Mast cell quantification and substance P immunoreactivity during the acute and chronic stages of pneumonic pasteurellosis in ruminants

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Mast cell quantification and substance P immunoreactivity during the acute and chronic stages of pneumonic pasteurellosis in ruminants

by

Rafael Ramirez-Romero

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology
Major Professor: Mark R. Ackermann

Iowa State University
Ames, Iowa
2000
This is to certify that the Doctoral dissertation of
Rafael Ramirez-Romero
has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor
Signature was redacted for privacy.

For the Major Program
Signature was redacted for privacy.

ο the Graduate College
To my Wife

María Dolores

And my Daughter

Cecilia

In memory of my Brother

Manuel
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The author is grateful to Universidad Autónoma de Nuevo León and CONACYT, México.
ABSTRACT

The objective of this work was to determine the numbers of stained mast cells (MCs) and substance P (SP) immunoreactivity during the initiation and progression of pulmonary lesions in a model of pneumonic pasteurellosis (PP) in ruminants. The first experiment demonstrated that, at 6 h after intrabronchial deposition of Mannheimia (Pasteurella) haemolytica in neonatal calves, MC numbers decreased in sites of severe lung damage. Prior administration of a sialyl Lewis mimetic (TBC1269) intended to prevent neutrophil infiltration had no effect on MC numbers, suggesting that MC degranulation is unaffected by selectin inhibition. In the second study, a single high dose of dihydrocapsaicin (DHC) administered to neonatal lambs depleted peptidergic nerves of SP. Depletion was measured in nasal septum, the site richest in SP fibers, and was estimated to be 85% of controls. In the upper and lower respiratory tract MC density increased progressively with age, from 3 to 21 days, but DHC-treated animals had significantly higher numbers of MCs; this suggests a functional association between MCs and SP fibers. Histamine content in the lung was similar to controls. The model is suitable for studies of the inflammatory response in the respiratory tract when the neurogenic contribution is reduced. In the third experiment, diminished numbers of MCs and histamine during the acute phase (1 day) of PP in sheep were associated with SP immunoreactivity in macrophages infiltrating the areas of severe damage, suggesting an extraneural source of SP. At 15 days, MCs were scarce at sites with pyogranulomatous foci, but increased in areas with interstitial pneumonia. Substance P immunoreactivity demonstrated that not only an extrinsic sensory innervation, but also an intrinsic contribution of pulmonary ganglion neurons, sustain the inflammatory response. At 45 days the fibroplastic changes in pleura, interlobular spaces, and interstitium were marked, as was the presence of bronchiolitis obliterans. These changes were associated with an increased number of MCs; however, the elevation in histamine did not correspond with the MCs increment. In areas of severe fibrosis SP immunoreactivity in nerve fibers was mild suggesting that the absence of SP during chronic PP hinders tissue repair.
CHAPTER 1. GENERAL INTRODUCTION

Introduction

*Pasteurella haemolytica* is commonly isolated from natural cases of fibrinous pleuropneumonia in cattle, which is the most characteristic lesion of pneumonic pasteurellosis (PP) (Whiteley et al., 1992; Ramírez-Romero and Brogden, 1995). This pneumonic syndrome has been rated the most economically important disease in the cattle industry in North America (De Alwis, 1993). The economic impact of PP is attributed to mortality, morbidity, and reduced productivity (Martin et al., 1981).

*P. haemolytica* has been renamed *Mannheimia haemolytica* (Angen et al., 1999). The bacterium possesses several virulence factors; however, the most important are leukotoxin (LKT) and lipopolysaccharide (LPS) (Ramírez-Romero and Brogden, 1995). Leukotoxin and LPS have the capability to induce tissue damage in the lung of ruminants (Whiteley et al., 1992). However, experimental studies in ruminants and laboratory animals have demonstrated that the LPS contributes more than LKT to the inflammatory process (Dungworth, 1993; Ramírez-Romero and Brogden, in press).

The pathogenesis of PP remains incompletely understood. Although the bacteria and their virulence factors are potent inducers of inflammation, there is evidence that the host inflammatory response is excessive and contributes to the pathogenesis of the severe lung damage (Ramírez-Romero and Brogden, in press). Recently, it has been proposed that two classical models of acute inflammatory response, the Arthus and Shwartzman reactions, may explain the pathogenesis of the characteristic lesions in PP (Ramírez-Romero and Brogden, in press). Nonetheless, the pathogenic mechanisms operating during the progression of the pulmonary lesions have not been fully defined.

Few studies have assessed the role of mast cells (MCs) in PP. Adusu et al. (1994) reported that MCs recovered from cattle infected experimentally with *M. haemolytica* release increased amount of histamine in vitro, and *M. haemolytica* LKT and LPS both cause an increase in the spontaneous release of histamine from isolated bovine lung parenchyma (Saban et al., 1997). However, there have been no studies on the influence of the acute and chronic inflammatory response induced by *M. haemolytica* on the numbers of MCs in the lung. Similarly, the role of substance P (SP), a neuropeptide that induces MC activation during neurogenic inflammation in the lung (Baluk, 1997), has not been defined in PP.
The main objective of this work was to determine the numbers of stainable MCs and SP immunoreactivity during the initiation and progression of the pulmonary lesions in a model of PP in ruminants.

Dissertation Organization
The dissertation is organized in an alternate format including six chapters composed of general introduction, a brief review of the literature, three papers suitable for publication, and general conclusions, respectively. The references have been presented at the end of each chapter. The manuscripts included correspond to three separate experiments. The first one has been accepted for publication in *Journal of Comparative Pathology*; the second has been submitted to *Regulatory Peptides*, and the third is prepared for submission to *Veterinary Pathology*. In all of them the first author is the Ph.D. Candidate Rafael Ramírez-Romero and Mark R. Ackermann, the Major Professor, is co-author.

In the first experiment, the number of stainable MCs was determined during the acute phase of *M. haemolytica* pneumonia in calves; additionally, the role of a compound that acts by competition as selectin inhibitor was included to determine its influence on MCs in vivo. During the second experiment, the effect of dihydrocapsaicin in depleting peptidergic fibers of SP was determined in neonatal lambs. Subsequently, the influence of SP depletion on MC density in the respiratory tract was determined. This experiment was designed to provide an animal model suitable for the study of respiratory tract inflammation in which neurogenic inflammation was diminished. The third experiment was carried out to quantify the relative relationships between histopathological changes in lung parenchyma, MC numbers, and SP production, during the acute, sub-acute, and chronic stages of PP in sheep. The results obtained from the three experiments provide evidence that MCs and SP are important components of the lung inflammatory response during initiation and progression of PP.

References


CHAPTER 2. LITERATURE REVIEW

Mast Cells

Mast cells are bone marrow-derived cells, carried as immature cells in the bloodstream, that complete their final steps of maturation after arrival in tissues (Metcalfe et al., 1997; Welle, 1997). Mast cells acquire their phenotypic characteristics in specific microenvironmental conditions of the tissues they colonize, and thus represent a widely heterogeneous population (Metcalfe et al., 1997; Welle, 1997). Traditionally described as the cells responsible for the severe effects of type I hypersensitivity, MCs are now recognized as orchestrators of the defense mechanisms, both acquired and innate (Galli et al., 1999; Galli, 2000). Mast cells possess an array of adhesion molecules and immunoglobulin receptors that provide them with the capability to react to multiple specific and non-specific stimuli (Metcalfe et al., 1997). In addition, MCs phagocytose bacteria and present antigens to lymphocytes (Malaviya et al., 1996b; Abraham et al., 1997; Mekori and Metcalfe, 1999), produce cytokines and release vasoactive compounds necessary to mount the inflammatory and immune responses (Metcalfe et al., 1997; Mekori and Metcalfe, 1999), and contain proteases and growth factors that act in remodeling tissues (Levi-Schaffer, 1995), among other functions.

At 15 days of gestation there are three different populations of precursor cells in the blood of fetal mice that can be separated by their expression of Thy-1 and c-Kit. These populations are Thy-1^−^ c-Kit^−^, Thy-1^+^ c-Kit^+^, and Thy-1^−^ c-Kit^−^, and correspond to multipotent hematopoietic stem cells, pro-thymocytes, and pro-mastocytes, respectively (Rodewald et al., 1996). This means that commitment to the MC lineage precedes tissue immigration and occurs earlier during hematopoiesis (Rodewald et al., 1996). In human beings MC precursors have the CD34^+^ marker and express also c-Kit^+^. Therefore, they are responsive to stem cell factor (SCF; also called mast cell growth factor, Steel factor, and c-Kit ligand) (Metcalfe et al., 1995; Broudy, 1997).

Thy-1 is a glycoprotein belonging to the immunoglobulin superfamily whereas CD34^+^ is a Ser/Thr-rich sialomucin, and both are commonly present in hematopoietic stem cells and influence their maturation. However, it is the presence of c-Kit, the receptor, and SCF, its ligand, that establish commitment and homing of the MC lineage (Metcalfe et al., 1995; Broudy, 1997). More recently it has been reported that eotaxin acts synergistically with SCF to promote the growth and differentiation of MC precursors...
Additionally, for optimal proliferation and differentiation, MCs require complementary factors such as interleukin (IL)-3, IL-4, IL-9, and IL-10, and nerve growth factor (NGF) (Metcalfe et al., 1995; Metcalfe et al., 1997). Conversely, granulocyte-macrophage colony-stimulating factor (GM-CSF) and transforming growth factor (TGF)-β inhibit MC differentiation (Metcalfe et al., 1995).

The early MC precursors contain characteristic cytoplasmic granules and express RNAs encoding mast cell-proteases but lack the high-affinity IgE receptor (FceRI) (Rodewald et al., 1996). The synthesis of the proteoglycan matrix, particularly heparin, is a pre-requisite to complete the adequate synthesis of granule components because improper sulfation of the matrix impedes the expression of proteases and probably other components (Humphries et al., 1999; Forsberg et al., 1999). Apparently, FceRI is a marker of terminal differentiation (Metcalfe et al., 1995).

Interleukin-3 is an important factor for MCs from murine and human origin, and can sustain, alone, the growth of MCs in vitro (Metcalfe et al., 1995; Metcalfe et al., 1997). It has been reported that withdrawal of IL-3 induces MC apoptosis but addition of SCF can prevent it (Metcalfe et al., 1995). The “rescue” effect of SCF is not fully understood but apparently is not mediated by bcl-2 (Metcalfe et al., 1995; Metcalfe et al., 1997). Conversely TGF-β inhibits the “rescue” effect of SCF when MCs are deprived of IL-3; the mechanism is probably related to the down-regulation of c-Kit expression induced by TGF-β (Metcalfe et al., 1995; Metcalfe et al., 1997). On the other hand, murine MCs express the Fas antigen and are susceptible to Fas-mediated apoptosis (Hartmann et al., 1997). More recently, it was reported that MCs also express the Fas ligand but cannot induce apoptosis in Fas-bearing cells because their Fas ligand is localized intracellularly (Wagelie-Steffen et al., 1998). An autocrine pathway of Fas-mediated apoptosis may regulate MC populations and specific stimuli may be required for surface expression of Fas ligand (Wagelie-Steffen et al., 1998).

Stem cell factor is normally produced in soluble and transmembrane forms and both forms are biologically active (Broudy, 1997). Stem cell factor is constitutively produced by endothelial cells and fibroblasts which display the transmembrane form and also release soluble SCF (Broudy, 1997). Additionally, SCF is also produced by keratinocytes and intestinal epithelial cells (Broudy, 1997), as well as MCs (de Paulis et al., 1999). Human lung MCs store SCF in their granules and after being released, the
protease chymase cleaves SCF giving a shorter protein that is still biologically active and thereby, may exert autocrine and paracrine actions (de Paulis et al., 1999).

The c-kit proto-oncogene encodes a growth factor receptor (c-Kit), with ligand-dependent tyrosine kinase activity and immunoglobulin (Ig)-like domains (Ig superfamily member), structurally related to the platelet-derived growth factor (PDGF) receptor (Timokhina et al., 1998). After being activated by the corresponding ligand (SCF), c-Kit dimerizes to permit its autophosphorylation and becomes able to bind and activate several molecules. c-Kit activation causes phosphorylation and activation of both an adaptor protein (Shc) and an exchange GDP/GTP (Vav) factor that allow incorporation and activation of Ras monomeric GTPases (Rac) (Timokhina et al., 1998). In addition, activated c-Kit permits association with phosphatidylinositol 3'-kinase (PI 3-kinase), phospholipase Cγ-1, an adaptor protein (Grb2), tyrosine phosphatases (SHP1 and SHP2), and Src kinases (Fyn, Lyn) (Timokhina et al., 1998). It has been demonstrated that after ligand-mediated c-Kit activation, the PI 3-kinase and Fyn pathways converge downstream to activate Rac1 and subsequently (RAF→MEK→MAPK), the protein kinase c-jun-N-terminal kinase (JNK); these signaling pathways are required during MC survival and proliferation (Timokhina et al., 1998).

Genetically MC-deficient mice have been very useful in understanding the role of c-Kit and SCF in MC maturation. The c-kit proto-oncogene is located at the W (white spotting) locus and mutations have relevant effects not only in hematopoiesis but also in gametogenesis and melanogenesis (Metcalfe et al., 1995; Metcalfe et al., 1997). The mutant mice W/W" suffer from hypoplastic anemia, hypopigmentation, and sterility, apart from lacking MCs (Castells, 1997). This is because hematopoietic cell precursors, germ cells, and melanocytes, all are derived from the neural crest and employ the same mechanism of cell growth and regulation. When W/W" mice receive a bone marrow transplant from normal mice (+/++) the deficiency in MCs is restored indicating that the deficiency resides in the MC precursors themselves (Metcalfe et al., 1995; Castells, 1997; Metcalfe et al., 1997). Conversely, Sl/Sf\(^d\) mutant mice, which show abnormalities similar to W/W" mice, cannot be restored from MC deficiency by +/+ bone marrow transplant; however, bone marrow from Sl/Sf\(^d\) can restore MC deficiency in W/W" mice, which implies, in this case, a deficiency in tissue microenvironmenal factors that regulate MC maturation (Metcalfe et al., 1995; Castells, 1997; Metcalfe et al., 1997). This is because a gene located at the Sl (Steel) locus encodes SCF. Although Sl/Sf\(^d\) mice have
a soluble form of SCF, the deficiencies in hematopoiesis (including lack of MCs), gametogenesis, and pigmentation, suggest that the transmembrane form of SCF is required for proper cellular growth and maturation (Broudy, 1997).

Considering their location and also their morphological and histochemical characteristics, mast cells are broadly assigned to one of two categories, mucosal mast cells (MMC) or connective tissue mast cells (CTMC) (Abraham et al., 1997; Metcalfe et al., 1997; Welle, 1997). Mucosal mast cells are primarily found in close proximity to the epithelium in the respiratory, urogenital and intestinal mucosa. Conversely, CTMC are found in the skin, peritoneal cavity, the muscular layer of the intestine and around venules in the lung. This classification was based in original studies in mice and rats and by analogy also has been employed in humans (Abraham et al., 1997; Metcalfe et al., 1997).

There are several differences in function between MMC and CTMC. For example, MMC contain chondroitin sulfate as the main matrix component and little histamine (<2 pg/ cell); these cells are sensitive to formaldehyde fixation and require special conditions for proper fixation and staining. Connective tissue mast cells contain heparin in their granules and large amounts of histamine (< 35 pg/ cell); these cells are formaldehyde resistant (Metcalfe et al., 1997; Welle, 1997). Mucosal mast cells are considered migratory cells with a short life span (half life < 40 days) whereas CTMC are tissue fixed cells that live longer (half life > 6 months) (Welle, 1997). In addition, the population of MMC expands remarkably during T cell-dependent immune responses whereas CTMC exhibit little or no T cell-dependence; CTMC occur in similar numbers in athymic nude and in euthymic mice (Metcalfe et al., 1997). Nonetheless, not all rat and mouse MCs observed in situ can be readily categorized and most of the tissues contain a mixed population including uncategorized MCs (Metcalfe et al., 1997). On the other hand, MMC can develop into CTMC and vice versa, depending on the microenvironmetal conditions in the tissue (Welle, 1997). It is generally accepted that murine MCs cultured in the presence of IL-3 become MMC whereas exposure to SCF or fibroblasts with or without IL-3 induces a CTMC phenotype (Welle, 1997).

The most effective markers for characterization of MC subpopulations are the proteases in their cytoplasmic granules. Neutral proteases (serine proteases) are quantitatively important and represent constituents restricted to MC granules (Metcalfe et al., 1997; Welle, 1997). Mast cell proteases have been classified as chymases or
tryptases because their chymotrypsin- and trypsin-like enzymatic activity (Welle, 1997). Moreover, tryptase and chymase are the main histochemical markers for recognition of MCs in tissue sections in humans (Metcalfe et al., 1997). Based on these markers, MCs have been classified in MCtc for those cells that contain tryptase, chymase and other proteases (carboxypeptidase and cathepsin G-like protease), and MCT for those MCs that contain only tryptase (Metcalfe et al., 1997; Welle, 1997). In humans, MCT predominate in the alveolar septa of the lung and in the small intestine mucosa; conversely, MCtc predominate in the skin and in the small intestine submucosa. Therefore, in terms of tissue localization, the human MCT correspond most closely to the rodent MMC whereas MCtc are much more related to rodent CTMC (Metcalfe et al., 1997). Tryptases and chymases have been recognized in MCs from rodents (Welle, 1997), cattle (Küther et al., 1998), dog (Kube et al., 1998), and horse (Welle et al., 1997). In sheep and goat, however, the MC proteases have a unique dual function being either tryptase- or chymase-like, depending on the microenvironmental conditions (Pemberton et al., 1997; Macaldowie et al., 1998).

Mast cell proteases are stored in the proteglycan matrix of MC granules in an active state but effectively stabilized at pH 5.5 in the cells. This stabilization is due to a strong ionic binding of the positive charged proteases with the highly negative charged proteoglycans, mainly heparin and chondroitin sulfate (Welle, 1997; Walls, 1998). After degranulation, however, the proteases are released by cationic exchange at neutral pH but probably exert their function only in close proximity to the site of release because subsequent attachment to extracellular matrix proteoglycans, such as heparan sulfate, is highly possible (Walls, 1998). It has been estimated that human lung MCs contain 11 pg/cell of tryptase and 2.6 pg/cell of chymase but skin MCs have higher amounts of these enzymes (Walls, 1998).

