THE UNIVERSITY OF CALGARY

Effects of Azithromycin on Human Neutrophils

by

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ABSTRACT

Pathogen virulence factors and the host inflammatory response cause tissue injury associated with respiratory tract infections. Azithromycin has demonstrated efficacy in treating these infections. We hypothesized that azithromycin may have anti-inflammatory properties, perhaps through the induction of neutrophil apoptosis. This study assessed the effects of azithromycin on human neutrophil 1) apoptosis, 2) phagocytosis by macrophages, 3) oxidative function, and 4) interleukin-8 production in the presence or absence of Streptococcus pneumoniae compared with penicillin, erythromycin, dexamethasone or saline. Azithromycin significantly induced neutrophil apoptosis but this effect was abolished in the presence of S. pneumoniae. Macrophage phagocytosis of azithromycin-treated neutrophils was significantly enhanced. Azithromycin did not affect neutrophil oxidative metabolism or interleukin-8 production. In summary, azithromycin induces neutrophil apoptosis without affecting neutrophil function, and enhances phagocytic clearance of these cells by macrophages. These properties may confer anti-inflammatory benefits to this antibiotic.
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I dedicate this to my Mom and Dad

who always support everything I do
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<td>ARDS</td>
<td>adult respiratory distress syndrome</td>
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<tr>
<td>CBA</td>
<td>Columbia blood agar</td>
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<td>CFU</td>
<td>colony forming unit</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>fMLP</td>
<td>formyl methionine leucine phenylalanine</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LT</td>
<td>leukotriene</td>
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<tr>
<td>MDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>'NBT</td>
<td>nitro-blue tetrazolium</td>
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<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-ADP-ribose-polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PI</td>
<td>propidium iodide</td>
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<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<td>PMN(s)</td>
<td>polymorphonuclear neutrophil(s)</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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CHAPTER 1: INTRODUCTION

1.1 The Pathogenesis of Respiratory Tract Infection

The lungs are exposed to more pathogenic organisms than virtually any other tissue [1]. Fortunately, the lungs have many mechanisms to remove, neutralize or kill pathogens such as mechanical, secretory and immune systems [2, 3]. Mechanical defenses include filtration by nasal vibrissae, the ciliated epithelial barrier, and the cough reflex. The mucociliary lining constitutes the secretory defense system. If a respiratory pathogen manages to evade these mechanisms and reaches the lungs, it will encounter resident pulmonary alveolar macrophages and other immune components such as polymorphonuclear neutrophils (PMNs) as a third line of defense.

Despite these defense mechanisms, bacterial colonization of the respiratory tract is a common cause of morbidity and mortality. The pathogenesis of bacterial infection in the respiratory tract involves both bacterial virulence factors and the host inflammatory response [4, 5]. The host inflammatory response is primarily driven by neutrophils. Despite being necessary for host defense, there is increasing evidence of the contribution by polymorphonuclear neutrophils to the severity of a number of inflammatory diseases such as streptococcal pneumonia [5-12]. In such pathologic circumstances, the potential for host tissue damage is clear: cytotoxic compounds such as elastase, hypochlorous acid, and oxygen radicals released by neutrophils at sites of inflammation can damage surrounding tissues, and induce necrosis in neighboring neutrophils [7, 11]. Necrotic neutrophils swell and burst as a result of metabolic collapse and failure to maintain ionic
homeostasis, releasing more cytotoxic compounds and pro-inflammatory mediators [13]. This can lead to severe host tissue damage and inflammation.

1.1.1 Bacterial Virulence Factors

Bacterial virulence factors initiate the host inflammatory response. Gram-negative bacteria have lipopolysaccharide (endotoxin) on the outermost layer of their cell wall that has antigenic properties and activates the inflammatory process [14]. Gram-positive bacteria have a large peptidoglycan layer that, along with associated lipoteichoic acids, can exert endotoxin-like effects. Virulence factors aid in bacterial colonization and protect the microorganism from host defenses enabling bacterial invasion and infection to occur [15].

1.1.1.1 Adhesion and Uptake

Adhesion of bacteria in the host localizes pathogenic bacteria to target tissues [15]. Adherence of bacteria to host cells and extracellular matrix components is aided by adhesins such as pili, fimbriae, and other adhesion molecules such as M protein of Streptococci and fibronectin-binding proteins of Streptococci and Staphylococci. Phosphorylcholine, a cell wall component of Streptococcus pneumoniae, binds the platelet-activating factor receptor on activated endothelial cells leading to adherence to and invasion of these cells. Teichoic acids aid adhesion of many Gram-positive bacteria to host epithelium fibronectin molecules [16]. Many pathogens produce virulence factors (such as EspA and EspB of Escherichia coli) that activate host cell signal transduction
pathways leading to expression of and activation of receptors on the host cell to which the bacteria can then bind.

Some bacteria such as *Salmonella* and *Shigella* enhance their uptake into nonphagocytic cells, enabling them to enter a protected environment or allowing them to pass through the epithelium [15]. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* avoid phagocytic uptake by injecting Yop proteins into phagocytic cells, paralyzing the actin cytoskeleton and dephosphorylating host proteins which impairs phagocytosis. *Pseudomonas aeruginosa* has this same effect on phagocytic cells via secretion of exoenzyme S which modifies G proteins involved in actin regulation.

1.1.1.2 Cytotoxins

Bacteria produce toxins which damage host cells by interfering with transmembrane signalling, by producing pores that alter the permeability of membranes resulting in lysis of host cells, or by altering cytosolic components of the host cell [15]. Some bacteria can induce apoptosis (programmed cell death) in host phagocytes, thereby preventing bacterial clearance [15, 17]. *Shigella* protein IpaB binds and activates interleukin-1β-converting enzyme (ICE), a cysteine protease which induces apoptosis when activated via cleavage of host cell substrates [15]. *Mycobacterium avium* induces apoptosis in human macrophages possibly via the generation of oxygen free radicals [17]. Attachment of *M. avium* to host cell surface receptors may activate second messengers resulting in oxidant production which could alter the redox status and activity of host enzymes. Diphtheria toxin, *Pseudomonas* exotoxin A, and cholera toxin induce apoptosis *in vitro*, although the mechanism differs in each case [15]. *Staphylococcus aureus* toxic
shock syndrome toxin-1 induces T cell apoptosis and inhibits immunoglobulin G (IgG) production. *S. pneumoniae* has extracellular toxins such as pneumolysin, neuraminidase, and purpura-producing factor which kill phagocytes and interfere with chemotaxis [3, 18].

1.1.1.3 Other Factors

Many intracellular organisms can prevent fusion of phagosomes with lysosomes [15]. Some intracellular pathogens, such as *Mycobacterium avium*, can inhibit acidification of the phagocytic vacuole preventing activation of lysosomal enzymes. *Streptococcus pneumoniae* and *Haemophilus influenzae*, two common causative agents of pneumonia, produce proteases that digest IgA [18]. The thick polysaccharide capsule of *S. pneumoniae* and the M protein within the cell wall of *Streptococcus pyogenes* help protect these organisms from being phagocytosed by macrophages and neutrophils [3, 18].

1.1.2 Inflammation

Inflammation is classified as acute on the basis of the persistence of injury, clinical symptoms and the nature of the inflammatory response [19]. Acute inflammation is characterized by the accumulation of fluid and plasma constituents in tissues, intravascular stimulation of platelets, and the presence of neutrophils. Chronic inflammation, on the other hand, is characterized by the presence of lymphocytes, plasma cells and macrophages.
The hallmarks of inflammation include redness, swelling, heat, pain and loss of function [20]. Inflammatory mediators released by cells at sites of infection cause vasodilation and increased blood flow and, in conjunction with increased vascular permeability, result in formation of inflammatory exudate and local edema.

The host immune system responds to bacterial products such as lipopolysaccharide (LPS) and formyl methionine leucine phenylalanine (fMLP) and an inflammatory response is generated [14]. A humoral response is produced that is mainly mediated via immunoglobulin G, which opsonizes the organism [2]. In conjunction with complement, IgG can also neutralize many viruses and toxins and lyse certain Gram-negative bacilli. Secretory IgA, localized at mucosal surfaces where most pathogenic microbes gain access to the body, serves an important protective function [21]. Secretory IgM is also present in mucosal secretions but in lower amounts than IgA. Secretory antibodies cross-link antigens and block attachment of bacteria to mucosal cells. IgM is the first immunoglobulin produced in response to infection (5 to 7 days after antigen exposure) but its concentration declines rapidly and IgG and IgA become more predominant. Previous exposure or immunization to an antigen results in a more rapid and stronger response by lymphocytes which produce antigen-specific immunoglobulins.

A cell-mediated immune response is also elicited and alveolar macrophages are the main effector cells. Lipopolysaccharide (endotoxin) activates macrophages, monocytes, endothelial cells, platelets, mast cells, neutrophils, lymphocytes and fibroblasts [14]. Alveolar macrophages process antigens that reach the lower respiratory tract and kill many microbes [2]. As discussed further, leukocyte recruitment is mediated by the generation of cytokines by lymphocytes, macrophages and other cells [12].
Leukocytes adhere to the endothelium and emigrate from the vasculature into the tissues in response to chemotactic gradients.

Spontaneous phagocytosis can occur when neutrophils come into contact with bacteria. Neutrophils also recognize and attach to pathogens via Fc receptors and complement receptors on the neutrophil surface [12]. Fc receptors bind to the Fc portion of immunoglobulin G molecules and complement receptors bind to C3b and C3bi found on the surface of opsonized bacteria. Phagocytosis of the pathogen ensues and microbicidal killing occurs within the phagolysosome of the neutrophil.

Mild acute inflammation caused by bacteria is usually resolved when the infection is cleared via host defense mechanisms (including the inflammatory response) or with antibiotics [20]. In pneumonia, the lung are filled with exudate, fibrin, bacteria and inflammatory cells. During the resolution phase of inflammation, this abnormal material is removed (mainly via the action of macrophages), and normal structure and function can be restored.

1.1.2.1 Mediators

Interleukin-1 (IL-1), tumor necrosis factor α (TNFα), leukotriene B4 (LTB4), and interleukin-8 (IL-8) are some of the major pro-inflammatory mediators involved in inflammation. Bacterial antigens, such as LPS, as well as host immune components stimulate the production and release of inflammatory mediators from many cell types [22]. Lipopolysaccharide activates macrophages and they secrete peptide and lipid mediators such as IL-1, TNFα, IL-6, thromboxane, leukotrienes, and platelet activating factor [14]. Reactions to LPS, infection and other inflammatory stimuli are mediated by
IL-1 and TNF [23]. IL-1 and TNF induce acute inflammation by inducing gene expression and synthesis of proteins in other cell types found in the lungs [23]. Both IL-1α and TNFα activate neutrophils. IL-1 is produced by various cell types in response to bacterial antigens and toxins, products of activated lymphocytes, complement and clotting components [23]. TNFα is produced by activated monocytes and macrophages, lymphocytes, endothelial cells and keratinocytes [23]. Neutrophils also release IL-1, TNFα and IL-8 [20]. IL-1 and TNF both cause an increase in IL-8 [24].

Mediators such as IL-8 and LTB₄ strongly amplify inflammation. IL-8 plays an important role in inflammation and clearance of pathogens by stimulating neutrophil chemotaxis and degranulation, and enhancing phagocytosis of opsonized particles [25-27]. Although individual neutrophils produce only small amounts of IL-8, their large number at sites of inflammation make neutrophils the most significant source of IL-8 in inflammatory processes [28]. Neutrophils also release LTB₄, another potent neutrophil chemoattractant responsible for potentiating inflammation [29].

