THE ROLE OF SUBSTANCE P IN BOVINE PNEUMONIA CAUSED BY *MANNHEIMIA HAEMOLYTICA*

by

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D.V.M., University of Missouri-Columbia, 1996

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
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Abstract

The bovine respiratory disease complex (BRDC) is a major concern for cattle producers in the United States and worldwide. One of the most costly and deadly components of BRDC is bovine pneumonic pasteurellosis (BPP) caused by *Mannheimia haemolytica*. The initial pulmonary inflammation associated with BPP is a characteristic serofibrinous exudation into the lung, which is believed to be induced by *M. haemolytica* virulence factors such as lipopolysaccharide (LPS) and leukotoxin (LKT) and host cytokines and chemokines such as tumor necrosis factor – α, interleukin – 1β, and interleukin – 8. However, these pulmonary changes often occur before virulence factors or cytokines are substantial components of the pulmonary microenvironment. Other proinflammatory molecules such as substance P (SP) may be involved in the pathogenesis of the peracute serofibrinous exudation of BPP. SP is an 11 amino acid long neuropeptide that is a neurotransmitter of pain that can be released from sensory nerves into tissues to cause neurogenic inflammation. Neurogenic inflammation is characterized by serofibrinous exudation and leukocyte activation. SP-like immunoreactivity was present in the airways, alveolar septa, macrophages, endothelium, and peribronchial nerves in both pneumonic and normal bovine lung; however, SP-like immunoreactivity was increased in pneumonic compared to normal bovine lung due to increased immunoreactivity in macrophages. SP and the combinations of SP with histamine and LPS increased the permeability of a calf pulmonary arterial endothelial cell line to Evans blue dye labeled albumin by 12.34%, 13.57%, and 22.03%, respectively compared to a cell control. Similarly, SP and the combination of SP and histamine increased the monolayer permeability of a bovine adrenal gland capillary endothelium by 8.27% and 16.69% compared to a cell control. The increase in permeability was due to endothelial cell shape change and the formation of intercellular gaps rather than cell death. However, SP does not increase the surface expression of the β_2 integrin CD18 (the *M. haemolytica* LKT receptor) on bovine neutrophils nor does it increase LKT-induced leukocytotoxicity of bovine peripheral blood leukocytes. These findings indicate that SP may be a contributor to BPP in association with other cytokines.

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Dedication

I would like to dedicate my dissertation to my wonderful family: Dad, Mom, Jerry, Mary, Jonah, Alex, Angie, Briana, and Josiah. I couldn't have done it without you all.

CHAPTER 1 - Literature Review

Bovine respiratory disease (BRD) is one of the most common and costly diseases of feedlot calves with estimated annual losses up to $1 billion in the United States (2, 18, 32, 57, 62, 110, 143, 147, 195). The average incidence of BRD in feedlots is estimated to be 14.4% (167). Some studies indicate that the risk of death attributable to BRD is increasing, with greater than half of all feedlot mortalities attributed to BRD (110). A major cause of severe pneumonia, deaths and economic loss associated with BRD is bovine pneumonic pasteurellosis (BPP) caused by *Mannheimia haemolytica* serotype 1 (2, 18, 32, 57, 143, 147). Thus, even small improvements in the ability to reduce the incidence of BPP could provide a substantial positive impact to beef cattle producers.

Bovine pneumonic pasteurellosis

Mannheimia haemolytica

Mannheimia haemolytica is a Gram-negative, nonmotile, non-spore-forming, oxidase positive, fermentative, facultative anaerobic coccobacillus within the family Pasteurellaceae (33, 71, 147, 200). *Mannheimia haemolytica* is considered a commensal pathogen in that it is part of the normal bacterial flora of the ruminant nasopharynx that can become an opportunistic pathogen when the host animal's immune system is compromised (2, 147, 200). It has numerous proposed virulence factors such as an adhesion, capsular polysaccharide, fimbriae, leukotoxin (LKT), lipopolysaccharide (LPS), lipoproteins, neuraminidase, outer membrane proteins, a serotype-specific antigen, sialoglycoprotease, and transferrin-binding proteins (147, 200). Of these, LKT and LPS are considered the two most important contributors to the virulence of the bacterium.

The bacterium was formerly classified as *Pasteurella haemolytica* and included two biotypes: biotype A and biotype T (147, 200). Biotype A contained 17 serotypes; all of which were renamed *Mannheimia haemolytica* except for serotype A11 (which was renamed *Mannheimia glucosida*). *Pasteurella haemolytica* biotype T became *P. trehalosi* and then *Bibersteinia trehalosi*.

Pathogenesis

The pathogenesis of BPP has been elucidated in general terms, but is still not completely understood, particularly in the initial stages of the disease. Cattle carry small numbers of both *M. haemolytica* serotypes 1 and 2 as part of their normal nasopharyngeal flora, with a predominance of serotype 2 (2, 147, 200). In the initial stages of BPP, *M. haemolytica* serotype 1 proliferates to large numbers in the nasopharynx, is aerosolized to the lung in numbers that overwhelm pulmonary defense mechanisms, colonizes and proliferates in the lower respiratory tract, and initiates a serofibrinous effusion into the alveoli that progresses to fibrinopurulent bronchopneumonia (2, 32). This is often associated with a stressful event in the calf's life such as shipping long distances, castration, dehydration, withholding food, or a predisposing viral or mycoplasmal infection that creates airway damage and damages pulmonary clearance mechanisms (2, 32, 143).

Mannheimia haemolytica leukotoxin

During the logarithmic phase of growth of *M. haemolytica* within the bovine lung, the bacterium produces and secretes its major virulence factor, LKT (147, 200). *Mannheimia haemolytica* LKT is a 102 kilodalton exotoxin classified within the RTX (repeats in toxin) family. Similar bacterial exotoxins include the *Escherichia coli* hemolysin, the *Actinobacillus pleuropneumoniae* and *A. suis* hemolysins and cytolysins, *Actinobacillus actinomycetemcomitans* leukotoxin, *Actinobacillus equuli* hemolysin, *Pasteurella aerogenes* Pax toxin, and *Bordetella pertussis* hemolysin.

Mannheimia haemolytica LKT interacts with the β_2 integrin CD11a/CD18 (LFA-1) on the surface of resident and recruited leukocytes (4, 45, 49, 81, 200). The LKT specifically binds to the β chain of LFA-1, CD18 and the binding domain is most likely within amino acids 1 to 291 of bovine CD18 (45, 60). The action of *M. haemolytica* LKT on host leukocytes is concentration-dependent. At higher concentrations, the interaction of LKT with bovine LFA-1 on leukocytes results in the formation of plasma membrane pores in the leukocytes leading to the efflux of potassium; the influx of sodium, calcium and water; cell swelling and lysis; and cell death by oncosis (35, 41, 147, 177, 200). Interaction of lower concentrations of LKT with LFA-1 on bovine leukocytes results in cell death by apoptosis (41, 147, 200). Leukocyte apoptosis initiated by LKT interaction with bovine LFA-1 is mediated through the intrinsic pathway of

apoptosis due to mitochondrial damage and the activation of caspase 9 (6). At very low concentrations of LKT, interaction of LKT with bovine LFA-1 triggers the leukocyte respiratory burst, leukocyte degranulation, and the synthesis of inflammatory cytokines such as tumor necrosis factor-α (TNF), interleukin-1β (IL-1), interleukin-8 (IL-8) and arachidonic acid (AA) metabolites (2, 111, 116, 147, 198, 200). Inflammatory cytokine synthesis by leukocytes after LKT binding is caused by translocation of nuclear factor-κB (NF- κB) to the nucleus due to increased intracellular calcium released from the endoplasmic reticulum and through calcium channels in the membrane (76, 200). Arachidonic acid (AA) metabolites can also be synthesized by bovine leukocytes after LKT binding by activation of phospholipase A_2 (PLA_2) (192, 200).

Mannheimia haemolytica lipopolysaccharide

The other major virulence factor of *M. haemolytica* is the LPS derived from the cell wall of the bacterium. The LPS of *M. haemolytica* interacts via Toll-like receptor 4 with alveolar macrophages, pulmonary intravascular macrophages, mast cells, recruited neutrophils, and endothelial cells to activate these cells and stimulate an inflammatory response (200). The activation of leukocytes, particularly macrophages, can result in TNF production, pyrexia, and hypotensive shock. *Mannheimia haemolytica* LPS interactions with endothelial cells can lead to increased vascular permeability and endothelial cell injury and death (54, 114, 134). In addition, LPS often forms a complex with LKT, which may enhance the leukocytotoxicity of LKT (108).

Host defenses and innate immune response to Mannheimia haemolytica

The earliest barrier to *M. haemolytica* colonization of the lung is the normal epithelium of the respiratory tract with its mucus layer and mucociliary apparatus that works to move particles trapped in the mucus out of the airway (2). The epithelium of the bovine respiratory tract can also produce antimicrobial peptides, such as β-defensins, anionic peptides, and cathelicidins to help eliminate *M. haemolytica* from the lung (2).

In the alveoli, *M. haemolytica*, LKT, and LPS can interact with pulmonary alveolar macrophages (PAMs) to initiate an acute inflammatory response. When PAMs are activated by *M. haemolytica* and its virulence factors, they synthesize and secrete TNF, IL-1, IL-8, and most likely other proinflammatory mediators such as leukotriene B4 (LTB4) and prostaglandin E2 (PGE2), which are increased in bovine lungs infected with *M. haemolytica* (2, 111, 116, 147, 198, 200). Mast cells are often also activated and degranulate to release histamine (2). These

inflammatory mediators and *M. haemolytica* LPS can activate endothelial cells causing them to increase expression of ICAM-1 mRNA (2). This leads to increased neutrophil adherence and infiltration of the lung, which is further enhanced by the inflammatory mediators released from alveolar macrophages and the presence of *M. haemolytica* and its products. Neutrophil infiltration can lead to increased Il-1 and IL-8 production and helps to eliminate the bacteria from the lung. However, the infiltration of neutrophils in the presence of LKT often leads to increased pulmonary damage. In fact, the activation of neutrophils by TNF, IL-1, gamma interferon (IFN-γ), and *M. haemolytica* LPS and LKT increased the expression of the β_2 integrins and neutrophil cytotoxicity when exposed to LKT (2, 99, 105, 106). The importance of neutrophils to pulmonary damage incurred during the disease process is emphasized by the fact that calves depleted of neutrophils prior to *M. haemolytica* inoculation have less pulmonary damage and vascular leakage than calves with an adequate peripheral neutrophil count (165).

Infection of the lung with *M. haemolytica* also results in a local procoagulant state within the lung. Activation of endothelial cells by *M. haemolytica* LPS and IL-1 induce tissue factor production to stimulate extrinsic coagulation and other procoagulant activities (2). During *M. haemolytica* pneumonia, PAMS express tissue factor and their procoagulant activity is increased 30- fold (2). In contrast, macrophage and neutrophil fibrinolytic activity is reduced 23-fold.

Lesions of Mannheimia haemolytica pneumonia

The macroscopic lesions of bovine pneumonia caused by *M. haemolytica* are cranioventral lobular to lobar fibrinopurulent bronchopneumonia with foci of coagulative necrosis and variable fibrinous pleuritis (Figure 1-1) (32). On cross-section, the lung is often purple to red and wet with prominent foci of necrosis (Figure 1-2). The interlobular septa are remarkably widened by fibrin and edema fluid.

Microscopically, the initial lesion begins as serofibrinous effusion into the alveoli (Figure 1-3) (32). This is followed by a massive infiltration of neutrophils into the alveoli (Figure 1-4). Because of the actions of *M. haemolytica* LKT, neutrophil infiltration is often associated with the formation of "oat cells" (degenerate/necrotic leukocytes) (Figure 1-5) and the formation of large foci of necrosis (Figure 1-6) that correspond to the necrotic areas seen macroscopically. As the lesions progress, there is often the formation of abscesses, sequestra of the necrotic tissue, and bronchiolitis obliterans (fibrous obstruction of bronchiole lumens) due to small airway damage.

Summary

Bovine pneumonic pasteurellosis caused by *M. haemolytica* is still the major cause of BRD, particularly in feedlots. *Mannheimia haemolytica* produces multiple virulence factors, but of most importance are LKT and LPS. *Mannheimia haemolytica* and its virulence factors initiate a process that starts as a serofibrinous effusion, followed by activation of host leukocytes to induce inflammatory mediator secretion that causes intense tissue-damaging fibrinopurulent bronchopneumonia that. Although the pathogenesis of the later stages of the lesion is relatively well characterized, the pathogenesis of the serofibrinous effusion in the early stages of *M. haemolytica* bronchopneumonia is not as well understood. The inflammatory mediator cascade associated with later stages of *M. haemolytica* pneumonia does not adequately explain the extensive initial effusion because there are very few bacteria and leukocytes present in the lung at this stage of infection. There may be other inflammatory mediators, such as substance P (SP), that may be responsible for the initial serofibrinous effusion in the absence of substantial numbers of *M. haemolytica* or leukocytes that can predispose to the initial proliferation of *M. haemolytica* in the lung.

Substance P and immunity

Substance P is a undecapeptide with the structure of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (74, 128, 139). Substance P is a neuropeptide that is classified as a tachykinin based on a common carboxy-terminal sequence of Phe-X-Gly-Leu-Met-NH_2 where X is a hydrophobic (Val or Ile) or aromatic (Tyr or Phe) amino acid. Substance P as well as most other tachykinins are encoded by the preprotachykinin I (PPT-I) gene. Substance P is synthesized as a larger protein and then enzymatically cleaved into the active peptide (74, 128, 139).

Substance P was first discovered in 1931 in equine brain and intestinal extracts as an unidentified hypotensive and spasmogenic agent (74, 128, 139). In 1970-1971, SP was isolated and purified from the bovine hypothalamus and its amino acid sequence was determined (74, 103, 128, 139). Further studies have shown that SP is widely distributed in both the central and peripheral nervous systems (73, 74, 128, 139). In the central nervous system, SP is not only a neurotransmitter of pain and various physiologic functions, but also serves as a modulator of behavioral and cognitive functions (131). These behavioral functions include those associated with stressful situations. For example, calves that vocalized continuously (a sign of distress in

cattle) during testicular manipulation in a castration model had higher plasma SP levels than calves that were silent during the process (36). In the peripheral nervous system, SP is localized in sensory nerves and neurons as a neurotransmitter of pain and in the neurons of the respiratory, gastrointestinal, and genitourinary tracts (128, 131). In addition, SP can be released from sensory nerves and leukocytes to initiate neurogenic inflammation and enhance the inflammatory process (128). Neurogenic inflammation is characterized by vasodilation and effusion of fluid from blood vessels (95).

Substance P receptor

The tachykinins as a group bind to at least three different receptors on target cells; neurokinin-1 (NK-1), neurokinin-2 (NK-2), and neurokinin-3 (NK-3) (74, 122, 128). These receptors are all members of the 7 transmembrane G-protein coupled receptor family, but each tachykinin receptor appears to be preferentially activated by a different tachykinin (74, 122, 128). SP preferentially activates NK-1, which is expressed on numerous target cells throughout the body including endothelial cells and leukocytes (58, 61, 72, 74, 142, 184).

Binding of SP to NK-1 on target cells results in rapid endocytosis of the NK-1 receptor allowing for desensitization of the target cell to SP signaling (74, 128). When the NK-1 receptor is stimulated by an agonist, phospholipase C is activated. Phospholipase C is then able to catalyze phosphatidylinositol 4, 5-biphosphate (PIP_2) into inositol 1, 4, 5-trisphosphate (IP_3) and diacylglyerol (DAG). IP_3 and DAG act as second messengers leading to calcium release from the endoplasmic reticulum to activate protein kinase C.

Substance P release from nerves and degradation within tissues

Substance P can be released from the sensory nerves in the peripheral nervous system into the tissues to initiate and enhance inflammation as well as influence vascular tone and permeability. This release is due to the activation of a specific receptor on the nerve termed transient receptor potential vanilloid subfamily, member 1 (TRPV1) (123, 151, 174, 175). TRPV1 has several exogenous and endogenous agonists that can potentially lead to the release of SP. In addition, several inflammatory molecules sensitize TRPV1 to reduce its threshold for activation. The most studied agonist is the vanilloid capsaicin, the pungent compound found in chili peppers (151, 174, 175). However, there are numerous other molecules and conditions that can activate/sensitize TRPV1, including numerous chemical irritants. These include,

resiniferatoxin and ovanil, venoms from jellyfish and spiders, bradykinin, nerve growth factor, anandamide, lipoxygenase products, arachidonic acid metabolites, leukotriene B4, adenosine and ATP, high temperatures above 43°C, and acidic pH less than 5.3 (151, 174, 175).

Normally within the tissues, SP is quickly degraded by various peptidases. The most common is CD10 [a.k.a., neutral endopeptidase, common acute lymphoblastic leukemia antigen (CALLA), enkephalinase, membrane metalloendopeptidase, or neprilysin] (150, 162, 164). CD10 is expressed in multiple cells throughout the body including pulmonary epithelium, endothelial cells, and neutrophils. In addition, endothelial cells secrete another peptidase capable of degrading SP called angiotensin-converting enzyme (ACE) (27, 150). In the absence of adequate CD10 activity (e.g., virus-mediated damage to pulmonary endothelium), the local tissue levels of SP that are released from the nerves can increase and lead to SP-induced vasodilation, increased vascular permeability, and neurogenic inflammation (197). The release of SP from nerves and its subsequent degradation within the tissue are summarized in Figure 1-7.

Substance P and innate immunity

The release of SP from sensory nerves into tissues can have profound effects on the local vascular and cellular innate immune responses by initiating neurogenic inflammation or by influencing ongoing acute inflammation (128). The prominent feature of neurogenic inflammation is vasodilation and fluid effusion. SP can cause vasodilation, increase vascular permeability, cause mast cell and neutrophil degranulation, activate neutrophils and macrophages, and increase proinflammatory molecule synthesis and release from leukocytes. The effects of SP on the innate immune system are summarized in Figure 1-8 and the text below.

Substance P and antimicrobial activity

Many neuropeptides, including SP, are similar in amino acid composition and structure to antimicrobial peptides suggesting that neuropeptides may have direct antimicrobial activity. In fact, SP has been found to have *in vitro* antimicrobial activity against *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus,* and *Candida albicans* (51, 66, 93).

Substance P and respiratory tract epithelium

The epithelium of the respiratory tract is innervated by SP-containing nerve fibers (55, 127, 128, 187). In particular, cattle contain SP immunoreactive nerve fibers in the airway epithelium, in the connective tissue beneath the epithelium and around blood vessels, and in the bronchial glands (127). Overall, these SP immunoreactive nerve fibers are few in number, and are more numerous in calves than in cows.

Exposure of a bovine bronchial epithelial cell line to SP increased neutrophil adhesion, presumably by increased affinity or avidity of ICAM-1 on the epithelial cells for β_2 integrins on the leukocytes (44). SP increases TNF, IL-6 and IL-8 production from human bronchial epithelial cells (188). In addition, mRNA for interleukins-l, -3, -5, -6, TNF, and IFN-y was increased in human nasal mucosa samples when exposed to SP (129).

Because SP-containing nerve fibers innervate the lung, it is possible that the release of SP increases the expression of antimicrobrial peptides in pulmonary epithelium. However, exposure of bovine tracheal epithelium to SP did not increase the expression of the β-defensin tracheal antimicrobial peptide in the epithelial cells (116).

Vascular effects of substance P

Substance P is a potent mediator of vascular changes. It can cause vasodilation and can increase vascular permeability allowing proteinaceous edema fluid to diffuse from the vasculature into the extracellular space (29, 30, 84, 88, 128). The vascular effects of SP may be due to its direct effects on endothelial cells or by causing mast cell degranulation resulting in the release of histamine or other inflammatory mediators that have vascular effects. Inflammatory mediators that increase vascular permeability, such as histamine and LPS, often enhance the SP-induced vascular permeability increase (86, 97). The direct vasodilatory effect of SP is mediated through endothelial cell production of nitric oxide, which diffuses to the vascular smooth muscle causing it to relax (88). The direct vascular permeability increase is caused by SP binding to endothelial cell NK-1 causing increased intracellular calcium resulting in endothelial cell contraction and formation of intercellular gaps (117, 179, 180, 186).

