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Anti-inflammatory effects of the macrolide antibiotic tilmicosin

by

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ABSTRACT

Pathogen virulence factors and pulmonary inflammation are responsible for tissue injury associated with respiratory failure in severe bacterial pneumonia, as seen in the bovine lung infected with *Pasteurella haemolytica*. This study assessed the effects of tilmicosin on pulmonary inflammation during a *P. haemolytica* infection *in vivo*, and the effects of tilmicosin in comparison to other drugs on peripheral bovine neutrophils *in vitro*. Wistar rats and Holstein calves were either 1) treated with tilmicosin before intratracheal infection with *P. haemolytica*, 2) infected and sham-treated, or 3) were unmanipulated controls. Bronchoalveolar lavages were obtained to assess *P. haemolytica* recovery, neutrophil function and apoptosis, and soluble inflammatory mediator (calves) levels. *In vitro*, neutrophils were assessed for induction of apoptosis by tilmicosin, by other antibiotics, or by dexamethasone in the presence or absence of *P. haemolytica*. Tilmicosin reduced *P. haemolytica* colonization, induced neutrophil apoptosis without affecting antibacterial activities, and inhibited synthesis of pro-inflammatory leukotriene B_4. In conclusion, tilmicosin's anti-inflammatory properties may limit the tissue injury associated with *P. haemolytica* pneumonia.

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LIST OF ABBREVIATIONS

PMN(s)	=	Polymorphonuclear neutrophil(s)
MPO	=	Myeloperoxidase
LTB_4	=	Leukotriene B_4
IL(s)	=	Interleukin(s)
TNF-α	=	Tumor necrosis factor - alpha
SC	=	Subcutaneous
CFU	=	Colony forming unit
BAL(s)	=	Bronchoalveolar lavage(s)
PBS	=	Phosphate-buffered saline
HBSS	=	Hank's balanced salt solution
PBS+	=	Phosphate-buffered saline containing calcium and magnesium
NBT	=	Nitro blue tetrazolium
SEM(s)	=	Standard error(s) of mean
TEM(s)	=	Transmission electron micrograph(s)
ELISA	=	Enzyme-linked immunosorbent assay

1. INTRODUCTION

1.1 PATHOGENESIS OF RESPIRATORY DISEASE

The lung is exposed to particulate matter present in inspired air which contains a variety of potentially harmful microorganisms. In response to this predicament, the lung has evolved both mechanical and immune defenses in preventing infections. Mechanical defenses constitute the first line of barriers and include the glottis, the cough reflex, airway secretions, and a mucociliary system that lines the entire surface of the upper airways (1). If the structural defenses of the lung are breached, infection may be prevented by the host's immune defense. Failure to control such infections may lead to pneumonia, an important cause of morbidity and mortality in most animal species and in man (2-5).

1.1.1 INFLAMMATION - CELLULAR COMPONENTS

Normally, mucosal infections caused by bacteria elicit a host inflammatory response that serves to isolate and destroy the invading pathogen. In the lung, the resident macrophages (alveolar macrophages) constitute the first line of innate immune defense. Alveolar macrophages phagocytose most of the particles and microorganisms reaching the lower respiratory tract (1,6-9). Alveolar macrophages also signal for the influx of other inflammatory cells, such as neutrophils (polymorphonuclear neutrophils, PMNs), to help stop the infection. This cellular interaction implicates the release of inflammatory mediators (1,9-13). Upon stimulation, PMNs roll, marginate, leave the blood vessel, and move chemotactically through the tissue towards the site of injury (9,12,14-23). Past studies have shown that the adherence between PMNs and the endothelium involves a series of PMN surface molecules such as integrins and selectins (12,15-16,24). The adhesion molecules on the endothelium also include selectins, and molecules belonging to the

immunoglobulin superfamily (12,16,25). It is the regulation of these surface molecules on either the PMNs or the endothelial cells that controls the rolling and margination of these leukocytes.

1.1.1.1 Neutrophils: Neutrophils represent the principal cell population involved in acute inflammation. Past studies have shown that intact PMN function is necessary for the eradication of acute pulmonary infection (26-32). The effective clearance of respiratory tract pathogens from the lung corresponds with and requires the rapid influx of PMNs into the lung (33). Neutrophils may release pro-inflammatory mediators such as leukotriene B_4 (LTB_4), Interleukin-1-alpha/-beta (IL-1α/β), and interleukin-8 (IL-8), as well as a variety of proteolytic compounds that are aimed at destroying the invading bacteria (8-13,34-39). These proteolytic compounds are stored within intracellular granules which include proteolytic enzymes (lysozyme, serine elastase, cathepsin G, proteinase 3, collagenase, gelatinase, heparinase), other enzymes (myeloperoxidase, MPO), integrins (Mac-1, CD11b/CD18), bactericidal proteins (lactoferrin, defensins, bactericidal/permeability-increasing protein, CAP37/azurocidin) and plasma proteins (albumin, immunoglobulin G, alkaline phosphatase, cytochrome b558, tetranectin) (9,35-37,40). Upon stimulation, the PMNs release these secretory products which are able to react with the pathogens as well as host tissue cells and matrix (9,35-37,41).

When the antioxidant and antiprotease systems are overwhelmed, the airways and gas-exchange units may sustain irreversible damage (4-5,23,41-44). Indeed, during chronic inflammation, PMNs may become major causes of host-mediated injury (4-5,9,21,23,41,44-48). The exaggerated release of proteolytic enzymes (4-5,41,49) and reactive oxygen species by PMNs play a major role in the destruction of the epithelium and endothelium (4-5,41,50-53). In the lung, PMN elastase has been implicated in the pathogenesis of such inflammatory lung diseases as Adult Respiratory Distress Syndrome (ARDS) and emphysema associated with alpha-1-protease inhibitor deficiency (4-5,41,55-

56). In ARDS, there is a marked increase in the number of PMNs implicating these leukocytes in the initiation and severity in acute lung injury (5,9,41,55-60). Although during bacterial pneumonia, the high counts in alveolar PMNs eventually clears, the associated acute lung injury may be due to the stimulation of PMNs to release myeloperoxidase, elastase, and pro-inflammatory mediators leading to a self-perpetuating cascade of events that ends in corresponding tissue damage.

The serine protease elastase uses elastin as its primary substrate but can also cleave types III and IV collagen, immunoglobulins, complement components, clotting factors, proteoglycans, fibronectin, and even intact cells (40,55,61-62). Neutrophils also possess the metalloproteases gelatinase and collagenase, which can degrade the extracellular matrix of host tissue, disrupt resident host cells, and stimulate further inflammation (20,35-37,40-42,63-66). Clearly, overwhelming release of these proteases has the potential to degrade both the invading pathogens as well as the surrounding tissue.

In addition to these intracellular compounds, PMNs can also undergo a respiratory burst releasing reactive oxygen metabolites such as superoxide anion, hydrogen peroxide, hypochlorous acid, and hydroxyl radical (19,37,43,67-68). Most of the superoxide anions are enzymatically converted to hydrogen peroxide. The PMNs can either detoxify hydrogen peroxide to water or produce hypohalous acids in the presence of halide and myeloperoxidase (19,37,43,67). Myeloperoxidase also has the ability to use chloride as a substrate to release large quantities of hypochlorous acid which is highly toxic to many biological substances (37,41,43,67). The hydroxyl radicals are formed by the combination of oxygen and hydrogen peroxide in the presence of iron (Fe^{3+}). The combination of hydrogen peroxide, hypochlorous acid, and hydroxyl radicals, represents a potent force aimed at destroying invading microorganisms. The combination of oxygen-independent and oxygen-dependent bacterial-killing mechanisms enable PMNs to act as efficient cellular defenders against invading pathogens.

1.1.2 INFLAMMATION - SOLUBLE COMPONENTS

Among the many potent pro-inflammatory compounds released during the inflammatory process, IL-1α, Tumor Necrosis Factor - alpha (TNF-α), IL-8, and LTB_4 play major roles in upregulating PMN function (2,9-12,23,69-70). Both IL-1α and TNF-α share a number of bioactivities, including the induction of fever, an increase of collagenase, increased fibroblast proliferation, induction of IL-1α production, and activation of PMNs (12,69-70). However, unlike TNF-α, IL-1α has the ability to activate both T and B cells (69). Tumor Necrosis Factor - alpha is produced in large amounts in the airspace of humans with bacterial pneumonia (71) and in the lungs of animals challenged with bacterial pathogens (8,72-75). Interleukin-8 plays a causative role in chronic lung inflammation and is one of the most potently effective molecules for eliciting PMN adhesion to endothelial cells and chemotaxis (1,11-12,19,23,76-80) as well as activating PMNs to release lysosomal enzymes (81-83). Leukotriene B_4 is an arachidonic acid metabolite that is generated via the 5-lipoxygenase pathway and is a potent stimulant of PMN aggregation/chemotaxis (10,12,19,84-86) and has been implicated in a variety of inflammatory lung diseases (1,13,34,87-88). The consistent observation of the accumulation of LTB_4 at inflamed mucosal sites has made it a reliable marker for inflammation.

1.1.3 REGULATION OF INFLAMMATION

Inflammation is tightly controlled where the body regulates the activity of many mediators, and uses several countermeasures such as antioxidants and antiproteases to prevent permanent damage to local tissues (4-5,41,43,47,89-91). Although studies have shown that PMNs are needed to promote recovery from acute lung injury (28), the influx of PMNs into the site of injury is carefully regulated (12). When the migration of PMNs needs to be blocked, the PMN activators are rapidly taken over by a number of compounds that inhibit

PMN migration including serum factors which inhibit C5a-induced PMN chemotaxis, and lipoxin A_4 (9,92-94).

1.1.4 BACTERIAL VIRULENCE FACTORS

One of the initiating factors of host inflammation is caused by bacteria and their virulence factors. In the constant battle between the host and the bacteria, both have evolved ways to defeat the other party. The host has developed defenses against the invading pathogens whereas the bacteria have developed ways to circumvent these host defenses and aid in their colonization. Virulence factors help the bacteria in colonizing the host and in establishing infection for its own survival. Adhesins (pili, fimbriae) enable some bacterial pathogens to adhere to host tissues, often resulting in internalization by either phagocytosis or invasion by the bacteria itself (95). Intracellular pathogens such as *Mycobacteria* avoid the process of lysosomal fusion with phagocytic vacuoles by preventing the acidification of the vacuole -- a prerequisite for lysosomal enzyme activation (95). It has been recently found that some bacteria induce host programmed cell death, or apoptosis (95-98). By inducing the apoptosis of host cells, especially effector cells (i.e. phagocytes), the invading pathogen could eliminate or downregulate the host's defense thus making it easier to colonize the host. Examples of bacteria that were shown to induce apoptosis in host cells (macrophages) include *Shigella flexneri* (97,99-101) and *Salmonella typhimurium* (102-103). Bacteria could also attack host cells through the production of toxins. Bacterial toxins can be divided into three groups: i) toxins acting at the plasma membrane interfering with transmembrane signaling, ii) toxins that alter membrane permeability forming pores inevitably lysing the host cell, and iii) toxins that act inside cells altering specific cytosolic targets (95). An example of a respiratory pathogen that produces an exotoxin cytotoxic to host phagocytes is *Pasteurella haemolytica* which is one of the etiologic agents of bovine pneumonic pasteurellosis.

1.2 PASTEURELLOSIS

Bovine pneumonic pasteurellosis provides an excellent model of multifactorial pathogenesis involving both bacterial virulence factors and the host inflammatory response. Also known as shipping fever, this devastating pulmonary disease of ruminants commonly occurs in calves placed in feedlots (104-114). One of the etiological agents of this disease is *Pasteurella haemolytica* - biotype A, serotype 1 (104,115-118). This disease is associated with the destruction of pulmonary tissue which leads to respiratory failure and death (104,106). The epidemiology of pneumonic pasteurellosis relates to many factors such as stress, weaning, inadequate nutrition, mixing and handling of animals, crowding and livestock transit, and weather (104,108-111,114). Pasteurellosis manifests clinically in the form of depression, lack of appetite, fever, occasional purulent discharge, coughing, and in the later stages, tachypnea and dyspnea (104,108-111,114). Post-mortem observations of calves that have died of pasteurellosis commonly reveal severe fibrin deposition and necrosis in pulmonary tissues (104,114,119-121).

1.2.1 PATHOGENESIS OF PASTEURELLOSIS

As with any bacterial disease, the pathogenesis of pneumonic pasteurellosis involves both host inflammation and bacterial virulence factors. Bacterial factors include endotoxins (115,122) and leukotoxins (123). Studies have shown that endotoxins purified from *P. haemolytica* caused PMN influx, fibrin exudation, and edema in alveolar spaces (124-127) as well as inducing TNF-α and IL-1β expression and secretion from bovine alveolar macrophages (122). Leukotoxins lyse macrophages and PMNs, thus rendering them unable to attack the invading bacteria (104,115,123). In addition, lysed leukocytes release their proteolytic compounds and pro-inflammatory mediators (104). This, in turn, causes

considerable damage to the local surrounding tissue, and overwhelms the ability of the resident alveolar macrophages to clear the invading pathogens leading to the influx of inflammatory cells, notably the PMNs (1,9-13,128-131). The local accumulation of PMNs plays a central role in the pathogenesis of bovine pneumonic pasteurellosis. Bacterial virulence factors such as leukotoxins not only impair the functions of PMNs, but also contribute to the acute and chronic infiltration of PMNs at the site of inflammation. The PMN influx leads to the release of large amounts of reactive oxygen species and proteolytic enzymes that target the invading bacteria, but concurrently damage the bronchial epithelium. These host products, compounded with bacterial leukotoxins, affect PMN viability and promote the local synthesis of potent pro-inflammatory mediators, including TNF-α and LTB_4 (122,129,132-134). Hence, both host and bacterial factors can contribute to delayed elimination of *P. haemolytica* and the subsequent uncontrolled self-perpetuating inflammation eventually leads to pulmonary failure.

