is delivered and bacterial killing occurs (95). However, earlier in the phagosome, when the respiratory burst has just begun, the pH is believed to be transiently lower (97). It is hypothesized that the lower pH would favor the conversion of $O_2^{\cdot -}$ and/or H_2O_2 to more toxic products such as $OH^{\cdot}$ prior to the delivery of MPO (184).

Since the mechanism of azide protection of *P. gingivalis* against neutrophil-mediated killing remains unknown, future studies are also warranted to determine the specific mechanism by which azide protects (e.g. whether by $OH^{\cdot}$ scavenging or by inhibiting $OH^{\cdot}$ generation).

It is also a question what effect azide has on MPO-independent, H_2O_2-mediated killing of *P. gingivalis*, in comparison to treatment with other potent $OH^{\cdot}$ scavengers or inhibitors (e.g. DMSO and thiourea, etc). It is hypothesized that azide would also inhibit *in vitro* H_2O_2-mediated killing of this pathogen, by inhibiting toxicity by H_2O_2 by the mechanisms previously discussed.

The question also still remains as to why subjects with CGD, whose neutrophils are incapable of killing *P. gingivalis*, rarely present with periodontal or other anaerobic infections. It is possible that the answer lies in the absence of the respiratory burst itself. For instance, the inability to reduce the microenvironment (i.e. rid oxygen) in CGD, despite its inability to kill, may prevent this strict anaerobe from growing and thus establishing an infection in healthy, aerated periodontal tissues.

It is also well-supported that ROS from the respiratory burst are the major contributors to the severe periodontal tissue destruction associated with aggressive periodontitis (185), so the absence of a respiratory burst in CGD may explain the absence of characteristic periodontal tissue damage. This is consistent with the findings of Gronert *et al* (186), which associated locally inflamed sites in subjects with localized aggressive periodontitis with excessive superoxide production. Additionally, this concept is consistent with the findings of Mydel *et al* (187), which suggested that the host respiratory burst paradoxically improved the survival of *P. gingivalis* in the mouse subcutaneous chamber model by enhancing local and systemic inflammation. Conversely, mice without a respiratory burst survived and were resistant to *P. gingivalis* infection, while mice with an intact NADPH oxidase succumbed to *P. gingivalis* challenge. Excessive neutrophil-generated inflammation in wild type mice, which was shown to be completely responsible for the morbidity and mortality associated with *P. gingivalis* infection, may not only have provided reduced oxygen conditions, but a source of nutrients for the growth of this anaerobic and assacharolytic bacterium.

Another explanation which may partially account for the lack of predisposition to periodontitis in subjects with CGD is long-term antibiotic use after diagnosis with CGD. This seems a less likely explanation, however, as subjects with other neutrophil disorders appear highly predisposed to developing severe periodontitis, despite aggressive antibiotic regimens (188).

It is also conceivable that the strict anaerobic metabolism of *P. gingivalis* may safeguard *P. gingivalis* in CGD, and that it is not killed by CGD neutrophils because it does not generate H_2O_2. It would be valuable to investigate whether this in fact is true. Furthermore, impaired bactericidal activity in CGD neutrophils, which are incapable of producing their own $O_2^{\cdot -}$ and H_2O_2, has been restored in the laboratory by the addition of a H_2O_2-generating system (189) (190) (191) (192) (193) (194). It would also be of interest to know whether a H_2O_2-generating system, such as 6-formylpterin (189), would restore bactericidal activity against *P. gingivalis* in CGD neutrophils.

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APPENDIX:

SUPPLEMENTAL DATA AND METHODS TO CHAPTER 2

Supplemental Table A.1. Neutrophil Phagocytosis of *P. gingivalis* is Antibody-Dependent and Oxygen-Independent

	% Phagocytosis, with Ab		% Phagocytosis, without Ab
Time (min)	**Aerobic**	**Anaerobic**	**Aerobic**
0	71 (5)	72 (6)	*1 (1)
10	79 (9)	80 (10)	*1 (3)
30	94 (6)	90 (10)	*10 (1)
60	97 (5)	96 (4)	*10 (1)
120	98 (3)	98 (3)	*10 (1)

Results are expressed as the % of total neutrophils involved in phagocytosis (three or more bacteria per neutrophil). Samples contained the presence of human complement, and either the presence or absence of hyperimmune rabbit antiserum (Ab). Experiments were performed at 37^{o}C under aerobic *vs.* anaerobic atmosphere, using normally competent neutrophils purified from the peripheral blood of healthy donors. Values are the means from three or more independent experiments, and values in parentheses are the standard deviations of those means. Asterisks indicate that the difference, compared to antibody-containing controls, is statistically significant ($p<0.05$).

Supplemental Table A.2. Description of CGD Subjects

Subject	Age	Gender	Genotype
CGD 1	10 mo	M	$gp91^{phox}$
CGD 2	12 yr	F	$p47^{phox}$
CGD 3	13 yr	M	$p47^{phox}$
CGD 4	10 yr	F	$p47^{phox}$
CGD 5	38 yr	F	$p47^{phox}$

Supplemental Table A.3. Neutrophil Oxidative Index by Flow Cytometry, and Vmax and Change in Optical Density over 30 min by Cytochrome *C* Reduction, Comparing CGD *vs.* Normal Neutrophils

	Flow cytometry	PMA[b]		fMLP[e]/cytochalasin b[f]	
Subject	NOI[a]	Vmax[c]	ΔmOD_{30min}[d]	Vmax[c]	ΔmOD_{30min}[d]
CGD 1	1	0	70	0	0
CGD 2	2	0	0	0	0
CGD 3	4	0	0	0	0
CGD 4	6	NT	NT	NT	NT
CGD 5	1	0	0	0	0
Controls	249 (95)	47 (20)	473 (335)	17 (2)	151 (28)

Control values are the means from normally competent neutrophils purified from the peripheral blood of four different healthy donors, and values in parentheses are the standard deviations of those means. Values for CGD Subjects 1-5 represent data obtained from a single sample. NT: not tested. Experiments were performed under aerobic atmosphere.

[a] NOI: neutrophil oxidative index (>30 defined as normal).

