PULMONARY EXPRESSION OF INFLAMMATORY CYTOKINES IN EXPERIMENTAL BOVINE PNEUMONIC MANNHEIMIOSIS

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ABSTRACT

Bovine pneumonic mannheimiosis (BPM), an acute fibrinonecrotic pleuropneumonia caused by *Mannheimia (Pasteurella) haemolytica*, remains a leading source of economic losses to North American beef and dairy industries. Current evidence indicates that pulmonary inflammatory responses, rather than the bacterium itself, are primarly responsible for the severe lung injury associated with disease. We therefore hypothesized that inflammatory cytokines participate in the pathogenesis of BPM, and that modulation of their expression may serve to prevent or reduce inflammatory lung injury.

The specific objective of Phase 1 of the project was to characterize patterns of TNFα, IL–1β, and IL–8 expression within the lungs of experimentally infected calves. All 3 cytokines were upregulated, and results demonstrated a spatial and temporal association between inflammatory cytokine expression and lung pathology, indirectly supporting the hypothesis that these mediators contribute to lung injury in BPM.

The objective of Phase 2 was to identify drugs capable of suppressing TNFα, IL–1β, and IL–8 gene and protein expression in bovine alveolar macrophages exposed to *M. haemolytica* lipopolysaccharide and leukotoxin in vitro. Compounds tested included dexamethasone, tetrahydropapaveroline, pentoxifylline, rolipram, SB203580, and thalidomide. Dose-dependent inhibition of cytokine secretion occurred in response to pretreatment with dexamethasone, tetrahydropapaveroline, pentoxifylline, rolipram, and SB203580. Dose-dependent inhibition of cytokine mRNA expression occurred in response to pretreatment

with dexamethasone, tetrahydropapaveroline, and pentoxifylline. Dexamethasone was the most effective inhibitor by far.

The objective of Phase 3 was to assess the ability of dexamethasone to ameliorate disease development in an in vivo experimental model of BPM. Clinical disease scores for DEX-treated calves were significantly lower than those for controls, and the percent lung volume exhibiting gross pneumonic lesions was significantly lower in DEX-treated calves (6.0% ± 1.1%) as compared to controls (68.9% ± 13.3%). Histopathological lesions were also less severe and extensive in DEX-treated calves.

Taken together, these findings support the hypothesis that pharmacological modulation of pulmonary inflammation may represent a novel approach to the prevention and treatment of BPM. Successful implementation of this strategy will require additional research to identify drug agents that target the expression of cytokines and other inflammatory mediators without compromising host immune responses.

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CHAPTER 1

GENERAL INTRODUCTION

Bovine pneumonic mannheimiosis (BPM), an acute fibrinonecrotic pleuropneumonia caused by *Mannheimia (Pasteurella) haemolytica*, affects cattle throughout the world. Despite recent progress in our understanding of the disease, it remains a leading source of economic loss to the North American beef and dairy industries. Estimates of annual losses attributable to infectious respiratory disease in the United States, of which pneumonic mannheimiosis is considered to be the most important clinical entity, range between $640 million and $4 billion, exceeding those from all other diseases combined. These losses are the combined result of treatment costs, death of affected animals, and impaired production performance among those that survive.

At present, prevention and treatment of BPM are based almost exclusively on systemic antibiotic therapy. However, mass medication of cattle with antibiotics poses some serious concerns. Widespread use of antibiotics appears to have contributed to the emergence of multiple antibiotic-resistant strains of *M. haemolytica*. In addition, mass medication increases the risk of unwanted drug residues in meat and milk intended for human consumption. Of even greater concern, however, is the fact that mass medication promotes the transfer of antibiotic resistance genes from animal pathogens to human bacterial pathogens, threatening our ability to control human infectious disease. Novel approaches to the management of BPM are therefore warranted.

Although *M. haemolytica* virulence factors such as leukotoxin (LktA) and lipopolysaccharide (LPS) are capable of inducing some degree of direct pulmonary pathology, there is mounting evidence that an uncontrolled host inflammatory reaction to the bacterium and its products is the principal cause of

lung injury. One of the hallmark histopathological features of BPM is extensive infiltration of the lungs by neutrophils, a process that begins within 2 hours of experimental infection. Depletion of neutrophils prior to experimental infection protects calves from subsequent lung injury, suggesting that this cell type is responsible for much of the pulmonary pathology associated with the disease. Recognition of the importance of neutrophils and the host inflammatory response in disease pathogenesis has prompted speculation that it may be possible to treat or prevent the disease through pharmacological modulation of pulmonary inflammatory responses.

The recruitment and activation of neutrophils are regulated by a complex network of interactions between cytokines, leukocytes, vascular endothelium, cellular adhesion molecules, and soluble activating and chemotactic factors. The inflammatory cytokines TNFα, IL-1β, and IL-8 play a central role in the initiation and orchestration of these interactions. In addition, they provoke the synthesis and release of a broad array of soluble inflammatory mediators. TNFa and IL–1b are pleiotropic early response polypeptides secreted by monocytes and macrophages in response to microbial pathogens and other stimuli. IL-8, a potent chemotactic and activating factor for neutrophils, is secreted by a variety of immune and nonimmune cell types, including monocytes, macrophages, fibroblasts, epithelial cells, and neutrophils. A growing body of evidence implicates inflammatory cytokines in the pathogenesis of BPM. Previous work in our laboratory and by others has demonstrated that *M. haemolytica* and its major virulence factors, LPS and LktA, are potent inducers of TNFα, IL–1β, and IL–8

genes and proteins in bovine alveolar macrophages. In addition, these cytokines are expressed within the lungs of naturally and experimentally infected cattle.

We hypothesize that inflammatory cytokines contribute to the pathogenesis of lung injury in BPM, and that pharmacological inhibition of their expression may prevent or reduce the inflammatory lung injury characteristic of the disease. Successful development and implementation of therapeutic strategies based upon cytokine inhibition or antagonism, however, will require a more comprehensive understanding of inflammatory cytokine expression during the early stages of disease development than is currently available. Therefore, the major goals of this thesis research were to (1) characterize patterns of TNFα, IL-1β, and IL-8 gene and protein expression within the lungs of experimentally infected calves, (2) identify drug agents capable of suppressing inflammatory cytokine expression by bovine alveolar macrophages exposed to *M. haemolytica* LPS and LktA in vitro, and (3) determine whether the most effective of these cytokine inhibitors is capable of reducing clinical disease and pulmonary pathology in vivo.

The specific objectives of the first research goal were to (a) characterize the kinetics of pulmonary TNFα, IL–1β, and IL-8 gene and protein expression in the initial 24 hours of experimental BPM; (b) compare patterns of cytokine expression in airways with those in lung lesions; and (c) identify major cellular sources of these cytokines within affected lung. Northern analysis was used to quantitate the expression of cytokine mRNA in bronchoalveolar lavage (BAL) cells and diseased lung parenchyma. Immunoreactive cytokines in BAL fluid and lung tissue extracts were measured by enzyme-linked immunosorbent assay

(ELISA). In situ hybridization was used to localize the expression of cytokine mRNA within lung tissues; numbers of positively staining cells were enumerated by quantitative morphometric techniques, and staining cells were identified by cell type on the basis of cell morphology and location.

The specific objective of the second research goal was to investigate the ability of six different pharmacological agents to inhibit in vitro expression of TNFα, IL–1β, and IL–8 genes and proteins by bovine alveolar macrophages exposed to *M. haemolytica* LPS and LktA. The drugs tested included (1) dexamethasone, a synthetic glucocorticoid; (2) tetrahydropapaveroline, an antioxidant compound; (3) pentoxifylline, a non-specific phosphodiesterase inhibitor; (4) rolipram, a type IV phosphodiesterase inhibitor; (5) SB203580, a bicyclic imadazole; and (6) thalidomide, an immunomodulatory sedative drug. These agents were selected on the basis of their demonstrated ability to suppress the production of one or more inflammatory cytokines in other experimental systems. Immunoreactive cytokines in the cell culture supernatant were measured using antigen-capture ELISA, and cytokine-specific gene expression was measured using Northern analysis.

The specific objective of the third research goal was to evaluate the ability of the most effective inhibitor of in vitro cytokine expression to ameliorate disease development in an in vivo experimental model of BPM. Disease was induced in the left lungs of six healthy Holstein calves by endobronchial administration of *M. haemolytica*. Four experimental calves received systemic therapy with a cytokine inhibitor prior to infection and in the early stages of disease, while two placebo-treated control calves received dose-matched

volumes of sterile saline. Clinical disease was characterized using a nonparametric scoring system, and the extent of gross pulmonary pathology affecting the left lung was calculated using morphometric methods. Histopathological lesions in the left lung of each calf were also characterized.

This thesis is organized and presented in six chapters. This general introduction is followed by a review of the literature pertinent to *M. haemolytica*, BPM, and inflammatory cytokines. The third, fourth, and fifth chapters detail the methods and results of the original research performed in the course of my graduate program. Each of these chapters is presented in the format required by the scientific journal in which it has been published, or to which it will be submitted. The format of Chapter 3 conforms to that of *Veterinary Pathology*, in which the original research was published. The format of Chapters 4 and 5 conforms to that of *Microbial Pathogenesis*, in which the original research was published. The sixth chapter is devoted to a general discussion of this research and its major conclusions.

CHAPTER 2

LITERATURE REVIEW

BOVINE PNEUMONIC MANNHEIMIOSIS

Introduction

Bovine pneumonic mannheimiosis (BPM) is an acute fibrinonecrotic pleuropneumonia that is common among cattle throughout North America, Europe, and the United Kingdom. The disease was first described in the United States in 1915 and in the United Kingdom in 1925.[113, 125, 144] The pathophysiology of the disease is complex and multifactorial, but it is generally accepted that *Mannheimia haemolytica* is the primary etiologic agent responsible for pulmonary pathology.[10, 69, 92, 224, 321, 328] Until recently, the disease was known as pneumonic pasteurellosis, reflecting the causative bacterium's prior classification within the genus *Pasteurella*.

Mannheimia haemolytica is a Gram-negative pleomorphic coccobacillus within the family Pasteurellaceae. It is a nonmotile aerobic organism whose colonies exhibit a zone of hemolysis when cultured on blood agar.[295] In the past, the organism was known as *Pasteurella haemolytica.* The genus *Pasteurella* was further divided into 2 biotypes, A and T, based on the ability to ferment arabinose and trehalose, respectively.[176] Twelve A serotypes and four T serotypes were recognized.[299] In 1990, using data from DNA-DNA hybridization studies, biochemical properties, and genetic analysis, the T biotypes were reclassified as a separate species named *Pasteurella trehalosi*. More recently, in 1999, using data from ribotyping, multi-locus enzyme electrophoresis, 16S ribosomal RNA sequence comparison, and DNA-DNA hybridization, the *P. haemolytica* A serotypes were reclassified into a new genus, *Mannheimia*, and the previously

recognized A serotypes of *P. haemolytica* were renamed *Mannhemia haemolytica*.[13] *M. haemolytica* serotypes 1 (ST1) and 6 (ST6) are the most common serotypes isolated from the lungs of cattle that die of BPM.

Pure cultures of *M. haemolytica* may be isolated from pneumonic lesions in naturally occurring cases. In addition, the disease can be reproduced experimentally in calves by intratracheal,[10] endobronchial,[311] or transthoracic intrapulmonic[211] inoculation of *M. haemolytica*, as long as a minimum of 5×10^9 colony-forming units of logarithmic phase organisms are delivered. In the absence of concurrent environmental stress and/or viral respiratory infection, it is difficult to reproduce the disease experimentally through intranasal inoculation of bacteria. Natural disease is therefore believed to be the result of a poorly characterized interaction between *M. haemolytica* and stressful management practices, extreme environmental conditions, and/or viral respiratory infection.

Despite recent progress in our understanding of the disease, BPM remains a leading source of economic losses to the North American beef and dairy industries. Estimates of annual losses attributable to infectious respiratory disease in the United States, of which pneumonic mannheimiosis is considered to be the most important clinical entity, range between $640 million and $4 billion, exceeding losses from all other diseases combined.[9, 142, 329, 331] Losses are the combined result of treatment costs, death of affected animals, and impaired production performance among those that survive.

Epidemiology

Pneumonic mannheimiosis affects cattle throughout North America, Europe, and the United Kingdom. In the United States and Canada, incidence is highest among weaned beef calves recently introduced into feedlots, but dairy calves are also commonly affected. Nursing beef calves, yearlings, and adult cattle develop the disease much less frequently. Despite the undisputed economic importance of BPM, surprisingly little has been established regarding the behavior of the disease at the population level. A review of the literature published prior to 1985 revealed that most feedlot studies were plagued by deficiencies and inconsistencies in case definitions and the methods used to determine morbidity and mortality rates.[143] Because of the difficulty and expense inherent in definitively diagnosing the causes of disease in feedlot cattle, the majority of those studies used crude mortality as an estimate of death losses due to shipping fever, and treatment rate as a measure of morbidity. The results of several necropsy surveys [120, 229] and at least one formal epidemiologic study,[231] however, indicate that crude mortality is unreliable as a surrogate measure of fibrinous pneumonia mortality and leads to erroneous conclusions regarding risk factors contributing to BPM.

There are currently no published reports of epidemiologic studies that specifically address BPM. An observational study of fatal fibrinous pneumonia in western Canadian feedlot calves conducted by Ribble *et al.* provides the most relevant information published to date.[230-234] Data were collected on 58,885 spring-born calves entering a single feedlot in southwestern Alberta between September 1 and December 31 over a 4-year period (1985-1988). The calves were

purchased at auction markets throughout western Canada and transported to the feedlot by truck. A complete necropsy examination was performed on all dead cattle within 24 hours of death, and a diagnosis of fatal fibrinous pneumonia was based on gross pathological evidence of acute lobar bronchopneumonia with a fibrinous exudate.

In this study, crude mortality ranged from 2.44% - 4.78% while mortality due to fibrinous pneumonia varied more than ten-fold between years (0.25% – 2.73%).[231] Proportionate mortality due to fibrinous pneumonia ranged from 10% - 57% between years, demonstrating that crude mortality is an inaccurate measure of fibrinous pneumonia mortality. Epidemic curves constructed for each of the 4 years revealed that peak mortality occurred approximately 16 days after arrival at the feedlot, and greater than 50% of all mortalities due to fibrinous pneumonia occurred within 3 weeks of arrival. Epidemic curves using the time of first treatment for all cases of fatal fibrinous pneumonia revealed that peak disease onset occurred within 8 days of arrival, and that 75% of the calves that died of fibrinous pneumonia were sick within 2 weeks of arrival. The consistent onset of fatal disease shortly after arrival at the feedlot suggests that the disease process may be well underway in affected calves prior to their installation in a home pen, and that preventive measures should be implemented at the time of arrival or possibly even before. When the incidence of fatal shipping fever was high (greater than 2%), disease was found to cluster within truckloads of calves and, in one year, within pens.[234] The study also documented extensive mixing of calves from different sources at the auction market, an interaction that is likely to influence, and possibly initiate, the development of fibrinous pneumonia.[232] The

study failed to identify an association between the risk of fatal disease and the distance over which calves were transported to the feedlot.[233]

Clinical signs

Affected cattle exhibit symptoms of acute bacterial bronchopneumonia and toxemia including fever, depression, inappetance, mucopurulent nasal discharge, ocular discharge, cough, and varying degrees of tachypnea and dyspnea.[220] Sudden death of affected animals without overt clinical signs may also occur. Chronically affected cattle may exhibit symptoms of chronic respiratory disease, weight loss, or impaired growth performance.

Pathology

Necropsy examination of affected cattle reveals acute fibrinonecrotic pleuropneumonia with an anteroventral lobar distribution.[106, 224] The pulmonary parenchyma may be extensively consolidated and exhibits multifocal coagulation necrosis. A marked fibrinous exudate is typically present on the pleural surface and within interlobular septae, and may be accompanied by a fibrinous pericarditis. Characteristic histopathological features of BPM include pronounced alveolar neutrophil influx and fibrin deposition; alveolar, interstitial, and interlobular edema; alveolar wall degeneration; platelet aggregation, neutrophil margination, and thrombus formation within alveolar capillaries; and extensive coagulation necrosis. Spindle-shaped "oat cells", which are believed to represent degenerate neutrophils, are commonly observed within the alveolar exudate. These gross and histopathological lesions have been reproduced

experimentally in calves through endobronchial,[311] intratracheal,[10, 264, 326] and transthoracic intrapulmonic[211] administration of logarithmic-phase *M. haemolytica*.

PATHOGENESIS OF BOVINE PNEUMONIC MANNHEIMIOSIS

Colonization and proliferation of *Mannheimia haemolytica*

Mannheimia haemolytica serotype 2 (ST2) is a normal inhabitant of the nasopharyngeal flora in healthy cattle and rarely causes disease.[101, 104] Exposure of cattle to stressful management practices (e.g. long distance transportation, overcrowding, food deprivation, or mixing of animals from different sources at auction), extreme environmental conditions, or viral respiratory infection, however, leads to selective proliferation of *M. haemolytica* ST1 and its rapid establishment as the dominant upper respiratory tract isolate.[102-104, 138] The specific host and bacterial factors mediating this shift from serotype 2 commensals to serotype 1 pathogens are currently unknown, but factors specific to serotype 1 may permit more extensive adherence and colonization of the upper respiratory tract mucosa.[117] Once colonization of the upper respiratory tract has been established, *M. haemolytica* ST1 invades the lower respiratory tract through repeated inhalation of bacteria in aerosol droplets.[119]

Virulence factors of *Mannheimia haemolytica*

Virulence factors are bacterial products that contribute to disease development through enhancement of bacterial colonization, invasion of host tissues, or impairment of host defenses. Virulence factors of *M. haemolytica* ST1 that contribute to the pathogenesis of pneumonic mannheimiosis in cattle include leukotoxin, lipopolysaccharide, capsular polysaccharide, fimbriae, outer membrane proteins, and neuraminidase. Of these, leukotoxin and lipopolysaccharide appear to be most important.

Leukotoxin

All known serotypes of *M. haemolytica* secrete leukotoxin (LktA), an exotoxic protein specific for ruminant leukocytes and platelets, during logarithmic-phase growth.[62, 140, 257, 259] It is a 105 kDa cytolysin within the Repeats in ToXin (RTX) family of Gram-negative exotoxins.[60, 73] Several lines of evidence derived from in vivo experiments support a central role for LktA in the pathogenesis of BPM. First, there is a positive correlation between the amount of LktA secreted by a challenge strain of *M. haemolytica* and the severity of experimental disease it induces.[10] Second, immunohistochemical studies showed that LktA is present within the inflammatory exudate in affected lungs, and is specifically associated with the membranes of degenerating leukocytes within alveoli.[325] Third, the clinical disease and pulmonary pathology induced by an isogenic leukotoxin deletion mutant strain of *M. haemolytica* are significantly less severe than those induced by the wild-type strain.[292] Fourth, a high level of

neutralizing serum antibody against LktA confers resistance to both natural and experimental disease.[54, 69, 71, 108, 258, 260, 272, 274]

The in vitro biological effects of LktA support these observations, and suggest that the toxin contributes to lung pathology through a variety of complex mechanisms. At high concentrations, the toxin impairs host pulmonary defenses by inducing cytolysis of neutrophils and macrophages, thereby preventing phagocytosis and bacterial killing by these cell types.[24, 28, 170] Lysis of neutrophils may also contribute to structural degradation of the lung through the extracellular release of reactive oxygen intermediates and proteolytic enzymes.[267] At subcytolytic concentrations, LktA activates macrophages[72, 129, 170, 337] and neutrophils[76, 124, 172], and induces the secretion of a variety of inflammatory mediators including reactive oxygen intermediates,[72, 171, 172] histamine,[124] eicosanoids,[59, 124, 317] nitric oxide,[337] and inflammatory cytokines.[129, 153, 336] In addition, exposure to subcytolytic concentrations of LktA reduces the respiratory burst capabilities of neutrophils[49] and inhibits the production of chemotactic factor by alveolar macrophages.[178] Leukotoxin may also contribute to pulmonary vascular injury in BPM, as hydroxyl radicals released from LktA-stimulated neutrophils mediated direct killing of bovine pulmonary artery endothelial cells in an in vitro coculture system.[171] Recent studies demonstrated that LktA induces apoptosis of leukocytes in vitro and in vivo, but the contribution of programmed cell death to disease pathogenesis remains unclear.[161, 279, 316]

The specific mechanisms by which LktA induces activation, cytolysis, and apoptosis of ruminant leukocytes have been the subject of active investigation for over 20 years. A comprehensive summary of the work in this field is beyond the

scope of this review, but several key findings are worthy of note. Early studies showed that the cellular effects of LktA are confined to leukocytes and platelets from ruminant species that are susceptible to *M. haemolytica* pneumonia, leading to the hypothesis that a specific receptor for LktA is present in susceptible cells, but not in resistant cells.[62, 70, 140, 257, 289] In addition, LktA-induced cytolysis and cellular activation are dependent on the presence of calcium in the extracellular culture medium and associated with a rise in intracellular [Ca^{2+}].[60, 61, 110] These observations led to speculation that LktA may act through the formation of cell membrane pores that permit an intracellular influx of calcium, and that leukocyte activation and cytolysis are in turn mediated through increased intracellular [Ca^{2+}]. Recent work conducted by our laboratory has confirmed that the effects of LktA are receptor-mediated, and that the receptor for LktA is the CD18 subunit of β2 integrins expressed on bovine leukocytes.[79, 82] Intracellular signaling following the interaction of LktA with LFA-1 results in activation of non-receptor tyrosine kinases, including Src and phosphotidylinositol kinases, and this activation is required for the LktA-induced elevation in intracellular [Ca^{2+}].[137]

Lipopolysaccharide

Lipopolysaccharide (LPS), or endotoxin, is the major structural component of the outer membrane of Gram-negative bacteria. The molecule interacts with membrane-bound CD14, soluble CD14, and β2 integrins on the surface of mammalian cells to activate signaling pathways that provoke a wide range of biological effects.[145, 195, 291, 301] Available evidence indicates that *M. haemolytica* LPS

contributes to the pathogenesis of BPM through a variety of complex mechanisms including the induction of inflammatory mediators, activation of the complement and coagulation cascades, enhancement of leukocyte adherence and infiltration, and direct toxic effects on the pulmonary endothelium.[328]

In vitro, *M. haemolytica* LPS stimulates bovine alveolar macrophages to secrete a broad array of inflammatory mediators including nitric oxide[337] and the inflammatory cytokines tumor necrosis factor-alpha (TNFα),[334] interleukin–1 beta (IL-1β),[334] and interleukin-8 (IL-8).[153, 196] It also stimulates lung parenchyma to release histamine and the eicosanoids leukotriene B4 (LTB4) and prostaglandin E_2 (PGE_2).[238] It is directly toxic to bovine pulmonary endothelial cells in vitro.[38, 213]

In vivo, immunohistochemical localization of LPS within the lungs of experimentally infected calves shows that it is released into the inflammatory exudate and is localized (1) in neutrophils within alveoli, the interstitial space, and capillaries; (2) in intravascular, interstitial, and alveolar macrophages; (3) in endothelial cells; and (4) on epithelial cell surfaces.[325] Endobronchial administration of purified *M. haemolytica* LPS alone is sufficient to cause neutrophil and fibrin exudation, pulmonary edema, and aggregation of platelets and neutrophils within capillaries.[327]

Lipopolysaccharide may also contribute to the pathogenesis of BPM through enhancement of the biological effects of LktA. Recent studies show that *M. haemolytica* LPS binds and stabilizes the LktA molecule,[77, 164] and that small amounts of LPS exert a synergistic effect on LktA-induced cytolysis and inflammatory cytokine induction.[154] Despite its important role in disease pathogenesis, however, LPS does not appear to be a significant protective

antigen. Although it is a potent immunogen, high levels of serum antibody against *M. haemolytica* LPS fail to confer resistance to experimental BPM.[68]

Capsular polysaccharide

Mannheimia haemolytica produces a serotype-specific polysaccharide capsule, or glycocalyx, during logarithmic-phase growth.[192] Although there is little evidence to support a major role for the capsule in disease pathogenesis, evidence from a variety of in vitro and in vivo studies indicates that capsular polysaccharide is involved in adherence of *M. haemolytica* to the respiratory mucosa, attraction of neutrophils to sites of bacterial colonization, inhibition of phagocytosis by neutrophils, and resistance to complement-mediated bacteriolysis.[67, 75, 193]

Fimbriae

Many Gram-negative bacteria possess cell-surface fimbriae, or pili, that function in bacterial adherence and the colonization of mucosal surfaces. These filamentous structures are comprised of protein adhesins that recognize and bind to specific glycoproteins or glycolipids on the epithelial cell surface. *M. haemolytica* A1 produces two different types of fimbriae; one is large (12 nm in diameter) and rigid, while the other is smaller (5 nm in diameter) and more flexible.[191-193] It is assumed that these structures enhance adherence of *M. haemolytica* to the respiratory epithelium, but the relative importance of fimbriae in the pathogenesis of BPM has not been determined. It is not known whether *M. haemolytica* serotypes other than A1 also produce fimbriae.

Outer membrane proteins

Mannheimia haemolytica produces several outer membrane proteins (OMP), including PomA, PomB, and Lpp38.[169, 212] These proteins serve as chemotactic factors and inhibit neutrophil adherence, phagocytic function, and intracellular killing in vitro.[133] They may also be important protective antigens, as there is a correlation between high levels of serum antibody against OMP and resistance to experimental BPM.[198] In addition, 3 major iron-regulated outer membrane proteins (IROMP) have been identified in *M. haemolytica* cultured under iron-restricted conditions.[189] Two of these, Tbp1 and Tbp2, are transferrin-binding proteins while the function of the third remains known. These proteins enhance bacterial colonization and invasion by allowing uptake of sufficient iron to support bacterial growth and LktA production in an iron-restricted host environment.[109, 189]

Neuraminidase

Neuraminidase secreted by *M. haemolytica* is believed to enhance bacterial colonization of the upper respiratory tract through alteration of the mucosal microenvironment. It catalyzes the removal of sialic acid from cell surface glycoproteins and decreases the viscosity of bovine respiratory mucus, undermining its protective effects and promoting adherence of *M. haemolytica* to the upper respiratory epithelium.[66, 280] In vitro, *M. haemolytica* A1 secretes greater amounts of neuraminidase than does *M. haemolytica* A2.[290]

Pulmonary inflammatory responses to *Mannheimia haemolytica*

While the clinical and pathological features of BPM have long been clearly defined, the specific cellular and molecular mechanisms contributing to their development are complex and incompletely understood. Interestingly, the bacterial infection itself does not appear to be directly responsible. Although *M. haemolytica* LktA and LPS are capable of inducing some degree of direct pulmonary pathology, there is mounting evidence that an uncontrolled host inflammatory reaction to the bacterium and its products is the principal cause of lung injury.

Alveolar macrophages are the major phagocytic defense mechanism of the terminal bronchioles and alveoli. When the lung is challenged with low numbers of bacteria or organisms of relatively low virulence, resident alveolar macrophages alone are capable of ingesting and killing the invading microbes.[223] If the bacterial burden is large (> 10^6 organisms) or if virulent Gram-negative organisms gain access to the alveolus, neutrophil recruitment is essential for effective containment and clearance of the infection. In addition to their phagocytic function, alveolar macrophages secrete a broad array of soluble mediators that regulate the responses of other pulmonary cell types.

Available evidence indicates that alveolar macrophages play a central role in the pulmonary inflammatory response to *M. haemolytica*. In vivo, neutrophils and fibrin may be observed adjacent to alveolar macrophages in affected lungs within 4 hours of experimental infection.[326] Similarly, aggregated platelets, neutrophils, and fibrin may be observed in close association with intravascular macrophages in the capillary bed.[326] In vitro, *M. haemolytica* and its products

activate bovine alveolar macrophages and elicit secretion of a variety of inflammatory mediators. At subcytolytic concentrations, LktA activates alveolar macrophages[170] and induces them to secrete reactive oxygen intermediates[72] and the inflammatory cytokines TNFα,[129, 154] IL-1β,[129] and IL-8.[129, 154] Purified *M. haemolytica* LPS is also a potent activator of these cells, and induces the secretion of nitric oxide[337], TNFα,[129, 153, 154, 278, 334] IL–1β,[129, 278, 334] and IL-8.[129, 153, 154, 196] Exposure of alveolar macrophages to heat-killed whole *M. haemolytica* also provokes the secretion of TNFα, IL-1β, and IL-8.[196, 197]

One of the hallmark histopathological features of BPM is extensive infiltration of the lungs by neutrophils. The early pulmonary response to *M. haemolytica* has been well characterized and studies show that neutrophil influx begins within 2 hours of experimental infection. [264, 265, 314, 322, 326] Neutrophil depletion prior to experimental infection protects calves from subsequent lung injury, indicating that this cell type is directly or indirectly responsible for most of the pulmonary pathology observed in the disease.[265, 322] The mechanisms by which neutrophils mediate lung pathology in BPM remain undefined but are likely to include oxidative injury and structural degradation due to extracellular release of reactive oxygen intermediates and proteolytic lysosomal enzymes.[267] Recognition of the importance of neutrophils in disease pathogenesis has prompted the investigation of specific factors governing their recruitment and activation within *M. haemolytica*-infected bovine lung.

The migration and activation of neutrophils in inflamed tissue are regulated by a complex network of interactions between cytokines, leukocytes, vascular endothelium, cellular adhesion molecules, and soluble activating and

chemotactic factors. The inflammatory cytokines TNFα, IL-1β, and IL-8 play a central role in the initiation and orchestration of these interactions. TNFα and IL–1β are pleiotropic early response polypeptides secreted by monocytes and macrophages in response to microbial pathogens and other stimuli.[155] IL-8, a potent chemotactic and activating factor for neutrophils, is secreted by a variety of immune and nonimmune cell types, including monocytes, macrophages, fibroblasts, epithelial cells, and neutrophils.[21, 150] Available evidence indicates that these cytokines also play an important role in pulmonary inflammatory responses, as discussed below.

INFLAMMATORY CYTOKINES IN DISEASE PATHOGENESIS

Introduction to cytokines

Cytokines are a diverse group of secreted regulatory proteins that control the survival, growth, differentiation, and effector functions of cells throughout the body. They encompass the families of peptides and proteins variously known as growth factors, colony-stimulating factors, interleukins, lymphokines, monokines, chemokines, and interferons, but are not usually considered to include the classical endocrine hormones.[203] Compared to the endocrine hormones, cytokines tend to act upon a wider spectrum of target cells, exert their biological effects at lower concentrations and for shorter periods of time, and operate locally in the tissues rather than systemically through the bloodstream.

Unlike the cells that produce endocrine hormones, those that produce cytokines are not organized into specialized glands.