1.3 THERAPY

Past studies have demonstrated the efficacy of certain chemical therapeutics for the treatment of inflammatory lung diseases. Mainly, these experiments have focused on the immunomodulation of the host response through the downregulation of the PMN-derived inflammation associated with these diseases. Compounds produced via the respiratory burst contribute to major cytotoxicity, thus therapeutic strategies using antioxidants have been shown to reduce lung injury (135). Other therapeutics have been shown to modulate PMN adhesion reducing the inflammation associated with endotoxin-induced pneumonia in rabbits (136). Additional studies have shown that LTB_4 receptor antagonists were able to significantly improve neutrophilic alveolitis, pulmonary edema, and arterial hypoxemia in a porcine model of acute lung injury (137) while other studies have shown that compounds

that block the 5-lipoxygenase pathway are beneficial in animal models of lung injury (138-140).

1.3.1 GLUCOCORTICOIDS

For the treatment of diseases, glucocorticoids have been used to prevent the damage or fibrosis that accompany inflammation (141-144). Glucocorticoids prevent PMN influx to a site of an inflammatory response by reducing PMN adhesion to blood vessel walls, suppressing PMN chemotaxis, and inhibiting the production of PMN-recruiting mediators (145-146). Other studies have shown that through the inhibition of enzymatic cleavage of arachidonic acid from precursor phospholipids by phospholipase A_2 (PLA_2), glucocorticoids block the release of arachidonic acid metabolites, notably the leukotrienes which are potent activators and chemoattractants of PMNs (145,147). By blocking arachidonic acid metabolism, glucocorticoids are potent anti-inflammatory agents which prevent the synthesis of major pro-inflammatory compounds such as LTB_4.

1.3.1.1 Dexamethasone: Dexamethasone, which is an extensively used glucocorticoid, dose-dependently inhibits superoxide production, and release of lactoferrin and lysozyme from human peripheral blood PMNs (148). Other studies have shown that dexamethasone reduces PMN chemotaxis and infiltration, and inhibits the synthesis of various inflammatory compounds such as LTB_4, IL-1, and elastase-alpha-1-proteinase inhibitor (149). It would seem that the use of anti-inflammatory compounds such as dexamethasone would prove useful in treating chronic inflammatory responses or preventing host-mediated tissue destruction.

1.3.1.2 Toxicity: The administration of glucocorticoids has a variety of negative side-effects. In general, two types of toxic effects are associated with the therapeutic administration of glucocorticoids (150). First, prolonged use of large doses of glucocorticoids results in several side effects including pituitary-adrenal suppression, fluid

and electrolyte disturbances, hyperglycemia and glycosuria, increased susceptibility to infections, peptic ulcers, osteoporosis, myopathy, behavioral disturbances, and cataracts (150). In addition, following withdrawal from glucocorticoid therapy, patients may develop fever, myalgia, arthralgia, and malaise (150).

In summary, while glucocorticoids may help alleviate the symptoms associated with inflammatory diseases, their negative side effects and the fact that they do not eliminate the source of the infection warrant the use of alternative therapy.

1.3.2 ANTIBIOTICS AND ANTIBACTERIAL COMPOUNDS

Classical therapy with antibacterial compounds such as antibiotics, still proves to be very effective in treating bacterial infections such as pneumonia. On the basis of their modes of actions on microorganisms, antibiotics and antibacterial compounds are classified as either group I bactericidals (e.g. penicillin, streptomycin) or group II bacteriostatics (e.g. tetracyclines, macrolides, sulfa drugs) (151). For the selection of antibiotics to treat diseases, clinicians need to consider the different properties of these drugs such as their pharmacodynamics and antibacterial spectra against the target pathogen. The diverse variety of antibiotics and antibacterial compounds allows the clinician to reach an informed decision to effectively treat infections based on the uniqueness of each compound. Based on their properties, antibiotics are classified into the following non-exhaustive list of families: penicillins, tetracyclines, cephalosporins, and macrolides. Other antibacterial compounds include the sulfa drugs or sulfonamides.

1.3.2.1 Sulfonamides: Sulfonamides contain the SO_2NH_2 group and prior to the discovery of penicillin, these drugs were extensively used for bacterial chemotherapy (152-153). Sulfonamides are bacteriostatic rather than bactericidal and are commonly delivered orally. Sulfonamides have been used for the treatment of animal respiratory diseases such as pneumonic pasteurellosis (152) and as synergistic formulations with trimethoprim

(108,154). However, treatment with sulfonamide boluses did not reduce morbidity rates when used for prophylaxis of bovine respiratory disease in calves (108, 154).

1.3.2.2 Penicillins and cephalosporins: Penicillin is one of the oldest and most extensively used antibiotics, and with the discovery of new broad-spectrum penicillins such as ampicillin and amoxicillin, antibacterial activity was expanded against gram-negative pathogens (151,155). The essential part of the penicillin molecule is the beta-lactam thiazolidine ring which is a common target of penicillin-resistant organisms that possess penicillinases such as beta-lactamases and acylases which inactivate the molecule (151,155). Penicillins are grouped into one natural group and three semisynthetic groups : i) natural penicillins (e.g. penicillin G) are produced by mold cultures and are rapidly hydrolyzed by gastric acid, ii) acid-resistant penicillins (e.g. phenoxymethyl or phenoxyethyl penicillin) resist gastric acid, iii) penicillinase-resistant penicillins (e.g. methicillin, cloxacillin) have substituents that sterically hinder the cleavage of the beta-lactam ring, and iv) broad-spectrum penicillins (e.g. ampicillin) have antibacterial activity against gram-positive and gram-negative bacteria (151). Penicillins are considered bactericidal and are mostly effective on bacteria during peak growth rate by interfering with bacterial cell wall synthesis through the inhibition of transpeptidase which is responsible for the cross-linking of adjacent peptidoglycan strands (151,155). Penicillin is not toxic to animal cells since the latter do not possess the peptidoglycan cell wall structures characteristic of bacteria. Penicillins are used for the treatment of pneumonic pasteurellosis (154).

Derived from the fungus *Cephalosporium* and chemically-related to penicillin, cephalosporins also possess the beta-lactam ring (155-156). As weak organic acids, cephalosporins are water soluble. Cephalosporins mainly concentrate in the serum since their diffusion into tissues is limited by poor lipid solubility (156). The poor lipid solubility may pose a problem when using cephalosporins for treatment during the later

and more persistent concentrations in tissue than in serum (165). The antibacterial spectrum of macrolides includes many gram-positive bacteria and some strains of *Listeria* (156,163). Macrolides are traditionally used for the treatment of respiratory tract infections (168). The most commonly known macrolide is erythromycin. Erythromycin inhibits bacterial protein synthesis by binding to the 50 S ribosomal subunit and is very effective against many gram-positive bacteria as well as *Pasteurella multocida* (156,161,164). Resistant bacteria fail to bind erythromycin (162). After 2 h, the highest concentrations of erythromycin are found in the liver, submaxillary glands, lungs, and kidneys (156). Within 4-6 h, serum concentrations of erythromycin rapidly decline which is corresponded by increased diffusion into most tissues (156); erythromycin diffuses readily into intracellular fluids (162,169-170). Recently, the development of novel macrolides derived from erythromycin such as roxithromycin, clarithromycin, dirithromycin, and azithromycin, have proven to be more effective *in vivo* than their parent compound (166,171). The advantages of these novel macrolides include higher tissue concentrations, increased stability in acidic conditions and longer half-lives which allow for better results with single doses (166). The development of new macrolide derivatives has also extended into animal health.

1.3.2.5 Tylosin: Structurally similar to erythromycin, tylosin is a bacteriostatic antibiotic derived from *Streptomyces fradiae* (156). Tylosin is effective against gram-positive bacteria that are generally susceptible to the macrolides, and organisms that are resistant to erythromycin are generally resistant to tylosin (156). Reasonable therapeutic concentrations of tylosin for pasteurellosis cannot be obtained in cattle (156). However, in goats with pleuropneumonia, treatment with tylosin prevented the appearance of lesions in lungs and lymph nodes as compared to untreated infected animals (156). In a comparison of duration in blood between tylosin and erythromycin, both antibiotics reached their peak concentrations after 1 h, however, erythromycin showed longer persistence (156). Past

studies have lead to the development of tylosin-related antibiotics and their derivatives. Derivatives of tylosin-related macrolides such as desmycosin have yielded useful properties that included a broad anti-microbial spectrum *in vitro*, particularly against *P. multocida* and *P. haemolytica*, *in vivo* efficacy, and improved tissue distributions (172). The antimicrobial activity against *Pasteurella* proved to be useful since these pathogens are a serious cause of respiratory illness in cattle. Through extensive experiments *in vivo* using models of bacterial pneumonia in calves and pigs, it was found that a cyclic aminoalkyl derivative of desmycosin known as tilmicosin was the best candidate for further development (172).

1.3.2.6 Tilmicosin: Tilmicosin, a semi-synthetic macrolide derived from tylosin, possesses a 16-membered lactone ring that is structurally similar to erythromycin (172). Several antimicrobial drugs such as sulfonamides, beta-lactams, tetracyclines, and tilmicosin are commonly used for the treatment of bovine pasteurellosis (107-112,154,158,161). Past studies showed evidence of *P. haemolytica* resistance to various antibacterial compounds such as penicillin and ampicillin (112,173), oxytetracycline (112,173-176), trimethoprim/sulfonamide and triple sulfonamides (112,173), and erythromycin (173). In light of this emergence of antibiotic resistance, the macrolide tilmicosin carried promises as an alternative antibacterial compound. *In vitro*, tilmicosin acts on a broad spectrum of bacteria, including the respiratory pathogens pasteurellae and mycoplasmas (107-112,177). Moreover, clinical studies have shown that tilmicosin was effective in treating bacterial pneumonia by preventing *P. haemolytica* (111,119) and *Mycoplasma bovis* colonization (111), improving clinical scores (110-111,113,119,161), lowering body temperature due to fever (113), reducing respiratory effort (109-110), lowering morbidity (112,154), lowering mortality (112-113), improving feed efficiency (110,154), and improving weight gains (107,112-113,154). Additional studies have shown that upon single injection of tilmicosin, serum concentrations decrease while

concentrations remain at therapeutic levels in the lungs of calves for long periods (111,119). This allowed the prescription of single injections for prolonged effective treatment, hence minimizing the stress associated with handling (110-112). To date, the macrolide tilmicosin is a very effective antibiotic for the treatment of bovine pneumonic pasteurellosis, and the success of this drug has been attributed to its pharmacodynamic concentration in appropriate tissues, and to its low inhibitory concentrations (107,109-113, 119,154,161,178).

1.3.3 PHARMACODYNAMICS

Pharmacodynamics is the study of the biochemical and physiological effects of drugs within the body (179). While pharmacodynamics is concerned with the mode of action of drugs, pharmacokinetics is the rate processes (absorption, distribution, metabolism, and excretion) in the handling of the drugs within the body (179). Many studies have focused on the pharmacological properties of antibiotics in an attempt to explain their efficacy in treating bacterial infections.

1.3.3.1 The Cellular/Extracellular ratio: The cellular uptake of drugs such as antimicrobial compounds depends on their physical and chemical properties. The uptake of drugs into cells determines their concentrations in both the serum and tissues. Some studies have investigated the uptake of antibiotics into phagocytes (PMNs and mononuclear phagocytes) where the cellular (intracellular) and extracellular levels were expressed as the C/E ratio 169-170). Results from these studies indicated that antibiotics such as penicillins and cephalosporins are not concentrated within cells (180). A few drugs such as the macrolides clarithromycin, roxithromycin, erythromycin, and azithromycin were found to reach higher intracellular concentrations (180-183).

1.3.3.2 Simple diffusion: Indeed as mentioned earlier, beta-lactam compounds have high water-solubility and poor lipid-solubility which prevents them from penetrating cellular

membranes (151). During a process known as simple diffusion, drugs cross the membrane and accumulate within the cell driven by a concentration gradient until equal concentrations of the drug are found on either side of the plasma membrane upon which flow will cease (180). Drugs that are able to concentrate within the cytoplasm would have to possess specific physical and chemical properties, such as size and lipid solubility, to diffuse across the cellular membrane (180). Charged or highly polar molecules do not readily cross the membrane (180). However, diffusion alone cannot explain the uptake of all antibiotics into cells.

1.3.3.3 Carrier-mediated transport: Drugs that undergo cellular endocytosis via transmembrane transport driven by energy or pH are possible mechanisms in which antibiotics enter cells (180). The two forms of carrier-mediated transport includes: i) facilitated diffusion where entering molecules flow down an energy-independent chemical gradient, and ii) active transport where molecules are carried across the membrane against a concentration gradient using an adenosine triphosphate (ATP) energy source (180).