Inflammatory mediators can be involved in the pathogenesis of many disease states. IL-1, TNFα, IL-8, and LTB₄ play a role in the pathogenesis of cystic fibrosis patients infected with *Pseudomonas aeruginosa* [30-34]. Increased levels of IL-1, TNFα, and IL-8 have been implicated in adult respiratory distress syndrome (ARDS) [35, 36].

1.1.2.2 Neutrophils

Neutrophils (polymorphonuclear leukocytes) originate and mature in bone marrow, are then released into the blood where they circulate for approximately ten hours, and can migrate from the vasculature into sites of infection where they can live up
to three days [20, 37-39]. Neutrophils are terminally differentiated cells that retain a smaller number of homeostatic pathways than their bone marrow precursors [40]. These cells are highly specialized for degranulation and oxidative metabolism.

Neutrophils are the principal cell type involved in acute inflammation. These cells are involved in phagocytic clearance of pathogens from infected tissues and thus are important in the resolution of infection. They are necessary in the removal of pathogens from the lung and their influx is regulated by chemoattractants and upregulation of adhesion molecules [7, 11, 37]. In humans, neutrophils comprise approximately 65% of circulating leukocytes but only 2% of leukocytes recovered from healthy broncho-alveolar spaces [37]. In healthy lungs, more than 90% of lung leukocytes are alveolar macrophages, while monocytes constitute only 5% of leukocytes in the circulation. This distribution of leukocytes in the lung reverses upon exposure to bacterial infection.

Neutrophils carry out their anti-bacterial function via oxygen-dependent or independent mechanisms. Firstly, when neutrophils are activated by pro-inflammatory mediators such as IL-1 or TNFα, membrane enzymes including NADPH oxidase, myeloperoxidase, and esterase are activated and result in the initiation of the respiratory burst [11, 41]. Reactive oxygen intermediates such as superoxide anion, hydrogen peroxide, hypochlorous acid and hydroxyl radical are produced during the respiratory burst of activated neutrophils. Oxygen is converted to superoxide, which is then converted to hydrogen peroxide. Neutrophil granules contain myeloperoxidase, which converts most of the hydrogen peroxide to hypochlorous acid if chloride is also present. Some hydrogen peroxide is also converted to water via catalase or to hydroxyl radicals if
iron is present. These reactive oxygen intermediates are involved in oxygen-dependent bacterial killing.

In addition, neutrophils have intracellular granules that contain proteolytic enzymes such as lysozyme, elastase, gelatinase, collagenase, cathepsin G, proteinase 3 and bactericidal proteins such as lactoferrin, defensins, bactericidal/permeability-increasing protein, and azurocidin [1, 11].

When neutrophils become activated during infection these enzymes, proteins and oxidants are released into the phagosome and the extracellular environment and aid the neutrophil in killing bacteria. Bacteria are killed by the oxidation and digestion of membrane phospholipids and glycoproteins [12]. Some of these same compounds can also act on host tissue components causing damage. In order to prevent such injury, antiproteases and antioxidants are produced by the host [11, 41]. Severe inflammatory states overwhelm this system and cause injury to host tissues. For example, patients with acute respiratory distress syndrome (ARDS) display abnormal antioxidant capacity in association with severe lung damage [41]. Oxygen radicals generated by neutrophils inactivate antiproteases and further increase proteolytic damage to the lung. Massive neutrophil infiltration seen in pneumonia results in the release of large quantities of oxidants, overwhelming antioxidants and antiproteases, which in turn leads to lung injury [12].

1.1.2.3 Macrophages

Bone marrow precursors differentiate into blood monocytes and migrate into tissues where they develop into macrophages [42]. Local production of chemotactic
factors, including fMLP and the complement-derived peptide C5 des arg, at the site of infection induces the immigration of monocytes into tissues [43]. Macrophages are important in the inflammatory process and the resolution of inflammation [44]. These cells are major effectors of the immune response against pathogens [12, 44]. Resident alveolar macrophages are the host’s first line of cellular defense against infections. Their defensive capabilities can be overwhelmed, however, by large numbers or highly virulent bacteria, and recruited neutrophils help defend the host in this case [12]. Besides their role in phagocytosis of bacteria, macrophages also function in antigen presentation to T lymphocytes [45]. Fragments of internalized pathogens bind to class II major histocompatibility complex proteins (MHC) which are then expressed on the cell surface [46]. Helper T cells with receptors specific for the antigen bind the MHC-antigen complex, become activated and secrete cytokines (such as IL-4) that stimulate B cells that have already encountered that particular antigen and display antigen fragments bound to MHC II. T cell receptors also bind to the antigen-MHC complex of these B cells. This results in activation of antigen-specific B cells. Activated B cells give rise to plasma cells that produce specific antibodies against the particular antigen. The phagocytosis of bacteria activates macrophages, which can then recruit neutrophils to sites of infection by secretion of neutrophil chemotactic factors such as IL-8 [2]. Macrophages are also responsible for clearing cellular debris from inflamed sites. Macrophage phagocytosis of dying cells is another mechanism whereby acute inflammatory reactions during pathological processes are physiologically controlled [47]. Indeed, macrophages have been implicated in the regulation of neutrophil-mediated damage as they rapidly phagocytose dead and dying neutrophils before the cell lyses and releases its cytotoxic
and pro-inflammatory compounds into the tissues, thus preventing damage to host tissues, inflammatory reactions and autoimmune responses (autoantibodies against nucleosomal DNA released from dead cells) [47]. Moreover, recent information suggests that during phagocytosis of apoptotic neutrophils, in contrast to phagocytosis of opsonized particles including bacteria, the production by macrophages of pro-inflammatory mediators, such as IL-1β, TNFα and IL-8, is inhibited [48]. These observations suggest that opsonin-mediated phagocytosis by macrophages activates inflammatory processes, while vitronectin/phosphatidylserine-mediated phagocytosis does not activate such processes. It is not yet known whether the inhibition of the production of pro-inflammatory mediators is due to binding of apoptotic cells to receptors on macrophages or to the uptake process. In the same study, increased production of transforming growth factor-β1 (TGF-β1), platelet activating factor (PAF), and prostaglandin E2 (PgE2) were also reported and appeared to mediate the decrease in pro-inflammatory cytokines. The mechanism by which phagocytosis of apoptotic cells stimulates production of TGF-B1, PAF, and PgE2 is not known nor is the mechanism by which these products reduce production of pro-inflammatory mediators.

1.2 Pneumonia

Pneumonia is one the most common community-acquired and nosocomial infections, and despite improved treatment strategies, the mortality rate remains high [49]. It is the fifth leading cause of death in the USA. In pneumonia, fibrin deposition congests the alveoli and bronchioles of the lungs [50, 51]. Severe inflammation causes pulmonary tissue damage that can ultimately lead to respiratory failure and death. Fever,
chills, headache, cough and chest pain accompany pneumonia. A number of pathogens are known to cause pneumonia, including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, adenovirus type III, influenza A and B viruses, rickettsiae, and fungi [50-52]. Combined infections of multiple respiratory pathogens are often seen [53, 54]. The most common bacterial lung infection in adults is streptococcal pneumonia caused by *Streptococcus pneumoniae* [3]. Bacteria are spread via contact with an infected person. A 7 to 10 day incubation period precedes the clinical manifestations of streptococcal pneumonia (chills, fever, and pain) [55]. The bacteria invade the body through the airways and reach the lungs where they multiply rapidly in the alveolar tissue. The antiphagocytic capsule of *S. pneumoniae* prevents phagocytosis by alveolar macrophages and other phagocytes, and the infection proceeds resulting in purulent exudate-filled alveoli. Neutrophil influx ensues and they accumulate in the infected alveoli and contribute to exudate formation. This proceeds for about a week during which anticapsular antibody is formed which usually results in recovery due to adequate phagocytosis and killing of the organism unless the disease is too severe or the host is immunocompromised. Particularly virulent strains of bacteria may overwhelm host defenses leading to respiratory failure.

*Streptococcus* was isolated in 55% of patients with pneumonia in one study [52]. Many normal healthy individuals carry streptococci in their upper respiratory tract, however, only a small proportion develop streptococcal pneumonia [52]. A study by Cherry et al. isolated both pathogenic bacteria and rhinovirus from 45% of patients,
perhaps indicating a viral infection as the initiating cause in bacterial pneumonia [53]. The large volume of mucous secretions in the nose and pharynx increase the chance of aspiration of bacteria past the epiglottal barrier where gravity can carry the bacteria into the alveoli. In another study, 46% of patients had an upper respiratory tract infection (possibly viral) in the week or so prior to the onset of bacterial pneumonia [52]. A viral source of the initial infection was difficult to confirm in most cases since viruses may only be shed for a few days and may not have been detectable by the time of hospitalization for the secondary bacterial pneumonia.

The annual rate of pneumococcal infections in the USA is approximately 500,000 cases in all age groups, but this rate can increase in certain populations [3, 55]. A major risk factor for contracting pneumonia is age; those over forty have a three to four times greater chance than those under twenty [3]. Hospitalization also increases the risk of contracting pneumonia [49]. Pneumonia causes 8% to 33% of all nosocomial infections making it the third most common nosocomial infection after urinary tract and surgical wound infections. Nosocomial infections are often caused by Gram-negative bacteria and are fatal in more than 50% of cases. The rate of respiratory tract infection is greatest in developing countries [56-58].

1.2.1 *Streptococcus pneumoniae*

*Streptococcus pneumoniae* is the most common causative agent of bacterial pneumonia in humans [50]. This organism appears as a lancet-shaped Gram-positive diplococcus that is found in the human nasopharynx and has no known environmental or animal reservoir [3]. *S. pneumoniae* is encapsulated and α-hemolytic. Asymptomatic
carriers are often more important in spreading the disease than those with acute infection since they are not isolated and treated [3]. A number of factors determine whether bacterial invasion and infection will occur such as degree of challenge, virulence factor expression, primary viral infection, host defenses and immune competence, nutritional status, alcohol intoxication [3]. *S. pneumoniae* has extracellular toxins such as pneumolysin, neuraminidase, and purpura-producing factor but, although proposed, the exact role of these in the pathology of pneumonia remains unclear [3, 55]. Hydrolytic enzymes such as neuraminidase and IgA1 protease may contribute to the colonization of the host [3, 18, 59]. Neuraminidase acts to kill phagocytes and interfere with their chemotaxis and the destruction of secretory IgA impairs opsonization and phagocytic clearance of the bacteria. *S. pneumoniae* strains carrying mutations in genes encoding for pneumolysin, autolysin and surface protein A demonstrate reduced virulence. Purpura-producing factor is a glycan-teichoic acid fragment generated by hydrolysis of the cell wall by autolysin [60]. Lipoteichoic acids aid in bacterial adhesion to host cells [16]. The primary determinant of pathogenicity is the polysaccharide capsule, which helps protect *S. pneumoniae* from being phagocytosed by macrophages and neutrophils [3].

### 1.3 Treatment

Several options are available for the treatment of bacterial pneumonia. Besides vaccines for the prophylaxis of pneumonia, therapeutic strategies such as antibiotics and anti-inflammatory drugs are available [3]. Drugs that modulate the immune response to infection have been studied experimentally, and several anti-inflammatory strategies
focus on preventing neutrophil-mediated tissue injury. Of course, the treatment of choice remains the use of antibiotics to eradicate the bacterial infection.

