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Initial Lung Lesions in Two Calves Experimentally Infected with *Haemophilus somnus*

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With 3 figures

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Summary

The initial lung lesions in two calves intrabronchially inoculated with *Haemophilus somnus* are described. The animals were euthanized within 7 h after challenge. The *in situ* location of *H. somnus* and accompanying lesions were examined by light microscopy, immunohistochemistry and transmission electron microscopy (TEM).

Inoculation with *H. somnus* resulted in the development of acute pulmonary lesions within 3.5 h. *H. somnus* antigen was demonstrated only within the luminal spaces of the airways and in one area of bronchio-associated lymphoid tissue (BALT). As observed by TEM, the bacteria were phagocytized by both neutrophils and alveolar macrophages. Antigen was never demonstrated in the pulmonary intravascular macrophages.

Introduction

Haemophilus somnus is the causal agent of a variety of bovine diseases, e.g. pneumonia, thrombotic meningoencephalitis and reproductive syndromes (HUMPHREY and STEPHENS, 1983). In Denmark, pneumonia is a major cause of losses in calf production and *H. somnus* is frequently isolated from pneumonic calf lungs (TEGTMEIER, 1996).

Lung lesions caused by *H. somnus* in calves following spontaneous (ANDREWS et al., 1985; BRYSON et al., 1990; TEGTMEIER, 1996) as well as experimental infections (GOGOLEWSKI et al., 1987; JACKSON et al., 1987; POTGIETER et al., 1988) have previously been described. Most experimental studies have focused on lesions developed at least 24 h after infection. Although cases of severe and often fatal pneumonia in naturally infected calves seem to develop rapidly, studies focusing on the pathogenesis of the initial lung lesions caused by *H. somnus* are not available. Moreover, the ultrastructural lesions related to *in vivo H. somnus* infection have not been described. However, THOMPSON and LITTLE (1981) observed widespread contraction and desquamation of endothelial cells with adherence of *H. somnus* bacteria *in vitro* when inoculated on bovine arterial endothelial cell cultures. Also, the possible expression of adhesion mechanisms, i.e. the formation of pili *in vivo*, has not been examined, whereas more *in vitro* studies have failed to demonstrate the presence of pili (STEPHENS and LITTLE, 1981; THOMPSON and LITTLE, 1981; WARD et al., 1984).

The aim of the present work was to study the early lung lesions in two calves experimentally infected with *H. somnus* and the possible *in vivo* expression of adhesion mechanisms.

Materials and Methods

Experimental design

Two 1 month old Red Danish dairy calves were purchased from a local closed herd free of *Salmonella* and bovine viral diarrhoea virus, as monitored by the Danish Veterinary Laboratory (DVL). The calves were acclimatized to the facilities in isolation stables for 1 week prior to challenge. The calves were anaesthetized with propofol (Rapinovet®, Schering-Plough, Farum, Denmark), and a sterile catheter (Kruuse 230755, Marslev, Denmark) was placed into the right main bronchus through the larynx.

The inoculum consisted of $\approx 10^{10}$ colony-forming units (CFU) of *H. somnus* bacteria suspended in 10 ml of a sterile physiological saline solution. For inoculation, a Danish *H. somnus* field strain (DVL 939/90), isolated from a case of bovine pneumonia, was used. The strain was previously characterized by biotyping, plasmid profiling, REA-patterns and ribotyping, and belongs to the major group of Danish *H. somnus* strains isolated from cases of calf pneumonia (FUSSING and WEGENER, 1993). The inoculum was deposited through the catheter, followed by 20 ml sterile saline and 30 ml air. After challenge, the calves were monitored clinically until euthanasia with an overdose of sodium pentobarbital given intravenously 3.5 h (calf no. 1) and 7 h (calf no. 2) after inoculation.

Gross pathology and microbiology

The calves were necropsied immediately after euthanasia and macroscopic lesions were recorded.

