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Mast cell density and substance P-like immunoreactivity during the initiation and progression of lung lesions in ovine *Mannheimia* (*Pasteurella*) *haemolytica* pneumonia

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To determine the density of mast cells (MCs) and the extent of substance P (SP) immunoreactivity during initiation and progression of pneumonic pasteurellosis (PP), 18 lambs were inoculated intrabronchially with *Mannheimia* (*Pasteurella*) *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. These pneumonic lesions had fewer numbers of MCs and reduced histamine content. Macrophages infiltrating some of the inflamed areas were strongly immunoreactive for SP. At 15 days, MCs remained scarce at sites where lung damage persisted, i.e. pyogranulomatous foci, but were increased in number in areas of interstitial damage. Pulmonary ganglion neurons were strongly immunoreactive for SP. By 45 days the fibrosing changes became more defined as pleural fibrosis, fibrosing alveolitis, alveolar epithelial hyperplasia and bronchiolitis obliterans. These lungs had increased numbers of MCs, but histamine content was not different from saline- and non-inoculated left lungs. Substance P immunoreactivity occurred only in nerves and was scarce and mild. This work demonstrates that MC density decreases initially with PP, but increases with progression of PP. SP fibres tend to be decreased during the initiation and at 45 days of PP, but other cells, such as macrophages and neuronal ganglion cells, produce substance P during progression of PP and thereby constitute an additional source of substance P.

Key words: *Mannheimia* (*Pasteurella*) *haemolytica*, pneumonic pasteurellosis, pathogenesis, mast cells, substance P.

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Introduction

In the lung, MCs orchestrate both the inflammatory and immune responses [1]. Mast cells produce an array of vasoactive compounds and cytokines necessary to mount the acute phase of inflammatory response. In addition, MCs are able to internalize bacteria and present antigens to immune cells [1]. During the acute inflammatory response in the lung, MCs are required for neutrophil infiltration and bacterial clearance [2]. Furthermore, MCs contain proteases and growth factors that make them an important component in chronic inflammatory responses such as chronic lung diseases [3, 4].

The unmyelinated C-fibres are an important component of the sensory innervation in the lung [5, 6]. These nerve fibres exert a functional connection with MCs through the neuropeptide substance P (SP) [7, 8]. Substance P acts on MCs, to induce MC activation [8]. Conversely, MC tryptase acts as a signal molecule, cleaving and activating a proteinase-activated receptor (PAR)-2 located at the surface of sensory nerves, exerting a positive feedback that induces release of sensory neuropeptides [9].

Substance P is the tachykinin responsible for neurogenic inflammation in the lung [10] and also influences the local immune response [11]. Although SP is mostly produced by unmyelinated C-fibres, this neuropeptide is also normally expressed by small numbers of thin myelinated Aδ afferent fibres [10, 12]. However, during persistent inflammation, additional Aβ fibres, which normally do not express SP, can acquire the capacity to express the neuropeptide [12, 13]. More recently it has been recognized that airway ganglion neurons produce SP and express NK-1R [14]. In addition, there are other extraneural sources of SP such as alveolar macrophages and neutrophils, particularly in lipopolysaccharide-inflamed lungs [15]. Substance P binds to a specific receptor, the neurokinin-1 receptor (NK-1R), and may also affect cells through direct activation of H protein [8].

Pasteurella haemolytica, recently renamed *Mannheimia haemolytica* [16], is the bacterium most commonly isolated from natural cases of fibrinous pleuropneumonia in cattle and sheep [17, 18]. The pathogenesis of pneumonic pasteurellosis (PP) is still poorly understood and most of the studies have emphasized the mechanisms operating during the acute stage, such as the Arthus and Shwartzman reactions [19]. However, the lung lesions remaining during the subacute and chronic stages, which are responsible for the decreased productivity of affected animals, have received little attention.

The aim of this work was to determine the extent to which MC density and SP production are related to acute and chronic lesions of PP.