Substance P and mast cells

Tissue mast cells are closely associated with nerve fibers, so they are in a unique position to be influenced by neuropeptides, such as SP, released from nerves. Interaction of SP with

human and rat mast cells results in degranulation of mast cells and release of stored mast cell inflammatory and vasoactive molecules such as histamine and serotonin (94, 128). In addition, mouse mast cells stimulated by SP increased TNF gene expression and protein secretion (128).

Substance P and neutrophils

Many neutrophil functions can be elicited or enhanced by exposure to SP. Substance P alone or in combination with LPS increases neutrophil attachment to endothelial cells, bovine bronchial epithelial cells, and alveolar epithelial cells (44, 47, 98). This effect was mediated by β_2 integrins, which are variably increased on the surface of neutrophils when they are treated with SP. Substance P is also a chemoattractant for neutrophils; it enhances neutrophil migration through a fibroblast barrier and primes neutrophils for the respiratory burst (85, 128, 138, 140). In addition, neutrophils exposed to SP degranulate, produce hydrogen peroxide, and can produce TNF, IL-1 and IL-6 (43, 128).

Substance P and eosinophils

Eosinophils can also be activated by SP. SP activation of eosinophils causes degranulation, release of arachidonic acid metabolites, and the release of superoxide anion (128). In some circumstances, (particularly eosinophils from allergic patients) SP can enhance the eosinophil chemotactic response to platelet-activating factor (PAF) and LTB4 (128). Eosinophils can also produce SP (128, 193, 194).

Substance P and macrophages

SP can activate and induce many proinflammatory changes in macrophages. SP is a chemotactic factor for human macrophages (26, 34, 79, 128). Macrophages exposed to SP have increased phagocytic capabilities and increase their production of reactive oxygen species to enhance the elimination of foreign material such as bacteria (67, 79, 149). SP induces macrophages to increase their production of the inflammatory cytokines TNF, IL-1, and IL-6 as well as arachidonic acid metabolites to augment the acute inflammatory process (79, 101, 102, 128). In addition, macrophages have been shown to possess NK-1 and to produce SP (58, 72, 149). Thus, SP production by macrophages may act as an autocrine or paracrine signal to further propagate the inflammatory process.

Substance P and adaptive immunity

In addition to playing a role in the innate immune response, SP has been implicated in enhancing adaptive immune responses. Mice deficient in the SP receptor did not develop a normal granulomatous response to *Schistosoma mansoni* and produced much less IFN-γ, IgG2a, and IgE than mice with a normal SP receptor (15). Mice treated with a NK-1 receptor antagonist were more susceptible to infection with *Salmonella* than untreated mice, with a decreased IFN-γ response in the treated mice (194). Also, mice deficient in the SP receptor had a decreased cytotoxic T-lymphocyte (CTL) response, decreased interleukin-12 (IL-12) synthesis and secretion, and increased viral load compared to normal mice when both were infected with murine γ-herpesvirus 68 (52). It has also been shown that SP can induce the production of IL-12 by murine macrophages (91) further emphasizing the role that SP can play in adaptive immunity.

Substance P also influences B- and T-lymphocytes. SP is a chemoattractant for lymphocytes and can enhance their attachment to endothelial cells (128, 189). Substance P is a B-lymphocyte differentiation cofactor that can increase immunoglobulin production, particularly the production of IgA (19, 133, 169). Substance P does increase the production of IgM from B cells, but the greatest increase is seen when SP is combined with LPS (133). With human and murine T-lymphocytes, SP enhances interleukin-2 (IL-2) production, enhances macrophage inflammatory protein-1β expression, and regulates INF-γ production (16, 17, 128. The natural killer cell activity of intraepithelial T-lymphocytes is increased when they are exposed to SP (40). Substance P can also stimulate the proliferation of both B- and T-lymphocytes (100, 136, 169). Both B- and T-lymphocytes express the NK-1 receptor, and T-lymphocytes can produce SP (28, 128, 170).

Summary

Substance P has multiple effects in initiating or enhancing innate and adaptive immunity (Table 1-1). Substance P has direct antimicrobial effects against some species of bacteria *in vitro*. It can elicit inflammatory cytokine and gamma interferon production, but not tracheal antimicrobial peptide production from respiratory epithelial cells. Substance P has profound vascular effects including the induction of vasodilation and increased vascular permeability that allows the effusion of protein-rich edema fluid from the vasculature. Mast cell histamine release and TNF production is induced by SP. Neutrophil attachment to endothelial and respiratory

epithelial cells is increased after exposure to SP. Substance P is also a chemoattractant for neutrophils, induces hydrogen peroxide production in neutrophils, increases cytokine synthesis in neutrophils, and primes neutrophils for the respiratory burst. Substance P causes eosinophils to degranulate, produce superoxide anion, and produce arachidonic acid metabolites. Substance P also enhances eosinophil chemotaxis induced by other chemotactic factors. Eosinophils can also produce SP. Substance P is a chemoattractant for macrophages, increases their cytokine synthesis, induces macrophages to synthesize arachidonic acid metabolites, causes oxygen free radical production, and increases macrophage phagocytosis. Macrophages also contain NK-1 and can produce SP. Both B- and T-lymphocytes contain NK-1, and T-lymphocytes can produce SP. Substance P increases lymphocyte attachment to endothelial cells, is a chemoattractant for lymphocytes, and can stimulate B- and T-lymphocyte proliferation. Substance P is a late co-differentiation factor for B-lymphocytes, and increases immunoglobulin production, particularly IgA. T-lymphocytes' cytokine production and natural killer abilities are enhanced after exposure to SP. Collectively, these findings indicate that SP can have multiple effects in promoting innate and adaptive immunity.

Substance P and respiratory disease

Substance P has been implicated in the pathogenesis of a wide variety of respiratory conditions including asthma, respiratory syncytial virus infection, and acute respiratory distress syndrome (11, 68, 128 183, 197). In both natural and experimental respiratory conditions that involve SP, there are usually increased levels of SP, upregulation of NK-1 receptors, or both. In humans, elevated concentrations of SP are present and NK-1 is up-regulated in patients with asthma and chronic obstructive pulmonary disease (84). Following immune stimulation in mice, NK-1 is upregulated on granulocytes and macrophages located perivascularly and peribronchially, and upon alveolar infiltrates within the lung (88). Substance P levels within BAL fluids are also increased, accompanied by increased numbers of inflammatory cells and elevated cytokines.

In many studies, the administration of NK-1 antagonists inhibits the inflammatory response. Systemic administration of a NK-1 antagonist has been shown to significantly reduce the numbers of inflammatory cells within the BAL fluids in a mouse model of pulmonary immune inflammation (88). In an immune complex model of lung inflammation characterized

by increased microvascular permeability and neutrophil influx, administration of antibody against NK-1 has been shown to inhibit these inflammatory changes (21). These studies indicate that the release of SP and increased levels of NK-1 within the lung are associated with inflammatory changes that are comparable with those of early stages of pneumonia.

Substance P and bovine respiratory disease

One of the earliest morphological changes in BPP is a dramatic serofibrinous effusion into alveoli (2, 32, 195). This extensive vascular leakage could be in response to a variety of direct or indirect acting vasoactive mediators, such as histamine, leukotrienes, TNF, IL-1, LPS, or SP. (2, 32, 195). Flooding of alveoli with fluid would contribute to reduced clearance of any bacterium in the inhaled air (e.g., *M. haemolytica*).

The distribution of SP-containing nerve endings in the respiratory tract has been investigated in various species, including ruminants (55, 127, 187). In normal calves SP-reactive nerve endings were most numerous in the nasal, laryngeal, and tracheal mucosa, and less numerous in the lung (127). Within the lung the SP-containing fibers were present in pulmonary epithelium, the connective tissue beneath the epithelium and around blood vessels and glands, but were sparse in smooth muscle layers. The SP-reactive nerve endings were most numerous in calves compared to cows. Capsaicin treatment of neonates caused a reduction in the number of SP fibers (127). A similar decrease with age in the number of respiratory SP-containing nerve fibers was reported in sheep (63). In neonatal lambs, dihydrocapsaicin depleted SP-fibers by 85% and caused an increase in the density of mast cells (144). However, in spite of the large numbers of mast cells in dihydrocapsaicin-treated lambs, pulmonary histamine content was no different than that in control lambs (144).

Direct biological effects of SP have been reported in *in vitro* studies. Bovine bronchial epithelial cells stimulated by SP and acetylcholine had increased production of eosinophil chemotactic factor and leukotriene B4 (96). Substance P mediated both direct and indirect contraction of bovine tracheal smooth muscle, with direct activation occurring in a manner similar to that caused by acetylcholine stimulation (39). The presence of NK-1 has been reported on bovine alveolar macrophages and exposure of alveolar macrophages to SP resulted in enhanced phagocytosis and TNF production compared to non-stimulated controls (149).

SP appears to be involved in the pathogenesis of bovine respiratory syncytial virus (BRSV) infection, a common predisposing factor to bovine respiratory disease and BPP (8). In other species, RSV infection can induce upregulation of NK-1 (92, 141, 183) and enhance SP-induced pulmonary inflammation (5, 68). Notably, a fusion protein of BRSV (virokinin) has biological properties and immunological reactivity similar to that of SP (201). In experimental infections of gnotobiotic calves using a wild type and mutant BRSV with defective virokinin production, there were less severe lesions induced by the mutant virus compared to the wild type virus (185). These studies suggest an enhancement of pulmonary inflammation in response to SP or SP-like activity associated with another important bovine respiratory pathogen that often precedes BPP.

Conclusions

Bovine pneumonic pasteurellosis caused by *M. haemolytica* is still a common and costly disease in beef cattle. Although much is known about the pathogenesis of BPP, there remains much to be learned particularly about the early stages of the disease when the lesion is mainly serofibrinous effusion without the presence of many *M. haemolytica* or leukocytes within the lung. In the absence of traditionally incriminated mediators of BPP (e.g., TNF, LKT, and LPS), other inflammatory mediators may be involved in the early stages of BPP.

Substance P has been shown to have a multitude of proinflammatory effects on components of both innate and adaptive immunity that can lead to initiation or enhancement of inflammation. Inflammation initiated by SP is termed neurogenic inflammation, the prominent feature of which is vasodilation and edema formation.

Substance P has been implicated in the pathogenesis of many respiratory diseases in various species. The numerous documented roles of SP in inflammation, notably, its association with stress-related conditions, pulmonary irritation, and serofibrinous effusion make it a prime candidate to have a role in the early pathogenesis of BPP. The question this project investigates is whether or not SP is a likely contributor to the pathogenesis of BPP. To investigate the potential role of SP in bovine pneumonia induced by *M. haemolytica* three hypotheses were established:

1. The quantity of SP is increased in *M. haemolytica*-induced pneumonia compared to normal bovine lungs.

2. Substance P is capable of inducing increased vascular permeability and vascular leakage in bovine vascular endothelial cells.
3. Substance P increases CD18 expression on bovine neutrophils and subsequent increased leukocytotoxicity upon exposure to *M. haemolytica* LKT.

Figures and Tables

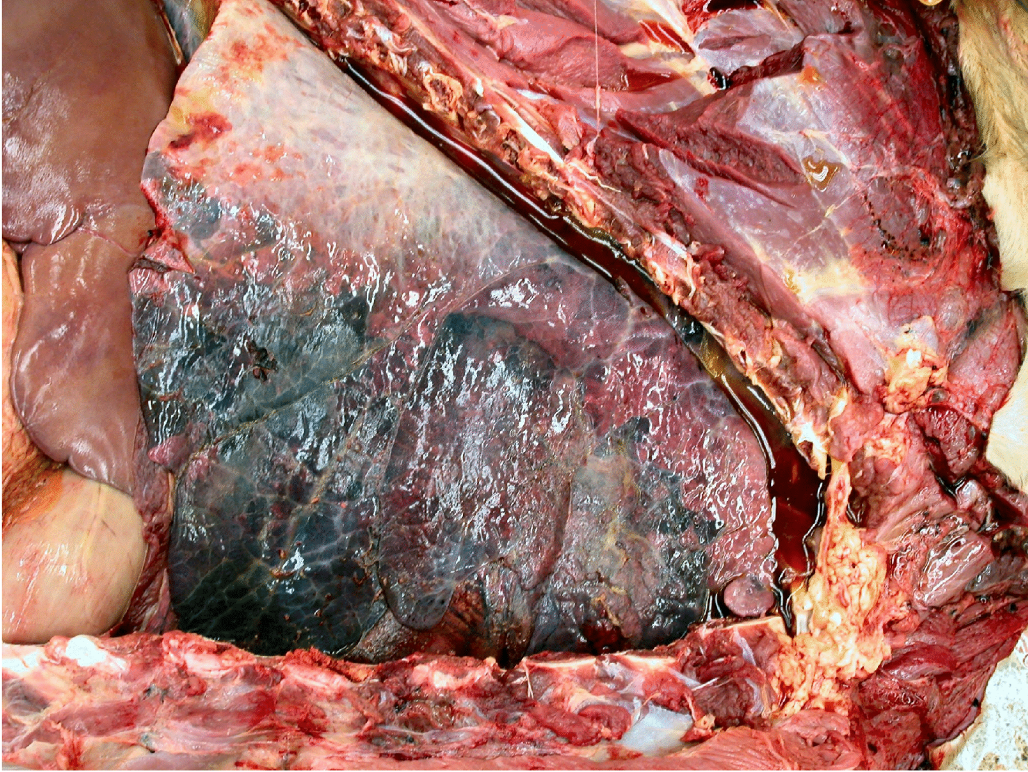

Figure 1-1 Bronchopneumonia in a feedlot calf caused by *Mannheimia haemolytica*.

Note the cranioventral consolidation, the fibrin on the pleura, and the fluid within the thorax.

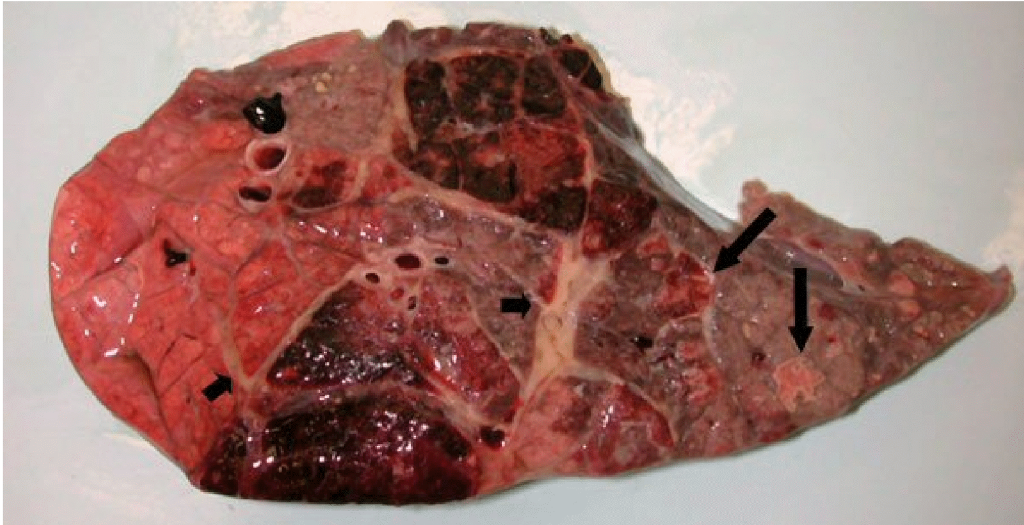

Figure 1-2 Cross-section of bronchopneumonia in a feedlot calf caused by *Mannheimia haemolytica*.

Note the multiple foci of necrosis indicated by the long arrows and the fibrin and edema in the interlobular septa indicated by the short arrows.

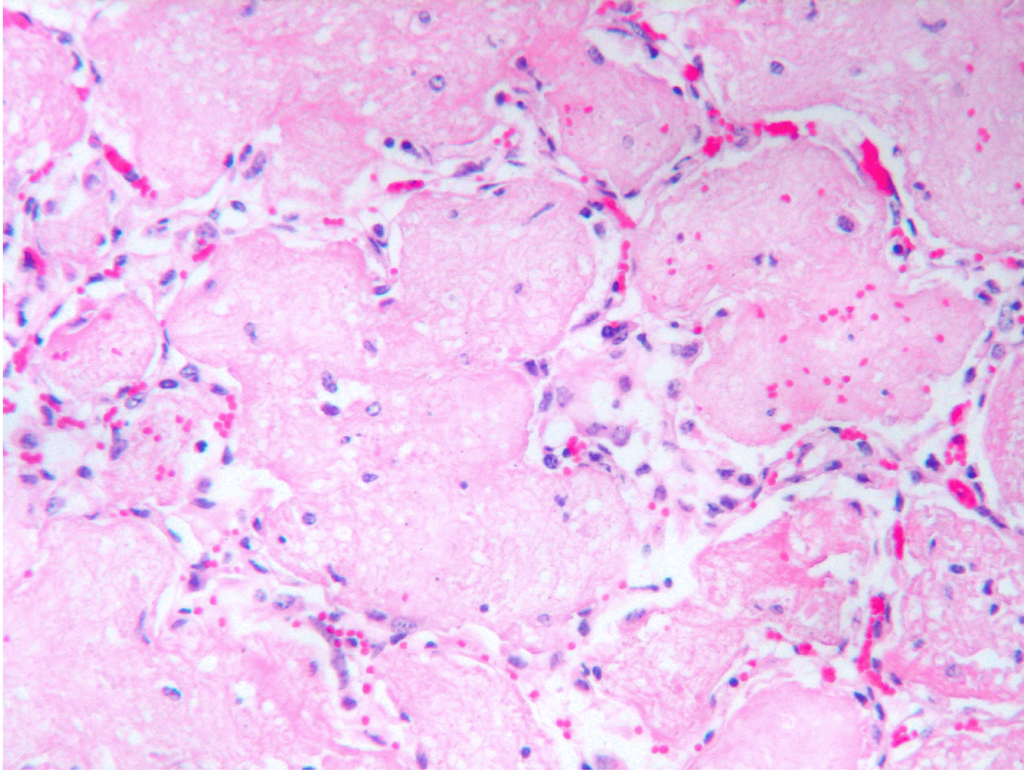

Figure 1-3 H&E stained section of peracute bronchopneumonia in a feedlot calf caused by *Mannheimia haemolytica*.

Note the fibrin and edema fluid in the alveoli and the paucity of inflammatory cells.