1.3.3.4 Subcellular compartmentalization: Antibiotics may be found preferentially within certain organelles such as lysosomes (180). Lysosomes have the ability to concentrate lysosomotropic weak bases, such as macrolides, by diffusion alone (180,184-185). In fact, almost one-third of intracellular macrolide concentrations is focused in lysosomes (182). As a result of this intra-lysosomal concentration, macrolides increase the lysosomal pH (180). The effect of this phenomenon on cellular physiology remains unknown.

1.3.3.5 Cellular physiology: The rate of drug penetration within the cell depends on the physiological characteristics of the cell. Activated phagocytes in the process of phagocytosing bacteria appear to correspond with higher rates of intracellular drug accumulation (166). Studies using macrophages showed higher lysosomal accumulation of drugs upon stimulation with phorbol esters, and macrophages also have the natural tendency to internalize an amount equivalent to their plasma membrane surface area every

30 minutes (186). Although macrophages have the ability for pinocytosis, PMNs do not have this capability, thus they would be poor carriers of antibiotics that have poor lipid solubility such as the beta-lactams (187).

1.3.3.6 Delivery of antibiotics by PMNs: As mentioned earlier, PMNs play an important role in host inflammation to fight off bacterial infections. The ability of PMNs to secrete antibacterial compounds has been recently investigated in the context of antibiotic transport. The potential benefits of PMNs transporting antibiotics include a higher concentration of the drugs at sites of injury and the achievement of therapeutic drug concentrations in restricted areas such as the cerebral spinal fluid and the aqueous humor (188). Another potential benefit would be the delivery of antibiotics to sites that are consolidated due to tissue necrosis caused by excessive inflammation. Tissue consolidation would block off the blood supply to the area thus preventing the distribution of serum-concentrated drugs such as the beta-lactam antibiotics. However, the concentration of macrolides within PMNs would be beneficial for the host since the PMNs have the ability to migrate through host tissues and matrices using their various proteases. Four factors must be considered for the mechanism of antibiotic delivery by PMNs: i) high concentrations of the antibiotic must be achieved within the PMN, ii) the antibiotic must not interfere with PMN function, iii) the antibiotic must remain at high levels within the PMN until it reaches its destination, and iv) the antibiotic must be released in active form (188). Beta-lactam antibiotics concentrate poorly within PMNs as exhibited by an C/E ratio of less than 1 (170,189). Macrolides such as erythromycin and azithromycin preferentially concentrate within PMNs exhibiting ratios of 7:1 to 16:1, and 79:1 respectively (183,190-192). Tilmicosin has also been shown in high concentrations within bovine PMNs (193). The concentration of antibiotics such as the macrolides within PMNs and other cells may lead to the alteration of certain components of the inflammatory cascade during a bacterial infection (168,187). Indeed, the evident success of antibiotics in improving the general health of the host have

that erythromycin shortens neutrophil survival by accelerating apoptosis *in vitro* (205). In the same study, other macrolide antibiotics such as clarithromycin, roxithromycin, and midecamycin also shortened neutrophil survival whereas the beta-lactams ampicillin and cefazolin, and the aminoglycoside gentamicin did not affect their survival *in vitro* (205). The relevance of this phenomenon for the infected lung has not been investigated.

1.3.5 APOPTOSIS, OR PROGRAMMED CELL DEATH

Vertebrate cells may die via two distinct processes: apoptosis or necrosis (206-210). In apoptosis, also known as programmed cell death, the cells undergo a series of dramatic morphological changes induced by internal or external stimuli. The induction of apoptosis is an important physiological mechanism. During immune selection, self-reactive thymocytes are disposed via apoptosis (211). In the intestine, enterocytes continually migrate to the tip of the villi, where they die via apoptosis (211). During embryonic development, human embryos use apoptosis to lose the webbing between digits (211). Although apoptosis is important to homeostasis, this mode of cell death is also implicated in pathology. In pathological cases, some apoptosis-inducing stimuli include DNA injury, cell membrane injury, mitochondrial injury, mild oxidative stress, cytotoxic T cell killing, viruses, and bacteria (212-213). Upon the initiation of apoptosis, the dying cell undergoes a period of membrane blebbing (212,214). These blebs are extensions of the cytosol and can be reversibly extruded and resorbed (212). The cytoplasm then condenses rapidly and irreversibly, progressing to an increase in cellular density, compaction of organelles, and condensation of nuclear chromatin forming dense granular caps underlying the nuclear membrane (212,214). Nuclear pores disappear while the proteinaceous fibrillar centre of the nucleolus separates from its surrounding shell of osmiophilic transcription complexes (212). At this time, the cell separates into a cluster of membrane-bound bodies (apoptotic bodies) containing intact organelles (212,214). Throughout the apoptotic process, the

cytoplasmic organelles remarkably remain intact (212). Eventually, apoptotic cells and bodies are, in turn, quickly phagocytosed by either macrophages or neighboring cells (212,214-215). This process ensures that the contents of these cells and organelles, often containing proteolytic and toxic compounds, are not released into the extracellular space thus preventing the development of a chronic inflammatory response and subsequent tissue injury (206,212-213,215-216). In contrast to apoptosis, necrosis involves cellular swelling, alteration of cellular and nuclear structures, maintenance of chromatin integrity and nuclear pores (212-213). The plasma membrane ruptures leading to osmotic lysis of the cell which releases its cytotoxic intracellular contents thus resulting in tissue damage and inducing an inflammatory response (212).

1.3.5.1 TNF and TNF receptor family: Studies have shown that TNF-α induces both apoptotic and necrotic cell death (217-220). A number of cytokines such as TNF are also known to induce apoptosis through non-transcriptional coupling of TNF receptors and interleukin-1β converting enzyme (ICE) activation. Cells possess a number of surface receptors belonging to the TNF receptor family that bind TNF-family ligands (212,218,220). Ligand binding to some of these TNF receptors initiates the formation of a complex known as the death initiating signaling complex (DISC) around the receptor (212).

1.3.5.2 PMNs and apoptosis: Mature PMNs rapidly die via apoptosis within 72 h (215,221). As mentioned earlier, PMNs play an important role during inflammation. Although old normal circulating PMNs may spontaneously die via apoptosis, studies have shown that the host's ability to resolve inflammation is achieved through PMN apoptosis (214,216). Recent evidence states that the major mechanism for the resolution of lung inflammation depends on apoptosis for the removal of extravasated PMNs at sites of injury (223). Several reports have shown that PMNs in the late stages of apoptosis lose their functional abilities including CD16 expression, degranulation, and generation of respiratory burst (216,221,224-225). The loss of CD16 expression would clearly compromise PMN

function since this is one of the Fc receptors (FcγRIII) which binds to immunoglobulins for the clearance of immune complexes, generation of respiratory burst, secretion of inflammatory mediators, and phagocytosis (12). These apoptotic PMNs are then phagocytosed by macrophages preventing the release of tissue-damaging compounds (215-216,226-227). The phagocytosis of apoptotic PMNs by macrophages plays a key role in the resolution of inflammation in a number of systems, including the lung (215-216,223-224,226-227). One mechanism by which macrophages recognize apoptotic PMNs is through the phosphatidylserine receptor (226,228). Apoptotic cells lose normal membrane asymmetry leading to the localization of phosphatidylserine to the outer leaflet of the plasma membrane (224,227,229-230). During apoptosis, the extracellular exposure of phosphatidylserine occurs early (230-231), preceding DNA fragmentation, plasma membrane blebbing, and loss of membrane integrity. Macrophages are not the only cells that may phagocytose apoptotic PMNs. Due to the potential disadvantageous consequence of the failure to clear apoptotic PMNs that may undergo secondary necrosis, the body has back-up cells to help in the phagocytosis of apoptotic PMNs. Studies have shown that semi-professional phagocytes such as smooth muscle-like glomerular mesangial cells and fibroblasts can ingest apoptotic PMNs (232-233).

1.3.5.3 Regulation of PMN apoptosis: The life span of PMNs can be extended through the inhibition of apoptosis by lipopolysaccharide (LPS), GM-CSF, and C5a which enhance PMN function (224-234). Phagocytosis of bacteria also prolonged the survival of PMNs *in vitro* (235). Other studies have shown that a prolonged exposure of PMNs to LTB_4 dose-dependently prevented PMN apoptosis *in vitro* (236). PMN apoptosis is also prevented in chronically inflamed sites which are characteristic of hypoxic diseased tissues (216). Among the molecules that induce PMN apoptosis, TNF-α accelerates PMN apoptosis *in vitro* (237-239). Other proteins related to TNF-α such as FasL induce PMN apoptosis *in vivo* (238-239).

1.3.5.4 Benefits of PMN apoptosis: Clearly, apoptosis represents a favorable process for limiting tissue injury and resolving inflammation. First, the cell membrane remains intact thus preventing the release of potentially injurious and pro-inflammatory agents. Second, PMN function is down-regulated through the prevention of degranulation, stimulated phagocytosis (240), and both selectin and integrin-mediated adhesion (216). Third, apoptotic PMNs are phagocytosed by macrophages without the leakage of potentially injurious PMN products. Finally, even upon maximal uptake of apoptotic PMNs, macrophages do not release pro-inflammatory mediators (enzymes and cytokines) that usually accompanies the ingestion of particles. In contrast, the ingestion of necrotic PMNs stimulates the macrophages to release pro-inflammatory mediators (216,241). In summary, PMN apoptosis is a beneficial process for the control of inflammation that accompanies many disease states. In the light of the overwhelming clinical efficacy of tilmicosin, this compound must have distinct, yet unknown benefits in addition to its antibacterial properties.

1.4 OBJECTIVES OF THIS PROJECT

The recent findings of macrolides inducing PMN apoptosis represents a novel potential therapeutic strategy in treating pneumonia. The aim of this project was to assess the effects of tilmicosin on bovine PMNs. Specific objectives were:

1. To describe the effects of tilmicosin on the function and apoptosis of pulmonary PMNs during a *P. haemolytica* infection *in vivo*.

2. To identify the effects of tilmicosin in comparison to other drugs on peripheral bovine PMNs *in vitro*.

2. METHODS AND MATERIALS

2.1 RAT MODEL - ANIMALS

Male Wistar rats (363.1 ± 9.4 g, n=18) were weight-ranked and randomly assigned to two experimental groups: 1) Infected sham-treated animals received subcutaneous (SC) doses of 25% propylene glycol vehicle at 18 h and 15 min before infection, and 2) Infected tilmicosin-treated animals received subcutaneous (SC) 30 mg tilmicosin (Micotil™, Provel, Guelph, ON, Canada) per kg body weight 18 h and 15 min before infection. Animals were housed in shoebox cages at the University of Calgary Life and Environmental Sciences Animal Resource Centre (LESARC) (20 ± 1°C, 40% humidity, 12:12 h photoperiods). Rats were given water *ad libitum* and fed commercial rat diet (Rat Diet, Lab Diet, The Richmond Standard, PMI Feeds Inc., St. Louis, MO). All animal care and experimental practices were conducted under the standards of the Canadian Council on Animal Care and approved by the University of Calgary Life and Environmental Science Animal Care Committee.

2.2 CALF MODEL - ANIMALS

Holstein calves were used in all experiments and were 2-3 week old after a 14 day period of acclimation. Animals (52.8 ± 5.2 kg, n=12) were weight-ranked and randomly assigned to two experimental groups: 1) Infected sham-treated animals received subcutaneous (SC) doses of 25% propylene glycol vehicle at 18 h and 15 min before infection, and 2) Infected tilmicosin-treated animals received SC 10 mg tilmicosin per kg body weight 18 h and 15 min before infection. A third group of unmanipulated control animals was used in parts of this study (n=2). Animals were housed indoors at the University of Calgary Life and Environmental Sciences Animal Resource Centre

(LESARC) (20 ± 1°C, 40% humidity, 12:12 h photoperiods). Calves were fed milk-replacer (Hi-NRG Calf Milk Replacer, DewDrop Milk Replacers, Edmonton, AB, Canada), non-medicated creep feed (20% Calf Starter Ration, Unifeed, Olds, AB, Canada), and hay *ad libitum* as well as given free access to water at all times. All animal care and experimental practices were conducted under the standards of the Canadian Council on Animal Care and approved by the University of Calgary Life and Environmental Science Animal Care Committee.

2.3 BACTERIA

Pasteurella haemolytica biotype A serotype 1 (strain B122) isolated from a steer that died from pneumonic pasteurellosis was used for infection in both animal models. Bacteria were grown overnight on Columbia blood agar (Difco Laboratories, Detroit, MI) containing 5% sterile defibrinated sheep blood at 35°C in a microaerophilic environment (5% CO_2). Cells were harvested and suspended in pyrogen-free phosphate-buffered saline (PBS, pH 7.2, 0.15 M NaCl). A bacterial concentration of 3x 10^8 cells per ml was used to infect the rats while 2x 10^8 cells per ml was prepared to infect the calves. The bacterial concentrations were estimated by using MacFarland nephelometry and confirmed by Colony Forming Unit (CFU) enumeration on Columbia blood agar. The bacterial suspension used to infect the calves was then diluted 1:10 vol:vol in pyrogen-free sterile PBS to a final volume of 100 ml.

2.4 OPERATING PROCEDURES: RATS

Following general halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, MTC Pharmaceuticals, Cambridge, ON, Canada) anaesthesia, a sterile two-inch 20 gauge catheter (Angiocath I.V. catheter/needle unit, Becton Dickinson Vascular Access, Sandy, UT) was inserted intratracheally via the oral cavity and 3x 10^8 *P. haemolytica* in 0.5 ml

inoculum was injected through the catheter with a 10 cc syringe. The catheter was removed and the rats were left to recover in shoebox cages. Animals were kept under observation throughout the entire experimental period.