Tissues from the right cranial lung lobe, the right diaphragmatic lung lobe and from the liver and spleen were examined bacteriologically.

Cultivation attempts for bacteria were performed according to standard laboratory procedures. In brief, tissue was cultured on four 5% bovine blood agar plates (Columbia agar base, Oxoid, Unipath Ltd, Basingstoke, UK) of which two contained polymyxin B sulphate (2500 IU/ml). Of the plates, two were incubated in normal atmosphere at 37°C and duplicate plates were incubated in an atmosphere of 10% CO_2 and 90% air at 37°C. All plates were inspected for growth after 16 h and 40 h. The identification of *H. somnus* was based on the growth of tiny white or yellow-white colonies on plates without polymyxin B sulphate incubated with 10% CO_2 and no growth in normal air, by Gram staining (negative), oxidase reaction (positive), catalase reaction (negative), production of indol and fermentation of glucose.

Other bacterial pathogens were identified according to standard laboratory procedures. In addition, lung tissue was also submitted for viral (bovine respiratory syncytial virus, parainfluenza-3 virus, bovine coronavirus, adenovirus, bovine virus diarrhoea virus) and mycoplasma examination according to standard laboratory procedures.

Histopathology and immunohistochemistry

Tissues from 16 areas of the lungs, the regional lymph nodes, tonsil, trachea, heart, brain, liver, spleen, kidney, and the gastrointestinal system were collected for histopathological and immunohistochemical examination. The tissues were fixed in 10% neutral buffered formalin, dehydrated in alcohol, embedded in paraffin wax and cut into sections 4–5 μm thick. All sections were stained with haematoxylin and eosin (HE) and in selected cases by Mallory's phosphotungstic acid haematoxylin (PTAH) stain for fibrin. Sections for immunohistochemistry were mounted on Star Frost® adhesive slides (Axel Johnson Lab System, Copenhagen, Denmark).

For the detection of *H. somnus* antigen in tissue sections, a peroxidase–antiperoxidase (PAP) technique previously developed for the *in situ* identification of the organism was applied. In brief, polyclonal rabbit serum raised against somatic fractions of *H. somnus* 939/90 bacteria was used as the primary reagent. The sensitivity and specificity of the primary immune serum and the PAP technique were examined and optimized by crossed immunoelectrophoresis and on experimentally infected murine tissues, respectively, as previously described (TEGTMEIER et al., 1995).

Transmission electron microscopy (TEM)

Multiple samples (> 15) of affected lung tissue were fixed in 2.5% glutaraldehyde in 0.13 M phosphate buffer for TEM. The tissue was postfixed in osmium tetroxide 1% in 0.13 M phosphate buffer and embedded in Vestopal-W® (Serva, Bie & Berntsen, Aarhus, Denmark). Ultrathin sections were contrasted with uranyl acetate and lead citrate prior to TEM examination, while the 1–2 μm thick survey sections were stained with toluidine blue.

Results

Clinical signs

Both calves developed symptoms in the first hours after challenge, i.e. coughing, increased respiration rate, abdominal respiration and depression.

In calf no. 1 the rectal temperature increased from 38.6°C to 39.1°C within 3.5 h after challenge, at which time it was euthanized. In calf no. 2, an initial increase in temperature (from 38.5°C to 38.8°C) was followed by a subsequent decrease to 37.5°C at 5 h post-infection, lasting until euthanasia 7 h after inoculation.

Gross pathology and microbiology

Calf no. 1. Acute pulmonary lesions were found in the right lung lobes and in the accesoric lobe. The lesions were characterized by areas of swollen tissue with a rubbery to firm consistency at palpation. On cross-section these areas were hyperaemic, oedematous with fibrinous exudation and ≈1–2 mm thick interstitial oedema. The trachea was hyperaemic with a foamy exudate and the regional lymph nodes were oedematous and swollen. *H. somnus* was recovered in pure culture from the lung lesions only. No virus or mycoplasma were detected.