Cytokines rarely resemble one another at the level of their primary sequence, but may be loosely organized on the basis of structural similarities at the secondary and tertiary levels.[127] Those that share significant structural features often interact with related receptors and provoke similar biological effects. Most cytokines are glycoproteins that are secreted through classical secretory pathways.[132] Membrane-bound forms have been recognized for many cytokines, however, and switching between soluble and membrane forms may represent an important regulatory event. In some cases, membrane forms of a cytokine are indispensable for normal development, with soluble forms being unable to substitute for them. In general, cytokines are not stored intracellularly, but are synthesized *de novo* in response to specific cellular stimuli. Their biological effects are typically short-lived, as induced expression is transient and secreted cytokines are susceptible to rapid degradation in the extracellular environment. The expression of most cytokines is tightly regulated rather than constitutive, and regulation occurs at all levels of gene expression.

The biological effects of cytokines are mediated through specific cell-surface receptor proteins that are expressed on virtually all known cell types. Many receptors are comprised of multiple subunits that are capable of both binding ligand and transducing intracellular signals through their intrinsic tyrosine kinase function. In addition, cytokine receptors may be associated with specialized intracellular proteins involved in signal transduction. Specific receptor subunits may be shared by several different receptors, and a given

receptor may bind more than one cytokine. Several receptors are converted into soluble binding proteins through proteolytic cleavage of the extracellular domain. At least four families of receptors may be defined on the basis of similarity in primary sequence, predicted secondary and tertiary structure, and biochemical function. These are the hemopoietin/interferon receptor family, the tumor necrosis factor/nerve growth factor family, the receptor kinase family, and the family of G-protein coupled, seven membrane-spanning receptors.[127]

Cytokines were initially thought to operate according to a simple "one producer cell – one cytokine – one target cell" model, but recognition of a high degree of pleiotropism and functional redundancy in their effects has led to the evolution of a much more complex model of cytokine action.[132] Individual cytokines frequently provoke different biological effects in different target cells. Furthermore, the responses of a particular target cell to a given cytokine are often highly context-specific, and may vary greatly depending on the cell's microenvironment, stage of development or activation, and concurrent exposure to other cytokines or regulatory substances. To complicate matters further, the effects of different cytokines often overlap; there are few cellular responses that are mediated by only one cytokine and many that can be induced by several different cytokines. The prevailing model, the "cytokine network", emphasizes these complex and context-specific patterns of interaction between cytokine-producing cells, secreted cytokines, target cells, and their induced responses.[22] In particular, cytokines often act through hierarchical cascades in which early-expressed factors activate target cells and trigger the production of one or more

additional cytokines that, in turn, provoke expression of further factors and establish complex regulatory feedback circuits.

Tumor necrosis factor-alpha

Introduction

Tumor necrosis factor-alpha (TNFα) was first discovered in 1975 as an endotoxin-induced serum factor that caused hemorrhagic necrosis of experimentally induced intradermal tumors in mice.[44] In 1986, the macrophage-derived factor cachectin, previously implicated in wasting syndromes associated with chronic disease states, was identified as an important mediator of tissue injury in septic shock.[32, 297] Subsequent cloning and sequencing of the genes encoding TNFα and cachectin confirmed that they were the same molecule.[31, 214] Historically, TNFα was also known as macrophage cytotoxin, macrophage cytotoxic factor, necrosin, hemorrhagic factor, and differentiation-inducing factor.[1]

Cellular sources

TNFα is secreted by macrophages, monocytes, hepatic Kupffer cells, neutrophils, B and T lymphocytes, NK cells, fibroblasts, mast cells, smooth muscle cells, astrocytes, epidermal cells, and adipocytes.[1, 271] Secretion is induced by a variety of stimuli including Gram-negative and Gram-positive bacteria and their products (e.g. lipopolysaccharide, muramyl peptides); viruses; mycoplasmas; immune complexes; complement; protein kinase C activators; reactive oxygen intermediates; and other cytokines such as interferon-gamma

(IFNγ), interleukin–2 (IL-2), granulocyte-macrophage colony stimulating factor (GM–CSF), tachykinins, bradykinin, and platelet activating factor (PAF).[1, 271]

Molecular features

In humans, mice, and rabbits, TNFα is synthesized as a 233 amino acid (25 kDa) precursor that is subsequently cleaved to a 157 amino acid (17 kDa) mature protein.[43, 105, 214] Except in mice, the protein is non-glycosylated.[43, 55, 105, 134] In vivo, soluble TNFα exists primarily as a homotrimer.[268] The amino acid sequences of human, murine, rat, rabbit, feline, caprine, bovine, and porcine TNFα are 70-90% homologous.[270] Interspecies homology at the genetic level is also high; human, murine, porcine, and rabbit cDNAs are 77-86% homologous.[55] Like many inflammatory cytokines, including IL-1β and IL-8, the 3'-untranslated region of TNFα mRNA contains an A/U-rich segment that increases message instability, contributing to transient expression.[43]

Receptors

High-affinity receptors for TNFα are expressed on all somatic cell types with the exception of erythrocytes.[2, 32, 128, 269, 300] Two distinct receptors, p55 (55 – 60 kDa) and p75 (75 – 80 kDa), have been characterized. The p55 form of the receptor is primarily expressed on epithelial cells, whereas the p75 form is expressed mainly on myeloid cells.[167, 246] TNFα and TNFβ (lymphotoxin) bind to both p55 and p75 with equal affinity. Both receptors are members of the tumor necrosis factor/nerve growth factor family of receptors.[127] The extracellular domains of both receptors are highly susceptible to cleavage by proteases

activated by kinases and phosphatase inhibitors, and the resulting soluble forms of p55 and p75 have been detected in cell-conditioned media, urine, serum, amniotic fluid, and synovial fluid.[3]

Endogenous inhibitors

Soluble forms of the p55 and p75 receptors inhibit the biological effects of TNFα.[96, 205, 252] Their capacity to neutralize cytokine is limited, however, and very high concentrations are required to protect against induced pathological effects in vitro and in vivo.[308] In addition, the production and secretion of TNFα is inhibited by a variety of endogenous substances including interleukin–4 (IL-4), interleukin–6 (IL-6), transforming growth factor-beta (TGFβ), vitamin D_3, and prostaglandin E_2.[1, 271]

Biological effects

TNFα is a pleiotropic cytokine involved in a wide range of physiological and pathological processes including inflammation, immunity, tissue metabolism, wound repair, and growth and development.[30] Its in vitro biological effects include inhibition of proliferation of certain tumor cells; antiviral effects against both DNA and RNA viruses; induction of endothelial cell adhesion molecules; induction of plasminogen activator and its inhibitor; neutrophil activation; myeloid cell differentiation; and induction of other cytokines.[29, 271] In vivo, TNFα is involved in host responses to viral, bacterial, and parasitic infections. It contributes to the pathogenesis of a variety of diseases including septic shock, rheumatoid arthritis, cerebral malaria, and multiple sclerosis. In

addition, it inhibits the growth of certain tumors and promotes growth and metastasis of others.[29, 271] This review will focus on the role of TNFα as an endogenous mediator of local and systemic inflammation.

Activated cells of the monocyte-macrophage lineage are the major source of TNFα.[31] The protein is not constitutively expressed under physiological conditions but is rapidly secreted following exposure to Gram-negative bacteria or LPS. Its in vivo biological effects are a combination of its direct effects on target cells bearing specific receptors for the molecule and indirect effects mediated by a multitude of TNFα-induced secondary cytokines and other inflammatory mediators. This is frequently referred to as the "cytokine cascade", in which TNFα is believed to be an important proximal mediator. An extensive body of literature supports a central role for TNFα in the initiation and regulation of local inflammatory responses throughout the body. Its effects are mediated largely through mechanisms that influence both the cellular and vascular components of inflammation. It affects the cellular component of inflammation by activating monocytes, neutrophils, and lymphocytes, and by regulating the local recruitment of these leukocytes through expression of TNFα-induced endothelial adhesion molecules and chemotactic cytokines. It affects the vascular component of inflammation by acting on endothelial cells to increase local blood flow and vascular permeability, and by inducing procoagulant activity. TNFα serves as an autocrine immunomodulator that activates macrophages and enhances their cytotoxic potential.[216] It is also chemotactic for monocytes, suggesting that its production at a site of infection or inflammation functions both to recruit additional macrophages and to activate those

macrophages already present. Experimental administration of TNFα into diverse organs has been reported to cause both acute neutrophilic[200, 302, 318] and chronic or mononuclear cell inflammation.[200]

While localized physiological expression of TNFα within the tissues plays an important role in host defense, systemic overexpression of this potent cytokine is deleterious to the host. Several lines of evidence support TNFα as a principal mediator of septic shock. First, serum concentrations of TNFα are frequently elevated in humans and laboratory animals with experimental or naturally occurring septic shock,[99, 312] and there is a correlation between increased serum levels and a poor clinical outcome.[99, 313] Second, administration of purified LPS to humans and laboratory animals provokes a rapid elevation of serum TNFα levels.[99, 184] Third, administration of purified recombinant TNFα to humans or experimental animals induces many of the pathophysiological alterations that are characteristic of septic shock, including hypotension, peripheral vasodilation, and ischemic organ damage.[226, 297] Fourth, administration of antibodies specific for TNFα protects against the lethality of LPS and gram-negative bacteremia [33, 180, 298]

Available evidence indicates that TNFα is also a principal mediator of local inflammation within the lung. Alveolar macrophages are the dominant cellular source of TNFα in the lung.[302, 319] Purified TNFα provokes pulmonary inflammation and increased vascular permeability following intratracheal[302] or intravenous administration.[277, 297] Furthermore, systemic increases in endogenous TNFα also provoke pulmonary injury. Endogenous TNFα induced by intraperitoneal injection of LPS in adjuvant-primed mice is associated with

pulmonary neutrophil influx, and this influx was inhibited by anti-TNF antibodies.[227] Similarly, hepatic ischemia and reperfusion in the rat is associated with increased levels of circulating TNFα that induce pulmonary edema and leukocyte accumulation.[64, 65]

The major target cells for TNFα within the lung include neutrophils, monocytes/macrophages, endothelial cells, and fibroblasts. Neutrophils are not normally present in significant numbers in the lung under physiological conditions, but become the dominant inflammatory leukocyte during acute bronchitis and pneumonia. Neutrophils express approximately 6000 high-affinity TNFα receptors per cell and are highly responsive to this cytokine.[255] TNFα upregulates neutrophil functions such as antibody-dependent cellular cytotoxicity, phagocytosis, hypochlorous acid production, and the release of oxygen radicals and lysosomal enzymes.[146, 243, 255, 324] TNFα also enhances neutrophil adhesion and phagocytosis through upregulation of receptors for complement components 1 and 3.[27]

In addition to serving as the major source of TNFα within the lung, monocytes and macrophages are important target cells for this cytokine. It activates them to enhance their bactericidal activity, express lipid mediators of inflammation, and amplify the inflammatory response through further elaboration of TNFα and other cytokines.[81, 210, 216]

Endothelial cells play an important role in the orchestration of inflammatory responses within the lung and other tissues. TNFα enhances local blood flow by inducing the endothelial expression of vasodilators such as prostaglandins and nitric oxide, which relax vascular smooth muscle.[219]

In addition, TNFα stimulates endothelial cells to express adhesion molecules that mediate leukocyte recruitment during inflammatory responses.[52, 219] It also amplifies the inflammatory response by upregulating endothelial cell production of TNFα, IL-1β, and IL-8.[201, 283]

Fibroblasts are present in large numbers beneath the epithelial lining of bronchi and in smaller numbers within the pulmonary parenchyma. TNFα exerts mitogenic effects on fibroblasts and contributes to the development of pulmonary fibrosis in experimental models of fibrosis.[217, 218] In addition, TNFα induces fibroblasts to express prostaglandin E_2 and chemotactic cytokines such as IL-8 and monocyte chemotactic protein.[140, 283]

Interleukin-1

Introduction

The term interleukin-1 (IL-1) encompasses two distinct peptides, IL–1α and IL-1β, which share receptors and biological properties. Several lines of evidence led to the discovery of IL-1. In 1949, a pyrogenic mediator (endogenous pyrogen) secreted by activated peritoneal exudate cells was described.[126] In 1971 and 1972, a peptide secreted by activated mononuclear phagocytes (lymphocyte activating factor) was identified as a costimulator of T lymphocyte proliferation.[111, 112] In 1984, a factor known as leukocyte endogenous mediator was described as a modulator of the acute phase inflammatory response.[141] These three factors were determined to be identical in 1984, and the name interleukin–1 (IL–1) was adopted.[83, 141] Historically, IL–1 was also known as catabolin,

osteoclast activating factor, hepatocyte stimulating factor, fibroblast activating factor, B cell activating factor, and haemopoietin–1.[89]

Cellular sources

IL-1 was originally described as a product of activated mononuclear phagocytes, but it is now clear that most nucleated cell types are capable of synthesizing the cytokine in response to an appropriate stimulus. Secretion may be induced by a variety of cell type-specific stimuli including LPS, TNFα, IL–1, leukotrienes, IFNγ, complement component 5a, muramyl dipeptide, phorbol myristate acetate, and phytohemagglutinin.[83, 209] Consistent with its central role in host defense, IL-1 acts upon a wide range of target cells including monocytes, macrophages, neutrophils, fibroblasts, endothelial cells, smooth muscle cells, hepatocytes, B and T lymphocytes, and keratinocytes.

Molecular features

IL-1α and IL-1β are synthesized as precursor proteins of approximately 270 amino acids (35 kDa) that are subsequently cleaved to 150 – 160 amino acid (17 kDa) mature proteins.[118, 168, 177] Neither cytokine exhibits the N-terminal signal sequence that is typical of secreted proteins.[15, 118, 168, 177] IL-1α appears to exist primarily as a cell-surface molecule that functions in cell-cell interactions, while IL-1β is secreted as a soluble extracellular mediator that can act locally or systemically.

Murine IL-1α and human IL-1β cDNAs were first cloned in 1984.[15, 168] Since then, IL-1 cDNAs from several other species have been cloned, including rats,

rabbits, swine, sheep, and cattle. A gene duplication that occurred approximately 270 million years ago is believed to be responsible for the divergence of the IL–1α and IL–1β genes.[341] The amino acid sequence of bovine IL–1α[162, 174] is 73%, 82%, and 97% homologous with murine,[118, 168] human,[177] and ovine IL–1α,[12] respectively. The bovine IL-1α cDNA is 62%, 71%, and 73% homologous with the murine, rabbit, and human sequences, respectively. The amino acid sequence of bovine IL–1β[174] is 69%, 69%, 76%, and 93% homologous with murine,[118] porcine,[131] human,[177] and ovine IL–1β,[12] respectively. Interspecies homology at the genetic level is also high; human, murine, porcine, ovine, and bovine cDNAs are 74-82% homologous.[131] By contrast, bovine IL–1α and IL–1β share only 23% homology.[174] Similar to TNFα and IL-8, the 3'–untranslated region of the IL–1β mRNA contains an A/U-rich segment that increases message instability, contributing to transient expression.[43]

Receptors

Most nucleated cell types express surface receptors for IL-1.[90] Two distinct receptors for IL-1 have been characterized. The type I receptor, p80 (80 kDa), binds IL–1α and IL-1β with equal affinity. This receptor is expressed predominantly on T lymphocytes and cells of mesenchymal origin, and is believed to mediate all of the known biological effects of IL-1.[263] The type II receptor, p60 (60 kDa), binds IL-1 but does not trigger intracellular signal transduction, and therefore inhibits the biological actions of IL–1 by preventing its interaction with the type I receptor. The type II receptor is encoded by a

separate gene, and is expressed predominantly on B lymphocytes and myeloid cells.[34, 53] Both receptor types also bind the IL–1 receptor antagonist (IL–1ra).

Endogenous inhibitors

IL-1 receptor antagonist (IL–1ra) is a soluble peptide that binds to both IL–1 receptor types but does not trigger intracellular signal transduction.[85, 251] It is structurally similar to IL-1, but is regulated independently and appears to function as a natural inhibitor of inflammatory responses. Infectious processes that stimulate increased levels of IL-1 are also associated with increased levels of endogenous IL-1ra, and induced expression of IL-1ra may greatly exceed that of IL-1β.[86] In addition, increased concentrations of IL-1ra may persist for longer periods of time than IL-1β, limiting the duration of the proinflammatory effect. In vivo, the administration of IL-1ra reduces the severity of infectious disease, perhaps through blockade of IL-1- and LPS-induced IL-8 production.[84]

The type II IL-1 receptor, which binds cytokine but does not signal intracellularly, inhibits the biological effects of IL-1β by preventing its interaction with the type I receptor.[263] It can do so as either a surface receptor or a soluble molecule subsequent to receptor shedding.[114]

Biological effects

IL-1α and IL-1β are pleiotropic cytokines involved in the initiation and regulation of local and systemic inflammatory responses.[83, 141] Like TNFα, IL–1 plays a central role in tissue repair and remodeling, in which it coordinates the responses of many cell types including endothelial cells, granulocytes,

osteoclasts, fibroblasts, hematopoietic cells, and lymphoid cells.[155] In addition, IL–1 appears to be an important mediator of septic shock. Experimental administration of lipopolysaccharide provokes increased circulating concentrations of IL-1β as well as increased local expression in a wide variety of tissues, especially the liver, spleen, and lung.[58] Administration of purified IL–1 to humans and laboratory animals induces clinicopathological alterations that mimic those of septic shock: fever, anorexia, neutropenia, and hypotension.[84] Likewise, administration of IL-1ra prevents lethal endotoxic shock in a variety of animal models.[5, 206]

The local inflammatory effects of IL–1 are due largely to its effects on leukocytes and the vascular endothelium, which collectively result in the recruitment and activation of leukocytes, particularly neutrophils, at sites of tissue infection and inflammation. Although the cytokine is not directly chemotactic, it upregulates the expression of adhesion proteins on leukocytes and endothelial cells,[199] and induces the expression of IL-8, the most potent chemotactic and activating factor for neutrophils, by a variety of cell types.[275, 283] IL–1 also primes neutrophils for oxidant production and induces the production of arachidonic acid metabolites, nitric oxide, and other soluble inflammatory mediators.

In addition to its local proinflammatory effects, IL–1 exerts several important systemic effects. It is a potent endogenous pyrogen, and an important proximal mediator of the hepatic acute phase response. In vitro and in vivo, IL–1 upregulates hepatocyte synthesis of fibrinogen, complement components,

metallothioneins, and coagulation factors. This effect is mediated largely through the intermediate production of IL-6.

The role of IL-1, if any, in the normal healthy lung is poorly defined. The cytokine does appear to play an important role in infectious and inflammatory disease of the lung, however. It may also play a role in regulating normal tissue repair and response to injury. In animal models, intravenous infusion of large amounts of IL-1 produces acute lung injury similar to adult respiratory distress syndrome.[116] In addition, intravenous administration of IL-1ra protects rabbits from fatality associated with endotoxin-induced shock.[206] These observations suggest that IL-1 may play an important role in the pathogenesis of the adult respiratory distress syndrome.

Increased IL-1 activity in the lungs of patients with pulmonary sarcoidosis is thought to contribute to the initiation and maintenance of the granulomatous inflammation that is characteristic of this disease.[294, 332] Available evidence also supports a role for IL-1 in the pathogenesis of other interstitial lung diseases, including idiopathic pulmonary fibrosis,[332] silicosis,[91] asbestosis,[122] and bleomycin-induced lung disease.[139] These observations suggest that IL–1 contributes to the pathogenesis of a variety of lung diseases in humans and laboratory species.

Interleukin-8

Introduction

Interleukin-8 (IL-8), a chemotactic cytokine (chemokine) with potent neutrophil activating properties, was independently isolated by several different

laboratories.[249, 305, 315, 338] Chemokines are broadly divided into three families (α, β, and γ) based on the presence and position of conserved cysteine residues. In members of the α family, the first two N-terminal cysteine residues are separated by another amino acid (CXC), while in those of the β family they are placed immediately next to each other (CC). By contrast, members of the γ family contain only one N-terminal cysteine residue (C). IL-8 is a member of the α-chemokine group, along with NAP-2, GRO-α, GRO-β, GRO-γ, ENA-78, and GCP-2. It is generally considered to be the most potent chemotactic and activating factor for neutrophils. Historically, IL-8 was also known as neutrophil activating protein-1 (NAP-1), monocyte-derived neutrophil chemotactic factor (MDNCF), and neutrophil activating factor.

Cellular sources

IL-8 is secreted by a wide variety of cell types including monocytes, tissue macrophages, neutrophils, fibroblasts, T lymphocytes, smooth muscle cells, epithelial cells, and endothelial cells.[304] The cytokine was originally isolated from peripheral blood monocytes stimulated with lipopolysaccharide or mitogen. Fibroblasts, smooth muscle cells, endothelial cells, and epithelial cells, however, produce IL–8 primarily in response to the cytokines TNFα and IL-1.[304]

Molecular features

Human,[181] porcine,[166] bovine,[153, 196] and rabbit[340] IL-8 cDNAs have been cloned, and approximately 75% sequence homology exists between species at the amino acid level. The cytokine is synthesized as a 99 – 103 amino acid precursor

protein with a putative signal sequence that is subsequently cleaved to yield a 72 – 80 amino acid (8 kDa) non-glycosylated mature protein.[303] At least four N-terminal variants of human IL-8 containing 69, 72, 77, and 79 amino acids have been described, of which the 72 amino acid form is most biologically active.[339] Interestingly, mice and rats do not appear to express IL–8.

Receptors

Two distinct IL-8 receptors have been characterized. The high-affinity (type I) IL-8 receptor, which is densely expressed on the surface of human neutrophils, is a dimeric glycoprotein consisting of a 59 kDa and a 67 kDa subunit.[240] It has been designated CDw128 and is a G-protein coupled receptor. The low-affinity (type II) IL-8 receptor also binds other members of the α-chemokine family.[47] To date, no endogenous inhibitors or IL-8 receptor antagonists have been identified.

Biological effects

IL-8 plays a key role in the accumulation of leukocytes, particularly neutrophils, at sites of inflammation. In vitro, is chemotactic for neutrophils, basophils, and T lymphocytes at nanomolar, nanomolar, and picomolar concentrations, respectively.[21, 156, 182] At nanomolar to millimolar concentrations, IL–8 provokes activation of neutrophils, which results in exocyctosis of lysosomal enzymes; initiation of the respiratory burst; elevated concentrations of intracellular ionized calcium; changes in cellular shape; production and release of arachidonic acid metabolites and leukotrienes; and upregulation of expression

of the cell-surface β2 integrin CD11b/CD18 complex.[286] IL–8 also activates primed basophils to release histamine and leukotriene C_4.[78] In vivo, intradermal or intraperitoneal injection of purified IL-8 induces a marked influx of neutrophils in rabbits and mice, and this neutrophil infiltration is associated with endothelial injury, plasma leakage, and edema formation.[107, 221] Systemic administration of the cytokine in rabbits also provokes significant granulocytosis.[305]

Available evidence indicates that IL-8 also plays a central role in the pulmonary inflammatory response. Several cell types within the lung are capable of producing IL–8, including intravascular, interstitial, and alveolar macrophages; endothelial cells; epithelial cells; fibroblasts; and smooth muscle cells.[21, 275, 281, 283, 284] Exposure of alveolar macrophages, monocytes, and endothelial cells to LPS, IL–1, or TNFα provokes time- and concentration-dependent IL–8 gene expression. By contrast, pulmonary smooth muscle cells, fibroblasts, and epithelial cells do not produce IL–8 in response to LPS, but instead require a specific host-derived signal in the form of TNFα and/or IL–1.

Recruitment of neutrophils from the pulmonary vasculature into the interstitium and alveoli is an important pathophysiological consequence of IL–8 production within the lung, since this event is accompanied by neutrophil activation. In normal healthy lungs, neutrophils represent fewer than 1% of bronchoalveolar lavage cells. By contrast, many acute and chronic lung diseases are associated with increased proportions and absolute numbers of neutrophils within the lung. Available evidence indicates that activated neutrophils within the lung contribute to lung injury in a wide variety of pulmonary diseases.

The adult respiratory distress syndrome (ARDS), a human pulmonary disorder with major systemic manifestations, is a common sequel to trauma, sepsis, and endotoxic shock.[26, 100, 188] The condition is characterized by diffuse alveolar-capillary membrane damage, increased pulmonary vascular permeability, extravasation of fluid within the interstitium and alveoli, and marked accumulation of neutrophils. Current evidence indicates that IL–8 contributes to the pathogenesis of ARDS through widespread recruitment and activation of neutrophils, which mediate much of the structural injury associated with the disease through extracellular release of reactive oxygen intermediates and proteolytic enzymes.

Inflammatory cytokines in models of pulmonary disease

Current evidence supports the theory that many of the pathophysiological manifestations of pulmonary diseases are due to local expression of cytokines and other soluble inflammatory mediators. Pulmonary overexpression of TNFα, IL–1β, and/or IL–8 is strongly implicated in the pathogenesis of several lung diseases affecting humans, including the adult respiratory distress syndrome,[88, 185, 235] cystic fibrosis,[35, 36, 148] pneumoconiosis,[310] and sarcoidosis.[17] Inflammatory cytokine overexpression is also associated with lung pathology in animal models of influenza pneumonia,[215] silicosis,[91] acute immune complex alveolitis,[319] and bleomycin-induced interstitial fibrosis.[217] Among domestic animals, TNFα, IL–1β, and IL-8 are implicated in the pathogenesis of swine influenza,[307] porcine pleuropneumonia caused by

Actinobacillus pleuropneumoniae,[16, 130] and *Corynebacterium pseudotuberculosis*-induced pyogranulomas in sheep.[94]

Inflammatory cytokines in bovine pneumonic mannheimiosis

A growing body of circumstantial evidence implicates inflammatory cytokines in the pathogenesis of bovine pneumonic mannheimiosis, prompting speculation that it may be possible to treat or prevent the disease through pharmacological modulation of cytokine expression. Heat-killed *Mannheimia haemolytica* and purified *M. haemolytica* LPS and LktA induce the expression of TNFα, IL-1β, and IL-8 mRNA and proteins by bovine alveolar macrophages in vitro.[153, 196, 197, 278, 334, 336] In addition, BPM is associated with the pulmonary expression of TNFα, IL-1β, and IL-8 in vivo.[46, 335] Because pulmonary cytokine expression was evaluated 2 – 4 days after experimental infection with *M. haemolytica* in these studies, however, it is not possible to draw definitive conclusions regarding a causative role for cytokines in disease pathogenesis, or even a temporal association between cytokine expression and the onset of clinical disease.

THERAPEUTIC MODULATION OF INFLAMMATORY CYTOKINES

Introduction

The discovery that inflammatory cytokines function as key mediators of tissue pathology was an important milestone in our understanding of disease

pathogenesis. Perhaps even more importantly, however, it identified cytokines as therapeutic targets whose expression could be manipulated with the goal of treating or preventing inflammatory disease. This is now an area of active research, and pharmaceutical companies throughout the world are developing drugs aimed at blocking the synthesis or biological actions of cytokines.

Effects mediated by inflammatory cytokines may be considered the end result of a complex series of events: activation of the cytokine-producing cell in response to a specific stimulus; intracellular signal transduction leading to the transcription of one or more cytokine genes; translation of cytokine-specific mRNA into polypeptide gene products; post-translational processing of cytokine proteins; conversion of inactive precursors to an active form; secretion of the active cytokine; binding of the cytokine to a specific receptor on the responding cell; intracellular signal transduction in the responding cell; and induction of a physiological response in the responder cell. In theory, interruption of this chain of events at any point may serve to prevent or reduce cytokine-induced inflammation. In practice, four major approaches have proven most effective: (1) inhibition of cytokine synthesis; (2) inhibition of processing to an active form; (3) inhibition of cytokine secretion; and (4) interference with binding to cytokine receptors.[111]

To date, most in vivo modulation studies have used the latter approach, in which cytokines are prevented from interacting with their specific receptors through neutralization by monoclonal antibodies, native or recombinant soluble receptors, or receptor antagonism. This approach has several important practical limitations, however. Monoclonal antibodies and recombinant receptors are

labor-intensive and expensive to produce, and must be administered by the intravenous route. Since they have a relatively short circulating half-life due to their inherent immunogenicity, they must be administered frequently, or even continuously, to maintain a therapeutic effect. Furthermore, their large size may limit distribution within tissues; if they cannot reach the site of cytokine expression, they will be unable to mediate a therapeutic effect. These limitations have shifted the recent emphasis in cytokine inhibition research toward low molecular weight synthetic inhibitors of cytokine synthesis. These compounds may hold more therapeutic promise since they are less expensive to manufacture, less restricted in their biodistribution, and can often be administered by the oral and intramuscular routes. In view of the overlapping pleiotropic effects of inflammatory cytokines, substances capable of inhibiting multiple cytokines are most likely to be efficacious.

Numerous compounds capable of suppressing inflammatory cytokine synthesis have been identified in recent years. In some cases the mechanism of action is known, while in others it remains to be determined. The major classes of cytokine inhibitors for which information about mechanism of action is available are discussed below.

Glucocorticoids

Glucocorticoids are among the most effective and widely used anti-inflammatory drugs, but their mechanism of action has been poorly characterized until recently. These agents, particularly dexamethasone, are potent inhibitors of cytokine synthesis at the transcriptional, post-transcriptional,

and translational levels.[7] Glucocorticoids inhibit the transcription of most inflammatory cytokine genes, including TNFα,[151, 187] IL–1,[8, 147] IL–6,[8, 342] and IL–8.[152] They also inhibit the synthesis of a wide range of immune cytokines, including IL–2, IL–3, IL–4, IL–5, IL–12, IFN-γ, macrophage colony-stimulating factor (M-CSF), granulocyte (G)–CSF, and granulocyte-macrophage (GM)–CSF.[7] Recent evidence indicates that glucocorticoids inhibit cytokine gene transcription by preventing nuclear translocation of NF–κB through upregulation of IκB, which traps NF–κB in the cytoplasm.[14, 247] Because NF–κB is a transcriptional activator for a wide range of genes associated with immune and inflammatory responses, including inflammatory cytokines, inhibition of its activity yields potent anti-inflammatory effects.

A variety of glucocorticoids have been used in an effort to modulate pulmonary inflammation in the management of bovine respiratory disease. Relatively few reports of the influence of glucocorticoids on the pathogenesis and clinical outcome of pneumonic mannheimiosis are available, however, and results do not uniformly support the efficacy of these agents. In two European studies, adjunctive therapy with corticosteroids was associated with improved outcomes in experimental models of BPM.[6, 97] Similarly, treatment of experimentally infected calves with a combination of ceftiofur and flumethasone (25 μg/kg/day IV for 3 days) resulted in reduced mortality and a quicker recovery as compared to no treatment or treatment with ceftiofur alone.[288] Addition of low-dose dexamethasone acetate (0.025 mg/kg IM q12h for 3 days) to an antibiotic treatment regimen slightly improved clinical scores in experimentally infected calves, but did not affect lung lesion scores 7 days post-

infection.[186] By contrast, addition of low-dose dexamethasone (20 mg IM once daily for 3 days) to an antibiotic regimen for treatment of naturally occurring bronchopneumonia in yearling feedlot cattle exacerbated clinical disease and provoked increased relapse and mortality rates.[57] However, case selection in this study was based solely on symptoms of clinical disease, which precludes differentiation of *M. haemolytica* pneumonia from other viral and bacterial causes of respiratory disease. In experimental models of other bovine bacterial pneumonias, dexamethasone pretreatment usually exacerbates disease. In experimental models of *Histophilus (Haemophilus) somnus* pneumonia, for example, administration of dexamethasone to calves prior to endotracheal inoculation of bacteria exacerbates morbidity, pulmonary pathology, and mortality.[51, 135]

Antioxidants

Reactive oxygen intermediates induce inflammatory cytokine gene expression through upregulation of NF–κB activity,[20, 248] leading researchers to speculate that antioxidant drugs may be used to modulate cytokine expression. Several antioxidants, including butylated hydroxyanisole (BHA),[50, 98] probucol,[4] tocopherol (vitamin E),[4] nordihydroguaiaretic acid (NDGA),[98] apomorphine,[98] and tetrahydropapaveroline (THP),[98] have been shown to inhibit inflammatory cytokine production by human and animal cell lines in vitro. Three of these, BHA, NDGA, and THP, inhibit the in vitro production of TNFα and IL-1 by colonic biopsies from human patients with inflammatory bowel disease.[225] In vivo, pretreatment of mice with THP suppresses LPS-induced production of

IL–1β by peritoneal macrophages, and pretreatment of mice with apomorphine suppresses systemic overproduction of TNFα and IL-1β in response to lethal challenge with lipopolysaccharide.[98] These observations suggest that antioxidant drugs may be useful in the treatment or prevention of diseases characterized by an overproduction of inflammatory cytokines, especially TNFα and IL-1β.