2.5 OPERATING PROCEDURES - CALVES

Following local lidocaine (Lido-2, Rafter 8 Products, Calgary, AB, Canada) anaesthesia, a sterile 14 gauge needle was inserted through a percutaneous incision into the trachea, and a sterile polyethylene catheter (Intramedic PE-90, Clay Adams, Parsippany, NJ) was threaded through the needle with the catheter tip extending approximately to the bifurcation of the trachea. The needle was removed and 10 ml of a 2x 10^7 *P. haemolytica*/ml suspension was injected through the catheter. The catheter was removed and the incision was sealed using tissue adhesive (Vet-Bond, 3M Products, St. Paul, MN). Rectal temperatures were taken from infected calves at t = -15 min, 3, 6, 9, 12, 15, 18, 21, and 24 h after infection. Animals were kept under observation throughout the entire experimental period.

2.6 BRONCHOALVEOLAR LAVAGE (BAL) - RATS

Two and a half hours following bacterial infection, the rats were euthanized with an overdose of sodium pentobarbitol (Somnotol, MTC Pharmaceuticals, Cambridge, ON, Canada). The trachea was exposed and a sterile plastic pipette tip adapted to a 3 cc syringe was used to collect BALs. Five sequential washings with 2 ml of pyrogen-free sterile PBS were collected in 15 ml centrifuge tubes. Leukocytes from the BAL were enumerated using a hemacytometer (Bright-Line Improved Neubauer, American Optical Corp., Buffalo, NY). Samples from the BAL were serially diluted, plated onto Columbia blood agar, and incubated overnight at 35°C for *P. haemolytica* enumeration. Bacterial clearance in tilmicosin-treated animals was determined by comparing bacterial numbers in the BAL to

the number of microorganisms recovered from the lungs of sham-treated animals. Another set of BAL samples (0.1 ml) was centrifuged for 10 min at 20 *g* onto a microscope slide with a cytospin apparatus (Shandon Southern Products Ltd., Cheshire, England). Cytocentrifuged specimens were fixed and stained with Diff Quik (Baxter Healthcare Corp., Miami, FL). Infiltration of PMNs from each sample was calculated as a percentage PMNs of total BAL leukocytes. The BAL was centrifuged for 10 min at 10°C and 200 *g*. The pellet was suspended in 6 ml Hanks Balanced Salt Solution (HBSS, Gibco HRL, Life Technologies Inc., Grand Island, NY) without calcium or magnesium.

2.7 BRONCHOALVEOLAR LAVAGE - CALVES

Three and 24 h following bacterial infection, BALs were collected by four sequential washings with 15 ml of pyrogen-free sterile PBS using the same surgical method as described above. Leukocytes from the BAL were enumerated using a hemacytometer. Samples from the BAL were serially diluted, plated onto Columbia blood agar, and incubated overnight at 35°C for *P. haemolytica* enumeration. Bacterial clearance in tilmicosin-treated animals was determined by comparing bacterial numbers in the BAL to the number of microorganisms recovered from the lungs of sham-treated animals. Another set of BAL samples (0.1 ml) was centrifuged for 10 min at 20 *g* onto a microscope slide with a cytospin apparatus. Cytocentrifuged specimens were fixed and stained with Diff Quik. Infiltration of PMNs from each sample was calculated as a percentage PMNs of total BAL leukocytes. The BAL was centrifuged for 10 min at 10°C and 200 *g*. The supernatant was aliquoted and stored at -70°C for later analyses. The pellet was suspended in 6 ml HBSS.

2.8 PMN PHAGOCYTIC ACTIVITY

For both animal models, cytospin slides were washed to remove free extracellular bacteria not associated with phagocytes, and the preparations were further evaluated with light microscopy to calculate a phagocytic index, i.e. the percentage of PMNs that have phagocytosed 1 or more bacteria.

2.9 PMN PURIFICATION

For both animal models, the pellet suspension (6 ml) was layered onto a gradient containing 3 ml Histopaque™ 1077 (Sigma Diagnostics, St. Louis, MO) on top of 3 ml of Histopaque™ 1119 (Sigma Diagnostics, St. Louis, MO). The preparation was centrifuged for 30 min at 700 *g* in a Sorvall General Laboratory Centrifuge-2 (GLC-2, Dupont Instruments, Newtown, CT). The PMN layer was collected and washed with 10 ml of HBSS. Purified cells were counted (300 cells) using a hemacytometer and degree of population purity was calculated from a cytospin slide. BAL leukocytes and purified PMNs were exposed to 0.1% trypan blue (Flow Laboratories Inc., McLean, VA) to assess membrane leakage (200 cells).

2.10 NITRO BLUE TETRAZOLIUM (NBT) REDUCTION

For both animal models, two samples of purified BAL PMN suspension (0.1 ml each) were spotted onto separate glass slides. The slides were incubated at 37°C in closed humid chambers for 15 min and rinsed with PBS containing calcium and magnesium (PBS+). One ml of a solution containing 2 mg/ml of nitro blue tetrazolium (NBT, Sigma Chemical Co., St. Louis, MO) and 0.1% dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO) in PBS+ was layered on the slides. The slides were incubated again at 37°C in closed humid chambers for 20 min. The slides were rinsed with PBS+, fixed with

absolute methanol, and counterstained with safranin (BDH Inc., Toronto, ON, Canada). Cells that have reduced NBT contain blue crystals and can be easily identified under light microscopy. Percentages of oxidatively active PMNs were counted from three distinct areas.

2.11 BOYDEN CHAMBERS

For the rat model, four purified PMN samples of 0.1 ml each were assessed for three parameters of PMN motility using Boyden chambers: random migration, chemotaxis, and chemokinesis. Boyden chambers were manufactured to accommodate two chambers separated by a 3 μm pore-sized membrane. Samples of 0.1 ml were layered on top of the membranes (Filter type SS, Millipore Corp., Bedford, MA). To assess for random migration, sterile pyrogen-free PBS was pipetted into the bottom and the upper chambers. For chemotaxis, sterile casein (Fisher Scientific Co., Fair Lawn, NJ) was pipetted into the bottom chamber only, and then topped up with sterile 1x PBS in the upper chamber. Chemokinesis was assessed by placing 2.5% non-immune rat serum in both chambers. The chambers were incubated for 1 hour at 37°C with 5% CO_2. After incubation, the membrane filters were recovered, fixed in absolute alcohol, stained with Mayer's hematoxylin, washed in distilled water, dehydrated in ethanol, stored overnight in xylene, and mounted between slide and coverslip. The depths of penetration by three leading PMNs in five different sites were determined with a micrometer incorporated into a Zeiss light microscope, and the means were calculated.

2.12 TRANSMISSION ELECTRON MICROSCOPY

For both animal models, samples taken from the BAL and purified PMN specimens were fixed in 5% glutaraldehyde in PBS (Electron Microscopy Sciences, Fort Washington, PA), post-fixed in 1% osmium tetroxide in PBS (JBS Supplies, J.B. EM Services Inc., Dorval,

PQ, Canada), dehydrated in ethanol, and embedded in Spurr low-viscosity medium (Electron Microscopy Sciences, Fort Washington, PA). Thin sections (80 nm) were stained with saturated uranyl acetate in 50% aqueous ethanol and 0.04% wt/vol lead citrate. Micrographs were obtained with a Hitachi 7000 transmission electron microscope at an acceleration voltage of 80 kV. Neutrophils with signs of apoptosis were identified according to the criteria of intact blebbed membranes, condensed nuclear chromatin, condensed perinuclear cytoplasm, and intact organelles (210).

2.13 PMN APOPTOSIS

Calf PMN apoptosis was measured in bovine BAL and circulating PMNs using a Cell Death Detection ELISA kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer's instructions. This quantitative sandwich-enzyme-immunoassay specifically measures the histone region (H1, H2A, H2B, H3, and H4) of mono- and oligo-nucleosomes which are released during apoptosis. The photometric development was monitored kinetically by reading the plate (THERMOmax™ microplate reader, Molecular Devices Corp., Menlo Park, CA) (405 nm) at various times: 1, 3, 6, 9, 12, 15, 38, and 53 min. Apoptosis was measured in triplicate from 10^5 PMNs of each group and expressed as the absorbance ratios of the infected and infected-tilmicosin cell lysates versus absorbances from unmanipulated controls arbitrarily set at 1.0. The detection limit for this ELISA is 10^2 apoptotic cells.

2.14 TUMOR NECROSIS FACTOR-α (TNF-α) ASSAY

In the calf model, the levels of soluble TNF-α from BAL supernatants were measured (THERMOmax™ microplate reader, Molecular Devices Corp., Menlo Park, CA) (450 nm) using a Predicta™ human TNF-α ELISA kit (Genzyme Diagnostics, Genzyme

Corporation, Cambridge, MA), according to the manufacturer's instructions. Human TNF-α has been shown to be cross-reactive with bovine TNF-α (242). The detection limit of this assay was 10 pg/ml.

2.15 LEUKOTRIENE B_4 (LTB_4) ASSAY

In the calf model, levels of LTB_4 in the BAL supernatants were measured (THERMOmax™ microplate reader, Molecular Devices Corp., Menlo Park, CA) (405 nm) using a competitive enzyme immunometric assay kit (Leukotriene B_4 enzyme immunoassay kit, Cayman Chemical Co., Ann Arbor, MI) performed according to the manufacturer's instructions. The specificity of the assay is 100% for LTB_4, 0.03% for 5(S)-HETE, and < 0.01% for LTC_4, LTE_4, LTD_4, and LTF_4, and has a detection limit of 7 pg/ml.

2.16 CIRCULATING PMN MODEL

2.16.1 PMN PURIFICATION

Peripheral blood was drawn into ACD vacutainers (ACD solution A, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) from the jugular veins of normal Holstein calves. The blood was pooled in two 50 ml polypropylene centrifuge tubes and spun at 1171 *g* in a IEC Centra-7R swinging bucket centrifuge (IEC 210 rotor, International Equipment Company, Needham Heights, MA) for 20 min at room temperature without braking. The plasma, buffy coat, and the top one-half of the erythrocyte pack was removed down to the 10 ml level. Then 20 ml of cold hypotonic lysis solution (10.6 mM Na_2HPO_4, 2.7 mM NaH_2PO_4) was added to the cell pack and gently mixed for 1 min. Isotonicity was restored through the addition of 10 ml of 3x hypertonic solution (10.6 mM Na_2HPO_4, 2.7 mM NaH_2PO_4, 462 mM NaCl). The mixture was centrifuged at 650 *g* for 10 min and the supernatant was discarded. The lysing procedure was repeated once again. After the final lysing step, the leukocyte cell pellet was resuspended in 10 ml of PBS (8.1 mM Na_2HPO_4,

1.47 mM KH_2PO_4, 2.68 mM KCl, 136.9 mM NaCl). The cell solution was poured into a 15 ml centrifuge tube and then centrifuged at 650 *g* for 10 min. The supernatant was discarded and the pellet was resuspended in 10 ml of 25 mM Hepes-buffered RPMI 1640 cell culture media (Sigma Chemical Co., St. Louis, MO). A cell concentration of 10^6 cells/ml in 45 ml was obtained through the use of a hemacytometer. The hemacytometer was also used to assess the percentage of cells that excluded trypan blue (Flow Laboratories Inc., McLean, VA). Differential cell counts were performed on cytospin preparations stained with Diff Quik (Baxter Healthcare Corp., Miami, FL).

2.16.2 EXPERIMENTAL DESIGN

For the analysis of apoptosis in cell populations (Cell Death Detection ELISA), the purified PMNs (10^6 cells/ml) were incubated with 5 or 0.5 µg/ml tilmicosin at 37°C and 5% CO_2 in the presence or absence of 10^7 *P. haemolytica* (B122)/ml for 2 h. For the comparative analysis of apoptosis in individual cells (Annexin-V-FLUOS), purified PMNs (10^6 cells/ml) were co-incubated with 0.5 µg/ml of tilmicosin (Micotil™, Provel, Guelph, ON, Canada), penicillin G (Sigma Chemical Co., St. Louis, MO), ceftiofur sodium (Excenel™, The Upjohn Company - Animal Health Division, Orangeville, ON, Canada), or oxytetracycline (Sigma Chemical Co., St. Louis, MO) for 2 h at 37°C and 5% CO_2. Controls were set up using 1x PBS instead of the antibiotics. An additional control was exposed to 10^{-8} M dexamethasone (Azium™, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada).

2.16.3 PMN APOPTOSIS

2.16.3.1 Nucleosome quantification ELISA: Neutrophil apoptosis was measured using a Cell Death Detection ELISA kit (Boehringer Mannheim GmbH, Germany) as described previously. Cells incubated with 1x PBS were used for comparisons of PMNs incubated

with tilmicosin only. Cells incubated with *P. haemolytica* were compared to PMNs incubated with both tilmicosin and bacteria.