Calf no. 2. An acute pulmonary lesion similar to the lesions observed in calf no. 1 was confined to the right diaphragmatic lung lobe (Fig. 1). In the cranial lung lobes, minor areas of chronic catarrhal bronchopneumonia were present. *H. somnus* was isolated from the affected lung areas, concomitant with a few *Pasteurella canis* biovar 2 bacteria. Also, *Mycoplasma dispar* and *Ureaplasma* spp. were cultured from the lung tissue, whereas the examination for viruses was negative.

Histopathology and immunohistochemistry

Lung. An exudative inflammation distributed in a lobular pattern was present in both calves. The dominating lesions were characterized as suppurative bronchiolitis and alveolitis, with a few necrotic foci scattered within the most severely affected areas. The alveoli were often filled with an amorphous eosinophilic exudate, fibrin and large numbers of erythrocytes (Fig. 2). Many neutrophils and some macrophages were also present. The interlobular septae and the lymphatic vessels were markedly distended by oedema fluid and inflammatory cells, especially neutrophils. A few areas of bronchio-associated lymphoid tissue (BALT) were markedly infiltrated with neutrophils in calf no. 1. The epithelial lining covering these areas was occasionally disrupted.

Within lesions, *H. somnus* antigen was regularly demonstrated immunohistochemically in the lumen of bronchi, bronchioles and alveoli. Sometimes antigen was observed in close association with the alveolar walls and especially alveolar macrophages. A few clumps of *H. somnus* antigen were observed in the distended lymphatic vessels and intracellular in BALT of calf no. 1. A few ulcerations of the epithelial lining in trachea with *H. somnus* antigen were also observed.

The areas with macroscopically visible chronic catarrhal bronchopneumonia in calf no. 2 were characterized by a suppurative bronchiolitis and alveolitis, peribronchiolar fibrosis, and hyperplasia of BALT.

In the pulmonary lymph nodes, the subcapsular and medullary sinuses contained a huge number of neutrophils. However, *H. somnus* antigen was not detected.

Other organs. A mild infiltration with neutrophils in the tonsillar crypts and a few microabscesses within the omasal laminae were recorded in both animals. *H. somnus* antigen was not detected in any extra pulmonary tissues.

TEM

In the alveolar walls, epithelial desquamation, perivascular oedema, and necrosis were present in both calves. Often the interalveolar capillaries were filled with leucocytes, pre-

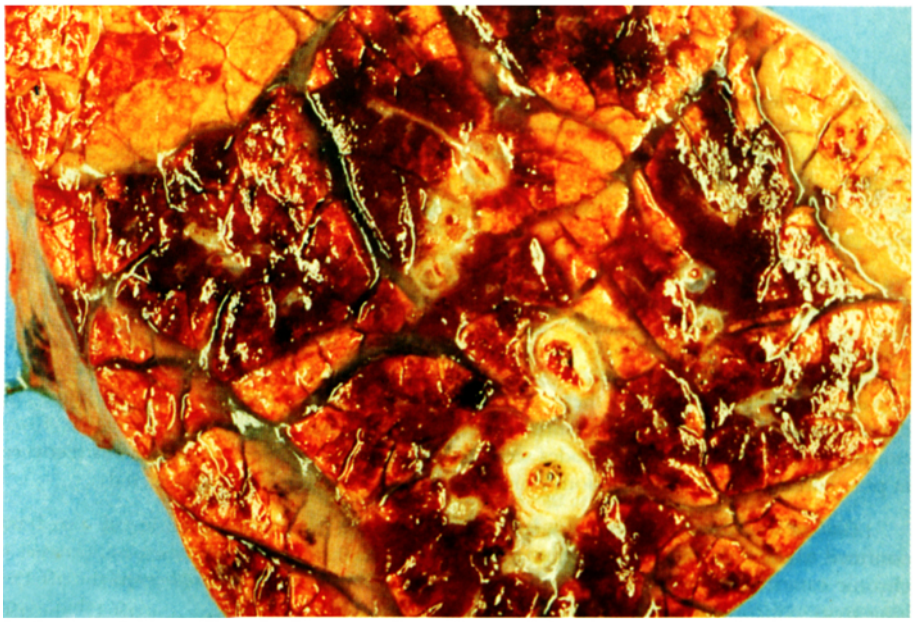

Fig. 1. Acute lesion with marked hyperaemia and interstitial oedema. Lung section from calf no. 2 euthanized 7 h after intrabronchial challenge with *Haemophilus somnus*.