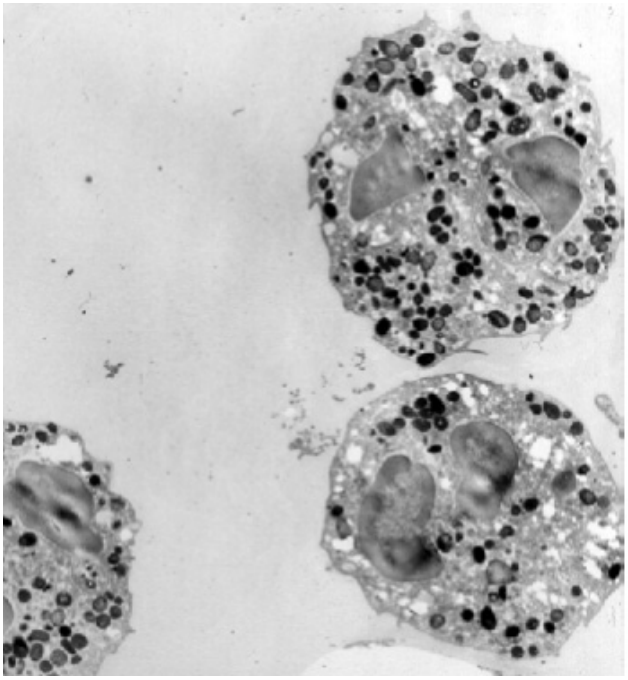
[b] PMA: phorbol myristate acetate (final concentration: $1x10^{-7}M/5x10^{6}$ neutrophils).

[c] Vmax: maximal velocity of superoxide production (mOD/min) calculated by SOFTMAX® software.

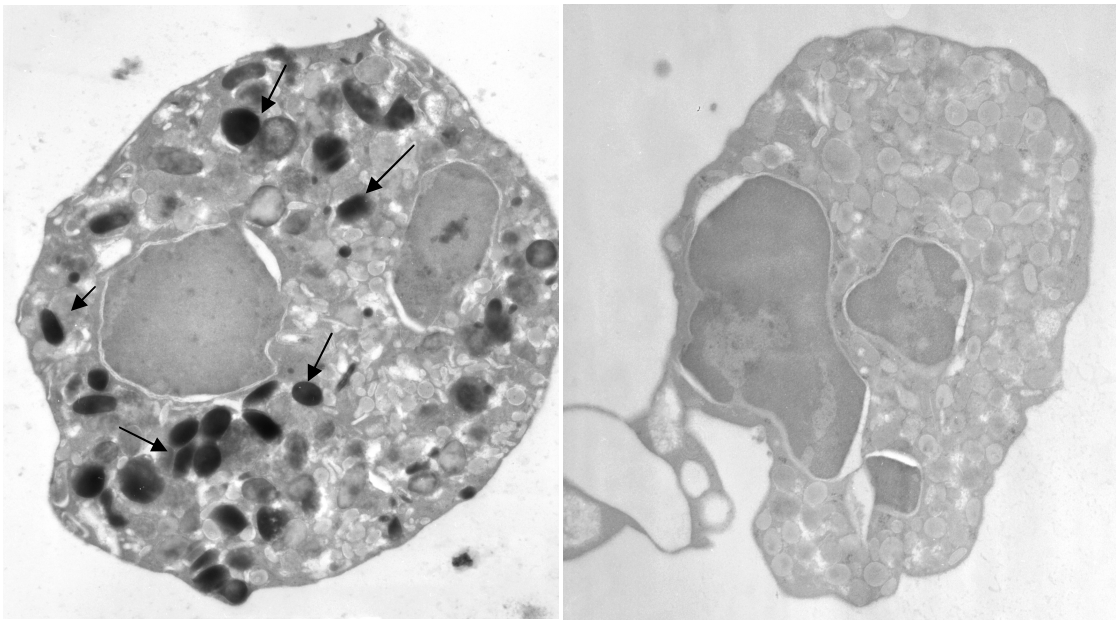
[d] ΔmOD_{30min}: change in optical density over 30 minutes (from time 0 to time 30 min).

[e] fMLP: N-formyl-methionyl-leucyl-phenylalanine (final concentration: $5x10^{-8}M/5x10^{6}$ neutrophils).

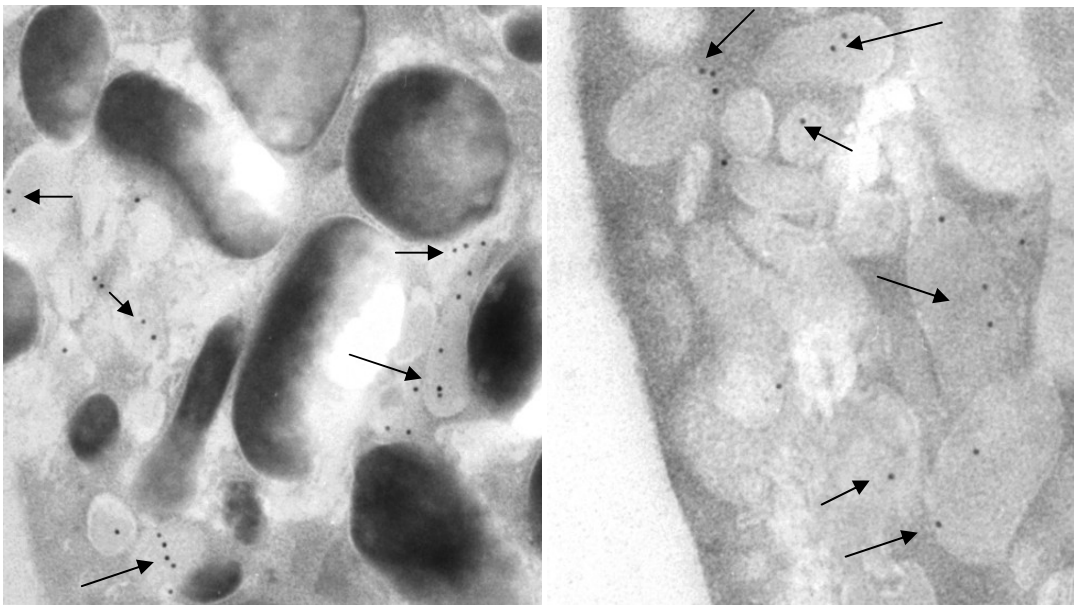
[f] Cytochalasin b (final concentration: $5\mu g\ mL^{-1}/5x10^{6}$ neutrophils).



Supplemental Figure A.1A. MPO-Positive Neutrophils from a Normally Healthy Human Donor. Transmission electron micrograph, representative of normal neutrophils from a healthy donor, showing histochemical staining for peroxidase-positive granules (original magnification x7500). Note the distribution and dark staining character of the peroxidase-positive granules. All neutrophils from donor controls demonstrated normal distributions of peroxidase-positive granules.