In humans there are two types of MC tryptases, α-tryptase and β-tryptase. Alpha-tryptase seems to be constitutively released and is normally found in the blood of healthy individuals but also in some pathological conditions such as mastocytosis. Conversely, β-tryptase is released during anaphylactic reactions (Walls, 1998). Tryptase promotes the inflammatory response because it degranulates neighboring MCs and is chemotactic for neutrophils and eosinophils (Walls, 1998). Also, tryptase can induce expression of IL-8 and upregulation of intercellular adhesion molecule-1 (ICAM-1) on epithelial cells (Welle, 1997; Walls, 1998). Tryptase cleaves high molecular weight kininogen (HMWK)
and thereby generate kinins; tryptase also inactivates fibrinogen and thereby limits the clotting reaction. All these activities could potentiate the inflammatory reaction (Welle, 1997; Walls, 1998). Moreover, tryptase degrades the neuropeptides vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP), probably as a regulatory mechanism during neurogenic inflammation (Walls, 1998). Tryptase is involved in tissue degradation and remodeling because it activates stromelysin and urokinase plasminogen activator, and cleaves fibronectin. In addition, tryptase acts as a growth factor for smooth muscle cells, fibroblasts, and epithelial cells, and induces collagen synthesis from fibroblasts (Welle, 1997; Walls, 1998). Finally, tryptase activates atrial natriuretic peptide (Walls, 1998).

A new role for tryptase has been recently identified. Trypsin and tryptase can activate by proteolysis a specific G protein-coupled receptor that belongs to the proteinase-activated receptors (PARs), thus acting as signaling molecules (Dery et al., 1998). There are three known members of this PARs family: PAR-1 and and PAR-3 are cleaved and activated by thrombin whereas PAR-2 is a receptor for trypsin-like enzymes (Dery et al., 1998). The mechanism of activation includes recognition of the G-protein-coupled receptor by the enzyme, cleavage of the receptor at a specific enzymatic site within the NH2-extracellular terminus, and exposure of a new NH2-terminus that acts as a tethered ligand that specifically binds and activates its own cleaved receptor (Dery et al., 1998). Evidence indicates that PAR-2 is most likely coupled to heterotrimeric Gαq or Gαo proteins (Dery et al., 1998). Proteinase-activated receptor-2 is expressed in epithelial, endothelial, smooth muscle cells, and fibroblasts in several tissues, including the airways (Akers et al., 2000), as well as in PMNs (Dery et al., 1998) and sensory nerves (Steinhoff et al., 2000). It is possible that the pro-inflammatory (including neurogenic inflammation) and mitogenic influences exerted by tryptase may be mediated by PAR-2 (Akers et al., 2000; Steinhoff et al., 2000).

Chymase has been involved in tissue inflammation and remodeling. For instance, rat chymase cleaves type IV and type VI collagens, fibronectin and vitronectin, and also activates stromelysin (Walls, 1998). Chymase, like tryptase, can cleave VIP and CGRP, but also substance P (SP) (Welle, 1997). Furthermore, chymase can convert IL-1β precursor into its active form and degrade IL-4 (Walls, 1998). In addition, chymase has a role in regulation of blood pressure because it converts angiotensin-I to angiotensin-II, independently, and even more efficiently than angiotensin-converting enzyme (Welle,
This chymase activity could be related with the high levels of angiotensin reported in some cases of acute asthma (Walls, 1998).

The W/W' MC-deficient mice have also been useful to understand the relevant role of MCs in bacterial clearance and influx of polymorphonuclear cells (PMN) into sites of infection. These mutant mice are more susceptible to an intranasal challenge with *Klebsiella pneumoniae* when compared with MC-competent littermate controls (Malaviya *et al.*, 1996a). Subsequently, MC-reconstituted W/W' mice exposed to the same challenge respond more favorably to the bacterial challenge and do not present the increased susceptibility (Malaviya *et al.*, 1996a). This improved response to challenge was related to an increased alveolar exudation of polymorphonuclears (PMNs) attributed to various inflammatory mediators released by MCs, particularly tumor necrosis factor alpha (TNF-α) (Malaviya *et al.*, 1996a).

Similarly, W/W' mice had increased mortality in a model of acute septic peritonitis (caecal ligation and puncture) (Echtenacher *et al.*, 1996). Reconstitution of these MC-deficient mice with cultured MCs substantially increased their resistance, whereas anti TNF-α antibody injected after the peritonitis procedure suppressed this MC-transferred resistance (Echtenacher *et al.*, 1996). The inflammatory response and the fibrin exudation mediated by MCs and MC-derived TNF-α, were believed to be the major factors in localizing and confining the bacterial infection, resulting in protective effects (Echtenacher *et al.*, 1996).

It has been suggested that bacteria can activate MCs by mechanisms distinct from those related to antigen recognition after opsonization (FcεR, FcγR, and receptors for complement fragments) (Dreskin and Abraham, 1999). Mast cells possess a wide range of receptor molecules in their membranes, some of which mediate recognition of bacterial constituents such as fimbriae and lipopolysaccharides (Abraham *et al.*, 1997; Dreskin and Abraham, 1999). Although these bacterial components induce MC activation, their specific receptors are still unidentified (Abraham *et al.*, 1997). On the other hand, MCs are able to phagocytose and kill bacteria and present bacterial antigens through class I MHC molecules to CD8+ T cells (Malaviya *et al.*, 1996b). Additionally, MCs express class II MHC molecules and present processed immunogenic peptides to CD4+ T cells (Mekori and Metcalfe, 1999). The presence of accessory molecules such as B7 and ICAM-1 on the surface of MCs is consistent with their function as antigen-presenting cells (Mekori and Metcalfe, 1999).
Mast cells are intimately related with the progression of the inflammatory response. During the acute stages MCs degranulate and become almost absent in sites of severe and acute inflammation (Cheville, 1994). The role of MCs during acute inflammation has been highlighted by the observation that MC degranulation prior to ischemia decreases ischemia-reperfusion injury in the small intestine of dogs (Boros et al., 1999). Similarly, in MC deficient mice (W/W and S/Sf), intravesical administration of LPS produced a mild reaction in comparison with normal controls (Bjorling et al., 1999). The inflammatory response occurs in animals lacking MCs but, as indicated, the response is inadequate because neutrophil recruitment is deficient; the defect is probably dependent on TNF-α which is one of the major cytokines stored preformed in MCs (Malaviya et al., 1996a; Echtenacher et al., 1996). On the other hand, the presence of neutrophils during a normal inflammatory response is very important to MCs because neutrophil defensins are potent MC secretagogues (Befus et al., 1999). In addition, activation of complement, particularly C3, is undoubtedly required during acute inflammation mediated by MCs (Prodeus et al., 1997).

During the chronic stages of inflammation, intralesional MCs proliferate (Metcalfe et al., 1997). In human beings MC hyperplasia is a characteristic of many chronic disorders in the skin and lung and is accompanied by fibrosis (Levi-Schaffer, 1995). In some cases, the MC-fibroblast relationship has been demonstrated in animal models such as pulmonary fibrosis in rats induced by bleomycin (Levi-Schaffer, 1995; Metcalfe et al., 1997). However, Okazaki et al. (1998) demonstrated that pulmonary fibrosis occurred when MC-deficient rats (Ws/Ws) were exposed to bleomycin. They concluded that MC hyperplasia is commonly associated with, but not the cause of fibrosis. Apart from the proliferative influence of MC proteases on fibroblasts and smooth muscle cells already mentioned, MCs contain many compounds that affect fibroblasts and have angiogenic effects. For example, heparin and histamine influence fibroblast proliferation, collagen synthesis and angiogenesis, and several cytokines and growth factors produced by MCs (IL-4, TNF-α, TGF-β1, NGF, basic fibroblast growth factor [bFGF]) have fibrogenic-angiogenic activity (Levi-Schaffer, 1995; Metcalfe et al., 1997). Mast cell-fibroblast heterotypic contact activates MCs to produce IL-4, which promotes fibroblast proliferation (Trautmann et al., 1998), and fibroblasts engulf MC granules directly (Levi-Schaffer, 1995). Similar to the inadequate inflammatory response observed in models of acute inflammation in MC-deficient mice (Malaviya et al., 1996a;
Echtenacher et al., 1996), the fibroplastic (and angiogenic) changes that occur during the chronic stages of an inflammatory response in MC-deficient rats, may be defective as well.

In normal human skin MCs have an important role in wound healing. By two days after incisional formation of a wound, MCs were largely absent in areas of necrosis and hemorrhage but were present in the surrounding viable tissue; however, between seven and fourteen days, MCs were increased in the fibrotic areas of the wound where the new matrix was formed (Trautmann et al., 2000). Trautmann and coworkers (2000) found that MCs were increased due to recruitment and did not replicate in situ. The chemotactic factor that promoted MC accumulation was monocyte chemotactic protein –1 (MCP-1) synthesized by macrophages and keratinocytes. Macrophage inflammatory protein-1α/β (MIP-1α, MIP-1β) and RANTES had no effect (Trautmann et al., 2000). In addition, MCs synthesized IL-4 which promotes fibroblast migration and proliferation, and fibroblast synthesis of collagen (Trautmann et al., 2000). The authors concluded that MCs are involved mostly in the late (anabolic) process of wound healing more than the earlier inflammatory (catabolic) period (Trautmann et al., 2000).

Tachykinins
Communication between the immune and neuroendocrine system is mediated by peptides and proteins that are used as intercellular messengers (Weigent and Blalock, 1997). For example, cells of the immune system, in both primary and secondary lymphoid organs, are able to produce hormones and neuropeptides (e.g., corticotropin, thyrotropin, growth hormone, prolactin, and catecholamines, among others) (Savino and Dardenne, 1995; Weigent and Blalock, 1997), which also can exert cytokine-like effects (Torres and Johnson, 1997). Conversely, classical endocrine glands, epithelial cells, as well as neurons and glial cells, can produce a variety of cytokines that exert hormone-like actions (Stadnyk, 1994; Savino and Dardenne, 1995; Smith, 1997; Weigent and Blalock, 1997; Benveniste, 1997). Moreover, receptors for hormones, neurotransmitters and cytokines are expressed in both cells of the immune and neuroendocrine systems (Garza Jr. and Carr, 1997; Walter, 1997; Weigent and Blalock, 1997).

The widespread innervation of lymphoid organs and the close association of nerves with lymphocytes, macrophages, dendritic cells, and MCs is a clear indication of the cell to cell interaction between the nervous and immune systems (Goetzl et al., 1995;
Maggi, 1997; Stevens-Felten and Bellinger, 1997). Stimulated sensory nerves,
particularly unmyelinated sensory nerve endings (C-fibers), release active neuropeptides
and simultaneously transmit sensory information (Brain, 1997; Maggi, 1997). Substance
P is one of the best-characterized neuropeptides, with demonstrated capability to induce
the neurogenic inflammatory response; SP is also able to act as a modulator of the
immune response (Maggi, 1997). More recently SP has been identified as an important

Substance P belongs to a family of small neuropeptides called tachykinins
(Otsuka and Yoshioka, 1993; Maggi, 1997; Nussdorfer and Malendowicz, 1998). The
name tachykinin refers to an ability to rapidly contract smooth muscle, as opposed to the
effect of bradykinin (Reynolds et al., 1997). The tachykinin family is a group of small
peptides characterized by the C-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH₂,
where X is either an aromatic (Phe) or branched aliphatic (Val) residue (Otsuka and
Yoshioka, 1993; Piedimonte, 1995; Maggi, 1997). Apart from SP, the other mammalian
tachykinins are neurokinin A (NKA) and neurokinin B (NKB) (Otsuka and Yoshioka,
1993; Piedimonte, 1995; Maggi, 1997).

Substance P and NKA are derived from the single preprotachykinin A (PPT-A)
gene after alternate splicing of the primary RNA transcript. Neurokinin B is derived from
a separate gene (PPT-B) (Piedimonte, 1995; Brain, 1997; Nussdorfer and Malendowicz,
1998). The alternate processing of the PPT-A gene primary transcript give several forms
of mRNAs, including the N-terminal extended forms of NKA: neuropeptide K (NPK) and
neuropeptide γ (NPγ) (Otsuka and Yoshioka, 1993; Piedimonte, 1995; Nussdorfer and
Malendowicz, 1998). Preprotachykinin A gene expression is highest in the central and
peripheral nervous systems whereas the PPT-B gene product occurs mostly in the brain
(Rameshwar and Gascón, 1997). Preprotachykinin-A gene expression has also been
demonstrated in non-neural cells such as Leyding cells of testis, endothelial cells,
lymphocytes, monocytes, macrophages, eosinophils, neutrophils, and keratinocytes
(Bae et al., 1999).

Substance P is present in all of the peripheral nerves that contain cutaneous or
visceral afferents and also in the central and peripheral projections of sensory neurons.
Locations of peripheral sensory nerve endings containing SP that are particularly
prominent include the varicose terminal-like structures associated with blood vessels
(including precapillary arterioles and venules) especially in epithelial surfaces, and free
nerve endings in epithelia of skin, cornea, respiratory and urogenital system. These structures are situated either just beneath the epithelium or extending to the epithelial surface. In addition, SP occurs in fascicles of varicose fibers and nerve endings in smooth muscle layers of visceral organs, and fibers in autonomic (parasympathetic, sympathetic pre- and paravertebral) ganglia where collaterals of afferent neurons make synaptic contacts with dendrites of the principal ganglion cells (Holzer, 1988).

Ganglion neurons in the myenteric and submucosal plexuses of the gastrointestinal tract also produce tachykinins and this should be expected to occur in airway ganglion neurons in the lung because of its embryonic development from the foregut (Dey et al., 1988). Nonetheless, the presence of SP in (intrinsic) airway ganglia has not been consistently recognized in the lung. The discrepancy could be explained by the fact that airway ganglia contain a heterogeneous population of neurons susceptible to adaptive functions under physiological and pathological conditions (Pérez Fontán et al., 2000).

The presence of tachykinins in primary and secondary lymphoid tissues is remarkable. Although the source of these mediators may be directly from the primary afferent nerves located in these lymphoid tissues (Stevens-Felten and Bellinger, 1997), the main sources appear to be endothelial cells, lymphocytes, eosinophils, macrophages and neutrophils. Preprotachykinin-A mRNA has been recognized in all of these cells (Maggi, 1997). Several factors influence the expression of the PPT-A gene, i.e., exposure to LPS upregulates whereas dexamethasone downregulates PPT-A gene expression in alveolar macrophages and pulmonary neutrophils (Killingsworth et al., 1997).

The agonistic activity of tachykinins is associated with the shared carboxy-terminal domain whereas the amino-terminal domain confers receptor specificity (Piedimonte, 1995). Three tachykinin receptors have been recognized, neurokinin 1-receptor (NK-1R), NK-2R and NK-3R. Although each of the tachykinin peptides can act as a full agonist on all three receptors if present at sufficiently high concentration, there is a preferential binding tendency. For example, SP, NK-A, and NK-B exhibit binding preferences for NK-1R, NK-2R and NK-3R, respectively (Piedimonte, 1995; Garza Jr. and Carr, 1997). Ligand binding studies indicate subtypes of each receptor which probably recognize different amino terminal domains in the peptides (Piedimonte, 1995).
Structurally the NK-R consists of an extracellular amino terminus, seven hydrophobic transmembrane helices with alternating extracellular and cytoplasmic loops and a cytoplasmic carboxy terminus. This structure is compatible with that of G protein-coupled receptors which mediate cell activation through $\beta$-AMP and a phosphatidylinositol pathway with a concomitant rise in cytosolic $Ca^{2+}$ (Piedimonte, 1995; Garza Jr. and Carr, 1997).

The NK-1R and NK-2R are expressed in bone marrow stromal cells and mediate regulatory actions on hematopoiesis (Rameshwar and Gascón, 1997). It has been suggested that SP and NKA mediate their hematopoietic effects through the induction of cytokines and their receptors in bone marrow stromal cells. Substance P exerts proliferative effects on hematopoiesis and its effects correlate with the induction of positive hematopoietic growth factors such as IL-3, IL-6, GM-CSF and SCF (Rameshwar and Gascón, 1997). Conversely, NKA exerts inhibitory effects on hematopoiesis and its actions correlate with the induction of two negative hematopoietic regulators, MIP-1$\alpha$ and TGF-β (Rameshwar and Gascón, 1997). The stimulatory effects on bone marrow progenitors is mediated by NK-1R (which preferentially binds SP) and its expression is influenced by cytokines, mainly IL-1, derived from bone marrow stromal cells stimulated by SP. Moreover, SP can induce the IL-1 type I receptor in stromal cells (Rameshwar and Gascón, 1997). Conversely, NK-2R (which binds preferentially NKA) downregulates the process (Rameshwar and Gascón, 1997).

The NK-1R and NK-2R receptors are also expressed in lymphocytes; their expression in T cells is associated with a modulatory influence on the immune response (McCormack et al., 1996; Maggi, 1997). The presence of NK-1R has been demonstrated mostly in T cells and under certain circumstances in B cells; the latter particularly in a human lymphoblastic cell line (IM-9) (Garza Jr. and Carr, 1997). In human peripheral blood NK-1R is expressed in T lymphocytes but not on B lymphocytes (Maggi, 1997). Approximately, 10% of CD8+ T cells and 18% of CD4+ T cells in circulating human blood express NK-1R (Torres and Johnson, 1997). Similarly, in rodents, T cells located in the spleen and Peyer's patches express NK-1R, as well as granuloma T lymphocytes in a murine model of schistosomiasis (McCormack et al., 1996; Maggi, 1997). There is evidence that expression of NK-1R in T cells is constitutive (McCormack et al., 1996).

Substance P activates T cells by inducing production of IL-2 (McCormack et al., 1996). Some studies documented the influence of SP to enhance the production of
immunoglobulins, particularly IgA and IgM but not IgG (Goetzl et al., 1995; Torres and Johnson, 1997). This promotion of the humoral response could be related with the expression of NK-R receptors on T cells and macrophages; thereby, the influence could be indirect due to the role of these cells in differentiation and maturation of B lymphocytes (Maggi, 1997; Torres and Johnson, 1997). Nonetheless, there is evidence of direct action of SP on B cells. Substance P alone is not a sufficient stimulus for immunoglobulin production but rather acts synergistically with other agents, e.g., LPS (Maggi, 1997).

