SHORT ARTICLES

ELECTRONMICROSCOPICAL OBSERVATIONS ON MYCOPLASMAS IN PNEUMONIC CALVES

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PLATES XXXII-XXXV

SEVERAL types of bovine pneumonia can be recognised pathologically, including cuffing pneumonia (Jarrett, 1956) and a significant association between *Mycoplasma dispar* and *Ureaplasma* spp. and cuffing pneumonia was demonstrated in a group of calves (Pirie and Allan, 1975). Both species have been shown to produce pneumonia experimentally (Gourlay and Thomas, 1969 and 1970; Gourlay *et al.*, 1976; Howard *et al.*, 1976), but microbiological and pathological studies on several groups of naturally infected calves suggested that *M. dispar* was the more important organism in cuffing pneumonia (Allan, Pirie and Selman, 1976).

Preliminary findings of an electronmicroscopical study of bronchial and lung tissue of calves were reported by Allan *et al.* (1976) and statistical examination of the data suggested that the presence of *M. dispar* in bronchial tissue, confirmed by laboratory culture, increased the probability of detecting mycoplasmas in pneumonic lungs by electronmicroscopy. This paper extends the earlier work on the ultrastructure of mycoplasmas in pulmonary tissue from calves with pneumonia and describes the changes in the bronchial epithelium in animals with cuffing pneumonia. In addition, the finding of mycoplasmas with an extracellular capsule, an ultrastructural feature associated with *M. dispar* (Howard and Gourlay, 1974), will be described.

MATERIALS AND METHODS

Twenty-eight calves aged 2-6 months with cuffing pneumonia were examined. The calves were members of four groups that had been studied microbiologically and pathologically by Allan *et al.* (1976). The samples for electronmicroscopical examination were collected from the right anterior lobe of all animals. Four normal calves were examined as controls. Fixation of specimens of bronchus and lung parenchyma in a solution containing 1.3% paraformaldehyde and 1.6% glutaraldehyde was followed by post-fixation in 1% osmium tetroxide and embedding in araldite. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined with an AEI 6B electron microscope.

In addition, lung tissue with lesions of cuffing pneumonia and from which mycoplasmas had been isolated was fixed and stained by the ruthenium red technique (Howard and Gourlay, 1974). This tissue was taken from six additional calves similar in age to those in the initial study. Duplicate samples of tissue from each animal were fixed in a mixture of 1.3% paraformaldehyde and 1.6% glutaraldehyde with post-fixation in 1% osmium tetroxide. Ultrathin sections of tissue stained with ruthenium red were examined directly in the AEI 6B electronmicroscope and also after double-staining with uranyl acetate and lead citrate.

RESULTS

When the 28 calves with cuffing pneumonia were examined microbiologically *M. dispar*, either alone or with other mycoplasmas, was recovered from the pulmonary tissue of 13; electronmicroscopical examination of the lung tissue of these 13 animals resulted in the detection of mycoplasmas in 10. Mycoplasmas other than *M. dispar* were recovered from

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11 of the remaining 15 calves and of these 11 animals five showed mycoplasmas in lung tissue on examination by the electron microscope. The four calves from which no mycoplasmas were isolated were examined electronmicroscopically and mycoplasmas were found in one.

Mycoplasmas detected by electronmicroscopy were always on the bronchial epithelium. In two cases a few mycoplasma cells were also seen in the alveolar tissue, usually as individual organisms in close association with neutrophils and macrophages in the alveolar air spaces. Occasionally they were detected within intracytoplasmic vacuoles in phagocytic cells but were never seen in the proximity of the alveolar epithelial cells.

Mycoplasmas were found on and between the cilia of the bronchial epithelium (fig. 1), often in layers two or three cells thick, giving the appearance of a microcolony on the epithelial surface. Most of the mycoplasma cells were oval to round although cells penetrating between the cilia appeared elongated, as if they had squeezed between these structures (fig. 1). The organisms were never seen within the cytoplasm of the epithelial cells. In many instances the ciliary surface was in close contact with the amorphous material on the outside of the mycoplasma cell (fig. 2). Cellular connections were not seen although a mycoplasma cell sometimes appeared to be engulfed by microvillous projections from the epithelium (fig. 3).