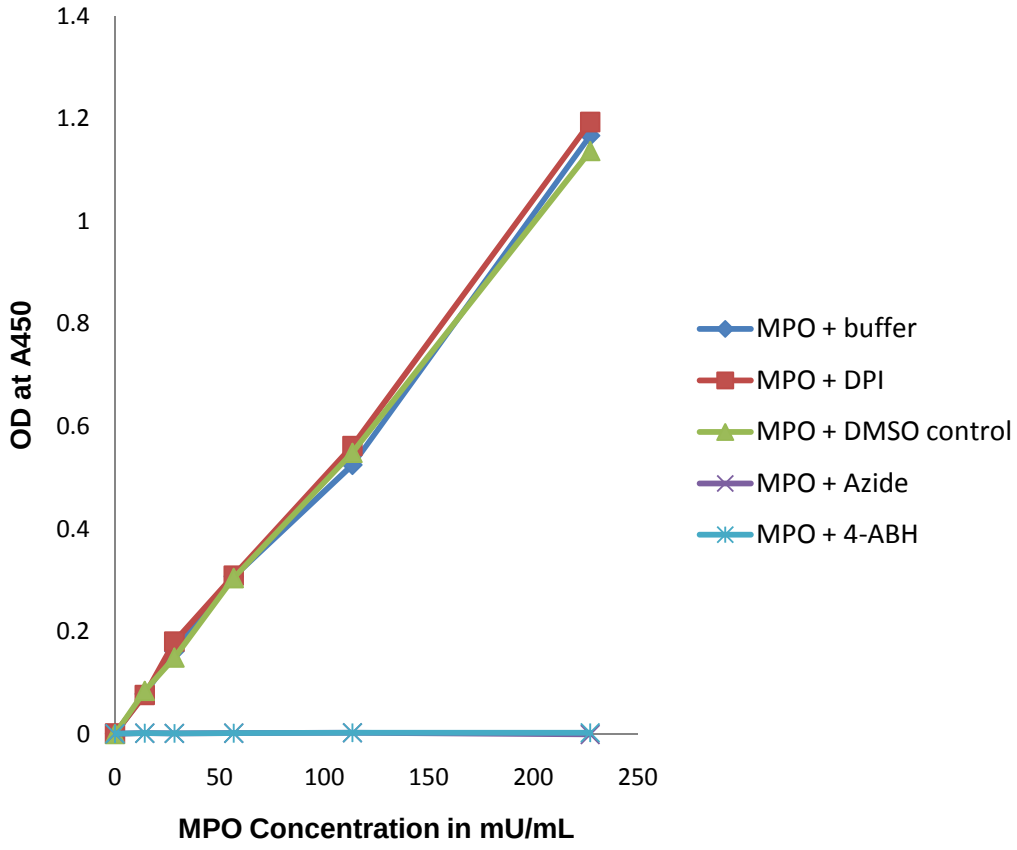


Supplemental Figure A.1B. Transmission Electron Micrographs of Representative Neutrophils from the Peripheral Blood of Subject MPO-PDM (left panel) Compared to MPO-DS (right panel). Right panel: Note the absence of peroxidase-positive granules in the neutrophils from MPO-DS, compared to that of MPO-PDM (arrows, left panel) and to that of normal neutrophils in Supplemental Figure A.1A. None of the neutrophils examined from subjects MPO-DS (right panel), MPO-DB or MPO-DF (not shown) contained peroxidase-positive granules. However, all subjects had dense distributions of granules and all contained granules that were positive for lactoferrin as determined by immunogold (Supplemental Figure A.1C and data not shown). Approximately 90% of the neutrophils from MPO-PDM (left panel) showed normal distributions of peroxidase-positive granule types; the remaining 10% of neutrophils had no discernible peroxidase-positive granule types.