The capability to respond specifically to SP could be related to the number of receptors expressed on the surface of the lymphocytes. It has been estimated that circulating T cells contains up to 7,000 sites for SP whereas splenic T and B cells have less than 200 (Garza Jr. and Carr, 1997). Furthermore, it has been proposed that B cells may also respond to SP by an NK-R receptor-independent mechanism. Apparently this mechanism requires relatively higher concentration of the peptide (μM) and could be operated as an additional pathway that employs a GTP binding protein, phospholipase D and MAP-Kinase (Garza Jr. and Carr, 1997). Substance P may act on B cells as a late differentiation cofactor (Maggi, 1997). Other actions of SP on lymphocytes include proliferation for T cells (Goetzl et al., 1995; Torres and Johnson, 1997), and promotion of lymphocyte homing (Goetzl et al., 1995). The latter is probably due to enhanced expression of ICAM-1 on endothelial cells and increased avidity of LFA-1 on lymphocytes (Vishwanath and Mukherjee, 1996).

Similarly, macrophages express the NK-1R and NK-2R receptors (Maggi, 1997). NK-1R receptor is expressed at a basal level in murine peritoneal macrophages but under exposure to LPS it is rapidly upregulated (Maggi, 1997). Alveolar macrophages also upregulate the NK-1R receptor after an immune-mediated lung inflammatory response (Kaltreider et al., 1997). As was already mentioned, macrophages are also a source of SP and its expression, particularly in alveolar macrophages, is upregulated by LPS (Killingsworth et al., 1997) or by an antigenic challenge in hypersensitized animals (Kaltreider et al., 1997).

Substance P stimulated macrophages can display a pro-inflammatory response that includes production of arachidonic acid metabolites, augmented oxidative metabolism and production of IL-1, IL-6 and TNF-α (Torres and Johnson, 1997). Apparently, the profile of the immune and inflammatory response depends on the
presence of NK-1R or NK-2R. In this way macrophages regulate their own function, and that of neighboring cells, in an autocrine and paracrine manner (Maggi, 1997).

Langerhans cells in the epidermis are in close association with peptidergic sensory nerve fibers and specifically bind SP. With this evidence it can be presumed that SP influences the epidermal immune response; apparently, by inhibiting the capability of Langerhans cells to present antigens (Stanieck et al., 1997). In the lung, dendritic cells are in close proximity to unmyelinated nerve fibers and bind SP. Dendritic cells display chemotactic activity toward graded concentrations of SP in vitro and SP promotes the accumulation of dendritic cells in vivo, but SP has no apparent effect on the antigen-presenting functions of dendritic cells (Kradin et al., 1997). More recently it has been demonstrated that dendritic cells produce SP and may stimulate T cell proliferation (Lambrecht et al., 1999).

NK-1R receptor has also been recognized in endothelial cells and granulocytes, particularly in neutrophils and eosinophils; thereby, these cells are both sources and targets for the proinflammatory and immunomodulatory effects of tachykinins (Killingsworth et al., 1997; Maggi, 1997; Rameshwar and Gascón, 1997). These cells are considered targets for therapeutic regulation of the inflammatory effects of tachykinins. From studies in vitro it has been demonstrated that tachykinins influence several actions of granulocytes, including chemotaxis, phagocytosis, augmented oxidative metabolism and generation of derivatives from the arachidonic acid, improve their antibody-dependent cell-mediated cytotoxicity, and favor the synthesis and release of IL-8 (Maggi, 1997; Torres and Johnson, 1997). However, most of the studies have revealed that larger concentrations (μM) of tachykinins, SP in most cases, are required in order to observe these effects (Maggi, 1997; Torres and Johnson, 1997). These tachykinin concentrations are probably far from those expected during physiological conditions in neurogenic inflammation (Maggi, 1997; Torres and Johnson, 1997). Nonetheless, the expression of adhesion molecules, an essential step for neutrophil extravasation and migration, has been shown to occur by the influence of SP on both PMNs and endothelial cells (Baluk et al., 1995). The expression of E-selectin, P-selectin and ICAM-1 was demonstrated in several lines of endothelial cells under the influence of SP whereas β2 integrins are expressed on PMNs (Maggi, 1997). Apparently, the adhesion of PMNs to SP-activated endothelial cells is mediated by a protein kinase C mechanism dependent on NK-1R (Tominaga et al., 1999).
The evidence of the role of a specific receptor for SP (NK-1R) is widely supported but many other factors could also have a direct influence on PMNs during the acute inflammatory response. In this respect, the role of NK-1R receptor is essential to initiate and promote the exudation of neutrophils into the alveolar space in a model of immune-complex alveolitis, because disruption of the gene expressing NK-1R results in a protective effect (Bozic et al., 1996). This observation confirms the role of NK-1R during neurogenic inflammation but the possibility of other stimuli involved, like complement activation and the consequent activation of MCs, is evident. Activation and release of pro-inflammatory mediators from other cells (macrophages and MCs) probably provide the major stimulus for granulocyte migration into the tissues during acute inflammation. However, the role of SP must be considered because NK-R receptors are present in all of the cellular components of the inflammatory response: endothelial cells, granulocytes, macrophages, and MCs. Furthermore, all, but MCs, express the PPT-A gene (Maggi, 1997).

There are several morphological studies that support the concept of a close relationship between MCs and sensory nerve endings containing SP and CGRP. This close proximity has been demonstrated in thymus, lymph nodes, dura mater, joints, airways, urinary bladder, intestine, and skin (Maggi, 1997; Metcalfe et al., 1997). Furthermore, during processes of chronic inflammation, there are more MCs and their contacts with nerve fibers increases (Metcalfe et al., 1997). In some cases, such as psoriatic lesions in humans, these increased contacts between nerve fibers and MCs are predominantly with SP and CGRP fibers (Naukkarinen et al., 1996).

As was indicated, MCs are one of the most important components of neurogenic inflammation. The influence of SP on MCs occurs through the specific receptor NK-R1 (Okada et al., 1999; Suzuki et al., 1999), and SP induces MC activation and degranulation at μM concentrations (Maggi, 1997; Metcalfe et al., 1997). Moreover, NKA also induces MC degranulation (Cross et al., 1997). Nonetheless, there is evidence that SP-induced MC degranulation could also involve an alternate mechanism. Physiological concentrations of SP (and NKA) may exert their effect by priming MCs through their NK-1R, making them more sensitive to degranulation via a tachykinin receptor-independent mechanism, like an electric field stimulatory effect (Hua et al., 1996).

More recently a new role of MCs in inducing neurogenic inflammation has been recognized. This mechanism includes MC-tryptase acting as signaling molecule on
specific PAR2 which are expressed in sensory nerves that release neuropeptides after activation (Brain, 2000). Steinhoff et al. (2000) have demonstrated co-localization of PAR2 and CGRP and SP in a large proportion of primary spinal afferent neurons. Furthermore, the edema resulting from employing PAR2 agonists is markedly inhibited by antagonists of the CGRP-receptor and NK-1R, as well as by ablation of spinal afferent C-fibers (Steinhoff et al., 2000).

From the previous report it is evident that the functional relationship of MCs and sensory nerves is reciprocal. Under physiological conditions MC activity is subjected to a positive control by neuropeptides which favors histamine release. Subsequently, a negative control is exerted by histamine acting on $H_3$ receptors located on sensory nerve endings, inhibiting the release of neuropeptides (Nemmar et al., 1999). Additionally, the neurotrophin NGF is produced by MCs (Leon et al., 1994; Aloe et al., 1999). This MC-production of NGF could explain the inductive effect on neurite formation in neurons maintained in culture under the influence of MCs (Blennerhassett, 1994). It has been suggested that during an inflammatory process, MCs could control the adaptive plasticity of the sensory nerves due to their ability to produce NGF (Leon et al., 1994).

The biological effects of tachykinins are modulated by enzymes expressed on the cell surfaces that degrade the peptide agonists in the proximity of their receptors. These membrane-bound peptidases are neutral endopeptidase (NEP, also called enkephalinase and common acute lymphocytic leukemia antigen, CD10/CALLA), angiotensin converting enzyme (ACE, also called kininase II) (Piedimonte, 1995), and dipeptidylpeptidase IV (DPP IV, also known as CD26) (Covas et al., 1997). The action of these peptidases is more evident by inhibiting the neurogenic inflammatory response in which NEP has a major role (Reynolds et al., 1997). The major source of NEP is the intact epithelium; thereby, epithelial damage may provide indirectly, an enhanced tachykinin effect (Piedimonte, 1995; Reynolds et al., 1997). On the other hand, CD10/CALLA and DPP IV/CD26 are also expressed as surface peptidases on T lymphocytes and probably play a modulatory role in the effects of SP on T cell function (Covas et al., 1997).

The term neurogenic inflammation describes the increase in vascular permeability induced by neuropeptides, mainly SP, released from stimulated sensory nerves. Neurogenic inflammation occurs in many organs and tissues but because the skin and the airways are highly innervated by nerve fibers that contain neuropeptides,
these sites are particularly prone to neurogenic inflammation (Baluk, 1997). Several irritant compounds induce neurogenic inflammation as well as antidromic electrical stimulation of sensory nerves; however, capsaicin has been employed as a valuable tool for induction of neurogenic inflammation (Holzer, 1988; Baluk, 1997). The compound capsaicin is a pungent substance contained in hot peppers of the genus Capsicum that induces release of SP from sensory nerve fibers (Govindarajan and Sathyanarayana, 1991).

During neurogenic inflammation, the stimulation of sensory nerves by capsaicin induces vasodilatation and promotes edema. The latter effect is mediated by the formation of gaps between the endothelial cells at the postcapillary level. Concomitantly, granulocytes (neutrophils and eosinophils) adhere to endothelial cells on postcapillary venules and eventually migrate into the inflamed tissues (Baluk, 1997; Brain, 1997; Maggi, 1997). During the initiation of the inflammatory process the neurogenic component of the inflammatory response is more predominant but later, the participation of other inflammatory mediators (vasoactive amines, cytokines and derivatives of the arachidonic acid) become more relevant (Baluk, 1997). Therefore, SP, the best characterized mediator of neurogenic inflammation, directly elicits an inflammatory response but also contributes indirectly by inducing release of vasoactive compounds from MCs (Baluk, 1997). In addition, tryptase released from degranulated MCs may exert a positive feedback through activation of PAR2 located on sensory nerves inducing a further release of SP and CGRP (Steinhoff et al., 2000).

Capsaicin interacts with specific vanilloid receptors, i.e. vanilloid receptor-1 (VR1), located on the entire membrane of the vanilloid-sensitive neurons (Szallasi and Blumberg, 1999). The VR1 is a vanilloid-operated nonspecific cation channel that, when activated, permits an influx of Na\(^+\), and predominantly Ca\(^{2+}\). This action drives the generation and propagation of an afferent nerve impulse and release of tachykinins (Szallasi and Blumberg, 1999). Because not only vanilloids but noxious heat and low pH also activate the VR1 channel, the current concept is that VR1 functions as a molecular integrator of noxious chemical and physical stimuli. It is possible that heat is the real opener for VR1 and that capsaicin and low pH serve to reduce the heat activation threshold of the receptor (Szallasi and Blumberg, 1999). Although MCs are involved during induction of neurogenic inflammation induced by capsaicin, their role was originally thought to be indirect due to released tachykinins and their consequent
activation (Baluk, 1997). However, recently, it has been determined that MCs also express the VR1 and can be directly affected by capsaicin (Biró et al., 1998).

The effects of capsaicin can be divided into three main types: excitation and release of tachykinins with a consequent acute inflammatory response, followed by a refractory state (desensitization) to a subsequent stimulus. This effect could be reversible if the dose is small and unique. However, if capsaicin is administered during certain periods and/or in large doses, an irreversible desensitization occurs due to neurotoxicity (Holzer, 1988; Baluk, 1997). Mechanisms proposed for capsaicin neurotoxicity include a massive Ca$^{2+}$ influx that leads to swollen mitochondria and activation of calpain, a Ca$^{2+}$-dependent protease; also, the large influx of Na$^+$ accompanied by a passive movement of chloride resulting in accumulation of water and osmotic lysis has been proposed. Another mechanism postulates that NGF deprivation causes neurotoxicity in neonatal animals because capsaicin blocks the intra-axonal transport of macromolecules, including NGF (Fox, 1995). In this regard, NGF has an important role in development and maintenance of neurons during neonatal life and could explain the high sensitivity of the newborn to capsaicin (Fox, 1995). In addition, there is an NGF-responsive element required for expression of the PPT-A gene and thus, in absence of NGF, SP (and NK-A) expression is down-regulated (Szallasi and Blumberg, 1999). More recently it has been reported that the neuronal damage induced by capsaicin in newborn rats occurs mostly by apoptosis and that the mechanism depends, at least partially, on deprivation of NGF (Sugimoto et al., 1998).

Evidence obtained from the use of tachykinins and the selective blockage of their receptors have demonstrated that vascular and airway smooth muscle cells and submucosal glands in the respiratory tract express NK-1R and NK-2R (Piedimonte, 1995). In the respiratory tract SP promotes ciliary beating, mucous secretion, pulmonary airway constriction, and an increase in the permeability of the lung microvasculature (Otsuka and Yoshioka, 1993; Piedimonte, 1995). In addition, it has been suggested that SP may modulate epithelial cell proliferation during lung development (Sunday, 1997). On the other hand, SP and NKA have the capability to induce proliferation of lung fibroblasts (Harrison et al., 1995).

From experiments in animal models of asthma, it has been generally accepted that tachykinins, particularly SP, might have a role in its pathogenesis (Lundberg, 1995; Piedimonte, 1995; Reynolds et al., 1997). The models propose that the tachykinin-
degrading enzymes (NEP, ACE) are decreased due to epithelial damage; this could result in an exaggerated and uncontrolled activation of the otherwise physiological mechanisms dependent on tachykinins. Another explanation could be an increase in tachykinin concentration due to augmented content of neuropeptides in nerve terminals or proliferation of the SP-containing nerves. Similarly, it has been proposed that a neurogenic overreaction could occur due to augmented expression of NKR (Piedimonte, 1995). Piedimonte and coworkers (1999) demonstrated an exacerbated response to SP in adult rats infected with respiratory syncytial virus due to increased expression of NK-1R in airway epithelium and walls of blood vessels; conversely, the NEP activity was not significantly affected. These results could explain why some respiratory viruses potentiate neurogenic inflammation and exacerbate asthma in children (Piedimonte, 1995).

In animal models of asthma the allergen challenge causes an immediate response characterized by airway smooth muscle contraction due to MC-derived mediators, mainly histamine and leukotrienes. Subsequently, during the delayed phase, interstitial edema and mucous secretion occurs as the result of the inflammatory response (Mashito et al., 1999). Apparently, neurogenic inflammation has a more important role during the delayed rather than the immediate asthmatic response (Mashito et al., 1999). It has been proposed that bradykinin may cause neurogenic inflammation during the delayed response due to a potent stimulatory action on C-fibers inducing them to release SP (Mashito et al., 1999). Conversely, in animal models of chronic bronchitis, the role of SP nerve fibers appears protective and limits or delays the inflammation occurring during chronic airway insult, probably as an adaptive response (Drazen et al., 1999; Long et al., 1999).

The prevailing concept of neurogenic inflammation in the lung is that SP and other neuropeptides are released from sensory nerve fibers that have their cell bodies located mainly in the nodose ganglion of the vagus with lesser numbers in the thoracic dorsal root ganglia (Baluk, 1997; Reynolds et al., 1997). Nonetheless, it has been convincingly demonstrated that airway (intrinsic) ganglion neurons in the lung produce SP and also express the NK-1R (Pérez Fontán et al., 2000). Indeed, syngeneic lung grafts, which lack (extrinsic) peptidergic sensory fibers, are able to mount an immune-complex mediated inflammatory response in the lung that depends on NK-1R activation (Pérez Fontán et al., 2000). Therefore ganglia-released tachykinins may act in a
paracrine and autocrine fashion to regulate neurogenic inflammation in the lung (Pérez Fontán et al., 2000).

References


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CHAPTER 3. REDUCTION OF PULMONARY MAST CELLS IN AREAS OF ACUTE INFLAMMATION IN CALVES WITH MANNHEIMIA (PASTEURELLA) HAEMOLYTICA PNEUMONIA

A paper accepted for publication (12/20/99) in Journal of Comparative Pathology


Summary
Mast cells in the left cranial pulmonary lobe of colostrum-deprived neonatal calves were quantified 2 and 6 h after intrabronchial inoculation with Mannheimia (Pasteurella) haemolytica A1. The mast cells were detected (1) immunohistochemically with a mouse anti-human mast cell tryptase monoclonal antibody, and (2) by metachromatic staining with low pH toluidine blue. A greater number of mast cells was demonstrated by the second method than by the first. At 6 h after inoculation, but not at 2 h, the number of mast cells was significantly reduced at the site of the main lesions. Treatment of calves with a sialyl Lewis mimetic (TBC1269) did not appreciably affect the results at 6 h.

Introduction
Apart from their role in the IgE-mediated immune response, the relevance of mast cells in non-specific pulmonary defence mechanisms is being recognized increasingly (Abraham et al., 1997). In the respiratory tract, mast cells are essential for bacterial clearance (Malaviya et al., 1996). Furthermore, mast cells produce an array of cytokines and vasoactive compounds that mediate the acute inflammatory response (Malaviya et al., 1996; Abraham et al., 1997; Metcalfe et al., 1997).

Pasteurella haemolytica A1, the bacterium most frequently isolated from natural cases of pneumonic pasteurellosis in cattle (Whiteley et al., 1992), has been recently reclassified as Mannheimia haemolytica (Angen et al. 1999). The pathogenesis of acute lung injury in bovine pneumonic pasteurellosis is complex and the inflammatory cells and their products have not been fully characterized (Whiteley et al., 1992). Few studies have assessed the role of mast cells in this pneumonic syndrome. However, Adusu et al. (1994) reported that mast cells recovered from cattle infected experimentally with M. haemolytica release increased amounts of histamine in vitro; and M. haemolytica
leukotoxin and lipopolysaccharide both cause an increase in the spontaneous release of histamine from isolated bovine lung parenchyma (Saban et al., 1997).

Although the distribution and quantification of mast cells in the bovine lower respiratory tract have been described (Chen et al., 1990; Küther et al., 1998), there have been no studies on the influence of the acute inflammatory response induced by *M. haemolytica* on the numbers of mast cells in the bovine lung. Kimman et al., (1989), however, reported a reduced number of mast cells in the lungs of cattle during the acute phase of natural infection with bovine respiratory syncytial virus. Cheville (1994) suggested that the number of recognizable mast cells decreases during an acute inflammatory response due to their degranulation.