1.3.1 Antibiotics and Antimicrobial Agents

There are many classes of antibiotic and/or antimicrobial drugs available to treat pneumonia including beta-lactamases, sulfonamides, fluoroquinolones, tetracyclines, and macrolides. The choice of drug depends on the specific organism isolated from sputum or blood, the pharmacodynamics of the drug and its antimicrobial spectrum, as well as clinical experience and an assessment of the patient's history.

1.3.1.1 Beta-lactams

Beta-lactam antibiotics include penicillins and cephalosporins [61]. They contain a beta-lactam ring with various substituents that lend different properties to the antibiotic. Beta-lactam antibiotics bind to penicillin-binding proteins and interfere with bacterial cell wall synthesis by inhibiting transpeptidase cross-linkage of adjacent peptidoglycans [62]. Additionally, beta-lactams inactivate an inhibitor of an autolytic enzyme in the bacterial cell wall, leading to lysis of the bacterium [61]. Bacterial resistance to beta-lactams is mainly due to the production of beta-lactamases, reduced outer membrane permeability (decreasing drug penetration; particularly in Gram-negative organisms), or modified penicillin-binding sites. Many strains of *S. pneumoniae* are resistant to β-lactams due to modification of the penicillin-binding proteins; *S. pneumoniae* does not produce a β-lactamase.
1.3.1.1 Penicillin

Penicillin G (benzylpenicillin) has been one of the most widely used antibiotics for treating streptococcal pneumonia, but growing bacterial resistance to penicillin has forced the development of other drugs [50, 51]. Penicillin contains a thiazolidine ring linked to the beta-lactam ring, which is the target of beta-lactamases and acylases from penicillin-resistant organisms [62]. Penicillin G is a natural penicillin that is produced by mold cultures. It is acid labile and bactericidal [61]. Semisynthetic penicillin derivatives (such as nafcillin, amoxicillin, ampicillin and piperacillin) have been produced which are acid-stable, more resistant to cleavage by beta-lactamases, or have an enhanced spectrum of activity against both Gram-positive and Gram-negative bacteria.

1.3.1.2 Cephalosporins

Cephalosporins are derived from *Cephalosporium* fungi. They are water-soluble and relatively acid-stable. Cephalosporins vary in their susceptibility to beta-lactamases [61]. Their usefulness in pneumonia may be limited since they have poor lipid solubility which hinders their diffusion into tissues. Many semisynthetic broad-spectrum cephalosporins, such as cefuroxime, have been produced by varying the side chains of the beta-lactam ring. They are effective against most Gram-positive and some Gram-negative organisms. Resistance to this class of drugs is increasing due to both plasmid-encoded and chromosome-encoded beta-lactamases. In fact, nearly all Gram-negative bacteria produce a beta-lactamase that hydrolyzes cephalosporins to a greater extent than penicillins.
1.3.1.2 Sulfonamides

Sulfonamides, such as sulfadiazine and sulfamethoxazole, are derived from sulfanilamide by substitution at the amide group [61]. Sulfanilimide is a structural analogue of p-aminobenzoic acid that is needed for folic acid synthesis in bacteria. DNA and RNA synthesis require folic acid. Sulfonamides compete with p-aminobenzoic acid for the enzyme dihydro-pterate synthetase and are bacteriostatic rather than bactericidal since they only inhibit bacterial growth. Sulfamethoxazole, in combination with trimethoprim (an antibiotic which acts as a folate antagonist), is called co-trimoxazole and is effective at much lower dosages. Co-trimoxazole is sometimes used to treat pneumonia.

1.3.1.3 Fluoroquinolones

Fluoroquinolones, such as ciprofloxacin and norfloxacin, are synthetic antibiotics that inhibit topoisomerase II (a DNA gyrase) and thus inhibit DNA transcription [61]. Although they are active against Gram-positive organisms, these antibiotics are especially active against Gram-negative bacteria. Ciprofloxacin is used for pneumonia caused by *Haemophilus influenzae*. One of the new drugs of choice for pneumonia is levofloxacin.

1.3.1.4 Tetracyclines

Originally, tetracyclines, such as oxytetracycline, were obtained from *Streptomyces* [61]. Newer tetracyclines, such as minocycline and tetracycline, are semisynthetic or synthetic. They are taken up by active transport and are distributed
throughout the body. They act by inhibiting protein synthesis by competing with aminoacyl tRNA for the A site on ribosomes. Tetracyclines have a very broad spectrum of activity against both Gram-positive and Gram-negative organisms but they are only bacteriostatic. Their usefulness is limited as many strains of bacteria have become resistant [61]. Resistance occurs due to defective uptake by bacteria; plasmid encoded genes lead to the development of energy-dependent efflux mechanisms which transport the drug out of the bacterium. Some bacteria also alter the ribosome binding site so the drug can not bind.

1.3.1.5 Macrolides

Macrolides have been used for many years for the treatment of various upper and lower respiratory infections [63]. The first macrolide, erythromycin, was introduced in 1952. Many derivatives of erythromycin have since been developed. One of these is azithromycin which will be discussed in detail in a later section.

1.3.1.5.1 Structure

Structurally, macrolides consist of a lactone ring structure with attached carbohydrate moieties. Macrolides have a variety of lactone ring structures but three main groups are the 14-membered macrolides (erythromycin, clarithromycin and roxithromycin), the 15-membered macrolides (azithromycin) and the 16-membered macrolides (josamycin, lincosamides, and streptogramins) [64]. Azithromycin is more correctly classified as an azalide due to the presence of a nitrogen atom into the macrolide ring.
1.3.1.5.2 Mode of action (antimicrobial activity)

Macrolides are generally considered to be bacteriostatic rather than bactericidal, and are active against a variety of Gram-positive and Gram-negative organisms [64]. The antibacterial activity of macrolide antibiotics is due to reversible binding of the drug to the bacterial 50S subunit of the 70S ribosome, thus inhibiting protein synthesis within the bacterial cell [65]. Macrolides stimulate dissociation of peptidyl tRNA from the ribosome by a variety of mechanisms including inhibition of translocation or peptidyl transferase reactions [66]. Some nonbacterial organisms such as Entamoeba histolytica and Toxoplasma gondii are also affected by macrolides but the mechanism of action is not known [64].

1.3.1.5.3 Resistance

Cross-resistance to azithromycin and erythromycin has been shown in many studies [64]. Enzymatic methylation of the ribosome binding site of macrolides is postulated to be responsible for resistance of organisms to macrolides by preventing macrolides from binding to the ribosome [67-70]. The methylase modifies the 50S ribosome subunit by N^6-methylation of adenine in 23S ribosomal RNA [70]. In addition to macrolides, this also confers resistance to lincosamides and streptogramin B antibiotics. The methylase can be constitutively produced by some bacteria such as Staphylococcus aureus and Staphylococcus epidermidis. Subinhibitory concentrations of the macrolide can also induce expression of the methylase in such bacteria as
**Streptococcus pneumoniae** and *Streptococcus pyogenes*. Plasmid pE194 specifies this type of resistance to macrolides.

Another mechanism of resistance involves the presence of the plasmid pNE24, which results in energy dependent efflux of 14- and 15-membered macrolides (but not 16-membered macrolides) from bacteria [71]. The energy dependent macrolide efflux pump maintains intracellular antibiotic concentrations below those necessary for binding of the macrolide to ribosomes. The efflux pump recognizes specific structural components of 14- and 15-membered macrolides and is able to remove several thousand macrolide molecules per second. Bacteria are thus able to maintain growth in the presence of antibiotic. Plasmid pNE24 has been detected in *Staphylococcus epidermidis*.

Other forms of resistance to macrolide antibiotics include hydrolysis of the macrolide ring by esterases, and phosphorylation or glycosylation of the macrolide [71].

### 1.3.2 Azithromycin

Standard treatments for lower respiratory tract infections include penicillins, cephalosporins, or erythromycin [61, 72]. Other agents such as sulfonamides, ciprofloxacin, or tetracyclines may also be prescribed. Penicillins are limited in their usage due to patient hypersensitivity reactions and bacterial resistance, and cephalosporins have poor activity against intracellular pathogens. Erythromycin was introduced over thirty years ago and has been considered the representative macrolide. Its disadvantages include need for frequent administration, acid instability, gastrointestinal disturbances and poor activity against *Haemophilus* species. This has led to the development of new macrolides. Azithromycin (CP-62,993; XZ-450) is a
semisynthetic macrolide antimicrobial drug of the azalide class. It is more acid-stable than erythromycin and demonstrates improved pharmacokinetic parameters [64]. Like other macrolides, the activity of azithromycin does, however, decrease in an acidic environment [73].

1.3.2.1 Structure

Azithromycin is derived from erythromycin. Its chemical structure is 9-deoxo-9A-methyl-9A-aza-9A-homo-erythromycin A [74]. Azithromycin has a 15-membered lactone ring structure with a tertiary amino group, attached to two sugars (see Appendix A) [64]. The amino substituent and expanded ring structure differentiates it from erythromycin (14-membered lactone ring). The enhanced acid-stability of azithromycin (300-fold increase over erythromycin) is related to these structural differences [75].

1.3.2.2 Pharmacodynamics and Antimicrobial Activity

The antimicrobial activity of azithromycin, like that of other macrolides, is due to inhibition of protein synthesis from the drug binding to the 50S subunit of the bacterial ribosomes [65]. Azithromycin has a similar spectrum of activity to other macrolides; it is active against many Gram-positive and Gram-negative bacteria. In addition to its bacteriostatic activity, azithromycin is also bactericidal against a number of bacterial species. Bactericidal activity is a decrease in “CFU concentration of at least 3 log_{10} CFU/mL or a 99.9% kill rate within 24 hours”[64]. Azithromycin is bactericidal against Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Bordetella species, Legionella
pneumophila, Moraxella catarrhalis, Chlamydia trachomatis, Chlamydia pneumoniae, Mycobacterium avium complex, and Borrelia burgdorferi [64]. Bacteria resistant to erythromycin are also usually resistant to azithromycin.

All macrolides exhibit a postantibiotic effect (PAE) which means bacterial regrowth is suppressed for a short period of time after exposure to an antimicrobial drug. The PAE is important to consider because drug levels may become lower than the minimum inhibitory concentration (MIC) in the interval between doses. Azithromycin has shown a postantibiotic effect of 1.7 to 3.9 hours for many respiratory tract pathogens [76].

The azalide azithromycin has proven to be more effective in the treatment of a number of respiratory tract infections compared to erythromycin, although both antibiotics have comparable antibacterial properties [77, 78]. Azithromycin has greater activity than erythromycin against Gram-negative bacteria and this is thought to be due to the extra positive charge from the methyl substituted nitrogen (structure-activity relationship) [79]. It may also be due to the high binding affinity for ribosomes [80]. Azithromycin also interacts with magnesium binding sites on outer membrane phospholipids, which serves to increase its membrane permeability and enhance its uptake [79]. The exact mechanism of uptake, however, has not been determined but is postulated to involve passive diffusion and lysosomal trapping [81, 82].