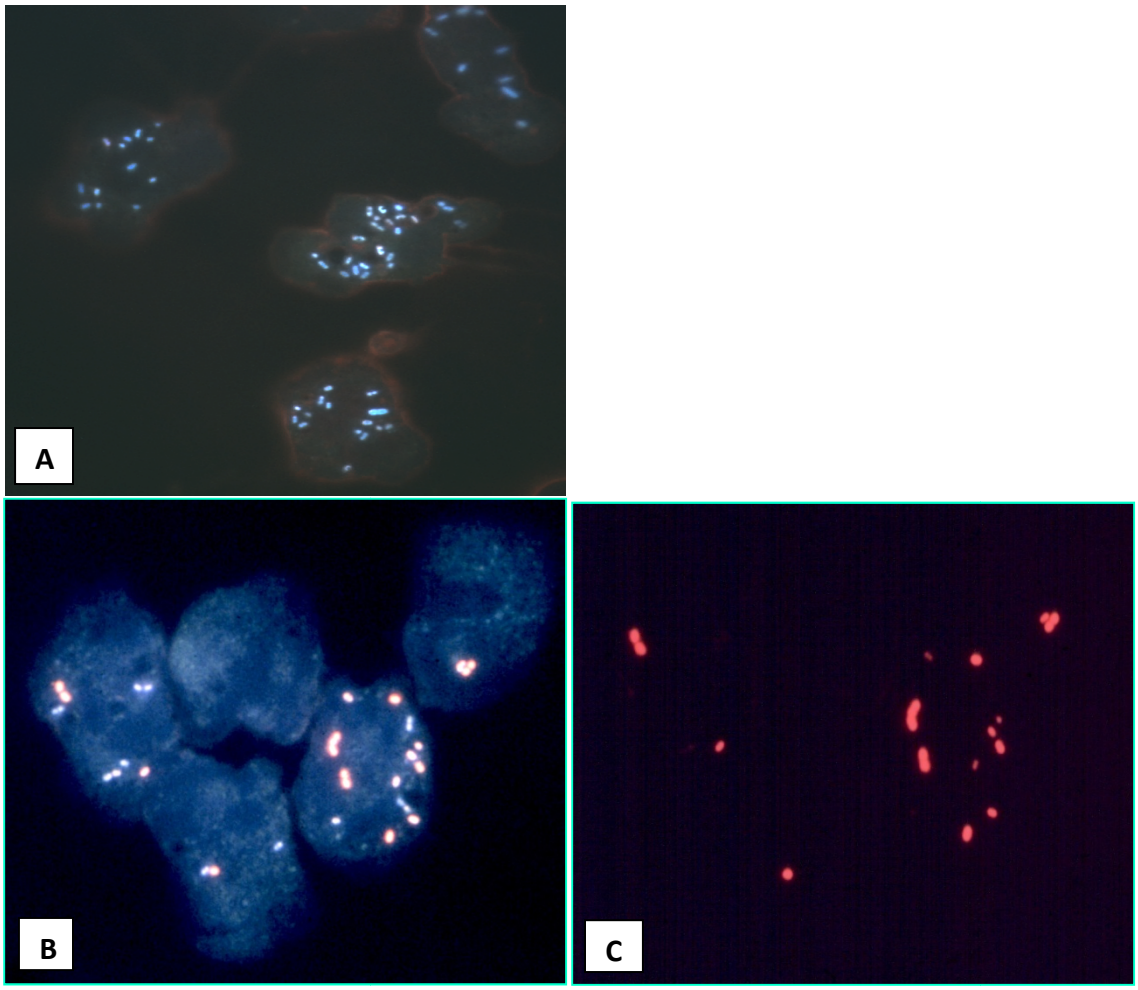


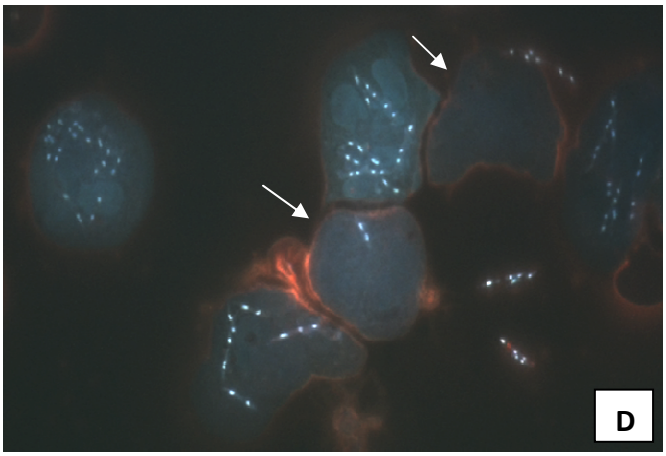
Supplemental Figure A.1C. Transmission Electron Micrographs of Representative Neutrophils from MPO-PDG, Histochemically Stained for Peroxidase and Immunogold-Labeled for Lactoferrin (arrows). Approximately 50% of neutrophils from MPO-PDG were peroxidase-negative (right panel), with the remainder having normal distributions of peroxidase-positive granules (left panel). All of the neutrophils from MPO-PDG contained lactoferrin-positive granules (arrows, right and left panels).

Supplemental Figure A.2. MPO Enzymatic Assay Testing Various Potential MPO Inhibitors. The following final concentrations were used: DPI: 20µM in less than 0.3mM or 0.1% (v/v) DMSO; DMSO: less than 0.3mM; sodium azide: 20µM; 4-ABH**:** 4mM.



Supplemental Figure A.3. DAPI/PI Fluorescence Microscopy of Neutrophil Phagocytosis and Killing of Opsonized *P. gingivalis* and *F. nucleatum* at 0 and 60 min. Neutrophils were purified from the peripheral blood of normally healthy donors and tested under aerobic atmosphere. DAPI fluorescence (blue color) indicates bacteria are alive, while PI uptake (red color) indicates bacteria are dead. Figures A (*P. gingivalis*) and D (*F. nucleatum*) represent samples obtained at initial time (0 min), while Figures B-C (*P. gingivalis*) and E-F (*F. nucleatum*) represent samples obtained at 60 min.

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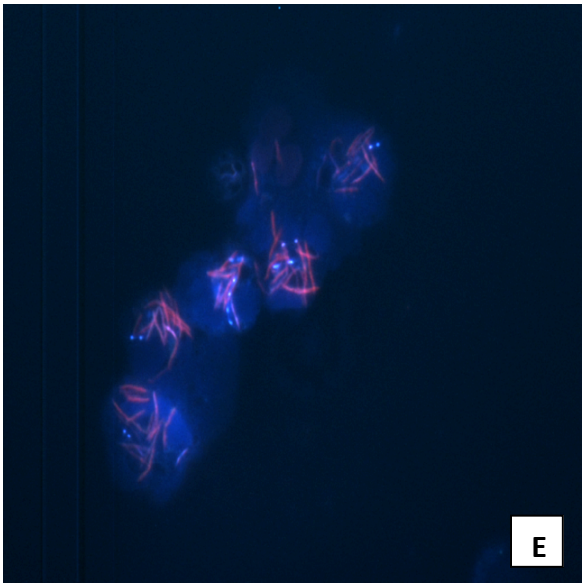
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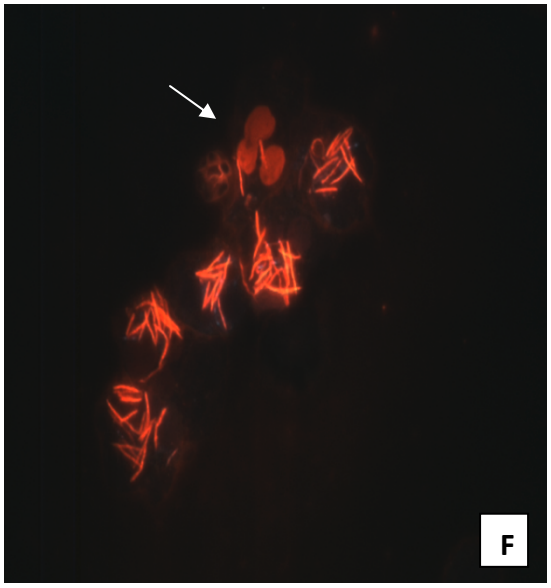
F

MATERIALS AND METHODS

Patient Selection

***Chronic Granulomatous Disease (CGD) Subjects*:** Five CGD subjects were included in this study and are characterized in Supplemental Table A.2. CGD1 was a ten month old male, who presented to UNC Hospital with a history of recurrent bacterial infections, most notably staphylococcal skin abscesses. CGD1 died two months later of a disseminating *Aspergillus* infection. CGD2, CGD3, and CGD4 were juveniles ranging between 10-13 years of age. All three reported a prior history of recurrent skin infections, abscesses, sore mouth, and sore gums. Oral examination revealed mild gingivitis in CGD3 and CGD4 (brother and sister), while CGD2 appeared to have excellent gingival health. CGD2, CGD3, and CGD4 had no tooth loss and no radiographic evidence of bone loss. CGD5 was a 38 year old female with a history of periodontal surgery. CGD5 was on a periodontal maintenance program and had a stable periodontal condition (2-5mm probing depths), little clinical inflammation, and excellent oral hygiene. CGD5 did not report a history of frequent intraoral ulcerations. Her periodontal condition rapidly deteriorated with highly inflamed gingival tissues with excessive bleeding and exudate, and probing depths of 6-9mm. This patient did not respond to antibiotics or to local irrigation treatments. Radiography showed moderate to severe generalized horizontal bone loss, but no missing teeth. Her purified neutrophils were determined incapable of reducing cytochrome *c* following either PMA or fMLP stimulation. She was subsequently diagnosed genetically as heterozygous $p47^{phox}$ deficient.