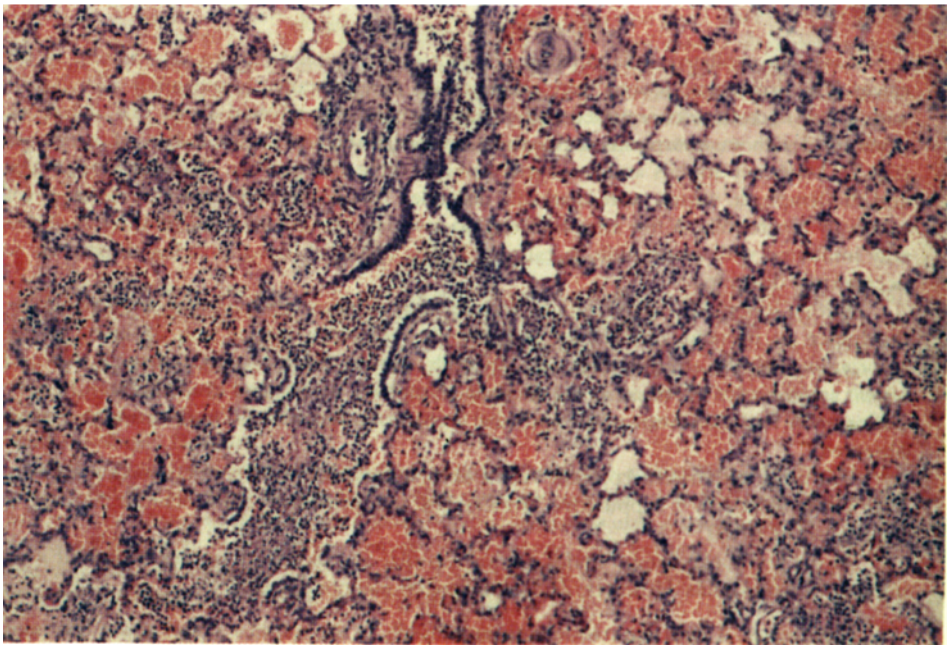

Fig. 2. Section from an acute lesion characterized by exudation of inflammatory cells (especially neutrophils), eosinophilic exudate, fibrin and large numbers of erythrocytes into the bronchiolar and alveolar lumina. Lung tissue from calf no. 1 euthanized 3.5 h after intrabronchial challenge with *Haemophilus somnus*. Haematoxylin and eosin (HE) stain, objective × 10.

dominantly neutrophils. In the alveolar spaces, exudation of proteinous and fibrinous fluids often containing erythrocytes and bacteria was observed. In such areas, inflammatory cells, primarily neutrophils and macrophages, were present. Occasionally bacteria could be demonstrated in different stages of division. Close adhesion to, or invasion of, epithelial cells by *H. somnus* was not observed, nor were pili or other adherent mechanisms. Bacteria were observed to be phagocytized by both neutrophils and pulmonary alveolar macrophages (Fig. 3), whereas they were not observed within the pulmonary intravascular macrophages.

Discussion

By light microscopy and TEM, neutrophils were seen to be the dominating cell infiltrating the early lung lesions. As determined by immunohistochemistry, *H. somnus* antigen was demonstrated only in areas of acute inflammation, in lymphatic vessels and in BALT. The uptake of *H. somnus* antigen by antigen-presenting cells and migration into the BALT tissue are predictable events in an aerogenic pulmonary infection. However, the illustration of uptake and transportation of the antigen from the bronchiolar lumen into the BALT within 3.5 h is notable.