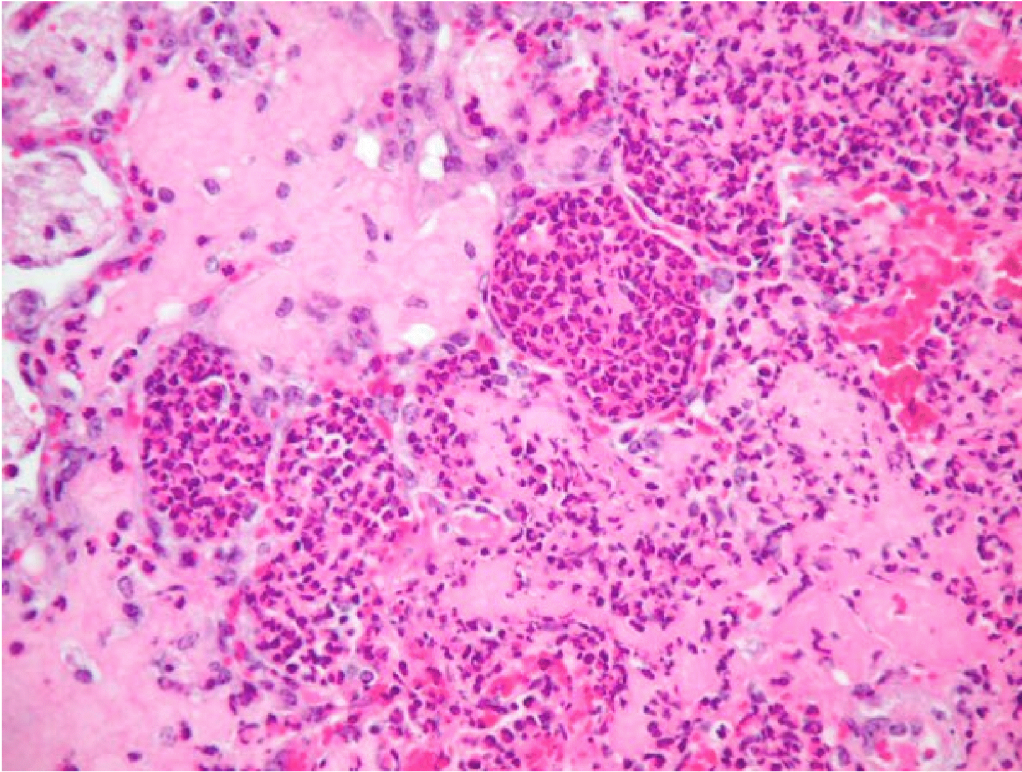

Figure 1-4 Acute fibrinosuppurative bronchopneumonia caused by *Mannheimia haemolytica* in a feedlot calf.

Note the infiltration of neutrophils, some of which are degenerate and have caused tissue damage.

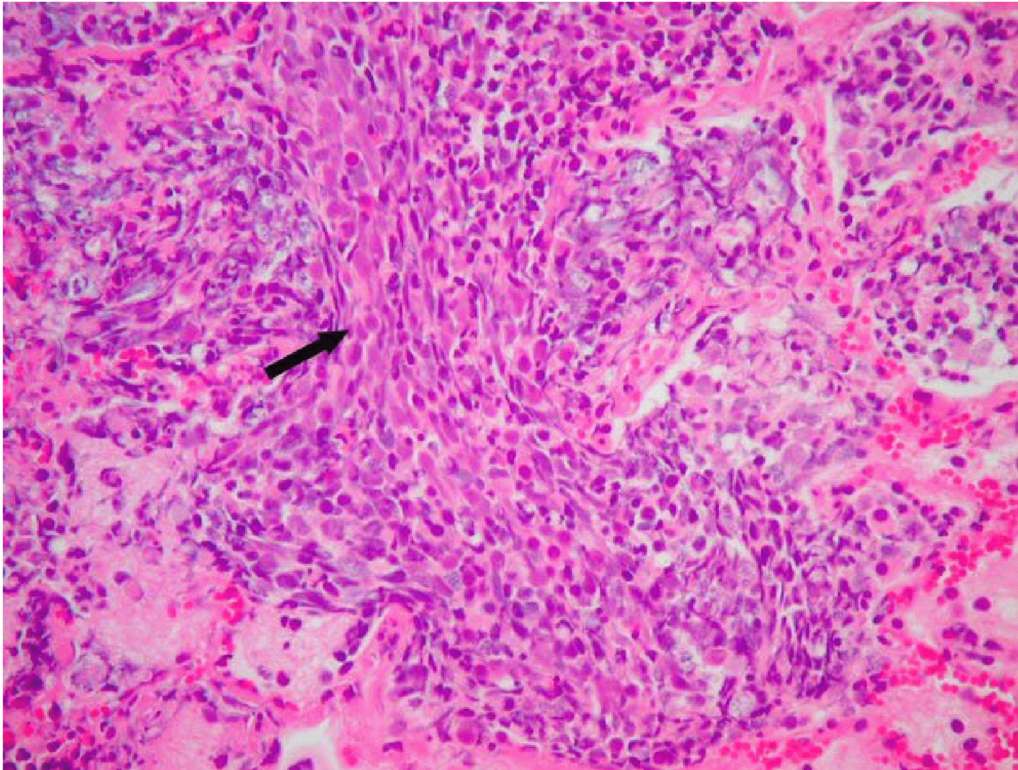

Figure 1-5 Acute fibrinosuppurative bronchopneumonia with "oat cells" caused by *Mannheimia haemolytica* in a feedlot calf.

The arrow points to the "oat cells".

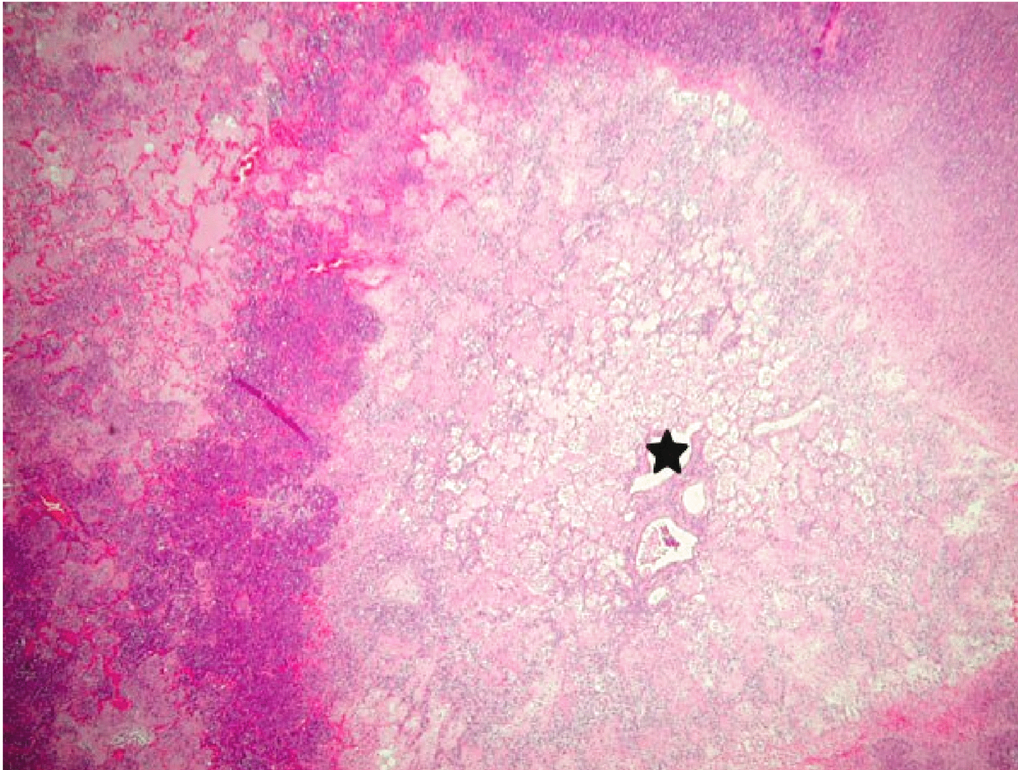

Figure 1-6 Acute fibrinosuppurative bronchopneumonia with necrosis caused by *Mannheimia haemolytica* in a feedlot calf.

The star is in the center of the necrotic focus.

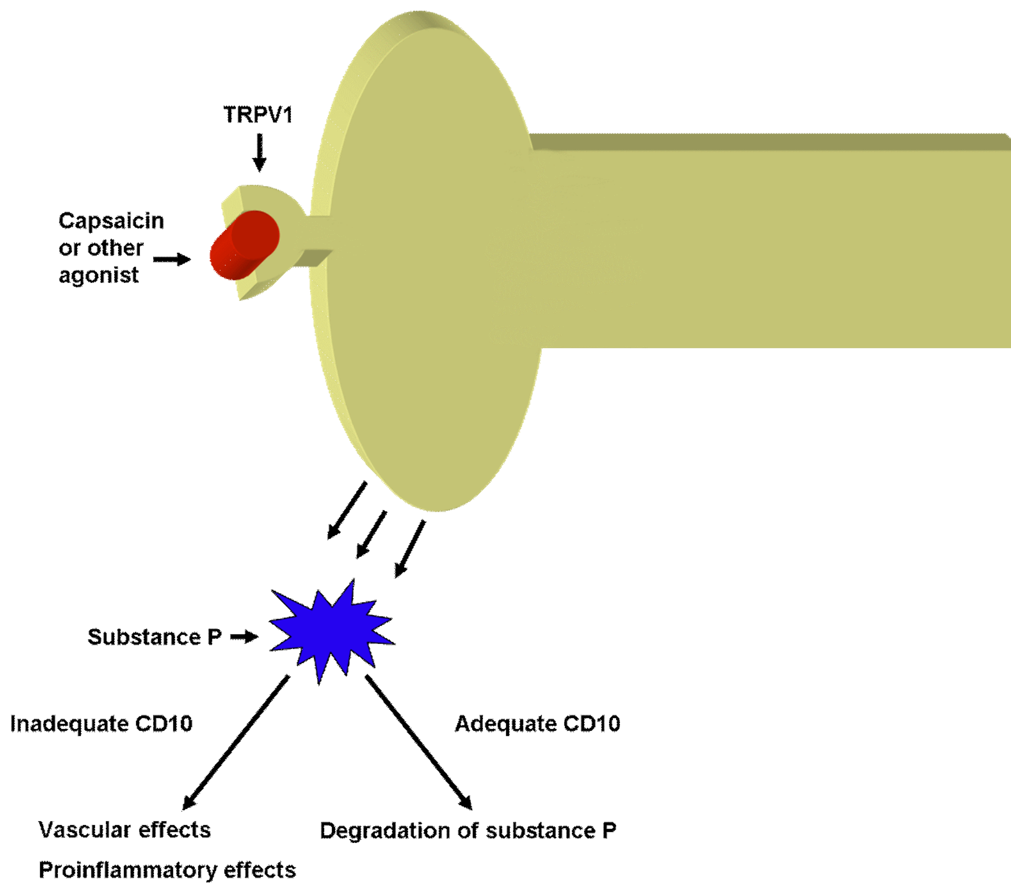

Figure 1-7 Summary of substance P release from peripheral nerve into the tissue and possible outcomes.

Figure 1-8 Summary of the action of substance P on the differing components of the innate immune response.

AA = arachidonic acid, PMN = neutrophil, SP = substance P.

Table 1-1 Action of substance P on the cells of the innate and adaptive immune responses.

Innate Immunity	Adaptive Immunity
Direct antimicrobial effects **Respiratory epithelium** • Cytokine secretion • Increased neutrophil adherence **Vascular effects** • Vasodilation • Increased vascular permeability **Mast cells** • Degranulation • Histamine release • Cytokine secretion **Neutrophils** • Increased adherence to endothelial and epithelial cells • Chemoattractant • Cytokine secretion • Degranulation • Hydrogen peroxide synthesis • Primes for respiratory burst **Eosinophils** • Produce superoxide anion • Arachidonic acid metabolite formation • Enhances chemotaxis induced by other chemotactic factors • Produce substance P **Macrophages** • Chemoattractant • Increase phagocytic ability • Cytokine secretion • Arachidonic acid metabolite formation • Oxygen free radical formation • Produce substance P	**Macrophages** • Produce IL-12 • Produce substance P • Previous actions in innate immunity **Lymphocytes** • Chemoattractant • Increased adherence to endothelium • B and T cell proliferation • Immunoglobulin production • Cytokine secretion from T cells • Enhance natural killer activity

CHAPTER 2 - Substance P – like immunoreactivity in normal and pneumonic bovine lung

Introduction

Bovine respiratory disease (BRD) is one of the most common and costly diseases of feedlot calves in North America and worldwide (2, 53, 57, 110, 121, 143, 146). *Mannheimia haemolytica* serotype 1 is the predominant cause of bovine pneumonic pasteurellosis (BPP) and is most often associated with severe pneumonia, deaths and economic loss associated with BRD (2, 18, 32, 38, 53, 57, 143). The typical lesion caused by M. *haemolytica* is fibrinopurulent bronchopneumonia. This lesion progresses from an initial serofibrinous exudation into alveolar lumens to neutrophil accumulation, parenchymal necrosis, and aggressive interlobular spread of inflammation (2, 18, 32, 57, 143). In cattle that survive the acute disease, chronic changes that may occur include abscesses, fibrosis, and bronchiolitis obliterans.

Many of the lesions of BPP are attributed to M. *haemolytica* virulence factors such as lipopolysaccharide (LPS) and leukotoxin (LKT) (2). However, lesions can also be initiated or exacerbated by host responses, such as the localized release of tumor necrosis factor – α (TNF), interleukin 1 – β (IL-1β), and interleukin – 8 (IL-8) (2, 165). The neuropeptide substance P (SP) is another host factor that may contribute to the lesion of BPP. Substance P is an 11-amino-acid neuropeptide found mainly in sensory nerves that can also be synthesized by inflammatory cells (128). Substance P serves mainly as a neurotransmitter of pain, but it can also be released into the tissue to initiate neurogenic inflammation (29, 46, 56, 75, 94, 95, 128, 148, 156). Substance P most notably results in serofibrinous effusion by inducing increased permeability of vascular endothelial cell. It has also been shown to attract and activate several types of leukocytes. Substance P has been implicated in the pathogenesis of various respiratory conditions of people and rodents, including allergic rhinitis, asthma, airway hyperresponsiveness, nonproductive cough, respiratory viral infection, sarcoidosis, idiopathic pulmonary fibrosis, and acute respiratory distress syndrome (11, 87, 128).

The purpose of this study was to determine if there are differences in the distribution and amount of SP within normal compared to pneumonic lungs from calves with BPP. This was

accomplished by investigating the presence and distribution of SP-like immunoreactivity (SP-LI) in histologic sections of normal lung and in lung with experimentally induced *M. haemolytica* pneumonia.

Materials and methods

Lung samples

Pneumonic and normal lung tissues were collected from twenty-one 500-600 pound beef calves and eight 200-300 pound Holstein calves that were part of independent studies on BPP. Calves were infected by endoscopically-guided intrabronchial instillation of *M. haemolytica* at the entrance of the right cranial lung lobe bifurcation and euthanized 3-5 days post-inoculation. Experimental lesions most typically occurred in and were collected from the right cranial apical, right caudal apical and right middle lung lobes. Normal lung tissue was collected from either the left caudal apical lung lobe of infected calves, or from the right apical or middle lobes of calves that were sham inoculated.

Histopathology and immunohistochemistry

Lung tissue was fixed in 10% buffered neutral formalin, routinely processed and stained with hematoxylin and eosin (H&E). For immunohistochemical staining, selected sections were sectioned at 4 μm, deparaffinized in xylene, and rehydrated in graded alcohols progressing to distilled water. Endogenous peroxidases were quenched with 3% H_2O_2 for 5 minutes and endogenous charged sites were blocked with protein blocking agent (Shandon-Lipshaw, Pittsburgh, PA) for 2 minutes, both at room temperature. Tissues were then sequentially incubated with a 1:1000 dilution of monoclonal mouse anti-SP primary antibody (Abcam, Cambridge, MA) overnight at 4°C, a 1:200 dilution of equine anti-mouse biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 15 minutes at room temperature, avidin-biotin-enzyme complex (Vector Labs) for 15 minutes, followed by the chromogen 3,3'-diaminobenzidine (DAB, Vector Labs). The slides were counterstained with Gill's 1 hematoxylin (Fisher Scientific). Control slides consisted of slides that were prepared where the primary antibody was replaced with mouse IgG1 isotype antibody (GeneTex, Irvine, CA) or where neither a primary or isotype antibody were used during incubation.

Image analysis

Slides that contained both pneumonic and more normal lung (defined as transitional lung tissue) were selected for evaluation as pneumonic and transitional tissue. Normal lung sections were confirmed to contain no histological abnormalities before being selected for evaluation. For each immunohistochemical slide, five tagged image file (TIF) photomicrographs (400x) were taken using a standard protocol. For pneumonic lung slides, the interface between pneumonic (defined as parenchyma that consisted of alveoli that were uniformly filled with either cellular or non-cellular exudates) and transitional (defined as parenchyma that consisted of alveoli that had predominately clear lumens or lumens with slight amounts or serous fluid) was identified. On the transitional side of the interface line, the tissue was divided into quadrants, and one photomicrograph from each quadrant was taken. At least one of these 4 photomicrographs was selected to include an airway larger than or equal to the size of a terminal bronchus. For normal lung tissue, the tissue was divided into quadrants and one photomicrograph was taken within each quadrant. At least one of these 4 photomicrographs was selected to include an airway larger than or equal to the size of a terminal bronchus. The quantity of immunostaining for each photomicrograph was determined by using morphometric software (ImageJ, National Institutes of Health). The color of the background counterstaining was separated from the color of the DAB deposition using a color deconvolution plugin (provided by G. Landini http://www.dentistry.bham.ac.uk/landinig/software/cdeconv/cdeconv.html#ruifrok) as previously described (153). The total mean gray value (MGV) of the 4 images for each calf was averaged. The final MGV value for each calf was calculated by subtracting the average MGV of lung stained with an isotype antibody and the null control from the MGV of the pneumonic and normal lung.

Various structures within the photomicrographs (airway epithelium, macrophages, alveolar septa, lymphatic/venular endothelium, and peribronchial nerve) were then evaluated for either the presence or absence of SP-immunostaining. The structure or region of interest (ROI) was outlined, and the MGV of the immunostaining in the ROI was measured. The MGV was recorded as MGV/area in pixels for the ROI. The final MGV for each component of each calf was calculated by subtracting the average MGV of lung stained with an isotype antibody and the null control from the MGV of the pneumonic and normal lung. The total MGV reported for each

measured structure was calculated as the average of the measured structure in all photomicrographs. A structure was considered to contain SP-immunostaining when it had a MGV less than the average of the isotype antibody stained tissue and the null control tissue (a negative MGV).

Statistics

Statistical analysis for the difference between pneumonic and normal lung was performed using a Student's T test. Statistical analysis for the difference between the structures in pneumonic and normal lung was conducted using a one-way ANOVA followed by Tukey's multiple comparison test. The difference in the staining in the pneumonic compared to the normal bovine lung was considered significant with a p-value < 0.05. Statistical analysis was performed using SAS 9.1.

Results

The MGV of SP-LI in pneumonic lung (-23.63 ± 12.24) was significantly less (p-value $<$ 0.0001) than normal lung (-10.50 ± 9.65). In the pneumonic lung, most SP-LI occurred in the 4 quadrants of the transitional area at the inflammation interface. However, the tissue evaluated on the more pneumonic side of the interface had similar reactivity (-23.43 ± 16.06), consisting of SP-LI in the infiltrating macrophages.

The average SP-LI (recorded as mean MGV) for each measured structure within the photomicrographs is listed in Table 2-1. There was decreased average MGV (increased SP-LI) in macrophages, airway epithelium and endothelial cells in pneumonic compared to normal lung. This increased SP-LI was different only for the macrophages ($p<0.05$). The average SP-LI was greater in normal compared to pneumonic lung for alveolar walls and peribronchial nerves.

The incidence of SP-LI for individual structures is listed in Table 2-2. SP-LI within macrophages (Figure 2-1) occurred about twice as often in pneumonic lungs as it did in normal lungs (macrophages were reactive in 89.7% of pneumonic lungs and 48.1% of normal lungs). In both pneumonic and normal lungs, there was SP-LI in the airway epithelium from the bronchi to the terminal bronchioles (Figures 2-2 to 2-4) with a higher incidence of SP-LI in pneumonic lungs compared to normal lungs (airway epithelium was reactive in 89.7% of pneumonic lungs and 61.1% of normal lungs). The incidence of SP-LI in the alveolar walls (Figure 2-5) was similar in both pneumonic and normal lungs. Substance P - like immunoreactivity within

endothelial cells lining venules and lymphatics (Figure 2-6) was more frequent in the pneumonic lungs than normal lung. In normal lung, SP-LI within peribronchial nerves (Figure 2-7) occurred about twice as frequently compared to pneumonic lung (42.55% in normal lungs compared to 22.2% in pneumonic lungs).