Results

Pathology

Gross lesions

Animals inoculated with *M. haemolytica* had severe lesions and lung consolidation in the right lung, particularly in the ventral area of the caudal part of the cranial lobe that averaged approximately 8 cm in diameter. Lungs from two animals, 15 days post-inoculation had abscesses. The control and non-inoculated (left) lobes lacked 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, neutrophil infiltration, hemorrhage and necrosis typical of PP. The low pH toluidine blue stain demonstrated a conspicuous absence of MCs in those areas with the most severe damage. Trichrome stain had no major changes in the distribution of connective tissue. By 15 days after bacterial inoculation the histopathological pattern of the lungs was more variable. The lungs of two animals had large foci of pyogranulomatous inflammation. In all animals, the fibrous connective tissue in interlobular septa and pleura was extremely increased and encroached on collapsed lobules. In addition, there was marked proliferation of large, cuboidal, alveolar epithelial cells mainly at terminal bronchioles and adjacent alveoli. These cells were supported by delicate strands of fibrous connective tissue that contained numerous lymphocytes and macrophages. The lesions in the other animal included in this group were characterized by alveolar and bronchiolar epithelialization with numerous macrophages

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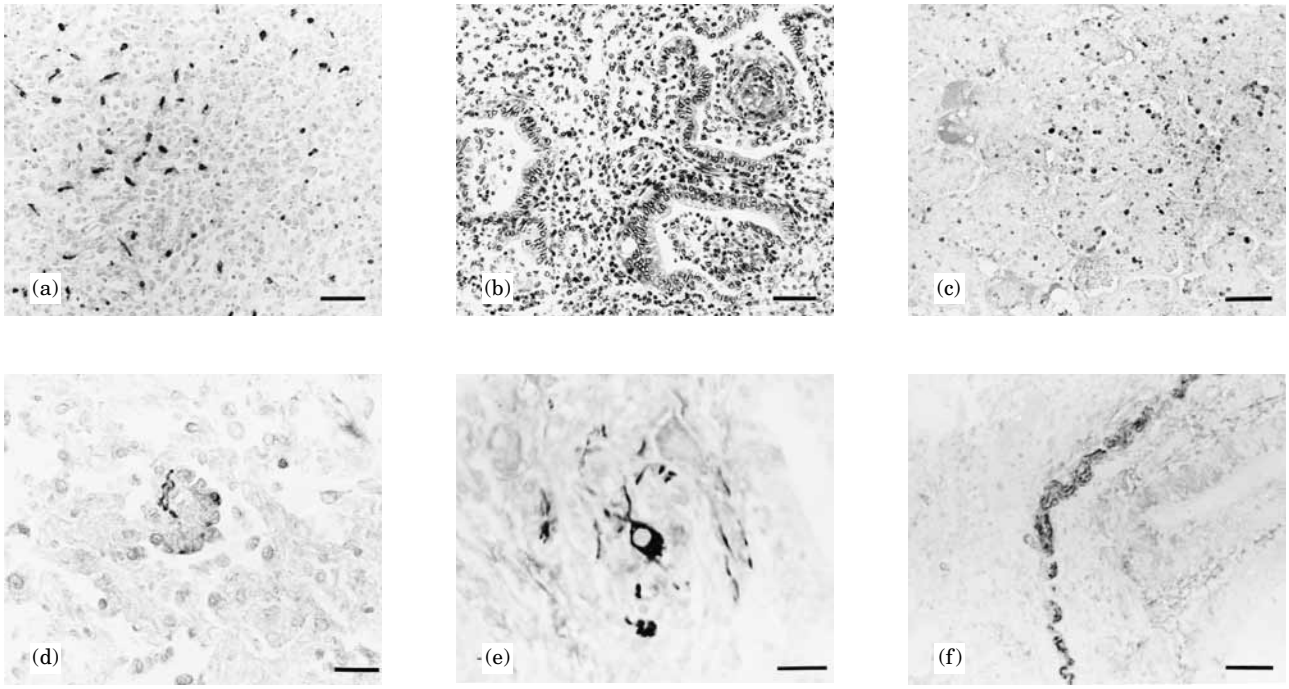