2.16.3.2 Translocation of phosphatidylserine: Neutrophil apoptosis in individual cells was determined using an Annexin-V-FLUOS staining kit (Boehringer-Mannheim GmbH, Germany) according to the manufacturer's instructions. This staining kit specifically measures phosphatidylserine which is highly translocated from the inner side of the plasma membrane to the external surface of the cell during apoptosis and necrosis. Apoptotic cells can be differentiated from necrotic cells by using a stain containing annexin-V-fluorescein which has high affinity for phosphatidylserine and propidium iodide which binds DNA. Briefly, the incubated cells were pelleted at 200 *g* for 5 min (Baxter Canlab Biofuge A, Heraeus Sepatech GmbH, Germany). The cells were then washed with 1 ml of pyrogen-free 1x PBS warmed at room temperature and centrifuged at 200 *g* for 5 minutes. The supernatant was discarded and the pellet was resuspended with 0.1 ml of a staining solution containing 20 µl of annexin-V-fluorescein, 20 µl of propidium iodide, and 1 ml of Hepes buffer. The cells were incubated in the dark for 15 min at room temperature. After incubation, the cells were washed with 0.1 ml of Hepes buffer warmed at room temperature and centrifuged at 200 *g* for 5 minutes. A wet mount of 15 µl of the stained cells was prepared and was visualized under fluorescence microscopy. The cells were visualized under fluorescence using a Zeiss Axiovert 25 CFL inverted reflected light microscope where apoptotic cells appeared solid green (FITC filter, excitation = 450 nm, emission = 490 nm), and necrotic cells appeared as both solid green (FITC filter) with red nuclei (Cy-3 filter, excitation = 535 nm, emission = 550 nm). The percentage of apoptotic cells were counted under ten different fields under 400X magnification. The highest and the lowest values were discarded and the remaining eight values were averaged to obtain a mean.

2.17 STATISTICAL ANALYSIS

Results were expressed as means ± SEMs and compared by one-way analysis of variance (ANOVA), followed by Tukey's test for multiple-comparison analysis where applicable. When results were expressed as percentages, all values underwent an arcsine transformation prior to statistical comparison and were transformed back to percentages for the expression of the means ± SEMs. For the comparison of two lines, linear regression analysis was used to statistically compare the two slopes. Levels at which the *P* value were less than 0.05 were considered to be significant.

3. RESULTS

3.1 CLINICAL OBSERVATIONS AND BACTERIAL RECOVERY

Rats treated with tilmicosin had significantly greater *P. haemolytica* clearance than sham-treated animals from infected lungs (Fig. 1). After 2.5 h of challenge, tilmicosin-treated rats had cleared 96% of bacteria compared to sham-treated animals.

Six hours following infection, peak rectal temperatures in untreated calves reached 105.8 ± 0.5 °F versus 104.2 ± 0.4 °F in tilmicosin-treated animals. The difference in rectal temperatures between both groups failed to reach statistical significance at any time during the study. Treatment with tilmicosin induced effective clearance of *P. haemolytica* from the lungs of infected calves (Fig. 2). After 3 h of infection, tilmicosin-treated calves had cleared 94% of bacteria compared to sham-treated animals. After 24 h, no live bacteria could be recovered from the BAL of tilmicosin-treated calves while sham-treated animals still harbored 4.8 ± 1.4 $\log_{10}$CFU/ml BAL. No bacteria were found in the BAL of control animals (data not shown).

3.2 PMN INFILTRATION, PHAGOCYTIC INDICES, OXIDATIVE FUNCTIONS, LOCOMOTION, AND MEMBRANE PERMEABILITY

PMN infiltration in the BAL of tilmicosin-treated rats (83.6% ± 1.4%) was significantly lower than in untreated animals (91.9% ± 0.9%) 2.5 h post-infection (Table 1). In unmanipulated control animals, the PMN population represents <5% of the total leukocyte

Figure 1 - *P. haemolytica* (log_{10}CFU/ml) in the BAL of sham-treated (■) or tilmicosin-treated rats (□) after 2.5 h post-infection. Values are means ± SEMs from 7 animals in each experimental group. $^{*}P < 0.01$ vs. sham-treated.

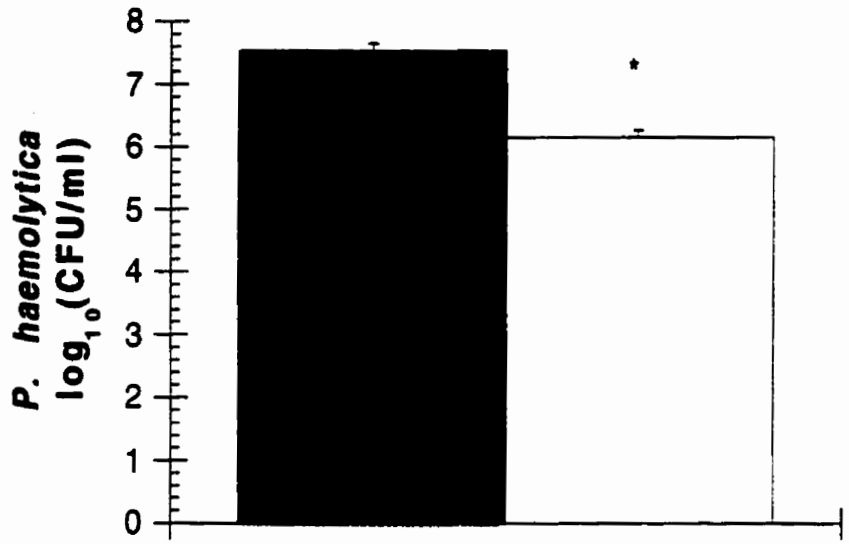
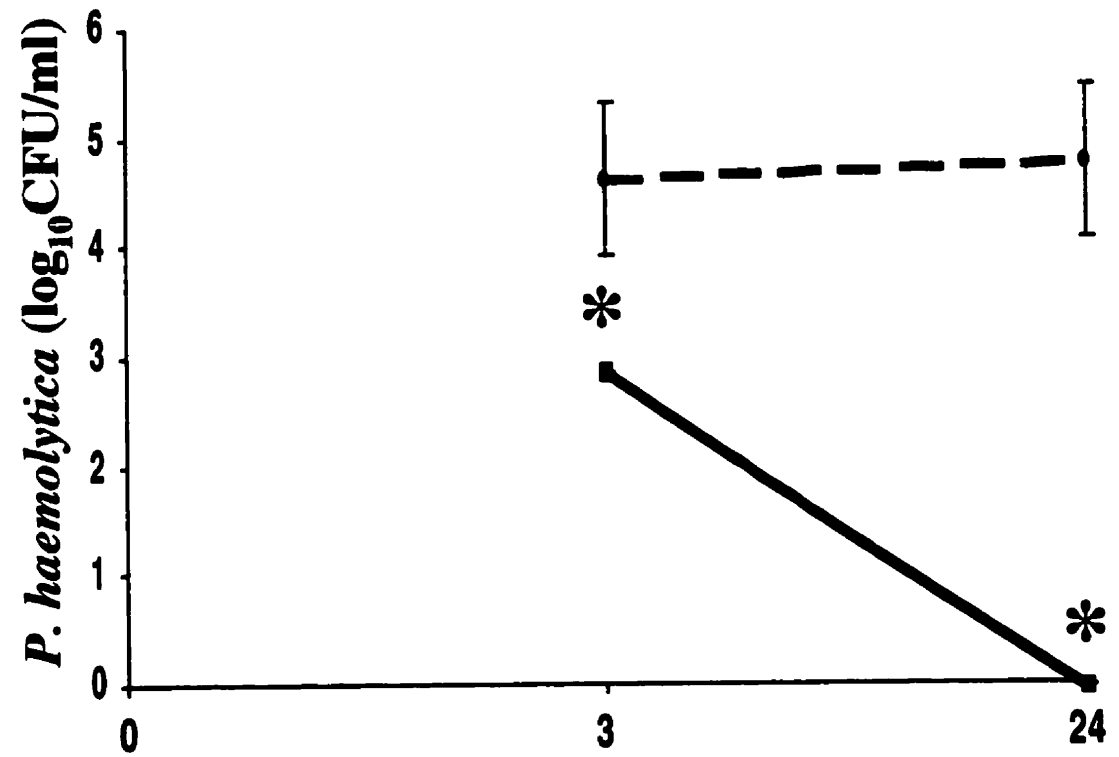

P. haemolytica
log10(CFU/ml)
8
7
6
5
4
3
2
1
0
*

Figure 2 - *P. haemolytica* recovery (log_{10}CFU/ml) in the BAL of sham-treated (— ●— —) and tilmicosin-treated (——■——) calves 3 and 24 h after bacterial infection. Values are means ± SEMs from 5 or 6 (3 h) and 3 (24 h) animals in each experimental group. Bacterial clearance in tilmicosin-treated animals was calculated as the percentage of cleared bacteria compared to the number of viable bacteria recovered from sham-treated animals. *$P < 0.05$ vs. sham-treated group.



clearance: **94%** **100%**

Table 1 - PMN counts and membrane integrity in BAL from sham-treated and tilmicosin-treated rats 2.5 h after *P. haemolytica* infection.

Rat group	% PMN	Log_{10}Total no. PMN	Trypan blue exclusion (%)
Sham-treated (n=9)	91.9 ± 0.9	7.13 ± 0.21	92.6 ± 0.8
Tilmicosin-treated (n=9)	83.6 ± 1.4[a]	6.92 ± 0.07[b]	84.4 ± 1.6[a]

a: $P < 0.01$ compared with values for sham-treated animals.

b: $P < 0.05$ compared with values for sham-treated animals.

Data are represented as means ± SEMs.

population in the rat lung (data not shown). PMNs from the BAL of tilmicosin-treated animals showed significantly decreased trypan blue exclusion, indicating reduced membrane permeability (Table 1).

More than 30% of PMN phagocytosed *P. haemolytica* in the BAL of infected rats. Phagocytic indices were not significantly different between the untreated and tilmicosin-treated groups (Table 2). The percentage of BAL PMNs that reduced NBT was greater than 90% upon infection with *P. haemolytica.* NBT reduction by PMNs was not significantly different between the two experimental groups (Table 2). Neutrophil random migration, chemotaxis, or chemokinesis were not statistically different between the two experimental groups (Table 2).

In the bronchoalveolar space of *P. haemolytica*-infected calves, PMN infiltration exceeded 90% PMNs by 3 h following infection and 70% by 24 h (compared with 1% as measured in the BAL of an unmanipulated control animal. PMN percentages were not significantly different between the BAL of tilmicosin-treated and those of untreated infected calves (Table 3). More than 10% of PMN had phagocytosed *P. haemolytica* in the BAL of infected calves, 3 h or 24 h post challenge. Phagocytic indices were not significantly different between experimental groups at either time (Table 3). The percentage of BAL PMNs that reduced NBT was increased to >90% by the *P. haemolytica* infection (compared to 49.8% in unmanipulated control calf, n=1). Reduction of NBT by PMNs (Table 3) was not significantly different between the tilmicosin-treated and sham-treated groups at either experimental time. Similarly, trypan blue exclusion was not different between the PMNs of tilmicosin-treated, sham-treated or control (86.6%, data not shown) animals (Table 3). In the BAL of tilmicosin-treated calves, a number of dying PMNs were found in the BAL exhibiting characteristic pyknotic nuclei (Fig. 3).

Table 2 - PMN phagocytic indices, PMN NBT reduction, and PMN motility in BAL of sham-treated and tilmicosin-treated rats 2.5 h after *P. haemolytica* infection.

Rat group	Phagocytic index (%)	NBT reduction (%)	PMN motility (μm/h)[a]		
			Random migration	Chemotaxis	Chemokinesis
Sham-treated	30.7 ± 5.1[a]	96.0 ± 3.0[b]	15.3 ± 3.5	88.8 ± 10.0	66.6 ± 6.8
Tilmicosin-treated	38.2 ± 7.0[b]	94.6 ± 0.8[c]	13.6 ± 3.8	84.1 ± 7.7	69.1 ± 7.7

a: n=7

b: n=8

c: n=9

d: n=5

Data are represented as means ± SEMs.

Table 3 - PMN percentages, phagocytic indices, NBT reduction, and membrane integrity in BAL from sham-treated and tilmicosin-treated animals 3 h and 24 h after *P. haemolytica* infection.

Challenge time and calf group	PMN (%)	Phagocytic index (%)	NBT reduction (%)	Trypan blue exclusion (%)
3 h				
Sham-treated (n=6)	92.7 ± 2.4	21.5 ± 2.7	93.4 ± 2.0[a]	90.5 ± 3.7
Tilmicosin-treated (n=6)	93.8 ± 1.7	16.5 ± 2.6	94.2 ± 3.0[b]	89.4 ± 2.2
24 h				
Sham-treated (n=3)	79.4 ± 6.3	18.6 ± 1.1	94.5 ± 1.5[c]	82.5 ± 3.5
Tilmicosin-treated (n=3)	74.9 ± 9.9	18.6 ± 3.2	97.3 ± 1.4	90.3 ± 1.9

a: n=5

b: n=3

c: One experiment failed to yield results for NBT reduction.

Data are represented as means ± SEMs.

Figure 3 - Photomicrograph of a cytospin preparation obtained from the BAL of a tilmicosin-treated calf 3 h following *P. haemolytica* infection. Note the dying PMNs with pyknotic nuclei (arrow heads). One such PMN is being phagocytosed by an alveolar macrophage (arrow), a common mechanism for elimination of apoptotic cells. Bar = 10 micrometers.

3.3 MICROSCOPY

In the BAL of tilmicosin-treated rats, there was an increased number of PMNs exhibiting pyknotic nuclei under light microscopy (data not shown). Ultrastructural evidence of apoptosis was exhibited by PMNs in the BAL of infected tilmicosin-treated rats (Fig. 4). Examination under electron microscopy allowed the detection of apoptotic cells with membrane blebbing, intact cellular membrane, nuclear chromatin condensation concurrent with nuclear membrane delamination, and cytoplasmic vacuolation. In addition, the cells contained intact mitochondria and other cellular organelles.