The aim of the present study was to investigate (1) the effect of *M. haemolytica* infection on the number of pulmonary mast cells, the work of Abraham et al. (1997) having already suggested that such cells react to gram-negative bacteria, and (2) the effect of a synthetic analogue (TBC1269) of sialyl Lewis (the natural ligand of the selectin family of leucocyte adhesion molecules), which competes with sialylated oligosaccharides, inhibiting the adhesion of selectins in vitro (Kogan et al., 1998), and might therefore exert an in vivo effect on mast cells.

**Materials and Methods**

*Animals and Treatments*

Colostrum-deprived, Holstein male calves aged 1-3 days were obtained from Iowa State University Dairy Farms and maintained in isolation rooms in accordance with the methods approved by the American Association for Accreditation of Laboratory Animal Care and the University Animal Care Review Committee. The animals were randomly assigned to five groups. Group 1 (three control animals) which received pyrogen-free saline, were killed at 2 h post-inoculation. Group 2 (four animals) were inoculated with $10^9$ colony-forming units (cfu) of *M. haemolytica* and killed at 2 h post-inoculation. Group 3 (three control animals) which received pyrogen-free saline, were killed at 6 h. Group 4 (four animals) were inoculated with $10^9$ cfu of *M. haemolytica* and killed at 6 h post-inoculation. Group 5 (four animals), which received TBC1269 (Texas Biotechnology Corp., Houston, Texas) in pyrogen-free saline (0.9%) intravenously (25 mg/kg) 30 min before and 2 h after inoculation with $10^9$ cfu of *M. haemolytica*, were killed at 6 h post-
inoculation. Euthanasia was carried out with an intravenous overdose of sodium pentobarbital.

**Intrabronchial Inoculation and Collection of Tissues**

Saline and *M. haemolytica* suspension, in volumes of 5 ml, were deposited in the left cranial bronchi by fiberoptic bronchoscopy as previously described (Brogden et al., 1995; Ackermann et al., 1996). Samples of lung from the animals receiving *M. haemolytica* inocula were collected from the site of the inoculum deposition (site 2) and from two adjacent sites, cranial (site 1) and caudal (site 3) to the lesion. Equivalent samples were collected from the control animals that had received saline. The samples were fixed in buffered 10% formalin.

**Histological and Immunohistochemical Examination**

The samples were embedded in paraffin wax, sectioned (4 μm) and stained with haematoxylin and eosin (HE), Giemsa, astra blue, toluidine blue, low pH toluidine blue, and immunohistochemically for tryptase. Except for HE, which was employed for evaluation of histological changes, the stains were used for the recognition of mast cells. Low pH toluidine blue and tryptase staining were carried out as described by Chen et al. (1990) and Küther et al. (1998), respectively.

Immunohistochemical examination was made with a primary monoclonal mouse antibody (anti-human skin mast cell tryptase) recommended for formalin-fixed, paraffin wax-embedded tissue sections (Mast Cell, AA1; Dako, Carpinteria, California). Briefly, the slides were heated in an oven for 30 min at 58°C, dewaxed in xylene and treated with a succession of graded alcohols. Subsequently, they were subjected to an antigen retrieval process which included heating the slides to boiling (c. 1.5 min) in citrate buffer (pH 6.0) followed by an extended heating (c. 95°C) for 10 min. They were then pre-digested with 0.1 % protease XIV (Sigma, St Louis, Missouri) at 37°C for 5 min. The primary antibody was applied at a 1 in 50 dilution and the slides were incubated for 1 h at room temperature. The secondary antibody, affinity-purified biotinylated goat anti-mouse (KPL, Gaithersburg, Maryland), was employed at 1 in 200 dilution and the slides were incubated for 30 min. The procedure included a 30- min treatment with supersensitive alkaline phosphatase (BioGenex, San Ramon, California) applied followed by two 30-min applications of Histomark Red (KPL, Gaithersburg, Maryland). All of these
steps were carried out in an OptiMax® Plus automated cell staining system (BioGenex). The slides were counterstained with haematoxylin for 20 sec, dehydrated through alcohols, cleared in Pro-Par (Anatech Ltd. Battle Creek, Michigan) and coverslipped. Negative controls omitting the primary antibody were included.

Procedure for Counting Mast Cells and Statistical Analysis
Mast cells were counted in 20 high-power (x 400) fields (equivalent to c. 3.694 mm²) and expressed in cells/mm², as described by Chen et al. (1990). The fields were randomly selected on each slide with no premeditated intention to avoid empty spaces except for the lumina of large airways or large blood vessels. A first counting procedure included the three sites (1, 2, and 3) in infected and control animals. There were usually two slides from each site, each slide bearing two lung sections, but only a single section, randomly selected, from each of sites 1-3, was used; the result for each animal was the mean value obtained from the three sites. In a second counting procedure sites 1 and 3 were excluded, mast cells being counted only in site 2; average values were obtained from counting three sections except in two animals from which only two sections were available.

The data were analysed in a completely random design with the model $Y_{ij} = \mu + r_i + a_{ij}$. Subsequently, a Tukey’s w procedure was used for specific comparisons (Steel et al., 1997).

Results
Gross Lesions and Histopathology
Macroscopically, all of the animals inoculated with M. haemolytica (groups 2, 4 and 5) showed characteristic lesions of pneumonic pasteurellosis, including consolidation with zones of haemorrhage and variable amounts of fibrin on the pleural surface. These lesions ranged from moderate to severe and were always confined to the site of deposition of the inocula. Animals killed at 6 h post-inoculation had the more severe changes. The main lesions, which varied in diameter from 4 to 8 cm, were confined to site 2; the adjacent areas (sites 1 and 3) showed mild congestion and oedema. Control animals (groups 1 and 3) had no lesions. Microscopically, site 2 in the lungs of the infected animals had a moderate to severe suppurative or fibrinosuppurative bronchopneumonia and extensive areas of alveolar oedema and haemorrhage; these
were multifocal, the foci sometimes coalescing. The lesions in the adjacent sites 1 and 3 were characterized by interstitial and alveolar oedema with neutrophils within the interstitium and bronchioles and adjacent alveolar spaces; these lesions were considered mild and had a diffuse distribution with interspersed areas of normal tissue. The control animals showed mainly normal tissue with some discrete areas of thickening of the alveolar septa due to interstitial oedema. There was no evidence of inflammatory response.

**Mast Cells**

The criteria for optimal detection of mast cells were (1) distinctive staining, enabling mast cells to be recognized at medium (x 100) power, and (2) absence of interfering background. The immunohistochemical procedure for tryptase proved to be the best method for producing distinctive staining, followed by low pH toluidine blue. Mast cells in sections treated immunohistochemically had deeply stained, bright red cytoplasm (Fig. 1B, inset), whereas low pH toluidine blue gave the characteristic metachromatic appearance of mast cell cytoplasm (Fig. 1A and B). When the two procedures were used on replicate sections from randomly selected animals, higher numbers of mast cells were detected ($P<0.01$) with the low pH toluidine blue stain. The analysis for comparison was a Student's t test, assuming independent samples and unequal variances (Steel et al., 1997). Tryptase staining yielded a result of $0.507 \pm 0.394$ mast cells/mm$^2$ (mean ± SD; $n=8$), whereas low pH toluidine blue yielded $5.210 \pm 2.655$ mast cells/mm$^2$ (mean ± SD; $n=8$). The latter method was therefore chosen for comparing the different animal groups.

In the comparison made by the first counting procedure (average of values from sites 1-3) analysis of variance (ANOVA) indicated differences between the groups of animals but not at a statistically significant level ($P>0.05$) (Table 1). However, the comparison made by the second counting procedure (average of values from site 2) revealed highly significant differences between groups ($P<0.005$). A posterior analysis of the data made with Tukey's $w$ procedure (Steel et al., 1997) demonstrated that calves inoculated with *M. haemolytica* and killed 6 h later (Groups 4 and 5) had reduced numbers of mast cells as compared with controls (Groups 1 and 3) ($P<0.05$). Calves in Group 2, also inoculated with *M. haemolytica* but killed 2 h later, also had reduced
numbers of mast cells but the reduction was not statistically significant ($P>0.05$). Differences were not detected between the infected groups (2, 4 and 5) (Table 2).

**Discussion**

The decreased number of mast cells in the lung of calves inoculated with *M. haemolytica* was restricted to the site of the main lesions. This decrease was statistically significant at 6 h post-inoculation but not as early as 2 h. Mast cells were particularly scarce in areas with severe damage, characterized by fibrinopurulent or fibrinohaemorrhagic pneumonic lesions. However, when less affected areas adjacent to the site of severe lesions were included in the comparisons, the numbers of mast cells were reduced but not to a statistically significant level. This suggests that the disappearance of mast cells due to degranulation during an acute inflammatory response (Cheville, 1994) is intimately related to the intensity of the lesion and the time of exposure. Furthermore, this confirms the relevant role of mast cells during an acute inflammatory response, particularly in the lung.

It has been postulated that mast cells represent one of the most important means of defence in the lung in both innate and immune responses (Abraham et al., 1997). In a mouse model of pneumonia produced by *Klebsiella pneumoniae*, mast cells were necessary for modulating the lung inflammatory response and bacterial clearance (Malaviya et al., 1996); these effects were mainly attributed to the release of tumour necrosis factor-alpha (TNF-α) from mast cell granules. Additionally, mast cells contain a vast array of pro-inflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-8 (IL-8) (Abraham et al., 1997; Metcalfe et al., 1997). Increased amounts of these cytokines have been demonstrated in the lungs of cattle with pneumonic pasteurellosis (Yoo et al., 1995; Caswell et al., 1998).

Chen et al. (1990) reported that with low pH toluidine blue, formalin and isotonic formalin–acetic acid fixation resulted in similar numbers of stained pulmonary mast cells. The same authors found that the number of mast cells was dramatically lower (c. 12 mast cells/mm$^2$) in 8-day-old calves than in adult cattle. In the present study c.7 mast cells/mm$^2$ were recognized in the controls, but the calves were younger than those examined by Chen et al. (1990).

Mast cell proteases are considered to be the most specific markers for mast cells (Metcalfe et al., 1997; Welle, 1997). Tryptase-positive mast cells form the great majority
 (>95%) of mast cells in the bovine lung (Küther et al., 1998; Jolly et al., 1999). For
demonstration of mast cell proteases, Küther et al. (1998) reported that the use of
Carnoy’s fixative was preferable to formalin fixation, but the difference was not
statistically significant in the lungs of adult cattle; the authors suggested that mast cells
from various tissues exhibited different sensitivity to formalin and that bovine mast cells
were relatively formalin-resistant. However, Küther et al. (1998) employed a polyclonal
rabbit anti-human mast cell tryptase antibody, whereas we used a monoclonal mouse
anti-human antibody. Nonetheless, the results in quality of staining were comparable
but, as already mentioned, the number of cells demonstrated immunohistochemically
was less than that stained by low pH toluidine blue. More recently, Jolly et al. (1999)
reported that a monoclonal mouse anti-human skin mast cell tryptase antibody worked
well in recognizing tryptase-positive mast cells in the bovine lung; however, as in the
present study, higher numbers of mast cells were detected with low pH toluidine blue
stain. Jolly et al. (1999) suggested that one of the reasons for this discrepancy was the
high specificity but lack of sensitivity of this monoclonal antibody; an alternative
possibility was that the type of tryptase produced by bovine mast cells was unable to
react readily with the monoclonal antibody employed.

Mast cell density and staining properties may be influenced by a number of
factors, including age and health. In the rat, mast cells have not been recognized in the
fetus (Jeffery, 1998) and are very scarce in the newborn (Wilkes et al., 1992). Mast cells
arrive in an immature state of differentiation in the tissues in which they complete their
maturation (Metcalfe et al., 1997). Here they first express proteoglycan (heparin); the
expression of the proteases occurs at the end of the process in mature mast cells
(Welle, 1997). It is possible, therefore, that the numbers of mature mast cells expressing
tryptase were low in the present study because of the young age of the calves.
Metachromatic staining of immature mast cells may be possible as the result of early
expression of a proteoglycan matrix in their granules.

In a model of viral bronchiolitis in rats infected with Sendai virus, increase in the
number of mast cells resulted from both a proliferation of pre-existing mast cells and an
augmented recruitment of circulating mast cell precursors, but a significant increase in
mast cells occurred only after day 14 post-infection (Sorden and Castleman, 1995).
Conversely, during acute episodes of naturally occurring bovine respiratory syncytial
virus infection, the numbers of pulmonary mast cells decrease (Kimman et al., 1989);
furthermore, the lungs of the virus-infected cattle contained an increased proportion of degranulated mast cells. Our observations were compatible with the results of Kimman et al. (1989).

Since the synthetic compound TBC1269 is a potent inhibitor of selectin-mediated cell adhesion \textit{in vitro} (Kogan, et al., 1998), we suspected that reducing the infiltration of neutrophils would have an effect on mast cell degranulation. This assumption is supported by recent work by Befus et al. (1999) who demonstrated that neutrophil defensins induce mast cell degranulation. However, the decreased numbers of mast cells in TBC1269-treated animals (Group 5) were not significantly different from those animals receiving the bacteria alone (Groups 2 and 4). Thus, TBC1269 did not prevent the initial degranulation of mast cells. Similarly, in a mouse model of mast-cell dependent inflammation in the skin, degranulation of mast cells was not prevented in mice genetically incapable of producing P- and E-selectins (de Mora et al., 1998).

**Acknowledgments**
The authors thank B. Smith for technical assistance, Drs Z. Radi and J. Caverly for the collection of tissues, Dr M. M. Welle for suggestions and comments concerning the immunohistochemical procedure, and Dr S. D. Sorden for advice on the procedure for counting mast cells. This work was supported in part by USDA/NRI Project # 970-2654. R. Ramírez-Romero is a graduate student receiving financial support from Universidad Autónoma de Nuevo León and CONACYT, México.

**References**


for the involvement of complement and mast cell mediators. *American Journal of Veterinary Research, 50*, 694-700.


Figure 1. Sections of lung corresponding to site 2, the site with the main lesion in an animal that received *M. haemolytica* (A) and its equivalent in a control that received saline (B), both killed at 6 h post-inoculation. In picture A, there is a single mast cell partially degranulated in an area with extensive consolidation. Low pH toluidine blue. x400. In picture B, there are several mast cells and no tissue damage. Low pH toluidine blue. x200. The inset shows a single tryptase-positive mast cell. Immunolabelling. x400.
Table 1
Numbers of mast cells (means obtained from sites 1-3) in calves of Groups 1-5

<table>
<thead>
<tr>
<th>Individual</th>
<th>1 (controls; n=3)</th>
<th>2 (infected; n=4)</th>
<th>3 (controls; n=3)</th>
<th>4 (infected; n=4)</th>
<th>5 (infected and TBC1269-treated; n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h after inoculation</td>
<td>6 h after inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>calves of Groups</td>
<td>calves of Groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.57</td>
<td>8.84*</td>
<td>7.12</td>
<td>4.96</td>
<td>4.14</td>
</tr>
<tr>
<td>2</td>
<td>6.58</td>
<td>2.25</td>
<td>6.49</td>
<td>4.14</td>
<td>3.66</td>
</tr>
<tr>
<td>3</td>
<td>4.60</td>
<td>3.99</td>
<td>5.95</td>
<td>2.25</td>
<td>3.03</td>
</tr>
<tr>
<td>4</td>
<td>….</td>
<td>4.78</td>
<td>….</td>
<td>2.52</td>
<td>1.92</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>6.25±1.51</td>
<td>4.96±2.79</td>
<td>6.52±0.58</td>
<td>3.46±1.29</td>
<td>3.18±0.95</td>
</tr>
</tbody>
</table>

* This animal showed the least severe lesions of all infected animals.
† These values were not significantly different from each other (ANOVA; P>0.05).
Table 2
Numbers of mast cells (means obtained from site 2) in calves of Groups 1-5

<table>
<thead>
<tr>
<th>Individual</th>
<th>2 h after inoculation</th>
<th>6 h after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>calves of Groups</td>
<td>calves of Groups</td>
</tr>
<tr>
<td></td>
<td>mean numbers of mast cells/mm² in site 2 at</td>
<td></td>
</tr>
<tr>
<td></td>
<td>calves of Groups</td>
<td>calves of Groups</td>
</tr>
<tr>
<td></td>
<td>2 h after inoculation</td>
<td>6 h after inoculation</td>
</tr>
<tr>
<td>1</td>
<td>8.11</td>
<td>9.06*</td>
</tr>
<tr>
<td>2</td>
<td>8.28</td>
<td>1.98</td>
</tr>
<tr>
<td>3</td>
<td>5.27</td>
<td>3.80</td>
</tr>
<tr>
<td>4</td>
<td>....</td>
<td>4.05</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>7.22±1.69</td>
<td>4.72±3.03</td>
</tr>
</tbody>
</table>

*This animal showed the least severe lesions of all infected animals.
†Values not sharing a superscript letter are significantly different (Tukey’s w test; P<0.05).
CHAPTER 4. DIHYDROCAPSAICIN TREATMENT DEPLETES PEPTIDERGIC NERVE FIBERS OF SUBSTANCE P AND ALTERS MAST CELL DENSITY IN THE RESPIRATORY TRACT OF NEONATAL SHEEP

A paper submitted for publication (12/13/99) to *Regulatory Peptides*

Rafael Ramírez-Romero, Jack M. Gallup, Ioana M. Sonea, Mark R. Ackermann

Abstract

In the present study we administered dihydrocapsaicin (DHC) to neonatal lambs to deplete C-fibers of neuropeptides. We measured the density of substance P (SP)-fibers in nasal septum to assess the effectiveness of the treatment at 3, 9, and 21 days. The numbers of mast cells in the upper and lower respiratory tract were determined at the same time points and histamine content was determined from lung tissue. DHC treatment depleted SP-fibers for up to the 21 day time point. This depletion was estimated as 85% in comparison with controls. In vehicle-treated lambs, the density of SP-fibers decreased progressively with age, but not to the degree of DHC-treated lambs whose SP-fibers were depleted from the initial 3-day measurement. In both vehicle- and DHC-treated lambs, numbers of mast cells increased progressively with time; however, the density of mast cells was augmented in the entire respiratory tract of DHC-treated animals. Apparently, DHC treatment exerts a single and initial effect in increasing mast cells whereas time maintains a continuous influence; both factors exert their influence independently. Despite large numbers of mast cells in DHC-treated animals, histamine content in the lung had similar levels as controls. Our study provides fundamental data for a better understanding of conditions that may influence defense mechanisms dependent on the mast cell-SP nerve axis in the respiratory tract.