Clinically, the antimicrobial activity of azithromycin appears to be enhanced by the ability of this compound to reach high tissue concentrations [64]. Azithromycin is more concentrated in tissues than in blood and accumulates intracellularly within monocytes, polymorphonuclear leukocytes and alveolar macrophages to concentrations
much greater than those in the extracellular environment [81-84]. This may enhance the antimicrobial activity of azithromycin against intracellular pathogens. Azithromycin has been shown to accumulate inside phagocytes much more effectively than erythromycin, and is primarily located within lysosomes [81, 85, 86]. Azithromycin does not impair the phagocytic function of cells and even enhances phagocytosis of *Staphylococcus aureus* at subinhibitory concentrations [81, 87]. Synergistic killing between azithromycin and phagocytes has been demonstrated in *vitro* for *S. aureus*, *L. pneumophila* and *H. influenzae* [84]. Azithromycin is released from phagocytes more slowly than erythromycin (within 24 hours versus 3 hours, respectively) and it is suggested that phagocytes deliver the drugs to sites of infection increasing tissue concentrations [88]. Fibroblasts also accumulate azithromycin (and release it more slowly than they release erythromycin) and may act as a slow release reservoir of antibiotic within tissues [89].

1.3.2.3 Pharmacokinetics

Animal models, healthy volunteers, and patients requiring antibiotics have been used to investigate the pharmacokinetics of azithromycin. As discussed previously, azithromycin is more acid-stable than erythromycin as the structural modifications block the normal degradation pathway. At pH 2, 10% decay takes 20 minutes for azithromycin but only 3.7 seconds for erythromycin [75].

Azithromycin is rapidly taken up from the blood into intracellular compartments of phagocytes and fibroblasts and is slowly released. Unlike other macrolides, azithromycin achieves high concentrations in tissues, including the lungs. High tissue concentrations are prolonged due to the long half-life of azithromycin [90]. This permits
a single daily dose regimen for most infections, which enhances compliance. Product labeling states that azithromycin should be taken on an empty stomach (as food decreases the oral bioavailability of the drug), but subsequent studies have found that food has no effect on the bioavailability of azithromycin [91, 92]. In healthy fasting volunteers, an oral bioavailability of 37% was shown when given a single 500 mg dose [93]. The low oral bioavailability is attributed to incomplete absorption rather than acid degradation or extensive first-pass metabolism [94]. A 500 mg dose of azithromycin gave a mean peak plasma or serum concentration ($C_{\text{max}}$) of 0.4 to 0.45 mg/L when measured 2.5 hours after administration to healthy fasting volunteers [93, 95]. Higher mean peak concentrations were reported for other macrolides and were achieved in a shorter time. The mean $C_{\text{max}}$ in healthy volunteers given 500 mg twice on day 1 and once on days 2 to 5 increased from 0.41 to 0.62 mg/L over five days [93].

Macrolides are dicationic yet they are still lipid soluble [64]. This results in extensive distribution in body fluids [96, 97]. The volume of distribution (Vd) is the volume of fluid required to contain the total amount of drug in the body at the same concentration as is present in the plasma [61]. The Vd for azithromycin has been reported to be 23 to 31 L/kg body weight, which is greater than the total amount of body water (0.55 L/kg body weight) indicating that the drug is stored in tissues and cells [97]. Delivery of azithromycin to tissues is accomplished via two mechanisms – direct uptake by tissues and uptake by phagocytic cells (targeted delivery) [90]. As discussed above, azithromycin is rapidly taken up and slowly released from many cell types leading to high local concentrations of drug [98].
In patients who received a single 500 mg dose, serum concentrations declined in a biphasic manner over three days post-administration and were between 1 and 9 mg/kg in gynecological tissue (ovary, fallopian tube, uterus, cervix, fibroid tissue), lung, peritoneal fluid, prostate, tonsil and urological tissue (testis, epididymis, vas deferens) [93, 99, 100]. Lower concentrations (0.2 to 1 mg/kg) were found in bone, fat, gastric mucosa, and muscle, but all concentrations were higher than those found in serum, reflecting the rapid uptake and concentration of azithromycin in tissues versus blood. Patients given a single 500 mg dose of azithromycin orally were also used to assess concentrations of azithromycin in the respiratory tract [101-103]. Broncho-alveolar macrophages, bronchial epithelial lining fluid, bronchial mucosa and sputum all achieved a maximum concentration within 48 hours, while this was reached in lung tissue within 96 hours, with concentrations ranging from 1.56 to 26.59 mg/L. These values represent high tissue or fluid : serum concentration ratios (52 : 1 to 1150 : 1). In patients, with respiratory infection, who received 500 mg on day 1, and 250 mg on days 2 to 5, the mean peak sputum concentration of azithromycin was 3.7 mg/L at 15 hours which represents a sputum : serum concentration ratio of 5.8 : 1 [74].

Equilibrium dialysis at pH 7.4 and 25°C was used to determine in vitro protein binding of [14C]-azithromycin in human serum [104]. Protein binding decreased as the azithromycin concentration increased; binding was 50% for azithromycin concentrations of 0.02 and 0.05 mg/L, 37% at 0.7 mg/L and 7% at 1 mg/L. Erythromycin and roxithromycin demonstrated higher binding than azithromycin (72% at 0.4 mg/L erythromycin and 96% at 2.5 mg/L roxithromycin) [104, 105]. Therefore, azithromycin has a greater proportion of drug in the free form that is then available for distribution to
infected tissues. Azithromycin, erythromycin, and roxithromycin are predominantly bound to $\alpha_1$-acid glycoprotein as suggested by low concentrations of drug displaying saturability [105, 106].

Azithromycin is mostly eliminated in the feces via transintestinal excretion, but a small amount leaves in the urine [107, 108]. Azithromycin is primarily eliminated in an unchanged form, although some biotransformation does occur. Biotransformation primarily occurs via N-demethylation of the desoamine sugar or the 9a position of the macrolide ring but hydrolysis and hydroxylation also occur [108].

Azithromycin (500 mg) showed a mean terminal elimination half-life ($t_{1/2}$) from serum of 9.6 to 14 hours when given orally and 35 to 40 hours when given intravenously [95, 104]. The $t_{1/2}$ for oral azithromycin (500 mg twice on day 1 and 500 mg once on days 2 to 5) increased from 11 to 14 hours on day 5 to 48 hours over 1 to 3 days after the last dose and to 57 hours over 1 to 6 days after the last dose [104].

The effect of age was compared in a nonblind parallel study using healthy elderly and young volunteers given 500 mg on day 1 and 250 mg on days 2 to 5 [109]. Mean maximum serum concentrations (~0.4 mg/L on day 1 and ~0.25 mg/L on day 5) and minimum serum concentrations (0.05 mg/L) were similar for young and elderly volunteers and there was no difference in urinary excretion. Area under the plasma concentration-time curve (AUC) and $t_{\text{max}}$ were significantly increased in the elderly. An inverse relationship was found between creatinine clearance and AUC indicating that dosage adjustment is not required in elderly people with mild renal impairment, only those with more severe renal impairment. Subsequent studies have confirmed that azithromycin can be safely used in children of all ages [110].
Healthy volunteers and patients with mild to moderate hepatic dysfunction did not display differences in pharmacokinetic parameters except for an increase in $t_{1/2}$ (68 versus 54 hours) suggesting that dosage adjustment, when mild to moderate hepatic impairment is present, is not necessary since azithromycin treatment is of short duration and tolerated favorably [111].

1.3.2.4 Efficacy in Animal Models

The efficacy of azithromycin in vivo has been assessed using animal models of infection. Mice infected with S. pneumoniae showed a higher rate of survival when given subcutaneous or oral azithromycin (25 – 50 mg/kg) either before or after infection than when given erythromycin [112]. Bacteria were completely cleared from lungs and blood in mice given two subcutaneous injections of azithromycin (25 mg/kg) at 48 and 65 hours post-infection but were not cleared in mice given erythromycin, clarithromycin, roxithromycin or spindamycin [113]. This result was consistent with greater concentrations of azithromycin that also remained longer in the lung. When azithromycin (25 mg/kg) was given orally 24 hours before infection with S. pneumoniae, recovery of bacteria after 6 hours of infection was reduced by greater than 99.9% compared to untreated controls [77]. In the same study, erythromycin (100 mg/kg) produced no difference compared to controls and roxithromycin (50 mg/kg) produced a 96% reduction in bacterial recovery compared to control values.

Intraperitoneal delivery of azithromycin (3.6 mg/kg/day) resulted in greater therapeutic efficacy than erythromycin (96 mg/kg/day) in a lethal L. pneumophila pneumonia model in guinea pigs. Survival rates were 100% with azithromycin and 83%
with erythromycin and clearance of bacteria was complete after two days with azithromycin whereas erythromycin did not clear the bacteria after six days [114]. Oral administration for two days in the same animal model resulted in 100% survival in the azithromycin group (3.6 mg/kg once daily) and in the clarithromycin group (28.8 mg/kg twice daily). Although the higher dose of clarithromycin resulted in similar survival rates, clarithromycin did not eradicate bacteria from the lungs as did azithromycin [115]. In another study, mice infected with \textit{K. pneumoniae} showed a 99% reduction in bacteria recovery from the lungs when given azithromycin (50 mg/kg/day) 24 hours before infection compared to the untreated control [77].

Taken together, the results generated from these animal studies demonstrate that the efficacy of azithromycin \textit{in vivo} is greater than that of many other macrolides.

### 1.3.2.5 Clinical Efficacy and Dosage

The clinical efficacy of azithromycin has been demonstrated in the treatment of lower and upper respiratory tract infections, skin and soft tissue infections, urethritis/cervicitis (1 g over 1 to 3 days), early Lyme disease and \textit{M. avium} complex infections (500 mg/day for 10 to 30 days) [64]. The efficacy of azithromycin is correlated with high tissue concentrations. Typical treatment regimens are a single or divided 500 mg dose on day one followed by either 250 mg/day for four more days or 500 mg/day for three more days. In both regimens, the azithromycin concentrations in respiratory tract tissues (> 2 mg/L) are above the minimum inhibitory concentrations (MIC) for key pathogens (\textit{Haemophilus influenzae}, azithromycin \text{MIC}_{90} of 0.5-2.0 mg/L; \textit{Streptococcus pyogenes}, azithromycin \text{MIC}_{90} of \leq 0.12 mg/L; \textit{Streptococcus pneumoniae},...
Moraxella catarrhalis, Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionella pneumophila, all with an azithromycin MIC\textsubscript{90} of \( \leq 2.0 \) mg/L) for up to ten days following treatment [98]. Small children should be given 10 mg/kg on day one and 5 mg/kg on days two to five and children weighing over 45 kg should be given the adult dosage via either regimen.

Standard regimens of azithromycin have been shown to have comparable efficacy compared to erythromycin, amoxicillin, cefaclor, amoxicillin/clavulanate, roxithromycin, and clarithromycin in the treatment of lower respiratory tract infections, although all the other drugs required more frequent and prolonged regimens than azithromycin to achieve this same efficacy [72, 116]. This suggests that azithromycin has a more potent clinical effect than the other drugs to which it was compared. In a study of patients with upper or lower respiratory tract infection given azithromycin (500 mg on day one and 250 mg on days 2 to 5), a 95% clinical cure rate and a 91% clearance of sputum bacteria was demonstrated [117]. A 91% clinical cure rate was recorded for patients with acute bacterial pneumonia given azithromycin (500 mg/day for 3 days) [118]. Studies have found that azithromycin is more effective than erythromycin in eradicating \( H. \) influenzae [74]. Clinical cure rates for azithromycin in comparative studies of lower respiratory tract infections ranged from 36% to 80% and clinical success rates (cure + improvement) ranged from 92% to 100% [64]. Azithromycin was better tolerated and showed a lower incidence of adverse effects compared to roxithromycin [72, 119]. Upper respiratory tract infections treated with azithromycin (500 mg on day 1 and 250 mg/day on days 2-5) showed clinical cure rates of 98% and a 96% clearance of bacteria [117]. Clinical cure rates for azithromycin ranged from 74% to 95% and clinical success rates ranged from
98% to 100% in comparative studies on the efficacy of azithromycin in upper respiratory tract infections [64]. Eradication rates for bacteria were greater than 89% and the clinical efficacy of azithromycin was demonstrated to be equivalent to the other antibiotics tested. Children given a total of 30 mg/kg of azithromycin over three or five days demonstrated clinical cure rates of 78% to 95% for those with acute otitis media and 86% to 93% in those with pharyngitis and/or tonsillitis [64]. The clinical cure rate of azithromycin was greater but clinical success rates were not different than those of amoxicillin in children with acute otitis media [120].

