Ultrastructural study of ovine pulmonary pasteurellosis: involvement of neutrophils and macrophages

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Summary. Pasteurellosis is a common infectious disease characterised by fibrinous pneumonia and involving neutrophils and macrophages. This study aimed to determine the timing and extent of the pathogenic involvement of these cell elements in lesions induced in experimentally-infected lambs.

A concentration of approximately 3×10^8 bacteria/ml. was inoculated into 30 two-month-old disease-free Merino lambs. Five lambs were assigned to each of five experimental batches, slaughtered on days 1, 3, 7, 11 and 15 following intratracheal inoculation, and to one control batch inoculated with a sterile solution. One control animal was slaughtered at the same time as each experimental batch.

More characteristic lesions occur in bronchioles, peribronchial tissue and alveoli and are characterised by fibrinous processes. From the start of the experiment, epithelial-cell disruption and loss of microvilli were apparent; cell debris, desquamate cells and bacterial elements were observed in bronchiolar lumina, embedded in a fibrillar granular material. Alveolar structures displayed fewer neutrophils and macrophages, containing phagocytic vacuoles. Laminar bodies were apparent in type II pneumocytes. The interseptal area contained similar cell types, as well as abundant edema. In the course of the experiment, macrophage numbers increased in all the areas involved, with signs of intense phagocytic activity. The final phase of the experiment was characterised by a mild interseptal infiltrate and by clear alveolar lumina.

Key words: Neutrophils, Macrophages, Pasteurellosis, Lung

Introduction

Pasteurellosis is a common infectious disease. The major agent is *Mannheimia haemolytica* (Alley, 1975), although other agents, including parainfluenza-3 virus

(Sharp et al., 1978; Davis et al., 1986), and bovine syncytial virus (Trigo et al., 1984) may also be involved in the process.

The disease is characterised by fibrinous pneumonia (Gilmour et al., 1986; Ackerman et al., 1991; Car et al., 1991). Experimental *M. haemolytica A1* infection prompts progressive ultrastructural damage to alveoli during the logarithmic stage of bacterial growth, evident in the presence of fibrin, neutrophils and bacteria (Westweber et al., 1990; Whiteley et al., 1991a,b). Alveolar macrophages and neutrophils are the cells involved in the early phases, and subsequently induce inflammation (Walker et al., 1980; Whiteley et al., 1991a,b; Cutlip et al., 1998) and macrophages neutralise Mannheimia by phagocytosis (Collins et al., 1983). Extensive damage is also reported in interseptal capillary endothelial cells, inducing a pathogenic process (Sharma et al., 1991) which subsequently recruits macrophages and neutrophils to act as cell mediators in triggering both the coagulation cascade (Bowersock et al., 1990) and interseptal and vascular fibrin deposition (Car et al., 1991; Whiteley et al., 1991b). The neutrophil-endothelial cell interaction is thus a crucial feature of vascular involvement, to the extent that neutrophil adherence to the capillary endothelium and the subsequent damage caused are two leading factors in the pathogenesis of pasteurellosis (Whiteley et al., 1991b). However, in addition to the direct action of *M. haemolytica*, other substances may also enhance endothelial damage; these include bacterial leukotoxin (Maheswaran et al., 1993), which causes edema and interseptal fibrin deposition. According to Sharma et al. (1991), endothelial damage is due to the contact of *Mannheimia* with the endothelium and to bacterial products, presumably capsular proteins and lipopolysaccharides (Breider et al., 1990; Confer et al., 1990; Whiteley et al., 1990; Brogden et al., 1995).

The involvement of alveolar macrophages in pasteurellosis is undisputed, since bacteria have been observed in alveolar macrophage phagosomes (Maheswaran et al., 1980; Ackerman et al., 1991). At high doses, some bacteria escape phagocytosis and produce leukotoxins, particularly during the logarithmic growth phase (Brown et al., 1997); these are responsible

for macrophage lysis (Benson et al., 1980) through pore formation, subsequent membrane permeabilization and apoptosis (Stevens and Czuprynski, 1996; Sun et al., 1999). This leukotoxin, produced by all types of *Mannheimia haemolytica*, is able to neutralize neutrophils and alveolar macrophages (Berggren et al., 1981), thus increasing the number of inflammatory mediators and leukocytic enzymes. So, neutrophils and macrophages are susceptible to leukotoxin (Berggren et al., 1981, Czuprynski et al., 1991).

This study aimed to determine the timing and extent of the pathogenic involvement of both neutrophils and alveolar macrophages, and to examine lesions produced by acute experimentally-induced ovine pasteurellosis.