1. Introduction

The relevance of mast cells in the defense mechanisms of the respiratory tract has been increasingly appreciated [1]. In the lung, mast cells are necessary for modulating the acute inflammatory response and clearance of bacteria [2]. Furthermore, mast cells are able to present antigens to lymphocytes favoring an interaction that modulates the immune response [3]. On the other hand, mast cells are closely related, physically and
functionally, to sensory nerve fibers in the respiratory tract [4-6]. The mast cell-nerve relationship, considered the prototype of the neuroimmune interaction, is provided by the neuropeptide substance P [7]. Substance P (SP) is a tachykinin released mostly from unmyelinated sensory nerves (C-fibers) that acts through a specific receptor on mast cells (NK-1 receptor), inducing their degranulation [7]. However, the communication is reciprocal and histamine released from mast cells can act on specific (H3) receptors located on C-fibers [6,8-10]. Abnormalities in this nerve-mast cell circuit have been postulated to occur in pathological states such as asthma [6,7].

Capsaicin and dihydrocapsaicin (DHC), the most potent of the capsaicinoids, are natural compounds present in hot peppers of the genus Capsicum, which confer the pungent taste to these fruits [11]. Capsaicinoids act through receptors, i.e. vanilloid receptor-1, located mostly in small primary sensory (afferent) neurons and their C-fibers, inducing them to release neuropeptides such as SP [12]. For this reason, capsaicin has been widely used to study the mechanisms of neurogenic inflammation [13,14]. The effects of capsaicin can be divided in three main types: a) excitation and release of tachykinins with a consequent acute inflammatory response, followed by b), a refractory (desensitization) and reversible state if the dose employed is small. However, if capsaicin is administered in large doses c), an irreversible desensitization due to neurotoxicity occurs [13]. Neonatal animals are particularly susceptible to the neurotoxic effects of capsaicin [11,13]. Capsaicin and DHC share the same pungent and neurotoxic properties [11].

Sheep have been proposed as a suitable model to study allergen-induced asthma in human beings [15,16] and tachykinins have a crucial influence on the response to allergen by sensitized sheep [16]. The density and location of mast cells in the lower respiratory tract of sheep [15] may influence the intensity of this response. In this report we studied the neurotoxic effects of DHC in neonatal lambs by assessing the area covered by SP fibers in the nasal mucosa; additionally, we determined the density of mast cells in the upper and lower respiratory tract and the histamine content of the lung. Our results provide fundamentals for the understanding of basic conditions that may influence the defense mechanisms dependent on the mast cell-substance P nerve axis in the respiratory tract.
2. Material and Methods

2.1. Animals and treatment protocols

Eighteen lambs, of either sex and mixed breed, 3 to 5 days old, were obtained from Iowa State University, Laboratory Animal Resources. The animals were maintained in isolation rooms in accordance with the methods approved by the American Association of Laboratory Animal Care. The experimental protocol was previously approved by the Iowa State University Animal Care Review Committee. The animals weighed 5.30 ± 0.38 kg (mean ± SEM) on the day of arrival. After 24 h, these animals were randomly assigned to two large groups; one group receiving DHC while the other received the vehicle without the compound. Subsequently, these groups were subdivided in accordance to the time of sacrifice at 3, 9, and 21 days after DHC or vehicle administration. This arrangement gave six groups, each one with three animals (see below the experimental design in section 2.6).

DHC (8-methyl-N-vanillylnonanamide; Sigma Chemical Co., St. Louis, MO) was dissolved in a 25 mg/ml solution composed of 80% pyrogen free saline (NaCl 0.9%), 10% Tween 20 (Sigma Chemical Co., St. Louis, MI) and 10% ethanol. The compound was initially mixed in ethanol to obtain its complete dissolution. The animals received DHC at a dose of 50 mg/kg or the vehicle alone, by several subcutaneous injections into the nape of the neck. Prior to DHC injections, the animals were deeply sedated with xylazine (0.6 mg/kg, intramuscularly). The animals received banamine (10 mg/lamb, intramuscularly) 45 min before DHC/vehicle injection to ameliorate the pain resulting from administration of the pungent compound. Subsequently, the animals also received isoproterenol (0.2 μg/kg, intramuscularly) to counteract the bronchoconstriction induced by DHC.

2.2. Euthanasia and collection of samples

Animals were euthanized with an overdose of sodium pentobarbital. The samples collected including nasal septum, ventral concha, trachea (cranial part), trachea (caudal part), and lung. The lung corresponded to two parts, one including intrapulmonary bronchi and the other a distal part of the lung. The samples were collected in duplicate and were fixed by immersion in 10 % neutral buffered formalin (48 h at room temperature) or in Carnoy's fixative (18 h at 4°C). The samples were processed routinely, embedded in paraffin wax, and sectioned at 4 μm. Additionally, similar
samples were collected in cryo-vials (Nunc CryoTube, Nalge Nunc Int. Naperville, IL) and maintained at -80°C for future histamine determinations.

2.3. Low pH toluidine blue and immunohistochemistry procedures

Tissues fixed in Carnoy's fixative were stained with low pH toluidine blue in order to recognize mast cells. The procedure yielded a pale bluish background and mast cells appeared deeply blue. This procedure has been employed to stain mast cells in the human lung [17].

The immunohistochemistry procedure for substance P was a modification from those methods described by Sakamoto et al. [18] and Sonea, et al. [19,20], in the brain and the lung, respectively. Substance P immunoreactivity was determined in slides from formalin-fixed tissues employing a rabbit anti-substance P primary antibody (Peninsula Laboratories, Inc., San Carlos, CA). Briefly, the slides were heated in an oven for 30 min at 58°C and deparaffinized in xylene and a succession of graded alcohols. Subsequently, they were subjected to peroxidase blocking by immersing the slides in a solution of 0.9% H₂O₂ during 40 min. After several rinses in buffer (Wash Buffer, BioGenex, San Ramon, CA) the slides were incubated at room temperature during 20 min with a solution containing 10% normal goat serum and 3% bovine serum albumin (Elisa-grade BSA, Sigma Chemical Co., St. Louis MO) dissolved in buffer. Then, the slides were incubated in an humidified and sealed chamber at 4°C during 72 h with the primary antibody diluted 1:10,000 in antibody diluent (Common Antibody Diluent, BioGenex, San Ramon, CA). The slides were rinsed several times and subjected to incubation at room temperature for one hour with the secondary antibody (affinity-purified biotin-labeled, Goat anti-Rabbit IgG (H+L), Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:200. After several rinses, the peroxidase-conjugated streptavidin reagent (Super Sensitive HRP, BioGenex, San Ramon, CA) was added (undiluted) for incubation at room temperature for one hour. Finally, slides were rinsed again and a metal enhanced DAB substrate that utilizes cobalt chloride and nickel chloride (ImmunePure® metal Enhanced DAB Substrate Kit, Pierce Chemical Co., Rockford, IL) was added for 15 min. The slides were then rinsed thoroughly in ultrapure water and, without counterstaining, they were dehydrated through alcohols, cleared in xylene, and coverslipped in Permount (Sigma Chemical Co.). The positive reaction at the site of antigen-antibody-peroxidase complex produced a dark brown/black
precipitate. Negative controls in which the primary antibody was omitted gave no immunostaining. Small intestine sections (ileum) from sheep were used as positive controls because these areas contain myenteric and submucosal plexuses with large numbers of immunoreactive nerve fibers and nerve cells bodies. Because we did not characterize the peptide reacting with our primary antibody and a cross-reaction with other related tachykinines (neurokinin A and neurokinin B) was possible, the term substance P-like immunoreactivity (SP-Li immunoreactivity) is used hereafter.

2.4. Quantitation of mast cells and image analysis for substance P-immunohistochemistry

For quantification of mast cells, slides containing two sections of each area of respiratory tract already mentioned were assessed by light microscopy. The slides were randomly assigned and mast cells density was determined by counting the numbers of mast cells present in 50 randomly chosen fields at 400 × magnification. The area provided is equivalent to 5.9 mm² [21]. The values obtained per lamb corresponded to counting the 50 fields in each one of the sections and then averaging these values. The values were expressed in mm².

Only the nasal septum, which is rich in SP-fibers, was used for image analysis of SP-Li immunoreactive fibers. The sections were examined with a Zeiss Axioskop fitted with Neoflur objectives, a 100-W light source, and a Sony DXC-3000A camera. The images were captured with Visilog Softwere (Noesis, St. Laurent, Quebec, Canada) on an SGI 02 workstation (Silicon Graphics, Inc., Mountain View, CA). Subsequently, images were edited on a Micron workstation with Photoshop (Adobe, Mountain View, CA).

For image analysis, the slides were randomly assigned without knowledge of the treatments. Each slide contained two sections of the nasal septum from each one of the animals. Ten fields randomly chosen from each one of the slides were captured. The magnification employed gave the same area (84266.12 μm²). The images containing SP-Li immunoreactive nerve fibers, including positive reaction encountered in nerve bundles, were edited by tracing exclusively the positive immunostained areas, procuring a close approximation to the shape. Subsequently, the images were thresholded for analysis. The values obtained from the ten fields were added; thereby, the value for
each animal was a total of the areas with SP-Li immunoreactivity (expressed in \( \mu m^2 \)) per 0.84 mm\(^2\). The values were adjusted proportionally to be expressed at 1 mm\(^2\).

2.5. Histamine determination

The content of histamine in the lung was measured employing an enzyme immunoassay (Histamine Enzyme Immunoassay Kit, Immunotech, Marseille, France). The lower limit of detection of this assay is 0.5 nM and the specificity is 100% to acylated histamine. Briefly, 15 mg of tissue were placed in a plastic tube containing 150\( \mu \)l of 0.2 N HClO\(_4\). The tissue was homogenized and centrifuged at 10,000 \( \times \) g for 5 min at 4\( ^\circ \)C. Supernatant was collected and added to an equal volume of 1M, pH 9.25 of K\(_2\)B\(_4\)O\(_7\). Subsequently, the sample was centrifuged again and the supernatant collected. Then the samples were acylated as suggested by the manufacturer. A microplate with antibody-coated wells were filled with the tissues' supernatants and incubated for 2 h at 4\( ^\circ \)C with shaking at 350 rpm. The wells were rinsed and the substrate was added for incubation during 30 min at room temperature. The reaction was stopped and the plate was read in an automated spectrophotometer at 410 nm. The histamine content was expressed in ng of histamine/g of wet weight of lung parenchyma.

2.6. Statistical analysis

Mast cell counts obtained from the different parts of the respiratory tract, as well as SP-Li immunoreactive fibers in nasal septum, and histamine content in the lung, were analyzed among the groups employing a completely random design with 2 x 3 factorial arrangement. The model was

\[ Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk} \]

In this model \( \alpha \) had two levels, vehicle- and DHC-treatments; \( \beta \) had three levels, 3, 9, and 21 days of euthanasia after treatments; \( (\alpha\beta) \) represents the interaction, and \( \epsilon \) the error [22]. After demonstrating statistical significance with a minimum of \( P < 0.05 \), the procedure was extended to recognize the simple effects' contribution in interactions \( (\alpha\beta) \) by an \( F \) test and/or the major contributions in levels of time \( \beta \) employing contrasts \( Q = \sum ci Yi \) [22]. The experimental design is presented in Table 1. Additionally, a model of linear regression, \( Yi = \alpha + \beta Xi + \epsilon \), was employed to analyze the areas covered by SP-Li immunoreactive fibers in nasal septa (\( Y \) dependent variable; the means of each group) of DHC- and vehicle-treated lambs and their probable relationship with days of euthanasia (\( X \).
independent variable) [22]. The same procedure was carried out with mast cells in the lung (Y dependent variable; the means of each groups).

3. Results

3.1. DHC administration
Immediately after DHC administration the lambs reacted with apnea lapses followed by rapid shallow breathing. The animals also exhibited swallowing movements. At this time those animals with the most severe reactions were administered an extra doses of xylazine and banamine. After this initial period that lasted approximately 20 min, the animals showed decreasing respiratory abnormalities and the swallowing movements were more common. Four hours later the lambs were conscious, most of them were in sternal position and few were already recuperated. By 6 h all animals appeared normal and had regained their appetites. In the following days the DHC-treated lambs appeared less active than controls but ate similarly to controls. No skin lesions were detected in the site of injection or in other tissues during post mortem examination.

3.2. Substance P-Li immunoreactive fibers depletion in nasal septum
After reviewing all of the slides processed for substance P-immunohistochemistry from different parts of the respiratory tract, it was determined that only those of the nasal septum had enough density of fibers to permit a quantitative comparison between the groups. The SP-Li immunoreactive nerve fibers in the nasal septa of DHC-treated lambs were decreased approximately 85% in comparison with those treated with vehicle (P< 0.005). In vehicle-treated animals the fibers were mostly distributed around submucosal glands and blood vessels (Figs. 1A and C). Also in many places, small fibers reached the epithelial surface. In DHC-treated animals the fibers were scarce and those remaining appear shortened (Figs. 1B and D). Although there was a tendency of reduced area of SP-Li immunoreactive fibers in vehicle-treated animals with age; the factorial experiment did not detect any significant contribution of time (P> 0.05) (Fig. 2). However, the linear regression model demonstrated a negative linear relationship between the area covered by the fibers and the time of euthanasia (P< 0.025; R²= 0.99), confirming a decrease in SP-Li fibers with time. Conversely, in DHC-treated animals, these SP-Li fibers remain depleted with no evidence of linear relationship (P> 0.05) (Fig. 2).
As was indicated, only the nasal septa had sufficient density of fibers to be compared by statistical procedures. Nonetheless, in vehicle treated animals SP-Li immunoreactive fibers were recognized in the different levels of the respiratory tract, particularly in the airways, and similarly associated to submucosal glands and blood vessels. In DHC treated animals, SP-Li immunoreactive fibers were extremely rare.

3.3. Mast cells density in the respiratory tract
The density of mast cells in the respiratory tract was modified by DHC treatment. Those animals receiving DHC had more mast cells in the respiratory tract than the controls treated with vehicle. The difference was statistically significant (P< 0.05 or higher significance levels) for all parts of the respiratory tract studied (nasal septum, cranial and caudal trachea, intrapulmonary bronchus, and lung; Figs. 3, 5, 6, 7, and 8, respectively), except for ventral conchae (P> 0.05) (Fig. 4). Similarly, the influence of time in increased numbers of mast cells was statistically significant, particularly at 21 days (P< 0.05 or higher significance levels), except for trachea (both cranial and caudal) which had values close to significance (P= 0.084 and P= 0.077, respectively). There was no interaction between treatments and time (P> 0.05), except in nasal septum in which this interaction showed significance (P< 0.05). Two representative fields of mast cells in the lung in vehicle- and DHC-treated animals are presented in Fig. 1E and F.

After determining that mast cells in the lung had a marked tendency to increase throughout time, a model of linear regression was applied to both vehicle- and DHC-treated animals. The model was adequate to demonstrate a positive linear regression relationship between number of mast cells and time of euthanasia in vehicle treated animals (P< 0.05; R²= 0.99). However, the same procedure showed a poor linear relationship between these variables (P= 0.079) in DHC-treated animals.

In all sections of the respiratory tract, mast cells were particularly abundant in the lamina propria and submucosa of the airways. Occasionally, mast cells were located subjacent to the epithelial layer. The lung had the highest numbers of mast cells and these were similarly abundant in the intrapulmonary airways as well as in the alveolar septa. Mast cells were also abundant in the adventitia of blood vessels, in the pleura, and in the interlobular septa.
3.3. Histamine content in the lung

After determining that mast cells were most numerous in the lung, the histamine determinations were carried out exclusively in this tissue. The content of histamine in the lung was not modified by DHC treatment. Although DHC treated animals had discrete and minimal increases of histamine at 9 and 21 days, respectively; these were not significant (P> 0.05). Similarly, the higher amount of histamine showed by both DHC and vehicle treated animals by day 21 was not significantly different from groups sacrificed at 3 and 9 days (P> 0.05) (Fig. 9).

4. Discussion

In the present study we recognized SP-Li immunoreactive fibers in the entire respiratory tract, both upper (nasal septum, ventral concha, cranial and caudal trachea) and lower (intrapulmonary bronchi, bronchioles, alveoli). However, only the nasal septum had a high enough density of SP-Li fibers suitable for a parametric comparison. A previous study in hamsters, guinea-pigs, rats, cats, and dogs, also demonstrated that SP fibers are sparse in the alveolar walls [23]. In the lung of horses, SP-Li fibers were more numerous in the large airways of the lung close to the hilus than caudal lobes and peripheral parenchyma [19,20]. In the trachea of sheep, SP fibers were present occasionally [24] whereas in the nasal mucosa of pig they were observed forming a dense network [4]. More recently, in a model of respiratory tract viral infection in rats, it was demonstrated that respiratory syncytial virus enhances the neurogenic inflammation in the upper and lower airways by increasing the SP (NK-1) receptor on target cells [25]. However, the capsaicin-induced neurogenic inflammation was significant only in the extrapulmonary airways of virus-infected rats, whereas the intrapulmonary airways responded to exogenous SP administration [25]. It has been suggested that capsaicin-sensitive C-fibers are more numerous in proximal airways and progressively decreases toward the distal parts [25]. During postnatal maturation of respiratory tract SP-fibers apparently decrease, i.e. it has been observed that SP-Li fibers were more numerous in neonatal foals than in adult horses [20]. We recognized a negative linear relationship between the area occupied by SP-Li immunoreactive fibers and time of euthanasia in vehicle-treated animals, which confirms the previous observation in horses.

It is widely accepted that capsaicin administered to neonatal animals in large doses, permanently depletes SP from primary afferent sensory C-fibers [11,13,26]. This
neurotoxic effect also occurs with DHC [11]. SP depletion occurs because of a massive degeneration of these fibers due to destruction of the corresponding peptidergic neurons [12,26]. It has been estimated that neonatal capsaicin treatment in rats provokes degeneration of up to 90% of unmyelinated primary afferent fibers [26]. Similarly, Carobi [27] found that this treatment destroys 70% of small and medium-sized afferent neurons in the vagal ganglia. In the present study, the area occupied by SP-Li immunoreactive fibers in the mucosa and submucosa of the nasal septum in lambs markedly decreased (85%) in DHC-treated animals in comparison with vehicle-treated controls. This data is in agreement with the previous work in rats [26]. Similarly, our data in vehicle-treated lambs is compatible with the values expressed in length of SP immunoreactive fibers in the nasal mucosa of normal rats [28]. Nonetheless, due to their naturally tortuous pattern with many varicosities, we suggest that data expressed in μm² is more accurate to describe, in parametric terms, the density of SP-Li immunoreactive fibers. Although we did not compare DHC treatment in older sheep, the results suggest that SP-Li fibers depletion is long lasting and probably life-long as has been demonstrated in rats.