Phosphodiesterase inhibitors

The cytokine-suppressive effects of phosphodiesterase (PDE) inhibitors have been studied extensively. These compounds inhibit TNFα production, but not IL–1, IL-6, or IL-8 production, at the transcriptional level by increasing intracellular concentrations of cyclic adenosine monophosphate (cAMP).[40, 95] Pentoxifylline (PTX), a methyl xanthine agent that acts as a general PDE inhibitor, exhibits significant anti-inflammatory effects. In vitro, PTX inhibits production of TNFα by peripheral blood monocytes,[202] peritoneal macrophages,[285] and alveolar macrophages.[179] In vivo, it reduces morbidity and mortality associated with sepsis or endotoxin challenge in mice, rats, and humans, probably through its ability to downregulate systemic TNFα production.[48, 63, 204, 245, 276] PTX also appears to exert direct anti-inflammatory effects on neutrophils. In LPS- or cytokine-stimulated neutrophils, PTX inhibits adherence, superoxide production, and granule release.[175, 239, 287] PTX also prevents TNFα-induced neutrophil mediated lung injury in vivo.[165, 333] In immune and inflammatory cells, including monocytes, mast cells, neutrophils, basophils, and eosinophils, PDE IV is the predominant cAMP metabolizing enzyme.[296] PDE IV-selective inhibitors such as rolipram are more potent

inhibitors of TNFα production than general PDE inhibitors such as pentoxifylline.

Bicyclic imidazoles

A novel group of pyridinyl-imidazole compounds that suppress the production of TNFα, IL-1β, and IL-6 in activated monocytes and macrophages has recently been introduced by researchers at SmithKline Beecham.[157] These compounds show activity in several experimental animal models of acute and chronic inflammation, and have been termed cytokine-suppressive anti-inflammatory drugs (CSAIDs). They inhibit cytokine production at the translational level through selective inhibition of p38 kinase, a serine/threonine kinase involved in cytokine biosynthesis; no effect on mRNA abundance or size/stability of the protein products has been identified.[159] Drugs of this class also suppress eicosanoid production through inhibition of cyclooxygenase and lipoxygenase.[160] The prototypical drug of this group, SK&F 86002 (dihydroimidazothiazoline), is a potent inhibitor of TNFα and IL-1 production in LPS-stimulated human monocytes in vitro.[158, 160] In vivo, bicyclic imidazoles inhibit TNFα production and reduce mortality in several animal models of septic shock.[19, 208, 266] Newer generations of these compounds, such as SB203580, feature more potent cytokine suppressive activity and reduced toxicity in vitro and in vivo. SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4–pyridyl) imidazole] is a potent inhibitor of inflammatory cytokine production in vivo in mice and rats, and reduces fatality in murine models of endotoxic shock.[18]

Thalidomide

Thalidomide is a nonbarbiturate sedative with anti-emetic properties that was commonly prescribed to pregnant women in the late 1950's. In 1961, however, the drug was abruptly withdrawn from the market when its use in early pregnancy was associated with catastrophic fetal abnormalities. Despite its tumultuous history, thalidomide has proven to be clinically useful in the management of chronic immunoinflammatory diseases such as lepromatous leprosy, tuberculosis, rheumatoid arthritis, and graft-versus-host disease. The drug's mode of action is incompletely understood, but its beneficial effects are now attributed, at least in part, to its ability to suppress TNFα production. In vitro, thalidomide inhibits TNFα secretion by human peripheral blood mononuclear cells stimulated with LPS or *Mycobacterium leprae* cell wall extract.[242] The drug also inhibits LPS-induced TNFα production by human alveolar macrophages harvested from patients with tuberculosis and other diseases characterized by macrophage activation.[293] In vivo, administration of thalidomide to patients with lepromatous leprosy is associated with reduced clinical symptoms and a corresponding reduction in serum TNFα concentrations.[241] Available evidence indicates that thalidomide suppresses cytokine production at the post-transcriptional level by reducing the stability of TNFα-specific mRNA.[194] These observations suggest that thalidomide may be useful in the prevention or treatment of diseases characterized by overproduction of TNFα.

NSAIDs in Modulation of Bovine Pulmonary Inflammation

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a diverse group of compounds with antipyretic, anti-inflammatory, and analgesic properties. They are widely used as an adjunct to antimicrobial therapy in an effort to modulate pulmonary inflammation in BPM and other bovine respiratory diseases. NSAIDs are often considered to be preferable to glucocorticoids in the management of infectious respiratory diseases of cattle, as they lack the immunosuppressive properties of the latter drugs. A variety of NSAID compounds have been investigated, including flunixin meglumine, carprofen, ketoprofen, and tolfenamic acid. Flunixin meglumine and tolfenamic acid significantly enhance clinical recovery in experimental models of viral pneumonia and BPM [253, 254] and in natural outbreaks of undifferentiated bovine respiratory disease.[80] In naturally occurring outbreaks of acute respiratory disease in housed calves, a single dose of carprofen (mean dose 1.4 mg/kg by subcutaneous injection) was equally effective as three daily doses of flunixin meglumine (mean dose of 2 mg/kg IV) as adjunctive therapy to antimicrobial treatment.[23] In an in vivo experimental model of BPM, ketoprofen (3 mg/kg/day for 4 treatments) exerted greater beneficial effects on respiratory rate, PaO_2, and gross lung lesions than did dexamethasone (0.016 mg/kg/day for 4 treatments).[41, 207] It is important to note, however, that the dose of dexamethasone used in these studies was very low, and usually considered insufficient to exert significant anti-inflammatory effects.

The mechanisms by which NSAIDs modulate pulmonary inflammation in cattle have been incompletely characterized, but are assumed to relate primarily to inhibition of cyclooxygenase, resulting in decreased production of

prostaglandins.[309] However, the anti-inflammatory and analgesic potency of these drugs does not always correlate with their activity as cyclooxygenase inhibitors,[74, 183] suggesting that they may exert anti-inflammatory effects through other mechanisms. For example, recent evidence suggests that carprofen, and to a lesser extent flunixin meglumine, exerts direct effects on NF–KB activation that are independent of their effects on cyclooxygenase.[39]

CHAPTER 3

PULMONARY EXPRESSION OF TNFα, IL-1β, AND IL-8 IN THE ACUTE PHASE OF BOVINE PNEUMONIC MANNHEIMIOSIS

INTRODUCTION

Bovine pneumonic mannheimiosis (BPM), an acute fibrinous pleuropneumonia caused by *Mannheimia (Pasteurella) haemolytica*, is a common and economically important disease of North American cattle. One of the hallmark histopathological features of BPM is extensive infiltration of the lungs by neutrophils. Neutrophil depletion prior to experimental infection with *M. haemolytica* protects calves from subsequent lung injury,[265, 322] indicating that this cell type is directly or indirectly responsible for most of the pulmonary pathology observed in the disease. The mechanisms by which neutrophils mediate lung pathology in BPM remain undefined but are likely to include oxidative injury and structural degradation due to extracellular release of reactive oxygen intermediates and proteolytic lysosomal enzymes.[267] Recognition of the importance of neutrophils in disease pathogenesis prompted our laboratory and others to investigate specific factors governing their recruitment and activation within *M. haemolytica*-infected bovine lung.

The migration and activation of neutrophils in inflamed tissue are regulated by a complex network of interactions between cytokines, leukocytes, vascular endothelium, cellular adhesion molecules, and soluble chemotactic factors. The inflammatory cytokines tumor necrosis factor-alpha (TNFα), interleukin-1 beta (IL-1β), and interleukin-8 (IL–8) play a central role in the initiation and orchestration of these interactions. TNFα and IL-1β are pleiotropic early-response polypeptides secreted by monocytes and macrophages in response to microbial pathogens and other stimuli.[155] IL-8, a potent chemotactic

and activating factor for neutrophils, is a C–X–C chemokine secreted by a variety of immune and nonimmune cell types, including monocytes, macrophages, fibroblasts, epithelial cells, and neutrophils.[21, 150]

Pulmonary overexpression of inflammatory cytokines is believed to contribute to the pathogenesis of several infectious and inflammatory lung diseases in humans, including adult respiratory distress syndrome,[88, 185] cystic fibrosis,[35, 148] and pneumoconiosis.[310] It is also associated with lung pathology in animal models of influenza pneumonia,[215] silicosis,[91] and immune complex alveolitis.[319] Among the domestic species, TNFα, IL–1β, and IL-8 are implicated in the pathogenesis of swine influenza,[307] porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae*,[16, 130] and *Corynebacterium pseudotuberculosis*-induced pyogranulomas in sheep.[94]

A growing body of circumstantial evidence implicates inflammatory cytokines in the pathogenesis of BPM, prompting speculation that it may be possible to treat or prevent the disease through pharmacological modulation of cytokine expression. Heat-killed *M. haemolytica* and purified *M. haemolytica* lipopolysaccharide (LPS) and leukotoxin (Lkt) induce the expression of TNFα, IL–1β, and IL-8 mRNA and proteins by bovine alveolar macrophages in vitro.[153, 196, 197, 278, 334, 336] In addition, BPM is associated with the pulmonary expression of TNFα, IL-1β, and IL-8 in vivo.[46, 335] Since these studies evaluated pulmonary cytokine expression 2 to 4 days after inoculation with *M. haemolytica*, it is difficult to draw definitive conclusions from them regarding a causative role for cytokines in disease pathogenesis. A comprehensive understanding of inflammatory cytokine expression during earlier stages of disease development would permit a

more accurate assessment of their role in disease pathogenesis, and is a necessary prerequisite for the development of therapeutic strategies based upon cytokine inhibition or antagonism.

The objectives of this study were to (1) characterize the kinetics of pulmonary TNFα, IL–1β, and IL-8 gene and protein expression in the first 24 hours of experimental BPM; (2) compare patterns of cytokine expression in airways with those in lung lesions; and (3) identify major cellular sources of these cytokines within affected lung. Northern analysis was used to quantitate the expression of cytokine mRNA in bronchoalveolar lavage (BAL) cells and diseased lung parenchyma. Immunoreactive cytokines in BAL fluid and lung tissue extracts were measured by enzyme-linked immunosorbent assay (ELISA). In situ hybridization was used to localize the expression of cytokine mRNA within lung tissues; numbers of positively staining cells were enumerated by quantitative morphometric techniques, and staining cells were identified by cell type on the basis of cell morphology and location.

An in vivo experimental model of BPM was used to characterize the pulmonary expression kinetics of tumor necrosis factor-alpha (TNFα), interleukin-1 beta (IL-1β), and interleukin-8 (IL-8) genes and proteins during the acute phase of disease development. Cytokine expression in bronchoalveolar lavage (BAL) fluid, BAL cells, and pneumonic lung parenchyma was quantitated by northern blot analysis, enzyme-linked immunosorbent assays (ELISA), and in situ hybridization at 2, 4, 8, 16, and 24 hours after endobronchial inoculation of *Mannheimia (Pasteurella) haemolytica*. Expression of TNFα, IL-1β, and IL-8 was significantly increased in the airways and lung lesions of infected calves as

compared to mock-infected controls. Although kinetic patterns varied, peak levels of cytokine mRNA occurred within 8 hours post-infection (PI) and peak cytokine concentrations occurred within 16 hours PI. In all samples, IL-8 was expressed to the greatest extent and TNFα was least expressed. Expression of TNFα was restricted to alveolar macrophages. Alveolar and interstitial macrophages produced IL-1β and IL-8 in the first 4 hours, and bronchial and bronchiolar epithelial cells were also significant sources of IL-8 during this period. By 8 hours PI, neutrophils were the dominant source of both IL-1β and IL-8. These findings demonstrate a spatial and temporal association between pulmonary expression of inflammatory cytokines and acute lung pathology, and indirectly support the hypothesis that cytokines contribute to inflammatory lung injury in BPM.

MATERIALS AND METHODS

Preparation of bacterial inoculum

Mannheimia haemolytica A1 strain 12296, a field isolate recovered from a yearling calf with fatal pneumonic mannheimiosis, was propagated in phenol red-free RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 2 mM L-glutamine as previously described.[10] Logarithmic phase cultures were diluted with additional medium to adjust the final concentration to 1×10^9 cfu/ml as determined by spectrophotometry.

Animals and induction of experimental disease

Eighteen healthy male Holstein calves between 4 and 7 weeks of age were purchased from the University of Minnesota Department of Animal Science. The calves were weaned at birth and raised in individual hutches, and were free of detectable serum antibodies against *M. haemolytica* surface antigens and Lkt as determined by ELISA using previously described methods.[273] Pneumonic mannheimiosis was induced in 15 calves using a well-characterized, reproducible experimental model developed in our laboratory.[10] Calves were sedated with xylazine (0.1 mg/kg IV) and positioned in sternal recumbency for the passage of a sterile fiberoptic bronchoscope into the left caudal lung lobe. With the tip of the endoscope wedged in a large bronchus, 5 ml (5×10^9 cfu) of logarithmic phase *M. haemolytica* was deposited into the airway, followed by 30 ml sterile LPS-free phosphate-buffered saline, pH 7.4 (PBS). Groups of three infected calves, randomly selected on the basis of birth order, were euthanatized at each of the following times post-infection (PI): 2, 4, 8, 16, and 24 hours. At necropsy, focally extensive regions of hemorrhage, interlobular edema, and consolidation consistent with pneumonic mannheimiosis were present in the left caudal lung lobe of all inoculated calves. Bacteriological cultures of pulmonary lesions yielded pure colonies of *M. haemolytica*. Three control calves received mock infections in which an equal volume of sterile culture medium was substituted for bacteria. These animals were euthanatized at 24 hours PI and exhibited no gross pulmonary pathology at necropsy.

Bronchoalveolar lavage and lung tissues

Bronchoalveolar lavage (BAL) fluid was collected from the right caudal lung lobe of all calves immediately prior to infection or mock-infection, and from the left caudal lung lobe at necropsy. Using a sterile fiberoptic endoscope, 60 ml of LPS-free PBS was infused into a large bronchus and immediately retrieved by gentle suction. This process was repeated with three additional 60-ml aliquots of PBS. Samples were pooled and centrifuged for 10 minutes at 400 × *g* (4°C) to separate fluid and cellular components. The supernatant was centrifuged again for 30 minutes at 15,000 × *g* (4°C) and stored at –80°C for quantitation of cytokines and urea. Bronchoalveolar lavage cells were washed with LPS-free PBS and lysed in 4 M guanidinium isothiocyanate containing 8 mM sodium citrate, 0.5% sodium lauroyl sarcosinate, and 8% (v/v) β–mercaptoethanol. Cell lysates were stored at –80°C for RNA extraction and northern blot analysis.

Non-lavaged lung tissues were collected from the left caudal lung lobe of all calves. Samples were taken from the margins of gross pneumonic lesions in infected calves, and from grossly normal lung in control calves. Tissue samples for in situ hybridization were fixed in neutral buffered formalin for 24 hours, stored at –20°C in 70% ethanol for 12 to 24 hours, processed, and embedded in paraffin by standard methods. Tissues for RNA extraction and northern analysis were snap-frozen in liquid nitrogen, homogenized in lysis buffer (4 M guanidinium isothiocyanate, 0.5% sodium lauroyl sarcosinate, 8 mM sodium citrate, and 8% (v/v) β–mercaptoethanol), and stored at –80°C. Tissue extracts were prepared by homogenizing 1 g of fresh lung tissue per 3 ml of PBS containing 0.05% Tween-20 and recovering the supernatant after centrifugation

for 10 minutes at 15,000 × *g* (4°C). Extracts were stored at -80°C for quantitation of cytokines by ELISA.

Plasmids

Bovine TNFα, IL-1β, and IL-8 cDNAs (488, 474, and 230 base pairs in length, respectively) were cloned and sequenced in our laboratory,[153, 334] ligated into pcDNA3 (Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* DH5α. A 1250-base pair human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA in pBluescript KS+ (Stratagene, La Jolla, CA) was the generous gift of Dr. M. Murtaugh (University of Minnesota, St. Paul, MN). All plasmids were purified by alkaline lysis using a commercial kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

RNA extraction and northern blot analysis

All solutions were treated with 0.1% diethylpyrocarbonate (DEPC) and glassware was baked overnight at 350°C before use. Total cellular RNA was extracted from BAL cells and lung tissues using the acid guanidinium thiocyanate and phenol-chloroform extraction method.[56] Ten micrograms of RNA from each sample was electrophoretically fractionated in a 1.2% agarose gel containing 6.5% formaldehyde, transferred to a neutral nylon membrane (Schleicher and Schuell, Keene, NH), and covalently linked to the membrane by ultraviolet illumination. Membranes were prehybridized at 45°C for 2 hours in solution containing 50% formamide, 5× saline sodium citrate (SSC),

5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 0.1 mg/ml yeast tRNA.

Gel-purified TNFα, IL-1β, IL-8, and GAPDH cDNA plasmid inserts were labeled with [α-^{32}P]dCTP by DNase/DNA polymerase I nick translation and unincorporated [α-^{32}P]dCTP was removed using Elutip–d affinity columns (Schleicher and Schuell). Labeled probe was added to prehybridization buffer at 2×10^6 cpm/ml and membranes were hybridized overnight at 45°C. Blots were washed to a stringency of 0.1× SSC/0.1% SDS at 50°C. Autoradiographs were prepared by exposing membranes to Kodak X–OMAT AR x-ray film (Eastman Kodak, Rochester, NY) with an intensifying screen for 1 to 3 days at –80°C. Phosphor screen autoradiographs were prepared (Phosphorimager SF, Molecular Dynamics, Sunnyvale, CA) and relative levels of cytokine-specific mRNA were quantified by densitometric analysis using ImageQuant software (Molecular Dynamics). Data were normalized to the expression of GAPDH mRNA. For each cytokine, values for infected calves were presented relative to mean normalized expression in mock-infected control calves.

TNFα ELISA

A capture ELISA was developed to quantitate immunoreactive TNFα in BAL fluid and lung extracts. Mouse monoclonal antibody 2C4 ascites and rabbit anti-TNFα antiserum were generously provided by Dr. T. H. Elsasser (USDA-ARS, Beltsville, MD), and purified recombinant bovine (rb) TNFα for use as a standard was generously provided by Dr. Dale Godson (Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan). All samples, standards, and

detection antibodies were diluted in PBS containing 10% (v/v) bovine serum albumin blocking concentrate (Kirkegaard & Perry, Gaithersburg, MD). Unless otherwise indicated, all reactions were conducted in a volume of 100 μl and plates were incubated at 37°C for 1 hour on a platform shaker. After each step, plates were washed five times with PBS containing 0.01% Tween-20. Monoclonal antibody 2C4 ascites diluted 1:1000 in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, and 3 mM sodium azide, pH 9.6) was adsorbed to 96-well ELISA plates (Costar Corp., Cambridge, MA) overnight at room temperature. After blocking nonspecific protein binding sites, samples and standards were added to plates. Samples were assayed in duplicate at two-fold dilutions from neat to 1:8. Rabbit anti-bovine TNFα antiserum diluted 1:2000 in blocking buffer was used for primary detection of bound cytokine, followed by secondary detection with horseradish peroxidase (HRP)-labeled polyclonal goat anti-rabbit IgG (Kirkegaard & Perry) at a dilution of 1:6000. The color substrate tetramethylbenzidine (Kirkegaard & Perry) was added and plates were incubated for 10 minutes at room temperature. The reaction was stopped with 100 μl 1 M phosphoric acid and absorbance was measured at 450 nm. For each plate, a standard curve was constructed using duplicate three-fold dilutions of rbTNFα. Sample dilutions yielding absorbance readings in the linear region of the standard curve were used to calculate cytokine concentrations by interpolation using SOFTmax PRO software (Molecular Devices, Sunnyvale, CA).

IL-1β ELISA

A capture ELISA was developed to quantitate immunoreactive IL-1β in BAL fluid and lung extracts. Mouse monoclonal antibody SA22 specific for bovine IL–1β was produced from hybridoma CRL-2052 (American Type Culture Collection, Manassas, VA) and purified by standard methods.[121, 222] Purified rbIL-1β for use as a standard was generously provided by Dr. Kathleen Heaney (Fort Dodge Animal Health, Princeton, NJ). Samples were assayed in duplicate at two-fold dilutions from neat to 1:8. Methods were as for the TNFα ELISA with the following exceptions. All samples, standards, and detection antibodies were diluted in PBS containing 10% (v/v) milk blocking concentrate (Kirkegaard & Perry). Purified monoclonal antibody SA22 in coating buffer (5 μg/ml) was adsorbed to plates overnight at room temperature. Rabbit anti-ovine IL–1β antiserum (Chemicon, Temecula, CA) diluted 1:1000 was used for primary detection and HRP-labeled goat anti-rabbit IgG diluted 1:4000 was used for secondary detection. Color development was allowed to proceed for 30 minutes before the reaction was stopped with 1 M phosphoric acid and absorbance was measured at 450 nm.

IL-8 ELISA

Purified rbIL-8 and mouse monoclonal antibody 170.13 specific for bovine IL-8 were produced and characterized in our laboratory. In brief, a cDNA encoding the mature bovine IL-8 protein was subcloned into the pET15b expression vector (Novagen, Madison, WI), transformed into *E. coli* BL21(λDE3)pLysS cells (Novagen), and expressed according to the

manufacturer's recommendations.[153] Histidine-tagged rbIL-8 was expressed, affinity purified on a Ni^{2+}-resin column (Novagen), and concentrated by dialysis against polyethylene glycol. Purified rbIL-8 was used to produce hybridomas and monoclonal antibodies by standard methods in collaboration with Immunochemistry Technologies (Bloomington, MN).[121] Monoclonal antibody 170.13 (IgG1) recognized both rbIL-8 and recombinant human IL-8 in western blots and neutralized the neutrophil chemotactic activity of rbIL-8 in vitro.

A capture ELISA was developed to quantitate immunoreactive IL-8 in BAL fluid and lung extracts. Methods were as for the TNFα ELISA with the following exceptions. Samples and standards were diluted in PBS containing 10% (v/v) milk blocking concentrate. Purified monoclonal antibody 170.13 in coating buffer (5 μg/ml) was adsorbed to plates overnight at room temperature. Bronchoalveolar lavage fluids were assayed in duplicate at two-fold dilutions from neat to 1:8 and lung extracts were assayed in duplicate at ten-fold dilutions from neat to 1:1000. Rabbit anti-ovine IL-8 antiserum (Chemicon) diluted 1:4000 in blocking buffer containing 5% normal mouse serum was used for primary detection and HRP-labeled goat anti-rabbit IgG diluted 1:6000 in blocking buffer was used for secondary detection. Color development was allowed to proceed for 20 minutes before the reaction was stopped with 1 M phosphoric acid and absorbance was measured at 450 nm.

Correction for variable dilution of BAL samples

Urea was used as an endogenous marker of dilution to calculate the extent to which epithelial lining fluid (ELF) from each calf was diluted during the BAL

procedure.[228] Blood for quantitation of serum urea nitrogen was drawn from the jugular vein of all calves immediately prior to euthanasia. Urea concentration in serum and BAL fluid was determined by enzymatic methods using a commercial kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Measured cytokine concentrations in BAL fluid were corrected for dilution and expressed as cytokine concentration per ml ELF.

In situ hybridization

All solutions were treated with 0.1% DEPC and glassware was baked overnight at 350°C before use. Plasmids containing TNFα, IL-1β, and IL-8 cDNA inserts were linearized by restriction digestion, and sense and antisense digoxigenin (DIG)-labeled RNA probes were synthesized by in vitro transcription using a commercial kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Labeled probes were ethanol-precipitated and dissolved in ultrapure water. Probe concentration was determined by dot blot hybridization and subsequent immunological detection using components of the DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

Sections of paraffin-embedded lung tissue (5 μm) were cut, transferred to Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), deparaffinized in xylene, and rehydrated through a series of graded ethanol baths to ultrapure water. Except where otherwise indicated, steps preceding hybridization were carried out at room temperature and slides were rinsed with ultrapure water between steps. Tissue sections were hydrolyzed in 0.2 N HCl for 20 minutes,

incubated in 0.15 M triethanolamine (pH 7.4) for 15 minutes, and transferred to 0.3% (v/v) Triton-X in PBS for 5 minutes. Tissues were permeabilized with 25 μg/ml proteinase K (Boehringer Mannheim) for 20 minutes at 37°C, acetylated with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0), and incubated in 2× SSC for 10 minutes at 70°C. Sections were then dehydrated through a series of graded ethanol baths, air-dried, and covered with 60 μl of hybridization buffer (50% deionized formamide, 7% dextran sulfate, 1× Denhardt's solution, 0.6 M NaCl, 0.05% SDS, 20 mM HEPES, 1 mM EDTA, 1 mg/ml poly[A], and 250 μg/ml yeast tRNA) containing 180 ng of DIG-labeled antisense riboprobe specific for TNFα, IL-1β, or IL-8. Siliconized glass cover slips were applied and sealed with rubber cement, and hybridization was then conducted overnight at 43°C. Negative control sections were hybridized with buffer containing an equal concentration of the corresponding sense riboprobe, or buffer containing no added riboprobe.

Non-specifically bound riboprobe was removed by enzymatic digestion and stringency washes as follows: 2× SSC for 30 minutes at 42°C; 50% formamide/2× SSC for 20 minutes at 52°C; 25 μg/ml ribonuclease A (Sigma) in enzyme buffer [10 mM Tris-HCl (pH 8.0), 0.5 M NaCl] for 30 minutes at 37°C; 1× SSC for 15 minutes at 37°C; and 0.1× SSC for 15 minutes at 37°C. Tissue sections were blocked for 30 minutes with Tris-buffered saline [0.1 M Tris-HCl (pH 7.4), 0.15 M NaCl] containing 3% normal sheep serum, then covered with sheep anti-DIG/alkaline phosphatase conjugate (Boehringer Mannheim) diluted 1:500 in blocking buffer and incubated for 1 hour at room temperature. After washing slides 3 times for 5 minutes each in Tris-buffered saline (TBS), they were

incubated for 15 minutes in TBS containing 4 mM levamisole to inactivate endogenous alkaline phosphatases. The color substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-indoyl phosphate (BCIP) were applied to sections and slides were incubated in the dark for 18 hours at room temperature. After a final rinse in ultrapure water, tissues were counterstained with nuclear fast red (Vector Laboratories, Burlingame, CA) and mounted with aqueous medium.

Morphometric analysis

Cells staining positive for cytokine mRNA in lung tissue were quantified using a combination of light microscopy and digital image analysis. For each calf, images of a minimum of three microscopic fields from each of at least three tissue sections (a minimum of nine fields per animal) were electronically captured using an Eclipse E800 microscope (Nikon, Melville, NY) equipped with a CoolCam 2000 digital video camera (Cool Camera Company, Atlanta, GA). Non-overlapping fields were selected at random along a line defining the long axis of the tissue section. Positively stained cells were identified by light microscopy. Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD) was used to mark and count stained cells in digital images, and to calculate the surface area of tissue fields analyzed. Results were expressed as the number of positively staining cells/mm^2 tissue.

Cells staining positive for cytokine mRNA in lung tissue were classified according to cell type. Fifty stained cells in each of at least three light microscopic fields per tissue section (three sections per animal) were identified by cell type on the basis of cell morphology and histological location. Fields were selected as for

morphometric analysis; additional non-overlapping fields were examined as needed to type a minimum of 450 cells per animal. Data from all calves in a given group were combined and results were expressed in terms of the percentage of total staining cells.

Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM). Data from all groups were analyzed using the Kruskal-Wallis test, and individual groups were compared with the Mann-Whitney *U* test. Differences were considered significant when $P < 0.05$.

RESULTS

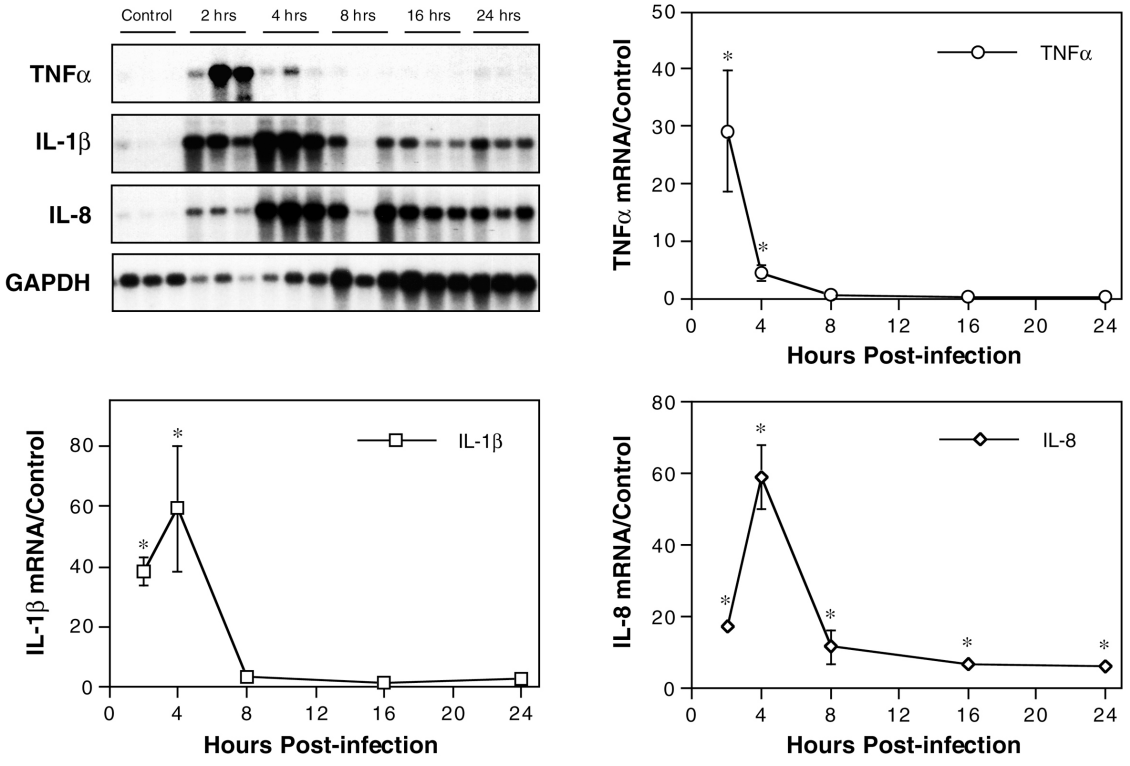
Kinetics of cytokine mRNA expression in BAL cells

Northern analysis was used to characterize inflammatory cytokine gene expression within airways during the first 24 hours of experimental pneumonic mannheimiosis. Steady-state levels of TNFα, IL-1β, and IL-8 mRNA in BAL cells collected 2, 4, 8, 16, and 24 hours PI were compared to those in cells from mock-infected control calves (Fig. 1). For all three cytokines, post-infection mRNA levels were significantly increased compared to controls ($P < 0.05$) and maximal levels occurred within 4 hours of disease onset. Peak levels of TNFα mRNA occurred at 2 hours PI and peak levels of IL-1β and IL-8 mRNA occurred at 4 hours PI. At maximum expression, levels of IL-1β and IL-8 mRNA were roughly

two-fold greater than those of TNFα mRNA. Expression of TNFα and IL-1β mRNA declined to control levels by 8 hours PI, but IL-8 mRNA levels were significantly increased throughout the 24 hour study period ($P < 0.05$). To rule out the possibility that experimental results were influenced by pre-existing differences in levels of gene expression between the groups of calves euthanatized at various time points, pre-infection BAL cells collected from the right lung of all calves were subjected to identical northern analysis. Results showed no significant differences in pre-infection cytokine mRNA expression between groups (data not shown).