Neutrophils in the BAL from infected tilmicosin-treated calves exhibited ultrastructural evidence of apoptosis (Fig. 5). Briefly, electron microscopy allowed detection of cells with characteristic plasma membrane blebbing without signs of loss of plasma membrane integrity, nuclear chromatin condensation coupled with nuclear membrane delamination, and cytoplasmic vacuolation. Concurrent with the above observations, cells contained intact mitochondria and other cellular organelles. These studies provided graphic evidence for apoptosis in BAL PMNs, and these observations indicated that apoptotic PMNs were found more commonly in the BAL of tilmicosin-treated animals than in untreated calves (data not shown). In order to obtain statistically reliable measurements of the comparative occurrence of this phenomenon in each group, quantitative ELISA was used to assess the occurrence of programmed cell death in this cell population.

3.4 PMN APOPTOSIS

For the calf model, comparative production of apoptotic nucleosomes by BAL PMNs from the three experimental groups of this study was measured using quantitative ELISA (Fig. 6). After 3 h of infection, PMN lysates from the BAL of tilmicosin-treated calves exhibited significant occurrence of apoptosis. These values were significantly higher ($P < 0.05$) than those measured in lysates from the same cell numbers of BAL PMNs of sham-

Figure 4 - Transmission electron micrograph of a BAL PMN obtained from a tilmicosin-treated rat 2.5 h after *P. haemolytica* infection. Features that are indicative of apoptosis are: intact plasma membrane with blebbing (small arrows), lysed nuclear membrane (arrow heads); chromatin condensation ($), and intact cytoplasmic organelles. (Bar = 1 micrometer).

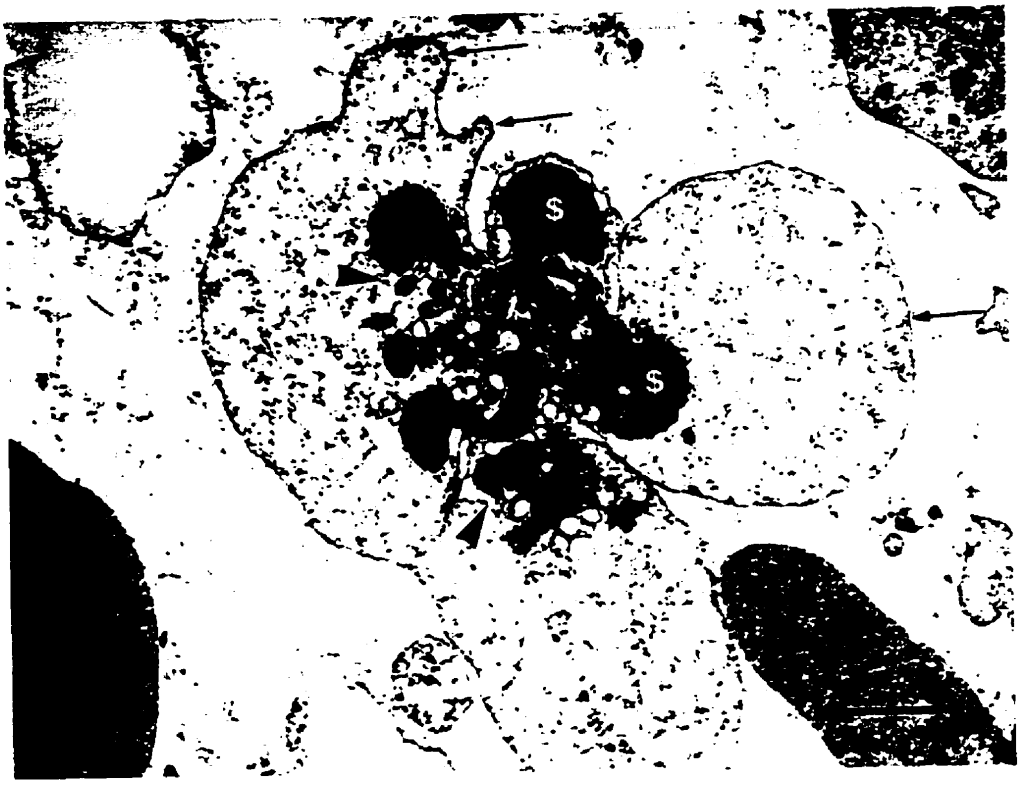

S
S

Figure 5 - Transmission electron micrographs of BAL PMNs obtained from tilmicosin-treated calves 3 h after *P. haemolytica* infection. A,B,C) Electron micrographs of apoptotic calf PMNs taken from the BAL of tilmicosin-treated calves. Apoptotic features are: intact plasma membrane with blebbing (small arrows), nuclear membrane delamination (arrow heads), condensed chromatin ($), intact cytoplasmic organelles and granules, and vacuolation of the cytoplasm (large arrows). (Bar = 1 micrometer).

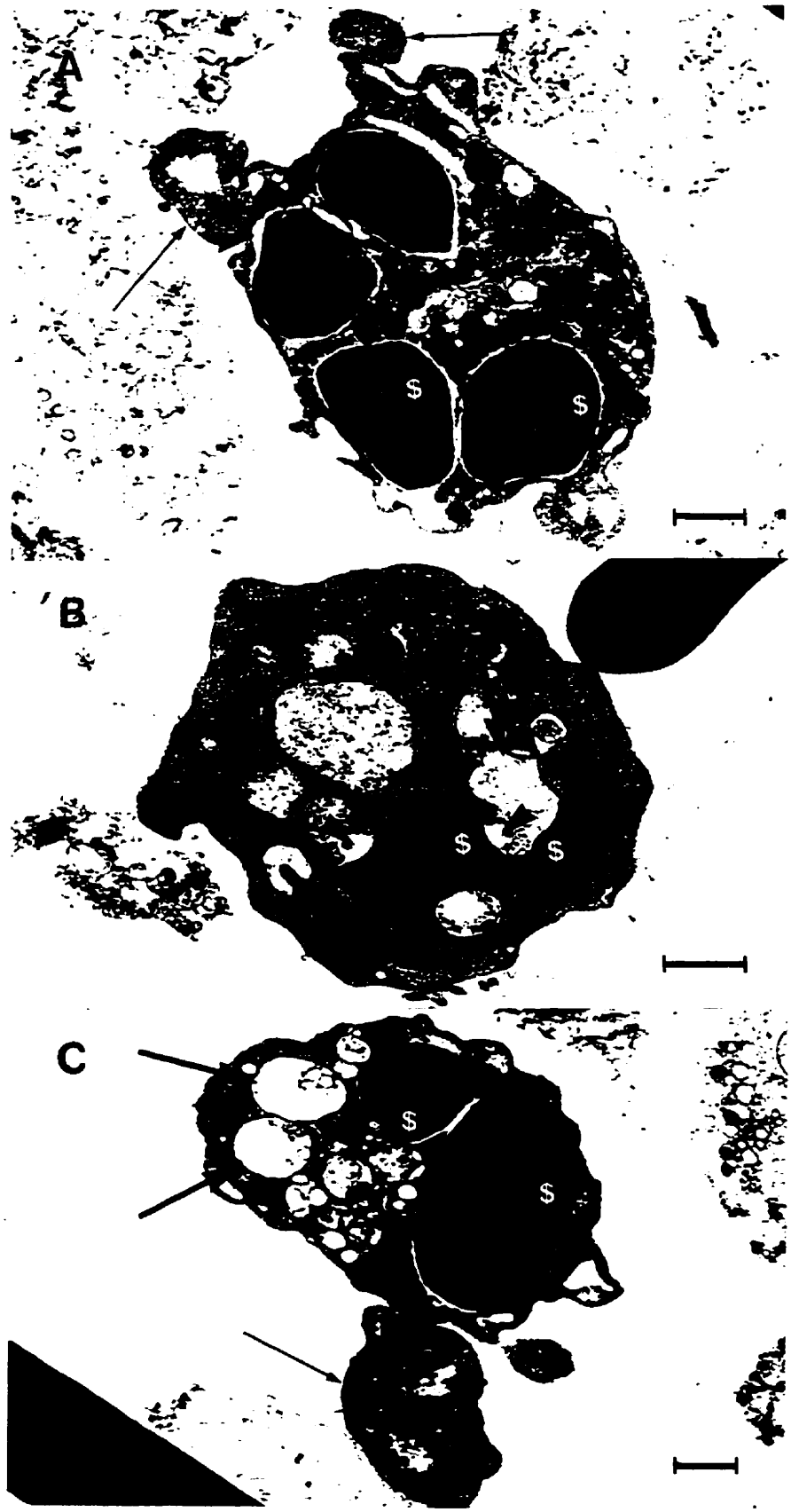
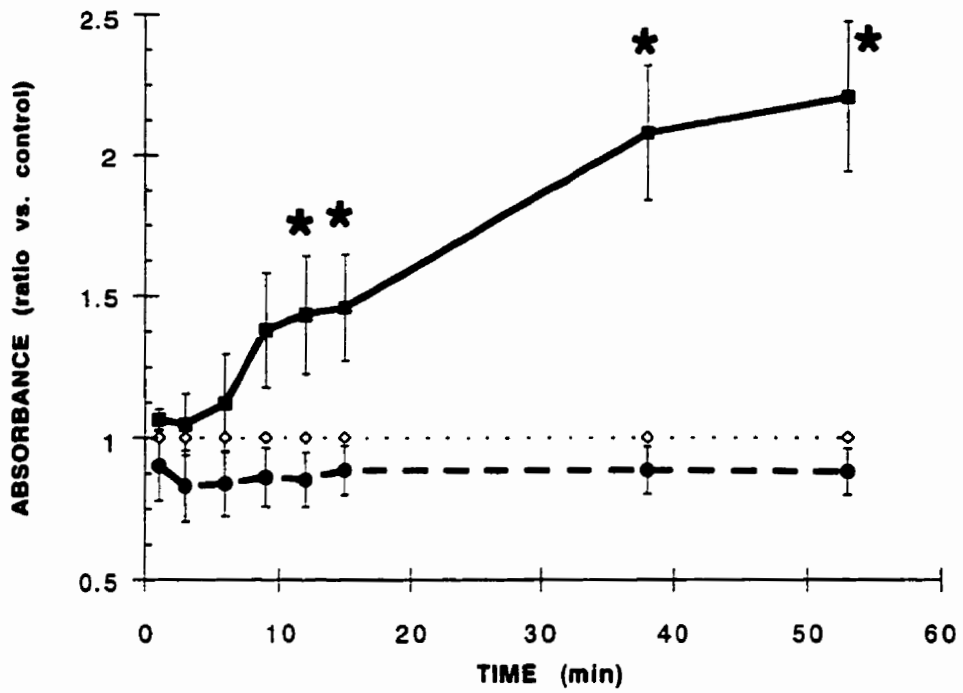
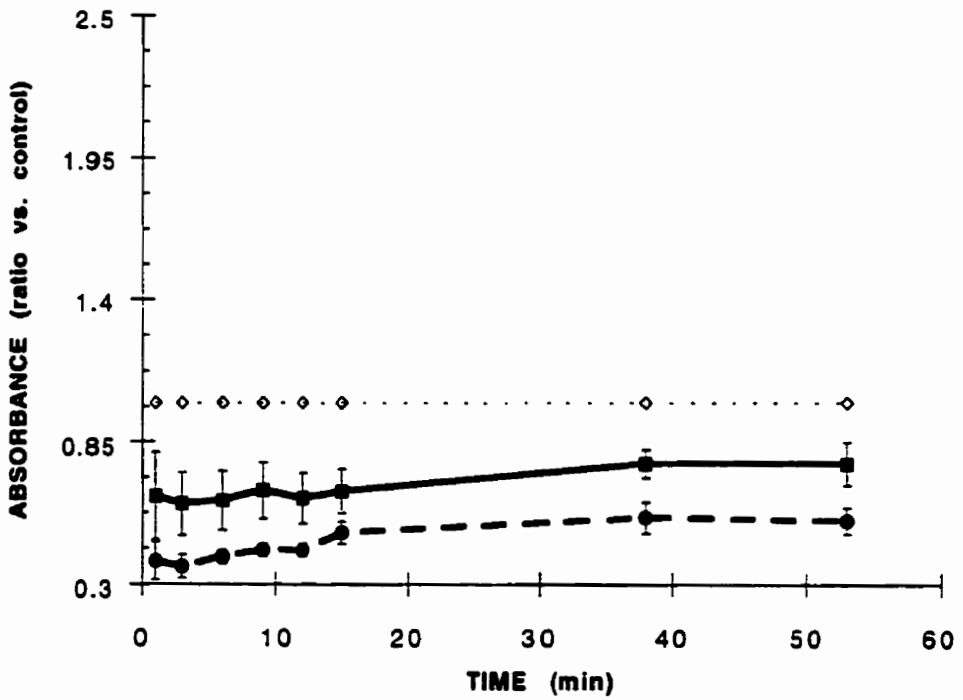

A
$
$
B
$
$
C
$
$

Figure 6 - Quantification of apoptosis in BAL PMNs of sham-treated (— ●— —) and tilmicosin-treated calves (—■—) at 3 h (A; n=5-6 per group) and 24 h (B; n=3 per group) after *P. haemolytica* infection. Values from 53 min of ELISA development are calculated as absorbance ratios versus values measured in BAL PMNs from unmanipulated controls (- - - - - -), which are arbitrarily set at 1.0. $*P < 0.05$ vs. sham-treated group.