Figure 1. (a) Lung of bacteria-inoculated animal killed at 15 days. There are numerous mast cells, some of them hypertrophied. Low pH toluidine blue. Bar = 80 μm. (b) Lung of bacteria-inoculated animal killed at 45 days. There are several tortuous bronchioles lined by hyperplastic epithelial cells. These airways are partially-filled with exudate and surrounded by fibrous connective tissue. In one airway there is a stalk of epithelium that extends into the airway lumen and is supported by fibrous connective tissue (bronchiolitis fibrosa obliterans). Mallory's trichrome. Bar = 80 μm. (c) Lung of bacteria-inoculated animal killed at 1 day. There are numerous immunoreactive leukocytes, mainly macrophages, infiltrating an area with extensive damage. Substance-P immunolabelling (no counterstain). Bar = 80 μm. (d) Lung of bacteria-inoculated animal killed at 15 days. There is a cluster of immunoreactive nerve fibres located in a bronchiolo–alveolar junction in an area of interstitial damage. Substance-P immunoreactivity (no counterstain). Bar = 20 μm. (e) 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 immunoreactivity (no counterstain). Bar = 80 μm. (f) Lung of bacteria-inoculated animal killed at 15 days. There is a large immunoreactive neuron and fragments of myelinated and unmyelinated nerve fibres immunoreacting in ths submucosa of a large bronchus. Substance-P immunolabelling (no counterstain). Bar = 20 μm.

in alveolar spaces. The toluidine blue special stain demonstrated a marked increased number of MCs [Fig. 1(a)].

Animals killed at 45 days after bacterial inoculation had a variable degree of chronic changes that included epithelial cell proliferation in alveoli and bronchioles. These small airways, which appeared tortuous, sometimes contained fibrous connective tissue forming organized masses inside lumen (bronchiolitis fibrosa obliterans) [Fig. 1(b)]. These areas alternated with others that had alveolar atelectasis or emphysema and others with no significant changes. The pleura and interlobular septa were markedly expanded by a dense connective tissue. Mast cells were numerous in these areas of fibrosis, however, they were even more numerous in those adjacent areas of alveoli and bronchioles with epithelial hyperplasia. Macrophages were the dominant cell population in the alveolar lumen with occasional clusters of neutrophils and cell debris.

Lung of control and non-inoculated (left lobe) lacked significant lesions. The density of MCs in controls as well as the non-inoculated lungs of animals that received the bacterial inoculum was typical for lambs of this age [20, 21]. The density and distribution of pulmonary fibrous connective tissue was also typical for ovine lung.

Mast cell quantification

The number of stained MCs was altered in bacterial inoculated animals. Those animals killed at 1 day after bacterial inoculation had fewer numbers of pulmonary MCs in their right (inoculated) lungs in comparison with the corresponding controls ($P<0.01$). Similarly, when the right and left (non-inoculated) lungs in this bacteria-inoculated group were compared, the right lungs had fewer MCs ($P<0.05$). In the case of animals killed at 15 days after bacterial inoculation, the numbers of MCs were not significantly different from controls killed at the same time period, or from the non-inoculated contralateral lungs. These bacteria-inoculated lungs had a high variability in the numbers of MCs, whereas one animal with interstitial pneumonia had higher numbers, the others with pyogranulomatous lesions had lower numbers. The bacteria-inoculated animals killed at 45 days after inoculation had higher numbers of MCs in their right lungs than the corresponding controls ($P<0.01$) as well as their non-inoculated contralateral lungs ($P<0.05$). Comparisons of the three time points within each treatment showed that only bacteria-inoculated lungs had significantly different numbers of MCs in their non-inoculated contralateral lungs ($P<0.05$). Comparisons of the three time points within each treatment showed that only bacteria-inoculated lungs had significantly different numbers of MCs ($P<0.005$). The MCs value of animals killed at 1 and 45 days were significantly different ($P<0.05$), however, both share similarities with the number of MCs of animals killed at 15 days (Fig. 2).