Figure 3-1. Kinetics of TNFα, IL-1β, and IL-8 mRNA expression in BAL cells from calves with acute pneumonic mannheimiosis

Inflammatory cytokine gene expression was measured by densitometric analysis of northern blots as described in Materials and Methods. For each cytokine, data were normalized to the expression of GAPDH mRNA and presented relative to mean normalized expression in BAL cells from mock-infected control calves. Values represent the mean ± SEM (n = 3). * $P < 0.05$ compared to mock-infected controls.



Kinetics of cytokine mRNA expression in pneumonic lung

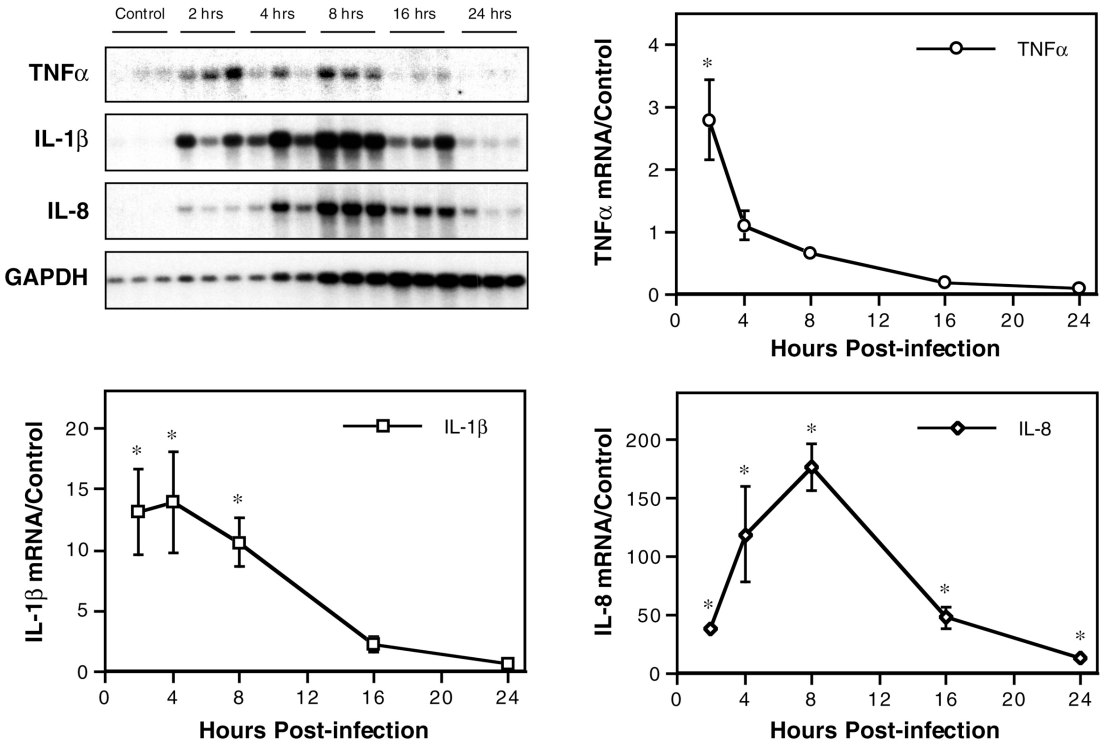
Northern analysis was used to characterize inflammatory cytokine gene expression within pulmonary lesions of pneumonic mannheimiosis during the first 24 hours of disease. Steady-state levels of TNFα, IL-1β, and IL-8 mRNA in lesional lung tissues collected at 2, 4, 8, 16, and 24 hours PI were compared to those in grossly normal lung tissues from mock-infected control calves (Fig. 2). For all three cytokines, post-infection mRNA levels were significantly increased compared to controls ($P < 0.05$) and peak levels occurred within 8 hours of disease onset. At maximum expression, levels of IL-1β and IL-8 mRNA were roughly 5- and 60-fold greater, respectively, than those of TNFα mRNA. By 24 hours PI, mRNA for each of the cytokines had declined to control (TNFα and IL-1β) or near-control (IL-8) values. To address the possibility that experimental results were influenced by differences in gene expression between the groups due to factors unrelated to *M. haemolytica* infection, tissues collected from the unaffected right lung of all calves at necropsy were subjected to identical northern analysis. Results showed no significant differences in cytokine mRNA levels between groups (data not shown).

Although overall kinetic patterns in lung lesions were similar to those in BAL cells, some important differences were observed. Upregulation of TNFα mRNA was 10-fold greater in BAL cells than in lung tissue, suggesting that these cells constitute the major cellular source of TNFα in affected lungs. At 2 hours PI, the time at which TNFα mRNA levels were highest in both samples, BAL cells are comprised largely of alveolar macrophages.[322] This finding provides indirect evidence that alveolar macrophages are important sources of TNFα in

M. haemolytica-infected bovine lung. Similarly, upregulation of IL-1β gene expression was 4-fold greater in BAL cells than in lung lesions, suggesting that BAL cells are important sources of IL-1β within affected lung. Since BAL cells consist largely of neutrophils at 4 to 8 hours PI,[322] the period during which peak IL-1β mRNA levels were observed, this finding provides indirect evidence that neutrophils are important pulmonary sources of IL-1β in BPM. Upregulation of IL-8 mRNA was 3-fold greater in lung lesions than in BAL cells, suggesting that pulmonary cell types not present in BAL fluid produce significant amounts of IL–8.

Figure 3-2. Kinetics of TNFα, IL-1β, and IL-8 mRNA expression in the lungs of calves with acute pneumonic mannheimiosis

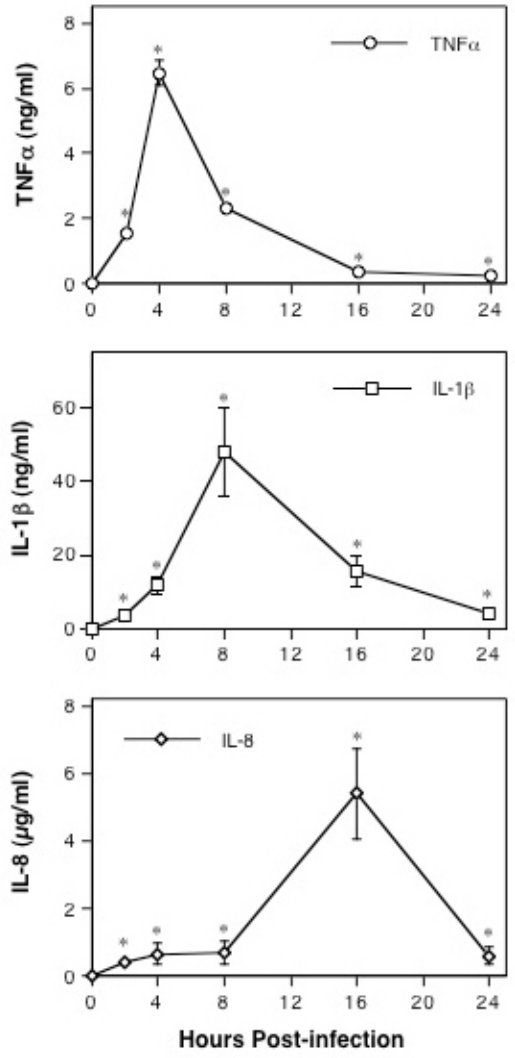
Inflammatory cytokine gene expression was measured by densitometric analysis of northern blots as described in Materials and Methods. For each cytokine, data were normalized to the expression of GAPDH mRNA and presented relative to mean normalized expression in lung tissues from mock-infected control calves. Values represent the mean ± SEM (n = 3). * $P < 0.05$ compared to mock-infected controls.



Kinetics of cytokine secretion in epithelial lining fluid

Enzyme-linked immunosorbent assays were used to characterize inflammatory cytokine secretion within airways. Concentrations of immunoreactive TNFα, IL-1β, and IL-8 in BAL fluid collected prior to infection and at 2, 4, 8, 16, and 24 hours PI were compared to those in BAL fluid from mock-infected controls. Urea was used as an endogenous marker of dilution to calculate the extent to which epithelial lining fluid (ELF) was diluted during BAL in each calf. Measured values were then corrected for dilution and expressed as cytokine concentration per ml ELF (Fig. 3). We consider this the best practical method by which to control for variable dilution of ELF constituents during BAL. Samples collected prior to inoculation with *M. haemolytica* and those from mock-infected controls contained no detectable TNFα, IL-1β, or IL-8. Concentrations of all three cytokines were significantly increased by 2 hours PI and remained elevated throughout the 24-hour study period ($P < 0.05$). Peak concentrations of TNFα (6.5 ± 0.4 ng/ml), IL-1β (48.0 ± 11.8 ng/ml), and IL-8 (5.4 ± 1.3 μg/ml) occurred at 4, 8, and 16 hours PI, respectively.

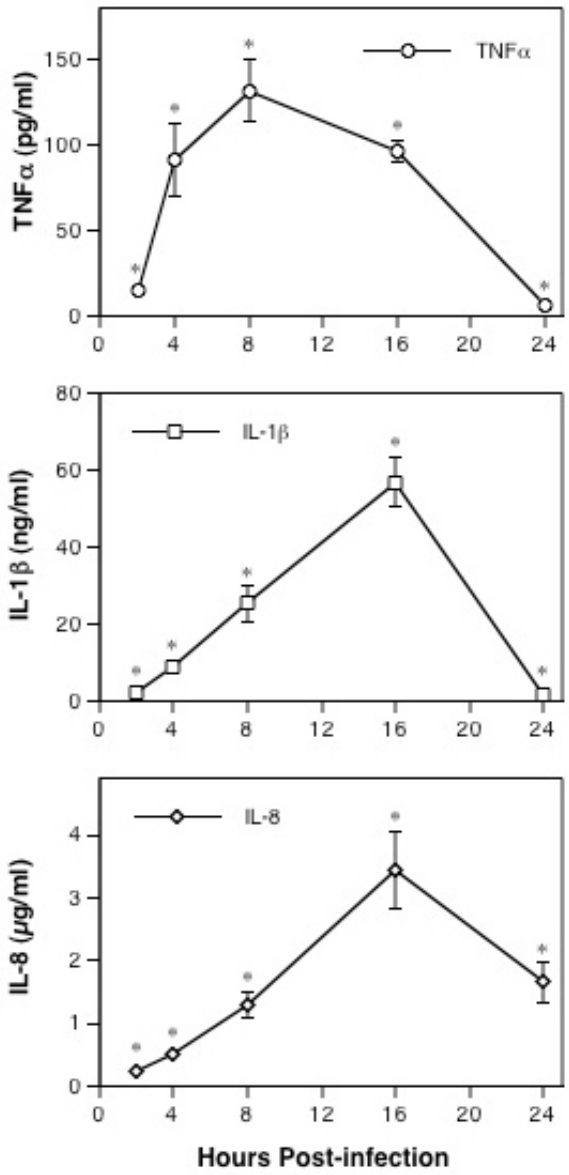
Figure 3-3. Kinetics of TNFα, IL-1β, and IL-8 secretion in the airways of calves with acute pneumonic mannheimiosis. Immunoreactive cytokines in BAL fluid were measured by antigen-capture ELISA as described in Materials and Methods. Urea was used as an endogenous marker of dilution to calculate the extent to which epithelial lining fluid (ELF) was diluted during the BAL procedure in each calf. Measured values were corrected for dilution and expressed as cytokine concentration per ml ELF. Values represent the mean ± SEM (n = 3). * $P < 0.05$ compared to mock-infected controls.



Kinetics of cytokine expression in pneumonic lungs

Enzyme-linked immunosorbent assays were used to characterize the expression of inflammatory cytokines within pulmonary lesions of pneumonic mannheimiosis. Concentrations of immunoreactive TNFα, IL-1β, and IL-8 in extracts of pneumonic lung collected 2, 4, 8, 16, and 24 hours PI were compared to those in extracts of grossly normal lung from mock-infected controls (Fig. 4). Control extracts contained 68.0 ± 6.0 ng/ml IL-8 but no detectable TNFα or IL-1β. As in ELF, concentrations of all three cytokines were significantly increased in tissue extracts by 2 hours PI and remained elevated throughout the 24-hour study period ($P < 0.05$). Peak concentrations of TNFα (131 ± 18 pg/ml) occurred at 8 hours PI, while peak concentrations of IL-1β (56.9 ± 6.5 ng/ml) and IL-8 (3.4 ± 0.6 μg/ml) occurred at 16 hours PI. Except that expression of TNFα and IL–1β was more prolonged in lung extracts as compared to ELF, overall kinetic patterns were similar. Relative concentrations in the two samples varied between cytokines, however. Whereas maximal concentrations of IL-1β and IL-8 were on roughly the same order of magnitude in either sample, TNFα concentrations were 50-fold higher in ELF than in lung extracts. This observation was consistent with the results of northern analysis and provides further evidence that TNFα expression in BPM occurs primarily within airways.

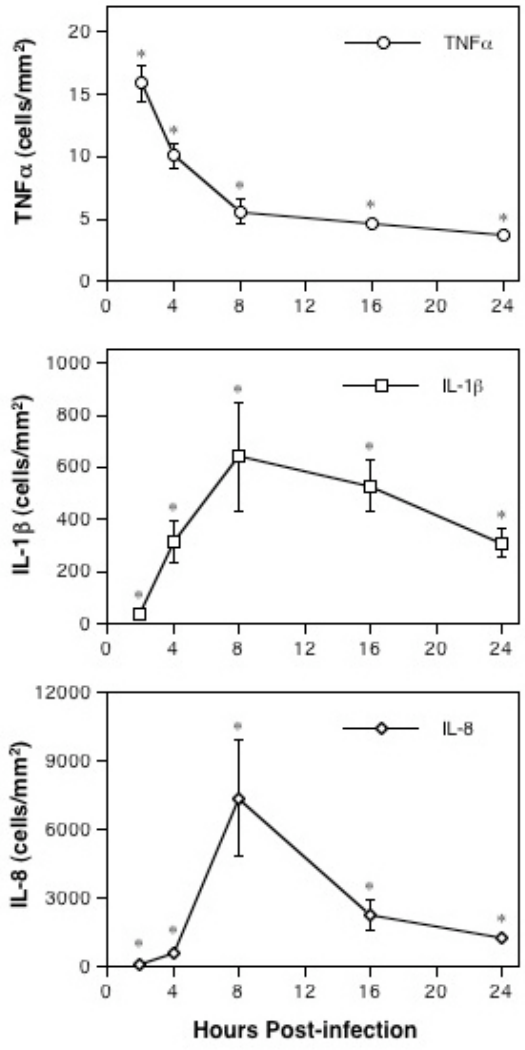
Figure 3-4. Kinetics of TNFα, IL-1β, and IL-8 expression in the lungs of calves with acute pneumonic mannheimiosis. Immunoreactive cytokines in extracts of lesional lung tissue were measured by antigen-capture ELISA as described in Materials and Methods. Values represent the mean ± SEM (n = 3). Extracts of mock-infected control lung tissue contained 68.0 ± 6.0 ng/ml IL-8 but no detectable TNFα or IL-1β. * $P < 0.05$ compared to controls.



In situ hybridization analysis of pulmonary cytokine mRNA expression

In situ hybridization with nonradioactive DIG-labeled riboprobes was used to localize inflammatory cytokine mRNA in infected and mock-infected lung tissues. Cells staining positive for TNFα, IL-1β, and IL-8 mRNA were enumerated by quantitative morphometric analysis using a combination of light microscopy and digital image analysis (Fig. 5). Tissue sections from mock-infected controls contained 2.6 ± 0.3 cells/mm^2 staining positive for TNFα mRNA and no detectable staining for IL-1β or IL-8 gene expression. In post-infection lung, the number of cells expressing mRNA specific for each of the cytokines was significantly increased throughout the 24 hour period following inoculation with *M. haemolytica* ($P < 0.05$). Peak numbers of cells expressing TNFα mRNA (15.9 ± 1.4 cells/mm^2) occurred at 2 hours PI, and peak numbers of cells expressing mRNA specific for IL-1β (640 ± 208 cells/mm^2) and IL–8 (7392 ± 2519 cells/mm^2) occurred at 8 hours PI. These data were consistent with the results of both northern analysis and ELISA, and provide further evidence that IL-8 is expressed to the greatest extent within *M. haemolytica*-infected lung while TNFα is least expressed.

Figure 3-5. Changes over time in the number of cells expressing TNFα, IL-1β, and IL–8 mRNA in the lungs of calves with acute pneumonic mannheimiosis. Pulmonary expression of cytokine mRNA was localized by in situ hybridization as described in Materials and Methods. The number of positively staining cells per mm^2 lung tissue was determined by quantitative morphometric analysis. Values represent the mean ± SEM (n = 3). Mock-infected control tissues contained 2.6 ± 0.3 cells/mm^2 staining positive for TNFα mRNA and no detectable staining for IL-1β or IL-8 mRNA. * $P < 0.05$ compared to controls.



In situ hybridization with TNFα antisense riboprobe revealed that pulmonary expression of TNFα mRNA was restricted to alveolar macrophages in both infected and mock-infected lungs (Fig. 6). This was the only cytokine for which mRNA-expressing cells were detected in control tissues. No staining was observed in lung tissues hybridized with TNFα sense riboprobe (Fig. 7). Hybridization of tissues with IL-1β antisense probe showed that at 2 hours PI, expression of IL-1β mRNA was localized to alveolar macrophages and cells within the alveolar septum, likely intravascular and/or interstitial macrophages (Fig. 8). Definitive identification of cell types at later time points was hindered by suboptimal tissue morphology, but comparison of serial sections routinely stained with hematoxylin and eosin confirmed that neutrophils within exudate in alveolar spaces and the lumina of bronchi and bronchioles became the dominant cellular source of IL-1β mRNA within 4 to 8 hours of disease onset (Fig. 9). Hybridization of lung tissues with IL-1β sense riboprobe yielded no detectable staining (Fig. 10). At 2 and 4 hours PI, IL–8 mRNA was detected in alveolar macrophages, macrophages within the alveolar septum, bronchial epithelial cells, bronchiolar epithelial cells, and neutrophils (Fig. 11). At 8 hours PI and later time points, the majority of staining for IL-8 mRNA occurred in neutrophils within alveolar exudate and exudate in the lumina of bronchioles and bronchi (Fig. 12). Staining was also observed in fibroblasts within interlobular septae. No staining was observed in tissues hybridized with IL-8 sense riboprobe (Fig. 13). A quantitative analysis of the cell types represented among cells staining positive for TNFα, IL-1β, and IL-8 at each time point was

performed. The results for IL-1β and IL–8 mRNA are summarized in Fig. 14. Expression of TNFα mRNA was restricted to alveolar macrophages.

Figure 3-6. Lung tissue from a *M. haemolytica*-inoculated calf, 2 hours post-infection. Blue-black cytoplasmic staining indicates that alveolar macrophages (arrows) express TNFα mRNA in the peracute phase of pneumonic mannheimiosis. In situ hybridization with antisense probe, nuclear fast red counterstain. Bar = 25 μm.

Figure 3-7. Lung tissue from a *M. haemolytica*-inoculated calf, 2 hours post-infection. No staining is evident in tissues hybridized with TNFα sense probe. In situ hybridization, nuclear fast red counterstain. Bar = 25 μm.

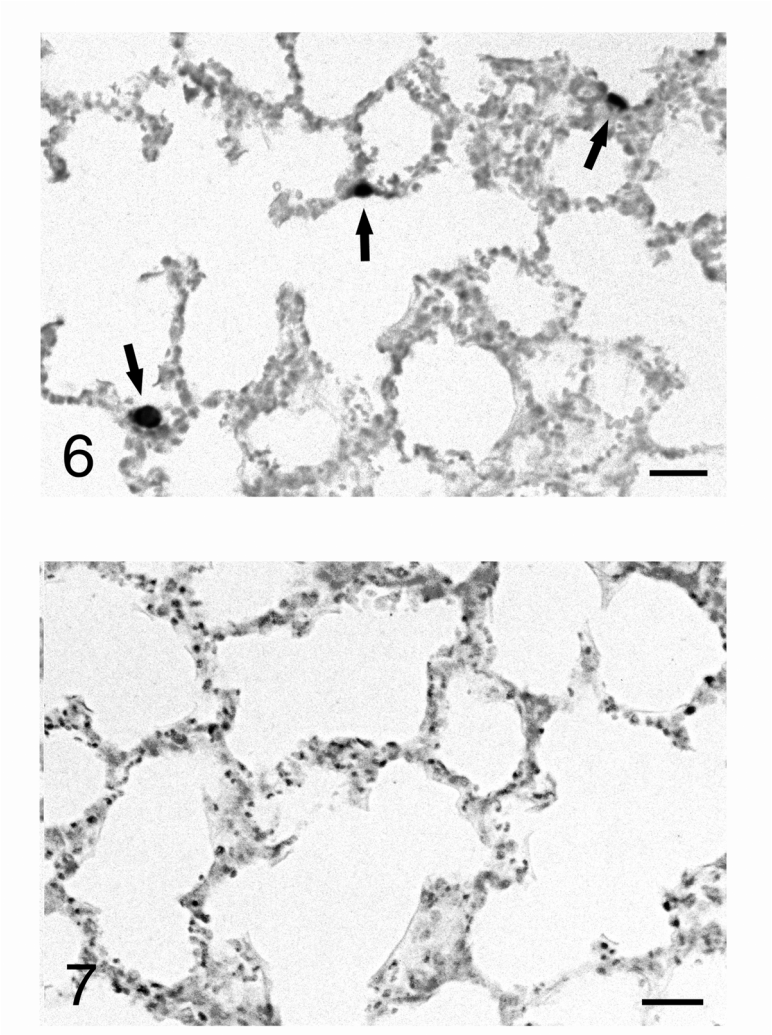


Figure 3-8. Lung tissue from a *M. haemolytica*-inoculated calf, 2 hours post-infection. Alveolar macrophages (arrows) and cells within the alveolar septum, likely intravascular macrophages, express IL-1β mRNA in the peracute phase of pneumonic mannheimiosis. In situ hybridization with antisense probe, nuclear fast red counterstain. Bar = 25 μm.

Figure 3-9. Lung tissue from a *M. haemolytica*-inoculated calf, 8 hours post-infection. Neutrophils within the alveolar exudate express IL-1β mRNA in the lesions of acute pneumonic mannheimiosis. In situ hybridization with antisense probe, nuclear fast red counterstain. Bar = 50 μm.

Figure 3-10. Lung tissue from a *M. haemolytica*-inoculated calf, 8 hours post-infection. No staining is evident in pneumonic lung tissues hybridized with IL-1β sense probe. In situ hybridization, nuclear fast red counterstain. Bar = 50 μm.

Figure 3-11. Lung tissue from a *M. haemolytica*-inoculated calf, 4 hours post-infection. Bronchiolar epithelial cells (arrows) and neutrophils within alveolar exudate and the bronchiolar lumen express IL-8 mRNA in the peracute lesions of pneumonic mannheimiosis. In situ hybridization with antisense probe, nuclear fast red counterstain. Bar = 50 μm.

Figure 3-12. Lung tissue from a *M. haemolytica*-inoculated calf, 8 hours post-infection. Extensive staining of neutrophils within the alveolar exudate reflects widespread expression of IL-8 mRNA in the lesions of pneumonic mannheimiosis. In situ hybridization with antisense probe, nuclear fast red counterstain. Bar = 100 μm.

Figure 3-13. Lung tissue from a *M. haemolytica*-inoculated calf, 8 hours post-infection. No staining is evident in pneumonic tissues hybridized with IL-8 sense probe. In situ hybridization, no counterstain. Bar = 50 μm.

FIGURES 3-8 THROUGH 3-13

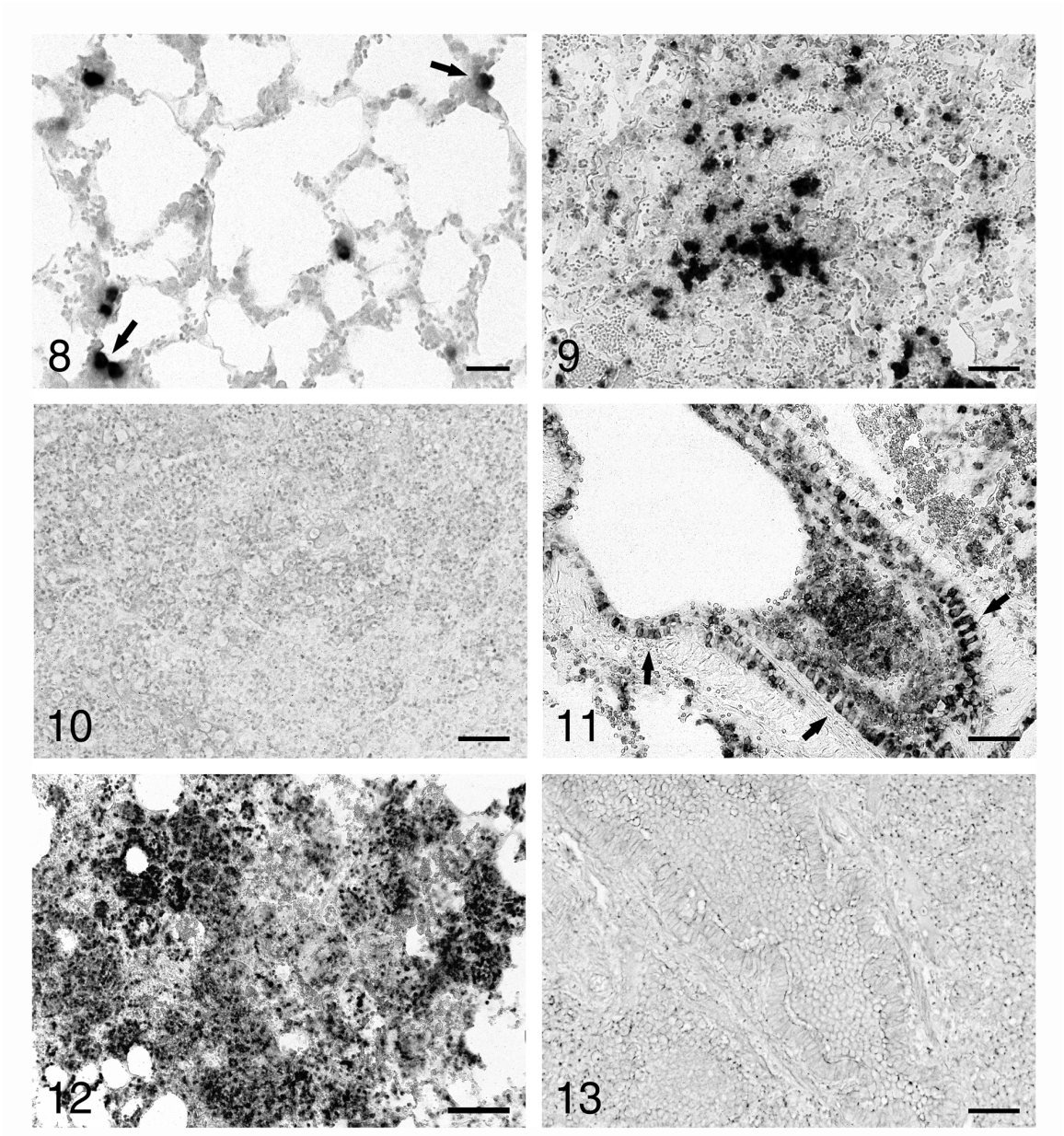
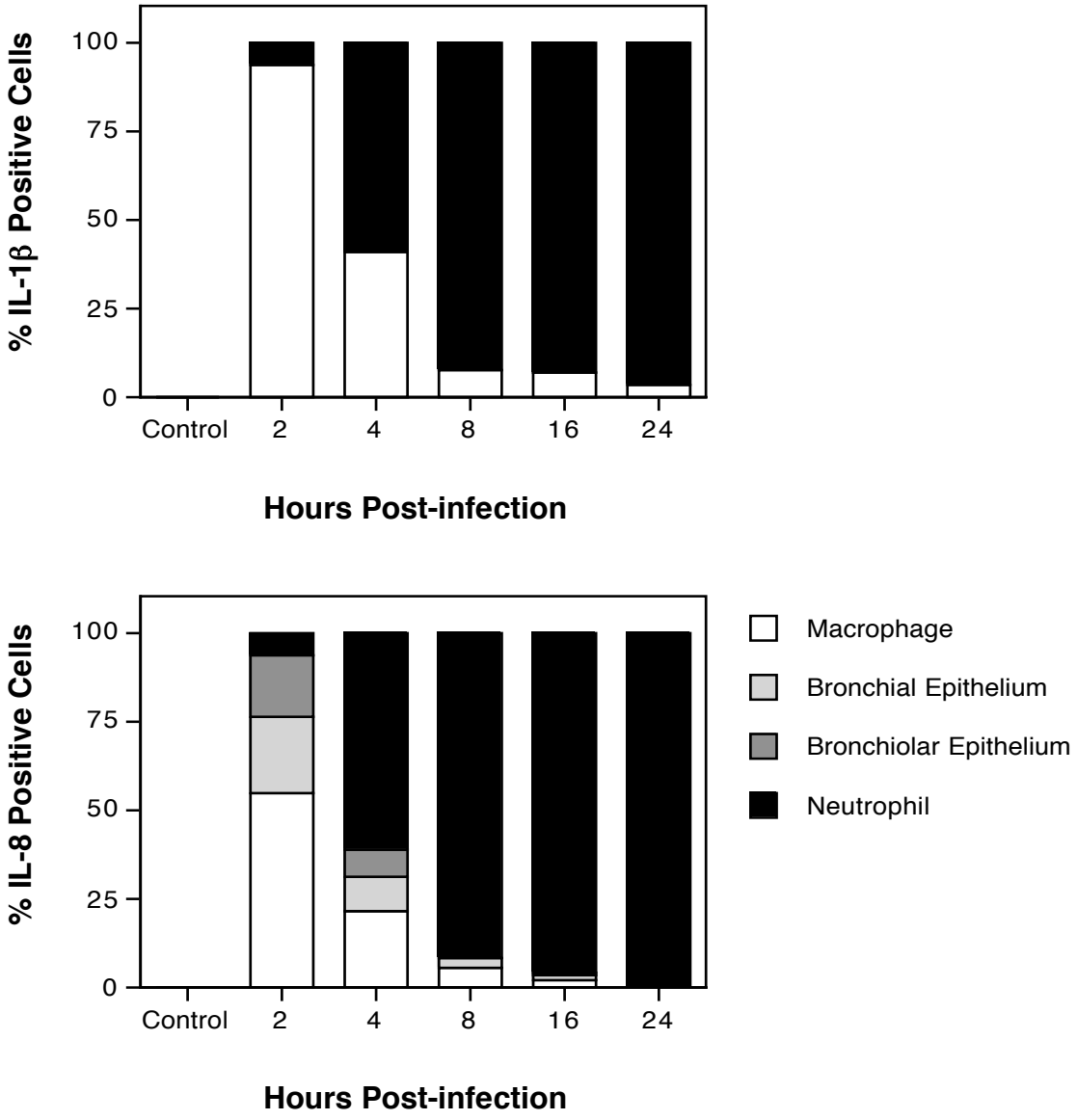


Figure 3-14. Cell types expressing IL-1β and IL-8 mRNA in the lungs of calves with pneumonic mannheimiosis. Pulmonary expression of cytokine mRNA was localized by in situ hybridization as described in Materials and Methods. Positively staining cells were identified by light microscopy on the basis of cell morphology and histological location.