Overall, clinical efficacy and pharmacological studies suggest that azithromycin displays comparable or greater efficacy in eradicating certain infections compared to other antibiotics, and that azithromycin requires a shorter and less frequent dosage schedule.

1.3.2.6 Tolerability

The overall adverse effect rate is 12% for azithromycin with the most frequent being gastrointestinal disturbances (9.6%) [diarrhea (2.6%), nausea (2.6%) and abdominal pain (2.5%)] and central and peripheral nervous system effects such as headaches and dizziness (1.3%) [121]. This rate is lower than those seen for amoxicillin/probenecid (54.5%), cefaclor (16.7%), doxycycline (15.8%) and erythromycin (20.4%). A study comparing the numbers of discontinuations of therapy demonstrated that azithromycin had a lower percentage of discontinuations of therapy compared to all the other drugs tested except for doxycycline [121]. There were no neurological, audiometric or ophthalmoscopic abnormalities detected nor did
phospholipidosis occur in this study. Patients with hypersensitivity to other macrolides should not be given azithromycin [121]. The adverse effects of azithromycin are similar in young and elderly people [121].

1.3.2.7 Drug Interactions

Drug metabolism in the body involves enzyme systems such as the mixed function oxidase system [61]. The enzyme cytochrome P450 is one of the key enzymes involved in this system. Hydroxylation by P450 often produces a reactive intermediate. Some drugs can induce this enzyme which can lead to an increase or a decrease in the metabolism of a second drug. Unlike erythromycin and many other macrolides, azithromycin does not induce or inhibit cytochrome P450 II A enzymes [122]. In part because of this, no pharmacokinetic interactions have been found for patients concomitantly receiving theophylline, cyclosporine, warfarin, cimetidine, carbamazepine, methyl prednisolone, terfenadine, oral contraceptives or zidovudine [121, 123-125]. Azithromycin should be taken 1 hour before or 2 hours after antacids containing aluminum and magnesium as this has been shown to reduce the peak serum concentrations but not the total absorption of azithromycin [104, 121]. Interactions between azithromycin and either ergotamine or digoxin have not been investigated but since erythromycin causes ischemia and peripheral vasospasm when given with ergotamine and erythromycin causes increased serum digoxin levels, co-administration is not recommended for ergotamine and azithromycin and digoxin levels must be monitored [64].
1.3.3 Anti-inflammatory Therapies

Anti-inflammatories can function to reduce the inflammation associated with pneumonia and prevent host-mediated damage but they do not eradicate the source of the infection. A number of anti-inflammatory strategies aim at down-regulating the neutrophil response to infection and inflammation. One example of anti-inflammatory drugs are the glucocorticoids that inhibit neutrophil emigration out of the vasculature and into sites of inflammation by decreasing neutrophil adherence to endothelium, inhibiting chemotaxis and inhibiting production of neutrophil chemotactic factors [126]. Inhibition of phospholipase A2 by glucocorticoids prevents the production of arachidonic acid derivatives such as leukotriene B4 that attract and activate neutrophils.

The anti-inflammatory glucocorticoid dexamethasone inhibits neutrophil superoxide generation, release of lactoferrin and lysozyme, emigration, chemotaxis, and production of LTB4 and IL-1 [127, 128]. Dexamethasone induces apoptosis of rat thymocytes, however, it inhibits apoptosis in human neutrophils [129, 130]. Dexamethasone decreases IL-8 production by macrophages pretreated with dexamethasone before exposure to lipopolysaccharide [131].

1.3.4 Immunomodulation by Antibiotics

Respiratory tract infections are commonly treated with macrolide antibiotics. Besides having antibacterial effects, many macrolides have been postulated to have anti-inflammatory properties that may enhance their efficacies in reducing illness. Azithromycin has proven to be more effective in the treatment of a number of respiratory
tract infections compared to erythromycin, another macrolide with comparable antibacterial properties [64].

A number of reports have suggested that macrolides, such as erythromycin, roxithromycin, and clarithromycin, have anti-inflammatory effects [7, 10, 28, 132-136]. Azithromycin may also have anti-inflammatory properties. Macrolides may exert this effect by modulating the production of cytokines including IL-1, IL-6, IL-8, and TNFα [7, 28, 132-139]. Macrolides may also alter functions of inflammatory cells [10, 140-142]. Some macrolides, such as azithromycin, roxithromycin, and clarithromycin, inhibit the respiratory burst of neutrophils [143-147]. It was recently demonstrated that induction of bovine neutrophil apoptosis by the veterinary macrolide tilmicosin may be associated with anti-inflammatory benefits [148]. Erythromycin, roxithromycin, clarithromycin, and midecamycin have also been demonstrated to induce neutrophil apoptosis [149]. Apoptotic neutrophils, unlike necrotic neutrophils, remain intact and are removed from tissues by phagocytes, thus preventing the release of pro-inflammatory products in situ [48, 150]. The induction of apoptosis by azithromycin has not been previously studied and its biological significance has yet to be uncovered.

1.4 Apoptosis

Cells can die via one of two forms of cell death, necrosis or apoptosis. Necrotic cell death results in release of intracellular contents leading to inflammation and extensive tissue damage [47].

Apoptosis, or programmed cell death, is a distinct form of cell death, both morphologically and biochemically. In apoptosis, the cell actively participates in its own
destruction. Apoptosis is characterized by a number of features: the membrane of an apoptotic cell remains intact, the cytoplasm and the nucleus becomes condensed, and the DNA is cleaved into mono- and oligo-nucleosomes, which are commonly used as indicators of apoptosis [150, 151]. In vivo, apoptosis is rapidly followed by uptake of apoptotic cells into phagocytic cells [48]. This process is involved in the regulation of cell turnover, tissue remodeling, regulation of the immune response, and resolution of inflammation [48, 152].

1.4.1 Induction

Apoptosis can be induced by the binding of an appropriate ligand to either a membrane receptor or a cytoplasmic receptor, which activates a second messenger system [17]. Various signals can induce apoptosis, such as the activation of receptors for Fas ligand or TNF, glucocorticoid treatment, UV irradiation, and serum or growth factor withdrawal (such as IL-3 or platelet-derived growth factor) [148, 153-157]. Although the stimuli may vary, one mechanism consistently implicated in apoptosis is the activation of a cascade of cytosolic proteases called caspases that initiate the changes associated with apoptosis [158]. In human cells, many substrates for caspases have been identified as DNA repair enzymes (poly-ADP-ribose-polymerase [PARP], and DNA-protein kinase), homeostatic proteins (protein kinase C, D4-GDI, and sterol regulatory element binding proteins), and structural proteins (nuclear lamins A and B, fodrin, and the nuclear mitotic apparatus protein [NuMA]) [40]. It is not known exactly how cleavage of these and other substrates leads to the apoptotic phenotype except that multiple homeostatic pathways are interrupted [40].
1.4.2 Neutrophil Apoptosis

Accumulation and bursting of necrotic neutrophils at the site of inflammation contribute to the propagation of inflammation during infectious pneumonia and other respiratory tract infections. Neutrophils recruited to sites of infection can undergo either necrosis or apoptosis (programmed cell death). Cytotoxic compounds such as elastase, hypochlorous acid, and oxygen radicals released by necrotic neutrophils recruited to sites of infection can damage surrounding tissues, and induce necrosis in neighbouring neutrophils [7, 159]. Neutrophils injured in this fashion further release cytotoxic compounds and pro-inflammatory mediators [13]. In contrast, apoptotic neutrophils may be eliminated without spilling pro-inflammatory and cytotoxic products in situ [48, 150]. Apoptosis is a less harmful process than necrosis since the membrane of an apoptotic neutrophil remains intact. In addition, apoptotic cells are more rapidly phagocytosed by macrophages, through the use of various surface-signaling molecules, including the exposure of phosphatidylserine on the outer leaflet of the cell membrane [160, 161]. Thus, apoptosis is a more desirable form of cell death since the preservation of membrane integrity and rapid clearance of apoptotic cells from the site of infection minimize host tissue damage and inflammation [132, 162, 163]. This could be important in the resolution of lung inflammation resulting from diseases such as streptococcal pneumonia.

1.4.3 Regulation of Apoptosis in Neutrophils

Activation of caspases (interleukin 1β-converting enzyme homologues) initiates apoptosis [40]. After isolation from blood, neutrophils undergo spontaneous apoptosis,
and thus neutrophil age is one factor involved in inducing apoptosis [40]. Forty-five percent of neutrophils incubated for 24 hours are apoptotic. Caspase-3 is the primary caspase involved in the initiation of the apoptotic cascade [40]. Active caspase-3 is detected in apoptotic neutrophils but not in freshly isolated neutrophils. Neutrophils contain a number of caspase substrates including D4-GDI, gelsolin, lamin B, and fodrin [40]. D4-GDI is a negative regulator of Rho GTPases, which may regulate the cytoskeletal and membrane alterations accompanying cell death [164, 165]. Cleaved D4-GDI is unable to bind GTPases thus allowing these GTPases to exert their effects. Cleavage and consequent activation of gelsolin, an actin-severing protein, results in a reduction in F-actin content and a disruption of cell structure leading to apoptosis [166]. Gelsolin cleavage also contributes to nuclear fragmentation. Lamin B is a nuclear matrix protein which when cleaved leads to the chromatin condensation characteristic of apoptosis [167]. Proteolytic processes during apoptosis make the surface of neutrophils pro-phagocytic and more readily identifiable by macrophages. Fodrin is a cytoskeletal component and its cleavage may be involved in membrane blebbing of apoptotic cells [168]. Fodrin cleavage has been linked to the exposure of phosphatidylserine on the outer membrane, which appears to be one mechanism by which phagocytic cells recognize and clear apoptotic neutrophils [169]. Phosphatidylserine, normally localized to the inner half of the bilayer of the cell membrane of neutrophils, is translocated to the outer half of the bilayer through the activation of scramblases and the downregulation of aminophospholipid translocase (which normally reinternalizes any phosphatidylserine that reaches the outer membrane) [161]. The receptor, 61D3, on macrophages may be involved in phosphatidylserine-dependent phagocytosis [170]. Uptake of apoptotic cells
also occurs via the vitronectin receptor and CD36 on macrophages, which interact with
neutrophils via thrombospondin and an as yet unidentified molecule on the surface of
apoptotic neutrophils, which does not appear to be phosphatidylserine [47, 170, 171].

1.5 Research Hypothesis and Objectives

While the clinical effectiveness of azithromycin may be due in part to its
pharmacokinetic properties, it may have anti-inflammatory properties in addition to its
antibacterial effects. It is my hypothesis that azithromycin may induce apoptosis in
human neutrophils more effectively than other antibiotics and hence help the host to
dispose of these cells without perpetuating local inflammation via enhanced macrophage
clearance of apoptotic neutrophils. The aim of this study was to determine the effects of
azithromycin on human neutrophil apoptosis, oxidative function and IL-8 production, in
the presence or absence of *S. pneumoniae*.