***MPO- Deficient Subjects*:** Five MPO-deficient subjects were included in this study, and were members of one family previously identified with MPO-deficiency. MPO-DS was a five year old Caucasian female, who presented with a history since

infancy of recurrent sinusitis (documented by sinus radiographs), bronchitis (associated with fever), and asthma. MPO-DS had frequent respiratory exacerbations, which were treated with both antibiotics and asthma therapy. She also had chronic headaches, treated with valproic acid and restoril. Her initial workup at UNC (age specific normal values for UNC laboratories shown parenthetically) included a hemoglobin of 11.3 g/dl (normal: 12.3-15.7 g/dl), hematocrit of 32.5% (normal: 38-47%), white blood count of 8.6 $x10^9$ cells/l (normal: 4-12 $x10^9$ cells/l), monocyte count of 0.5 $x10^9$ cells/l (0.7-1.5 $x10^9$ cells/l), and an eosinophil count of 0.2 $x10^9$ cells/l (0.3-0.8 x 10^9 cells/l). Serum immunoglobulins included IgA of 25 mg/dl (normal: 40-320 mg/dl), IgM of 26 mg/dl (normal: 25-210 mg/dl), and an IgG of 319 mg/dl (normal: 400-1400 mg/dl) and an IgG4 of 11 mg/dL (normal: 0-138 mg/dL). IgE was <10 IU and her skin test negative. Her oxidative burst index was 151 (>30 considered normal). Delayed type sensitivity skin tests were normal, as were lymphocytic marker studies for CD3, CD4, CD8, CD19, and CD56. Pneumococcal vaccine was administered and her pre- and post- immunization titers (performed at Scripps) obtained on the day of immunization and four weeks post-immunization for each serotype (protective considered >2.0) were: serotype 4: <0.1 and <0.1, serotype 6b: <0.1 and <0.1, serotype 14: <0.2 and <0.2, serotype 18c: <0.1 and 0.1, serotype 19f: <0.3 and <0.3 and serotype 23b: <0.1 and <0.1. She then underwent a course of IVIG (0.4 g/kg monthly), with gradual improvement in her sinopulmonary status, such that she had no hospitalizations for the 12 months following initiation of IVIG. After one year, she was taken off IVIG therapy for six weeks and immunized again with diphtheria, tetanus, and pneumococcal vaccines. Post- immunization titers to diphtheria were 1:19,683, tetanus 1:59,049, and pneumococcal titers were: serotype 4: 0.5, serotype 6b: 0.6, serotype 14: 2.2, serotype 18c: 0.9, serotype 19f: 1.1 and serotype 23b: 0.7. During the time she was off IVIG, she began having respiratory

episodes with temperature of 38.2 or less, which were treated with antibiotics. IVIG treatment was reinitiated.

MPO-DB, the brother of MPO-DS, presented to UNC Hospital at age two with a history of chronic sinusitis (documented by sinus radiographs), which did not resolve with an initial 21-day treatment with amoxicillin and clavulanic acid. He then underwent treatment with clindamycin for 14 days without improvement and eventually required concurrent treatment with clindamycin and amoxicillin + clavulanic acid. He had recurrent episodes of sinusitis. His initial workup at UNC (age specific normal values for UNC laboratories shown parenthetically) included a hemoglobin of 12.8 g/dl (12.3-15.7 g/dl), hematocrit of 34.9% (38-47%), white blood count of 7.6 $x10^9$ cells/l (4-12 x 10^9 cells/l), neutrophil count of 2.6 $x10^9$ cells/l (2-6 x 10^9 cells/l), lymphocyte count of 4.2 $x10^9$ cells/l (2-5 x 10^9 cells/l), monocyte count of 0.2 $x10^9$ cells/l (0.3-1.1 x 10^9 cells/l), and an eosinophil count of 0.3 $x10^9$ cells/l (0.2-2.0 x 10^9 cells/l). Serum immunoglobulins included an IgA of 30 mg/dl (25-240 mg/dl), IgM of 48 mg/dl (25-210 mg/dl), and an IgG of 532 mg/dl (325-1300 mg/dl), with an IgG1 level of 353 mg/dl (290-1065 mg/dl), IgG2 of 59 mg/dl, IgG3 of 38 mg/dl (4-71 mg/dl), and an IgG4 of 23 mg/dl (0-90 mg/dl). IgE was <10 IU and he was skin test negative for allergy. His oxidative burst index was 151 (>30 considered normal). Delayed type sensitivity skin tests were normal, as were lymphocyte marker studies for CD3, CD4, CD8, CD19, and CD56. Pneumococcal vaccine was administered and titers obtained four weeks later to each serotype were: serotype 4: 0.8, serotype 6b: 0.1, serotype 14: <0.2, serotype 18c: 2.1, serotype 19f: <0.3 and serotype 23b: 0.2. IVIG treatment was started and the need for antibiotic treatment for sinusitis subsided. After four months of treatment, IVIG was stopped and pre- and post- pneumococcal immunization titers for each serotype were found to be: serotype 4: 0.1 and 0.1, serotype 6b: <0.1 and <0.1, serotype14: <0.2 and <0.2,

serotype18c: 0.6 and 0.5, serotype19f: <0.3 and <0.3 and serotype 23b: <0.1 and <0.4. MPO-DB is now receiving IVIG**.**

The mother (MPO-PDM) and maternal grandmother (MPO-PDG) to MPO-DS and MPO-DB both reported problems with sinopulmonary infections, especially sinusitis. They did not choose to undergo treatment. The father (MPO-DF) had a normal health history, despite complete MPO-deficiency. Five gender- and race-matched normally healthy donors were included as controls for the determination of neutrophil MPO- and lactoferrin-granule profiles. Purified neutrophils (buffy coat from MPO-DB) from peripheral blood from each of these subjects were examined by transmission electron microscopy for peroxidase-positive and lactoferrin-positive granule staining (see methods below).

Transmission Electron Microscopy

Purified neutrophils or buffy coat from selected MPO-deficient subjects and controls were examined simultaneously by histochemistry for peroxidase-positive granule content and by immunogold-labeling for lactoferrin-containing granules. Cell preparations were fixed in 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for three hours, then rinsed in the 0.1M cacodylate buffer overnight. Histochemical staining for MPO was performed according to the methods of Pelliniemi (1) and Castaneda (2) *et al*. Briefly, cells were incubated in 5mg diaminobenzidine (DAB) contained in 10mL 0.05M Tris-HCl buffer and 0.1mL 1% H_2O_2 for 30 min, rinsed and amplified with 2% OsO_4 for one hour. Cell suspensions were dehydrated in 50% and 70% ethanol for five min each, and then transferred to a 2:1 mixture of LR White resin and 70% ethanol for four hours. The samples were then infiltrated in LR White resin overnight at room temperature, transferred to gelatin

capsules and cured for three days at 45°C. Sections were cut on an ultramicrotome (60-90nm) and placed on nickel grids.