DISCUSSION

Recent investigations implicate inflammatory cytokines in the pathogenesis of BPM. In this study, we demonstrated early and sustained expression of TNFα, IL–1β, and IL-8 mRNA and proteins within the airways and lung lesions of cattle experimentally infected with *M. haemolytica*. By contrast, samples collected from mock-infected control animals and the unaffected lung of infected cattle exhibited little or no such expression. Our findings are consistent with earlier reports identifying an association between lung pathology and increased pulmonary expression of inflammatory cytokines in BPM.[46, 335] Since the present study addresses the peracute and acute phases of disease development, however, it provides stronger evidence in support of a causative role for TNFα, IL-1β, and IL-8 in disease pathogenesis. This report also extends previous work by characterizing the kinetics of pulmonary cytokine expression during the initial 24 hours of disease, comparing patterns of expression in airways with those in lung parenchyma, and identifying the major cellular sources of inflammatory cytokines within pneumonic lung.

The results obtained by northern analysis, ELISA, and in situ hybridization were closely correlated. Expression of TNFα, IL–1β, and IL–8 in the airways and lung parenchyma of infected calves was significantly upregulated at both gene and protein levels. In all samples, IL-8 mRNA and proteins were upregulated to the greatest extent and those for TNFα were upregulated the least. Northern blot and ELISA analyses suggested that TNFα gene and protein expression occurred predominantly within airways, and in situ hybridization

studies confirmed that mRNA expression was localized to alveolar macrophages. Expression of IL-1β and IL-8 genes and proteins, by contrast, was more generalized. Alveolar and interstitial macrophages were important early sources of both IL-1β and IL–8, and bronchial and bronchiolar epithelial cells were significant sources of IL-8 in the first 4 hours PI. Neutrophils, however, became the dominant source of both IL-1β and IL-8 within 4 to 8 hours of disease onset. These findings demonstrate a spatial and temporal association between pulmonary expression of inflammatory cytokines and acute lung pathology, and indirectly support the hypothesis that cytokines contribute to inflammatory lung injury in BPM.

Previous studies measured pulmonary cytokine expression 2 to 4 days following endobronchial inoculation of *M. haemolytica* and did not address changes over time.[46, 335] In this study, we showed that cytokine upregulation occurs much earlier in the course of disease development than was previously recognized. TNFα, IL–1β, and IL–8 mRNA and proteins were significantly increased in all samples by 2 hours PI. Although kinetic patterns varied, peak levels of mRNA for all cytokines were achieved within 8 hours PI and peak cytokine concentrations occurred within 16 hours PI. By 24 hours PI, mRNA specific for all three cytokines declined to control or near-control values. Although cytokine concentrations in airways and lung lesions remained elevated throughout the study period, they were significantly decreased at 24 hours PI compared to peak values achieved earlier. These observations suggest that TNFα, IL-1β, and IL-8 may exert their greatest pathogenic effects within 16 hours of disease onset.

The specific mechanisms, if any, by which inflammatory cytokines mediate lung injury in BPM await clarification. It is reasonable to assume, however, that their biological effects in bovine lung parallel those recognized in other systems. In most mammalian models TNFα, IL-1β, and IL-8 are central components of a complex cytokine network that initiates, amplifies, and sustains the inflammatory response in tissue. Available evidence also supports the importance of this network in coordinating acute inflammatory responses within the lung.[237, 275] TNFα and IL-1β are pleiotropic early-response mediators that establish cytokine cascades through autocrine and paracrine activation of a broad array of cell types.[155, 256] They initiate neutrophil transmigration and activation by upregulating the expression of adhesion molecules on neutrophils and microvascular endothelium.[42, 282] Though not directly chemotactic for neutrophils, both TNFα and IL-1β induce the secretion of IL–8, the most potent neutrophil chemotactic and activating factor, and other chemokines by a variety of cell types.[25, 237, 275, 281-283] In addition to their roles in neutrophil recruitment, TNFα, IL–1β, and especially IL-8 promote neutrophil-mediated tissue injury by stimulating neutrophil degranulation and the extracellular release of arachidonic acid metabolites, toxic oxygen radicals, and proteolytic enzymes.[37, 87, 93, 244, 250, 255]

Our findings indicate that IL-8 is the dominant inflammatory cytokine expressed within the lungs during the acute phase of BPM. Throughout the 24 hour period following inoculation of *M. haemolytica*, IL-8 was expressed in much greater quantities than either TNFα or IL-1β. At 2 hours PI, the earliest time point studied, concentrations of IL-8 in ELF were already roughly 250- and 100-fold greater than those of TNFα and IL-1β, respectively. In extracts of

lesional lung parenchyma at the same time point, concentrations of IL-8 were roughly 17,000- and 100-fold greater than those of TNFα and IL-1β, respectively. Abundant pulmonary expression of IL-8 is classically considered to be a downstream event that is dependent, at least in part, on the prior secretion of early-response cytokines such as TNFα and IL–1β.[150, 237, 275] Our findings do not exclude the possibility that a similar cascade of interactions is a necessary prerequisite for IL-8 production in BPM, but they do indicate that the critical events in that cascade must occur well before 2 hours PI. These observations, together with the results of our kinetic analyses, have at least two important implications for therapeutic strategies based upon modulation of inflammatory cytokines. First, pharmacological agents that inhibit the synthesis of IL-8 or antagonize its biological effects are likely to prove more effective in the management of BPM than those targeting only TNFα or IL-1β. Second, anti-cytokine agents may have to be administered very early in the course of disease, or possibly even prior to colonization of the lung by *M. haemolytica*, in order to prevent or interrupt inflammatory lung injury.

In conclusion, we have demonstrated a correlation between early pulmonary expression of TNFα, IL–1β, and IL-8 and the development of acute BPM, further substantiating a role for these mediators in disease pathogenesis. Inflammatory cytokines may therefore represent drug targets that could be pharmacologically modulated in the management of this important disease of cattle. The results reported here, however, suggest that such strategies may have to be implemented very early in the course of disease if they are to be effective. Since pulmonary expression of IL-8 was much greater than that of TNFα and IL–

1β at all time points studied, anti-cytokine agents targeting this mediator may prove to be most useful in the prevention and treatment of BPM.

ACKNOWLEDGEMENTS

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CHAPTER 4

PHARMACOLOGICAL INHIBITION OF *MANNHEIMIA HAEMOLYTICA* LIPOPOLYSACCHARIDE- AND LEUKOTOXIN-INDUCED CYTOKINE EXPRESSION IN BOVINE ALVEOLAR MACROPHAGES

INTRODUCTION

Bovine pneumonic mannheimiosis (BPM), an acute fibrinous pleuropneumonia caused by *Mannheimia (Pasteurella) haemolytica*, is a common and economically important disease of North American cattle. One of the hallmark histopathological features of BPM is extensive infiltration of the alveolar spaces by neutrophils. Neutrophil depletion prior to experimental infection with *M. haemolytica* protects calves from subsequent lung injury [265, 322], indicating that this cell type contributes to the severe pulmonary pathology that is characteristic of this disease. The specific mechanisms by which neutrophils mediate lung pathology in BPM remain undefined but are likely to include oxidative injury and structural degradation due to the extracellular release of reactive oxygen intermediates and proteolytic lysosomal enzymes [267]. Recognition of the importance of neutrophils in disease pathogenesis prompted us and others to investigate specific factors governing their recruitment and activation within *M. haemolytica*-infected bovine lung.

The migration and activation of neutrophils within inflamed tissue are regulated by a complex network of interactions between cytokines, leukocytes, vascular endothelium, cellular adhesion molecules, and soluble chemotactic factors. Current evidence indicates that the inflammatory cytokines tumor necrosis factor-alpha (TNFα), interleukin–1 beta (IL–1β), and interleukin–8 (IL–8) play a central role in the initiation and orchestration of these interactions. TNFα and IL–1β are pleiotropic early response polypeptides secreted by monocytes and macrophages in response to microbial pathogens, tissue trauma, and other

stimuli [155]. IL–8, a potent chemotactic and activating factor for neutrophils, is a C-X-C chemokine secreted by a variety of immune and non-immune cell types including monocytes, macrophages, fibroblasts, epithelial cells, and neutrophils [21, 150].

Pulmonary overexpression of inflammatory cytokines is believed to contribute to the pathogenesis of several infectious and inflammatory lung diseases in humans, including asthma [261, 262], adult respiratory distress syndrome [88, 185], cystic fibrosis [35, 148], and pneumoconiosis [310]. Cytokine overexpression is also associated with lung pathology in animal models of influenza pneumonia [215], silicosis [91], and immune complex alveolitis [319]. Among the domestic species, TNFα, IL–1β, and IL–8 are implicated in the pathogenesis of swine influenza [307], porcine pleuropneumonia caused by *Actinobacillus pleuropneumoniae* [16, 130], and *Corynebacterium pseudotuberculosis*-induced pulmonary pyogranulomas in sheep [94].

A growing body of evidence implicates inflammatory cytokines in the pathogenesis of BPM, prompting speculation that it may be possible to treat or prevent the disease through pharmacologic modulation of cytokine expression. Heat-killed *M. haemolytica*, as well as purified *M. haemolytica* lipopolysaccharide (LPS) and leukotoxin (LktA), induce the expression of TNFα, IL–1β, and IL–8 genes and proteins in bovine alveolar macrophages *in vitro* [153, 196, 197, 278, 334, 336]. In addition, BPM is associated with pulmonary expression of these cytokines *in vivo* [45, 46, 335]. Furthermore, *in vivo* studies conducted in our laboratory recently showed that pulmonary expression of TNFα, IL–1β, and IL–8 is significantly increased within 2 hours of experimental infection [173].

The purpose of this study was to evaluate the ability of six pharmacological agents to suppress the expression of TNFα, IL–1β, and IL–8 genes and proteins in bovine alveolar macrophages (AM) exposed to *M. haemolytica* lipopolysaccharide (LPS) and leukotoxin (LktA) *in vitro*. The compounds tested included dexamethasone (DEX), tetrahydropapaveroline (THP), pentoxifylline (PTX), rolipram (ROL), SB203580 (SB), and thalidomide (THL). These agents were selected on the basis of their demonstrated ability to suppress the production of one or more inflammatory cytokines in other experimental systems. Cytokine expression was induced by the addition of purified *M. haemolytica* LPS and LktA to AM cell cultures following pretreatment with inhibitor compounds. Secretion of TNFα, IL–1β, and IL–8 proteins into the cell culture supernatant was measured using enzyme-linked immunosorbent assays, and steady-state accumulation of cytokine-specific mRNA was measured by northern blot analysis. Dose-dependent inhibition of cytokine secretion occurred in response to pretreatment of AM with DEX (TNFα, IL–1β, IL–8), THP (TNFα, IL–1β, IL–8), PTX (TNFα, IL–1β, IL–8), ROL (TNFα, IL-1β), and SB (TNFα, IL–8). Significant dose-dependent inhibition of cytokine mRNA expression occurred in response to pretreatment with DEX (TNFα, IL–1β, IL–8), THP (TNFα, IL–1β, IL–8), and PTX (TNFα). DEX was the most effective inhibitor by far; pretreatment with this compound yielded greater than 95% inhibition of cytokine gene and protein expression over a broad range of concentrations. These findings demonstrate that DEX, THP, PTX, ROL, and SB are capable of suppressing inflammatory cytokine secretion by bovine AM *in vitro*. If

pulmonary cytokine secretion may be similarly inhibited *in vivo*, anti-cytokine therapy may represent a novel strategy for the management of BPM.

RESULTS

Effect of inhibitors on cytokine protein secretion

Enzyme-linked immunosorbent assays were used to measure inflammatory cytokines in cell culture supernatants 24 h following exposure to a combination of *M. haemolytica* LPS (100 ng/mL) and LktA (2 LU/mL). Concentrations of immunoreactive TNFα, IL–1β, and IL–8 in supernatants from cultures treated with cytokine inhibitors 30 min prior to stimulation were compared with those from cultures receiving no pretreatment, and with cultures receiving pretreatment with the inhibitor vehicle only. Significant ($P < 0.05$) dose-dependent inhibition of AM cytokine secretion was observed in response to pretreatment with dexamethasone (Fig. 1a; TNFα, IL–1β, IL–8), tetrahydropapaveroline (Fig. 2a; TNFα, IL–1β, IL–8), pentoxifylline (Fig. 3a; TNFα, IL–1β, IL–8), rolipram (Fig. 4; TNFα, IL–1β), and SB203580 (Fig. 5; TNFα, IL–8). Of these, dexamethasone was the most effective inhibitor (Fig. 1a); it suppressed greater than 95% of the secretion of all three cytokines over a broad range of concentrations (10 nM – 100 μM). Inhibition of IL–8 secretion by tetrahydropapaveroline occurred only at 100 μM, the highest concentration tested. Inhibition of IL–8 secretion by pentoxifylline occurred only at 2 mM and 1 mM, the highest concentrations tested. Although SB203580 suppressed the

production of TNFα and IL–8 at concentrations between 100 nM and 10 μM, these doses also triggered increased secretion of IL–1β well beyond that provoked by the vehicle in which it was dissolved, DMSO (Fig. 5). Pretreatment with thalidomide exerted no significant effect on cytokine production, although its vehicle, DMSO, significantly increased the secretion of both TNFα and IL–1β by stimulated AM (Fig. 6).

Effect of inhibitors on cytokine mRNA expression

Northern blot analysis was used to characterize TNFα, IL–1β, and IL–8 gene expression in bovine alveolar macrophages (AM) 8 h following exposure to *M. haemolytica* LPS and LktA. Steady-state levels of inflammatory cytokine mRNA in cultures treated with cytokine inhibitors 30 min prior to stimulation were compared with those in cultures receiving no pretreatment, and with those in cultures receiving pretreatment with the inhibitor vehicle only. Significant ($P < 0.05$) dose-dependent inhibition of steady-state cytokine mRNA accumulation was observed in response to pretreatment with dexamethasone (Fig. 1b; TNFα, IL–1β, IL–8), tetrahydropapaveroline (Fig. 2b; TNFα, IL–1β, IL–8), and pentoxifylline (Fig. 3b; (TNFα). Dexamethasone was the most effective inhibitor of cytokine gene expression (Fig. 1b); it suppressed accumulation of mRNA specific for all three cytokines by approximately 95% over a broad range of concentrations (10 nM – 100 μM). Tetrahydropapaveroline significantly suppressed TNFα mRNA levels at concentrations between 137 nM and 100 μM, while significant suppression of IL–1β and IL–8 mRNA was only observed at the

highest concentration tested, 100 μM (Fig. 2b). Pentoxifylline pretreatment caused dose-dependent suppression of TNFα mRNA at concentrations between 500 μM and 2 mM (Fig. 3b). These findings suggest that dexamethasone, tetrahydropapaveroline, and pentoxifylline regulate inflammatory cytokine production at the level of transcription or mRNA stability. Pretreatment of AM with rolipram or SB203580 exerted no significant effect on gene expression (data not shown), suggesting that these agents suppress cytokine production through post-transcriptional mechanisms. Pretreatment with thalidomide had no effect on cytokine mRNA levels at any dose (data not shown).

Figure 4-1. Effect of dexamethasone pretreatment on induced secretion of TNFα, IL–1β, and IL–8 by bovine alveolar macrophages (panel A) and steady-state accumulation of cytokine-specific mRNA (panel B). Immunoreactive cytokines in cell culture supernatants were measured by antigen-capture ELISA as described in Materials and Methods. Measured cytokine concentrations were expressed as a percentage of those detected in positive control cultures. Inflammatory cytokine gene expression was measured by densitometric analysis of northern blots. Gene expression data were normalized to the expression of GAPDH mRNA and the accumulation of cytokine mRNA was expressed as a percentage of that observed in positive control cultures. Values represent the mean ± SEM (n=3). $^{*}P < 0.05$ compared to cultures treated with *M. haemolytica* LPS (100 ng/mL) and LktA (2 LU/mL) in the absence of any cytokine inhibitors.

FIGURE 4-1

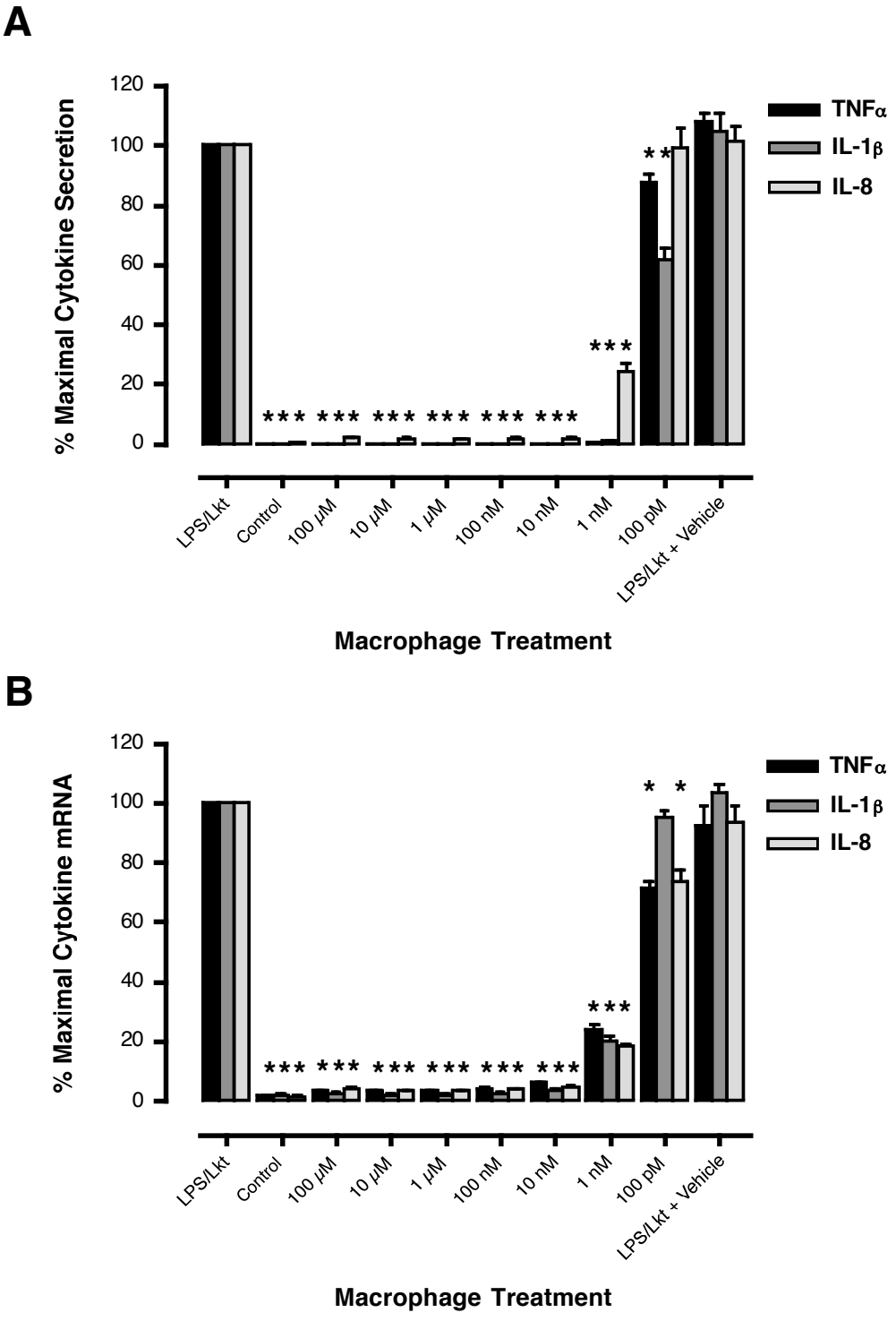
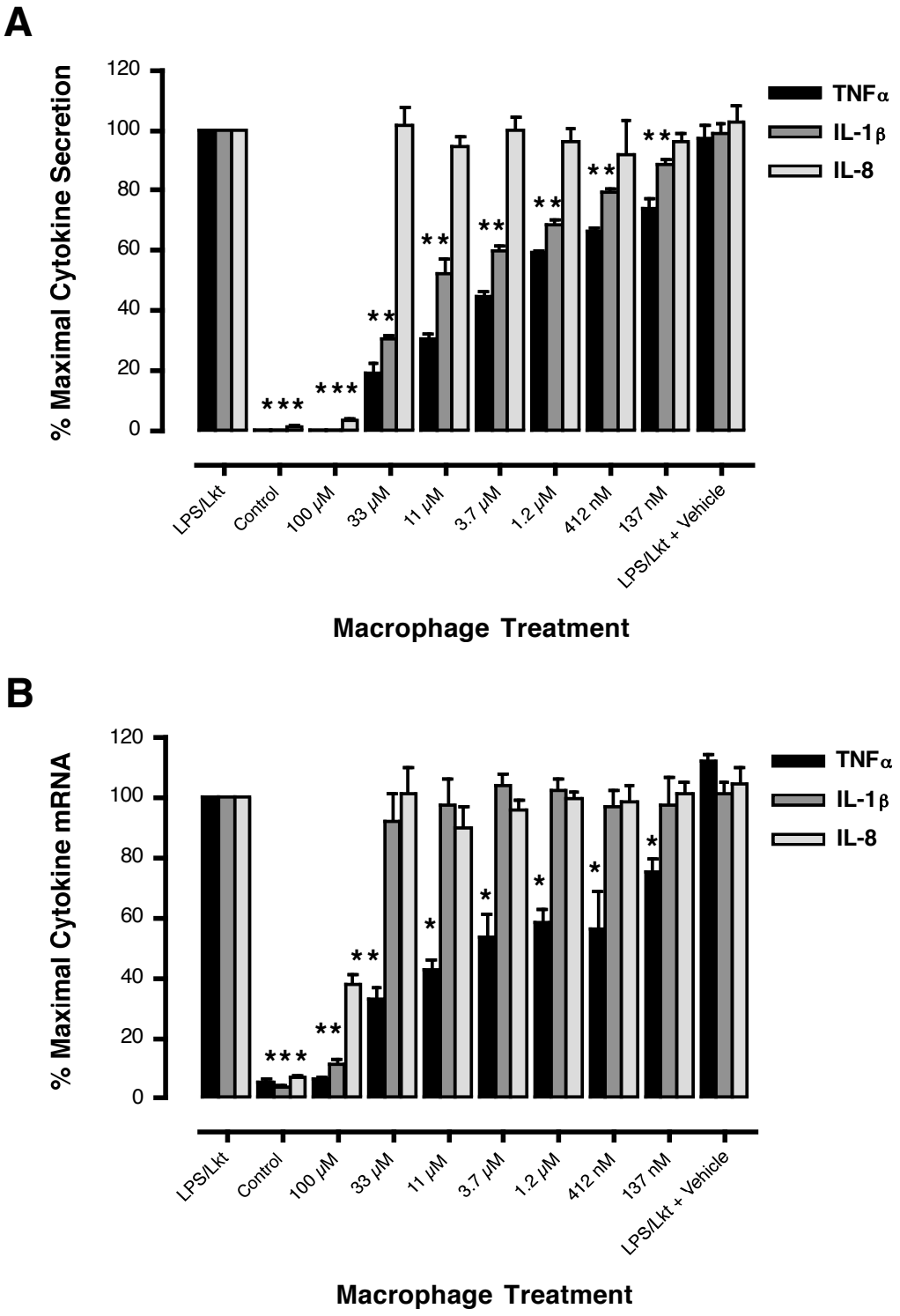

A
% Maximal Cytokine Secretion
120
100
80
60
40
20
0
TNFα
IL-1β
IL-8
LPS/Lkt
Control
100 μM
10 μM
1 μM
100 nM
10 nM
1 nM
100 pM
LPS/Lkt + Vehicle
Macrophage Treatment
B
% Maximal Cytokine mRNA
120
100
80
60
40
20
0
TNFα
IL-1β
IL-8
LPS/Lkt
Control
100 μM
10 μM
1 μM
100 nM
10 nM
1 nM
100 pM
LPS/Lkt + Vehicle
Macrophage Treatment

Figure 4-2. Effect of tetrahydropapaveroline pretreatment on induced secretion of TNFα, IL–1β, and IL–8 by bovine alveolar macrophages (panel A) and steady-state accumulation of cytokine-specific mRNA (panel B).

Immunoreactive cytokines in cell culture supernatants were measured by antigen-capture ELISA as described in Materials and Methods. Measured cytokine concentrations were expressed as a percentage of those detected in positive control cultures. Inflammatory cytokine gene expression was measured by densitometric analysis of northern blots. Gene expression data were normalized to the expression of GAPDH mRNA and the accumulation of cytokine mRNA was expressed as a percentage of that observed in positive control cultures. Values represent the mean ± SEM (n=3). * $P < 0.05$ compared to cultures treated with *M. haemolytica* LPS (100 ng/mL) and LktA (2 LU/mL) in the absence of any cytokine inhibitors.

FIGURE 4-2


A
% Maximal Cytokine Secretion
120
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80
60
40
20
0
TNFα
IL-1β
IL-8
LPS/Lkt
Control
100 μM
33 μM
11 μM
3.7 μM
1.2 μM
412 nM
137 nM
LPS/Lkt + Vehicle
Macrophage Treatment
B
% Maximal Cytokine mRNA
120
100
80
60
40
20
0
TNFα
IL-1β
IL-8
LPS/Lkt
Control
100 μM
33 μM
11 μM
3.7 μM
1.2 μM
412 nM
137 nM
LPS/Lkt + Vehicle
Macrophage Treatment

Figure 4-3. Effect of pentoxifylline pretreatment on induced secretion of TNFα, IL-1β, and IL–8 by bovine alveolar macrophages (panel A) and steady-state accumulation of cytokine-specific mRNA (panel B).

Immunoreactive cytokines in cell culture supernatants were measured by antigen-capture ELISA as described in Materials and Methods. Measured cytokine concentrations were expressed as a percentage of those detected in positive control cultures. Inflammatory cytokine gene expression was measured by densitometric analysis of northern blots. Gene expression data were normalized to the expression of GAPDH mRNA and the accumulation of cytokine mRNA was expressed as a percentage of that observed in positive control cultures. Values represent the mean ± SEM (n=3). *$P < 0.05$ compared to cultures treated with *M. haemolytica* LPS (100 ng/mL) and LktA (2 LU/mL) in the absence of any cytokine inhibitors.

FIGURE 4-3

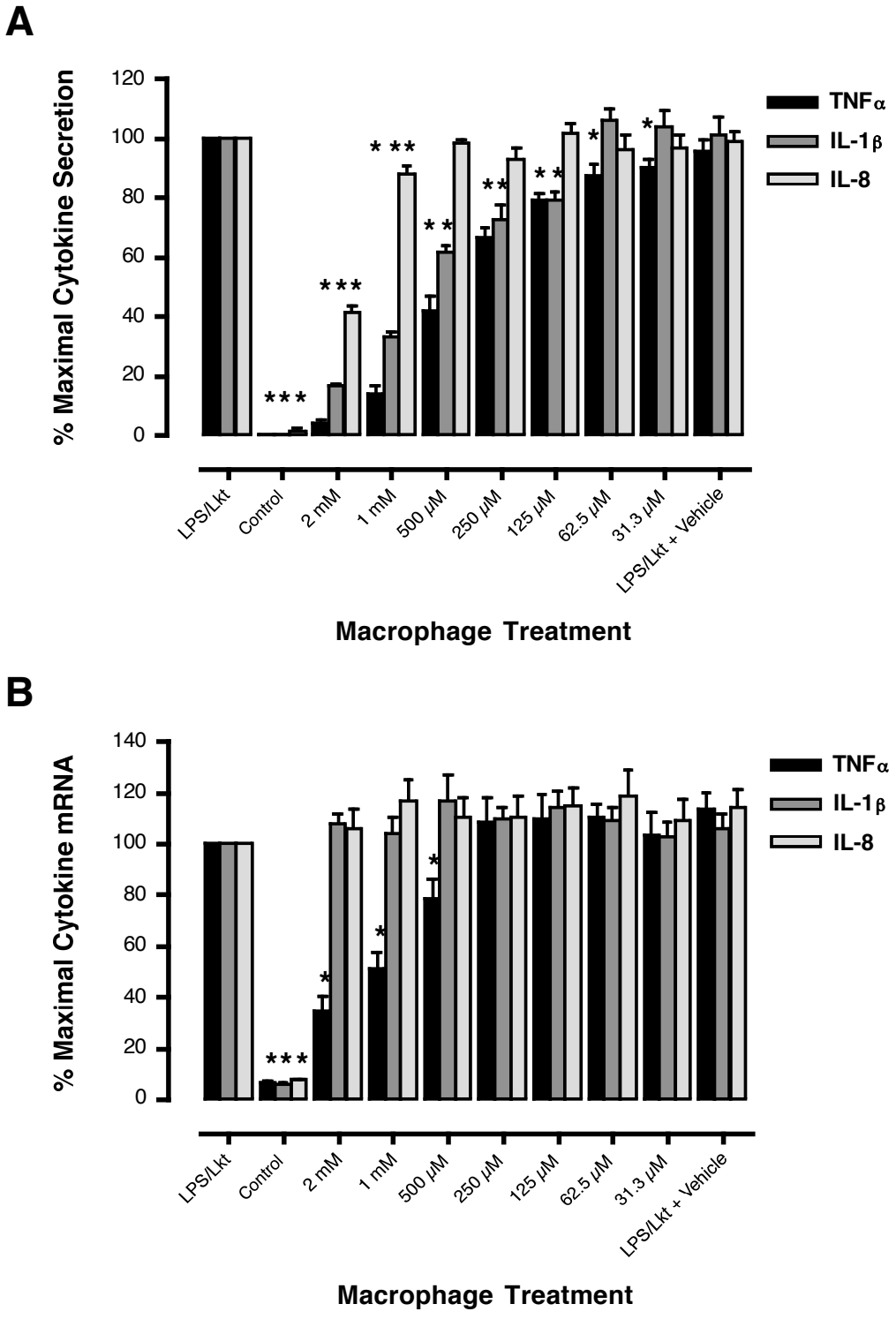

A
% Maximal Cytokine Secretion
120
100
80
60
40
20
0
TNFα
IL-1β
IL-8
LPS/LKt
Control
2 mM
1 mM
500 μM
250 μM
125 μM
62.5 μM
31.3 μM
LPS/Lkt + Vehicle
Macrophage Treatment
B
% Maximal Cytokine mRNA
140
120
100
80
60
40
20
0
TNFα
IL-1β
IL-8
LPS/LKt
Control
2 mM
1 mM
500 μM
250 μM
125 μM
62.5 μM
31.3 μM
LPS/Lkt + Vehicle
Macrophage Treatment

Figure 4-4. Effect of rolipram pretreatment on induced secretion of TNFα, IL–1β, and IL-8 by bovine alveolar macrophages.