Specific objectives of this study are:

1. To assess the induction of neutrophil apoptosis by azithromycin in comparison to
   other drugs, in the presence or absence of *Streptococcus pneumoniae*.
2. To investigate the effect of azithromycin on the phagocytosis of neutrophils by
   macrophages.
3. To determine the effects of azithromycin on neutrophil function (oxidative
   metabolism, IL-8 production) in comparison to other drugs, in the presence or
   absence of *Streptococcus pneumoniae*. 
CHAPTER 2: METHODS AND MATERIALS

2.1 Drugs

Azithromycin (Pfizer Canada Inc., Montreal, PQ, Canada), penicillin-G (Sigma Diagnostics, St. Louis, MO, USA), and erythromycin (Sigma) were dissolved in pyrogen-free phosphate buffered saline (PBS, pH 7.1) and diluted to a concentration of 0.05 µg/mL. Dexamethasone (Schering Canada Inc., Pointe-Claire, PQ, Canada) was diluted in PBS (pH 7.1) to a concentration of 0.02 µg/mL.

2.2 Bacteria

*Streptococcus pneumoniae* 14259 (human clinical isolate obtained from Dr. R. R. Read, Foothills Hospital, Calgary, AB, Canada) was grown overnight on Columbia blood agar (CBA) plates at 37°C. Bacterial cells were collected, suspended in PBS (pH 7.1), and diluted to approximately $1.5 \times 10^8$ bacteria/mL using a 0.5 McFarland standard (Dalynn Laboratory Products, Calgary, AB, Canada). The final concentration of viable bacteria ($\sim 1 \times 10^8$ CFU/mL) was calculated via colony forming unit (CFU) counts of serial dilutions on CBA after a 24 h incubation at 37°C. Bacteria were used either live or as sonicates (4 h, on ice, 85% duty cycle, output control setting 4, W-350 Sonifier®, Branson Ultrasonics Corporation, Danbury, CT, USA).

2.3 Subjects

Male and female subjects were recruited from volunteers at the University of Calgary. Only those not taking medications were included in these studies. Throughout
this study more than 10 different donors were used and at least three different donors were used to obtain data for the individual experiments.

2.4 Neutrophils

Blood was collected by venipuncture from healthy human volunteers and collected in sodium heparin Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Neutrophils were purified by density gradient centrifugation using Histopaque™ 1119/1077 (Sigma). Whole blood was layered onto a gradient of Histopaque™ 1077 (3 mL) that was underlayered with Histopaque™ 1119 (3 mL). After centrifugation (1500×g, 30 min, Beckman J-6B centrifuge, Beckman Instruments, Palo Alto, CA, USA), the mononuclear cell layer was removed and discarded. The neutrophil layer was removed, washed in 10 mL of 1× Hanks’ balanced salt solution without calcium and magnesium (HBSS, Gibco BRL, Life Technologies Inc., Grand Island, NY, USA), and centrifuged (1500×g, 10 min). Contaminating erythrocytes were lysed by resuspending the pellet in 1 mL of sterile distilled water for 30 seconds, and osmolarity was restored by adding 1 mL of 2× HBSS (Gibco BRL). After washing in 10 mL of 1× HBSS, purified cells were resuspended in either HEPES-buffered RPMI 1640 (Sigma) supplemented with 0.05μg/mL L-glutamine, Ca²⁺/Mg²⁺-free PBS (pH 7.1) (for NBT assay) or 1× HBSS (for macrophage phagocytosis) to a final concentration of 10⁵ to 10⁶ cells/mL depending on the experiment. Cells were counted in a haemacytometer (Spencer Bright-Line Improved Neubauer, American Optical Corp., Buffalo, NY, USA), and viability was assessed by 0.1% trypan blue (Gibco BRL) exclusion. The cell suspension (100 μL) was
centrifuged (20×g, 10 min) onto a microscope slide with a cytospin (Shandon Southern Producers Ltd., Cheshire, England), fixed and stained with Diff Quick (Baxter Healthcare Corp., Miami, FL, USA) and neutrophil purity was assessed by direct microscopy differential leukocyte counts. Neutrophils were incubated (37°C, 5% CO₂) for various times with PBS (pH 7.1) (controls), azithromycin (0.05 μg/mL), penicillin (0.05 μg/mL), erythromycin (0.05 μg/mL), dexamethasone (0.02 μg/mL), or tumor necrosis factor-α (TNFα, R&D Systems, Minneapolis, MN, USA, 5 ng/mL), with or without live Streptococcus pneumoniae or lysate from S. pneumoniae.

2.5 Monocytes/Macrophages

Human peripheral blood monocytes can be cultured in vitro. They become activated both by the isolation process from whole blood and by attachment to tissue culture plastic surface [172]. This adherence in the presence of serum mimics the immigration of monocytes through the vasculature into the site of infection and primes the monocyte for inflammatory responses [173]. The activated monocytes differentiate into macrophages when serum is present in the medium [174]. As the monocytes differentiate they increase in size and phagocytic activity, and many biochemical and morphological changes occur [172]. Human leukocytes contain esterases which hydrolyze aliphatic and aromatic esters [45, 175]. Macrophages contain a nonspecific esterase that can be detected by addition of the substrate α-naphthyl acetate coupled to a diazonium salt, followed by counter-staining with methyl green. Hydrolysis of the substrate produces an insoluble coloured azo dye. Macrophages show a diffuse
red/brown staining of the cytoplasm with a green nucleus (positive for nonspecific esterase), while monocytes, which produce very little nonspecific esterase, appear green (negative for esterase).

Blood was collected by venipuncture from healthy human volunteers and collected in Vacutainer® CPT™ cell preparation tubes with sodium citrate (Becton Dickinson). Monocytes were purified by density centrifugation (2000×g, 30 min). The mononuclear cell layer was removed, washed in 1× HBSS (10 mL), and centrifuged (300×g, 15 min). The pellet was resuspended in 1× HBSS (10 mL) and centrifuged (300×g, 10 min). The pellet was resuspended in Iscove’s modified Dulbecco’s medium (MDM) (Gibco BRL) to a final concentration of 6 × 10⁶ cells/mL. Cells were counted in a haemacytometer, and viability was assessed by trypan blue (0.1%) exclusion. Monocytes (1 mL) were incubated (37°C, 5% CO₂) in 24-well tissue culture plates (Costar, Cambridge, MA, USA) for 60 min after which non-adherent cells (mainly lymphocytes) were removed by washing 3 times with 1× HBSS (37°C, 1 mL) [176]. Monocyte purity was assessed by staining cytospin preparations with Diff Quick and direct microscopy differential leukocyte counts. Monocytes were cultured in 1 mL Iscove’s MDM containing 10% fetal bovine serum (Sigma) and were allowed to differentiate into macrophages for seven days. The media was replaced every 2-3 days by aspiration.

Macrophage differentiation was ascertained by mature cell morphology (size and shape changes) after being stained with Diff Quick and by a positive reaction for nonspecific esterase. Nonspecific esterase staining was done as previously described
Briefly, after cells were fixed, they were incubated with esterase substrate solution containing α-naphthyl acetate (Sigma) and hexazoatized pararosalanine (Sigma) for 45 min at 37°C. Slides were counter-stained for 2 min with 1% methyl green and examined by light microscopy.

2.6 CFU Counts

*S. pneumoniae* was incubated with PMNs and drugs (37°C, 5% CO₂) at a ratio of 10:1 for 2 h and 6 h, plated on CBA plates after serial dilutions, and colonies were counted after 24 h incubation at 37°C.

2.7 Annexin-V/Propidium Iodide Labelling

Neutrophil apoptosis and necrosis were assessed by double staining with FITC-conjugated annexin-V and propidium iodide (PI) using an Annexin-V FLUOS kit (Boehringer-Mannheim GmbH, Germany), according to manufacturer’s instructions. Annexin binds with high affinity to phosphatidylserine which is translocated from the inner half of the bilayer to the outer half of the membrane bilayer during both apoptosis and necrosis [159]. Necrotic cells are differentiated by staining with propidium iodide. After 1 hour co-incubation with or without drugs and with or without bacterial lysate (4:1 bacteria to cell ratio), PMNs (5.5 × 10⁶ cells/mL) were centrifuged (Baxter Canlab Biofuge A, Heraeus Sepatech GmbH, Germany) (200×g, 5 min), then washed with 1 mL PBS (pH 7.1). Cells were centrifuged again, resuspended in 100 μL of FITC-conjugated annexin-V and propidium iodide staining solution, and incubated for 15 minutes in the
dark. Samples were then centrifuged (200×g, 5 min) and resuspended in PBS (pH 7.1), and a wet mount of 15 μL was observed by epifluorescent microscopy (450 nm and 535 nm) (Leitz Aristoplan, Germany) at 400× magnification and the percentages of apoptotic and necrotic cells were determined from ten different fields per preparation. The high and low counts were discarded and the other 8 were averaged.

As a positive control for the annexin-V/PI labelling system, PMNs were incubated with 5 ng/mL (approximately 100 U/mL) recombinant human TNFα in PBS (pH 7.1) with 0.1% bovine serum albumin for 1, 2, and 3 hours. TNFα at this concentration is known to induce neutrophil apoptosis [177].

2.8 Cell Death ELISA

Neutrophil apoptosis was also assayed via ELISA. Neutrophils (10^5 cells/mL) were co-incubated for 6 hours with or without drugs and with or without live bacteria (10:1 bacteria to cell ratio) and stored at -70°C for later analysis. Samples were assayed for apoptosis with a Cell Death Detection ELISA kit (Boehringer Mannheim), which detects the histone region (H1, H2A, H2B, H3, and H4) of mono-nucleosomes and oligo-nucleosomes that are formed during apoptosis. The plates were read at 405 nm (THERMOMax™ microplate reader, Molecular Devices Corp., Menlo Park, CA, USA) at intervals for 58 min, starting 1 min after addition of substrate. Results were expressed as the ratio of the sample absorbance versus the mean absorbance of the vehicle-treated control.
2.9 Macrophage Phagocytosis

PMNs (6 ×10⁶ cells/mL) were incubated for 1 hour with or without azithromycin and then centrifuged (minimum speed, 5 min, microcentrifuge). The pellet was washed by resuspending in 1× HBSS (1 mL) and centrifuged (minimum speed, 5 min). This pellet was resuspended in 1× HBSS to the original cell volume of 300 μL. The PMNs were added to the wells containing 7 day old cultured macrophages and incubated for 1 hour (37°C, 5% CO₂). PMNs were obtained from the same donor as described above. The wells were washed three times with 1× HBSS (37°C, 1 mL) and stained with Diff Quick. Three counts of 100 cells were done on each well to determine the number of macrophages containing phagocytosed PMNs. A total of 2400 macrophages were counted. Only macrophages that had completely surrounded a PMN were counted.