The organisms were pleomorphic (figs. 1, 2 and 3) with characteristics typical of the Mycoplasmatales: there was a triple-layered unit membrane enclosing the cell with no evidence of a cell wall, densely-stained ribosomes lay at the cell periphery and aggregates of nuclear material (nucleoids) were present in the clear central area of the cell with electron-dense strands stretching towards the periphery. Amorphous material could be detected around some organisms giving an almost beaded appearance to the cell surface (figs. 2 and 3). When pulmonary tissue from the six additional pneumonic calves was cultured, *M. dispar* was recovered from three animals either alone or with other mycoplasmas; mycoplasmas with an extracellular capsule were detected by electronmicroscopy in all three calves. The extracellular capsule was recognised as dark staining material around the mycoplasma cells (fig. 4) situated between the cilia of the bronchial epithelium and also in the lumen. The capsule appeared to be diffuse with no obvious structure (fig. 5). Mycoplasmas other than *M. dispar* were recovered in culture from two of the remaining three calves, but no mycoplasmas were seen by electronmicroscopy in the three animals.

Ultrastructural changes in the cells of the pulmonary tissue were evident, mainly in the bronchial epithelium. The changes detected in all 34 calves with cuffing pneumonia were similar whether or not mycoplasmas had been detected by cultural methods or electronmicroscopy. Loss of cilia was common (fig. 6) leaving only the basal bodies at the cell apices. The most obvious feature was loss of the regular contour of the epithelial surface due to protrusions of the apical cytoplasm into the bronchial lumen. The protrusions were often extensive (fig. 7) and mycoplasmas were sometimes seen close to them. Distended mitochondria, vacuoles or other organelles were occasionally seen in these protrusions (fig. 7) but some contained very little cytoplasmic material and consisted of granular material in which the rudiments of cilia could be recognised. The mitochondria in many of the bronchial epithelial cells were distended (fig. 6) with loss of cristae and total organelle disruption in some cells. Cytoplasmic vacuoles were seen in many bronchial cells (fig. 6).

Neutrophils packed with electron-dense granules were frequently seen passing through the epithelium towards the bronchial lumen (fig. 6). An increase in the number of goblet cells was common; they were tightly packed with mucinogen granules and were seen being passed into the bronchial lumen (fig. 8).

DISCUSSION

The information provided by electronmicroscopical examination of lung tissue from calves with pneumonia is limited because the species of mycoplasma cannot be identified. Allan *et al.* (1976) suggested that mycoplasmas could be detected more readily by electronmicroscopy if *M. dispar* was present; in pneumonic tissue from which members of the genus other than *M. dispar* were cultured it was often difficult to recognise mycoplasmas. This may be because *M. dispar* is more closely associated than the other species with the bronchial epithelium.

MYCOPLASMAS IN PNEUMONIC CALVES

FIG. 1.—Mycoplasmas situated on and between the cilia of bronchial epithelial cells of a pneumonic calf. Paraformaldehyde and glutaraldehyde followed by osmium tetroxide fixation (PGOT). $\times 21\ 000$.

FIG. 2.—Mycoplasmas in contact with the cilia of the bronchial epithelium of a pneumonic calf. Amorphous material is evident on the external surface of the mycoplasma membrane. PGOT. $\times 48\ 000$.

MYCOPLASMAS IN PNEUMONIC CALVES

FIG. 3.—Mycoplasmas associated with the bronchial epithelium in pneumonic calves. A microvillous projection has surrounded approximately one-third of an organism. PGOT. $\times 40\,000$.

FIG. 4.—Mycoplasmas and bronchial epithelium fixed and stained, by the ruthenium red technique. Dark capsular material is clearly seen around the mycoplasma cells. Glutaraldehyde and osmium tetroxide fixation with ruthenium red. $\times 12\,000$.

MYCOPLASMAS IN PNEUMONIC CALVES

FIG. 5.—Bronchial epithelium and mycoplasmas fixed and stained by the ruthenium red technique followed by uranyl acetate and lead citrate staining. Electron-dense capsular material is present around the mycoplasma cells. $\times 48\,000$.

FIG. 6.—Bronchial epithelium of a calf with pneumonia and a pulmonary mycoplasma infection. Note the loss of cilia and the basal bodies at the cell apex (arrows). A neutrophil is seen infiltrating between the epithelial cells. PGOT. $\times 7000$.

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FIG. 7.—Two non-ciliated bronchial epithelial cells, with a protrusion of the apical cytoplasm containing the electron-dense granules of the cell. The microvilli are not present on the protrusion or on part of the luminal surface of the other cell. Mycoplasmas are situated near the protrusion. PGOT. $\times 16\,000$.

FIG. 8.—A portion of bronchial epithelium containing goblet cells close to each other from a pneumonic calf with mycoplasma infection. The mucin granules, of different electron density, are tightly packed in the goblet cells and spill on to the epithelial surface and into the lumen. The intervening ciliated cell shows degenerative changes. PGOT. $\times 4800$.