Material and methods

Inoculum

For preparation of bacterial inoculum, *Mannheimia haemolytica* serovar 2 No. ATCC 29694 was inoculated onto blood agar base (Difco), previously sterilised at 121 °C for 15 minutes with later addition of 5% sterile defibrinated sheep blood (Oxoid). After 24 hours' incubation at 37 °C, it was grown in brain heart infusion medium (Difco) in order to obtain a high bacterial concentration, and incubated for a further 24 hours at 37 °C with constant shaking. Bacterial concentration was calculated, and adjusted to Standard Tube 1 on the McFarland scale (0.1 ml of 1% barium chloride and 9.9 ml of 1% sulphuric acid), equivalent to a concentration of roughly $3x10^8$ bacteria/ml. The concentration was confirmed by counting colony-forming units (CFUs) in the previously-adjusted suspension. Five tubes containing 9 ml sterile saline solution (distilled water + 0.85 NaCl) were used, providing serial dilutions of 1/10, 1/100, 1/1000, 1/10000 and 1/100000. Subsequently, 0.1 ml of each dilution was added to blood agar in a Petri plate and incubated at 37 °C for 24 hours. The colonies on each plate were then counted (CFU = no. colonies/plate x 1/dilution x10). The arithmetic mean confirmed a concentration similar to that obtained using McFarland's turbidimetric scale. Lambs were inoculated intratracheally following sedation with 0.3 ml of xylazine (Rompún, Bayer); each lamb receiving 20 ml of suspension.

Experimental groups

Inoculated groups

Twenty-five 2-month-old male and female Merino lambs weighing roughly 20 kg were used. Prior to commencing the study, control analyses were performed to confirm that all animals were free of infectious and contagious disease. The previous day to the inoculation, blood was extracted and the serum was obtained by coagulation. This was used by ELISA and tube agglutination, obtaining a value zero. Also, nasal swabs

were cultured in blood agar, incubated at 37 °C for 48 hours; these cultures were negative. The animals were fed *ad libitum* with cereal feed, hay and concentrate. The lambs were grouped into five experimental batches, each group containing five animals. Experimental batches were slaughtered at 1, 3, 7, 11 and 15 days postinoculation (dpi) by intravenous inoculation of a lethal dose of sodium thiopental. Following necropsy, lung samples were fixed in 5% glutaraldehyde in phosphate buffer, postfixed in 2% buffered osmium tetroxide and embedded in araldite (Durcapan A.C.M.). Sections 200 and 400 nm thick were contrasted using 2% uranyl acetate and lead citrate and viewed through a JEOL JEM. 100SX electron microscope.

Control group

Five 2-month-old male and female Merino lambs weighing roughly 20 kg were used. Prior to commencing the study, control analyses were performed similar to those of the inoculated groups. The lambs received 20 ml of apyretic sterile saline solution and were slaughtered at the same time as each experimental batch. The treatment of lung samples was similar to that of the inoculated animals.

Results

Control group

Lungs of these animals showed histological characteristics considered inside a range of normality
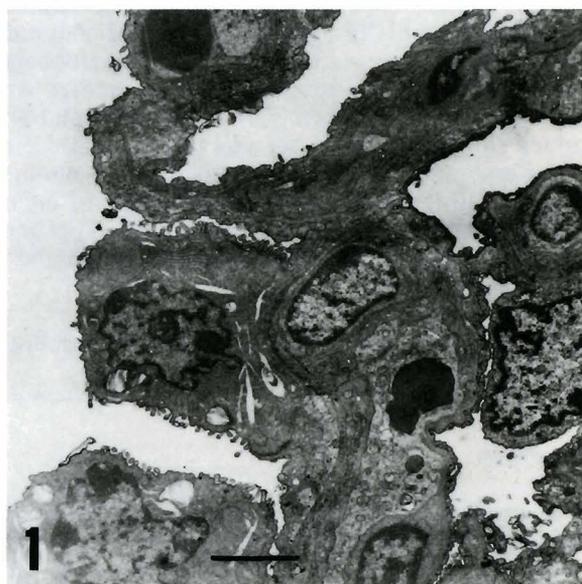
Fig. 1. Sheep. Control. Detail of alveolar parenchyma in which lumens are shown covered by epithelial cells and type II pneumocytes. Capillaries and some interstitial cells are noted in the interseptum. Size Bar: 2µm.

from 1 dpi until 15 dpi (Fig. 1). Bronchiolar epithelium, lumen and subepithelial space were intact and the presence of cell elements representative of inflammatory processes were not noted.