Discussion

This study demonstrated a clear difference in SP-LI in normal bovine lungs compared to pneumonic lungs that were induced by experimental infection with *M. haemolytica*. In previous studies of normal bovine lung, SP immunoreactive nerve fibers were found in the airway epithelium, in the connective tissue beneath the epithelium and around blood vessels, and in the peribronchial glands (127). We also demonstrated reactivity in these tissues in normal, as well as pneumonic lung. The difference between normal and pneumonic lung was due predominately to an increased quantity and incidence of SP-LI in alveolar macrophages within pneumonic compared to normal lungs. These results are similar to those found in sheep experimentally infected with *M. haemolytica*, where there was also a predominance of SP-LI in alveolar macrophages within pneumonic compared to normal lungs (145). Also in sheep, as in the current study, there was a decreased incidence of SP-LI in peribronchial nerves of pneumonic lungs compared to normal lungs (145). This decrease may be a reflection of depletion of SP from nerve endings in response to noxious stimuli associated with inflammation. The increased SP-LI associated with alveolar macrophages in pneumonic bovine lungs may represent binding of the SP released by local nerves, local production by the alveolar macrophages, or both. The transition in SP distribution from acutely inflamed to less actively inflamed lungs was demonstrated in sheep inoculated with *M. haemolytica* (145). The strong SP-LI within alveolar macrophages present during acute stages of ovine pneumonia waned over time, so that most immunoreactivity in later stages of pneumonia was present within nerve fibers and nerve cell bodies within ganglia.

Macrophages from many species have been shown to produce and be activated by SP to secrete inflammatory cytokines, produce reactive oxygen species, and increase phagocytosis (10, 26, 58, 67, 72, 101, 102, 120, 128, 132, 154). Bovine alveolar macrophages also become activated upon exposure to SP (149). Exposure of bovine alveolar macrophages to SP resulted in pro-inflammatory events, including increased phagocytosis and TNF production compared to

non-stimulated controls. Because alveolar macrophages are one of the first inflammatory cells exposed to *M. haemolytica* and its virulence factors, autocrine signaling of alveolar macrophages by SP may be involved in the inflammatory response initiated by alveolar macrophages when they are exposed to *M. haemolytica*. In addition, paracrine signaling of other leukocytes and endothelial cells by SP released from macrophages can influence the progression of BPP by increasing vascular permeability to contribute to the serofibrinous exudate characteristic of the disease and by attracting and activating resident pulmonary and peripheral blood leukocytes. These collective findings suggest a role for SP in the pathogenesis of BPP, alongside other cytokines and virulence factors shown to be involved in the progression of the disease.

SP has been implicated in the pathogenesis of other respiratory conditions including asthma, respiratory syncytial virus infection, and the acute respiratory distress syndrome (11, 68, 128, 183, 197). In both natural and experimental respiratory conditions that involve SP, there are usually increased levels of SP, upregulation of NK-1 receptors, or both. Cutaneous burn-induced acute lung inflammation in mice resulted in increased SP production and SP neurokinin-1 (NK-1) receptor activity, which was associated with enhanced lung damage (163). Mice with hydrogen sulfide-induced pneumonia exhibited increased plasma and lung SP levels leading to pulmonary neutrophilic inflammation and increased lung levels of TNF and IL-1β (12). In a hepatic ischemia/reperfusion pulmonary inflammation model in mice there were increased SP levels in bronchoalveolar lavage (BAL) fluid, which corresponded to the time of greatest neutrophil influx into the lungs (130). SP was also increased in BAL fluids of pigs exposed to aerosolized citric acid as a model to induce coughing (119). Following immune stimulation in mice, SP levels within BAL fluids were increased along with increased numbers of inflammatory cells and cytokines (84). These changes corresponded with an upregulation of NK-1 receptors on granulocytes and macrophages located perivascularly and peribronchially, and within alveolar infiltrates within the lung. In humans, elevated concentrations of SP are present in patients with asthma and chronic obstructive pulmonary disease, and NK-1 is up-regulated during pulmonary inflammation (84). Also, higher levels of immunoreactivity for SP was found in BAL fluid and sputum of people with asthma, chronic bronchitis, or after ozone exposure (69, 125, 182).

Studies in various species have demonstrated a relationship between SP and respiratory syncytial virus (7, 92, 141, 184). Capsaicin-induced SP release from sensory nerves enhanced respiratory syncytial virus pneumonia in rats (7). RSV infection can induce upregulation of NK-

1 and enhance SP-induced pulmonary inflammation (92, 141, 183). SP also appears to be involved in the pathogenesis of bovine respiratory syncytial virus (BRSV) infection, a common predisposing factor to BRD and BPP (8). A fusion protein of BRSV (virokinin) has biological properties and immunological reactivity similar to that of SP (201). In experimental infections of gnotobiotic calves using a wild type and mutant BRSV with defective virokinin production, there were less severe lesions induced by the mutant virus compared to the wild type virus (185). These studies suggest an enhancement of pulmonary inflammation in response to SP or SP-like activity associated with another important bovine respiratory pathogen that often precedes BPP.

This study demonstrates an increased amount of SP in experimental lesions of BPP compared to normal lungs. Based on these findings, and the role of SP in respiratory conditions in other species, SP should be regarded as another host factor that contributes to the pathogenesis of this important cause of BRD.

Figures and Tables

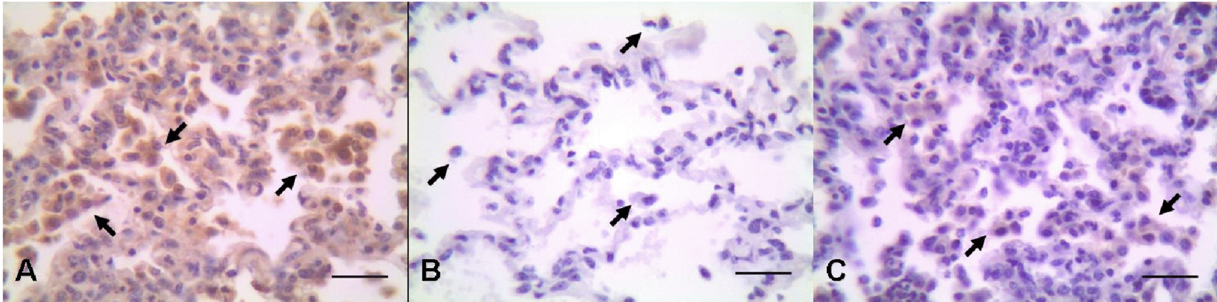

Figure 2-1 Substance P-like immunoreactivity in macrophages of pneumonic and normal lung.

A. Moderate to strong substance P-like immunoreactivity in macrophages of pneumonic bovine lung stained with a monoclonal mouse anti-human substance P antibody. Arrows point to macrophages. Bar = 30 µm. B. Little to no substance P-like immunoreactivity in macrophages of normal bovine lung stained with a monoclonal mouse anti-human substance P antibody. Arrows point to macrophages. Bar = 30 µm. C. Slight immunoreactivity in macrophages of pneumonic lung stained with a mouse IgG1 isotype antibody. Arrows point to macrophages. Bar = 30 µm.

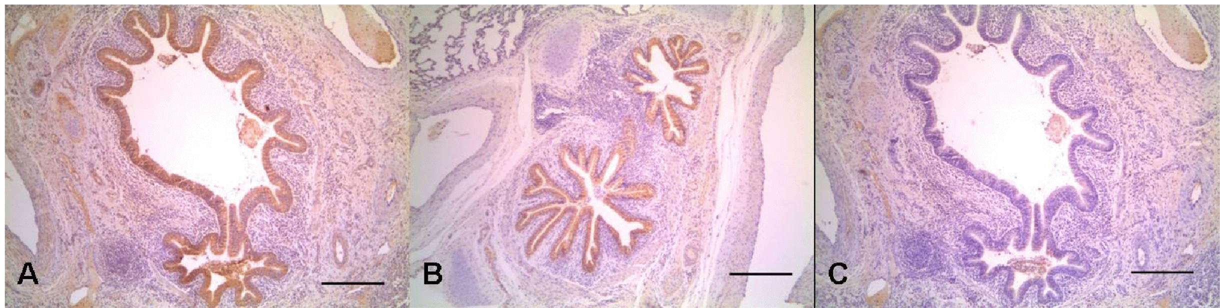

Figure 2-2 Substance P-like immunoreactivity in a bronchus of pneumonic and normal lung.

A. Moderate to strong substance P-like immunoreactivity in a bronchus of pneumonic bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 250 µm. B. Moderate substance P-like immunoreactivity in a bronchus of normal bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 250 µm. C. Little to no immunoreactivity in a bronchus of pneumonic lung stained with a mouse IgG1 isotype antibody. Bar = 250 µm.

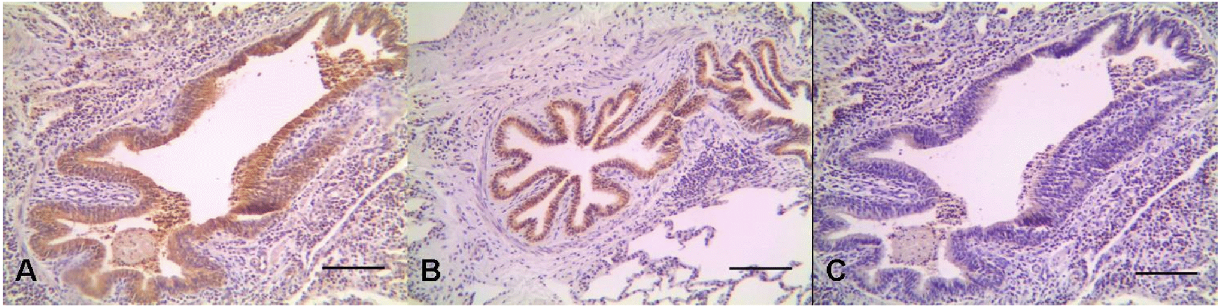

Figure 2-3 Substance P-like immunoreactivity in a bronchiole of pneumonic and normal lung.

A. Moderate to strong substance P-like immunoreactivity in a bronchiole of pneumonic bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 100 µm. B. Moderate to strong substance P-like immunoreactivity in a bronchiole of normal bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 100 µm. C. Little to no immunoreactivity in a bronchiole of pneumonic lung stained with a mouse IgG1 isotype antibody. Bar = 100 µm.

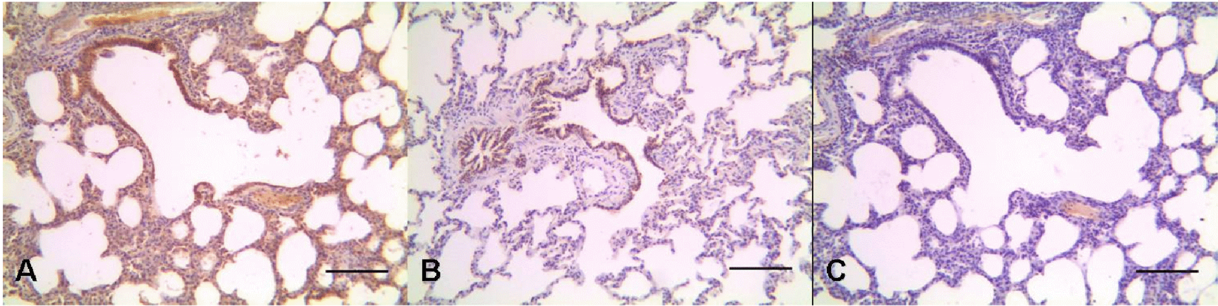

Figure 2-4 Substance P-like immunoreactivity in a respiratory bronchiole of pneumonic and normal lung.

A. Moderate to strong substance P-like immunoreactivity in a respiratory bronchiole of pneumonic bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 100 µm. B. Moderate to strong substance P-like immunoreactivity in a respiratory bronchiole of normal bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 100 µm. C. Little to no immunoreactivity in a respiratory bronchiole of pneumonic lung stained with a mouse IgG1 isotype antibody. Bar = 100 µm.

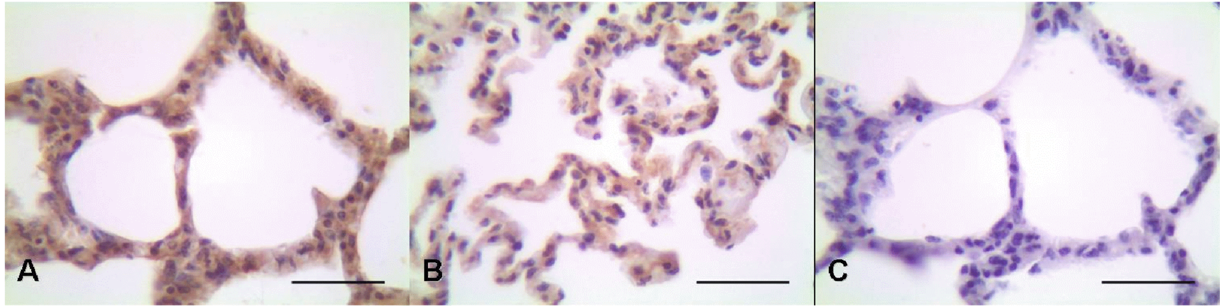

Figure 2-5 Substance P-like immunoreactivity in the alveolar walls of pneumonic and normal lung.

A. Moderate to strong substance P-like immunoreactivity in the alveolar walls of pneumonic bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 50 µm. B. Moderate substance P-like immunoreactivity in the alveolar walls of normal bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 50 µm. C. Little to no immunoreactivity in the alveolar walls of pneumonic lung stained with a mouse IgG1 isotype antibody. Bar = 50 µm.

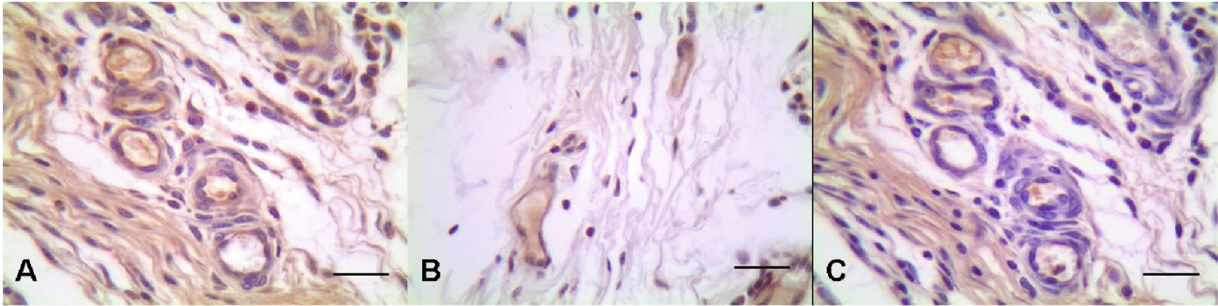

Figure 2-6 Substance P-like immunoreactivity in the endothelial cells of venules and lymphatic vessels of pneumonic and normal lung.

A. Moderate substance P-like immunoreactivity in the endothelial cells of venules of pneumonic bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 30 µm. B. Moderate substance P-like immunoreactivity in the endothelial cells of lymphatic vessels of normal bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 30 µm. C. Little to no immunoreactivity in the endothelial cell of venules of pneumonic lung stained with a mouse IgG1 isotype antibody. Bar = 30 µm.

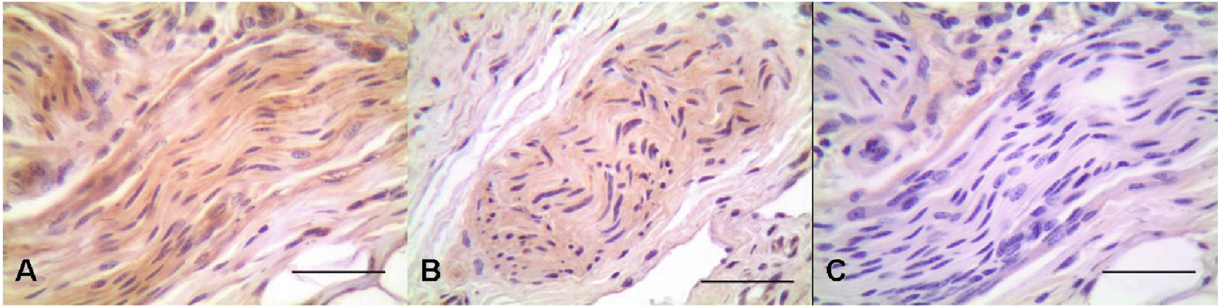

Figure 2-7 Substance P-like immunoreactivity in the peribronchial nerves of pneumonic and normal lung.

A. Moderate substance P-like immunoreactivity in a peribronchial nerve of pneumonic bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 50 µm. B. Slight to moderate substance P-like immunoreactivity in a peribronchial nerve of normal bovine lung stained with a monoclonal mouse anti-human substance P antibody. Bar = 50 µm. C. Little to no immunoreactivity in a peribronchial nerve pneumonic lung stained with a mouse IgG1 isotype antibody. Bar = 50 µm.

Table 2-1 Mean ± standard deviation substance P – like immunoreactivity of structures of pneumonic and normal bovine lung

	Mean Gray Value	
	Pneumonic lung (29 calves)	**Normal Lung (54 Calves)**
Structure		
Macrophages	-0.010156 ± 0.007582*	0.002100 ± 0.011565*
Airway epithelium	-0.000057 ± 0.000047	-0.000014 ± 0.000067
Alveolar wall	-0.000007 ± 0.000151	-0.000071 ± 0.00030
Endothelium	0.000680 ± 0.003371	0.001547 ±0.00433
Peribronchial nerve	0.000608 ± 0.001196	0.000350 ±0.000860

*p-value <0.05

Table 2-2 Incidence of substance P – like immunoreactivity per structure in pneumonic and normal bovine lung

	Pneumonic lung	**Normal lung**
Structure		
Macrophages	26/29 (89.7%)	26/54 (48.1%)
Airway epithelium	26/29 (89.7%)	33/54 (61.1%)
Alveolar wall	17/29 (58.6%)	29/54 (53.7%)
Endothelium	15/29 (51.7%)	22/54 (40.7%)
Peribronchial nerve	4/18 (22.2%)	12/28 (42.9%)

CHAPTER 3 - Effect of substance P on the permeability of bovine endothelial cell monolayers

Introduction

Bovine respiratory disease (BRD) is one of the most common and costly diseases of feedlot calves in the United States and worldwide (53, 110, 121, 143, 146). A major cause of severe pneumonia, deaths and economic loss associated with BRD is bovine pneumonic pasteurellosis (BPP), caused by *Mannheimia haemolytica* serotype 1 (18, 32, 38, 57, 143). BPP often occurs in young calves experiencing one or more of several environmental stressors (25, 32, 143). These environmental stressors are believed to cause a switch in the normal nasopharyngeal flora from *M. haemolytica* serotype 2 to the more pathogenic *M. haemolytica* serotype 1 (2, 32, 143, 148, 200). *M. haemolytica* serotype 1 proliferate in the nasopharynx and are aerosolized to the lower respiratory tract in numbers that overwhelm pulmonary defenses.

Proliferation of *M. haemolytica* serotype 1 in the lungs results in release of *M. haemolytica* serotype 1 virulence factors, two of which are lipopolysaccharide (LPS) and leukotoxin (LKT) (2, 32, 38, 143, 148, 200). *M. haemolytica* serotype 1 LPS increases vascular permeability and cause injury to and death of bovine endothelial cells (22, 32, 114, 143). Alveolar macrophages exposed to *M. haemolytica* LPS and LKT release inflammatory cytokines, such as tumor necrosis factor – α (TNF) and interleukin – 1 (IL-1), and chemokines such as interleukin – 8 (IL-8) (2, 32, 76, 111, 118, 198). Both TNF and IL-1 can increase the permeability of bovine endothelial monolayers (23, 59, 112, 152). Interleukin – 8 primarily attracts neutrophils, which can result in endothelial damage when neutrophils degranulate. The interaction between LPS and LKT with host endothelial cells and inflammatory cells is believed to result in the exudation of fluid and fibrin into alveolar lumens that is characteristic of the initial stages of BPP (2, 18, 32, 57, 143). This serofibrinous effusion may be due to the direct effects of *M. haemolytica* serotype 1 LPS or LKT on endothelial cells or it may occur indirectly through inflammatory cytokine release from alveolar macrophages.