Lambs treated with DHC had an augmented density of mast cells in all areas of the respiratory tract studied, except ventral concha. However, mast cell homing and/or proliferation do not appear to be drastically modified by treatment with DHC, because density of mast cells show similar patterns in both DHC- and vehicle-treated animals. Mast cells are scarce in the newborn rat [29] and have a progressive pattern of lung homing and/or proliferation that reach the peak at 18 weeks in mice [30]. This is in agreement with our results that show a major influence of time in the number of mast cells. In the lung, which had the highest numbers of mast cells, the density of mast cells throughout time occurs with a positive linear pattern. Although this linear relationship was decreased in DHC-treated lambs; a linear relationship remains evident. Taking in account values of mast cells in the lung of three 4 month-old sheep employed in a different experiment (mean 134.26 ± 7.84 SEM), and comparing them with a predicted value from our calculated regression in vehicle-treated animals, provide compatible results. In sheep, mast cell numbers most likely continuously increase up to adult age because Chen, et al. [15] reported values of 210 mast cells/mm² in the lung. On the other hand, except for nasal septa, in which an interaction between time and DHC treatment was detected, the two factors behave independently. This means that DHC treatment was a single effect that provoked increased numbers of mast cells but does
not alter their pattern of homing and/or proliferation in the respiratory tract; in both, vehicle- and DHC-treated animals, progressive increases of mast cell density is influenced by time. It is important to mention that conventional mice have increased numbers of mast cells in comparison to specific pathogen-free and germ-free animals in the nasal mucosa [31]. Thereby, increases of mast cells throughout time may reflect a time-related exposition to antigens that naturally challenge mucosal surfaces such as the respiratory tract. In these sites mast cells function as orchestrators of the acute inflammatory response [1].

When capsaicin is administered to adult rats and pigs, there is no modification in the number of mast cells [5]. However, neonatal rats treated with capsaicin have increased numbers of mast cells in the lung [6,32]. The latter is in agreement with our results. It has been suggested that one or more factors released from afferent C-fibers might exert a selective negative control on proliferation of mast cells that is lost after denervation [33]. Alternatively, after capsaicin treatment, the massive release of neuropeptides (SP, neurokinin A, somatostatin, and calcitonin-gene-related peptide among others) which colocalize in C-fibers [12] along with neurotrophins such as nerve growth factor, may promote mast cell proliferation [33]. Additionally, cytokines released from degranulated mast cells (tumor necrosis factor-α, interleukins 3, 4, 5, 8, and 13 among others) may also promote their own proliferation [33]. Nerve growth factor could be present in high amounts during this particular situation because mast cells are also important extraneural producers of this neurotrophin [34]. Nerve growth factor administered to neonatal rats induces an increase in mast cell density in peripheral tissues [34]. In the present study, the increased availability of these factors was probably a single event that occurred after DHC treatment and could explain the initial augment in the numbers of mast cells. Paradoxically, the values of histamine in the lung of lambs were similar in DHC and vehicle treated animals, despite the increased number of mast cells detected. Compatible findings have been reported by Dimitriadou, et al. [6] who encountered similar levels of mast cell proteases and histamine synthesis in capsaicin-treated and control rats, although capsaicin treated rats had higher numbers of mast cells. This was interpreted as an individual mast cell reduction in proteases and histamine synthesis due to capsaicin denervation, and suggests that mast cells require intact nerve fibers in order to express complete activity [6].
Mast cells arrive to the tissues in an immature state and require several factors to complete their maturation in situ [36]. After DHC treatment, the absence or imbalance in these trophic factors could have influenced both, mast cells and their tissue environment, impeding an appropriate histamine (and probably other mast cell products) synthesis as normally occurs in mature mast cells. Conversely, high number of mast cells in certain conditions such as parasitic infections in rats, are accompanied by an increase in mast cell-nerve contacts [35] and an augmented response to neuroinflammatory inducers [36]. Under these conditions, the increased numbers of mast cells correlate with high amounts of histamine and proteases simply because the nerve-mast cell axis is completely functional [6]. It has been proposed that, under physiological conditions, mast cells activity is subjected to a positive control by neuropeptides which favors histamine release. Subsequently, a negative control is exerted by histamine acting on H3 receptors on sensory nerve endings, inhibiting release of neuropeptides [6,8-10]. This regulatory negative-feedback loop was clearly reduced in DHC treated animals.

Acknowledgements

We thank B. Smith and M. Carter for technical assistance. We are also grateful to Professor T.B. Bailey for suggestions and comments about the statistical procedures. This work was supported in part by USDA/NRI Project # 970-2654. R. Ramírez-Romero receives financial support from CONACYT and Universidad Autónoma de Nuevo León, México.

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**Table 1**

Experimental Design

<table>
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<tr>
<td>3 days (b1)</td>
<td>9 days (b2)</td>
<td>21 days (b3)</td>
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Fig. 1. Representative fields of nasal septum (A-D) and lung (E, F) from vehicle- and DHC- treated lambs. A, distribution of SP-Li immunoreactive fibers in close association to submucosal glands in vehicle-treated lamb, killed at 3 days. B, similar area in a DHC-treated lamb killed at the same period showing few shortened fibers that remained after treatment. C, SP-Li immunoreactive fibers around a small artery in a vehicle-treated lamb killed at 3 days. D, similar area in a DHC-treated lamb killed at the same period showing no immunoreactivity. Nasal septum, x 400, immunohistochemistry. E, distribution of mast cells in the lung of a vehicle-treated lamb killed at 21 days. F, a comparable area in a DHC-treated lamb killed at the same period showing an increased number of mast cells. Lung, x 160, low pH toluidine blue.
Fig. 2. Substance P-Li immunoreactive fibers in nasal septum. Values correspond to the area covered by SP-Li immunoreactive fibers in \( \mu m^2/mm^2 \) and are expressed as mean ± SEM for each group. DHC (a2) treatment is different from vehicle (a1) \((P< 0.005)\). There is no influence of factor B \((P> 0.05)\). There is a negative linear regression relationship between the area covered by the fibers and time of euthanasia \((P< 0.025; R^2 = 0.99)\). There is no evidence of linear relationship in DHC treated animals \((P> 0.05)\).
Fig. 3. Mast cells in nasal septum. Values correspond to numbers of mast cells/mm$^2$ and are expressed as mean ± SEM for each group. DHC (a2) treatment is different from vehicle (a1) (P< 0.05). Day 21 (b3) is different from days 3 and 9 (P< 0.005). Factors A and B interact (P< 0.05); the simple effects contributing to this interaction correspond to DHC (a2) and day 21 (b3).
Fig. 4. Mast cells in ventral concha. Values correspond to numbers of mast cells/mm² and are expressed as mean ± SEM for each group. Factor A, treatments, have no influence (P > 0.05). Day 21 (b3) results different from days 3 and 9 (P < 0.005).
Fig. 5. Mast cells in trachea (cranial). Values correspond to numbers of mast cells/mm² and are expressed as mean ± SEM for each group. DHC treatment (a2) is different from vehicle (a1) (P< 0.005). There is no influence of factor B (P> 0.05).
Fig. 6. Mast cells in trachea (caudal). Values correspond to numbers of mast cells/mm² and are expressed as mean ± SEM for each group. DHC treatment (a2) is different from vehicle (a1) (P< 0.005). Factor B has no influence (P> 0.05).
Fig. 7. Mast cells in intrapulmonary bronchus. Values correspond to numbers of mast cells/mm² and are expressed as mean ± SEM for each group. DHC (a2) treatment is different from vehicle (a1) (p< 0.005). Day 21 (b3) results different from days 3 and 9 (P< 0.005).
Fig. 8. Mast cells in lung. Values correspond to numbers of mast cells/mm² and are expressed as mean ± SEM for each group. DHC (a2) resulted different from vehicle (a1) (P< 0.01). Day 21 (b3) is different from days 3 and 9 (P< 0.005). There is a direct linear regression relationship between number of mast cells and time of euthanasia in vehicle treated animals (P< 0.05; $R^2 = 0.99$). DHC treated animals have a poor linear relationship between these variables (P< 0.1).
Fig. 9. Histamine content in the lung. Values correspond to histamine in ng/g wet lung and are expressed as mean ± SEM for each group. There is no influence of factor A (P > 0.05) or factor B (P > 0.05).
CHAPTER 5. MAST CELL QUANTIFICATION AND SUBSTANCE P-LIKE IMMUNOREACTIVITY DURING THE INITIATION AND PROGRESSION OF LUNG LESIONS IN OVINE MANNHEIMIA (PASTEURELLA) HAEMOLYTICA PNEUMONIA

A paper submitted for publication (3/8/00) to Veterinary Pathology

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Abstract. In order to determine the numbers of stained mast cells (MCs) and extent of substance P (SP) immunoreactivity during initiation and progression of Mannheimia (Pasteurella) haemolytica pneumonia, eighteen lambs were inoculated intrabronchially with M. haemolytica or saline and lung tissue was collected at 1, 15, and 45 days post inoculation (n=3, each group). Additionally, the left (non-inoculated) contralateral lungs in bacteria-inoculated animals were collected as controls. At 1 day after bacterial inoculation the lungs had typical M. haemolytica lesions. Stainable MCs were absent in these areas, and histamine content was reduced. Leukocytes infiltrating some of these affected areas, most likely macrophages, were strongly immunoreactive for SP, suggesting an important extraneural contribution to the inflammatory process. At 15 days, MCs remain scarce at sites where lung damage persisted, i.e. pyogranulomatous foci, but become hyperplastic in areas of interstitial damage. Substance P immunoreactivity indicated that not only an extrinsic sensory innervation, but also an intrinsic contribution of pulmonary ganglion neurons, sustains the inflammatory response. By 45 days the fibrosing changes become more defined such as pleural fibrosis, fibrosing alveolitis, and bronchiolitis obliterans. These lungs had increased numbers of MCs; however, the histamine content did not correspond to this increased population. Substance P immunoreactivity appeared only in nerves and was mild. The diminished SP immunoreactivity during the chronic stages of lung damage may correspond to a lack of protective effects elicited by SP, including regulation of inflammatory response and promotion of epithelial repair. This work demonstrates that during the acute stage of pneumonic pasteurellosis (PP) the sites with the most severe lung lesions have reduced numbers of stained MCs and extraneural SP immunoreactivity. Whereas in chronic stages, the areas of epithelial hyperplasia have increased numbers of MCs and SP fibers immunoreactivity is decreased.
Key words: immunohistochemistry; *Mannheimia (Pasteurella) haemolytica*; mast cells; pathogenesis; pneumonic pasteurellosis; acute, subacute, and chronic pulmonary lesions; substance P.

Mast cells (MCs) are necessary for both the acquired and innate defense mechanisms. In the lung, MCs orchestrate both the inflammatory and immune responses. Mast cells produce an array of vasoactive compounds and cytokines necessary to mount the acute phase of inflammatory response; additionally, MCs are able to internalize bacteria and present antigens to immune cells. During the acute inflammatory response in the lung, MCs are required for neutrophil infiltration and bacterial clearance. However, MCs also contain proteases and growth factors that make them an important component in chronic inflammatory responses such as chronic lung diseases.

The unmyelinated C-fibers are an important component of the sensory innervation in the lung. These fibers are in close apposition to MCs, and through the neuropeptide substance P (SP) acting on its specific receptor (neurokinin-1 receptor [NK-1R]) located on the surface of MCs, exert a functional connection to induce MC activation. Conversely, MC-tryptase can exert a positive feedback by acting as a signal molecule, cleaving and activating a proteinase-activated receptor (PAR)-2 located at the surface of sensory nerves, and inducing release of sensory neuropeptides, including SP.

 Substance P is the tachykinin responsible for neurogenic inflammation in the lung and other tissues. Although the main source of SP are unmyelinated C-fibers, this neuropeptide is also normally expressed by small numbers of thin myelinated Aδ afferent fibers. However, during persistent inflammation, additional Aβ fibers, which normally do not express SP, can acquire the capacity to express the neuropeptide. More recently, an important contribution of airway ganglion neurons that produce SP and express NK-1R during immune-mediated neurogenic inflammation in the lung has been recognized. In addition, there are other extraneural sources of SP such as alveolar macrophages and neutrophils, particularly in lipopolysaccharide-inflamed lungs.

The pathogenesis of the severe lung damage that characterizes pneumonic pasteurellosis (PP) is still poorly understood and most of the studies have emphasized in the pathogenic mechanisms operating during the acute stage of this pneumonic
syndrome. However, the lung lesions remaining during the sub-acute and chronic stages, which are responsible for the decreased productivity of affected animals, have received little attention.

The objective of this work was to determine the extent to which the development of chronic PP affects the density of MCs and SP production, both of which are key elements in chronic lung diseases of man.

**Materials and Methods**

**Experimental animals**

Eighteen sheep, of both sexes and mixed breed, three months old, were obtained from Iowa State University, Laboratory Animal Resources. The animals were maintained in isolation rooms in accordance with the procedures approved by the American Association of Laboratory Animal Care. The Iowa State University Animal Care Review Committee approved this protocol previously. After 24 h, the animals were randomly assigned to two large groups; one group receiving *Mannheimia* (*Pasteurella*) *haemolytica* while the other received saline. Subsequently, these large groups were subdivided in accordance to the time of sacrifice at 1, 15, and 45 days after inoculation. This arrangement gave six groups, each one with three animals (Table 1).

**Inoculation**

*Mannheimia haemolytica* (strain 82-25; originally recovered from a natural case of PP in sheep) was grown in tryptose broth medium for 3 h at 37°C and then suspended in pyrogen-free saline (PFS; Baxter healthcare Corp., Deerfield, Ill) to achieve $1 \times 10^8$ colony-forming units (CFU)/ml. The bacteria-inoculated animals received 5 ml of the bacterial inoculum into the right cranial lobe bronchus whereas the controls received the same volume of PFS. The inoculation was carried out employing a fiber-optic bronchoscopy as previously described.

**Euthanasia and collection of lung samples**

Animals were euthanized with an intravenous overdose of sodium pentobarbital. Samples were collected from the site of inoculum deposition in the right lung; additionally, from those animals that received the bacterial inoculum, the equivalent site in the non-inoculated (left) lung, was also collected. Samples were obtained in duplicate to be fixed by immersion in 10% neutral buffered formalin (48 h at room temperature) or Carnoy's fixative (18 h at 4°C). Furthermore, small pieces of lung tissue from the same
areas were collected in cryo-vials (Nunc CryoTube, Nalge Nunc Int. Naperville, IL) and maintained at -80°C for histamine determinations and bacteriological studies.

**Histopathology, mast cell staining and quantitation**

Tissues fixed in Carnoy's fixative were processed routinely, embedded in paraffin wax, and sectioned at 4 μm. Serial sections were stained with H & E, Mallory's trichrome, and low pH toluidine blue. The special stains were employed for recognition of connective fibrous tissue and MCs, respectively. Low pH toluidine blue yielded a pale bluish background where MCs appeared deeply blue. This procedure has been used to stain MCs in the human lung.23

For quantification of MCs, slides containing two sections of the right lung were assessed by light microscopy. The slides were randomly assigned and MCs density was determined by counting the numbers of MCs present in 50 randomly chosen fields at 400X magnification. The area provided is equivalent to 5.9 mm².30 The values obtained per sheep corresponded to counting the 50 fields in each one of the sections and then averaging these values. A similar procedure was carried out in the left, non-inoculated lungs of the animals that received the bacterial inoculum. The values were expressed as MCs/mm².

**Histamine determination**

The content of histamine in the lung was measured employing an enzyme immunoassay (Histamine Enzyme Immunoassay Kit, Immunotech, Marseille, France). The lower limit of detection of this assay is 0.5 nM and the specificity is 100% to acylated histamine. Briefly, 50 mg of tissue were placed in a plastic tube containing 500μl of 0.2 N HClO4. The tissue was homogenized and centrifuged at 10,000 × g for 5 min at 4°C. Supernatant was collected and added to an equal volume of 1M, pH 9.25 of K₂B₄O₇. Subsequently, the sample was centrifuged again and the supernatant collected. Then the samples were acylated as suggested by the manufacturer. A microplate with antibody-coated wells were filled with the tissues' supernatants and incubated for 2 h at 4°C with shaking at 350 rpm. The wells were rinsed and the substrate was added for incubation during 30 min at room temperature. The reaction was stopped and the plate was read in an automated spectrophotometer at 410 nm. The histamine content was expressed in ng of histamine/g of wet weight of lung tissue.
**Substance P immunohistochemistry**

Tissues fixed in neutral buffered formalin were also processed routinely and serial sections were stained with H & E or left unstained to carry out the substance P immunohistochemical procedure. The immunohistochemical procedure was a modification from those methods described by Sakamoto et al.\textsuperscript{46} and Sonea et al.\textsuperscript{47,48} in the brain and the lung, respectively. Substance P immunoreactivity was determined employing a rabbit anti-SP primary antibody (Peninsula Laboratories, Inc., San Carlos, CA). Briefly, the slides were heated in an oven for 30 min at 58° C and deparaffinized in xylene and a succession of graded alcohols. Subsequently, they were subjected to peroxidase blocking by immersing the slides in a solution of 0.9% H\textsubscript{2}O\textsubscript{2} during 40 min. After several rinses in buffer (Wash Buffer, BioGenex, San Ramon, CA) the slides were incubated at room temperature during 20 min with a solution containing 10% normal goat serum and 3% bovine serum albumin (Elisa-grade BSA, Sigma Chemical Co., St. Louis MO) dissolved in buffer. Then, the slides were incubated in a humidified and sealed chamber at 4°C during 96 h with the primary antibody diluted 1:10,000 in antibody diluent (Common Antibody Diluent, BioGenex, San Ramon, CA). The slides were rinsed several times and subjected to incubation at room temperature for one hour with the secondary antibody (affinity-purified biotin-labeled, Goat anti-Rabbit IgG (H+L), Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:200. After several rinses, the peroxidase-conjugated streptavidin reagent (Super Sensitive HRP, BioGenex, San Ramon, CA) was added (undiluted) for incubation at room temperature for one hour. Finally, slides were rinsed again and a metal enhanced DAB substrate that utilizes cobalt chloride and nickel chloride (ImmunePure© metal Enhanced DAB Substrate Kit, Pierce Chemical Co., Rockford, IL) was added for 15 min. The slides were then rinsed thoroughly in ultrapure water and, without counterstaining, they were dehydrated through alcohols, cleared in xylene, and coverslipped in Permout (Sigma Chemical Co.). The positive reaction at the site of antigen-antibody-peroxidase complex produced a dark brown/black precipitate. Negative controls in which the primary antibody was normal rabbit serum gave no immunostaining. Small intestine sections (ileum) from sheep were used as positive controls because these areas contain myenteric and submucosal plexuses with large numbers of immunoreactive nerve fibers and nerve cells bodies. Because we did not characterize the peptide reacting with our primary antibody and a
cross-reaction with other related tachykinines was possible, the term SP-like (SP-Li) immunoreactivity is used hereafter.