Post-embedding immunolocalization of lactoferrin was performed based on a procedure described by Esaguy *et al* (3). All washing, incubation, and fixation procedures were accomplished by immersing the grids face down in drops of the different solutions. Briefly, the grids were washed with water for 10 min, rinsed with PBS/1% BSA x 2 for five min at room temperature or 37°C, washed with PBS/BSA x 3 for five min, and incubated with 10nm gold conjugated Protein A/antibody (affinity-purified rabbit anti-human lactoferrin IgG prepared in this laboratory) in PBS/BSA (1:20, v/v) for 45 min. The grids were then rinsed with PBS/BSA x 3 for five min, PBS x 2 for five min, fixed with 2% glutaraldehyde in 0.1M phosphate buffer for 10 min, rinsed with water x 3 for five min, and post-stained in uranyl acetate and lead citrate. Sections were viewed with a Philips CM12 transmission electron microscope operating at 80kV accelerating voltage.

Flow Cytometry Assay

The intracellular production of hydrogen peroxide was measured in individual stimulated neutrophils by flow cytometry using the procedures reported by Rothe *et al* (4) (5). An Oxidative Burst Kit was purchased from New Concept Scientific Ltd (Burlington, Ontario). One ml of blood was collected in EDTA from the five CGD subjects and controls. 100μL of whole blood was suspended in a microcentrifuge tube containing 900μL of phosphate buffered saline (PBS) and dihydrorhodamine 123 (DHR 123, final concentration 2.5 μg/ml). The suspension was incubated in a shaking water bath for 15 min at 37°C. 10μL of phorbol myristate acetate (PMA, final concentration 100ng/mL) was added and the neutrophils were incubated for another 15 min at 37°C.

Control samples containing no DHR123 or no PMA were run in parallel. The neutrophils were collected after centrifugation (300xg, five min), red blood cells were lysed with lysing buffer, and the remaining neutrophils were washed again with wash buffer and fixed in 0.5mL of 1% paraformaldehyde. Flow cytometric analysis was performed on 5,000 neutrophils using a Becton Dickinson FACScan® flow cytometer. Neutrophil Oxidative Index (NOI) was calculated by dividing the mean fluorescence of the sample with both DHR123 and PMA, by the mean fluorescence of the sample without PMA. An NOI > 30 was considered to be normal, and an NOI < 30 was considered an absence of respiratory burst, and suggested CGD.

Neutrophil Superoxide Production Assay

The extracellular release of superoxide anions by neutrophils upon stimulation with fMLP (N-formyl-methionyl-leucyl-phenylalanine) in the presence of cytochalasin b or with PMA was measured using a microtiter spectrophotometric assay based on the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* (6). A continuous rate-determination (Vmax or maximal velocity) and total superoxide production assay (ΔOD_{30min}) were employed to examine neutrophil oxidative burst functions. Briefly, stock solutions of SOD (Boehringer Mannheim, GmbH, Germany) and cytochrome *c* (Sigma Chemical Co., St. Louis, MO) were made using HBSS buffer without metals. fMLP and PMA (Sigma Chemical Co., St. Louis, MO) were made in 100% DMSO (dimethyl sulfoxide, Sigma Chemical Co.) and diluted to the appropriate working concentration just before use. The final concentration of DMSO in the assay mixture was <0.1% (v/v). All experiments were carried out at least in triplicate. Each test well contained $5x10^6$ neutrophils, 0.11μM cytochrome *c*, 0.6 mM $CaCl_2$, 0.5 mM $MgCl_2$, and either fMLP ($5x10^{-8}$M) + cytochalasin b (5μg/ml), or PMA ($1x10^{-7}$M). Control wells additionally contained

100μg SOD. HBSS without metals was added to adjust the final volume to 100μL. Superoxide production by neutrophils after stimulation with either fMLP/cyt b or PMA was assessed by measuring the change in absorption at $A_{\lambda 550nm}$ every 10 seconds for 30 min in a Vmax kinetic microplate reader (Molecular Devices, Menlo Park, CA) at room temperature. Agitation was applied before each reading using an auto vibrating plate holder. The microplate reader was connected to a PC with Softmax software (Molecular Devices, Menlo Park, CA) allowing Vmax estimates, OD (optical density) change measurement, and curve fittings. An absence of cytochrome *c* reduction, as demonstrated by a Vmax of zero, and a negligible OD change over 30 min, was considered an absence of respiratory burst (inability to produce superoxide), and suggested CGD.

MPO Assay

MPO enzymatic activity was tested using a microtiter spectrophotometric assay on titered human neutrophil MPO (Athens Research and Technology Inc, Athens, GA) (frozen as 30μL aliquots of 20U/mL stock in 50mM sodium acetate pH 6 + 100mM NaCl), using a substrate kit from Kirkegaard & Perry Laboratories (#50-76-00), in the presence and absence of various inhibitors (azide at 20μM FC, 4-ABH at 4mM FC, DPI at 20μM FC, and DMSO at 0.03% (v/v) or 0.28mM FC). Briefly, 50μL of substrate, mixed in equal parts (25μL of 1.7mM 3,3'5,5'-tetramethyl benzidine (FW 240.35), in N,N',dimethylforamide phosphate buffer (Kirkegaard & Perry Labs) + 25μL of 0.02% (v/v) H_2O_2 in citric acid buffer) was added per well, in triplicate. On a 15 s interval, 10μL of MPO (serially diluted two-fold) with or without inhibitor (pre-incubated for one min) were added to microtiter dish wells; 10μL of 0.05M KH_2PO_4 (pH 7) was used as the blank. The reaction was allowed to proceed for 15 min. Adhering to the 15 s interval, the reaction was stopped after 15 min by adding 50μL of 1M phosphoric acid per well.

Endpoints were read at $A_{\lambda 450nm}$ and were plotted as a function of OD at $A_{\lambda 450nm,}$ (y axis) and MPO concentration (x axis), in the presence and absence of the various potential MPO inhibitors. Positive values indicated MPO enzymatic activity.