2.10 Nitro Blue Tetrazolium (NBT) Assay

The oxidative function of PMNs (10⁶ cells/mL) co-incubated for 1 hour with or without drugs and with or without bacterial lysate (10:1 bacteria to cell ratio) was assessed by NBT reduction. Cells were incubated in chamber slides (Nalge Nunc International, Naperville, IL, USA) for 20 minutes with nitro blue tetrazolium (NBT, 2 mg/mL, Sigma) in the presence or absence of 2 μg/mL phorbol 12-myristate 13-acetate (PMA) (Sigma). Cells were fixed with methanol and counterstained with safranin (1%). The percentage of oxidatively active cells with blue formazan crystals in their cytoplasm was calculated following direct microscopic examination and counting under oil immersion (1000×) with a light microscope (Zeiss, Germany).
2.11 Interleukin-8 Assay

PMNs (2×10^7 cells/mL) were incubated for 2 hours with drugs, then washed in PBS (pH 7.1) and incubated (24 h, 37°C, 5% CO₂) with or without *S. pneumoniae* lysate (10:1 bacteria to cell ratio). Cells were then centrifuged (800xg, 10 min) and the supernatant collected. IL-8 production was assessed by a Quantikine™ human IL-8 sandwich enzyme immunoassay (R&D Systems) according to the manufacturer’s instructions. This assay is specific for natural and recombinant human IL-8. No significant cross-reactivity or interference has been observed for other human or mouse interleukins or other factors tested. The sensitivity of this kit is 10 pg/mL.

2.12 Statistical Analysis

Results were expressed as mean ± SEM and compared by one-way analysis of variance (ANOVA) followed by Tukey’s test for multiple comparison except for the macrophage phagocytosis results which were analyzed using Fisher’s Exact test with a 2-sided P value and a chi-square approximation. *P*<0.05 was considered significant.
CHAPTER 3: RESULTS

3.1 Cell Isolation

The success of the cell isolation procedures was determined via examination of the viability and purity of the resultant populations. The viability of purified PMNs incubated in PBS (pH 7.1), HBSS, or RPMI 1640 was >95%, and cell purity was >98%. The purity of the isolated mononuclear cells was >98% and viability was >97%. Monocytes accounted for ~40% of the mononuclear cells. Contaminating cells in the purified monocytes were mainly lymphocytes and the occasional neutrophil, but due to the short lifespan of neutrophils none remained after the third day of incubation [44]. Contaminating lymphocytes in the monocyte preparation were removed by washing following the initial 1 hour incubation in the well plates, as lymphocytes do not adhere to the wells [176].

3.2 CFU Counts

CFU counts were done to determine whether the antibacterial activity of the antibiotics was altered by the presence of neutrophils and whether S. pneumoniae was susceptible to the antibiotics tested. After 6 hours of co-incubation with PMNs, azithromycin, penicillin, and erythromycin significantly decreased S. pneumoniae numbers compared to controls (Figure 1). At 6 hours, bacterial numbers in preparations containing azithromycin were significantly lower than those exposed to other antibiotics. Incubation with dexamethasone did not affect bacterial numbers.
3.3 Neutrophil Apoptosis

To assess the anti-inflammatory potential of azithromycin, I looked at the induction of apoptosis in neutrophils. To determine whether azithromycin induces neutrophil apoptosis two systems were used – annexin-V/PI labelling and Cell Death Detection ELISA. The annexin-V/PI fluorescent labelling system allowed discrimination of apoptotic from necrotic cells (Figure 2A and Figure 2B). TNFα induced neutrophil apoptosis in a time-dependent fashion (Figure 3). In the absence of *S. pneumoniae* lysate, azithromycin induced neutrophil apoptosis at levels similar to those seen in cells incubated with TNFα for 3 h (Figure 4A). Cells incubated with penicillin, erythromycin, or dexamethasone were not different from vehicle controls. Addition of bacterial lysate inhibited the induction of apoptosis by azithromycin (Figure 4B).

In an attempt to explain this inhibition of apoptosis by bacterial lysate, cell necrosis was assessed to see if neutrophil necrosis was higher in the samples containing lysate. As shown in Figure 5, *S. pneumoniae* did not affect neutrophil necrosis. In addition, neutrophil recovery was not significantly different between cells incubated with saline (7.43×10⁶ ± 4.61×10⁵ cells/mL), azithromycin (6.06×10⁶ ± 4.81×10⁵ cells/mL), *S. pneumoniae* (6.88×10⁶ ± 7.04×10⁵ cells/mL) or azithromycin and *S. pneumoniae* together (7.15×10⁶ ± 3.31×10⁵ cells/mL).

The induction of neutrophil apoptosis by azithromycin was also detected by ELISA at 6 h in the absence of *S. pneumoniae* (Figure 6A). The colorimetric reaction was rapid enough to be detected after 1 minute of incubation with reagent. As was observed with annexin-V labelling, co-incubation with *S. pneumoniae* abolished this
effect (Figure 6B). Incubation of neutrophils with penicillin, erythromycin, or
dexamethasone did not affect neutrophil apoptosis in the presence or absence of S.
pneumoniae.

3.4 Macrophage Phagocytosis

Apoptotic neutrophils are rapidly phagocytosed in vivo by phagocytes, a process
that disposes of these cells without perpetuating local inflammation. To determine
whether the increased number of apoptotic cells seen in azithromycin-treated neutrophil
populations contributed to an increased uptake of these cells by macrophages, I used
monocyte-derived macrophages. Monocyte differentiation into macrophages was
determined using two techniques: mature cell morphology and nonspecific esterase
staining. On day 0, all cells looked like typical monocytes (cell size less than 13 μm, a
round nucleus, and a thin ring of cytoplasm). Cell morphology (cell size greater than 13
μm, a bean shaped nucleus, and much larger cytoplasmic area) revealed differentiation of
70% on day 2, 96% on day 5, and 98% on day 7. Nonspecific esterase staining showed
differentiation of 79% on day 2, 92% on day 5, and 98% on day 7 (Figure 7). These
measures indicate that a large population of differentiated macrophages was present on
day seven.

Macrophages which had ingested neutrophils were identified via light microscopy
(Figure 8). Macrophage phagocytosis of neutrophils after 1 hour was found to be
significantly increased for azithromycin-treated PMNs compared to control PMNs
(Figure 9).
3.5 Oxidative Function and IL-8 Production

To determine whether the induction of apoptosis by azithromycin affects the function of neutrophils, I looked at oxidative function and IL-8 production. The ability to produce reactive oxygen intermediates for microbial killing is an essential function of neutrophils. None of the drugs affected oxidative function in resting (not exposed to PMA) or stimulated (exposed to PMA) neutrophil populations (Figure 10A). Similarly, the drugs did not affect oxidative function when co-incubated with bacterial lysate (Figure 10B).

Neutrophils produce chemotactic IL-8 in order to recruit more neutrophils to sites of infection. Exposure of neutrophils to *S. pneumoniae* lysate promoted synthesis of IL-8 (Figure 11). IL-8 secretion was not affected by any of the antibiotics in the presence or in the absence of *S. pneumoniae* lysate compared to controls (Figure 11). In contrast, dexamethasone significantly inhibited *S. pneumoniae* lysate-induced IL-8 synthesis.
Figure 1. *S. pneumoniae* recovery (log\(_{10}\) CFU) after 2 h and 6 h co-incubation of PMNs with azithromycin (---■---■), penicillin (- -●- -), erythromycin (- -×- -) and dexamethasone (---▲---). Antibiotic treatments are significantly different from control (---●---) and dexamethasone at both times (n=5; experiment was repeated 3 times). * P<0.05 compared with control. △ P<0.05 azithromycin compared with all groups.
Figure 2. Light micrographs (400×) of PMNs stained with the annexin-V/propidium iodide system. A) Bright field micrograph illustrating several PMNs, and B) fluorescence micrograph of the same field showing an apoptotic PMN (arrow) and two necrotic PMNs (arrowheads); viable neutrophils seen on the bright field micrograph were not labelled.
Figure 3. Apoptosis in PMNs treated with 5 ng/mL TNFα for 1, 2, and 3 h (n=4; experiment was repeated 2 times), determined by annexin-V/PI labelling. The bar labelled control represents the mean of the control values (n=6) from 1, 2, and 3 h. * $P<0.05$ compared with control.
Figure 4. Apoptosis in neutrophils determined by annexin-V/PI labelling after 1 h incubation with azithromycin (Azi), penicillin (Pen), erythromycin (Ery), or dexamethasone (Dex). A) Without lysate, B) with *S. pneumoniae* lysate (4 CFU:1 PMN). TNFα sample is the 3 h incubation value from Figure 2. * $P<0.05$ compared with control. ** $P<0.05$ compared with control and other drugs ($n \geq 5$; experiment was repeated 5 times).
Figure 5. PMN necrosis in samples incubated with azithromycin (Azi), penicillin (Pen), erythromycin (Ery), or dexamethasone (Dex) and with (black bars) or without (white bars) *S. pneumoniae* lysate determined by annexin-V/PI labelling. No significant difference between any samples (n≥5; experiment was repeated 5 times).
Figure 6. Apoptosis detected by Cell Death ELISA, after 6 h incubation. A) Without *S. pneumoniae*. B) with live *S. pneumoniae*. Absorbance readings were plotted as a ratio of the sample absorbance to the control, which was set at 1. Azithromycin (---□---), penicillin (——●——), erythromycin (——•×•) and dexamethasone (——△——), control (——◆——). *P<0.05 compared with control and dexamethasone (n ≥ 5; experiment was repeated 4 times).
Figure 7. Light micrograph (400×) of monocytes/macrophages stained for nonspecific esterase. Positively stained cells (monocyte-derived macrophages) appear red and negatively stained cells (undifferentiated monocytes) appear green.
Figure 8. Light micrograph (400×) of macrophages incubated with PMNs for one hour. A macrophage that has phagocytosed a neutrophil (arrow) and a non-phagocytosed PMN (arrowhead) are seen.
Figure 9. Phagocytosis of azithromycin-treated PMNs by macrophages after 1 h, following a 1 h incubation of PMNs with azithromycin or HBSS (control). *$P<0.05$ versus control (experiment was repeated 3 times; a total of 2400 macrophages were counted). No error bars are shown as values are discrete numbers and not a mean.
Figure 10. Oxidative activity determined by the percentage of resting (white bars) and PMA-stimulated (black bars) PMNs able to reduce NBT. Samples incubated 1 h with azithromycin (Azi), penicillin (Pen), erythromycin (Ery), or dexamethasone (Dex) and A) without S. pneumoniae lysate or B) with S. pneumoniae lysate. In A) and B), all stimulated groups are significantly different from all resting groups. No significant difference between drug-treated and control samples within any group (n=5; experiment was repeated 3 times).
Figure 11. IL-8 production by PMNs incubated 24 h with (black bars) or without (white bars) *S. pneumoniae* lysate following a 2 h incubation with azithromycin (Azi), penicillin (Pen), erythromycin (Ery), or dexamethasone (Dex). No significant difference was observed between antibiotic-treated and control samples within groups treated with or without lysate (*n*≥4; experiment was repeated 2 times). *P*<0.05 versus sham-treated control.
CHAPTER 4: DISCUSSION

My study investigated the effects of azithromycin on human circulating polymorphonuclear neutrophils. My results indicate that azithromycin induces neutrophil apoptosis without affecting the oxidative metabolism or IL-8 production of these cells in vitro (Figures 4A, 6A, 10, and 11). Exposure of neutrophils to azithromycin significantly increased in vitro phagocytic uptake of these cells by macrophages (Figure 9). Co-incubation of neutrophils with S. pneumoniae inhibited the induction of apoptosis by azithromycin (Figure 4B, and 6B).

Co-incubation of live S. pneumoniae with antibiotics (azithromycin, erythromycin, and penicillin) and neutrophils confirmed that the antibacterial effects of these antibiotics are not altered by the presence of neutrophils, and that azithromycin exhibits effective antibacterial properties (Figure 1). After six hours of incubation azithromycin was more effective in eradicating S. pneumoniae than the other antibiotics. My observations are consistent with those published in other reports [77, 78].