**Additional studies**

Small pieces of the right lung were collected in cryo-embedding medium (OCT compound, Miles Inc., Elkhart, IN) and stored at -80°C. This material was subsequently sectioned in a cryostat for an immunohistochemical procedure to detect *Mycoplasma ovipneumoniae* by indirect immunofluorescence employing a rabbit anti *M. ovipneumoniae* antiserum. The procedure is a modified protocol from the technique described by Amanfu, et al.⁵

Samples of blood were taken from each animal the day after arrival and the day scheduled for euthanasia after inoculation. Passive hemagglutination titers to *M. haemolytica* lipopolysaccharide were assessed from these samples.⁴⁰

Samples maintained at -80°C were used for serial dilution and plated on sheep blood agar (TSA/sheep blood agar; Remel, Lenexa, KS) to determine the number of CFU per mg of lung tissue. The identification of the bacterial isolates was carried out by conventional bacteriological procedures.

**Statistical Analysis**

The numbers of MCs as well as histamine determinations were subjected to statistical comparisons. Each group receiving the bacterial challenge were compared with the corresponding control at the same time period employing a Student's t test, assuming independent samples and equal variances.⁵⁰ Additionally, in the bacterial challenged groups, the non-inoculated (left) lungs were compared against the inoculated (right) ones with a paired Student's t test.⁵⁰

**Results**

**Pathology**

Gross lesions. Animals killed at 1 day post-inoculation with *M. haemolytica* had severe lesions of hemorrhage and lung consolidation in the right lung; particularly at the ventral area of the caudal part of the cranial lobe. The lesions had a diameter of 8 cm approximately. One animal also had mild lesions on the dorsal area of the cranial part of the cranial lobe and on the pulmonary hilium. In two animals the pleural surface covering the affected areas had a coarse appearance with delicate strands of fibrin.
The post-mortem exam of animals that received the bacterial inoculum and killed 15 days after revealed marked fibrous adhesions between parietal and visceral pleura on the surface of the caudal part of the cranial lobe. The lungs showed a depressed surface with grayish areas of discoloration and a rubbery to firm texture. One animal also showed fibrous adhesions between pleura and pericardium. Sections of the affected lungs contained abscesses with extensive areas of necrosis in two animals.

At 45 days after *M. haemolytica* inoculum, the lesions were more variable; the lungs of two animals showed irregular depressed areas on the surface with grayish discoloration located mainly at the caudal part of the cranial lobe but also extending toward the dorsal part and to the hilium. These areas were rubbery in texture and were alternating with areas of normal appearance and texture in the lung. The pleura covering the affected and adjacent areas appeared thickened. In one animal there were marked fibrous adhesions between the parietal and visceral pleura with extensive areas of pleural thickness. The lung was markedly rubbery in texture. No exudate was recognized in these lesions.

The macroscopic lesion in controls were practically absent except in two cases in which discrete areas of grayish discoloration were present at the cranial part of the cranial lobe. The non-inoculated (left) lungs were absent of lesions in all of the animals.

Microscopic lesions. The lungs of animals killed 1 day post-inoculation with *M. haemolytica* had extensive areas of proteinaceous edema, hemorrhage, and necrosis typical of PP. In the most affected areas the alveolar architecture was markedly distorted due to luminal accumulation of a sero-fibrinous exudate and leukocytes. In many areas there were swirls of necrotic neutrophils and macrophages occupying the alveolar lumina (Fig. 1A). Bronchioles were also plugged with neutrophils and cell debris. Pleural surface contained fibrin and was also infiltrated by neutrophils. Alveolar capillaries and some small blood vessels had fibrin thrombosis and adjacent edema. In two cases were recognized small clusters of cocobacillary bacteria associated with the alveolar lesions. The pattern of affected areas showed mostly a lobular distribution where interlobular septa appeared distended due to edema, fibrin and hemorrhage. One animal in this group had a mild hyperplasia of peribronchiolar lymphoid tissue in some areas. The low pH toluidine blue stain demonstrated a conspicuous absence of MCs in those areas showing the most severe damage (Fig. 1B). Trichrome stain showed no major changes in the pattern of connective tissue distribution.
By 15 days after bacterial inoculation the histopathological appearance of the lungs were more variable. The lung of two animals had large foci of pyogranulomatous reactions composed of lymphocytes, plasma cells and large (sometime binucleated) foamy macrophages (Fig. 2A), and limited by fibrous tissue (Fig. 2B). In some of these pyogranulomatous foci remnants of limiting bronchial/bronchiolar epithelium was still discernible. Furthermore, few bacterial coccobacilli colonies were recognized in some of these lesions. Mast cells were practically absent in the core of the pyogranulomatous foci and scarce in the margins. The connective fibrous tissue in some interlobular septa and pleura was extremely increased to the point of encroaching on some collapsed lobules (Fig. 3). In addition there was a marked proliferation of large and cuboidal alveolar epithelial cells mainly at terminal bronchioles and adjacent alveoli. These areas were commonly intervened by delicate strands of fibrous connective tissue with numerous lymphocytes and macrophages intermingled (Fig. 4). The lamina propria and submucosa of large bronchi were markedly infiltrated by lymphocytes and plasma cells. Additionally, these animals showed some areas with a mild hyperplasia of peribronchiolar lymphoid tissue. The lesions in the other animal included in this group were more interstitial in character. The lesions were less severe and characterized by alveolar and bronchiolar epithelialization with abundant macrophages exuding into the alveolar spaces (Fig. 5A). The toluidine blue special stain demonstrated surprisingly an increase in the number of MCs (Fig. 5B). Pleura and interlobular spaces also showed a marked fibroplasia.

Animals killed at 45 days after bacterial inoculation had a variable degree of chronic changes. One animal had discrete areas of interstitial pneumonia with epithelial proliferation in alveoli and bronchioles. These areas alternated with others that had alveolar atelectasis or emphysema and still others with no significant changes at all. Nonetheless the pleura and interlobular septae were thickened due to fibrous tissue. Similarly, some interlobular small blood vessels had medial hypertrophy and adventitial proliferation of fibrous tissue (Fig. 6). This animal also had a mild hyperplasia of peribronchiolar lymphoid tissue. The other two animals also had interstitial lesions but the proliferation of epithelium, particularly at bronchioles, was always accompanied by a marked infiltration of lymphocytes and plasma cells. Additionally, these small airways, which appeared segmented and tortuous, sometimes contain fibrous connective tissue forming organized masses inside the lumen (bronchiolitis fibrosa obliterans; Fig. 7).
These fibrosing changes extend into the surrounding parenchyma. The pleura and interlobular septa were markedly expanded by a dense connective tissue that supported well developed blood vessels and nerves (Fig. 8A). Mast cells were numerous in these areas of fibrosis; however, they were even more numerous in those adjacent areas of parenchyma showing the interstitial changes, and around the hyperplastic bronchioles (Fig. 8B). Macrophages were the dominant cell population in the alveolar lumen with occasional clusters of neutrophils and cell debris occluding bronchioles and adjacent alveoli. Some arteries adjacent to bronchioles had marked hypertrophy of the media that occludes their lumen and also perivascular lymphocytes and plasma cells infiltrates. These cells also infiltrated the lamina propria and submucosa of large bronchi.

Most of the lung of controls lacked lesions. However, three had mild hyperplasia of peribronchiolar lymphoid tissue with no other significant changes. Similarly, four of the non-inoculated (left) lungs of animals that received the bacterial inoculum had the same discrete lymphoproliferative changes. The density of MCs in controls as well as the noninoculated lungs of animals that received the bacterial inoculum was typical for lambs of this age. The density and distribution of pulmonary connective fibrous tissue was also typical for ovine lung.

**Mast cell quantitation**

The number of MCs was altered in bacterial inoculated animals. Those animals killed at 1 day after bacterial inoculation had decreased numbers of pulmonary MCs in their right (inoculated) lungs in comparison with the corresponding controls (Student's t test; P< 0.01). Similarly, the inoculated (right) lungs had lower numbers of MCs than the non-inoculated (left) lungs in the bacteria-inoculated group (Student's t test; P< 0.05). In the case of animals killed at 15 days after bacterial inoculation, the number of MCs were no different from controls killed at the same time period (Student's t test; P> 0.05), neither the non-inoculated lungs had different numbers than the inoculated ones (Student's t test; P> 0.05). It is important to mention that in this bacteria-inoculated group there was a high variability in the numbers of pulmonary MCs. Whereas one animal with interstitial pneumonia had higher numbers of MCs, the others with pyogranulomatous lesions had lower numbers. Finally, the bacteria-inoculated animals sacrificed at 45 days after inoculation had higher numbers of MCs in their right lungs than the corresponding controls (Student's t test; P< 0.01) as well as their non-inoculated contralateral lungs (Student's t test; P< 0.05). These results are presented in Fig. 9.
Histamine determinations

Bacteria-inoculated animals killed at 1 day had reduced values of histamine in their inoculated lungs than controls (Student's t test; \( P < 0.001 \)) as well as lower than their noninoculated contralateral lungs (Student's t test; \( P < 0.01 \)). However, neither the bacteria-inoculated groups euthanized at 15 days nor those killed at 45 days had significant differences in these comparisons (Student's t test; \( P > 0.05 \)). Nonetheless, at 45 days, the values of histamine in the bacteria-inoculated lungs were higher and close to significance when compared with both controls (Student's; \( P = 0.055 \)) and their contralaterals (Student's; \( P = 0.052 \)). Figure 10 presents these results.

Substance P-like immunoreactivity

At day 1 after bacterial inoculation the SP-Li immunoreactivity appeared mostly in leukocytes surrounding or enmeshed in the areas of most severe damage (Fig. 11). These cells reacted strongly, although not as much as nerve fibers. Serial sections stained with H&E showed that most of the cells were macrophages. The immunoreactivity of nerve fibers associated with the airways and blood vessels appeared decreased, particularly in areas of most severe damage.

By day 15 after bacterial inoculation the SP-Li immunoreactivity was mostly confined to nerve fibers. However, the large macrophages surrounding the areas of necrosis in the pyogranulomatous foci had a mild immunoreactivity. Many of the nerve fibers immunoreacted strongly and appeared thicker in areas in which they normally were faint in controls. For example, thicker SP-Li immunoreactive fibers were seen in some distorted terminal bronchioles (Fig. 12), around small blood vessels (Fig. 13) and in alveolar walls. Furthermore, some small bronchioles had also irregularly thickened immunoreactive fibers (Fig. 14), and occasionally enlarged myelinated fiber bundles that were immunoreactive (Fig. 15). Some large peribronchial ganglion neurons located in the submucosa of few bronchi had an intense immunoreaction (Fig. 16). All of these changes were observed in areas in which the pulmonary damage appeared with interstitial pattern. The SP-Li immunoreactivity occurs more scant in areas of extreme parenchymal fibrosis and was rare in areas of pleural and interlobular fibrosis.

At 45 days after bacterial inoculation the SP-Li immunoreactivity was confined to nerve fibers in a very similar way as in the previous group. However, in some areas of interstitial-fibrosing damage the fibers appeared less numerous and had a variable
degree of immunostaining; few appeared strongly immunolabelled but most of them showed mild immunoreactivity.

The lung of controls and the corresponding non-inoculated lungs in bacteria-inoculated animals had a pattern of immunoreactivity similar to that described in horses (Sonea, et al., 1994a,b). The immunoreaction was present in small nerve fibers that had the typical varicosities of unmyelinated C-fibers in the lamina propria and submucosa of bronchi and in the adventitia of adjacent blood vessels. Bundles of myelinated fibers also had several enmeshed immunoreactive fibers. However, the immunolabelling was not present in peribronchial neuronal cell bodies. Similarly, small unmyelinated fibers and some myelinated ones also had immunoreactivity around small bronchioles and small blood vessels. The immunoreactivity was extremely rare in terminal bronchioles and alveoli and when it was recognized showed very tiny nerve fibers. Nonetheless, the immunoreactive nerve fibers showed a more consistently pattern of distribution and stronger immunoreaction than bacteria-inoculated lungs.

**Additional studies**

*Mycoplasma ovipneumoniae* immunofluorescence. Some of the lungs of control animals as well as bacteria-inoculated sheep had a positive reaction to *M. ovipneumoniae*. In groups killed at 1 day after inoculation one control was weak positive and two bacteria-inoculated animals were weak positive and positive, respectively. One animal in the bacteria-inoculated group killed at 15 days was positive whereas two controls were positive. The groups sacrificed at 45 days after inoculation had one positive animal each. There was no massive accumulation of antigen in any of the positive sections, and the positive reaction was not related to infiltrating or exudating cells.

Serology. All of the animals showed positive titers to *M. haemolytica* LPS at arrival. Titers oscillated between 4 to 32 with an average mean and SEM of 18.88±2.66. After challenge the control groups maintained their titers whereas the bacteria-inoculated groups changed. At 1 day after bacterial inoculation the animals showed a decrease in the titer; 18.66±7.05 at arrival and 12±4 at time of euthanasia. At 15 days the titer increased; 18.66±7.05 at arrival and 48±16 at time of euthanasia. And at 45 days the titer showed a discrete increase; 45.33±18.66 at arrival and 52±8.10 at time of euthanasia.
Bacteriology. *M. haemolytica* was recovered from bacteria-inoculated animals at 1 day and 15 days after challenge but not at 45 days. The lungs of animals killed at 1 day had 80 and 400 CFU/mg of lung tissue. Only samples from two animals were available for this study in this group. Bacteria-inoculated animals killed at 45 days had 40, 3500, and 580 CFU/mg lung tissue. The first value correspond to the animal with an interstitial pattern of pneunonic lesions whereas the other values correspond to animals with pyogranulomatous abscesses. The bacteriological studies from saline-inoculated controls lacked *M. haemolytica* colonies.

**Discussion**

We have been working with a model of PP in ruminants employing *Mannheimia (Pasteurella) haemolytica* inoculated intrabronchially by fiber-optic bronchoscopy. This procedure reproduces typical lesions located mainly in the site of inoculum deposition. Moreover, as was observed in the present work, the procedure results in a lesion confined to the inoculated lung and no involvement of the contralateral lung. This characteristic permits the bacteria-inoculated animals to develop a focal lesion of PP that evolve as probably occurs in natural infections. The time points for euthanasia were chosen as representative of the acute, sub-acute, and chronic stages of the pneumonic lesion.

The acute lesions observed at 1 day after bacterial inoculation were characterized by severe serofibrinous alveolitis and bronchopneumonia, extensive areas of coagulative necrosis with presence of swirls of oat cells obliterating the alveolar spaces, and thrombosis of capillaries and small blood vessels. These microscopic changes have been considered the hallmark of the acute stage of PP. Recently, it has been proposed that these changes may occur by two different pathological mechanisms that can act alone or in combination, depending on the circumstances; these are the Arthus and the Shwartzman reactions. Both mechanisms could, eventually, activate the complement system allowing the participation of MCs, neutrophils, and macrophages, with release of many pro-inflammatory compounds, including cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-8. The final result is an exacerbated host inflammatory response. Apparently, the bacterial lipopolysaccharide is a more important inducer of lung inflammation and tissue damage than the leukotoxin. The decrease in the serum titers against *M.*
haemolytica lipopolysaccharide in bacteria-inoculated animals killed at this time point could represent a consumption of specific antibodies during the acute inflammatory response. On the other hand, the bacteria counts in the lungs of these animals appear low considering the severe lung damage observed, supporting the idea of an exacerbated host inflammatory response. Preliminary work in this laboratory demonstrated that at 6 h after intrabronchial inoculation of *M. haemolytica* in neonatal calves, those areas in the lung with severe damage have decreased numbers of MCs and MCs that were still recognizable appeared degranulated. In the present study in sheep, MCs were conspicuously absent at areas of acute lung damage at 1 day after bacterial inoculation. The overall number of MCs in these areas was statistically reduced in comparison with saline-inoculated controls and non-inoculated contralateral lungs in bacteria-inoculated animals. This supports Cheville’s suggestion that during acute inflammation recognizable MCs decrease due to their degranulation. Mast cells are considered the orchestrators of the acute inflammatory response in the lung due to their content of preformed pro-inflammatory compounds, particularly TNF-α. Moreover, it has been reported that MCs recovered from cattle infected with *M. haemolytica* release increased amount of histamine *in vitro*; and that bacterial leukotoxin and lipopolysaccharide both cause an increase in spontaneous release of histamine from isolated bovine lung parenchyma. The amount of histamine in the bacteria-inoculated lungs was statistically lower than the values in saline-inoculated controls and the non-inoculated contralateral lungs of bacteria-inoculated animals, confirming the previous reports.

It was an unexpected and striking finding that leukocytes infiltrating those areas of severe lung damage had SP-Li immunoreactivity. Conversely, the nerve fibers associated with airways and blood vessels located in areas of severe damage reacted only moderately probably due to an early depletion of neuropeptides. In rats it has been demonstrated that alveolar macrophages normally express the preprotachykinin-I gene mRNA which encodes SP and neurokinin A; however, during lung inflammation induced by lipopolysaccharide, the preprotachykinin gene-I mRNA is expressed two to fourfold and SP can be recognized by immunocytochemistry. Thus, it is logical to assume that in our study the induction of pulmonary inflammation by *M. haemolytica* exerted a similar stimulus that resulted in recognition of SP-Li immunoreactivity in infiltrating leukocytes. Although serial sections stained with H&E demonstrated that alveolar macrophages
were the main cell type immunoreacting, neutrophils could also be reacting because these cells also express the preprotachykinin gene-I mRNA and immunoreact to SP in lipopolysaccharide-inoculated lungs of rats. Moreover, eosinophils also synthesize, store, and release substance P. Therefore, in addition to sensory C-fibers, inflammatory cells infiltrating the lung might be an important source of tachykinins, as has been suggested by Killingsworth et al. This extraneural production of SP may contribute to an inflammatory imbalance that favors severe lung damage. On the other hand, it is possible that the moderate reaction observed in nerve fibers could be related to degranulated MCs because trypsin-like enzymes contained in their granules can activate sensory nerves through PAR2, inducing release of sensory neuropeptides.