In peracute stages of BPP there are relatively few *M. haemolytica* serotype 1 bacteria present in the lung relative to the severity of the vascular leakage. The overall cellular inflammatory response at this stage of pneumonia is also minimal. Therefore, the pathology

associated with peracute BPP may not entirely be the result of *M. haemolytica* serotype 1 and its virulence factors. Another mediator of vascular permeability in acute inflammation that is capable of inducing serofibrinous effusion is substance P (SP). Substance P is an 11-amino-acid neuropeptide found mainly in sensory nerves that can be synthesized by inflammatory cells (128). SP is a neurotransmitter of pain that can also be released into tissue to cause neurogenic inflammation, which is characterized by serofibrinous effusion (29, 30, 46, 56, 75, 94, 95, 98, 128, 148, 156). Substance P has been implicated in the pathogenesis of various respiratory conditions of people and rodents, including asthma, respiratory viral infection, allergic rhinitis, airway hyperresponsiveness, nonproductive cough, sarcoidosis, idiopathic pulmonary fibrosis, and acute respiratory distress syndrome (11, 87, 128).

In this study we investigated the role of SP in inducing increased permeability in bovine endothelial cells. We evaluated the effect of SP alone, as well as in combination with other vasoactive cytokines on the permeability of bovine endothelial cell monolayers *in vitro*.

Materials and methods

Cells and reagents

A pulmonary macrovascular cell line (bovine pulmonary arterial endothelial cells; CPAE cells), and a microvascular cell line (bovine adrenal gland capillary endothelial cells; EJG cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The endothelial cells were cultured according to ATCC instructions using Eagle's Minimum Essential Medium (ATCC, Manassas, VA) with 20% fetal bovine serum (Hyclone, Logan, UT) for the CPAE cells and Eagle's MEM with 10% fetal bovine serum for the EJG cells. Cells were used for experimentation between the 5th and 15th passage (CPAE cells), and the 8th and 14th passages (EJG cells). Cell lines were tested every 1-2 months for maintenance of endothelial cell properties based on their cobblestone morphology, formation of capillary tubes on the basement membrane material Matrigel™ (BD Biosciences, Bedford, MA), expression of CD31 (AbD Serotec anti-ovine CD31 antibody, Raleigh, NC) after culture on Matrigel™ (BD Biosciences, San Jose, CA), and the presence of the coagulant protein Factor VIII in the supernatant detected by a colorimetric assay (Chromogenix, Lexington, MA).

Bovine recombinant TNF-α (TNF) was purchased from Endogen (Rockford, IL). *Escherichia coli* O111:B4 lipopolysaccharide (LPS), substance P (SP), and histamine were

purchased from Sigma-Aldrich (St. Louis, MO). The concentrations of the reagents used were 10 ng/ml TNF, 100 ng/ml *E. coli* O111:B4 LPS, 1 nM SP, and 10 micromolar histamine. These same concentrations were used for the combinations of SP with TNF, SP with LPS and SP with histamine. The TNF, LPS, and histamine were suspended in Eagle's MEM with 10% FBS, aliquoted and frozen at -80° C (TNF) and -20° C (LPS and histamine). The SP was suspended in 0.1 M acetic acid with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), aliquoted, and frozen at -20° C. The SP aliquots were diluted in Eagle's MEM with 10% FBS and used fresh for each experiment.

Monolayer permeability

Endothelial cells were grown to confluent monolayers (3-4 days for CPAE cells and 3-7 days for EJG cells) in 12-well Transwell® culture system plates (Corning Life Sciences, Corning, NY) at 37° C in CO_2. The culture medium in the upper and lower chambers was replaced by fresh Eagle's MEM with 10% FBS 24 hours prior to each experiment. An experiment consisted of adding 250-µl of each of the 7 treatments (TNF, LPS, histamine, SP, SP + TNF, SP + LPS, or SP + histamine), a cell control (Eagle's MEM with 10% FBS), and a diffusion control (Eagle's MEM with 10% FBS in a well without cells) to the upper chamber of wells for 1 hour at 37° C in 5% CO_2. Then, 250-µl of Eagle's MEM with 4% bovine serum albumin containing 1 mg/ml of Evans blue dye (Sigma-Aldrich, St. Louis, MO) was added to the upper chamber of these same wells for 1 hour. Optical densities (OD) of triplicate samples from the lower chamber of each well were then determined at 630 nm absorption, averaged and compared against a standard curve to estimate albumin concentration. The albumin diffusion/cm^2/hour (diffusion) for each well was calculated by the formula:

$$\text{Diffusion} = [\text{albumin}]/\text{growth area } (1.12 \text{ cm}^2)/\ 1 \text{ hour.}$$

The percent increase in diffusion induced by the treatments (% diffusion) was calculated by the formula:

$$\%\ \text{diffusion} = \{(\text{diffusion}_{\text{treatment}} - \text{diffusion}_{\text{cell control}})/\ \text{diffusion}_{\text{diffusion control}}\}\ \text{X}\ 100$$

where $\text{diffusion}_{\text{cell control}}$ represented zero permeability and $\text{diffusion}_{\text{diffusion control}}$ represented 100% permeability.

Experiments were repeated until significance for the positive controls was attained (significance of 0.05 and a power of 0.90).

Scanning electron microscopy (SEM)

Three- hundred thousand CPAE and EJG cells were grown to confluence on separate 22 mm diameter collagen coated glass coverslips (BD Biosciences, San Jose, CA). The monolayers were incubated as before with either TNF, LPS, histamine, SP, SP + TNF, SP + LPS, or SP + histamine, or Eagle's MEM with 10% FBS alone for 2 hours, washed, and then fixed in 2.5% gluteradehyde/2% paraformaldehyde. Monolayers were then washed, fixed with osmium, dehydrated, mounted on silver stubs, and sputter coated with gold. Samples were examined using a Hitachi H300 scanning electron microscope. The samples were examined for the presence or absence of endothelial cell shape change and the difference in intercellular gap size and morphology between treatment and control samples.

Cytotoxicity

Endothelial cell monolayers in 24-well plates were incubated with treatments as described above, or with Eagle's MEM with 10% FBS (control) for 2 hours in a humidified incubator at 37° C with 5% CO_2. Supernatants from each well were collected and evaluated for lactate dehydrogenase (LDH) enzyme activity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) following the manufacturers instructions. Briefly, 50-µl of supernatant from 7 treatment wells, 50-µl of supernatant from completely lysed cells, and 50-µl of supernatant from a cell control were placed in triplicate onto 96-well plates. Background controls on the plate included 50-µl of each reagent, 50-µl of Eagle's MEM plus 10% FBS, and 50-µl of Eagle's MEM plus 10% FBS containing cell lysing reagent. Fifty-µl of the kit's reconstituted Substrate Mix was added to each well and the plate was covered, protected from light, and incubated at room temperature for 30 minutes. After incubation, 50-µl of stop solution was added to each well and OD at 490 nm was determined. The mean OD of each treatment triplicate, control triplicate, and background OD for each reagent and Eagle's MEM plus 10% FBS was calculated. The OD for the treatment and control wells was calculated by subtracting the background reagent and Eagle's MEM + 10% FBS OD from the treatment and

control well OD. The OD of the treatments and control were compared to the OD of the completely lysed cells to calculate the percentage total LDH release from the treatment and control cells to estimate endothelial cell membrane damage. The percent of the total LDH release was calculated by the formula:

$$\% \text{ total LDH release} = (OD_{\text{Treatment or Spontaneous cell release}} / OD_{\text{Lysed cells}}) \times 100.$$

Statistics

Statistical analysis was performed using a one-way ANOVA followed by a Dunett's multiple comparison test. Treatments were determined to be significant with a p-value < 0.05. Statistical analysis was performed using SAS 9.1.

Results

Monolayer permeability

There was an increase in endothelial cell monolayer permeability due to the addition of SP alone or in combination with other mediators. Figure 3-1 shows the mean percentage increases in BSAE diffusion across CPAE cell monolayers for SP, TNF, LPS, histamine, and the combination treatments. The combination treatment of SP + LPS induced the greatest increase in CPAE cell monolayer permeability (mean 22.03% above control). Increases in CPAE cell monolayer permeability in response to SP, SP + histamine, TNF and LPS were similar (means 12.34%, 13.57%, 12.67% and 13.13%, respectively above control). Histamine decreased the monolayer permeability of this cell line (mean -6.51% less than control). In addition, the combination treatment of SP + TNF had a very small increase in monolayer permeability (mean 2.99% above control) that was less than either TNF or SP alone.

The mean percentage increases in BSAE diffusion across EJG cell monolayers for SP, TNF, LPS, histamine, and the combination treatments are presented in Figure 3-2. The combination treatment of SP + histamine had the greatest percentage increase of BSAE diffusion compared to the cell control (mean 16.69% above control). SP and histamine alone increased the percentage of BSAE diffusion by approximately half this amount (mean 8.27% and 8.10%, respectively above control). The combinations of SP + LPS and SP + TNF, and LPS and TNF

alone induced slight increases in the percentage of BSAE diffusion across the endothelial cell monolayer.

Scanning electron microscopy (SEM)

The CPAE cell monolayers exposed to LPS, TNF, SP, SP + LPS, and SP + histamine had endothelial shape change. Additionally, there was formation of intercellular gaps in CPAE cell monolayers (Figure 3-3). Substance P, histamine, and SP + histamine caused endothelial shape change and the formation of intercellular gaps in EJG cell monolayers (Figure 3-4). Results are shown only for the treatments that increased monolayer permeability and caused intercellular gaps.

Cytotoxicity

The percentage of total LDH release from CPAE endothelial cells induced by the treatments is shown in Figure 3-5. LPS was the only reagent that significantly increased the percentage of total LDH release (mean 14.30%) when compared to spontaneous LDH release from control cells (mean 9.55%). TNF alone (mean 10.43%) and SP + LPS (mean 11.24%) slightly increased LDH release compared to controls. SP (mean 8.06%), histamine (mean 6.52%), SP + TNF (mean 6.95%), and SP + histamine (mean 6.96%) induced less LDH release compared to controls. The percentage of total LDH release from EJG endothelial cells spontaneously released from a cell control or that was induced by the reagents is shown in Figure 3-6. For EJG cells, LPS significantly increased the percentage total LDH release (mean 21.12%) compared to spontaneous LDH release from controls (mean 9.74%). In addition, SP + LPS (mean 17.98%) and SP + TNF (mean 18.00%) induced increased LDH release compared to spontaneous LDH release. Histamine (mean 2.91%), SP (mean 1.01%), TNF (mean 4.31%), and SP + histamine (2.71%) had less LDH release compared to LDH release from control cells.

Discussion

These experiments showed that SP enhanced bovine endothelial cell monolayer permeability either alone or in combination with other permeability increasing cytokines in two bovine endothelial cell lines. The increase in endothelial cell monolayer permeability was due to endothelial cell shape change and the formation of intercellular gaps, and was not caused by increased endothelial cell cytotoxicity. These results are similar to results in other species in

which SP-associated also increased vascular permeability. In rats and guinea pigs SP administration resulted in edema in multiple organs, including the skin and airways (46, 75, 97, 156). In humans and mice, SP induced edema in the skin (29, 30, 46, 56).

Increased vascular permeability induced by SP can be a result of the direct action of SP on endothelial cells through NK-1 or indirectly through mast cell degranulation, induction of arachidonic acid metabolite synthesis by monocytes/macrophages, or by secretion of inflammatory cytokines by monocytes/macrophages (11, 20, 11, 128). The vascular permeability induced by SP is often enhanced by the presence of other vascular permeability increasing molecules such as histamine and LPS (86, 97). In guinea pig airways, LPS enhanced neurogenic plasma exudation (97). Histamine and SP also caused a synergistic increase in capillary permeability measured by plasma exudation in rat skin (86). In this study, histamine and SP in combination doubled the percentage of BSAE diffusion compared to histamine or SP treatments alone. In contrast, the SP and TNF combination reduced BSAE diffusion to less than the diffusion induced by either agent alone. This result is similar to that reported in a study of vasodilation in rat skin where TNF did not enhance the effects of SP and prevented the synergistic effects of SP and IL-1 (70). Decreased permeability of arterial endothelial cells exposed to histamine has also been previously observed (77, 176).

Agents that increase vascular permeability allow fluid to escape the vasculature between endothelial cells by inducing conformational changes in the actin cytoskeleton initiated by increased cytosolic Ca^{2+} concentrations (117, 179, 180, 186). Certain intracellular signaling molecules, such as cyclic adenosine monophosphate (cAMP), are associated with stabilization of the endothelial barrier resulting in a reversal of or decrease in vascular permeability (117, 186). In fact, decreased permeability of arterial endothelial cell monolayers treated with histamine are associated with increased intracellular cAMP concentrations (77, 176). Based on the morphologic changes observed in bovine endothelial monolayers in response to SP, it is possible that SP increased bovine endothelial cell monolayer permeability to BSAE by increasing cytosolic Ca^{2+} either alone or synergistically with LPS and histamine. The decrease in vascular permeability seen with the combination of SP+TNF versus either agent alone in CPAE cells could be due to decreased cytosolic Ca^{2+}, increased cytosolic cAMP, or both. Another possibility is that the two agents chemically inhibit one another in the supernatant.

Psychological or local pulmonary stressors are important components of BPP and other respiratory conditions (11, 14, 25, 32, 143, 147, 199). These stressors may increase the amounts of SP in the lung, which can enhance pulmonary inflammation. In mice, stress increased the amount of SP in sensory neurons innervating the lung as well as increasing the severity of allergen-induced airway inflammation (83). Stress-exacerbated allergic airway inflammation in mice was mediated by NK-1 (82). Respiratory syncytial virus exposure in rats and mice results in increased expression of NK-1 as well as increased levels of SP compared to levels in non-infected lungs (68, 92). In guinea pigs, SP was significantly increased in plasma and bronchoalveolar lavage (BAL) fluids following electric shock stress (181). These changes in pulmonary SP and NK-1 may be due to activation of intrapulmonary afferent nerves containing SP by inhaled irritants or other noxious stimuli, production by inflammatory cells, or both.

There have been several studies on the changes in pulmonary SP associated with acute lung inflammation in ruminants. Increased levels of SP and NK-1 were present in experimental *M. haemolytica* pneumonia in sheep (63, 145). We have extended these studies to cattle and found that SP-like immunoreactivity is more intense in *M. haemolytica*-infected pneumonic lung compared to normal bovine lung (see chapter 2). In pneumonic lung this SP-reactivity was localized to airway epithelium, and was also present in macrophages, alveolar septa, and occasionally lymphatic and venular endothelial cells.

If SP has the same vascular permeability effect *in vivo* as it does *in vitro*, then SP could play a role in initiating or enhancing the serofibrinous effusion present in the initial stages of BPP. This effusion into pulmonary alveoli could lead to decreased bacterial clearance, increased *M. haemolytica* serotype 1 proliferation, and the severe inflammatory response typical of advanced cases of BPP.

Figures and Tables

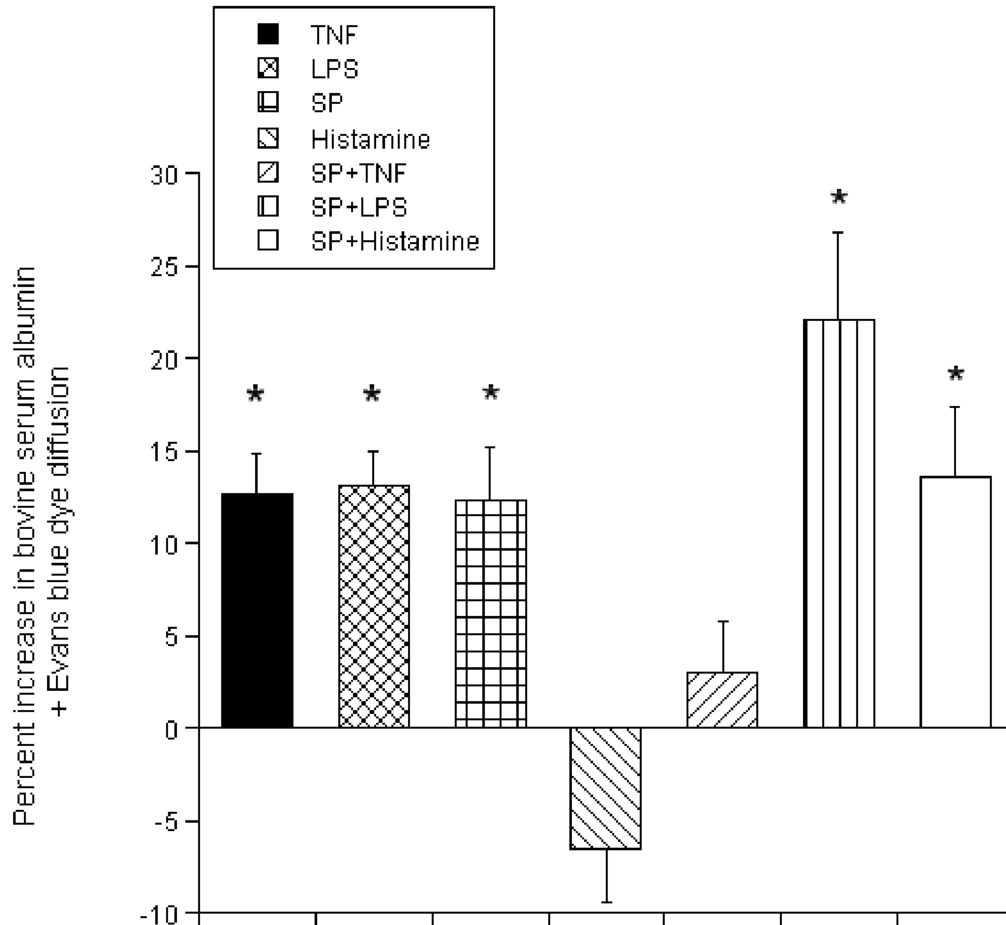

Figure 3-1 The percent change in albumin diffusion through CPAE endothelial cell monolayers. The monolayers were treated with 10 ng/ml TNF, 100 ng/ml LPS, 1 nM SP, 10 μM histamine and combinations of SP+TNF, SP+LPS, and SP+histamine. Data are reported as mean +/- standard error of the mean of the percent change in albumin concentration in the lower chamber of a Transwell® insert of treated monolayers versus a cell control monolayer. The cell control monolayer was set as zero percent. Random diffusion in a blank well was set as one hundred percent. n = 13. * p-value < 0.05. TNF = bovine recombinant Tumor Necrosis Factor – α. LPS = *Escherichia coli* O111:B4 lipopolysaccharide. SP = substance P.