An extracellular capsule has been detected on *M. dispar* in pure cultures grown in broth and stained by the ruthenium red technique (Howard and Gourlay, 1974). *Mycoplasma mycoides* var. *mycoides* is the only other bovine mycoplasma known to have an extracellular capsule and the recognition of capsulated organisms in the pneumonic tissue examined here suggests that they are *M. dispar*.

Studies of mycoplasmas from the bovine respiratory tract in explant cultures from foetal bovine trachea (Thomas and Howard, 1974) revealed that these organisms became established and multiplied in the explant cultures, but only *M. dispar* produced cytopathic effects causing progressive sloughing of ciliated epithelial cells and patchy flattening of the epithelial layer. The damage to the epithelium only occurred when large numbers of actively growing *M. dispar* organisms were in close association with the cilia.

The association of pathogenic mycoplasmas with the bronchial epithelium has been described in other animals, both in experimental infections (Organick, Siegesmund and Lutsky, 1966; Kohn, 1971; Baskerville, 1972) and in studies of explant cultures (Collier and Clyde, 1971; Collier and Baseman, 1973). The mycoplasmas were seen on and between the cilia; intracellular organisms were never seen, although Organick *et al.* (1966) and Kohn (1971) described *M. pulmonis* in the intercellular spaces and within intracytoplasmic vacuoles in mouse and rat bronchial epithelium respectively. Ciliated epithelium appears to be a predilection site for mycoplasmas in the respiratory tract, possibly because the specific secretions of the cells produce a suitable environment for growth (Collier, 1972). In addition, and possibly more important, mycoplasmas below the mucous layer and between the cilia may be protected from many of the host's natural and acquired defence mechanisms.

Mycoplasmas were seldom present in the alveolar tissue of calves with pneumonia and then only as single organisms. This may have been due to the rapid removal and destruction of the organisms in the alveolar tissue by neutrophils and macrophages as described in experimental infections with *M. pulmonis* (Organick *et al.*, 1966). Neutrophils are known to contain mycoplasmacidal factors such as myeloperoxidase and there may be enhanced phagocytosis as a result of the action of complement or antibody.

Ultrastructural changes were evident in the bronchial epithelium of calves in which mycoplasmas were detected. Sloughing of cilia and degenerative changes in the bronchial epithelial cells were seen. Intracytoplasmic alterations including distension and disruption of the mitochondria were present and fairly extensive cytoplasmic vacuoles were formed. In addition, protrusions of the apical cytoplasm from epithelial cells were found. Similar ultrastructural changes have been recorded in experimental mycoplasma infections in other species and also in tracheal organ cultures infected with mycoplasmas (Organick *et al.*, 1966; Kohn, 1971; Baskerville, 1972; Collier, 1972). Hyperplasia of goblet cells in the bronchial epithelium and an alteration in the composition of bronchial mucus was noted in calves with cuffing pneumonia (Allan, Pirie and Wheeldon, 1977). In the present study numerous goblet cells were seen in the bronchial epithelium.

The electronmicroscopical examination of pulmonary tissue from calves with pneumonia has shown that (1) mycoplasmas could be demonstrated, (2) their location appeared to be similar to that found in mycoplasma infections of other hosts, (3) many of the ultrastructural changes in the respiratory tract of these calves were similar to those reported in mycoplasma infection in other hosts, and (4) mycoplasmas with an extracellular capsule could be demonstrated.

SUMMARY

Mycoplasma dispar was recovered from 13 of 28 calves with cuffing pneumonia and mycoplasmas were seen by electronmicroscopical examination of lung tissue from 10 of the 13 animals. Mycoplasmas were detected by electronmicroscopical examination of lung tissue from five of 11 calves that yielded mycoplasmas other than *M. dispar* on culture. Mycoplasmas were detected in one calf with pneumonia from which organisms could not be cultured. These results indicated a significant association between the isolation of *M. dispar* and the detection of mycoplasmas by electronmicroscopy.

Pulmonary tissue from six additional pneumonic calves with cuffing pneumonia was

fixed and stained by the ruthenium red technique. *M. dispar* was cultured from three of these calves and electronmicroscopical examination of pulmonary tissue from them demonstrated mycoplasmas with an extracellular capsule similar to that seen in pure cultures of *M. dispar*. These organisms were neither seen nor isolated from the pulmonary tissue of the other three calves.

The mycoplasmas were seen in close contact with the bronchial epithelium but never intracellularly. The cellular changes seen in the bronchial epithelium of the pneumonic calves were loss of cilia and protrusions of the apical cytoplasm of epithelial cells into the bronchial lumen; intracytoplasmic changes consisted of distension and disruption of the mitochondria and vacuole formations in the cytoplasm of the cells of the bronchial epithelium.

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