Previous studies have suggested that macrolides have anti-inflammatory effects, but the mechanisms of this action remain unclear [7, 10, 28, 132-136]. A number of these studies have focused on the effects of macrolides on cytokine production [7, 28, 132-139]. Other studies have suggested that macrolides modulate the functions of polymorphonuclear leukocytes, macrophages, and lymphocytes, as well as altering the functions of airway secretory cells and epithelial cells [10, 140-142]. Although still controversial, findings from these previous studies indicate that erythromycin, roxithromycin and clarithromycin may reduce the production of pro-inflammatory IL-1,
IL-6, IL-8 and TNF. In addition, erythromycin derivatives, including azithromycin, roxithromycin and clarithromycin, are known to inhibit the oxidative response of neutrophils in a time and concentration dependent fashion [143-147]. My results indicate that after two hour exposure to azithromycin, the release of IL-8 by a population of human neutrophils remains unchanged, despite the commitment of some cells to undergo programmed cell death (Figure 11). Moreover, results from my study indicate that one hour exposure to azithromycin is insufficient to reduce oxidative metabolism of human neutrophils (Figure 10). Taken together, these observations indicate that the effects of azithromycin on IL-8 production as well as oxidative metabolism are time and concentration dependent (as the other studies used different drug concentrations and exposure times than my study).

Dexamethasone is known to reduce IL-8 production by LPS-treated human peripheral blood monocytes and alveolar macrophages [131]. In my study, dexamethasone significantly inhibited Streptococcus pneumoniae lysate-induced IL-8 synthesis by neutrophils (Figure 11). In vivo, a reduction in IL-8 could be an important strategy to reduce the inflammatory lung injury caused by neutrophils. IL-8 is a major chemotactic cytokine for neutrophils, and macrophages and monocytes are the major producers of this cytokine during the inflammatory response [28]. The large numbers of neutrophils at sites of inflammation, however, make these cells an additional important contributor of IL-8 [28, 178]. The results of my study, however, indicate that the efficacy of azithromycin is not dependent on the reduction of pro-inflammatory IL-8 (Figure 11).

Accumulation and necrosis of neutrophils in tissues contribute to the propagation of inflammation in a number of inflammatory diseases, including infectious pneumonia.
In contrast, apoptotic neutrophils may be eliminated without spilling pro-inflammatory products \textit{in situ}. A recent report indicates that erythromycin, roxithromycin, clarithromycin, and midecamycin shorten neutrophil survival by accelerating apoptosis [149]. The biological significance of this observation has yet to be uncovered. It is shown in my study that after one hour exposure to azithromycin, apoptosis may be detected in human neutrophils via annexin-V labelling of exposed phosphatidylserine (Figure 4A). Additionally, neutrophils exposed to azithromycin for one hour exhibited an increased level of phagocytic uptake by human monocyte-derived macrophages (Figure 9). Only activated macrophages are able to phagocytose apoptotic neutrophils. Human peripheral blood monocytes cultured \textit{in vitro} become activated both by the isolation process from whole blood and by attachment to tissue culture plastic surfaces in the presence of serum [172]. The adherence mimics the immigration of monocytes through the vasculature into the site of infection and primes the monocyte for inflammatory responses [173]. Macrophages use phosphatidylserine to recognize and phagocytose certain populations of apoptotic cells. The macrophage receptor, 61D3, binds to phosphatidylserine on apoptotic cells [170]. Additionally, the vitronectin receptor and CD36 on macrophages interact with apoptotic neutrophils via thrombospondin and a surface molecule (which does not appear to be phosphatidylserine) [47, 170, 171]. The results of my study suggest that apoptotic neutrophils may also use the phosphatidylserine-dependent mechanism for uptake into macrophages since both phosphatidylserine exposure (which annexin-V binds) and phagocytic uptake of neutrophils by macrophages were increased in azithromycin-treated neutrophils.
I hypothesize that azithromycin may induce apoptosis in human neutrophils more effectively than other antibiotics and hence help the host dispose of these cells without perpetuating local inflammation. Consistent with this hypothesis, it was recently shown that tilmicosin, a macrolide used in the treatment of bovine pneumonic pasteurellosis, has anti-inflammatory benefits which are associated with neutrophil apoptosis and inhibition of local leukotriene $\text{B}_4$ release [148]. In addition, it is well established in the literature that physiological removal of apoptotic neutrophils in the inflamed lung in vivo promotes the resolution of inflammation [179, 180]. Neutrophil influx must be limited and neutrophils that are no longer necessary must be removed in order for the resolution of inflammation to occur [179]. Macrophage clearance of apoptotic neutrophils prevents tissue destruction by the cytotoxic intracellular contents of neutrophils.

Annexin-V labelling detects the exposure of phosphatidylserine on the outer leaflet of the cell membrane, an early event in cell death [159, 181]. In this system, apoptosis is distinguished from necrosis by propidium iodide labelling, which enters only cells with a compromised membrane. The experimental assessment of apoptosis was validated by using TNFα as a positive control (Figure 3) [177]. My results indicate that human neutrophils exposed to azithromycin for one hour in vitro were committed to apoptotic death, while erythromycin, penicillin and dexamethasone showed no induction of apoptosis in neutrophils (Figure 4A). In addition, the formation of mono- and oligonucleosomes, a late event in apoptotic cell death, was detected in neutrophils via ELISA after six hours of incubation with azithromycin (Figure 6A).

In another study, erythromycin was shown to induce apoptosis in neutrophils in vitro [149]. The fact that this was not observed in my study may be explained by the
different incubation times and antibiotic concentrations; the previous study assessed the induction of neutrophil apoptosis after twenty-four hours of incubation, and used a minimum concentration of 1 μg/mL [149]. My findings suggest that azithromycin induces neutrophil apoptosis more quickly and at lower drug concentrations than erythromycin. Neutrophils have a higher affinity for azithromycin than erythromycin. Additional studies will help assess whether this difference in affinity accounts for the more rapid induction of neutrophil apoptosis by lower concentrations of azithromycin compared to erythromycin [64]. The anti-inflammatory glucocorticoid dexamethasone has been reported to inhibit neutrophil apoptosis [130], but this effect was not seen in my study. Again, as inhibition of apoptosis in human PMNs was observed after twenty-four hours of exposure to dexamethasone, the six hour incubation used in my study may not have been sufficient to significantly inhibit apoptosis [130]. Regardless, my study demonstrates that azithromycin induces apoptosis in human neutrophils to levels greater than those observed for other drugs and that azithromycin-treated neutrophils show a greater uptake by macrophages (Figures 4A, 6A, and 9). The induction of apoptosis may be responsible for the increased phagocytic uptake of azithromycin-treated neutrophils via recognition of apoptotic cells by a phosphatidylserine-dependent mechanism. I postulate that this mechanism may at least in part contribute to the clinical efficacy of this macrolide in the treatment of infectious pneumonia by limiting the ability of neutrophils to damage surrounding tissues and preventing further amplification of inflammation.

The mechanism by which azithromycin induces apoptosis in neutrophils remains unknown and is an area that requires further study. It may be that azithromycin binds to and activates receptors such as the Fas receptor or the TNF receptor that have been
implicated in the induction of the apoptotic cascade of events [153, 154, 182]. Alternatively, azithromycin may enter neutrophils and induce apoptosis by activating a nuclear or cytosolic receptor. It has been suggested that the loss of lipid asymmetry is linked to activation of the Fas system [161]. The mechanism by which the enzymes involved in phosphatidylserine translocation become activated or inhibited is not known but has been postulated to involve increased cytosolic calcium concentrations in erythrocytes and platelets [183]. Alternatively, the mechanism by which TNFα results in neutrophil apoptosis is postulated to involve the generation of reactive oxygen intermediates [184].

The effect of azithromycin on other cell types involved in the mediation of inflammatory lung injury is another area that may give additional information about the clinical efficacy of azithromycin in resolving pulmonary infection.

My results suggest that the apoptosis-inducing effect of azithromycin is abolished when neutrophils are co-incubated with *Streptococcus pneumoniae* lysate (Figures 4B, and 6B). Levels of necrosis were not different between samples with or without lysate, indicating that the low apoptotic level was not due to an increase in necrosis (Figure 5). The mechanisms whereby *S. pneumoniae* products may reverse or inhibit the apoptotic signal remain to be investigated. In one study, the LPS of Gram-negative bacteria has been shown to inhibit TNFα-induced apoptosis and spontaneous apoptosis in neutrophils *in vitro*, possibly by inducing synthesis of proteins which are involved in regulating the apoptotic pathway or by production of cytokines such as IL-1β and TNFα [185]. TNFα (in concentrations under 0.1 U/mL), and IL-1β inhibit apoptosis. In another study, Staphylococcal enterotoxins (superantigens) have been shown to increase production of
LTB₄, and LTB₄ is able to delay neutrophil apoptosis [186]. Thus, superantigen may act indirectly to inhibit neutrophil apoptosis. *In vivo*, superantigen may also inhibit neutrophil apoptosis through its effects on cytokine production by other immune cells such as monocytes and lymphocytes [186]. It remains to be shown whether other Gram-positive bacteria have components that also inhibit apoptosis, and whether this effect may contribute to the difficulty in treating infections caused by these organisms. Further studies are also needed to assess whether the inhibition of azithromycin-induced apoptosis observed in my study occurs in the presence of other bacterial species, including *Haemophilus influenzae*, another respiratory tract pathogen which azithromycin is more effective against than *S. pneumoniae* [64]. A paper recently suggested that macrolides might significantly reduce the inflammatory injury associated with the presence of *H. influenzae* in the lower respiratory tract, via unknown mechanisms [134].

In summary, azithromycin induces apoptosis in human neutrophils more effectively than erythromycin or penicillin (Figures 4A and 6A). Phagocytic uptake of azithromycin-treated neutrophils by macrophages is enhanced (Figure 9), which in turn may confer a significant anti-inflammatory benefit to this drug. The oxidative function of resting or stimulated neutrophils and the production of IL-8 remain unchanged (Figures 10 and 11), indicating that these functions are unaffected in the early stages of azithromycin-induced apoptosis. I postulate that the clinical efficacy of azithromycin in the treatment of lower respiratory tract infections is due, at least in part, to committing neutrophils to apoptotic death, hence reducing the amount of cytotoxic and pro-inflammatory compounds released by these cells in the infected lung. Whether inhibition
of neutrophil apoptosis by \textit{S. pneumoniae} contributes to the difficulty in treating infections caused by this pathogen requires further investigation.

Results from my study open the way to a number of directions for future research. A summary of these include:

1) Exploring the mechanism by which azithromycin induces apoptosis in neutrophils.

2) Determining if the higher affinity of azithromycin for neutrophils is responsible for inducing apoptosis more rapidly and at lower drug concentrations than erythromycin.

3) Examining the effect of azithromycin on other inflammatory cells.

4) Determining whether azithromycin-induced neutrophil apoptosis and enhanced phagocytosis by macrophages occur \textit{in vivo}.

5) Exploring the biological significance and clinical importance of azithromycin-induced apoptosis \textit{in vivo}.

6) Elucidating the mechanism by which \textit{Streptococcus pneumoniae} reverses or inhibits the induction of apoptosis by azithromycin and determining whether this inhibition occurs in the presence of other species of bacteria.

7) Determining whether the inhibition of apoptosis by \textit{Streptococcus pneumoniae} contributes to the difficulty in treating streptococcal pneumonia.
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APPENDIX A: Structure of Azithromycin and Erythromycin

(Aadapted from reference 64)