A


2.5
2
1.5
1
0.5
ABSORBANCE (ratio vs. control)
0
10
20
30
40
50
60
TIME (min)
*
*
*
*

B


2.5
1.95
1.4
0.85
0.3
ABSORBANCE (ratio vs. control)
0
10
20
30
40
50
60
TIME (min)

treated animals. The values obtained from sham-treated calves were not different from baseline controls (Fig. 6A). At 24 h post-infection, apoptosis levels were not different between any experimental groups (Fig. 6B).

3.5 SOLUBLE TNF-α

Concentration of soluble TNF-α in BAL supernatants of each calf group was measured 3 h after infection; the time at which PMN apoptosis was observed in the tilmicosin-treated group. Soluble TNF-α concentration was not significantly different between any groups (control = 8.6 ± 1.5 pg/ml; infected = 19.6 ± 4.1 pg/ml; infected-tilmicosin = 18.8 ± 8.1 pg/ml).

3.6 LTB_4 SYNTHESIS

Concentrations of LTB_4 were measured from the BAL of infected calves (Fig. 7). After 3 h of infection, the levels of BAL LTB_4 were not statistically different between untreated-infected and tilmicosin-treated infected calves. In contrast, 24 h post-infection, BALs from tilmicosin-treated animals contained significantly less ($P < 0.05$) LTB_4 than those of untreated calves.

3.7 CIRCULATING PMN MODEL

3.7.1 NUCLEOSOME QUANTIFICATION ELISA

The production of apoptotic nucleosomes by peripheral PMNs incubated with 5 μg/ml or 0.5 μg/ml tilmicosin in the presence or absence of *P. haemolytica* was measured using quantitative ELISA (Fig. 8). Tilmicosin at either concentration, in the presence or the absence of *P. haemolytica*, induced a five-fold increase in apoptosis compared to controls after 2 h of incubation (Fig. 8A,B). Linear regression analysis indicated that there was no

Figure 7 - LTB_4 synthesis in the BAL of sham-treated (■) or tilmicosin-treated (□) calves 3 h and 24 h after *P. haemolytica* infection. $*P < 0.05$ vs. tilmicosin-treated group.

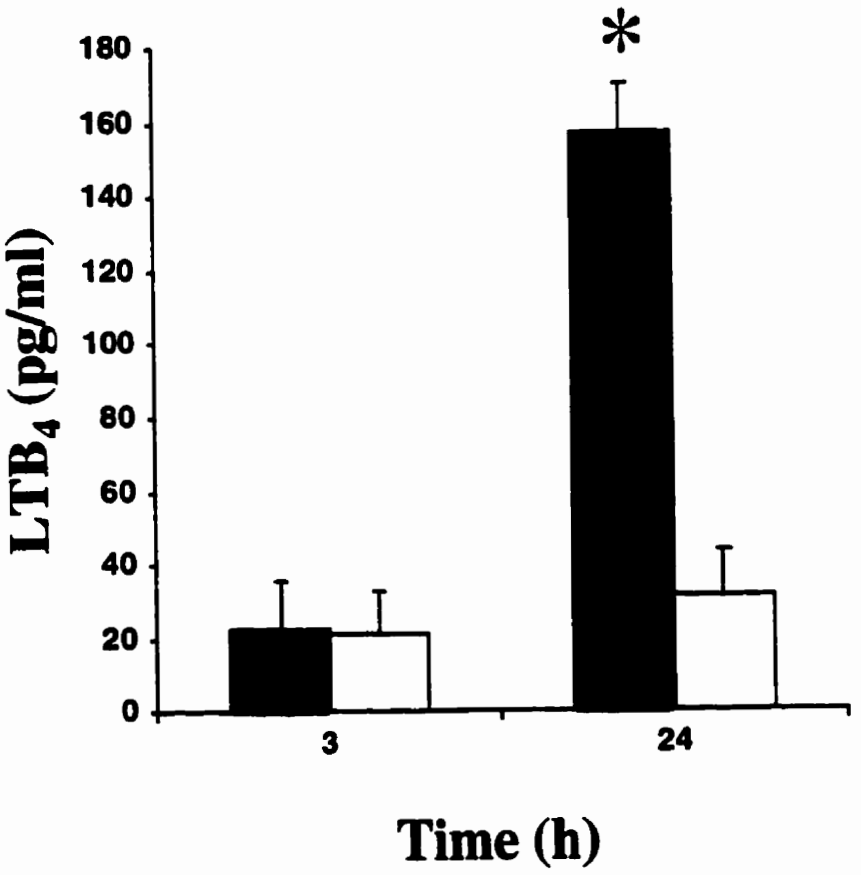
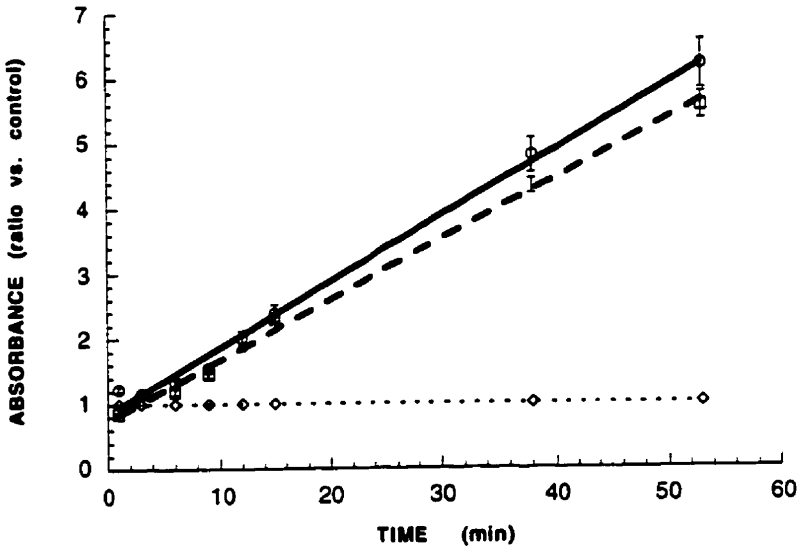
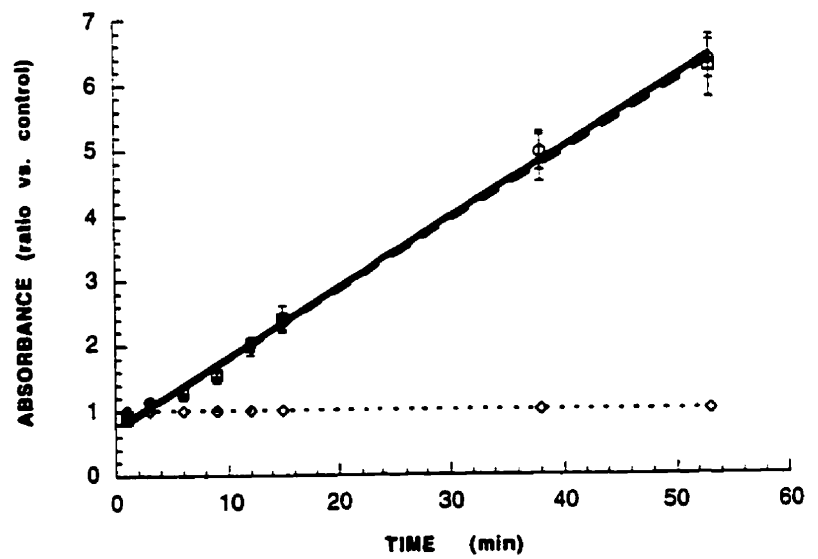

*
180
160
140
120
100
80
60
40
20
0
LTB4 (pg/ml)
3
24
Time (h)

Figure 8 - Quantification of apoptosis in peripheral bovine PMNs incubated with 5 μg/ml (A) or 0.5 μg/ml (B) tilmicosin in the presence (—■—) or in the absence (– ● –) of *P. haemolytica*. Values from 53 min of ELISA development were calculated as absorbance ratios versus values measured in BAL PMNs from controls incubated with 1x PBS (- - - - - -) arbitrarily set at 1.0.

A


ABSORBANCE (ratio vs. control)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
TIME (min)

B

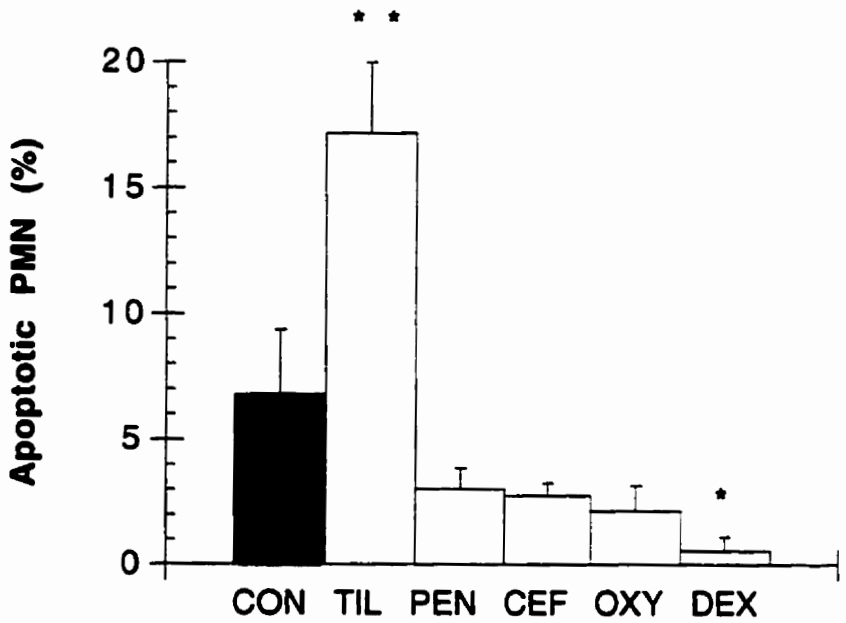

ABSORBANCE (ratio vs. control)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
TIME (min)

significant difference between the slopes of the lines expressing the induction of PMN apoptosis by tilmicosin alone and with tilmicosin and *P. haemolytica*.

3.7.2 TRANSLOCATION OF PHOSPHATIDYLSERINE

Induction of apoptosis in peripheral PMNs incubated with various drugs was quantitated using Annexin V - propidium iodide labeling and fluorescence microscopy (Fig. 9). Significantly ($P < 0.01$) more PMNs were apoptotic when exposed to tilmicosin for 2 h than in the control group. Conversely, dexamethasone significantly ($P < 0.05$) inhibited PMN apoptosis compared to controls. Penicillin, ceftiofur, or oxytetracycline did not affect apoptosis in PMNs.

Figure 9 - Percentage of apoptotic peripheral bovine PMNs incubated for 2 h with either 1x PBS (CON), 0.5 μg tilmicosin (TIL), 0.5 μg penicillin (PEN), 0.5 μg ceftiofur (CEF), 0.5 μg oxytetracycline (OXY), or 10^{-8} M dexamethasone (DEX) (n=3 per group). Values are means ± SEMs from 3 per experimental group. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control.


Apoptotic PMN (%)
20
15
10
5
0
* *
*
CON
TIL
PEN
CEF
OXY
DEX

4. DISCUSSION

In an attempt to assess the anti-inflammatory potential of tilmicosin, this study investigated the effects of this macrolide antibiotic on PMNs. The results indicate that in addition to accelerated *P. haemolytica* clearance from the lung, induction of PMN apoptosis by tilmicosin is an important mode of action of this antibiotic as opposed to others such as penicillin, ceftiofur, or oxytetracycline. This effect is associated with a significant anti-inflammatory benefit, as indicated by reduced accumulation of LTB_4 in the infected lungs of tilmicosin-treated calves. However, while tilmicosin induced PMN apoptosis, it did not affect their phagocytic activity, oxidative function, and motility.

This study established an *in vivo* rat model of pasteurellosis to investigate bronchoalveolar PMNs during pulmonary inflammation. This model helped establish useful experimental parameters prior to studying the bovine model of pasteurellosis. Past studies have indicated the clinical efficacy of tilmicosin in calves infected with *P. haemolytica* (107,109-113,119,154,161). No studies have been published on the direct effects of tilmicosin on the bronchoalveolar PMNs during a *P. haemolytica* infection. Findings from this study have shown that in tilmicosin-treated rats, clearance of *P. haemolytica* was significantly increased after 2.5 h of infection. Tilmicosin-induced bacterial clearance and patterns of rectal temperatures in treated calves measured in the present study are consistent with these earlier observations establishing the outstanding clinical efficacy of tilmicosin in *P. haemolytica*-infected calves. The total disappearance of detectable *P. haemolytica* from the BAL of calves infected for 24 h further underscores the potent antibacterial properties of tilmicosin.