Inoculated groups

At 1 dpi, lesions consistent with fibrinous bronchopneumonia affected bronchioles, peribronchial tissue and alveoli. Significant damage was observed (Fig. 2) in bronchiolar epithelium, lumen and subepithelial space. The bronchiolar epithelium was disrupted; cells displayed a highly-vacuolated cytoplasm and an almost total loss of microvilli, and some necrotic

cells were observed. The bronchial lumen contained cell debris, desquamate cells and bacterial elements (Fig. 3), together with granular and moderately electron-dense material; fibrillar material was also occasionally observed. These findings persisted until 11 dpi, diminishing partially by 15 dpi. There was evidence of edema in the subendothelial space, and changes were apparent both in vascular lumina and within endothelial cells. Platelet aggregates, macrophages containing phagosomal bodies (Fig. 4) and lymphocyte elements were frequently observed in capillary lumina. Vascular endothelium displayed vacuolization, cell degeneration and partial cell loss; marked edema was apparent in the adventitia.
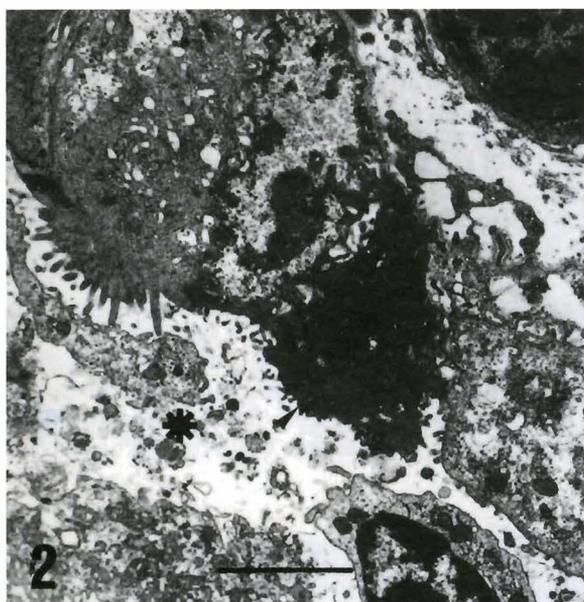
Fig. 2. Experimental pasteurellosis. Sheep 1 day postinoculation (dpi). Bronchiolar lumina with desquamate phenomena (asterisk) and necrotic rests (arrowheads). Size bar: 2 μm.

Fig. 3. Experimental pasteurellosis. Sheep 1 dpi. Bacterial elements (arrows) and cell debris in bronchial lumen. Size bar: 2 μm.
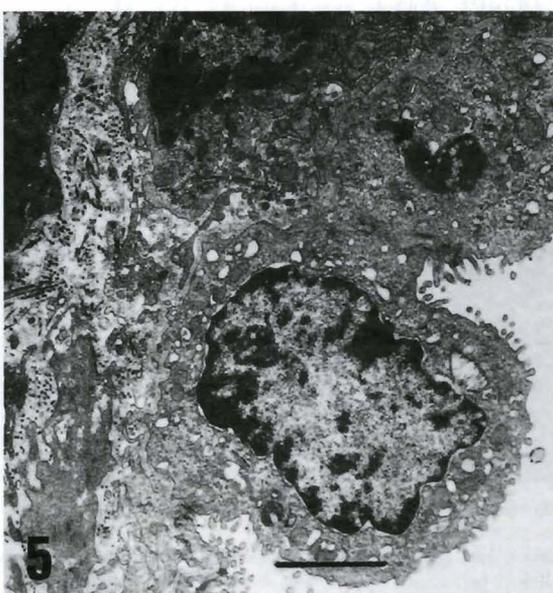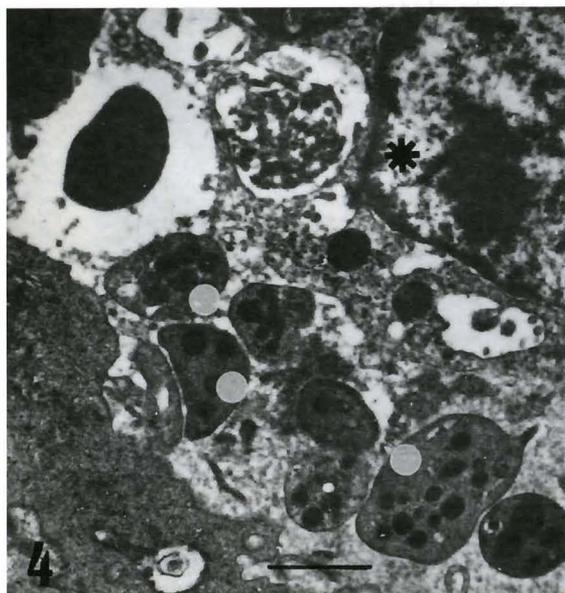
Fig. 4. Experimental pasteurellosis. Sheep 3 dpi. Platelet aggregates (circles) and macrophages (asterisk) in capillary lumen. Size bar: 2 μm.