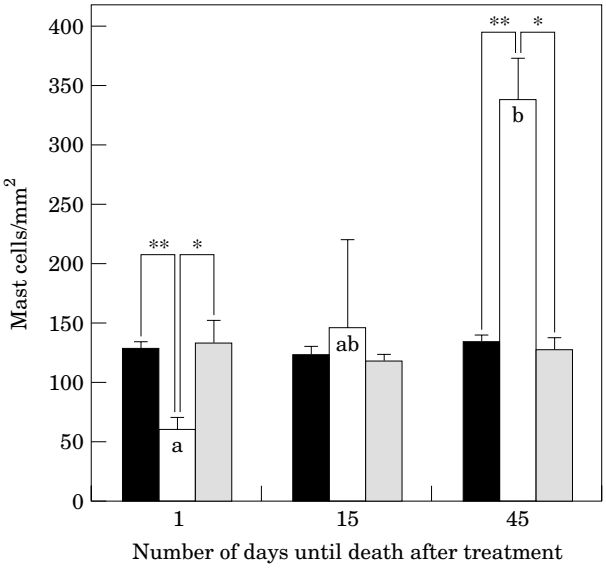

Figure 2. Mast cell density in the lung. (■) Saline, (□) *M. haemolytica*, (▨) non-inoculated. ** denotes $P<0.01$; * denotes $P<0.05$. At 1 day, bacteria-inoculated animals had a lower number of MCs in lung than saline-inoculated control animals (**$P<0.01$). Also, the bacteria-inoculated (right) lungs 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 (*$P<0.05$). In addition, there was a significant difference in MC numbers in bacteria-inoculated lungs over time. The numbers of MCs in animals killed at 1 (a) and 45 days (b) were significantly different ($P<0.05$), however, both values were not significantly different from MC at 15 days (ab).

Histamine determination

Bacteria-inoculated animals killed at 1 day post-inoculation had reduced concentrations of histamine in their inoculated lungs compared to controls ($P<0.001$), as well as compared to their non-inoculated contralateral lungs ($P<0.01$). However, neither the bacteria-inoculated groups killed at 15 days or those killed at 45 days had significant differences in these comparisons. Nonetheless, at 45 days, the concentrations of histamine in the bacteria-inoculated lungs were higher and closer to significance when compared with both controls ($P=0.055$) and the contralateral lobes ($P=0.052$). Comparisons of the three time points within each treatment showed that only bacteria-inoculated lungs had significantly different concentrations of histamine ($P<0.005$). The histamine content of animals killed at 45 days was significantly different from those of animals killed at 1 and 15 days ($P<0.05$) (Fig. 3).

Substance *P*-like immunoreactivity (SP-Li)

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. 1(c)]. These cells reacted strongly, although less than nerve fibres. Serial sections stained with H&E showed that most of the cells were macrophages. The immunoreactivity of nerve fibres associated with

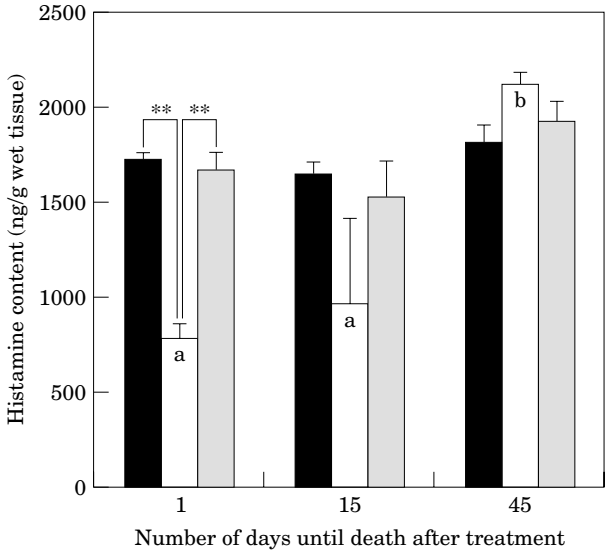

Figure 3. Histamine content in the lung. (■) Saline, (□) *M. haemolytica*, (▨) non-inoculated. ** denotes $P<0.01$; * denotes $P<0.05$. At 1 day, bacteria-inoculated animals had a lower amount of histamine in their lungs than saline-inoculated control animals (**$P<0.001$). Also, the bacteria-inoculated (right) lungs had lower amount of histamine than the non-inoculated (left) lungs in bacteria-inoculated animals (**$P<0.01$). In addition, there were significant differences in histamine content over time. The histamine content of animals killed at 45 days (b) was significantly higher than those values of animals killed at 1 (a) and 15 days (a) ($P<0.05$).

the airways and blood vessels was decreased, particularly in areas of most severe damage.

