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Lesions in lambs experimentally infected with bovine respiratory syncytial virus

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Summary. An experimental model was designed to characterize lesions in the lung of lambs inoculated with bovine respiratory syncytial virus (BRSV). 25 Merino lambs of both sexes, with a live weight of 17±3 Kg, received an intratracheal inoculation of 20 ml saline solution containing $1.26x10^6$ $TCID_{50}$ BRSV (strain NMK-7) per ml. Lambs were laughtered 1, 3, 7, 11 and 15 postinoculation days (PID), and histopathological, immunohistochemical and electron microscopic studies were performed. Results reflected a series of lesions, the most noteworthy of which were bronchiolitis obliterants with destruction of the mucociliary apparatus, the presence of syncytial cells in alveoli and a progressive interstitial reaction. BRSV antigen was detected in lung samples. These changes might be expected to decrease the efficiency of respiratory tract defence mechanisms, rendering the lung parenchyma susceptible to opportunist bacterial infection.

Key words: BRSV, Ovine, Lesions, Lung

Introduction

Speculation regarding the role of respiratory syncytial virus (RSV) in acute respiratory infections in children began in the early 1960s, and since then RSV has frequently been linked to fatal pneumonia and bronchiolitis in children (Armstrong et al., 1962). Indeed, it is currently regarded as the main cause of hospitalisation in western children under one year old (Stott and Taylor, 1985).

RSV is a major pathogen of the lower respiratory tract of young cattle (Bryson et al., 1983; Kimman et al., 1989). Bovine and human RSV strains are currently considered to be antigenically related (Bruguère-Picoux et al., 1985). Although greater susceptibility has been reported in cattle, RSV may also affect other domestic species, particularly small ruminants (Berthiaume et al., 1973).

Complement-fixing antibodies against RSV (Berthiaume et al., 1973), and neutralizing antibodies to bovine respiratory syncytial virus (BRSV) (Smith et al., 1975) in sheep serum have been reported. BRSV can infect lambs and cause mild disease (Lehmkuhl et al., 1979a,b).

In our work we shall describe lesions in the lung of lambs experimentally infected with BRSV.

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Materials and methods

Animals

25 gnotobiotic merino lambs of roughly 17±3 kg live weight, each received an intratracheal inoculation of 20 ml BRSV diluted to a concentration of $1.26x10^6$ $TCID_{50}$/ml. The lambs were housed in an isolation barn.

Virus inoculate was prepared using primary bovine foetal kidney (BFK) cell cultures infected with a BRSV stock strain NMK-7, to which Minimun Essential Medium with 15% bovine foetal serum was added; the final culture wås incubated at 37 °C in a CO_2 atmosphere.

Ten control animals were inoculated with an identical volume of uninfected BFK cell culture. They were kept in another isolation box.

The lambs were necropsied at 1, 3, 7, 11 and 15 days after inoculation (PID). Lambs were anaesthetized intravenously with sodium pentobarbital (25-35 mg/kg) and were sacrificed by exsanguination and induction of pneumothorax. Immediately after death, lungs were subjected to gross examination.

Histopathology

Samples of all lobes (normal and consolidated areas) were fixed in 10% neutral formalin saline and processed by routine paraffin-embedding methods. Sections, 5 µm thick, were cut and stained with haematoxylin and eosin (HE), touluidine blue (TB) and Masson's trichrome

(TM) for routine morphological studies.

Immunoperoxidase staining procedure for detection of BRSV antigens

An Avidin-Biotin-Peroxidase complex (ABPC) was carried out on deparaffinized and trypsinized lung samples. Sections were incubated in diluted (1:50) normal swine serum for 15 minutes to reduce background and were incubated in diluted (1:1000) rabbit anti-RSV for 3 hours at 20 °C. Diluted (1:500) biotinylated swine anti-rabbit IgG was placed on the sections for 30 minutes, and sections were then incubated with diluted (1:50) ABPC reagent for 1 hour. Sections were incubated in diaminobenzidine solution for 5 minutes and were counterstained lightly with Mayer's haematoxylin. The positive controls included BRSV-infected BFK cell cultures (the cytopathic effect was evident in the formation of numerous syncytia) and noninfected BFK cell cultures. Test control sections were also stained using nonimmune rabbit serum as first layer.