Dungworth and López coincide in mentioning that in animals surviving an acute episode of PP a complete resolution is rare and the process commonly includes sequelae such as bronchiolitis obliterans, bronchiectasia, pulmonary sequestra and abscesses, pulmonary fibrosis, and chronic pleuritis with fibrous pleural adhesions. In the present study all of these changes were found in animals killed at 15 and 45 days after bacterial inoculation. However, in all of them, the interstitial component characterized by alveolar and bronchiolar epithelialization was an important component. Although some of the lungs included in these groups (as well as the groups killed at 1 day) reacted positively to *M. ovipneumoniae* by immunohistochemistry, the reaction did not show a massive accumulation of antigen and was neither associated with interstitial cellularity or exudation. This was interpreted as a short-term infection; possibly secondary to the bronchoscopy procedure during bacterial or saline intrabronchial deposition. Additionally, the histological changes, particularly the peribronchial and perivascular lymphoid hyperplasia, considered characteristic of *Mycoplasma spp.* infection, were barely seen in these animals. Therefore, the interstitial changes are most likely the result of *M. haemolytica* infection. This assumption is reinforced with the lack of significant histological changes in saline-inoculated controls and non-inoculated contralateral lungs in bacteria-inoculated animals.

At 15 days after bacterial infection, the animals had two patterns of sequelae, one with a prominent interstitial change characterized by alveolar and bronchiolar epithelialization, accompanied by large numbers of MCs and high amount of histamine. Conversely, the others had pyogranulomatous foci arising from bronchiectactic airways that predominated over the interstitial pattern, with lower numbers of MCs and also lower
amount of histamine. This wide variability resulted in no statistical difference when these values were compared with the corresponding saline-inoculated controls neither the contralateral non-inoculated lungs in the bacteria-inoculated animals. Sorden and Castleman\(^\text{49}\) have demonstrated in a model of viral bronchiolitis in rats infected with Sendai virus, that a significant increase in MCs occurs only after day 14 post-infection, which is in agreement with our findings in one of the animals. However, our observations indicate that whereas the inflammatory reaction remains active and associated with tissue damage, such as the pyogranulomatous foci accompanied by the highest numbers of CFU, MCs remain absent in these areas. Nonetheless, the extensive areas of fibrosing alveolitis and the fibroplastic changes in pleura and interlobular spaces were consistently seen at this time point.

Because the pyogranulomatous foci were delineated by well-differentiated fibrous tissue and MCs were scarce in these areas, we can assume that in certain circumstances fibrosis occurs first and independently from MCs. This observation has been previously reported in the lungs of patients that survived acute stages of adult respiratory distress syndrome. In these cases fibrosis appears before MCs hyperplasia.\(^\text{27}\) Additionally, rats genetically deficient in MCs were still capable of mounting a fibroplastic lesion in their lungs.\(^\text{36}\) Nonetheless, MC infiltration has been widely associated with chronic lung disorders in humans.\(^\text{39}\) MCs contain proteases and growth factors that stimulate lung fibroblast proliferation and synthesis of type I collagen,\(^\text{19,25}\) and MCs-fibroblast heterotypic contacts induce MC synthesis of interleukin-4 which augments fibroblast proliferation.\(^\text{53}\) On the other hand, fibroblasts also influence MCs due to production of growth factors and particularly stem cell factor, which is necessary for MC maturation.\(^\text{20,21}\) In our study, at 15 days after bacterial inoculation, MC hyperplasia occurred mostly in those areas with interstitial pattern and was always accompanied by epithelializing changes and abundant macrophage infiltration in alveolar spaces. This pattern of MCs proliferation may represent a functional relationship between MCs and epithelial cells to favor epithelial proliferation. Mast cell tryptase is also a potent mitogen for epithelial cells in the respiratory tract.\(^\text{9}\)

At 15 days after bacterial infection the SP-Li immunoreactivity had a different pattern of reaction from the previous group. Here the large macrophages bordering those areas with pyogranulomatous reaction had mild immunoreactivity. However, the unmyelinated fiber innervating alveoli, terminal bronchioles, small blood vessels and
bronchi appeared irregularly thickened with a moderate to intense immunoreactivity. Similarly, large myelinated nerve bundles in bronchioles and bronchi, which in some cases appeared enlarged, also contain numerous immunoreactive fragments. The presence of some large peribronchial ganglion neurons which were associated with an intense immunoreaction, similar to that observed in ganglion cells in the myenteric and submucosal plexuses in the small intestine, was a striking feature. When compared with the lungs of saline-inoculated controls and with the non-inoculated contralateral lungs of bacteria-inoculated sheep, the overall impression for immunoreacting nerve fibers in bacteria-inoculated lungs was that of an irregular thickness and enlargement with variable intensity of reaction but with a normal pattern of distribution.

It has been described that during persistent injury in muscles and articular ligaments, an increase in SP-immunoreactive nerve fibers occurs. And in the urinary bladder, an organ highly innervated by afferent neurons of C-fiber type located mainly in the dorsal root ganglia, chronic inflammation induces their somal hypertrophy. In the lungs of asthmatic individuals the reports have been contradictory; one study reports an increase in SP nerve fibers but others did not confirm this finding. Although in some areas of these bacteria-infected lungs we recognized a trend compatible with hypertrophy, we cannot assume this criterion because we did not carried out a morphometric-quantitative evaluation. In the respiratory tract SP nerve fibers occur with highest density at the nasal septum, whereas the lung has few fibers and most of them are associated with large airways. For this reason, an accurate analysis of density of SP nerve fibers in the lung is probably not feasible.

We can suggest, however, that in our model at 15 days, there is some degree of phenotypic switch as was demonstrated by the unusual SP-Li immunoreactivity of some neuronal cell bodies associated with large airways. In general terms, it is widely accepted that the majority of SP nerve fibers originating from the lung have their cell bodies located in the nodose ganglion of the vagus with lesser numbers in the thoracic dorsal root ganglia. Substance P immunoreactivity has not been recognized in ganglionic neurons of the respiratory tract in normal horses. In the cat, however, neurons of airway ganglia express SP immunoreactivity, and explants of airway walls have demonstrated an alteration in phenotypic expression of neuropeptides when maintained in culture. Ganglion neurons in the myenteric and submucosal plexuses of the gastrointestinal tract are a rich source of tachykinins and this should be expected to
occur in the lung because its embryonic development from the foregut. Nonetheless, the presence of SP in intrinsic airway neurons in the lung has not been consistently recognized. The discrepancy could be explained by the fact that airway ganglia contain a heterogeneous population of neurons susceptible to adaptive functions under physiological and pathological conditions.

In certain models of inflammatory pain there is a phenotypic switch in primary sensory neurons which normally do not express SP but become committed during persistent inflammatory injury. Apparently, this transcriptional and posttranslational plasticity is induced mainly by nerve growth factor, and mast cells are an important source of nerve growth factor. Thus, as has been demonstrated in models of inflammatory pain, the expression of SP-Li immunoreactivity in some ganglion neurons recognized here, may represent a novel expression of SP to sustain the inflammatory reaction. Pérez Fontán and coworkers have demonstrated recently that syngeneic lung grafts, which lack (extrinsic) peptidergic sensory innervation, still produce SP and NK-1R by intrinsic airway neurons. Furthermore, these transplanted lungs were able to mount an immune-mediated pulmonary inflammatory response that corresponded to SP production and was attenuated by NK-1R antagonists.

By 45 days after bacterial inoculation the fibrotic pattern of alveolitis and bronchiolitis become more pronounced as did the fibrosing changes in pleura and pleural adhesions. None of the animals sacrificed at this time point had significant exudative changes although macrophages were still abundant in the alveolar spaces in some areas. At this time point, the alveolar and bronchiolar changes were defined more precisely as fibrosing alveolitis and bronchiolitis fibrosa obliterans, respectively, although these changes were present to a lesser degree at 15 days after bacterial challenge. The increased numbers of pulmonary MCs were maintained and represented by a higher amount of histamine in comparison with the saline-inoculated controls and the non-inoculated contralateral lungs of bacterial-inoculated sheep. Nonetheless, this trend of histamine increase was not statistically significant. Maturation of MCs is a process that occurs mediated by many environmental conditions in the tissue, and during conditions of proliferation MCs mature asynchronously and their granules contain variable amounts of histamine. Thus, it is possible that during the chronic stages of lung inflammatory response the higher number of MCs included a large population of immature cells with lower amount of histamine. Recognition of immature MCs by toluidine blue is possible
because the expression of proteoglycan matrix in their granules occurred first and regulates the content of histamine, proteases and other granule components.\textsuperscript{19,24}

The SP-Li immunoreactivity in bacteria-inoculated animals at 45 days after challenge occurred with a decreased pattern of distribution in comparison with bacteria-inoculated animals killed at 15 days. Also, the intensity of the immunostaining was variable, but mostly decreased. Moreover, in none of the lungs was there any positive immunolabelling of peribronchial neuronal bodies. This group also lacked any extraneural SP-Li immunoreactivity. According to bacteriological studies no viable \textit{M. haemolytica} were recovered from these lungs indicating that, in absence of the infectious agent, the chronic lesions persist. In animal models of chronic bronchitis, it has been demonstrated that, in contrast to being detrimental, SP nerve fibers exert a beneficial adaptive response during chronic injury favoring a regulatory action on the inflammatory response.\textsuperscript{16,28} The lowered pattern of SP nerve fibers distribution as well as the low immunoreaction observed in these bacteria-inoculated lungs may reflect a deficient function of C-fibers and therefore a tendency to maintain or increase the lung injury.

**Acknowledgements**

We thank B. Smith, Dr. R.F. Rosenbusch, and J.M. Fosse for technical assistance. This work was supported in part by USDA/NRI Project # 970-2654. R. Ramirez-Romero receives financial support from CONACYT and Universidad Autónoma de Nuevo León, México.

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<th>Treatments</th>
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*Mannheimia haemolytica* (strain 82-25) was grown in tryptose broth medium for 3 h at 37°C and then suspended in pyrogen-free saline to achieve $1 \times 10^8$ CFU/ml. The bacteria-inoculated animals received 5 ml of the bacterial inoculum into the right cranial lobe bronchus by fiber-optic bronchoscopy whereas the controls received the same volume of PFS. Additionally, the left (non-inoculated) lungs of the bacteria-inoculated animals were also employed as contralateral negative controls.
Fig. 1. Lung of bacteria-inoculated animal killed at 1 day. The alveolar spaces contain serofibrinous exudate and are partially-filled with macrophages and neutrophils. In some areas the cellular exudate forms typical swirls of oat cells (A). H&E stain. Bar=40μm. There is one mast cell partially degranulated in this field that corresponds to a serial section of the same area presented previously (B). Low pH toluidine blue stain. Bar=40μm.

Fig. 2. Lung of bacteria-inoculated animal killed at 15 days. There are many large and vacuolated macrophages, some of them binucleated, in the margins of a necrotic area in a pyogranulomatous abscess (A). H&E stain. Bar=40μm. Serial section of the same area to demonstrate that the pyogranulomatous abscesses were limited by fibrous connective tissue (B). Mallory's trichrome stain. Bar=60μm.

Fig. 3. Lung of bacteria-inoculated animal killed at 15 days. The proliferated but unorganized fibrous connective tissue in pleura and interlobular septum encroaches a collapsed pulmonary lobule. Mallory's trichrome. Mallory's trichrome stain. Bar=60μm.

Fig. 4. Lung of bacteria-inoculated animal killed at 15 days. There is proliferation of epithelial cells at terminal bronchioles and alveoli. The parenchyma is intervened by strands of connective fibrous tissue. There are also many infiltrating leukocytes, mostly lymphocytes and macrophages with few clusters of neutrophils. Mallory's trichrome. Bar=μm.
Fig. 5. Lung of bacteria-inoculated animal killed at 15 days. There is a thickened appearance of alveolar septa due to epithelial proliferation. Numerous macrophages are occupying the alveolar spaces (A). H&E stain. Bar=40μm. The corresponding serial section of this area demonstrate a large number of mast cells, some of them hypertrophied (B). Low pH toluidine blue. Bar=40μm.

Fig. 6. Lung of bacteria-inoculated animal killed at 45 days. Two small arteries in an interlobular septum are accentuated by medial hypertrophy and fibrous connective tissue proliferating in the adventitia. Mallory’s trichrome. Bar=40μm.

Fig. 7. Lung of bacteria-inoculated animal killed at 45 days. There are several proliferated and tortuous bronchioles with hyperplastic epithelium partially-filled with exudate at different stages of organization. The lesion was classified as bronchiolitis fibrosa obliterans. Mallory’s trichrome. Bar=40μm.

Fig. 8. Lung of bacteria-inoculated animal killed at 45 days. The pleura is thickened due to a massive proliferation of dense fibrous connective tissue. The adjacent parenchyma is atelectatic and the bronchioles appear proliferated and tortuous (A). Mallory’s trichrome. Bar=60μm. The corresponding serial section shows the distribution of the proliferated mast cells. Most of these cells reside in lung parenchyma or proliferate around small bronchioles whereas few of them infiltrate the fibrous connective tissue at pleura (B). Low pH toluidine blue. Bar=60μm.
Fig. 9. Mast cell density in the lung. Values are expressed as mean ± SEM. At 1 day, bacteria-inoculated animals had significantly lower number of MCs in their lungs than saline-inoculated control animals (**P< 0.01). Also, the bacteria-inoculated (right) lungs had lower numbers of mast cells than the non-inoculated (left) lungs in bacteria-inoculated animals (*P< 0.05). At 45 days the lungs of bacteria-inoculated animals had higher numbers of MCs than saline-inoculated controls (**P<0.01) as well as their non-inoculated left lungs (*P<0.05)
Fig. 10. Histamine content in the lung. Values are expressed as mean ± SEM.

At 1 day, bacteria-inoculated animals had significantly lower amount of histamine in their lungs than saline-inoculated control animals (**P<0.001). Also, the bacteria-inoculated (right) lungs had lower amount of histamine than the non-inoculated (left) lungs in bacteria-inoculated animals (**P<0.01).
Fig. 11. Lung of bacteria-inoculated animal killed at 1 day. There are numerous immunoreactive leukocytes, probably macrophages, infiltrating an area with extensive damage. Substance-P immunolabelling (no counterstain). Bar=40 µm.

Fig. 12. Lung of bacteria-inoculated animal killed at 15 days. There is a cluster of immunoreactive nerve fibers located in a bronchiolo-alveolar junction in an area of interstitial damage. Substance-P immunolabelling (no counterstain). Bar=20 µm.

Fig. 13. Lung of bacteria-inoculated animal killed at 15 days. There is a thickened immunoreactive nerve fiber adjacent to a small blood vessel in an area of interstitial damage. Substance-P immunolabelling (no counterstain). Bar=20 µm.

Fig. 14. Lung of bacteria-inoculated animal killed at 15 days. There is a fragmented and thickened nerve fiber adjacent to a small bronchiole. Substance-P immunolabelling (no counterstain). Bar=20 µm.

Fig. 15. Lung of bacteria-inoculated animal killed at 15 days. There is an enlarged bundle of myelinated nerves containing many immunoreactive fragments around a bronchiole. Substance-P immunolabelling (no counterstain). Bar=40 µm.

Fig. 16. Lung of bacteria-inoculated animal killed at 15 days. There is a large immunoreactive neuron and fragments of myelinated and unmyelinated nerve fibers immunoreacting in the submucosa of a large bronchi. Substance-P immunolabelling (no counterstain). Bar=20 µm.
CHAPTER 6. GENERAL CONCLUSIONS

Mast cells and SP have a relevant role during initiation and progression of pulmonary lesions in PP. The following supports this argument:

The decreased numbers of stained MCs in sites of severe and acute lung damage, typical of PP, is a consistent finding and suggests the participation of MCs during early events of lung inflammation, particularly at 6h. Previous administration of a sialyl Lewis mimetic (TBC1269) intended to decrease PMN infiltration during the acute phase of lung damage has no effect on MC numbers suggesting that MC degranulation occurs in spite of selectin inhibition.

In the second study, a single high dose of DHC administered to neonatal lambs depleted peptidergic nerves from SP. This depletion was estimated to be 85% of controls, when measured in the nasal septum, which is the site richest in SP nerve fibers. In the upper and lower respiratory tract MC density increased progressively with age, from 3 to 21 days, but DHC-treated animals had significantly higher numbers of MCs in comparison with controls. However, histamine content in the lung was similar. A functional association between MCs and SP nerve fibers is suggested. The model is suitable for experiments designed to study the inflammatory response in the respiratory tract when the neurogenic contribution is diminished. It can be used to critically test the effect of SP fibers in PP and other inflammatory diseases of the lung, such as asthma and chronic obstructive pulmonary disease.

In the third study, a diminished number of pulmonary MCs and amount of histamine during the acute phase of PP in sheep, was associated with SP immunoreactivity in macrophages infiltrating the areas of severe damage, suggesting an extraneural contribution of SP during the acute (24h) stage of the lung lesions. At 15 days, MCs remain scarce at sites where lung damage persisted, i.e. pyogranulomatous foci, but become hyperplastic in areas of interstitial damage. At this time point, SP immunoreactivity suggested that an extrinsic, but also intrinsic contribution of SP immunoreactive nerves sustain the inflammatory response in the lung. By 45 days the fibroplastic changes in pleura, interlobular spaces, and interstitium were marked as was the presence of bronchiolitis obliterans. These changes were associated with increased numbers of MCs; however, elevation in histamine content did not correspond to this increment. In areas of severe fibrosis SP immunoreactivity in nerve fibers was mild.
suggesting that the absence of SP during the chronic lung damage hinders complete tissue repair. This work suggests that a dysregulation of the MC-SP nerve fiber axis may contribute to the epithelial hyperplastic changes. Additional studies are needed to test whether this dysregulation prevents the complete resolution of chronic pulmonary lesions.