By day 15 after bacterial inoculation the SP-Li immunoreactivity was mostly confined to nerve fibres. However, the large macrophages surrounding the areas of necrosis in the pyogranulomatous abscesses had a mild immunoreactivity. Many of the nerve fibres immunoreacted strongly, were thicker, and in the tortous bronchiolo–alveolar junctions [Fig. 1(d)], around small blood vessels and in alveolar walls. Furthermore, some affected small bronchioles had also irregularly thickened immunoreactive unmyelinated fibres. Occasionally, enlarged myelinated fibre bundles had many immunoreactive fragments [Fig. 1(e)]. Some large peribronchial ganglion neurons located in the submucosa of a few bronchi had an intense immunoreaction [Fig. 1(f)]. All of these changes were observed in areas where there was epithelial cell proliferation. The SP-Li immunoreactivity was more scant in areas of extreme parenchymal fibrosis and was rare in areas of pleural and interlobular fibrosis.

The SP-Li immunoreactivity in bacteria-inoculated animals at 45 days after challenge had a decreased pattern of distribution with variable intensity, that was mostly mild. Moreover, none of the lungs had immunoreactivity of peribronchial neuronal bodies. This group also lacked any extraneural SP-Li 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 [22, 23] and sheep [21]. The immunoreaction was present in small nerve fibres that had the typical varicosities of unmyelinated C-fibres in the lamina propria and submucosa of bronchi and in the adventitia in the lamina propria and submucosa of bronchi and in the adventitia of adjacent propria and submucosa of bronchi and in the adventitia of adjacent blood vessels. Bundles of myelinated fibres also had several enmeshed immunoreactive fibres. The immunoreactivity was not present in peribronchial neuronal cell bodies. Similarly, small unmyelinated fibres and some myelinated fibres 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 fibres. Nonetheless, the immunoreactive nerve fibres showed a more consistent pattern of distribution and stronger immunoreaction than bacteria-inoculated lungs.

Additional studies

Immunofluorescence

Some of the lungs of control animals as well as bacteria-inoculated sheep had immunofluorescence for *Mycoplasma ovipneumoniae*. There was no massive accumulation of antigen in any of the positive sections or was there a positive reaction related to infiltrating or exudating cells.

Serology

All of the animals had positive titres to *M. haemolytica* at arrival that ranged 4–32 with an average mean and SEM of 18.88 ± 2.66. After challenge the titres of control animals remained unchanged whereas the titres of bacteria-inoculated animals varied (Table 1).

Table 1. Average serum titres to *M. haemolytica* for experimental groups

	Titre		
Group	Day 1	Day 15	Day 45
M. haemolytica	18.9±2.7	48.0±16.0	52.8±8.1
Saline	18.9±2.7	18.9±2.7	18.9±2.7

Table 2. Isolation of *M. haemolytica* from lung of experimental groups

	Average cfu/g *M. haemolytica*		
Group	Day 1	Day 15	Day 45
M. haemolytica	240	1340	0
Saline	0	0	0

Bacteriology

M. haemolytica was recovered from bacteria-inoculated animals at 1 day and 15 days after challenge but not at 45 days or lungs of saline-inoculated controls (Table 2).

Discussion

The extent of mast cell alterations in subacute and chronic PP has not been previously determined. The increases in MCs at 15 and 45 days post-inoculation in our study is similar to increases seen by Sorden and Castleman in rats infected with respiratory syncytial virus [29]. The increases in MCs at 15 and 45 days occurred mostly in those areas with epithelializing changes in pulmonary alveoli and bronchioles. This pattern of MC proliferation may represent a functional relationship with epithelial cells because MC tryptase is a potent mitogen for epithelial cells in the respiratory tract [30]. Although there was increased deposition of fibrous connective tissue at these timepoints, the role of MC on fibroplasia is not fully understood. Fibrosis can occur independent of MC [31–33], however, MC contain proteases and growth factors that stimulate lung fibroblast proliferation and synthesis of type I collagen [3, 4]. MCs-fibroblast heterotypic contacts induce MC synthesis of interleukin-4, which augments fibroblast proliferation [34, 35].