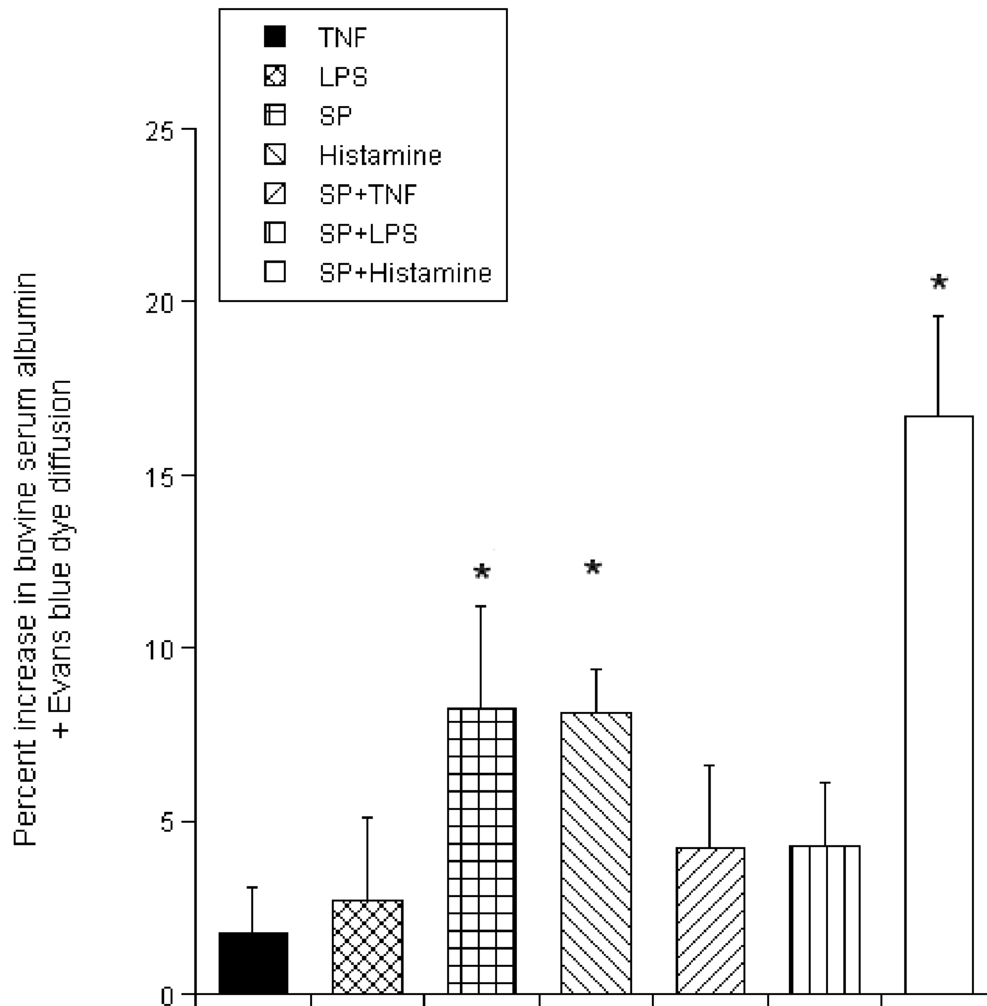

Figure 3-2 The percent change in albumin diffusion through EJG endothelial cell monolayers. The monolayers were treated with 10 ng/ml TNF, 100 ng/ml LPS, 1 nM SP, 10 µM histamine and combinations of SP+TNF, SP+LPS, and SP+histamine. Data are reported as mean +/- standard error of the mean of the percent change in albumin concentration in the lower chamber of a Transwell® insert of treated monolayers versus a cell control monolayer. The cell control monolayer was set as zero percent. Random diffusion in a blank well was set as one hundred percent. n = 15. * p-value < 0.05. TNF = bovine recombinant Tumor Necrosis Factor – α. LPS = *Escherichia coli* O111:B4 lipopolysaccharide. SP = substance P.

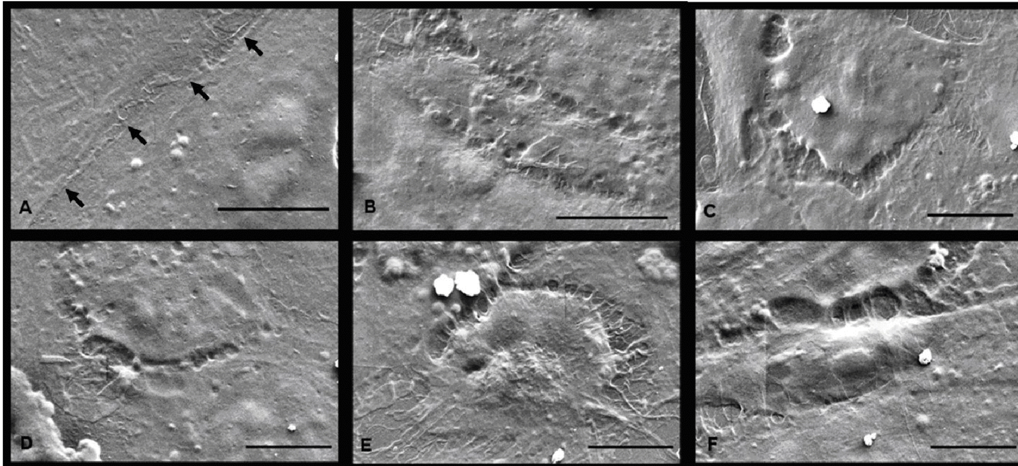

Figure 3-3 Scanning electron photomicrographs of CPAE cell monolayers.

A. Intercellular junction between CPAE cells in the control sample. Arrows indicate the intercellular junction. Bar = 10μ. B. Intercellular junction and gaps between CPAE cells after treatment with TNF. Bar = 10μ. C. Intercellular junction and gaps between CPAE cells after treatment with LPS. Bar = 10μ. D. Intercellular junction and gaps between CPAE cells after treatment with SP. Bar = 10μ. E. Intercellular junction and gaps between CPAE cells after treatment with SP + LPS. Bar = 10μ. F. Intercellular junction and gaps between CPAE cells after treatment with SP + histamine. Bar = 10μ. TNF = bovine recombinant Tumor Necrosis Factor – α. LPS = *Escherichia coli* O111:B4 lipopolysaccharide. SP = substance P.

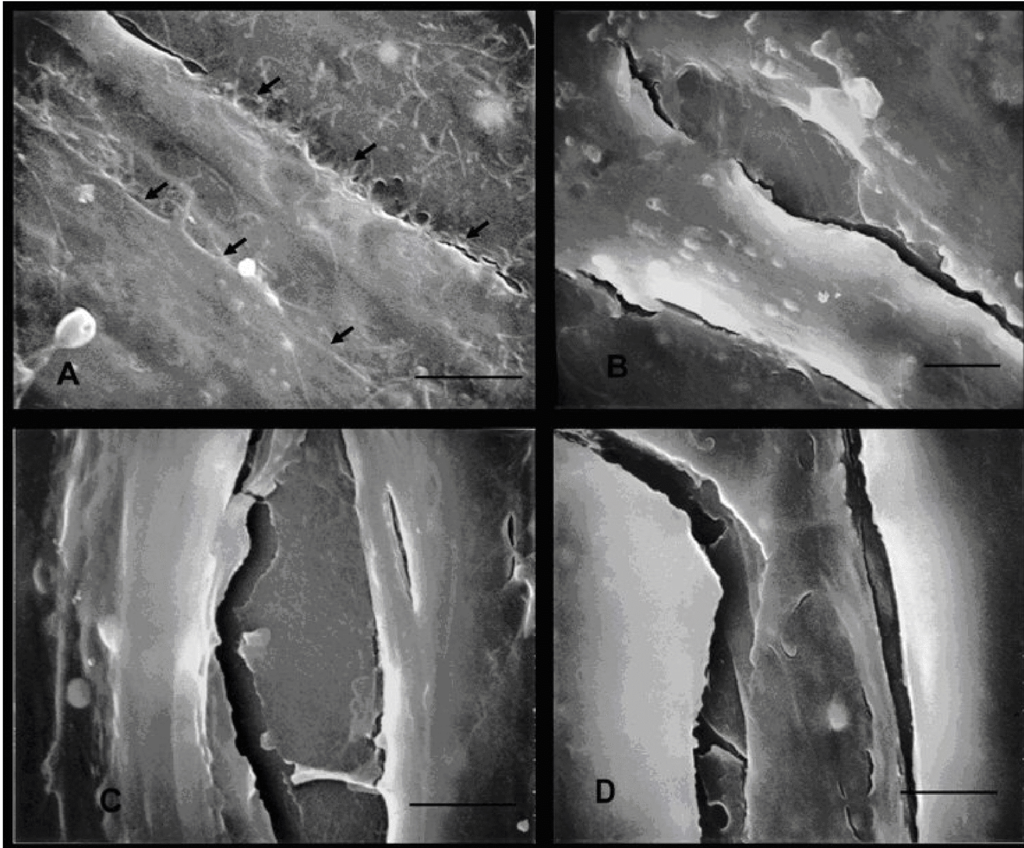

Figure 3-4 Scanning electron photomicrographs of EJG cell monolayers.

A. Intercellular junction between EJG cells in the control sample. Arrows indicate the intercellular junction. Bar = 10μ. B. Intercellular junction and gaps between EJG cells after treatment with SP. Bar = 10μ. C. Intercellular junction and gaps between EJG cells after treatment with histamine. Bar = 10μ. D. Intercellular junction and gaps between EJG cells after treatment with SP + histamine. Bar = 10μ. SP = substance P.

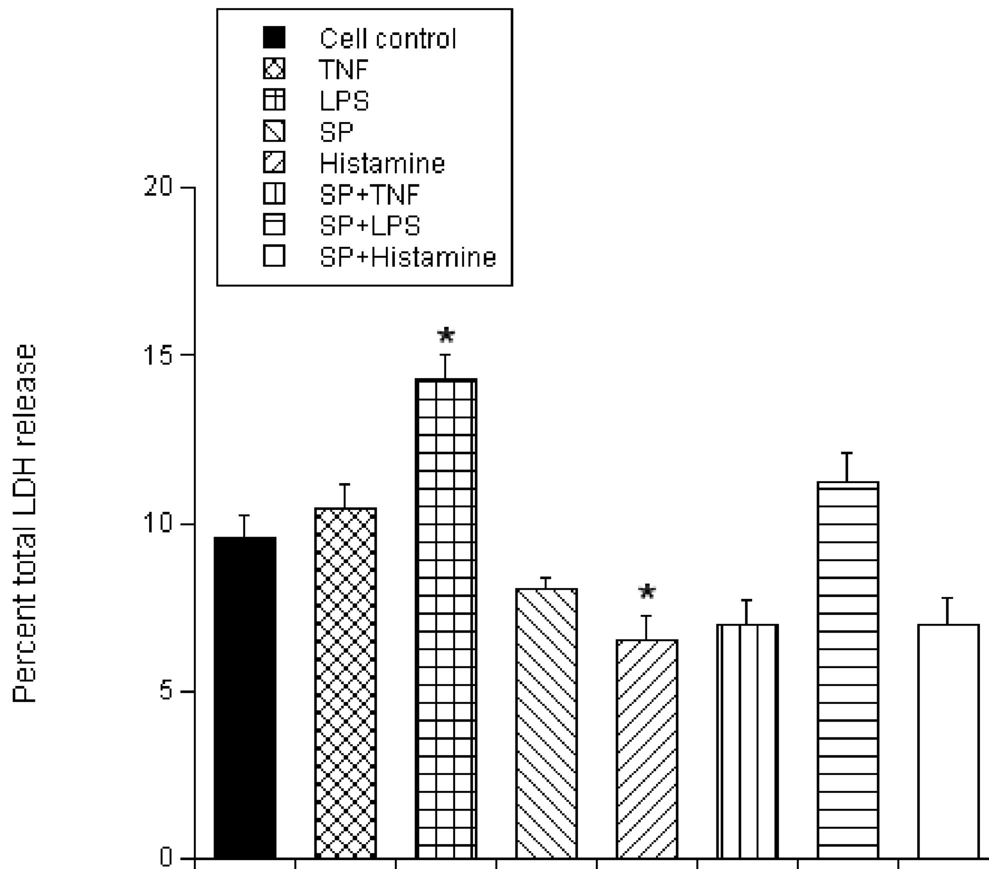

Figure 3-5 The percent of total LDH release from CPAE endothelial cell monolayers.
The monolayers were treated with 10 ng/ml TNF, 100 ng/ml LPS, 1 nM SP, 10 μM histamine and combinations of SP+TNF, SP+LPS, and SP+histamine. Data are reported as mean +/- standard error of the mean of the percent total LDH release. LDH release from completely lysed cells was set as total LDH released. Treatments were compared to LDH spontaneously released from a cell control. n = 4. * p-value < 0.05. TNF = bovine recombinant Tumor Necrosis Factor – α. LPS = *Escherichia coli* O111:B4 lipopolysaccharide. SP = substance P.

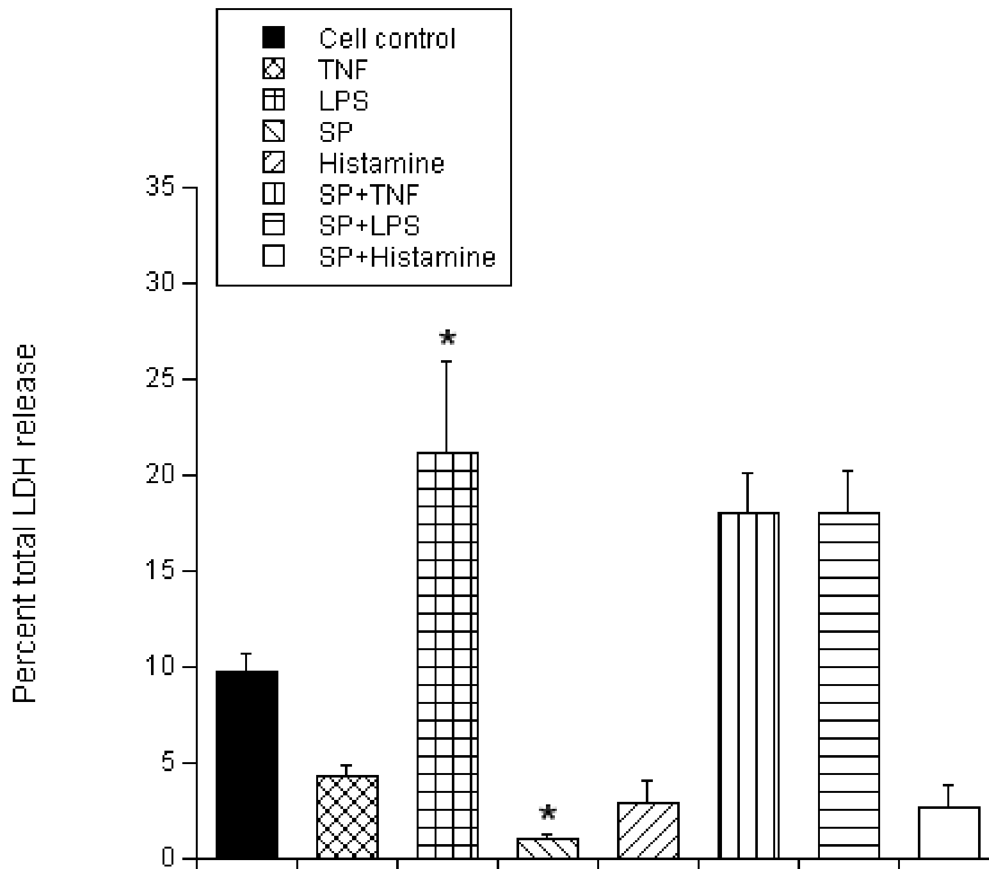

Figure 3-6 The percent of total LDH release from EJG endothelial cell monolayers.
The monolayers were treated with 10 ng/ml TNF, 100 ng/ml LPS, 1 nM SP, 10 µM histamine and combinations of SP+TNF, SP+LPS, and SP+histamine. Data are reported as mean +/- standard error of the mean of the percent total LDH release. LDH release from completely lysed cells was set as total LDH released. Treatments were compared to LDH spontaneously released from a cell control. n = 4. * p-value < 0.05. TNF = bovine recombinant Tumor Necrosis Factor – α. LPS = *Escherichia coli* O111:B4 lipopolysaccharide. SP = substance P.

CHAPTER 4 - The role of substance P on surface expression of CD18 on bovine neutrophils and *Mannheimia haemolytica* leukocytotoxicity

Introduction

Bovine respiratory disease (BRD) is one of the most common and costly diseases of feedlot calves in the United States and worldwide (2, 18, 32, 57, 110, 143, 147). A major cause of severe pneumonia, deaths and economic loss associated with BRD is bovine pneumonic pasteurellosis (BPP), caused by *Mannheimia haemolytica* serotype 1 (2, 18, 32, 57, 143, 147). Proliferation of *M. haemolytica* in the lungs results in release of *M. haemolytica* virulence factors, two major ones of which are lipopolysaccharide (LPS) and leukotoxin (LKT) (2, 32, 143, 147). *M. haemolytica* LKT binds to its receptor the β subunit (CD18) of the β_2 integrins on ruminant leukocytes to cause specific leukocyte oncosis and apoptosis (4, 6, 35, 41, 45, 49, 60, 81, 109, 173, 200). These changes are due to the creation of a channel within the leukocyte membrane leading to an efflux of potassium and influx of sodium and calcium that causes cell swelling and osmotic lysis, similar to the mechanism of complement-mediated cell lysis (177, 200). *M. haemolytica* LKT and LPS also interact with intravascular and alveolar macrophages to cause the release of proinflammatory molecules, such as tumor necrosis factor – α (TNF), interleukin – 1 (IL-1), interleukin – 8 (IL-8), and leukotriene B_4 (LTB_4) (2, 147, 200). The proinflammatory molecules as well as *M. haemolytica* LPS and LKT can prime neutrophils and recruit them into the lung, which involves increased surface expression of CD18 on the neutrophil membranes (64, 99, 105, 106). This often results in increased leukocytoxicity caused by *M. haemolytica* LKT.

Another mediator of acute inflammation that can influence expression of CD18 is substance P (SP). Substance P is an 11-amino-acid neuropeptide found mainly in sensory nerves that can be synthesized by inflammatory cells (128). SP is a neurotransmitter of pain that also can be released into the tissue to enhance the inflammatory response (128). It has been shown to prime neutrophils for the respiratory burst, induce neutrophil chemotaxis, cause neutrophil degranulation, increase neutrophil migration through a fibroblast barrier, and increase neutrophil

adherence to endothelial cells and bovine bronchial epithelial cells (37, 44, 47, 65, 85, 113, 128, 138, 140, 159). The adhesive properties with endothelium and bronchial epithelium are mediated by β_2 integrins. In addition, the combination of SP and LPS increased neutrophil adhesion to alveolar epithelial cells mediated by the β_2 integrins (98). It is unclear whether substance P alone can increase β_2 integrins, particularly CD11b/CD18, on neutrophils (98, 162). However, it has been shown that SP in combination with LPS can increase expression of CD11b/CD18 on human neutrophils (98, 162). This is particularly evident after inhibition of CD10, the peptidase that degrades SP.

In this study we evaluated the effect of SP alone and in combination with other proinflammatory agents on the expression of CD18 on the surface of bovine leukocytes and the subsequent effect of *M. haemolytica* LKT on the stimulated leukocytes.

Materials and methods

Isolation of peripheral blood leukocytes

Approximately 25-ml of blood was collected via jugular venipuncture in acid citrate dextrose (ACD) blood collection tubes (BD, Franklin Lakes, NJ) from clinically healthy feedlot calves ranging from weaning to slaughter weight. Blood was centrifuged at 300 g at 4°C for 30 minutes, plasma was aspirated, and erythrocytes were lysed with sterile double distilled water and returned to isotonicity with 2x PBS containing 1% bovine serum albumin (BSA) similar to a previously described procedure (31). After centrifugation and decanting of the supernatant, the lysis procedure was repeated. The isolated peripheral blood leukocytes were washed 3 times with PBS containing 1% BSA. The leukocytes were suspended in Eagle's minimum essential medium (ATCC, Manassas, VA) containing 10% fetal bovine serum (Hyclone, Logan, UT).