Supplemental Table A.4. *Staphylococcus aureus* Sensitivity to Propidium Iodide at 1 hr. Results are expressed as the number of log_{10} reductions in recoverable CFU/mL. Propidium iodide (PI) concentrations are expressed in µg/mL FC. Samples contained a starting inoculum of ~10^6 CFU/mL. Since the limit of detection was 2.0 log_{10} CFU/mL, a maximum of four log reductions could be detected; >4 indicates no recoverable CFU on replicate plating.

PI Concentration (µg/mL)	Log_{10} Reductions in CFU/mL			
	Strain 25923	Strain 29213	Strain 14154	Strain 27659
0	0	0	0	0
1.88	0	2	0	1
3.75	1	3	1	2
7.5	2	4	2	3
15	3	>4	3	4
30	4	>4	4	>4

RESULTS AND DISCUSSION

Because subjects with CGD are characteristically susceptible to *Staphylococcus aureus* infections, we ideally wanted to include *S. aureus* in our neutrophil killing studies along with *P. gingivalis.* It was hypothesized that like *P. gingivalis*, neutrophil-mediated killing of *S. aureus* is dependent on the function of the NADPH oxidase. Because absolutely no killing of *P. gingivalis* occurred by CGD neutrophils or by normal neutrophils in the presence of DPI or under anaerobiosis (as detected by the complete absence of PI uptake), it was of interest to see how *S. aureus* compared. However, in preliminary experiments, it was discovered that optimal and sub-optimal PI concentrations killed *S. aureus*, as detected by immediate PI uptake under fluorescence microscopy and logarithmic reductions in CFU/mL by plating. Therefore, it was not possible to include *S. aureus* in our phagocytosis and killing studies using DAPI/PI.

It is important to note that the current understanding is that PI, a small cationic dye, is excluded by the cytoplasmic membranes of live bacteria, and enters bacteria upon loss of membrane integrity, associated with death. As a result of membrane damage, PI enters cells with an increased membrane permeability to molecules ≥2nm in diameter, and binds to double-stranded nucleic acids and fluoresces red at an excitation/emission maxima of 535/617nm (7) (8). Two common applications of PI permeability by bacteria involve quantification by flow cytometry as an indicator of membrane damage and associated lethality, and the detection of intracellular bacterial killing by phagocytes using dual-color fluorescence microscopy. Used both ways, PI permeability (PI uptake) is thought to be a reliable indicator of bacterial killing, as confirmed by plating (9) (10) (11) (12). However, as suggested by Williams *et al* (13), PI staining has not been extensively tested on bacteria, and was developed for small eukaryotic cells.

Four strains of *S. aureus* (strains 25923, 29213, 14154, and 27659) were tested (Supplemental Table A.4), as well as other *Staphylococcus* species including *S. epidermidis* and *S. saprolyticus* (data not shown), with a variety of PI concentrations. All *Staphylococcus* species and strains tested were killed by sub-optimal PI concentrations; i.e. concentrations below which were optimal for distinguishing live from dead bacteria using DAPI/PI fluorescence microscopy. In our hands, ~7.5µg/mL PI (FC) was optimal based on color intensity and complete staining of heat-killed *S. aureus*, *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans* alike (at 10^6 bacteria/mL) under both DAPI and PI filtrations. Furthermore, this concentration of PI had no effect on the viability of live *P. gingivalis*, *F. nucleatum*, or *A. actinomycetemcomitans,* as detected by plating and the absence of PI uptake (data not shown).

Williams *et al* (13) determined a similar concentration (5µg/mL PI) to be optimal for the fluorescence of *E. coli, B. subtilis,* and *Pseudomonas* species, based on PI staining of heat-killed bacteria and no effect on viability; but suggested that several factors, including the PI concentration used and inherent membrane permeabilities of viable bacteria, can impact PI staining and lead to false conclusions. For instance, this study suggested that too high of PI concentrations can falsely stain viable but "leaky" bacteria; while too low of PI concentrations can give heat-killed bacteria, confirmed to be non-viable by plating, the appearance of being unstained, unevenly stained, or weakly stained (13). The comparison of PI permeability with plating, as observed by this group (13) and others (14), therefore indicate the two are not always consistent with each other. It has thus been suggested that leakiness of cells, when too high of PI concentrations are used, is artifactual and does not necessarily indicate death (13).

Hilliard *et al* (14) also observed that the uptake of PI due to membrane damage occurs rapidly and may precede the event of death in bacteria. This may be consistent with our observations, as logarithmic reductions in CFU/mL were not immediately

detected upon plating *S. aureus* in the presence of ≤120µg/mL PI at 0 time (data not shown), though *S. aureus* was immediately fluorescent with as low as 0.12µg/mL PI, under PI filtration (data not shown). Though <7.5µg/mL PI was not enough PI present to penetrate all *S. aureus* cells (at 10^6 bacteria/mL) at 0 time, a certain percentage were detected with bright red intensity at 0 time with as low as 0.12µg/mL PI, under PI filtration. Therefore, PI permeability by fluorescence was a more sensitive indicator of a moribund-state than was plating.

Our results suggest that with as low as 3.75µg/mL PI, all *S. aureus* strains tested experience one or more logarithmic reductions (which correspond to a ≥90% reduction in viability) in recoverable CFU/mL at one hour (Supplemental Table A.4), and are highly permeable to PI at this concentration at 0 time (all were various shades of red under PI filtration). At concentrations of ≥7.5µg/mL PI, all *Staphylococcus* tested fluoresced bright red in entirety at 0 time under both DAPI and PI filtrations. Furthermore, in our hands, no other genuses have shown sensitivity like this to PI, which appears unique for *Staphylococcus* species. Yet, to our knowledge, there are no reports that have described a hypersensitivity of *Staphylococcus* to PI. This is particularly confounding because PI has been used as a tool in multiple studies to investigate the killing of *S. aureus*– some of which have used the same strains as reported here, and all of which have used PI concentrations (e.g. 2 - 100µg/mL PI) which are reported here to be lethal to *S. aureus*. While this certainly warrants further investigation, we hope that killing studies using PI with *S. aureus* have not been misinterpreted due to a hypersensitivity of this bacterium to PI.

Similar to the hypersensitivity of *S. aureus* to sub-optimal concentrations of PI, preliminary studies (data not shown) also found a hypersensitivity of *Streptococcus pyogenes* with DAPI, as confirmed by plating, at concentrations much below that which is required for optimal visualization of DAPI fluorescence (45µL of 7.5µg/mL). To our

knowledge, this too has never before been reported, despite the common use of DAPI with this organism.