The lack of a significant increase in histamine at 15 and 45 days contrasts the increase in MCs present at these timepoints. In the lung, local environmental conditions influence maturation of MCs [34], and this most likely occurs asynchronously for each MC [42]. It is possible that higher numbers of MCs in the chronic pneumonic lesions (45 days) includes a large population of immature MC that contain low amounts of histamine. Recognition of immature MCs with low histamine content by toluidine blue is not possible, because the expression of the proteoglycan matrix in their granules occurs prior to maturation and regulates the content of histamine, proteases and other granule components [43].

The reduction in MCs and decreased levels of histamine in areas of severe lung damage at 1 day after bacterial inoculation are consistent with another study in calves [24]. However, our histamine findings differ from a study demonstrating an increase in histamine released from MCs recovered from cattle infected with *M. haemolytica* [25] and from a study in which bacterial leukotoxin and lipopolysaccharide both caused an increase in spontaneous release of histamine from isolated bovine lung parenchyma [26].

It was an unexpected and striking finding that leukocytes, likely macrophages, infiltrating areas of severe lung damage had SP-Li immunoreactivity. In rats, alveolar macrophages normally express the preprotachykinin-I gene mRNA which encodes SP and neurokinin A; however, during lung inflammation induced by lipopolysaccharide, preprotachykinin gene-I mRNA expression is increased two- to four-fold and SP can be recognized by immunohistochemistry [15]. 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 with immunoreactivity, neutrophils can also be a source of SP in rats [15, 27]. Thus, in addition to sensory C-fibres, inflammatory cells infiltrating the lung may be an important source of tachykinins. This extraneural contribution may favour an inflammatory imbalance that results in severe lung damage [15] accompanied by fibroplasia [28]. The nerve fibres associated with airways and blood vessels located in areas of severe damage

reacted only moderately, probably due to an early depletion of neuropeptides.

Alterations in SP-immunoreactive nerve fibres can be seen with musculoskeletal injury [36, 37] and in some studies of asthma [38]. Although we recognized a trend of increased SP-Li immunoreactivity of nerve fibres in some areas of interstitial damage at 15 days after bacterial challenge, we did not carry out a morphometric-quantitative evaluation. In the respiratory tract, SP nerve fibres occur with highest density in the nasal septum, whereas the lung has few fibres and most of them are associated with large airways [21–23]. Therefore, an accurate analysis of density of SP nerve fibres in the lung was not feasible.

SP-Li immunoreactivity in neuronal bodies at 15 days post-inoculation, but not in control animals, was an unusual and striking finding and may represent a phenotypic switch in substance P production by these cells. In general, it is widely accepted that the majority of SP nerve fibres within the lung have their cell bodies located extrapulmonary in the nodose ganglion of the vagus nerve with lesser numbers in the thoracic dorsal root ganglia [5, 6]. The presence of SP in intrapulmonary neurons has not been consistently recognized [14]. Horses [22, 23, 39] and sheep [21] lack SP in intrapulmonary neurons, whereas it is present in cats [40]. In certain models of inflammatory pain there is a phenotypic switch of primary sensory neurons, which normally do not express SP, to produce SP during persistent inflammatory injury [12, 13]. Apparently, this transcriptional and post-translational plasticity is induced mainly by nerve growth factor [12, 13], and MCs are an important source of nerve growth factor [41]. Thus, as has been demonstrated in models of inflammatory pain [12, 13], the expression of SP-Li immunoreactivity in many nerve bundles and some ganglion neurons recognized here may represent a novel or augmented expression of SP to sustain the inflammatory reaction.