Flow cytometry

Five- hundred thousand leukocytes in 100-µl were placed into 14 wells of a V-bottom 96 well plate. The leukocytes were treated with either 10 ng/ml bovine recombinant tumor necrosis factor – α (TNF) (Endogen, Rockford, IL), 100 ng/ml *Escherichia coli* O111:B4 lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO), SP (Sigma-Aldrich, St. Louis, MO) or SP in combination with TNF and LPS. Leukocytes were incubated for 1 hour at 37°C in 5% CO_2. The leukocytes were washed 3 times with PBS containing 2% horse serum and 0.1%

sodium azide. The leukocytes were then incubated with 50-µl of a 1:50 dilution of a monoclonal mouse anti-bovine CD18 primary antibody (Abcam, Cambridge, MA) on ice for 45 minutes. The leukocytes were washed 3 times with the previous buffer then incubated with 50-µl of a 1:50 dilution of a goat anti-mouse FITC- labeled secondary antibody (AbD Serotec, Raleigh, NC) on ice in the dark for 45 minutes. Leukocytes were washed 2 times with PBS containing 0.1% sodium azide, suspended in 2% formaldehyde, and stored overnight at 4°C. The leukocytes were analyzed for 10,000 events with a Becton Dickinson FACSCalibur (BD Biosciences, etc). Control samples included using either a mouse IgG1 isotype antibody (GeneTex, Irvine, CA) as the primary antibody or samples incubated without exposure to either a primary or isotype antibody. The region containing neutrophils in a control leukocyte sample was gated based on cell size (forward scatter) and cell granularity (side scatter) using the dot plots of the leukocytes. The mean fluorescent intensity (MFI) of the gated neutrophils for each treatment was determined using histograms. The percentage increase in MFI for each treatment compared to basal CD18 expression on neutrophils was calculated by

$$MFI_{treatment}/MFI_{control} \text{ X } 100$$

where basal MFI of CD18 expression on neutrophils was set as 100%.

Preparation of Mannheimia haemolytica leukotoxin

Mannheimia haemolytica serotype 1 was cultured on blood agar plates (Remel, Lenexa, KS) and incubated overnight at 37°C in 5% CO_2**.** Two- hundred and fifty- ml of brain heart infusion (BHI) broth (BD Biosciences, San Jose, CA) was inoculated with *M. haemolytica* and incubated at 37°C with 100 oscillations/minute for 5 hours. The BHI broth was centrifuged and the supernatant was decanted and mixed with ammonium sulfate ($AmSO_4$; Sigma Aldrich, St. Louis, MO; 313 grams/liter). The supernatant with $AmSO_4$ was stirred overnight at 4°C, centrifuged, and the pellet was suspended in Eagle's MEM with 10% FBS (M. *haemolytica* leukotoxin (LKT) preparation).

Leukocytotoxicity Assay

Isolated leukocytes were suspended in 5 ml of Eagle's MEM containing 10% FBS and 100-µl was placed in the wells of a V-bottom 96 well plate. The plate was centrifuged and the media was replaced with either 10 ng/ml bovine recombinant tumor necrosis factor – α (TNF)

(Endogen, Rockford, IL), 100 ng/ml O111:B4 *Escherichia coli* lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO), SP (Sigma-Aldrich, St. Louis, MO) or SP in combination with TNF and LPS. The leukocytes were incubated for 1 hour at 37°C in 5% CO_2 followed by the addition 100-µl of a 1:10 dilution of LKT incubated at 37°C in 5% CO_2 for 45 minutes. After centrifugation, 50-µl of the supernatants from each well were collected and evaluated for lactate dehydrogenase (LDH) enzyme activity using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) following the manufacturers instructions. Briefly, 50-µl of supernatant from 5 treatment wells, 50-µl of supernatant from a LKT alone well, 50-µl of supernatant from a heat-inactivated LKT well, 50-µl of supernatant from completely lysed cells, and 50-µl of supernatant from a cell control were placed in triplicate onto 96-well plates. Background controls on the plate included 50-µl of each reagent, 50-µl of Eagle's MEM containing 10% FBS, and 50-µl of Eagle's MEM containing 10% FBS and cell lysing reagent. Fifty-µl of the kit's reconstituted Substrate Mix was added to each well and the plate was covered, protected from light, and incubated at room temperature for 30 minutes. After incubation, 50-µl of stop solution was added to each well and the optical density (OD) at 490 nm was determined. The mean of the OD of each treatment triplicate, control triplicate, and background OD of each reagent and Eagle's MEM containing 10% FBS was calculated. The OD for the treatment and control wells was calculated by subtracting the background reagent and Eagle's MEM containing 10% FBS OD from the treatment and control well OD. The ODs of the treatments were compared to the ODs of the LKT to calculate a ratio of increased LKT-induced cytotoxicity from the treatment leukocytes and the leukocytes exposed to LKT alone. The ratio of increased LKT cytotoxicity was calculated by the formula:

$$\text{Optical density}_{\text{treatments}} / \text{Optical density}_{\text{leukotoxin}}$$

where optical $\text{density}_{\text{leukotoxin}}$ was set as the baseline with a ratio of 1.

Statistics

Statistical analysis was performed using a one-way ANOVA followed by a Dunett's multiple comparison test. Treatments were determined to be significant with a p-value < 0.05. Analysis was performed using SAS 9.1.

Results

Bovine neutrophil CD18 surface expression

The flow cytometry dot plot for bovine leukocytes and the area gated as bovine neutrophils is shown in Figure 4.1. Representative fluorescent intensity for surface CD18 expression on neutrophils after TNF, LPS, SP, SP + LPS, and SP + TNF treatments are shown in Figures 4.2-4.6. Figure 4.7 shows the mean neutrophil CD18 MFI for TNF, LPS, SP and the combinations of SP with TNF and LPS.

TNF increased the MFI the greatest over baseline (mean 114%; $p< 0.05$). The MFI for the combination of SP + TNF (mean 110%; $p< 0.05$) and for LPS alone (mean 108%; $p<0.05$) were also different than the background MFI. SP alone (mean 99%) and SP + LPS (mean 107%) did not increase MFI for CD18 surface expression on neutrophils.

Mannheimia haemolytica – induced leukocytotoxicity of bovine peripheral blood leukocytes

Figure 4.8 shows the mean increase in leukocytotoxicity of peripheral blood leukocyte in response to *M. haemolytica* LKT s following treatment with TNF, LPS, SP, SP + TNF, and SP + LPS. TNF was the only reagent that increased LKT-induced leukocytotoxicity (mean 1.37 times LKT alone). Neither SP nor the combinations of SP with TNF or LPS increased LKT-induced leukocytotoxicity.

Discussion

The study shows that SP alone does not increase bovine neutrophil CD18 surface expression and does not enhance *M. haemolytica* LKT-induced cytotoxicity of leukocytes. SP in combination with TNF, but not LPS did enhance neutrophil CD18 expression, but not *M. haemolytica* LKT-induced leukocytotoxicity. However, this increase was less than that with TNF alone.

Other studies on the ability of SP to directly increase expression of the β_2 integrins have yielded mixed results. In a previous report, SP alone did not increase the expression of the integrin CD11b/CD18 on human neutrophils, but SP plus LPS did increase the expression of this integrin on neutrophils (98). However, other authors have reported that SP alone increased the

expression of CD11b/CD18 on human neutrophils, particularly following inhibition of CD10 (the peptidase that degrades SP) (162).

Although SP alone did not increase CD18 expression on bovine neutrophils and subsequent *M. haemolytica* LKT leukocytotoxicity, this does not eliminate a role for SP as a participant with other cytokines in interacting with bovine neutrophils. The combination of SP with TNF and to a lesser extent LPS did result in increases in CD18 expression, but these were less than the increases with TNF or LPS alone. Cytokine interactions are complex often with confounding results when mixed experimentally. For example, SP treatment primed human neutrophils for superoxide anion production following formyl-methionyl-leucyl-phenylalanine (FMLP) exposure (160). However, superoxide anion production induced by TNF was strongly inhibited when the neutrophils were pretreated with SP (160). In the same study, human neutrophils pretreated with SP increased neutrophil interleukin – 8 production induced by both FMLP and TNF. Another study showed that neutrophils primed with granulocyte-macrophage colony stimulating factor (GM-CSF) plus TNF and granulocyte colony stimulating factor (G-CSF) plus TNF followed by FMLP application produced a greater respiratory burst in human neutrophils compared with any agent alone (90). However, priming human neutrophils with the combination of GM-CSF and G-CSF followed by FMLP application did not increase the respiratory burst above priming with GM-CSF alone (90).

SP might play a role in the innate immune response of the bovine lung to *M. haemolytica* in ways unrelated to enhancement of LKT leukocytotoxicity. There was increased SP in macrophages in pneumonic bovine lung versus normal bovine lung (see chapter 2). SP has been shown to increase the phagocytic ability of bovine alveolar macrophages as well as increase their TNF production (149). Substance P has also been shown to increase production of the inflammatory cytokines TNF, interleukin–6 and interleukin-8 by human bronchial epithelial cells (188). Also, mRNA for interleukins-1β, -3, -5, -6, TNF, and interferon-γ was increased in human nasal mucosa samples following exposure to SP (129). The inflammatory cytokines TNF, interleukin -1β, interleukin-2, and interleukin-6 can be produced by rat T-lymphocytes, macrophages, and neutrophils when exposed to SP (43). Pretreatment of mice with leukotriene and TNF antagonists prior to SP injection into the skin inhibited SP-induced neutrophil migration into the skin indicating that SP can cause local production or release of leukotrienes and TNF (155). Substance P alone or in combination with LPS increases neutrophil attachment

to endothelial cells, bovine bronchial epithelial cells, and alveolar epithelial cells (44, 47, 98). Substance P is also chemoattractant for neutrophils, enhances neutrophil migration through a fibroblast barrier, and primes neutrophils for the respiratory burst (37, 85, 113, 128, 138, 140, 159).

In this study we were unable to detect any direct relationship between SP, increased CD18 expression by neutrophils, or LKT-induced leukocytotoxicity. Any interaction between SP and bovine neutrophils during the onset and progression of BPP most likely involves the participation of other cytokines or proinflammatory molecules. The effects of these interactions may include attracting and priming neutrophils, inducing inflammatory cytokine production from alveolar macrophages and bronchial epithelial cells, and increasing the phagocytic capability of alveolar macrophages.

Figures and Tables

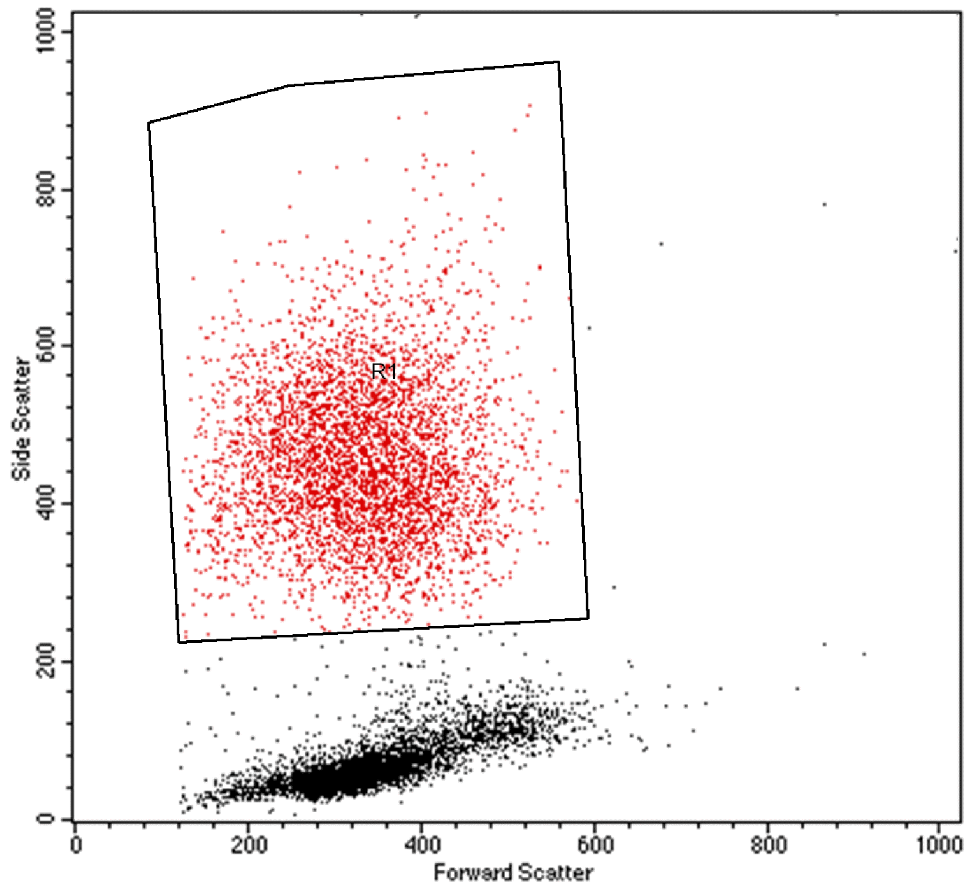

Figure 4-1 Bovine peripheral leukocytes dot plot with gated neutrophils.

The neutrophils in blood are highlighted in red in the flow cytometry dot plot. Analysis of 10,000 events.

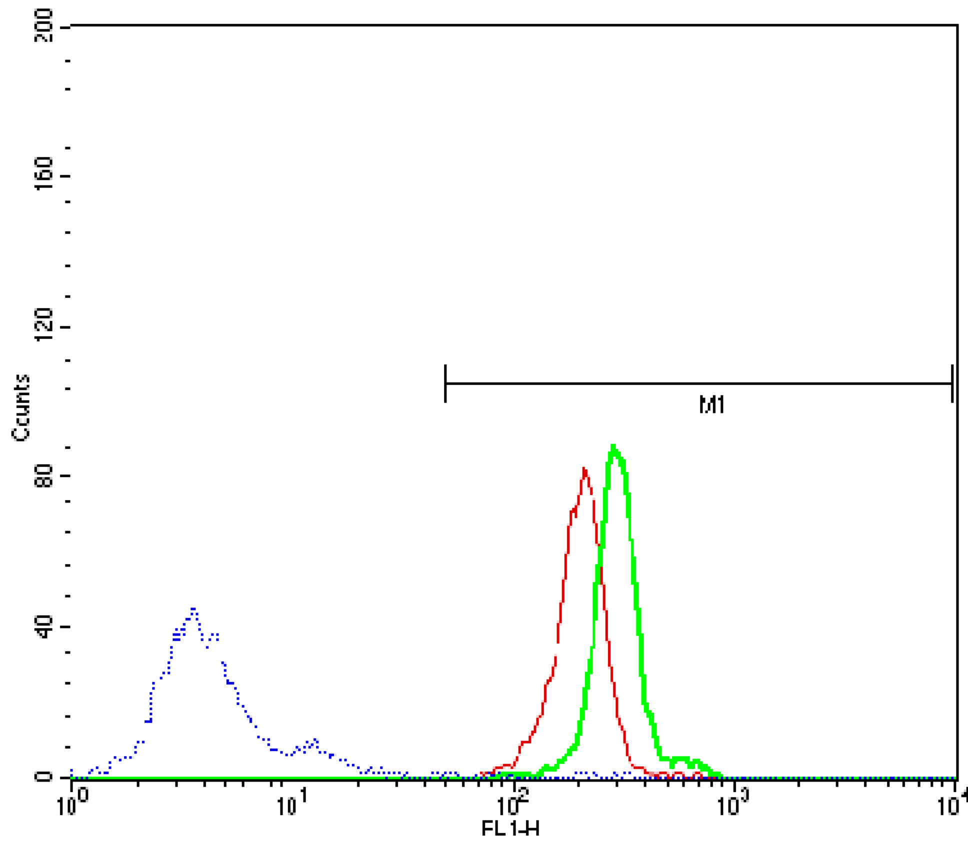

Figure 4-2 Neutrophil CD18 median fluorescent intensity when neutrophils are treated with 10 ng/ml bovine recombinant tumor necrosis factor -α (TNF) versus control neutrophils.

Analysis of 10,000 events gated on region of neutrophils. TNF-α treated neutrophils in green, control neutrophils in red, primary anti-CD18 antibody replaced with an isotype antibody in blue.

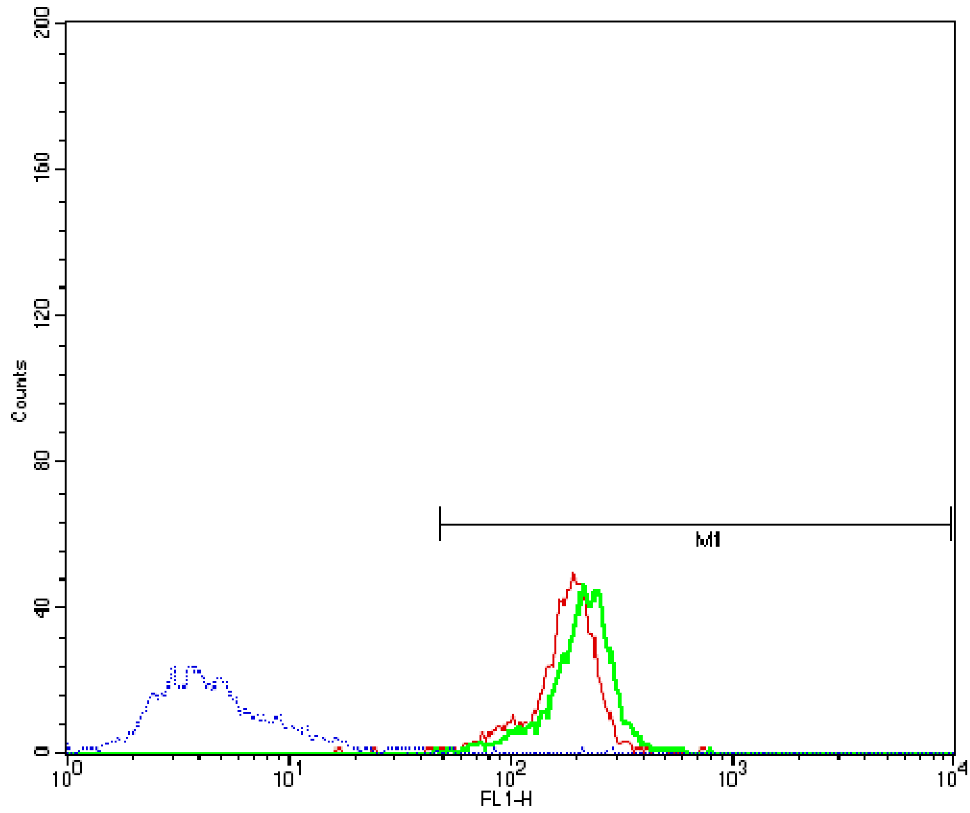

Figure 4-3 Neutrophil CD18 median fluorescent intensity when neutrophils are treated with 100 ng/ml *Escherichia coli* O111:B4 lipopolysaccharide (LPS) versus control neutrophils.

Analysis of 10,000 events gated on region of neutrophils. LPS treated neutrophils in green, control neutrophils in red, primary anti-CD18 antibody replaced with an isotype antibody in blue.