As in the previous *in vitro* studies of the ultrastructure of *H. somnus* (STEPHENS and LITTLE, 1981), we failed to demonstrate pili in the present *in vivo* study. THOMPSON and LITTLE (1981) have described adhesion of *H. somnus* to bovine arterial endothelial cell cultures. However, in our study, adhesion or invasion by *H. somnus* of different types of epithelial cells was not observed. The close contact of *H. somnus* antigen to alveolar walls occasionally observed by light immunohistochemistry, could not be confirmed by TEM, although multiple areas were examined. In a previous immunohistochemical study of spontaneously infected calf lungs, *H. somnus* antigen was described as being located within the cytoplasm of airway epithelial cells,

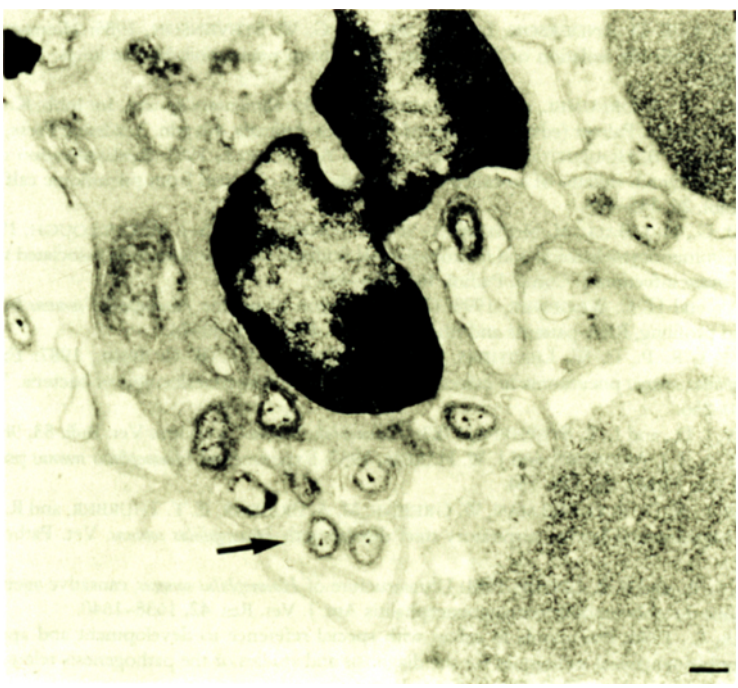

Fig. 3. Bacteria undergoing phagocytosis (arrow) by an alveolar macrophage in the alveolar lumen. Lung tissue from calf no. 2 euthanized 7 h after intrabronchial challenge with *Haemophilus somnus*. Bar = 0.6 μm.

which was speculated as a source leading to chronic persistence of infection (BRYSON et al., 1990). In the present study, *H. somnus* could not be demonstrated intracellularly in epithelial cells. This could be explained by the early stage of infection, or that the apparent intracellular location observed by immunohistochemistry was an optical artefact.

P. canis biovar 2 has previously been isolated from pneumonic bovine lungs (BISGAARD et al., 1991), and might (eventually concomitantly with the isolated *M. dispar* and/or *Ureaplasma* spp.) be the causative organism of the chronic bronchopneumonia in the cranial lobes of calf no. 2. These chronic lesions might have enhanced the ability of *H. somnus* to colonize in the lung. However, the degree of influence seems to be minor as the inoculum was deposited in the diaphragmatic lobe in which the acute lesions developed. The mild neutrophilic infiltration in the tonsils and the few omasal microabscesses are considered as occasional observations (BARKER et al., 1993) without any significance for this study.

As the number of animals was restricted to an absolute minimum, any general conclusions should not be drawn from the present study. However, valuable information on the early pathogenesis has been obtained which might contribute to the understanding of the disease.

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