Dungworth [17] and López [18] agree that in animals surviving an acute episode of PP, complete resolution of lung lesions is rare. The parenchymal changes commonly include sequelae such as bronchiolitis obliterans, bronchiectasia, pulmonary sequestra and abscesses, pulmonary fibrosis and chronic pleuritis with fibrous pleural adhesions [17, 18]. In the present study, all of these changes were found in animals killed at 15 and 45 days after bacterial inoculation. In addition, alveolar and bronchiolar epithelialization was a prominent and consistent feature. Although some of the lungs included in these groups (as well as the groups killed at day 1) contained antigen to *M. ovipneumoniae*, the reaction was minimal and not associated with the *M. haemolytica* lesions. In addition, histological changes considered characteristic of *Mycoplasma* spp. infection [17] were rarely seen in these animals and control animals lacked such lesions. Therefore, the changes are most likely the result of *M. haemolytica* infection. According to bacteriological studies, no viable *M. haemolytica* were recovered from the lungs of animals killed at 45 days. Thus, even in absence of the infectious agent, the chronic lesions persist.

Based in our findings, we propose that the extraneural sources (macrophages) of SP during the acute phase of PP (day 1) create an inflammatory imbalance that favours an excessive degranulation of MCs. Then, the excessive mediators released and the augmented production of SP promote tissue damage and fibrosis. Subsequently, during the sub-acute phase (15 days), SP is produced not only by extrinsic sensory neurons, but also by intrinsic pulmonary (ganglionic) neurons, probably to sustain an adequate balance between inflammatory reaction and immune response. At this time point MCs proliferate and promote the epithelializing changes that characterize this stage. Finally, during the chronic phase (45 days), when the fibrotic changes are more defined and MCs are numerous, the diminished SP immunoreactivity may allow enhanced inflammatory reactions. Future studies are needed to address the expression and distribution of the NK-1R during the acute and chronic lesions in PP.

Materials and Methods

Experimental animals

Eighteen sheep, of both sexes and mixed breed, 3 months old, were obtained from Iowa State University, Laboratory Animal Resources. The animals had been weaned previously and adapted to a commercially available grower ration. These animals were arbitrarily assigned to two large groups, which were maintained in entirely climate controlled isolation rooms until their killing. After 24 h, one group received *Mannheimia* (*Pasteurella*) *haemolytica* while the other received saline. Subsequently, these large groups

Table 3. Experimental design

Treatment	Time of death		
	1 day	15 days	45 days
*M. haemolytica**	n = 3	n = 3	n = 3
Saline	n = 3	n = 3	n = 3
Non-inoculated**	n = 3	n = 3	n = 3

* *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 1×10^8 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 pyrogen-free saline (PFS).
** Additionally, the left (non-inoculated) lungs of the bacteria-inoculated animals were used as contralateral negative controls.

were subdivided in accordance to the time of death at 1, 15, and 45 days after inoculation. This arrangement resulted in six groups, each one with three animals (Table 3). The Iowa State University Animal Care Review Committee approved this protocol.

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, IL, U.S.A.) to achieve 1×10^8 cfu/ml [46]. 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 fibre-optic bronchoscopy as previously described [46, 47].

Collection of lung samples

Animals were killed with an intravenous overdose of sodium pentobarbital. Samples were collected from the site of inoculum deposition in the right cranial lung lobe. One sample was proximal to hilum and included the cranial bronchus, the other was distal and included the apex of the caudal part of the cranial lobe. Additionally, from those animals that received the bacterial inoculum, similar samples from the contralateral, cranial left lobe were also collected. Samples were obtained in duplicate to be fixed by immersion in 10% neutral buffered formalin (48 h at 4°C). Furthermore, small pieces of lung tissue from the same areas were collected in cryo-vials (Nunc CryoTube, Nalge Nunc International, Naperville, IL, U.S.A.) 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. No less than two serial sections were stained with H&E, Mallory's trichrome, and low pH toluidine blue. The special stains were employed for recognition of fibrous connective 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 respiratory tract of sheep [21].