Fig. 5. Experimental pasteurellosis. Sheep 7 dpi. Vacuolization and disruption of alveolar epithelium. Size bar: 2 μm.

The most severe lesions were observed in airway lobules. From 1 dpi onwards, alveolar ducts displayed marked vacuolization and partial loss of epithelium (Fig. 5). A severe and highly-active alveolar infiltrate comprised trilobar neutrophils, lysosomal bodies and phagocyte structures. Infiltrate also contained a smaller number of macrophages. Damage to the alveolar epithelium particularly involved type II pneumocytes, which displayed secreting laminar bodies (Fig. 6). The interseptal space was enlarged due to the presence of edema and infiltrate consisting mainly of neutrophils, lymphocytes and macrophages.

This process was observed throughout the study, and was most significant at 7 and 11 dpi, by which time alveolar structures displayed a pronounced infiltrate leading to disruption of airway lobules. The lumina of both alveolar ducts and alveoli contained numerous neutrophils and macrophages, the latter adhering to the alveolar wall. A similar infiltrate was detected in the interseptal area and in the peribronchial space, where numerous macrophages displayed highly-vacuolised cytoplasm and lysosomal structures. Both neutrophils and macrophages displayed phagocytic vacuoles containing possible bacterial residue. Over the following days, macrophage infiltrate was observed in alveolar lumina and airway lobule ducts. This infiltrate eventually took the form of largely-homogeneous macrophage populations with occasional interspersed neutrophils. Macrophages were also abundant in the interseptal space, prompting increased interseptal thickness, and thus discapacitating the respiratory membrane. Ultrastructurally, macrophages appeared highly active (Fig. 7). Macrophage cytoplasm contained numerous primary lysosomes and residual bodies, as well as secondary lysosomes and a highly electron-dense hyaloplasm. Cytoplasmic membrane displayed frequent filopodia, giving rise to marked phagocytic activity. Although these lesions persisted until the end of the study, by 11 and 15 dpi pneumonic areas were evident only in a mild interseptal macrophage infiltrate; alveolar lumina were relatively clear. Bacterial cultures showed growth in all the treated animals, but this is more abundant with more patent lesions.

Discussion

M. haemolytica serovar 2 (also called *P. haemolytica* serovar 2 biovar A) is responsible for numerous respiratory processes in ruminants (Angen et al., 1997) characterized by pneumonia in sheep of all the ages (Gilmour, 1980; Gilmour et al., 1986) in contrast to biovar T of *M haemolytica*, which causes septicemia overall in old animals (Gilmour, 1978; Sneath and Stevens, 1990) or chronic pneumonias which are caused by this agent combined with *Mycoplasma ovipneumoniae* (Gilmour et al., 1979). *M. haemolytica* serovar 2, either by intratracheal inoculation (Haritini et al., 1987) or by aerosol (Fedde et al., 1986), prompts changes in the bronchiolar epithelium and in peribronchiolar structures from 1 dpi onwards; bacterial products act directly on capillaries, and subsequently on neutrophils (Breider et al., 1991; Maheswaran et al., 1993; Ramirez and Brogden, 1995). Exponential bacterial growth, studied by cultures taken of the
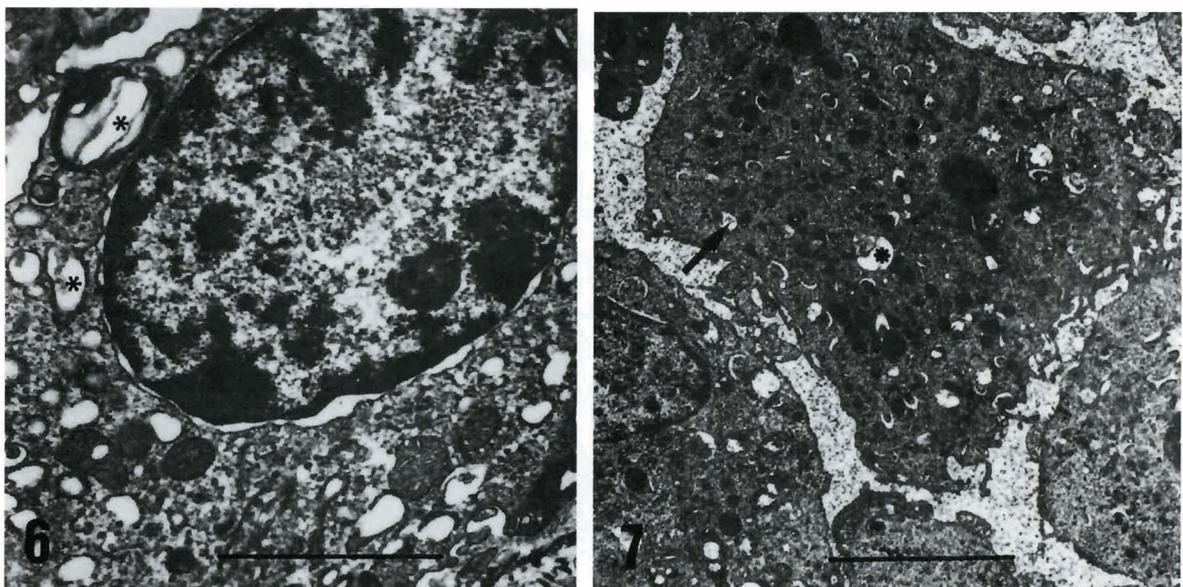
Fig. 6. Experimental pasteurellosis. Sheep 7 dpi. Type II pneumocytes containing multilaminar bodies (asterisk). Size bar: 2 μm.