Immunoreactive cytokines in cell culture supernatants were measured by antigen-capture ELISA as described in Materials and Methods. Measured cytokine concentrations were expressed as a percentage of those detected in positive control cultures. Values represent the mean ± SEM (n=3). *$P < 0.05$ compared to cultures treated with *M. haemolytica* LPS (100 ng/mL) and LktA (2 LU/mL) in the absence of any cytokine inhibitors.

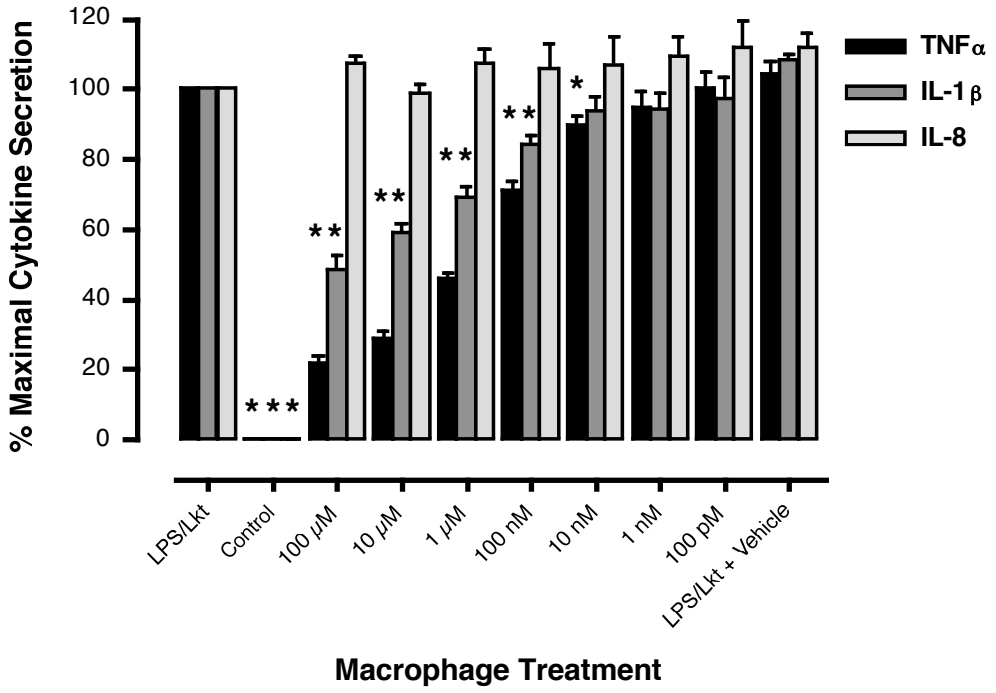


Figure 4-5. Effect of SB203580 pretreatment on induced secretion of TNFα, IL–1β, and IL–8 by bovine alveolar macrophages.

Immunoreactive cytokines in cell culture supernatants were measured by antigen-capture ELISA as described in Materials and Methods. Measured cytokine concentrations were expressed as a percentage of those detected in positive control cultures. Values represent the mean ± SEM (n=3). $^*P < 0.05$ compared to cultures treated with *M. haemolytica* LPS (100 ng/mL) and LktA (2 LU/mL) in the absence of any cytokine inhibitors.

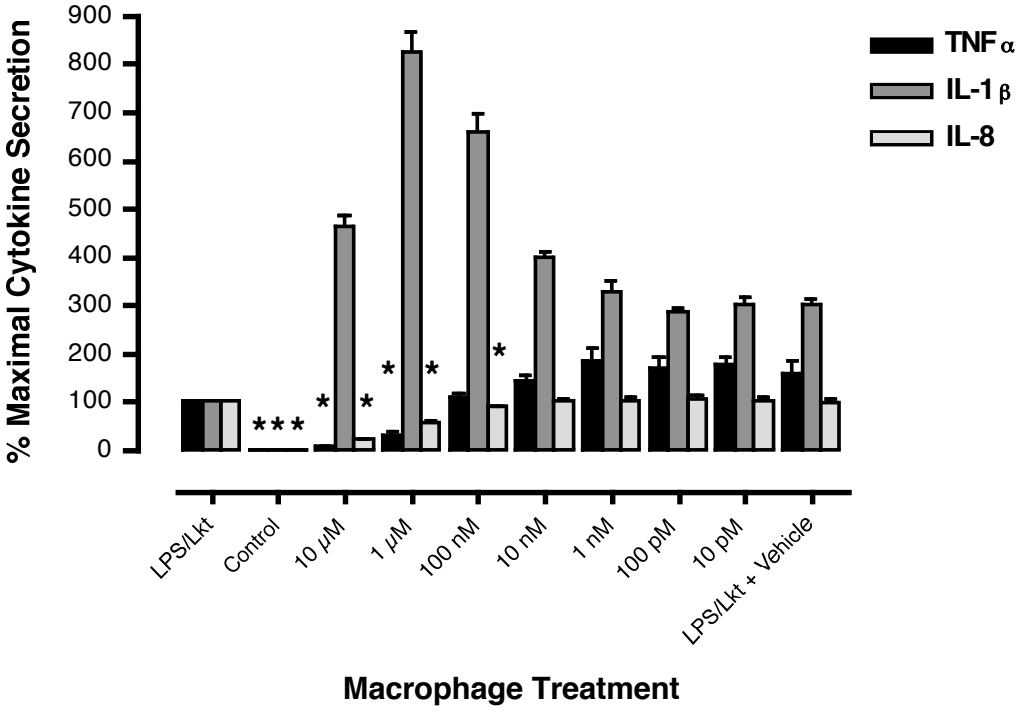
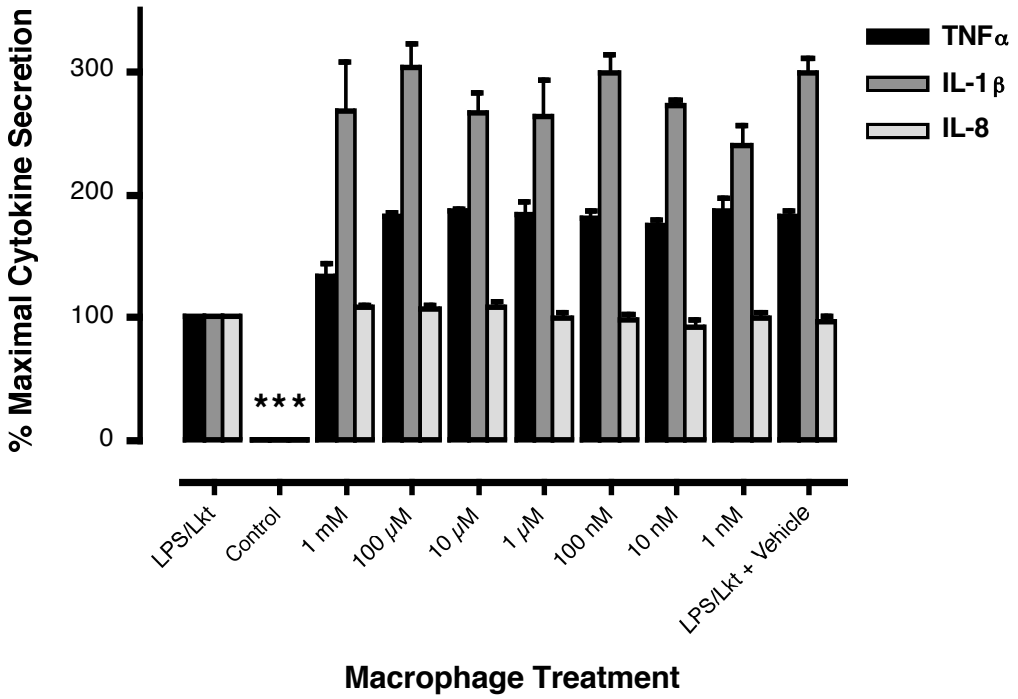


Figure 4-6. Effect of thalidomide pretreatment on induced secretion of TNFα, IL-1β, and IL–8 by bovine alveolar macrophages.

Immunoreactive cytokines in cell culture supernatants were measured by antigen-capture ELISA as described in Materials and Methods. Measured cytokine concentrations were expressed as a percentage of those detected in positive control cultures. Values represent the mean ± SEM (n=3). $^{*}P < 0.05$ compared to cultures treated with *M. haemolytica* LPS (100 ng/mL) and LktA (2 LU/mL) in the absence of any cytokine inhibitors.



DISCUSSION

Bovine pneumonic mannheimiosis (BPM) remains a leading source of economic loss to the North American beef and dairy industries. Recent investigations indicate that inflammatory cytokines contribute to the pathogenesis of inflammatory lung injury in BPM through a combination of mechanisms including neutrophil recruitment, leukocyte activation, and the induction of a broad array of inflammatory mediators. Inflammatory cytokines may therefore represent therapeutic targets that could be pharmacologically modulated with the goal of treating or preventing BPM. Each of the agents tested in this study inhibits production of TNFα, IL–1β, and/or IL–8 in a variety of experimental systems. Their effects in a bovine system, however, have not been evaluated previously. In this study, we demonstrated that dexamethasone, tetrahydropapaveroline, pentoxifylline, rolipram, and SB203580 are capable of suppressing production of one or more of these inflammatory cytokines by AM *in vitro*. Thalidomide failed to inhibit secretion of any of the cytokines under investigation.

Glucocorticoids are among the most effective and widely used anti-inflammatory drugs, but their mechanism of action has been poorly characterized until recently. These agents, particularly dexamethasone, are potent inhibitors of cytokine synthesis at the transcriptional, post-transcriptional, and translational levels [7]. In most mammalian models, glucocorticoids inhibit the transcription of most inflammatory cytokine genes, including TNFα [151, 187], IL-1β [8, 147], and IL–8 [152]. They also inhibit synthesis of a wide range of immune

cytokines, including IL–2, IL–3, IL–4, IL–5, IL–12, IFN–γ, macrophage colony-stimulating factor (M-CSF), granulocyte (G)-CSF, and granulocyte-macrophage (GM)–CSF [2]. Recent evidence indicates that glucocorticoids inhibit cytokine gene transcription through upregulation of IkB, which traps NF-KB in the cytoplasm and prevents its translocation into the nucleus [14, 247]. Because NF-KB is a transcriptional activator for a wide range of genes associated with immune and inflammatory responses, including inflammatory cytokines, inhibition of its activity yields potent anti-inflammatory effects. In the study reported here, dexamethasone was by far the most effective inhibitor studied; pretreatment yielded greater than 95% inhibition of cytokine gene and protein expression over a broad range of concentrations. These findings are consistent with results obtained in wide variety of mammalian systems.

The observation that reactive oxygen intermediates provoke inflammatory cytokine gene expression through upregulation of NF-KB activity [20, 248] led researchers to hypothesize that antioxidant drugs may be used to modulate cytokine expression. Several antioxidants, including butylated hydroxyanisole (BHA) [50, 98], probucol [4], tocopherol (vitamin E) [4], nordihydroguaiaretic acid (NDGA) [98], apomorphine [98], and tetrahydropapaveroline (THP) [98], inhibit inflammatory cytokine production by human and animal cell lines *in vitro*. Three of these compounds (BHA, NDGA, and THP) inhibit *in vitro* synthesis of TNFα and IL–1 by colonic tissue samples from human patients with inflammatory bowel disease [225]. *In vivo*, pretreatment of mice with THP suppresses LPS-induced production of IL–1β by peritoneal macrophages, and pretreatment of mice with apomorphine suppresses systemic overproduction of TNFα and IL–1β

in response to lethal challenge with LPS [98]. These observations suggest that antioxidant compounds may be useful in the management of diseases characterized by the overproduction of inflammatory cytokines, especially TNFα and IL–1β. In this study, THP pretreatment yielded dose-dependent inhibition of induced secretion of TNFα, IL-1β, and, to a lesser extent, IL–8 by bovine AM. Inhibition of TNFα and IL–1β was statistically significant over a broad range of concentrations (137 nM – 100 μM). Northern blot analysis revealed significant suppression of mRNA specific for all three cytokines at 100 μM, the highest dose tested, and significant suppression of TNFα mRNA alone at concentrations between 137 nM and 33 μM. These findings suggest that THP regulates the production of TNFα at the level of transcription or mRNA stability, while IL–1β is regulated at the post-transcriptional level. Although suppression of IL–8 secretion and mRNA accumulation was observed at 100 μM, the highest dose tested, it is possible that this was due to nonspecific, and possibly toxic, effects of the agent on cellular gene expression and protein synthesis.

Two different phosphodiesterase (PDE) inhibitors, pentoxifylline and rolipram, were investigated in this study. The cytokine-suppressive effects of PDE inhibitors have been studied extensively. These compounds inhibit TNFα production, but not generally IL–1, IL-6, or IL-8 production, at the transcriptional level by increasing intracellular concentrations of cyclic adenosine monophosphate (cAMP) [40, 95]. Pentoxifylline (PTX), a methyl xanthine agent that acts as a general PDE inhibitor, exhibits significant anti-inflammatory effects. *In vitro*, PTX inhibits production of TNFα by peripheral blood monocytes [202], peritoneal macrophages [285], and alveolar macrophages [179]. *In vivo*, it reduces

morbidity and mortality associated with sepsis or endotoxin challenge in mice, rats, and humans, probably through its ability to downregulate systemic TNFα production [48, 63, 204, 245, 276]. PTX also appears to exert direct anti-inflammatory effects on neutrophils. In LPS- or cytokine-activated neutrophils, PTX inhibits adherence, superoxide production, and granule release [175, 239, 287]. PTX also ameliorates TNFα-induced neutrophil-mediated lung injury *in vivo* [165, 333]. In this study, pretreatment of bovine AM with pentoxifylline caused significant dose-dependent inhibition of TNFα, IL–1β, and IL–8 secretion. TNFα was suppressed to the greatest extent and over the broadest range of PTX concentrations (31.3 μM – 2 mM). IL-1β suppression occurred at doses from 125 μM – 2 mM, while IL-8 suppression occurred only at 1 mM and 2 mM, the highest concentrations tested. Consistent with the results of other studies, Northern blot analysis revealed significant dose-dependent suppression of TNFα mRNA, indicating that PTX inhibits this cytokine at the level of transcription of mRNA stability. By contrast, IL–1β and IL–8 were inhibited at the post-transcriptional level by this agent.

In immune and inflammatory cells, including monocytes, mast cells, neutrophils, basophils, and eosinophils, PDE IV is the predominant cAMP metabolizing enzyme [296]. In most models, PDE IV-selective inhibitors such as rolipram are more potent inhibitors of TNFα production than general PDE inhibitors such as pentoxifylline. In this study, rolipram inhibited the secretion of both TNFα and IL–1β in a dose-dependent manner, consistent with previous studies. However, inhibition of TNFα was exerted at the post-transcriptional level, suggesting that inflammatory cytokine regulation by rolipram may be mediated by different mechanisms in bovine AM than in other cellular systems.

Additional studies will be required to characterize these differences in more detail.

A novel group of pyridinyl-imidazole compounds that suppress the production of TNFα, IL-1β, and IL-6 in activated monocytes and macrophages has recently been introduced by researchers at SmithKline Beecham [157]. These compounds show activity in several experimental animal models of acute and chronic inflammation, and have been termed cytokine-suppressive anti-inflammatory drugs (CSAIDs). They inhibit cytokine production at the translational level through selective inhibition of p38 kinase, a serine/threonine kinase involved in cytokine biosynthesis; no effect on mRNA abundance or size/stability of the protein products has been identified [159]. Drugs of this class also suppress eicosanoid production through inhibition of cyclooxygenase and lipoxygenase [160]. The prototypical drug of this group, SK&F86002 (dihydroimidazothiazoline), is a potent inhibitor of TNFα and IL-1 production in LPS-stimulated human monocytes *in vitro* [158, 160]. *In vivo*, bicyclic imidazoles inhibit TNFa production and reduce mortality in several animal models of septic shock [19, 208, 266]. Newer generations of these compounds, such as SB203580, feature more potent cytokine suppressive activity and reduced toxicity *in vitro* and *in vivo*. SB203580 [4–(4–fluorophenyl)–2–(4–methylsulfinylphenyl)–5–(4–pyridyl) imidazole] is a potent inhibitor of inflammatory cytokine production *in vivo* in mice and rats, and reduces fatality in murine models of endotoxic shock [18]. Our findings were consistent with studies in that pretreatment of bovine AM cultures with SB203580 suppressed the secretion of TNFα and IL–8 at higher doses (100 nM – 10 μM). In our experiments, however, the agent also provoked

increased secretion of IL–1β at these doses, suggesting that p38 kinase may be regulated differently in bovine AM than in previously studied cell types. These experiments, unlike previous studies, used *M. haemolytica* LktA in addition to LPS as a stimulus for inflammatory cytokine expression. Therefore, our findings might also be attributed, at least in part, to a different pattern of cellular activation and intracellular signaling.

Thalidomide is a nonbarbiturate sedative with anti-emetic properties that was commonly prescribed to pregnant women in the late 1950's. The drug was withdrawn from the market in 1961, when its use in early pregnancy was associated with catastrophic fetal abnormalities. Despite its tumultuous history, thalidomide has proven to be clinically useful in the management of chronic immunoinflammatory diseases such as lepromatous leprosy, tuberculosis, rheumatoid arthritis, and graft-versus-host disease. The drug's mode of action is incompletely understood, but its beneficial effects are attributed, at least in part, to its ability to suppress TNFα production. *In vitro*, thalidomide inhibits TNFα secretion by human peripheral blood mononuclear cells stimulated with LPS or *Mycobacterium leprae* cell wall extract [242]. The drug also inhibits LPS-induced TNFα production by human alveolar macrophages harvested from patients with tuberculosis and other diseases characterized by macrophage activation [293]. *In vivo*, administration of thalidomide to patients with lepromatous leprosy is associated with reduced clinical symptoms and a corresponding reduction in serum TNFα concentrations [241]. Available evidence indicates that thalidomide suppresses cytokine production at the post-transcriptional level by reducing the stability of TNFα-specific mRNA [194]. These observations suggest that thalidomide

may be useful in the prevention or treatment of diseases characterized by overproduction of TNFα. In this study, however, pretreatment of bovine AM with thalidomide exerted no significant effect on the expression of TNFα, IL–1β, and IL–8 genes or proteins. Although significant increases in TNFα and IL–1β were measured in supernatants from treated cultures, these effects were mediated by the vehicle, DMSO, rather than thalidomide itself.

Recent work conducted in our laboratory indicates that IL–8 is the dominant inflammatory cytokine expressed within the lungs during the acute phase of the disease [173]. Throughout the 24 hour period following experimental infection, IL-8 was secreted in much greater quantities than either TNFα or IL-1β. At 2 hours post-infection, the earliest time point examined, concentrations of IL-8 in epithelial lining fluid were already roughly 250- and 100-fold greater than those of TNFα and IL–1β, respectively. In extracts of lesional lung parenchyma at the same time point, concentrations of IL–8 were approximately 17,000- and 100-fold greater than those of TNFα and IL–1β, respectively. Furthermore, in situ hybridization studies indicated that IL–8 gene expression occurred in a much wider variety of cell types than was the case for the other two cytokines. TNFα gene expression was restricted to alveolar macrophages, while IL–1β gene expression was limited to neutrophils, alveolar macrophages, and interstitial macrophages. By contrast, widespread IL–8 gene expression was observed in alveolar macrophages, interstitial macrophages, neutrophils, bronchial and bronchiolar epithelium, and pulmonary fibroblasts.

These observations, together with current evidence supporting the central role played by neutrophils in disease pathogenesis, have at least three important

implications for therapeutic strategies based on modulation of inflammatory cytokines. First, pharmacologic agents targeting all three cytokines, with particular emphasis on IL–8, are likely to be more effective than those targeting only TNFα and/or IL–1β. Second, agents that target production of cytokines by a broad range of cell types, particularly in the case of IL–8, are most likely to be effective than those which suppress cytokine production by a single cell type such as the alveolar macrophage. Third, anti-cytokine agents may have to be administered very early in the course of disease, possibly even prior to colonization of the lung by *M. haemolytica*, to prevent the development of inflammatory lung injury. As such, this strategy may be more appropriate for prevention of BPM than its treatment. In this study, dexamethasone was an effective inhibitor of all three cytokines in bovine alveolar macrophages, and results of studies in other models confirm its efficacy in other cell types. Nevertheless, this agent's profound suppressive effects on host defenses and specific immune responses make it poorly suited for use in the context of naturally occurring disease in the feedlot. Additional research is therefore indicated to identify pharmacologic agents with more specific effects on inflammatory cytokine production.

In conclusion, this study demonstrated that *M. haemolytica* LPS- and LktA-induced expression of TNFα, IL–1β, and IL–8 by bovine alveolar macrophages may be inhibited by pretreatment with dexamethasone, tetrahydropapaveroline, pentoxifylline, rolipram, and SB203580. Of these agents, only dexamethasone effectively suppressed the production of all three cytokines. If pulmonary secretion of these inflammatory cytokines, particularly IL–8, may be effectively

inhibited *in vivo*, anti-cytokine therapy may represent a novel strategy for the management of pneumonic mannheimiosis in cattle. Practical implementation of this strategy, however, will require further research to identify effective cytokine inhibitors that exert minimal collateral effects on host immune responses.

MATERIALS AND METHODS

Recovery and isolation of bovine alveolar macrophages

Healthy male Holstein calves between 4 and 8 weeks of age were humanely euthanatized by intravenous administration of barbiturate. After removal of the trachea and lungs, alveolar macrophages were collected by lung lavage using four to six 250 mL aliquots of sterile endotoxin-free phosphate-buffered saline (PBS), pH 7.4. Lavage fluid was filtered through sterile gauze and centrifuged at 400 × *g* (4°C) for 10 minutes to separate fluid and cellular components. The supernatant was discarded and cells were washed three additional times by repeated centrifugation and suspension in fresh PBS. Approximately 1×10^7 cells were plated onto 6-cm tissue culture dishes (Costar Corp., Cambridge, MA) in 3.5 mL Dulbecco's modified Eagle medium (Celox Laboratories, Minneapolis, MN) supplemented with 2% fetal bovine serum, 5 mg/mL glucose, 4 mM L–glutamine, 14 mM HEPES, 0.1 mM non-essential amino acids, 100 IU/mL sodium penicillin G, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. Cells were allowed to adhere for 3 h at 37°C in a

humidified atmosphere containing 5% CO_2, after which the culture medium and nonadherent cells were removed and 2 mL fresh medium was added to each plate. Adherent cells isolated according to this protocol are ≥ 95% alveolar macrophages (AMs) as determined by nonspecific esterase staining, and ≥ 98% viable as determined on the basis of trypan blue exclusion. Purified AMs were incubated at 37°C/5% CO_2 for a minimum of 36 hours to ensure that cells were quiescent prior to the induction of inflammatory cytokine expression. All solutions and materials were sterile and free of endotoxin contamination.

Induction of inflammatory cytokine expression

Purified bovine AMs in cell culture were treated with dexamethasone (DEX), pentoxifylline (PTX), rolipram (ROL), tetrahydropapaveroline (THP), SB203580 (SB), or thalidomide (THL) at a range of concentrations 30 min prior to induction of cytokine expression with *M. haemolytica* LPS and LktA. Cytokine inhibitors were added to cultures in 250 μL medium; after swirling to mix, plates were incubated at 37°C/5% CO_2 for 30 min. Purified *M. haemolytica* LPS and LktA were added to cultures in an additional 250 μL medium to yield final concentrations of 100 ng/mL LPS and 2 LU/mL LktA. Preliminary experiments conducted to optimize the combined dose of LPS and LktA demonstrated that this dose elicited maximal levels of TNFα, IL–1β, and IL–8 mRNA and immunoreactive proteins at 8 h and 24 h, respectively (data not shown). Positive control cultures were treated with LPS/LktA in the absence of any cytokine inhibitors, and negative control cultures were treated with culture medium

alone. To investigate the possibility that observed cytokine suppression was caused by the vehicle in which each inhibitor was dissolved, additional control cultures were treated with culture medium containing equal volumes of vehicle. All experiments were conducted in triplicate.

Twenty-four hours following stimulation with LPS/LktA, culture supernatants were harvested, centrifuged at 10,000 × *g* (4°C), and stored at -80°C for quantitation of cytokine proteins by ELISA. Samples for northern blot analysis of cytokine mRNA were harvested 8 h following stimulation with LPS/LktA. The culture medium was removed and adherent cells were lysed in 2 mL Trizol® reagent (Invitrogen, Carlsbad, CA). Nonadherent cells were captured by centrifugation of the culture medium for 10 min at 400 × *g* (4°C) and lysed in 1 mL of lysate from the corresponding culture plate. After combining lysates from adherent and nonadherent cells, samples were mixed thoroughly, incubated at room temperature for 5 min to permit complete dissociation of nucleoprotein complexes, and stored at -80°C until RNA extraction could be performed.

Lipopolysaccharide (LPS) was purified from *M. haemolytica* strain 12296 using the phenol-water extraction method as previously described [323, 334], and the bioactive endotoxin content of this preparation determined using the chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD). *Mannheimia haemolytica* leukotoxin (LktA) was purified from logarithmic-phase cultures by preparative SDS-PAGE as previously described [171, 336]. The bioactivity of this preparation was measured with a colorimetric XTT reduction assay using BL–3 bovine lymphoma cells as targets as previously described [236]. Purified LktA

was contaminated with a small amount of endotoxin as determined using the chromogenic *Limulus* amebocyte lysate assay, and this was taken into account when calculating combined doses of LPS and LktA for induction of inflammatory cytokine expression.

Plasmids

Bovine TNFα, IL-1β, and IL-8 cDNAs (488, 474, and 230 base pairs in length, respectively) were cloned and sequenced in our laboratory [153, 334], ligated into pcDNA3 (Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* DH5a. A 1250-base pair human glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA in pBluescript KS+ (Stratagene, La Jolla, CA) was the generous gift of Dr. M. Murtaugh (University of Minnesota, St. Paul, MN). All plasmids were purified by alkaline lysis using a commercial kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

RNA extraction and northern blot analysis

All solutions were treated with 0.1% diethylpyrocarbonate (DEPC) and glassware was baked overnight at 350°C before use. Total cellular RNA from bovine alveolar macrophages was extracted from Trizol® lysates according to the manufacturer's instructions. Ten micrograms of RNA from each sample was electrophoretically fractionated in a 1.2% agarose gel containing 6.5% formaldehyde, transferred to a neutral nylon membrane (Schleicher and Schuell, Keene, NH), and covalently linked to the membrane by ultraviolet illumination. Membranes were prehybridized at 45°C for 2 hours in solution containing 50%

formamide, 5× saline sodium citrate (SSC), 5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 0.1 mg/mL yeast tRNA.

Gel-purified TNFα, IL-1β, IL-8, and GAPDH cDNA plasmid inserts were labeled with [α-^{32}P]dCTP by DNase/DNA polymerase I nick translation and unincorporated [α-^{32}P]dCTP was removed using Elutip–d® affinity columns (Schleicher and Schuell). Heat-denatured labeled probe was added to prehybridization buffer at 2 × 10^6 cpm/mL and membranes were hybridized overnight at 45°C. Blots were washed to a stringency of 0.1× SSC/0.1% SDS at 50°C. Autoradiographs were prepared by exposing membranes to Kodak X–OMAT AR x-ray film (Eastman Kodak, Rochester, NY) with an intensifying screen for 1 to 3 days at –80°C. Phosphor screen autoradiographs were prepared (Phosphorimager SF, Molecular Dynamics, Sunnyvale, CA) and relative levels of cytokine-specific mRNA were quantified by densitometric analysis using ImageQuant software (Molecular Dynamics). Cytokine gene expression data were normalized to the expression of GAPDH mRNA and steady-state accumulation of cytokine mRNA was expressed as a percentage of that observed in positive control cultures.

TNFα ELISA

A capture ELISA was developed to measure immunoreactive TNFα in alveolar macrophage culture supernatants. Mouse monoclonal antibody 2C4 ascites and rabbit polyclonal anti-TNFα antiserum were generously provided by Dr. T. H. Elsasser (USDA-ARS, Beltsville, MD). Purified recombinant bovine (rb) TNFα for use as a standard was generously provided by Dr. Dale Godson

(Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan). All samples, standards, and detection antibodies were diluted in PBS containing 10% (v/v) bovine serum albumin blocking concentrate (Kirkegaard & Perry, Gaithersburg, MD). Unless otherwise indicated, all reactions were conducted in a volume of 100 μL and plates were incubated at 37°C for 1 hour on a platform shaker. After each step, plates were washed five times with PBS containing 0.01% Tween-20. Monoclonal antibody 2C4 ascites diluted 1:1000 in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, and 3 mM sodium azide, pH 9.6) was adsorbed to 96-well ELISA plates (Costar Corp., Cambridge, MA) overnight at room temperature. After blocking nonspecific protein binding sites, samples and standards were added to plates. Samples were assayed in triplicate at three-fold dilutions (1:10, 1:30, and 1:90). Rabbit anti-bovine TNFα antiserum diluted 1:2000 in blocking buffer was used for primary detection of bound cytokine, followed by secondary detection with horseradish peroxidase (HRP)-labeled polyclonal goat anti-rabbit IgG (Kirkegaard & Perry) at a dilution of 1:6000. The color substrate tetramethylbenzidine (Kirkegaard & Perry) was added and plates were incubated for 7 minutes at room temperature. The reaction was stopped with 100 μL 1 M phosphoric acid and absorbance was measured at 450 nm. For each plate, a standard curve was constructed using duplicate three-fold dilutions of rbTNFα. Sample dilutions yielding absorbance readings in the linear region of the standard curve were used to calculate cytokine concentrations by interpolation using SOFTmax PRO software (Molecular Devices, Sunnyvale, CA). Measured TNFa concentrations were expressed as a percentage of that observed in positive control cultures.