MATERIALS AND METHODS

Bacterial Growth and Storage:

The aerotolerant anaerobe *S. aureus* was grown at 36^{o}C under aerobic atmosphere, in 9mL volumes of non-reduced Wilkins-Chalgren (WC) Anaerobe Broth Medium (Oxoid Ltd, Basingstroke, Hampshire, England) in glass test tubes, without shaking. WC was the standard medium used to grow all bacteria (for consistency), and worked well for the growth of strict anaerobes as well as aerotolerant anaerobes that are capable of growing in the presence of oxygen alike. In order to minimize mutation, bacteria were freshly grown from minimally passaged, aliquoted frozen stocks each week before use in experiments. Briefly, 100µL aliquots of *S. aureus* (grown in WC to mid-exponential phase or to ~0.5 optical density (OD) at $A_{\lambda 660nm}$) were stored in 1mL glass vials containing 0.5mL sterilized skim milk (Sigma, St. Louis, MO), at -80^{o}C until use. Individual frozen aliquots were thawed at room temperature and recovered by inoculation into 9mL volumes of WC. From this, 1 drop (using a sterile plastic transfer pipette) was inoculated into fresh 9mL volumes of WC, and from this, 3-5 drops were inoculated into a second 9mL volume of WC, and from that, 3-5 drops were inoculated into a third 9mL volume of WC, and placed into the aerobic incubator for growth. These broth cultures took approximately 18-24 hr to initially recover from frozen stock in WC. Upon initial visible growth, broth cultures were serially diluted (1 drop and several 10-fold dilutions from that) into fresh 9mL volumes of WC. Bacteria were harvested from overnight cultures (~18 hr) using the dilution that best fit early- to mid-exponential phase with an $A_{\lambda 660nm}$ of 0.2-0.6. The starting inoculum also assured that the test bacteria used in all experiments had undergone a minimum of five doublings. Individual frozen aliquots were checked for purity by streaking for isolation on reducible Brucella blood agar plates (Anaerobe Systems, Morgan Hill, CA), incubated aerobically at 36^{o}C. A

morphologically pure, β-hemolytic culture on blood agar assured purity and was confirmed by Gram stain (agar and broth cultures).

Bacterial Labeling with DAPI

One 9mL overnight culture (~18 hr) of *S. aureus* in early- to mid-exponential phase ($A_{\lambda 660nm}$ of 0.2-0.6) was divided into six 1.7mL polypropylene centrifuge tubes (VWR International, West Chester, PA), and centrifuged for four min at 10,000xg. Supernatants were discarded, and bacteria were concentrated into one tube by resuspending pellets serially in one 1mL volume (q.s. to 1.5mL) of Hanks Balanced Salt Solution (HBSS) (GIBCO, Grand Island, NY) without metals (calcium or magnesium) or phenol red, followed by 4', 6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co, St. Louis, MO) labeling (45µL of 7.5µg/mL) for ten min at room temperature, without motion in the dark. This was followed by three washes (four min at 10,000xg) in 1.5mL of fresh HBSS without metals. After the fourth and final spin, the pellet was resuspended in 0.75mL of HBSS with calcium and magnesium (with metals), and adjusted with HBSS with metals to a final $A_{\lambda 660nm}$ =0.2 (approximately 10^8 CFU/mL), using a spectrophotometer (Beckman, Fullerton, CA).

Dual-Color Fluorescence Killing Assay

The dual-color fluorescence phagocytosis and killing assay using DAPI/PI first described by Kalmar *et al* (15) and modified by Cutler *et al* (16) (17) was intended to be used to assess neutrophil phagocytosis and killing of *S. aureus*, in comparison to *P. gingivalis*. DAPI is a vital stain, which binds to A/T-rich regions of double-stranded DNA, and forms a highly fluorescent complex (blue color) when bacteria are alive (18). Propidium iodide (PI) is a red fluorescing viability dye, which is excluded by the

cytoplasmic membranes of live bacteria and enters the bacteria upon loss of membrane potential, associated with death (19).

In summary, DAPI-labeled *S. aureus* (100µL at $A_{\lambda 660nm}$ =0.2) were opsonized with 12.5µL of active human complement and 12.5µL of heat-inactivated (at 56^{o}C for 30 min just prior to use) human complement (gently mixed), in a 1.7mL polypropylene centrifuge tube (VWR International) for 10 min in a 37^{o}C Fisher Isotemp dry bath (Fisher Scientific Company, Fairlawn, NJ), with rocking. DAPI-labeled *P. gingivalis* was included as a control, and opsonized with human complement (12.5µL) and hyperimmune rabbit antiserum (12.5µL), as previously described. After opsonization, 10µL of PI, and 125µL of HBSS without metals (in place of neutrophils) were added and gently resuspended, initiating the start of the experiment (time 0). In testing the sensitivity of *S. aureus* to a variety of PI concentrations, PI was titered using 2-fold dilutions (in deionized water, the diluent of PI from stock), from 120µg/mL through 0.12µg/mL FC. The standard FC of PI normally used in this assay is 7.5µg/mL (10µL).

At the prescribed time intervals, 30µL aliquots were removed (after gently resuspending) and cytospun (Shandon Inc., Sewickley, PA) for 3 min at 850rpm onto pre-cleaned microscope slides (VWR International). Once dried (≤1 min after spin finished), glass cover slips (VWR) were mounted with cyanoacrylate (Krazy Glue™), and slides were examined by epi-illumination UV microscopy with oil immersion (63x) on a Zeiss Axioskop to access bacterial viability in the presence of DAPI/PI. Approximate fluorescence excitation/ emission maxima for DAPI and PI, when bound to DNA, were 358/461 and 535/617, respectively.

At the prescribed time intervals, 10µL aliquots were also removed (after gently resuspending) and plated by delivering 10µL of sample (spot assayed) to the surface of non-reduced WC agar (Oxoid Ltd), for growth and bacterial enumeration. To assess bacterial viability in the presence of DAPI/PI, compared to controls (no PI +/- DAPI),

detection of logarithmic differences in recoverable CFU was sufficient. Logarithmic reductions in bacterial densities per spot were estimated by comparison to controls, which were serially diluted 10-fold. Logarithmic reductions in recoverable CFU/mL were considered significant; i.e. ≥1 log reduction was considered significant and equated to a ≥90% reduction in CFU/mL, while <1 log reduction was not considered significant. After plating, agar surfaces were briefly allowed to dry in ambient air (dried within 5 min of plating), and were quickly placed into the aerobic incubator (for *S. aureus*) or anaerobic chamber incubator (for *P. gingivalis*) for growth. Plates were incubated at 36^{o}C, and recoverable CFU were countable after 4-6 days for *P. gingivalis,* and after 1 day for *S. aureus*.

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