For quantification of MCs, two slides containing the corresponding sections of the right lung (proximal and distal parts of the cranial lobe) were assessed by light microscopy. The slides were assessed blindly and MC density was determined by counting the numbers of stained MCs present in 50 different fields at 400× magnification. The procedure has been referred previously to count MCs in the respiratory tract of sheep [21]. The fields were chosen by chance with no premeditated intention to avoid empty spaces except for the lumina of large airways or large blood vessels [21]. The area provided by these fields is equivalent to 5.9 mm^2 [21]. The values obtained from each sheep corresponded to counting the 50 fields in each one of the two slides 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/mm^2.

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 500 µl of 0.2 N $HClO_4$. 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 1 M, pH 9.25 of $K_2B_4O_7$. 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 was filled with the tissue 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

Replicate sections from routinely processed formalin-fixed tissues were stained with H&E and with immunoperoxidase stain for SP. The immunohistochemical procedure has been employed to stain the SP nerve fibres in the respiratory tract of sheep [21]. Substance P immunoreactivity was determined employing a rabbit anti-SP primary antibody (Peninsula Laboratories, Inc., San Carlos, CA, U.S.A.). 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_2O_2 for 40 min. After several rinses in buffer (Wash Buffer, BioGenex, San Ramon, CA, U.S.A.) the slides were incubated at room temperature for 20 min with a solution containing 10% normal goat serum and 3% bovine serum albumin (Elisa-grade BSA, Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in buffer. Then, the slides were incubated in a humidified and sealed chamber at 4°C for 96 h with the primary antibody diluted 1:10 000 in antibody diluent (Common Antibody Diluent, BioGenex, San Ramon, CA, U.S.A.). The slides were rinsed several times and subjected to incubation at room temperature for 1 h with the secondary antibody (affinity-purified biotin-labelled, Goat anti-Rabbit IgG [H + L], Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) diluted 1:200. After several rinses, the peroxidase-conjugated streptavidin reagent (Super Sensitive HRP, BioGenex) was added (undiluted) for incubation at room temperature for 1 h. 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, U.S.A.) 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 normal rabbit serum gave no immunostaining. Small intestine sections (ileum) from sheep were used as positive controls because these areas contain mysenteric and submucosal plexuses with large numbers of immunoreactive nerve fibres and nerve cells bodies. As we did not characterize the peptide reacting with our primary antibody and a cross-reaction with other related tachykinins was possible, the term SP-like (SP-Li) immunoreactivity is preferred.

Additional studies

Small pieces of the right lung were collected in cryo-embedding medium (OCT compound, Miles Inc., Elkhart, IN, U.S.A.) and stored at −80°C. This material was subsequently sectioned in a cryostat and prepared to detect *Mycoplasma ovipneumonia* by indirect immunofluorescence employing a rabbit anti-*M. ovipneumoniae* antiserum. The procedure is a modified protocol from the technique described by Amanfu *et al*. [48]. Only an inconsistent and minimal amount of antigen was present and sections lacked significant lesions of *M. ovipneumoniae* infection.

Samples of blood were taken from each animal the day after arrival to isolation rooms and the day scheduled for death. Passive hemagglutination titres to *M. haemolytica* lipopolysaccharide were assessed from these samples [49].

Samples maintained at −80°C were used for serial dilution and plated on sheep blood agar (TSA/sheep blood agar; Remel, Lenexa, KS, U.S.A.) to determine the cfu/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 concentrations were separately subjected to statistical comparisons. Each group receiving the bacterial challenge was compared with the corresponding control at the same time period employing a two-tailed Student's *t*-test [50]. The procedures were carried out assuming independent samples and equal variances, except for comparisons at 15 days where an *F*-test showed unequal variances and therefore, a Student's *t*-test for unequal variances was utilized [50]. Additionally, in the bacterial challenged groups, the non-inoculated (left) lungs were compared with the inoculated (right) lungs using a two-tailed paired Student's *t*-test [50]. An analysis of variance (one-way classification) was used to compare the different time points within each treatment [50]. When a significant *F* value was recognized a subsequent Tukey's *w*-test was employed to identify the differences among the means at the different time points [50]. The level of significance for all of the procedures was $P = 0.05$.

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