IL-1β ELISA

A capture ELISA was developed to measure immunoreactive IL-1β in alveolar macrophage culture supernatants. Mouse monoclonal antibody SA22 specific for bovine IL–1β [222] as produced from hybridoma CRL-2052 (American Type Culture Collection, Manassas, VA) and purified by standard methods [121, 222]. Purified rbIL–1β for use as a standard was generously provided by Dr. Kathleen Heaney (Fort Dodge Animal Health, Princeton, NJ). Samples were assayed in triplicate at three-fold dilutions (1:3, 1:9, and 1:27). Methods were as for the TNFα ELISA with the following exceptions. All samples, standards, and detection antibodies were diluted in PBS containing 10% (v/v) milk blocking concentrate (Kirkegaard & Perry). Purified monoclonal antibody SA22 in coating buffer (5 μg/mL) was adsorbed to plates overnight at room temperature. Rabbit anti-ovine IL–1β antiserum (Chemicon, Temecula, CA) diluted 1:1000 was used for primary detection and HRP-labeled goat anti-rabbit IgG diluted 1:4000 was used for secondary detection. Color development was allowed to proceed for 30 minutes before the reaction was stopped with 1 M phosphoric acid and absorbance was measured at 450 nm. Measured IL-1β concentrations were expressed as a percentage of that observed in positive control cultures.

IL-8 ELISA

Purified rbIL-8 and mouse monoclonal antibody 170.13 specific for bovine IL-8 were produced and characterized in our laboratory. In brief, a cDNA encoding the mature bovine IL-8 protein [153] was subcloned into the pET15b expression vector (Novagen, Madison, WI), transformed into *E. coli*

BL21(λDE3)pLysS cells (Novagen), and expressed according to the manufacturer's recommendations. Histidine-tagged rbIL-8 was expressed, affinity purified on a Ni^{2+}-resin column (Novagen), and concentrated by dialysis against polyethylene glycol. Purified rbIL-8 was used to produce hybridomas and monoclonal antibodies by standard methods in collaboration with Immunochemistry Technologies (Bloomington, MN) [121]. Monoclonal antibody 170.13 (IgG1) recognized both rbIL-8 and recombinant human IL-8 in western blots and neutralized the neutrophil chemotactic activity of rbIL-8 in vitro.

A capture ELISA was developed to measure immunoreactive IL-8 in alveolar macrophage culture supernatants. Methods were as for the TNFα ELISA with the following exceptions. Samples and standards were diluted in PBS containing 10% (v/v) milk blocking concentrate. Purified monoclonal antibody 170.13 in coating buffer (5 μg/mL) was adsorbed to plates overnight at room temperature. Samples were assayed in triplicate at five-fold dilutions (1:100, 1:500, and 1:2500). Rabbit anti-ovine IL-8 antiserum (Chemicon) diluted 1:4000 in blocking buffer containing 5% normal mouse serum was used for primary detection and HRP-labeled goat anti-rabbit IgG diluted 1:6000 in blocking buffer was used for secondary detection. Color development was allowed to proceed for 20 minutes before the reaction was stopped with 1 M phosphoric acid and absorbance was measured at 450 nm. Measured IL-8 concentrations were expressed as a percentage of that observed in positive control cultures.

Statistical analysis

Relative steady-state accumulation of cytokine mRNA and secretion of cytokine proteins were expressed as the mean ± standard error of the mean (SEM). Data were analyzed using Student's unpaired *t*-tests, and differences were considered significant when $P < 0.05$.

ACKNOWLEDGEMENTS

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CHAPTER 5

PROTECTIVE EFFECT OF DEXAMETHASONE IN EXPERIMENTAL BOVINE PNEUMONIC MANNHEIMIOSIS

INTRODUCTION

Bovine pneumonic mannheimiosis (BPM), an acute fibrinous pleuropneumonia caused by *Mannheimia haemolytica*, is a common and economically important disease of North American cattle. One of the hallmark histopathological features of the disease is extensive infiltration of the lungs by neutrophils, and clinical and experimental studies provide circumstantial evidence that neutrophils mediate lung injury in BPM. The early pulmonary response to *M. haemolytica* has been well characterized, and studies show that neutrophil influx begins within 2 hours of experimental infection. [264, 265, 314, 322, 326]. Neutrophil depletion prior to experimental infection with *M. haemolytica* reduces the severity of lung damage [265, 322], indicating that this cell type participates in disease pathogenesis. The mechanisms by which neutrophils mediate lung pathology in BPM remain poorly defined but are likely to include oxidative injury and structural degradation of lung tissue due to extracellular release of reactive oxygen intermediates and proteolytic lysosomal enzymes from activated neutrophils [267].

The migration and activation of neutrophils in inflamed tissue are regulated by a complex network of interactions between cytokines, leukocytes, vascular endothelium, cellular adhesion molecules, and soluble chemotactic factors. The inflammatory cytokines tumor necrosis factor-alpha (TNFα), interleukin–1 beta (IL–1β), and interleukin–8 (IL–8) play a central role in the initiation and orchestration of these interactions. TNFα and IL–1β are pleiotropic early response polypeptides secreted by monocytes and macrophages in

response to microbial pathogens and other stimuli [155]. IL–8, a potent chemotactic and activating factor for neutrophils, is a C–X–C chemokine secreted by a variety of immune and nonimmune cell types, including monocytes, macrophages, fibroblasts, epithelial cells, and neutrophils [21, 150]. Recent studies indicate that bovine IL–8 is a potent chemoattractant for neutrophils and plays a key role in the genesis of lung injury associated with BPM [45, 46, 173].

Recognition of the importance of neutrophils and the host inflammatory response in disease pathogenesis prompted speculation that it may be possible to treat or prevent the disease through pharmacological modulation of pulmonary inflammatory responses. At present, treatment of BPM is based almost exclusively on systemic antibiotic therapy. Furthermore, metaphylactic administration of long-acting antibiotics to calves on arrival at the feedlot has become a commonplace preventive measure; this strategy effectively reduces morbidity and mortality during the early feeding period in calves incubating the disease [190, 306]. Nevertheless, mass medication of cattle with antibiotics also poses some serious concerns. Current evidence indicates that widespread use of antibiotics may have contributed to the emergence of multiple antibiotic-resistant strains of *M. haemolytica* [11, 320]. In addition, mass medication increases the risk of unwanted drug residues in meat and milk intended for human consumption. Of even greater concern, however, is the fact that mass medication promotes the transfer of antibiotic resistance genes from animal pathogens to human bacterial pathogens, threatening our ability to control human infectious disease [163, 330]. Novel approaches to the control of BPM are therefore warranted.

Since cytokines play a central role in the migration and activation of neutrophils, we hypothesize that pharmacological inhibition of their expression may prevent or reduce the inflammatory lung injury characteristic of the disease. Results presented in Chapter 3 of this dissertation characterized the ability of six different pharmaceutical agents to suppress *in vitro* production of TNFα, IL–1β, and IL–8 by bovine alveolar macrophages exposed to *M. haemolytica* LPS and LktA. In these studies, dexamethasone, a synthetic glucocorticoid with potent anti-inflammatory effects, was the most effective inhibitor of cytokine synthesis.

The specific objective of this study was to determine whether systemic therapy with dexamethasone sodium phosphate, a potent inhibitor of inflammatory cytokine synthesis, ameliorates disease development in an *in vivo* experimental model of BPM. Disease was induced in the left lungs of six calves by endobronchial administration of *Mannheimia haemolytica*. Four experimental calves were treated intravenously with dexamethasone sodium phosphate (DEX; 2 mg/kg 6 h prior to infection, 2 mg/kg immediately prior to infection, and 1 mg/kg q 12 h thereafter), while 2 placebo-treated control calves received dose-matched volumes of sterile saline. Clinical disease was characterized using a nonparametric scoring system, and the extent of gross pulmonary pathology affecting the left lung 48 h post-infection (PI) was determined using morphometric methods. Disease scores for DEX-treated calves were significantly lower than those for placebo-treated controls at all time points beyond 2 h PI ($P < 0.05$), and the percent volume of the left lung exhibiting gross pneumonic lesions was significantly lower in DEX-treated calves (6.0% ± 1.1%) as compared to controls (68.9% ± 13.3%), $P < 0.05$. In addition, histopathological lesions were

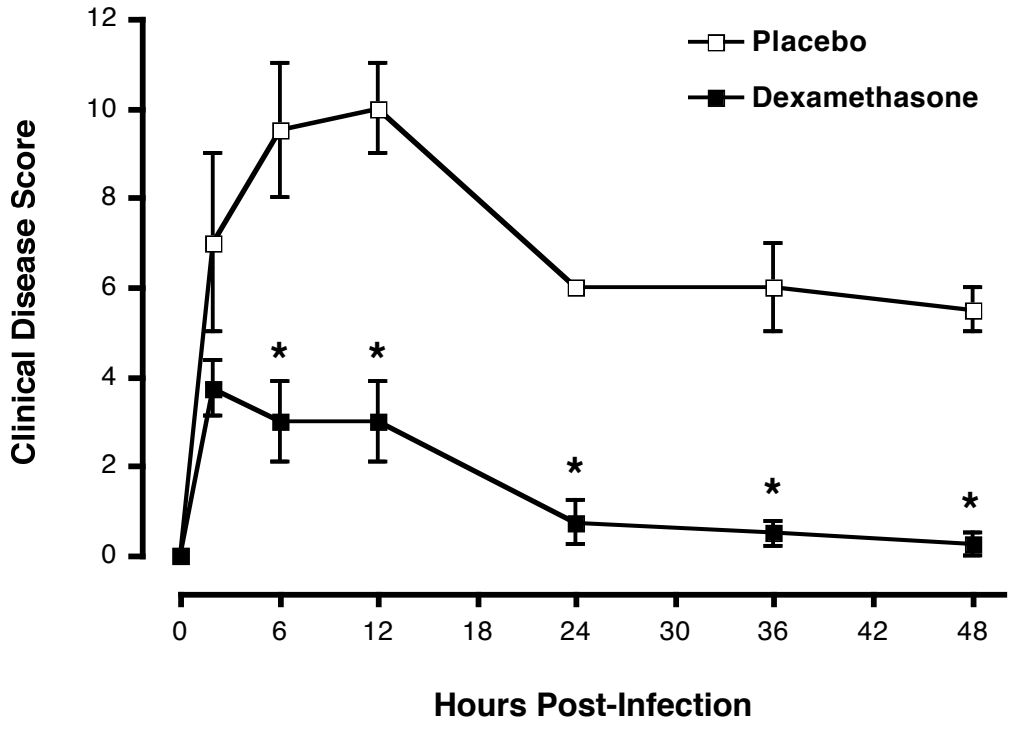
less severe and extensive in DEX-treated calves. These findings indicate that pharmacological modulation of pulmonary inflammation may represent a novel approach to the prevention of BPM. Successful implementation of this strategy will require additional research to identify drug agents that target the expression of cytokines and other inflammatory mediators without compromising host immune responses.

RESULTS

Clinical disease scores

Physical examinations of each calf were conducted immediately prior to experimental infection with *M. haemolytica* and at 2, 6, 12, 24, 36, and 48 hours post-infection. All calves were clinically normal (clinical score = 0) prior to the induction of experimental disease. Within 2 hours of infection, all calves developed clinical symptoms of disease (Fig. 1). Disease scores for dexamethasone-treated calves, however, were significantly lower than those for placebo-treated controls at all time points beyond 2 hours post-infection ($P < 0.05$). By 24 hours post-infection, the dexamethasone-treated calves exhibited near-normal clinical status, with mean clinical scores remaining below 1 throughout the remainder of the study period. By contrast, mean clinical scores of the control calves remained at approximately 6 during this period.

Figure 5-1. Clinical disease scores in calves treated with dexamethasone (n=4) versus placebo-treated controls (n=2). Physical examinations of each calf were performed immediately prior to experimental infection and at 2, 6, 12, 24, 36, and 48 hours post-infection. Symptoms of clinical disease were allocated points according to the following scoring system: body temperature greater than 103.5°F (2 points); inappetance (1 point); lethargy or depression (1 point); marked weakness or recumbency (2 points); moribund state (3 points); cough (1 point); nasal discharge (1 point); respiratory rate greater than 60 breaths per minute (1 point); dyspnea (2 points); and abnormal breath sounds on thoracic and tracheal auscultation (1 point). Clinical scoring was performed by an investigator unaware of the experimental group to which each calf was assigned. Values represent the mean ± SEM. * $P < 0.05$ compared to placebo-treated controls.



Gross pulmonary pathology

All calves were humanely euthanatized at 48 hours post-infection and post mortem examinations were conducted. Gross pulmonary lesions consistent with pneumonic mannheimiosis were identified in all except one calf, but lesions in dexamethasone-treated calves were consistently less severe than those observed in placebo-treated controls. The left lungs of the two control calves were enlarged (1014 g and 938 g) and extensively consolidated (Fig. 2). Marked interlobular edema and fibrinous pleuritis were present in both calves, and numerous fibrinous adhesions were present between the visceral and parietal pleura. On cut surface, extensive regions of hemorrhage, consolidation, and coagulation necrosis were evident within the caudal lung lobes (Fig. 3). By contrast, the left lungs of the four dexamethasone-treated calves were much less enlarged (439 g, 350 g, 483 g, and 622 g) with little or no interlobular edema (Fig. 4). On cut section, pneumonic lesions were less marked and extensive than those in the placebo-treated calves (Fig. 5). Regional consolidation and a small amount of pleural effusion without fibrin were evident in Calves 1 and 2. In Calf 3, gross abnormalities were limited to mottled discoloration of the lung surface, small focal areas of consolidation, and localized areas of atelectasis within the caudal lobe. The left lung of Calf 4 was grossly normal. Pure cultures of *M. haemolytica* were isolated from the left lungs of all calves.

The left lung of each calf was fixed, sectioned, and the percent volume of the left lung exhibiting gross pneumonic lesions was determined using morphometric techniques as previously described with minor modifications [18]. Calculated values were significantly lower in dexamethasone-treated calves

(6.0% ± 1.1% of total left lung volume) as compared to placebo-treated controls (68.9% ± 13.3% of total left lung volume), $P < 0.05$.

Figure 5-2. Left lungs of placebo-treated control calves (n=2). Experimental disease was induced by endobronchial inoculation of 5×10^9 CFU *Mannheimia haemolytica* within the left caudal lung lobe. In both calves, the left caudal lung lobe was grossly enlarged and extensively consolidated, with marked interlobular edema.

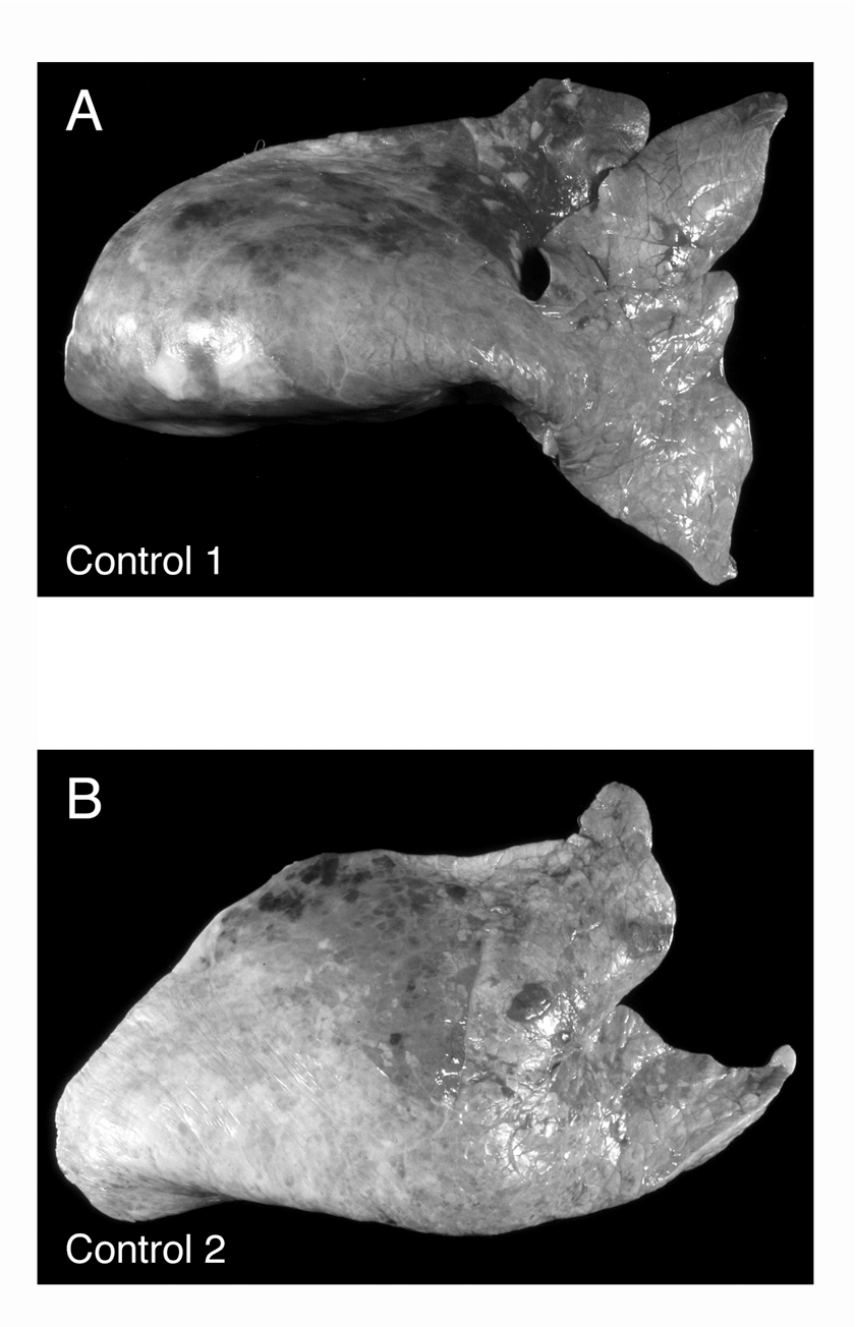
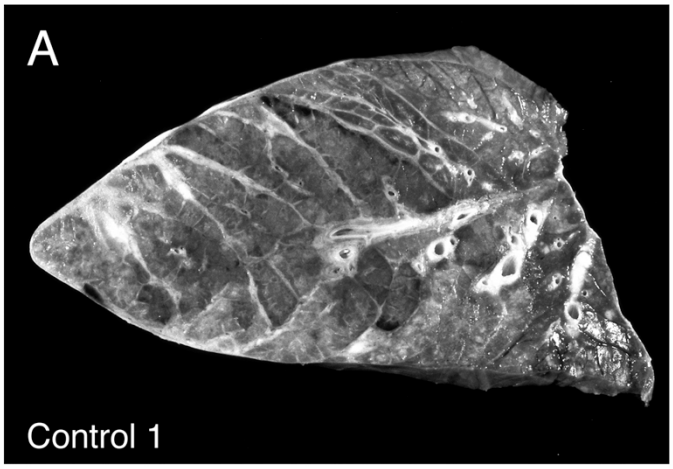


Figure 5-3. Cut surfaces of left caudal lung lobes from placebo-treated control calves (n=2). Experimental disease was induced by endobronchial inoculation of 5×10^9 CFU *Mannheimia haemolytica* within the left caudal lung lobe. The left lung of each calf was fixed by perfusing the airways with 10% neutral buffered formalin at a constant pressure of 30 cm H_2O for 48 hours. Fixed lungs were sliced into 1 cm thick serial sections. In both calves, extensive regions of hemorrhage, consolidation, interlobular edema, and coagulation necrosis were evident on cut sections of the left caudal lung lobe.



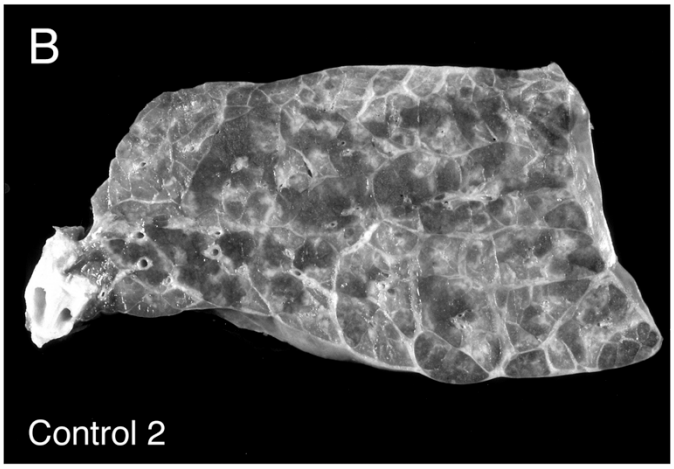


Figure 5-4. Left lungs of calves treated with dexamethasone (n=4)

Experimental disease was induced by endobronchial inoculation of 5×10^9 CFU *Mannheimia haemolytica* within the left caudal lung lobe. Calves were treated with dexamethasone in the form of dexamethasone sodium phosphate according to the following protocol: 2 mg/kg IV 6 hours prior to experimental infection; 2 mg/kg IV immediately prior to infection; and 1 mg/kg IV every 12 hours thereafter until a final treatment at 36 hours post-infection. The left caudal lung lobes were minimally enlarged as compared to placebo-treated controls, with little or no interlobular edema.

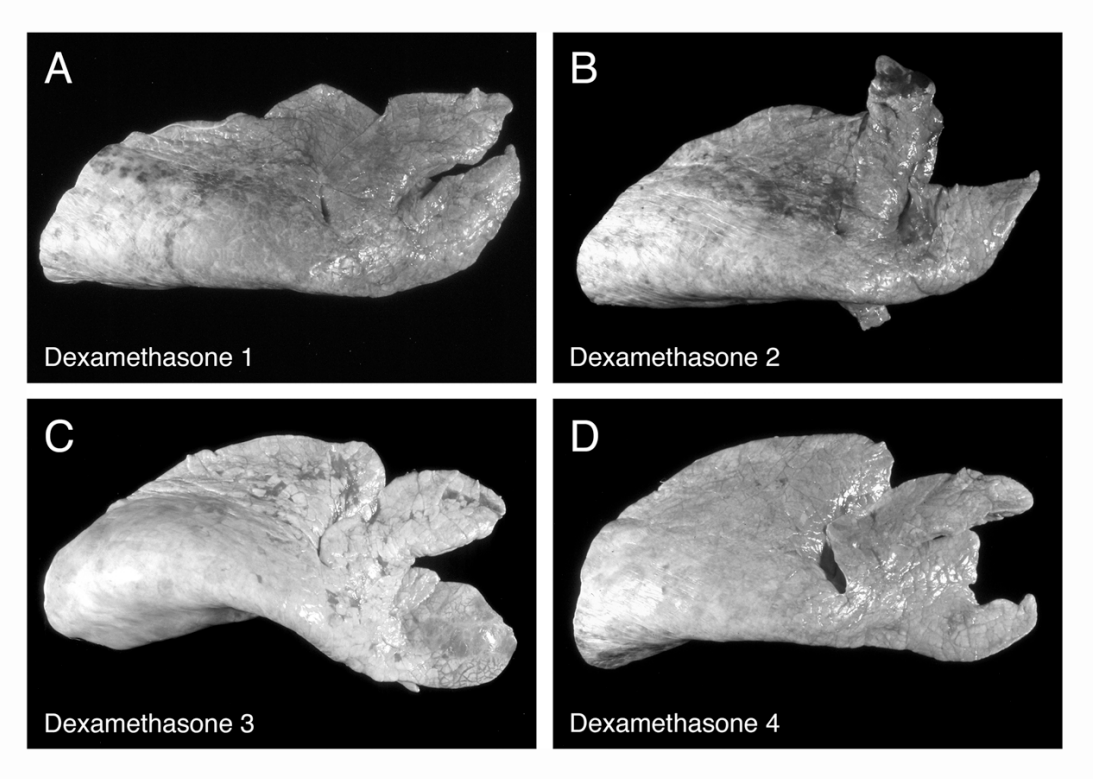
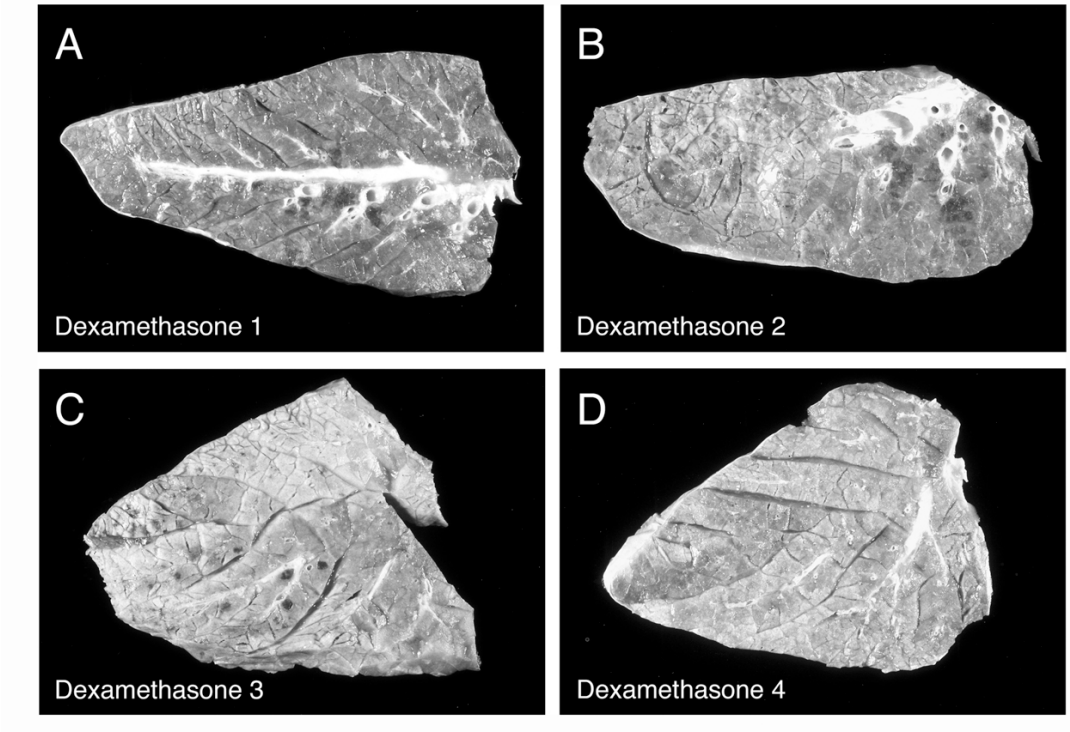


Figure 5-5. Cut surfaces of left caudal lung lobes from dexamethasone-treated calves (n=4). Experimental disease was induced by endobronchial inoculation of 5×10^9 CFU *Mannheimia haemolytica* within the left caudal lung lobe. Calves were treated with dexamethasone in the form of dexamethasone sodium phosphate according to the following protocol: 2 mg/kg IV 6 hours prior to experimental infection; 2 mg/kg IV immediately prior to infection; and 1 mg/kg IV every 12 hours thereafter until a final treatment at 36 hours post-infection. The left lung of each calf was fixed by perfusing the airways with 10% neutral buffered formalin at a constant pressure of 30 cm H_2O for 48 hours. Fixed lungs were sliced into 1 cm thick serial sections. Gross pneumonic lesions are limited to focal regions of consolidation and atelectasis within the left caudal lung lobes, which appear as small dark areas in the photographs.



Pulmonary histopathology

Light microscopic examination of pulmonary tissues from placebo-treated control calves revealed severe and extensive lesions characteristic of bovine pneumonic mannheimiosis. In both control animals, pulmonary alveoli and interstitial spaces contained a marked accumulation of fibrin, edema fluid, and degenerative neutrophils (Fig. 6a, Fig. 6b). Many "oat" or "swirling" cells, which represent neutrophils in an advanced state of degeneration, were present within alveolar spaces (Fig. 6c, Fig. 6d). Extensive necrosis of alveolar walls was evident, with widespread hemorrhage into the interstitium and alveolar spaces (Fig. 6b). Interlobular septae were markedly thickened and infiltrated with degenerative neutrophils, edema fluid, and fibrin (Fig. 6e). Vascular lesions observed in the interstitium and interlobular septae included extensive thrombosis and fibrinoid necrosis of venules, neutrophilic infiltration of septal arterial walls, and endothelial cell swelling in large arteries. Extensive necrosis and hyperplasia of the bronchial epithelium was observed, and bronchioles contained abundant fibrinous exudate and neutrophilic casts (Fig. 6f).

Microscopic lesions consistent with pneumonic mannheimiosis were also observed in the four calves in the dexamethasone-treated group, but these were less severe and extensive than those observed in placebo-treated controls. Whereas lesions in the control calves involved the majority of the left caudal lung lobe, lesions in the dexamethasone-treated calves exhibited a patchy lobular distribution. Occasional lobules were severely affected, but even the worst lesions in these animals were milder than those observed in controls. Inflammation of the alveoli, interstitium, and interlobular septae was less severe,

with comparatively little fibrin deposition, neutrophil influx, edema, hemorrhage, and necrosis (Fig. 7a). Neutrophils in the alveoli, interstitium, and interlobular septae lacked the degenerative changes typical of those observed in controls and "swirling" cells were rare (Fig. 7b). Thrombosis, fibrinoid necrosis, and endothelial cell swelling of pulmonary vessels were not observed. Bronchial epithelial necrosis was limited to focal regions, less exudate was present within bronchi and bronchioles, and airway exudate contained fewer neutrophils and reduced fibrin as compared to controls (Fig. 7c). In Calf 4, histological lesions were limited to focal regions of alveolar neutrophil influx without edema fluid or fibrin deposition (Fig. 7d).

Figure 5-6. Lung tissues from placebo-treated control calves, 48 hours post-infection. Experimental disease was induced by endobronchial inoculation of 5×10^9 CFU *Mannheimia haemolytica* within the left caudal lung lobe. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin using routine methods. 5 μm tissue sections were cut and stained with hematoxylin and eosin for light microscopic examination.

Figure 5-6a. Pulmonary alveoli and interstitial spaces contain a marked accumulation of fibrin, edema fluid, and degenerative neutrophils. Bar = 200 μM.

Figure 5-6b. Pulmonary alveoli and interstitial spaces contain a marked accumulation of fibrin, edema fluid, and degenerative neutrophils. Necrosis of alveolar walls is also evident, with hemorrhage into the alveoli and interstitial spaces. Bar = 100 μM.

Figure 5-6c. Extensive infiltration of pulmonary alveoli with degenerative neutrophils, including elongated spindle-shaped "oat" cells characteristic of pneumonic mannheimiosis. Bar = 40 μM.

Figure 5-6d. Extensive infiltration of pulmonary alveoli with degenerative neutrophils, including elongated spindle-shaped "oat" cells characteristic of pneumonic mannheimiosis. Necrosis and physical disruption of alveolar walls is also evident. Bar = 50 μM.