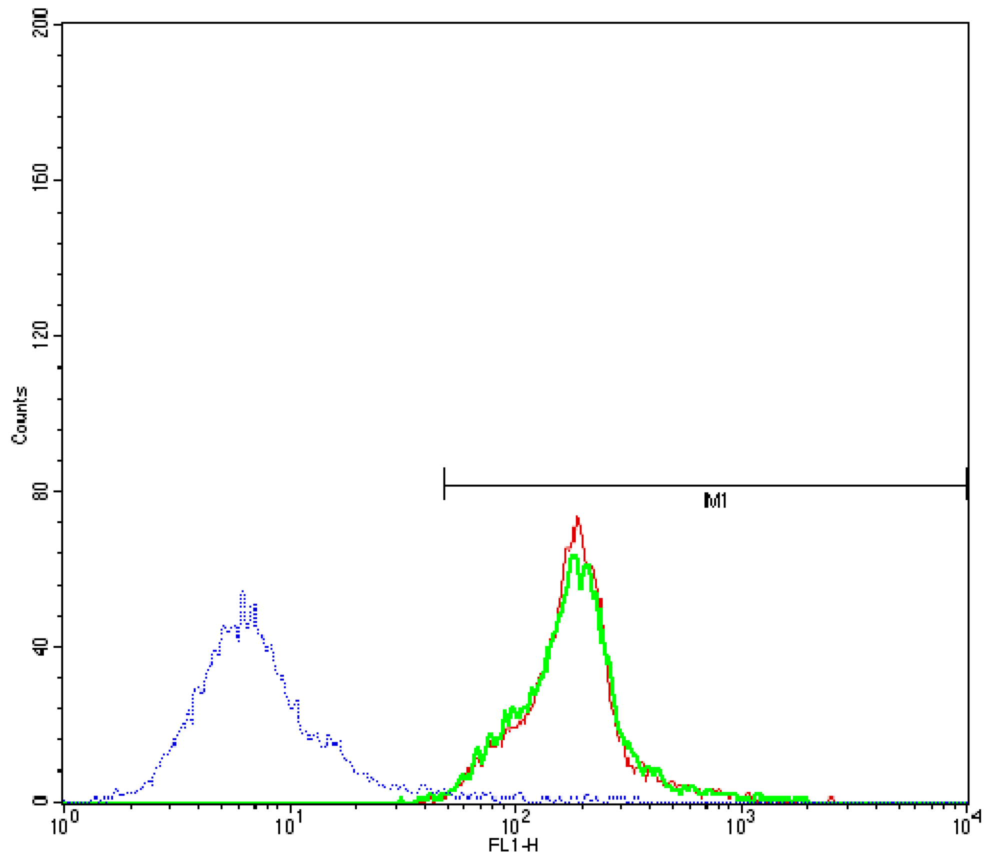

Figure 4-4 Neutrophil CD18 median fluorescent intensity when neutrophils are treated with 1 nM substance P (SP) versus control neutrophils.

Analysis of 10,000 events gated on region of neutrophils. SP treated neutrophils in green, control neutrophils in red, primary anti-CD18 antibody replaced with an isotype antibody in blue.

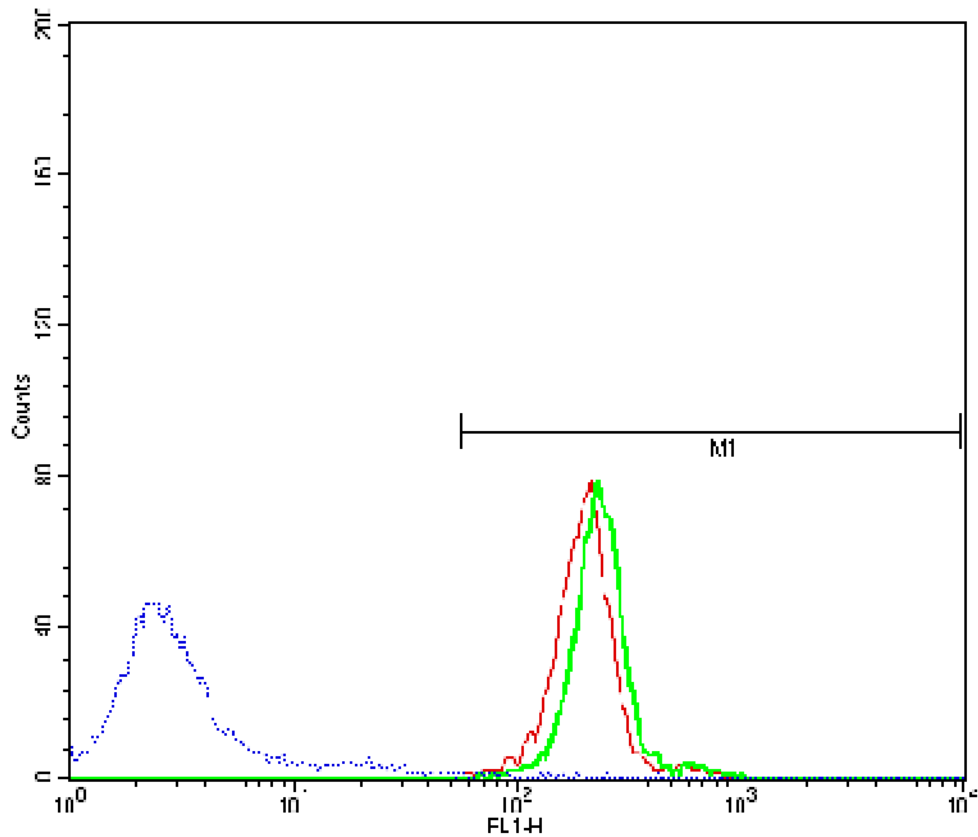

Figure 4-5 Neutrophil CD18 median fluorescent intensity when neutrophils are treated with 1nM substance P (SP) + 100 ng/ml *Escherichia coli* O111:B4 lipopolysaccharide (LPS) versus control neutrophils.

Analysis of 10,000 events gated on region of neutrophils. SP + LPS treated neutrophils in green, control neutrophils in red, primary anti-CD18 antibody replaced with an isotype antibody in blue.

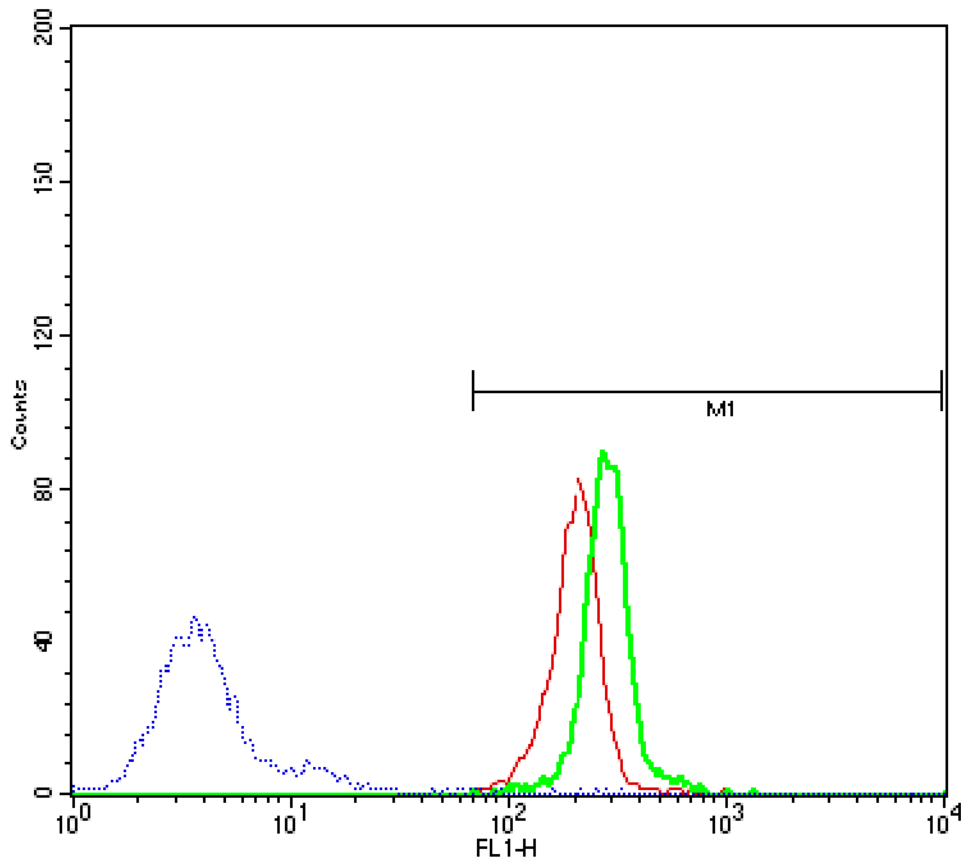

Figure 4-6 Neutrophil CD18 median fluorescent intensity when neutrophils are treated with 1 nM substance P (SP) + 10 ng/ml bovine recombinant tumor necrosis factor -α (TNF) versus control neutrophils.

Analysis of 10,000 events gated on region of neutrophils. SP + TNF-α treated neutrophils in green, control neutrophils in red, primary anti-CD18 antibody replaced with an isotype antibody in blue.

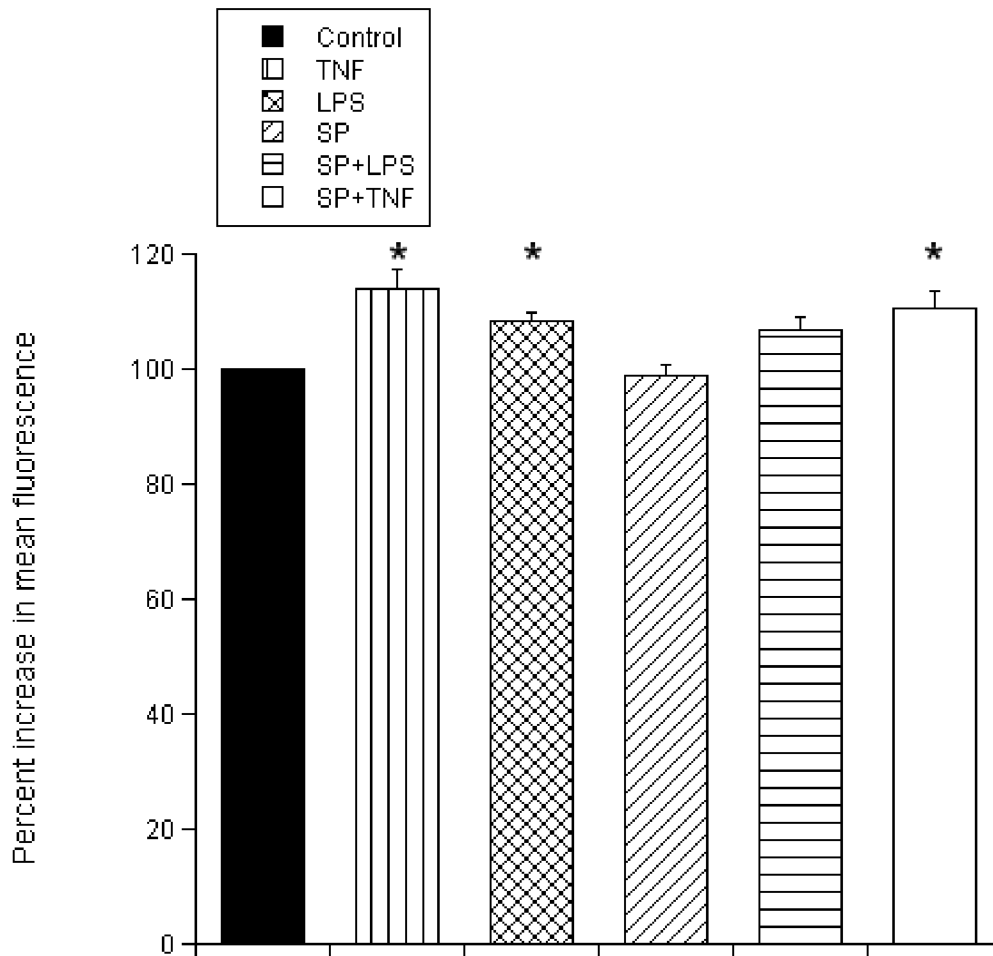

Figure 4-7 Mean fluorescent intensity of CD18 on bovine neutrophils.

The neutrophils were treated with 10 ng/ml TNF, 100 ng/ml LPS, 1 nM SP, and combinations of SP+LPS, and SP+TNF. Data are reported as mean +/- standard error of the mean relative to control neutrophils, which were set as 100% expression of CD18. N=13. * = p-value < 0.05.

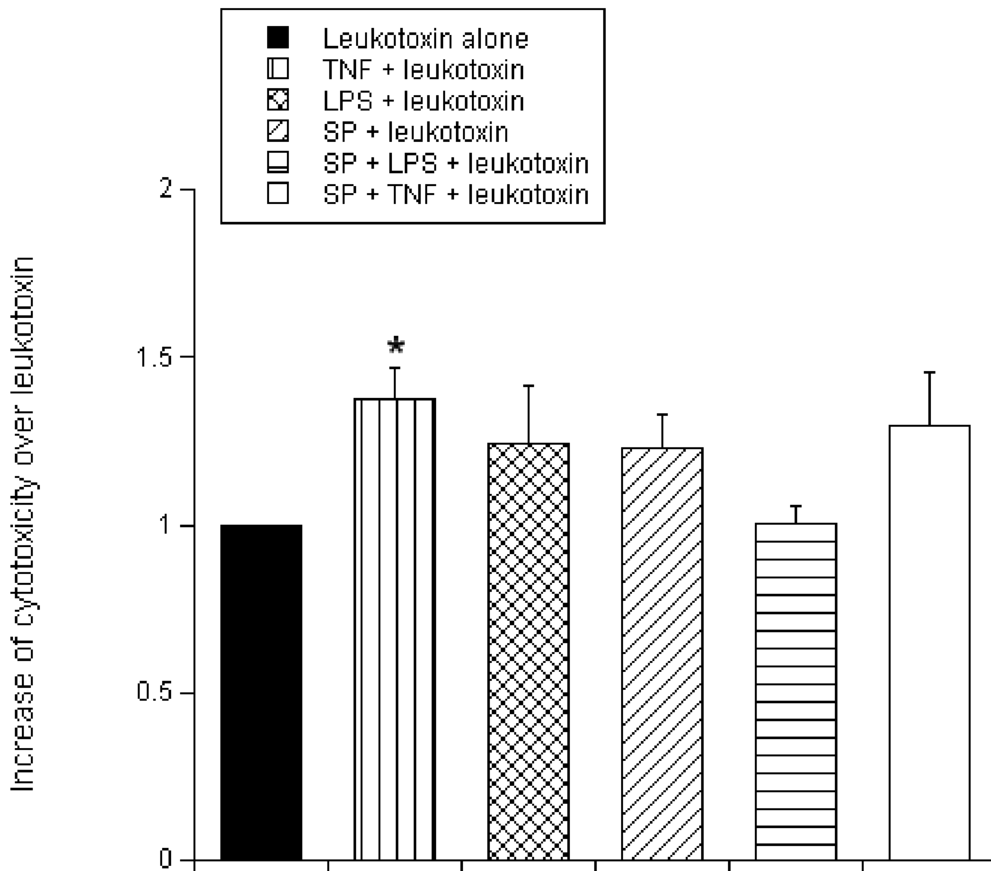

Figure 4-8 ***Mannheimia haemolytica*** **leukotoxin (LKT) - induced cytotoxicity of bovine peripheral blood leukocytes relative to LKT alone.**

The leukocytes were treated with 10 ng/ml TNF, 100 ng/ml LPS, 1 nM SP, and combinations of SP+LPS, and SP+TNF. Data are reported as mean +/- standard error of the mean relative to LKT alone. N=10. * = p-value < 0.05.

CHAPTER 5 - Conclusions

The question these studies investigated was whether or not SP plays a role in the pathogenesis of BPP. Three hypotheses were established to study the relationship between BPP and SP:

1. The quantity of SP is increased in *M. haemolytica*-induced pneumonia compared to normal bovine lungs.
2. Substance P is capable of inducing increased vascular permeability and vascular leakage in bovine vascular endothelial cells.
3. Substance P increases CD18 expression on bovine neutrophils and subsequent increased leukocytotoxicity upon exposure to *M. haemolytica* LKT.

The results of the studies showed two of these hypotheses to be correct. There was increased substance P-like immunoreactivity (SP-LI) in pneumonic lesions induced by *M. haemolytica* compared to normal bovine lung. Additionally, it was shown that SP alone or in combination with LPS and histamine can increase bovine endothelial cell monolayer permeability to albumin. However, under the conditions of the experiments that were conducted SP did not activate bovine neutrophils to increase CD18 surface expression. Neither did SP increase the leukocytotoxicity of *M. haemolytica* leukotoxin after treatment of bovine peripheral blood leukocytes with SP.

The difference in SP-LI in pneumonic lesions induced by *M. haemolytica* and normal bovine lung was due to increased SP-LI within macrophages in the pneumonic lung. This is similar to sheep where SP-LI was found mainly in macrophages in acute pneumonia caused by *M. haemolytica* (145). In addition, macrophages within human sputum have been found to contain SP (58). This evidence suggests that macrophages may be an important source of SP in pneumonia. In addition, there was increased SP-LI and increased incidence of SP-LI in peribronchial nerves in normal bovine lung compared to pneumonic lung. It is possible that the SP in the peribronchial nerves was released into the tissues to enhance inflammation in the pneumonic lung. The role of SP in the inflammatory process of *M. haemolytica* pneumonia may

be most pertinent in the peracute stages. The characteristic lesion of peracute *M. haemolytica* pneumonia is a serofibrinous effusion into the alveoli in the absence of many inflammatory cells or *M. haemolytica* bacteria (2, 32). In the absence of other common mediators of these changes, SP is a strong candidate for inducing and/or participating in the production of the hallmark serofibrinous effusion of peracute BPP (95).

The secretion of SP by alveolar macrophages could contribute to the changes in vascular permeability and the subsequent serofibrinous effusion. Local release of SP from nerve endings in the lung is another potential source of SP in the early stages of BPP. Human and rat endothelial cells express NK-1 indicating that SP can act directly on endothelial cells. Substance P has been shown to be capable of inducing increased vascular permeability and edema formation in multiple species including humans, rats, guinea pigs and mice (29, 30, 46, 56, 75, 94, 95, 97, 128, 148, 156). The SP-induced increased vascular permeability can be enhanced when other permeability increasing agents, such as LPS and histamine, are combined with SP (86, 97). The data in Chapter 3 show that SP alone or in combination with LPS and histamine increases the permeability of bovine endothelial cell monolayers to albumin in an *in vitro* model of increased vascular permeability. Substance P increased the endothelial cell monolayer permeability through endothelial cell shape change and the creation of intercellular gaps rather than increasing endothelial cytotoxicity. If SP acts on bovine endothelial cells *in vivo* as it does *in vitro*, macrophages or local nerve endings present in the lung during *M. haemolytica* might secrete SP that induces increased vascular permeability due its effects on endothelial cells.

Treating bovine neutrophils with SP did not increase CD18 surface expression on the cells or increase *M. haemolytica* LKT killing of bovine leukocytes. Though these data disproved our 3rd hypothesis, the interactions between leukocytes, SP, and other cytokines are complex and may require more elaborate studies to clearly define the roles of each. Bovine alveolar macrophages exposed to SP have increased phagocytic ability and produce TNF (149). Substance P has also been shown to increase production of the inflammatory cytokines TNF, IL–6 and IL-8 by human bronchial epithelial cells (188). The inflammatory cytokines TNF, IL -1β, and IL-6 can be produced by rat T-lymphocytes, macrophages, and neutrophils when exposed to SP (43). The production of these inflammatory cytokines by other cell types could play an important role in the subsequent activation of neutrophils. Substance P has been shown to have direct effects on neutrophils in other species, resulting in increased adherence of human

neutrophils to endothelial cells and bovine bronchial epithelial cells (44, 47). Lipopolysaccharide in combination with SP increased human neutrophil adherence to alveolar epithelial cells and induced the release of Il-1β and TNF (98). Substance P induced chemotaxis of and hydrogen peroxide production by human neutrophils (85, 128, 140, 159). In addition, SP primed human neutrophils for the respiratory burst (37,113, 128, 138, 160).

The data obtained in this study provide indirect evidence using immunohistochemistry and *in vitro* endothelial experiments of potential links between SP and the pathogenesis of BPP. Specific findings of the study were that SP is more prevalent in macrophages in pneumonic bovine lung compared to normal bovine lung and that bovine endothelial cells respond to SP by changing shape, forming intercellular gaps and increasing permeability through an endothelial monolayer. The release of SP from macrophages in the lung and pulmonary nerves could cause *in situ* increased vascular permeability in the lung resulting in protein-rich edema fluid diffusing from the vasculature causing a serofibrinous effusion. This effusion could be a key event in the reduced pulmonary clearance of the bacteria in the critical early stages of BPP, and the subsequent progression of infection and inflammation. In addition, SP may be part of the inflammatory cytokine cascade that activates and recruits neutrophils and other leukocytes into the lung parenchyma in the early stages of BPP.

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