The central role played by PMNs in clearing the lung of invading bacteria is well established. Recruitment of PMNs also represents a pivotal mechanism of protection

against *P. haemolytica* (128), and experimental infections in other model systems have suggested that accelerated bacterial clearance from the immune lung may be due to enhanced PMN infiltration and functions (84). Consistent with these observations, infiltration of PMNs into the bronchoalveolar space of infected rats exceeded that of unmanipulated control animals. The predominance of PMNs (over 80%) in the bronchoalveolar space indicated that infection with *P. haemolytica* in rats induced significant inflammatory response. In tilmicosin-treated rats, bronchoalveolar PMN infiltration exceeded 80%, but was less than sham-treated rats that had over 90%. The reduction of PMN infiltration in tilmicosin-treated rats was not due to downregulation of PMN motility as assessed by Boyden chamber studies. Indeed, experiments on PMN random migration, chemotaxis, and chemokinesis indicated that these parameters were not significantly different between both experimental groups. It was also found that in the rat model, tilmicosin decreased PMN membrane permeability after 2.5 h of bacterial infection. In contrast, results from the calf model indicate that tilmicosin does not affect PMN infiltration or membrane permeability. It remains to be shown whether the discrepancy of these observations is due to the different kinetics of the models or to species-dependent phenomena. The assessment of PMN functions such as phagocytic activity, oxidative metabolism, and motility in the rat model revealed that there was no significant difference between the two experimental groups, which implies that the tilmicosin-induced reduction of bacterial colonization was not due to enhanced antibacterial functions of the PMNs. This is further supported by the findings from the calf study which showed that PMN infiltration, phagocytic function and oxidative metabolism were not altered in tilmicosin-treated calves. Recent studies have suggested that macrolides may adversely affect leukocytes in the inflamed respiratory tract. Erythromycin, as well as roxithromycin, inhibit PMN recruitment in the LPS-stimulated rat trachea, and erythromycin was recently shown to impair PMN chemotaxis in the lungs of foals (202,204). Also, azithromycin and

clarithromycin impair the phagocytic activity of human peripheral PMNs (243). In contrast, results from this study demonstrate that tilmicosin treatment does not alter the primary infiltration, phagocytic function, oxidative metabolism or membrane integrity of PMNs in the infected calf lung.

Tilmicosin accumulates in high amounts within PMNs (193). This recent finding may partly explain the high concentrations of tilmicosin within infected lung tissues (111,119,244). Moreover, transport of the antibiotic by PMNs to the site of infection may significantly contribute to the increased clearance of *P. haemolytica* from the lungs of treated animals.

The central driving force of the inflammatory response and tissue injury in many mucosal systems is the chronic accumulation of PMNs (4-5,9,21,23,41,44-48). The body has developed ways of controlling the inflammatory response to limit the irreversible tissue damage that may occur during chronic inflammation. The body regulates inflammation through the use of anti-inflammatory cytokines, antiproteases and antioxidants (4-5,41-43,89-91), as well as inhibiting PMN activity (9,92-94). Another way of limiting PMN-mediated tissue injury is the pre-programming of PMNs for apoptosis (216,223,245). Among all the leukocytes in the body, the PMN has the shortest life span with approximately 72 h in the body (215,221). Neutrophils are continually regenerated via maturation from progenitor pools (18), eliminated through apoptosis, and ultimately phagocytosed by macrophages (215-216,226-227). Findings from this study indicate that the enhanced induction of PMN apoptosis by an antibiotic may be beneficial in chronic inflammatory states, as seen in bovine pasteurellosis. During a *P. haemolytica* infection, release of a soluble heat-labile leukotoxin by the bacteria destroys PMNs, hence promoting the release of pro-inflammatory products in surrounding tissues and further amplifying inflammation in the lung (115,123,129,130). Clearly, the self-perpetuating necrosis of PMNs at the site of inflammation is a central driving force for the pathogenesis of

pneumonic pasteurellosis. Apoptosis is a more subtle mode of cell death. Unlike necrosis, apoptotic PMNs may be discarded without releasing their pro-inflammatory contents *in situ*. In apoptosis, cells characteristically translocate phosphatidylserine to the outer portion of the phospholipid bilayer, exhibit membrane blebbing and chromatin condensation, maintain intact organelles, show DNA fragmentation and shedding of nucleosomes, all ultimately leading to the phagocytosis of the apoptotic cell by neighboring macrophages (212,214-215). Recent investigations found that erythromycin and other macrolides may induce apoptosis in PMNs *in vitro* (205). It was hypothesized that tilmicosin may stimulate PMNs to undergo apoptosis in the inflamed lung *in vivo*, hence helping the host to dispose of these cells without promoting further inflammation. In the rat model, electron microscopy studies yielded graphical evidence of the increased induction of apoptosis in bronchoalveolar PMNs of tilmicosin-treated animals. Results from the calf study unequivocally demonstrate that BAL PMNs recovered from the lungs of tilmicosin-treated calves contain increased amounts of apoptotic nucleosomes when compared to PMNs from sham-treated animals, in which the extent of apoptosis is not different from unmanipulated controls. Occurrence of programmed cell death in PMNs from tilmicosin-treated calves was confirmed under transmission electron microscopy, which provided more evidence of the hallmarks of cell apoptosis. Interestingly, the data suggests that tilmicosin-induced apoptosis in the inflamed lung is an acute effect which cannot be detected 24 h post-treatment. The findings also demonstrate that higher levels of apoptosis were measured in PMNs isolated from lungs of treated calves which harboured significantly less bacteria than sham-treated animals, and that occurrence of apoptosis was not different between PMNs from sham-treated infected animals and cells from unmanipulated controls. Taken together, these data suggest that induction of neutrophil apoptosis by *P. haemolytica* itself, albeit measurable *in vitro* (132), may only affect an insignificant proportion of cells in the

inflamed lung *in vivo*. Conversely, the addition of tilmicosin results in a highly significant induction of the cell death mechanism.

Recent evidence indicates that normal human PMNs are highly susceptible to Fas-induced death (221,238,246). Fas is a type I membrane protein member of the TNF/nerve growth factor family, which is involved in apoptosis through interactions with anti-Fas immunoglobulin M or FasL (220-221). FasL is a type II protein member of the TNF family, which includes TNF-β and TNF-α. Tumor necrosis factor - alpha is a potent pro-inflammatory cytokine released in large amounts by alveolar macrophages upon pulmonary colonization by pathogens (8,69,71-75), and this cytokine also has the capability to induce PMN apoptosis (217-220). In an attempt to determine whether PMN apoptosis in the infected lungs of tilmicosin-treated animals was associated with a concurrent increase in BAL TNF-α concentrations, bronchoalveolar samples were collected 3 h post-infection (i.e. when apoptotic PMNs were detected), and were assessed for TNF-α content. Soluble TNF-α concentrations were not significantly different between experimental groups. This suggests that tilmicosin induces apoptosis in pulmonary PMNs independently of increased soluble TNF-α levels in the bronchoalveolar space. The kinetics of TNF-α synthesis as well as the patterns of cell-bound TNF-α in the lung need to be further investigated in this experimental model.

Following appropriate stimulation, PMNs, macrophages as well as other cell types produce LTB_4, an arachidonic acid metabolite synthesized via the 5-lipoxygenase pathway (10,12-13,19,84-86). This eicosanoid is one of the most potent chemoattractants for PMNs. The implication of LTB_4 in the self-perpetuating inflammatory injury of various chronic disorders has been well-documented (5,13,34,55,87-88,247-248). Therefore, accumulation of this metabolite has become a reliable marker of inflammation. In the inflamed lung infected with *P. haemolytica*, the principal source of LTB_4 is the PMN (134,249). Recent findings have shown that PMN infiltration in the lungs of tilmicosin-

treated calves experimentally infected with *P. haemolytica* was drastically decreased when compared to the inflamed tissue recovered from sham-treated animals (119). Consistent with these observations, results from this study show that 24 h post-infection, severe inflammation in the lungs of sham-treated infected calves is associated with a more than seven-fold increase in BAL LTB_4 compared with baseline concentrations measured 3 h after infection. In contrast, this eicosanoid did not accumulate in the BAL of tilmicosin-treated animals, and at 24 h post-infection LTB_4 levels in these calves were markedly less than those measured in sham-treated calves. Results clearly indicate that tilmicosin-induced apoptosis of PMNs is associated with a subsequent reduction of pro-inflammatory LTB_4 synthesis in the bronchoalveolar space. This suggests that tilmicosin aids in the treatment of pasteurellosis by inducing bronchoalveolar PMN apoptosis during the acute phase of the infection, and by attenuating LTB_4 synthesis once the infection proceeds to the chronic stage. In turn, this inhibition may prevent the self-perpetuation of inflammation in the lung.

Although the above studies have revealed the beneficial properties of tilmicosin within the context of infection, the results from the *in vivo* studies could not answer specific questions on the pro-apoptotic properties of tilmicosin. For example, these studies could not answer whether the induction of apoptosis was due to the drug alone, or whether tilmicosin could induce apoptosis in the absence of *in vivo* parameters, nor could the experiments distinguish the anti-inflammatory properties of tilmicosin from its antibacterial properties. In order to answer these questions, an *in vitro* model using peripheral bovine PMNs was established.

First, experiments assessed the release of nucleosomes by apoptotic bovine PMNs co-incubated with two concentrations of tilmicosin in the presence or absence of *P. haemolytica*. Results from these studies show that tilmicosin equally induces apoptosis in bovine PMNs at either drug concentration, and regardless of the presence or absence of *P.*

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IMAGE EVALUATION
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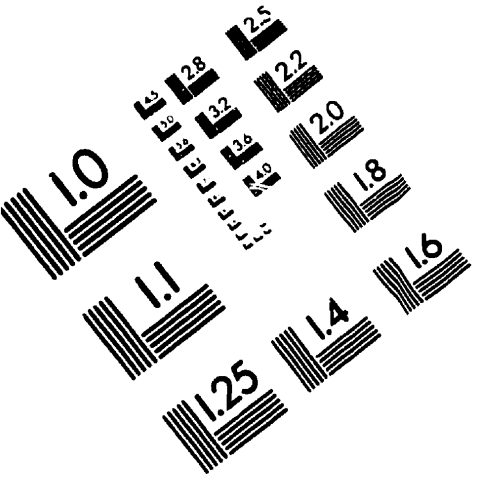

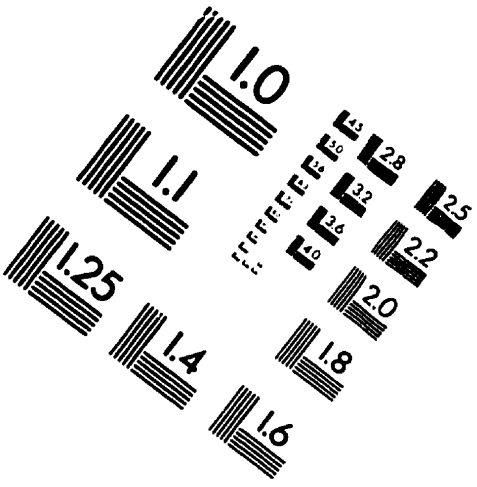

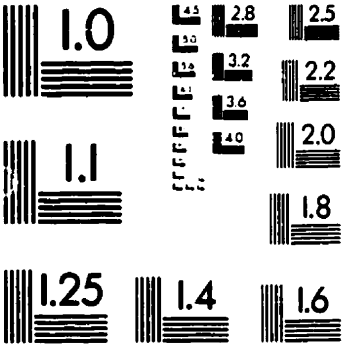

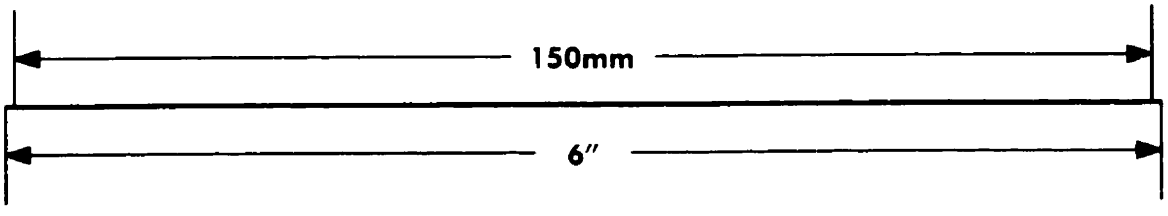

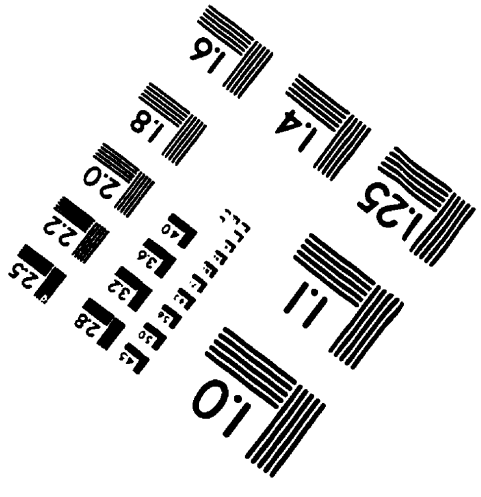

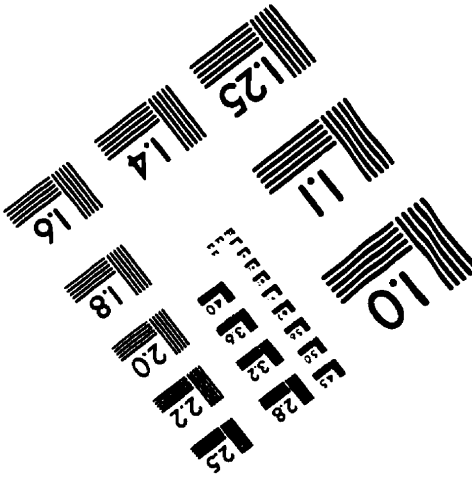

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