Figure 5-6e. Interlobular septae (between arrows) were markedly edematous and infiltrated with fibrin and degenerative neutrophils. Bar = 100 μM.

Figure 5-6f. Bronchi and bronchioles contained abundant fibrinous exudate and degenerative neutrophils. Bar = 100 μM.

FIGURE 5-6

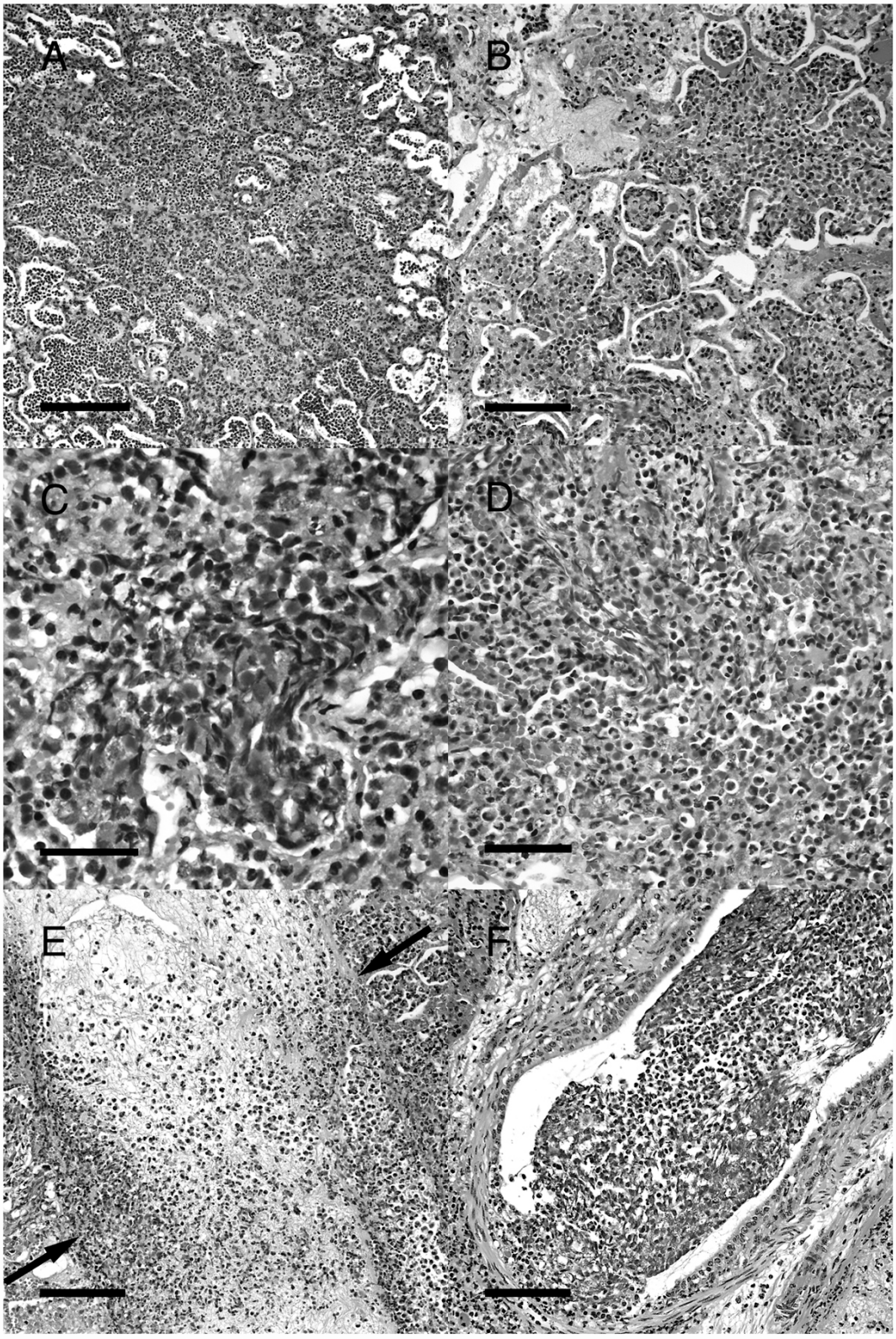


Figure 5-7. Lung tissues from dexamethasone-treated control calves, 48 hours post-infection.

Experimental disease was induced by endobronchial inoculation of 5×10^9 CFU *Mannheimia haemolytica* within the left caudal lung lobe. Calves were treated with dexamethasone in the form of dexamethasone sodium phosphate according to the following protocol: 2 mg/kg IV 6 hours prior to experimental infection; 2 mg/kg IV immediately prior to infection; and 1 mg/kg IV every 12 hours thereafter until a final treatment at 36 hours post-infection. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin using routine methods. 5 μm tissue sections were cut and stained with hematoxylin and eosin for light microscopic examination.

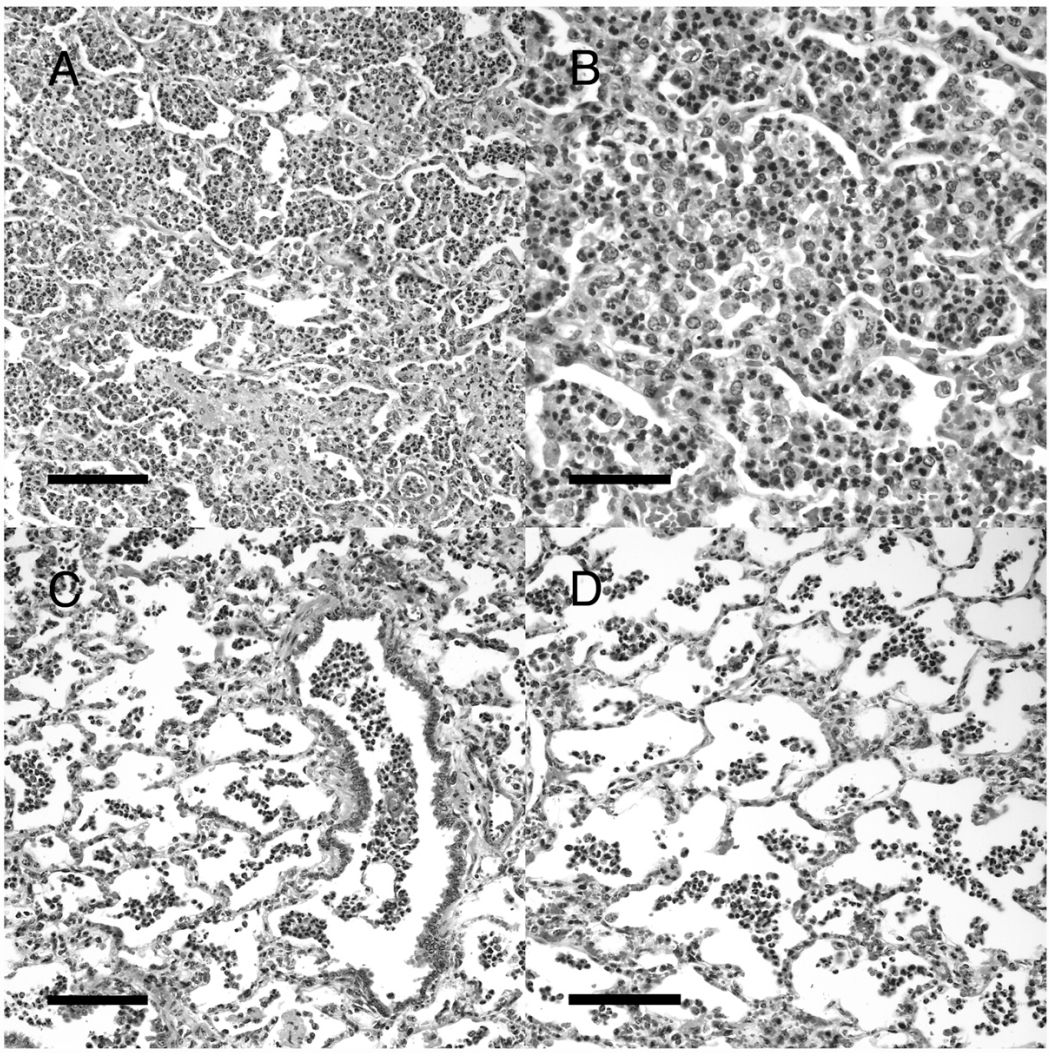
Figure 5-7a. Pulmonary lesions in dexamethasone-treated calves were milder than those observed in placebo-treated controls. Even in the worst lesions, the alveoli and interstitium contained comparatively little edema and fibrin deposition. Bar = 100 μM.

Figure 5-7b. Although pulmonary alveoli and interstitial spaces contained a significant accumulation of neutrophils, they lacked the degenerative changes typical of those observed in control calves. Necrosis, physical disruption, and hemorrhage of alveolar walls were rarely evident. Bar = 50 μM.

Figure 5-7c. Bronchi and bronchioles contained inflammatory exudate in the most severely affected regions, but this was less severe than in control animals. The airway exudate contained comparatively less fibrin, and neutrophils in the exudate lacked the degenerative changes typical of those observed in control calves. Bar = 100 μM.

Figure 5-7d. In Calf 4, histological lesions were limited to focal regions of alveolar neutrophil influx without edema fluid or fibrin deposition. Neutrophils lacked the degenerative changes typical of those observed in control calves. Bar = 100 μM.

FIGURE 5-7



DISCUSSION

In this study, we demonstrated that treatment of calves with dexamethasone sodium phosphate, a synthetic glucocorticoid with potent anti-inflammatory effects, markedly reduced both clinical disease and pulmonary pathology in an *in vivo* experimental model of *M. haemolytica*-induced pneumonia. Clinical disease scores of dexamethasone-treated calves were significantly lower than those of controls for all time points beyond 2 hours post-infection, and all treated calves returned to near-normal clinical status by 24 hours post-infection. These results were closely correlated to the gross and histopathological findings; pulmonary lesions in dexamethasone-treated calves were less extensive and severe than those in the placebo-treated group. Taken together, these findings support the hypothesis that host inflammatory responses are primarily responsible for much of the lung pathology characteristic of pneumonic mannheimiosis, and suggest that pharmacological modulation of pulmonary inflammation may represent a novel approach to the management of this important disease of cattle.

While dexamethasone and other glucocorticoids are often used as an adjunct to antibiotic therapy in the management of bovine respiratory disease, relatively few reports of their specific effects on the pathogenesis and clinical outcome of pneumonic mannheimiosis are available, however, and results do not uniformly support the efficacy of these agents. In two European studies, adjunctive therapy with corticosteroids was associated with improved outcomes in experimental models of BPM [6, 97]. Similarly, treatment of

experimentally infected calves with a combination of ceftiofur and flumethasone (25 μg/kg/day IV for 3 days) resulted in reduced mortality and a quicker recovery as compared to no treatment or treatment with ceftiofur alone [288]. Addition of low-dose dexamethasone acetate (0.025 mg/kg IM q12h for 3 days) to an antibiotic treatment regimen slightly improved clinical scores in experimentally infected calves, but did not affect lung lesion scores 7 days post-infection [186]. By contrast, addition of low-dose dexamethasone (20 mg IM once daily for 3 days) to an antibiotic regimen for treatment of naturally occurring bronchopneumonia in yearling feedlot cattle exacerbated clinical disease and provoked increased relapse and mortality rates [57]. However, case selection in this study was based solely on symptoms of clinical disease, which precludes differentiation of *M. haemolytica* pneumonia from other viral and bacterial causes of respiratory disease. In experimental models of other bovine bacterial pneumonias, dexamethasone pretreatment usually exacerbates disease. In experimental models of *Haemophilus somnus* pneumonia, for example, administration of dexamethasone to calves prior to endotracheal inoculation of bacteria exacerbates morbidity, pulmonary pathology, and mortality [51, 135].

The design of this study did not address the specific mechanisms by which dexamethasone reduced clinical disease and pulmonary pathology. Possible mechanisms include direct antibacterial effects on *M. haemolytica* and/or suppression of pulmonary inflammatory responses. As glucocorticoids are not known to exert direct antibacterial effects *in vitro* or *in vivo*, the latter mechanism is more likely to account for the effects observed in this study. These agents are among the most effective and widely used anti-inflammatory drugs; their

efficacy reflects their broad effects on cytokines, leukocytes, and soluble inflammatory mediators, many of which are believed to contribute to the pathogenesis of BPM. They are potent inhibitors of cytokine synthesis at the transcriptional, post-transcriptional, and translational levels.[7] Glucocorticoids inhibit the transcription of most inflammatory cytokine genes, including TNFα [151, 189], IL-1 [8, 147], IL-6 [8, 342], and IL-8 [152]. They also inhibit the synthesis of numerous immune cytokines, including IL–2, IL–3, IL–4, IL–5, IL–12, IFN–γ, macrophage colony-stimulating factor (M–CSF), granulocyte (G)–CSF, and granulocyte-macrophage (GM)–CSF [7]. Recent evidence indicates that glucocorticoids inhibit cytokine gene transcription by preventing nuclear translocation of NF–κB through upregulation of IκB, which traps NF–κB in the cytoplasm [14, 247]. Because NF–κB is a transcriptional activator for a wide range of genes associated with immune and inflammatory responses, including inflammatory cytokines, inhibition of its activity yields potent anti-inflammatory effects.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a diverse group of compounds with antipyretic, anti-inflammatory, and analgesic properties. They are widely used as an adjunct to antimicrobial therapy in an effort to modulate pulmonary inflammation in BPM and other bovine respiratory diseases. NSAIDs are often considered preferable to glucocorticoids in the management of infectious respiratory diseases of cattle, as they lack the immunosuppressive properties of the latter drugs. A variety of NSAID compounds have been investigated, including flunixin meglumine, carprofen, ketoprofen, and tolfenamic acid. Flunixin meglumine and tolfenamic acid significantly enhance

clinical recovery in experimental models of viral pneumonia and BPM [253, 254] and in natural outbreaks of undifferentiated bovine respiratory disease [80]. In naturally occurring outbreaks of acute respiratory disease in housed calves, a single dose of carprofen (mean dose 1.4 mg/kg by subcutaneous injection) was equally effective as 3 daily doses of flunixin meglumine (mean dose of 2 mg/kg by IV injection) as adjunctive therapy to antimicrobial treatment [23]. In an *in vivo* experimental model of BPM, ketoprofen (3 mg/kg/day for 4 treatments) exerted greater beneficial effects on respiratory rate, PaO_2, and gross lung lesions than did dexamethasone (0.016 mg/kg/day for 4 treatments) [41, 207]. It is important to note, however, that the dose of dexamethasone used in these studies was very low, and usually considered insufficient to exert significant anti-inflammatory effects.

The mechanisms by which NSAIDs modulate pulmonary inflammation in cattle have been incompletely characterized, but are assumed to relate primarily to inhibition of cyclooxygenase, resulting in decreased production of prostaglandins [309]. However, the anti-inflammatory and analgesic potency of these drugs does not always correlate with their activity as cyclooxygenase inhibitors [74, 183], suggesting that they may exert anti-inflammatory effects through other mechanisms. For example, recent evidence suggests that carprofen, and to a lesser extent flunixin meglumine, exerts direct effects on NF–KB activation that are independent of their effects on cyclooxygenase [39].

In conclusion, the results of this study suggest that modulation of pulmonary inflammatory responses, particularly through suppression of inflammatory cytokine expression, may represent a novel approach to the

management of pneumonic mannheimiosis in cattle. Successful implementation of this strategy, however, will require additional research to identify pharmacological agents that target the production of cytokines and other inflammatory mediators without compromising host immune responses.

MATERIALS AND METHODS

Experimental design

Experimental pneumonic mannheimiosis was induced in six healthy Holstein calves by endobronchial administration of *Mannheimia haemolytica*. Calves were randomly assigned to two experimental groups. Four experimental calves (Group 1) received intravenous injections of dexamethasone prior to experimental infection and every 12 hours thereafter according to the protocol described below. Two placebo-treated control calves (Group 2) received dose-matched volumes of sterile saline IV according to the same schedule. Clinical disease in each calf was scored at 0, 2, 6, 12, 24, 36, and 48 hours post-infection. Calves were humanely euthanatized 48 hours after the induction of experimental disease, and the percent volume of the left lung exhibiting gross pulmonary pathology was calculated using morphometric methods. Histopathological lesions from the left lung of each calf were also characterized.

Preparation of bacterial inoculum

Mannheimia haemolytica serotype 1 strain 12296, a field isolate recovered from a yearling calf with fatal pneumonic mannheimiosis, was propagated in phenol red-free RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 2 mM L-glutamine as previously described [10]. Logarithmic phase cultures were diluted with additional medium to adjust the final concentration to 1×10^9 CFU/ml as determined by spectrophotometry. The inoculum was stored on ice for a maximum of 30 minutes until its subsequent administration to calves.

Animals and induction of experimental disease

Six healthy male Holstein calves between 7 and 10 weeks of age were purchased from the University of Minnesota Department of Animal Science. The calves were weaned at birth and raised in individual hutches, and were free of detectable serum antibodies against *M. haemolytica* surface antigens and leukotoxin as determined by ELISA using previously described methods [273]. Twenty-four hours prior to experimental infection, an intravenous catheter (Becton Dickinson; Sandy; UT) was placed in the right jugular vein of each calf. Pneumonic mannheimiosis was induced in all calves using a well-characterized, reproducible experimental model developed in our laboratory [10]. Briefly, calves were sedated with xylazine (0.2 mg/kg IV) and positioned in sternal recumbency for the passage of a sterile fiberoptic bronchoscope into the left lung. With the tip of the endoscope wedged in the left main-stem bronchus, 5 ml (5×10^9 CFU) of logarithmic phase *M. haemolytica* was deposited within the airway, followed by

30 ml sterile LPS-free phosphate-buffered saline, pH 7.4. Following withdrawal of the endoscope, inoculated calves were positioned in left sternal recumbency during recovery from sedation.

Four experimental calves (Group 1) were treated with dexamethasone in the form of dexamethasone sodium phosphate (4 mg/ml; American Regent Laboratories, Inc.; Shirley, NY) according to the following protocol: 2 mg/kg IV 6 hours prior to experimental infection; 2 mg/kg IV immediately prior to infection; and 1 mg/kg IV every 12 hours thereafter until a final treatment at 36 hours post-infection (5 treatments in total). Two placebo-treated control calves (Group 2) received dose-matched volumes of sterile saline IV according to the same schedule.

Scoring of clinical disease

Physical examinations of each calf were performed immediately prior to experimental infection and at 2, 6, 12, 24, 36, and 48 hours post-infection. Symptoms of clinical disease were allocated points according to the following scoring system: body temperature greater than 103.5°F (2 points); inappetance (1 point); lethargy or depression (1 point); marked weakness or recumbency (2 points); moribund state (3 points); cough (1 point); nasal discharge (1 point); respiratory rate greater than 60 breaths per minute (1 point); dyspnea (2 points); and abnormal breath sounds on thoracic and tracheal auscultation (1 point). Higher clinical scores therefore reflected a greater degree of clinical illness as determined on the basis of demeanor, appetite, body temperature, and specific

symptoms of respiratory disease. Clinical scoring was performed by an investigator unaware of the experimental group to which each calf was assigned.

Quantitation of gross pulmonary pathology

All calves were humanely euthanatized 48 hours following the induction of experimental disease. The left lungs were removed at post mortem examination, and the percent lung volume exhibiting gross pathological lesions of pneumonic mannheimiosis was determined using a morphometric technique as previously described with minor modifications [115]. Briefly, the left lung of each calf was fixed by perfusing the airways with 10% neutral buffered formalin at a constant pressure of 30 cm H_2O for 48 hours. Fixed lungs were sliced into 1 cm thick serial sections, and then the outlines of each section and the gross pneumonic lesions within it were traced onto transparent acetate sheets. Tracings were then scanned into a public domain image analysis application, NIH Image (National Institutes of Health; Bethesda, MD), which was used to measure areas representing both the total serial section and the gross pneumonic lesions within it. Measured areas for each serial section were used to calculate the volume of each lung and the grossly pneumonic regions within it using Simpson's rule:

$$V = \left(\frac{1}{3}\right)h\left[\left(A_0 + A_n\right) + 4\left(A_1 + A_3 + \ldots + A_{n-1}\right) + 2\left(A_2 + A_4 + \ldots + A_{n-2}\right)\right]$$

where V is the total or pneumonic lung volume, h is the thickness of each slice in centimeters, and A_0, A_1, A_2, A_n represent measured lung or pneumonic lesion areas for lung slices 0, 1, 2, n. These values were then used to calculate the

percent volume of the left lung exhibiting gross pulmonary pathology in each calf.

Lung tissues from representative gross lesions in each calf were collected for histopathological evaluation. Tissue samples were fixed in 10% neutral buffered formalin and embedded in paraffin using standard techniques. After routine processing, 5 μm tissue sections were stained with hematoxylin and eosin for light microscopic examination.

Statistical analysis

Clinical scores and the percent volume of the left lung exhibiting gross pneumonic lesions were expressed as the mean ± standard error of the mean (SEM). Data from dexamethasone- and placebo-treated calves were compared using Student's unpaired *t*-tests. Differences were considered significant when $P < 0.05$.

ACKNOWLEDGEMENTS

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CHAPTER 6

GENERAL CONCLUSIONS

Despite recent progress in our understanding of bovine pneumonic mannheimiosis (BPM), it remains a leading source of economic loss to the North American beef and dairy industries. Recent studies indicate that an uncontrolled host inflammatory reaction to the bacterium and its products is the principal cause of lung injury in the disease. Earlier work in our laboratory and others showed that *M. haemolytica* and its major virulence factors, LPS and LktA, are potent inducers of TNFα, IL–1β, and IL–8 genes and proteins in bovine alveolar macrophages. In addition, these cytokines are expressed within the lungs of naturally and experimentally infected cattle. At the outset of this project, we therefore hypothesized that these inflammatory cytokines contribute to the pathogenesis of lung injury in BPM, and that pharmacological inhibition of their expression may prevent or reduce the inflammatory lung injury characteristic of the disease. Without a deeper understanding of cytokine expression within infected lung, however, it would be impossible to develop and implement therapeutic or preventive strategies based on modulation of one or more of these cytokines.

Therefore, the central goals of this thesis research were to (1) characterize patterns of TNFα, IL–1β, and IL-8 gene and protein expression within the lungs of experimentally infected calves, (2) identify drug agents capable of suppressing inflammatory cytokine expression by bovine alveolar macrophages exposed to *M. haemolytica* LPS and LktA *in vitro*, and (3) determine whether the most effective of these cytokine inhibitors is capable of reducing clinical disease and pulmonary pathology *in vivo*.

The specific objectives of the first research goal were to (a) characterize the kinetics of pulmonary TNFα, IL–1β, and IL-8 gene and protein expression in the initial 24 hours of experimental BPM; (b) compare patterns of cytokine expression in airways with those in lung lesions; and (c) identify major cellular sources of these cytokines within affected lung. Our studies demonstrate early and sustained expression of TNFα, IL–1β, and IL-8 mRNA and proteins within the airways and lung lesions of experimentally infected calves, providing stronger evidence in support of a causative role for TNFα, IL–1β, and IL–8 than was previously available. In all samples, IL-8 mRNA and proteins were upregulated to the greatest extent and those for TNFα were upregulated the least. TNFα gene and protein expression occurred predominantly within airways, and in situ hybridization studies confirmed that mRNA expression was localized to alveolar macrophages. Expression of IL–1β and IL–8 genes and proteins, by contrast, was more generalized. Alveolar and interstitial macrophages were important early sources of both IL–1β and IL–8, and bronchial and bronchiolar epithelial cells were significant sources of IL–8 in the first 4 hours post-infection (PI). Neutrophils, however, became the dominant source of both IL–1β and IL–8 within 4 to 8 hours of disease onset. These findings demonstrate a spatial and temporal association between pulmonary expression of inflammatory cytokines and acute lung pathology, and indirectly support the hypothesis that cytokines contribute to inflammatory lung injury in BPM.

In this study, we also showed that cytokine upregulation occurs much earlier in the course of disease development than was previously recognized. TNFα, IL–1β, and IL–8 mRNA and proteins were significantly increased in all

samples by 2 hours PI. By 24 hours PI, mRNA specific for all three cytokines declined to control or near-control values. Although cytokine concentrations in airways and lung lesions remained elevated throughout the study period, they were significantly decreased at 24 hours PI compared to peak values achieved earlier. These observations suggest that TNFα, IL–1β, and IL–8 may exert their greatest pathogenic effects within 16 hours of disease onset.

Our findings demonstrated for the first time that IL–8 is the dominant inflammatory cytokine expressed within the lungs during the acute phase of BPM. Abundant pulmonary expression of IL–8 has traditionally been considered a downstream event that is dependent, at least in part, on the prior secretion of early-response cytokines such as TNFα and IL–1β. If that is the case, our results indicate that the critical events in that cascade must occur well before 2 hours PI. These observations, together with the results of our kinetic analyses, have at least two important implications for therapeutic strategies based upon modulation of inflammatory cytokines. First, pharmacological agents that inhibit the synthesis of IL–8 or antagonize its biological effects are likely to prove more effective in the management of BPM than those targeting only TNFα or IL–1β. Second, anti-cytokine agents may have to be administered very early in the course of disease, or possibly even prior to colonization of the lung by *M. haemolytica*, in order to prevent or interrupt inflammatory lung injury.

The specific objective of the second research goal was to investigate the ability of six different pharmacological agents to inhibit *in vitro* expression of TNFα, IL–1β, and IL–8 genes and proteins by bovine alveolar macrophages (AM) exposed to *M. haemolytica* LPS and LktA. The drugs tested included

(1) dexamethasone, a synthetic glucocorticoid; (2) tetrahydropapaveroline, an antioxidant compound; (3) pentoxifylline, a non-specific phosphodiesterase inhibitor; (4) rolipram, a type IV phosphodiesterase inhibitor; (5) SB203580, an inhibitor of p38 kinase; and (6) thalidomide, an immunomodulatory sedative drug. The results of these studies demonstrated that dose-dependent inhibition of cytokine secretion occurred in response to pretreatment of AM with dexamethasone, tetrahydropapaveroline, pentoxifylline, rolipram, and SB203580. Significant dose-dependent inhibition of cytokine mRNA expression occurred in response to pretreatment with dexamethasone, tetrahydropapaveroline, and pentoxifylline. Of these, dexamethasone was the most effective inhibitor by far; pretreatment with this compound yielded greater than 95% inhibition of cytokine gene and protein expression over a broad range of concentrations. Although each of the remaining agents other than thalidomide were capable of suppressing the production of one or more cytokine to some extent, only dexamethasone suppressed all 3 cytokines.

The specific objective of the third research goal was to evaluate the ability of the most effective inhibitor of *in vitro* cytokine expression, dexamethasone, to influence disease development in an *in vivo* experimental model of BPM. We would have preferred to use an agent whose biological effects were more strictly limited to the inhibition of inflammatory cytokine expression, but our preliminary experiments failed to identify a drug whose ability to suppress TNFα, IL–1β, and IL–8 was equal to that of dexamethasone. Although glucocorticoids have been used as an adjunct to therapy with antibiotics in an effort to reduce pulmonary inflammation in affected cattle, their efficacy in this

regard remains controversial and concerns about their ability to suppress immune responses persist. Our study demonstrated that systemic thereapy with comparatively high doses of dexamethasone prior to and in the early stages of disease development markedly reduced both clinical disease and pulmonary pathology in an *in vivo* experimental model of BPM. Clinical disease scores of dexamethasone-treated calves were significantly lower than those of controls for all time points beyond 2 hours post-infection, and all treated calves returned to near-normal clinical status by 24 hours post-infection. These results were closely correlated to the gross and histopathological findings; pulmonary lesions in dexamethasone-treated calves were less extensive and severe than those in the placebo-treated group. Taken together, these findings support the hypothesis that host inflammatory responses are primarily responsible for much of the lung pathology characteristic of pneumonic mannheimiosis, and suggest that pharmacological modulation of pulmonary inflammation may represent a novel approach to the management of this important disease of cattle. Further research to delineate the role of inflammatory cytokines and other pulmonary inflammatory mediators in the pathogenesis of BPM is therefore indicated.

It is important to note that the design of this study did not address the specific mechanism(s) by which dexamethasone reduced clinical disease and pulmonary pathology. The anti-inflammatory effects of glucocorticoids are not due solely to suppression of inflammatory cytokine expression, but also to extensive effects on leukocytes and a wide range of soluble inflammatory mediators, many of which are believed to contribute to the pathogenesis of BPM. While dexamethasone treatment effectively reduced lung injury in this

experimental model, we do not recommend that it be used to prevent naturally occurring disease in the feedlot. *Mannheimia haemolytica* is only one of numerous viral and bacterial pathogens threatening cattle in this environment, particularly in the period immediately following arrival. Administration of dexamethasone at the relatively high doses used in this study would be likely to interfere with immune responses to these organisms, leading to increased morbidity and mortality. Nevertheless, the results of this study warrant further research addressing alternative methods by which to modulate pulmonary inflammation in *M. haemolytica*-infected bovine lungs.

In conclusion, the results of this thesis research have broadened our understanding of the kinetics and cellular sources of inflammatory cytokine expression within the lungs of cattle with pneumonic mannheimiosis. In addition, these studies provide compelling evidence to support the hypothesis that host inflammatory responses may be primarily responsible for much of the lung pathology characteristic of the disease. Future research should now focus on defining pulmonary inflammatory responses in greater detail, identifying additional cytokines or other mediators contributing to that inflammation, and identifying pharmacological agents capable of either suppressing the expression of specific inflammatory cytokines or antagonizing interactions with their cellular receptors.

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APPENDICES

APPENDIX A:

Veterinary Pathology

An International Journal of Natural and Experimental Disease
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1925 Coffey Road **Columbus, OH 43210**
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November 16, 2001

Dr. Christie Malazdrewich
Department of Clinical and Population Sciences
225 Veterinary Teaching Hospitals
College of Veterinary Medicine
University of Minnesota
1365 Gortner Ave.
St. Paul, MN 55108

Dear Dr. Malazdrewich :

This letter, on behalf of the American College of Veterinary Pathologists, authorizes you to incorporate the following manuscript into your Ph.D. dissertation: "Pulmonary expression of tumor necrosis factor alpha, interleukin-1 beta, and interleukin-8 in the acute phase of bovine pneumonic pasteurellosis" (Veterinary Pathology, 38: 297-310, 2001). This permission is for the one-time reproduction of the manuscript in written form and in English. We request that the source of the manuscript be fully acknowledged.

Sincerely,
Donna F. Kusewitt

Donna F. Kusewitt, D.V.M., Ph.D.
Editor-in-Chief, *Veterinary Pathology* (2000-2004)

APPENDIX B:

ELSEVIER

16 September 2004 Our ref: HG/mm/sept 04.J069

Professor Christie Malazdrewich
University of Minnesota
Malazoo002@tc.umn.edu

Dear Professor Malzdrewich

MICROBIAL PATHOGENESIS, *Vol 36, 2004,pp 159-169, Malazdrewich et al, "Pharmacological inhibition..."*
Vol 36, 2004, pp 227-236, Malazdrewich et al, "Protective effect of..."

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