Fig. 7. Experimental pasteurellosis. Sheep 11 dpi. Neutrophils and macrophages containing numerous primary lysosomes (arrow) and residual bodies (asterisk), as well as secondary lysosomes and a highly electron-dense hyaloplasm. Size bar: 2 μm.

diseased lungs, commenced at 1 dpi in bronchiolar and alveolar lumina, and lasted until 3 dpi, numbers thereafter becoming negligible. Observation of bacteria and bacterial products interspersed with cell debris, fibrin and lymphocyte elements suggests direct lesion of both bronchiolar and alveolar epithelia, as reported by Collins et al. (1983); the presence of fibrin indicates triggering of the coagulation cascade, as reported by Bowersock et al. (1990) and Whiteley et al. (1991b). The start of the process is also marked by epithelial damage, evident in visible cell degeneration. Direct action of LPS on interseptal capillaries marked the start of a pneumonic process involving peribronchial and interseptal structures, and characterised by edema and fibrin deposits which were also observed in alveoli. These lesions continued until 7 dpi, suggesting the possibility of process feedback up to 15 dpi. The pneumonic process is therefore triggered by two forms of bacterial action: one caused directly by bacteria, acting on terminal bronchiolar and alveolar epithelium; and the other caused by bacterial products because lesions have been shown without bacterial colonies and others with bacterial elements. Bacterial action directly involves neutrophils which, following migration, are responsible for capillary damage (Whiteley et al., 1991b), edema formation and interseptal fibrin deposition (Westweber et al., 1990; Maheswaran et al. 1993). Bacterial toxins cause neutrophil degeneration and subsequent migration (Whiteley et al., 1991a) and also macrophage activation; activation occurred midway through this study, thus prolonging the pneumonic process (Sharma et al., 1991) and giving rise to feedback inflammation. Attention is drawn to the joint involvement of degenerative cell debris from type II pneumocytes and alveolar epithelial cells in the development of these lesions. Although it is considered that the role of neutrophils and macrophages are asseseed at 2, 4 and 6 hours dpi (Ackermann et al., 1996, 1999), in this study both have been observed even at 15 dpi with significant alterations.

Degeneration of the alveolar epithelium and the direct bacterial action are cofactors in the development of fibrinous processes; these persist due to macrophage involvement in the second half of the process and to feedback prompted by epithelial and interseptal damage. For this reason, it would appear that although the process triggered by *Mannheimia* initially arises from the interaction of bacteria with the bronchiolar and alveolar epithelia, its subsequent development is due to neutrophil action, with macrophage-mediated feedback bacause damaged neutrophils liberate chemotactic factors which promote the presence of more cells.

Finally, it should be noted that the pneumonic process observed in this study was triggered only by *Mannheimia haemolytica*; no other associated factor prompted major histopathological alterations. These findings suggest that the mild pneumonic reaction still evident at 11 and 15 dpi is due, in part, to the feedback described previously. Therefore, natural infections may involve other elements, probably of bacterial or viral origin, which complicate histopathological events and thus complicate the disease itself.

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