Transmission electron microscopy (TEM)

Sections of lung were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, for 2 days, post-fixed with osmium tetroxide, and embedded in epoxy resin. Ultra-thin sections were cut, routinely stained with lead citrate and uranyl acetate, and examined with a transmission electron microscope.

Results

Gross lesions

Control group lungs were apparently normal, with no detectable gross lesions.

Gross lesions in the experimental group were similar throughout the study, varying only in extent; lesions started to resolve from 11 PID onwards. In affected lobes, areas of consolidation alternated with emphysematous areas.

Microscopic findings

Catarrhal bronchiolitis observed on 1 PID was associated with granulocyte infiltration of the bronchiolar lumen. No necrosis of respiratory epithelia was detected, and there was no evidence of inclusions or syncytial cells. The interalveolar septum was thickened, with pronounced interstitial edema (Fig. 1) and moderate cell reaction.

Bronchial and bronchiolar epithelia in animals slaughtered 3 PID revealed erosive phenomena related to the epithelial hyperplasia of surface cells. Emigrating neutrophils and mononuclear cells were frequently observed interspersed among epithelial cells. Peribronchial lymphoid infiltrate was also detected (Fig. 2). Alveolar exudate consisted of neutrophils, mononuclear cells and multinucleate giant cells which formed the syncytia. There was considerable thickening of the interalveolar septae, due to the presence of edema and granulocyte and monocyte infiltration.

By 7 PID, bronchiolitis was accompained by epithelial cell necrosis. Hyperplasia was conspicuous in areas of marked necrosis, and early stages of re-epithelisation were apparent.

Exudate in the bronchiolar lumina consisted of desquamated cells and necrotic debris. Syncytial cells (Fig. 3) varied considerably in shape; spherical, columnar and even pedicular shapes were recorded. The number of nuclei per syncytium ranged from 4 to 15; nuclei were centrally located. Evidence of edema and the infiltration of mononuclear cells and neutrophils was observed in the interalveolar septum. This coupled with the presence of multinucleate syncytial macrophages led to the appearance of numerous areas of lung consolidation and the obliteration of respiratory lumina.

Severe bronchial, bronchiolar and alveolar damage was visible by 11 PID. Bronchial and bronchiolar epithelia showed clear signs of re-epithelisation, with the appearance of squamous and mononuclear cells in bronchiolar lumina. Bronchial and bronchiolar lumina contained lymphocytes, macrophages and plasma cells (Fig. 4). Moderate peribronchial lymphoid hyperplasia was also observed. Lung parenchyma showed clear focal areas of consolidation due to bronchiolitis obliterans and the alveolar collapse caused by infiltration of macrophages, lymphocytes and giant cells into lumina. Syncytial cells were observed sporadically. Emphysema and atelectasis alternated consistently throughout areas of lung parenchyma where no intense inflammatory reaction was observed.

Animals slaughtered at 15 PID presented a marked interstitial inflammatory reaction, with considerable septal thickening (Fig. 5). Bronchial and bronchiolar lumina contained largely cellular exudate. Intense bronchial and/or alveolar hyperplasia was also observed. Alveolar lumina contained moderate amounts of lymphoplasmocellular exudate and macrophage infiltrate, though no syncitial cells were observed. A peribronchial or peribronchiolar lymphoid reaction was evident. Lung consolidation was less marked than in animals slaughtered earlier.

Antigen localization

BRSV antigen was detected in bronchial and bronchiolar epithelial cells (Fig. 6), in bronchial mucous cells and in alveolar epithelial cells at 3 and 7 PID. Intense staining was also observed at alveolar macrophages, interstitial mononuclear cells (Fig. 7) and syncytial cells from 3 to 15 PID. Antigen was commonly detected in exudate within bronchial, bronchiolar (Fig. 8) and alveolar lumina. Specific staining was absent in the negative control.

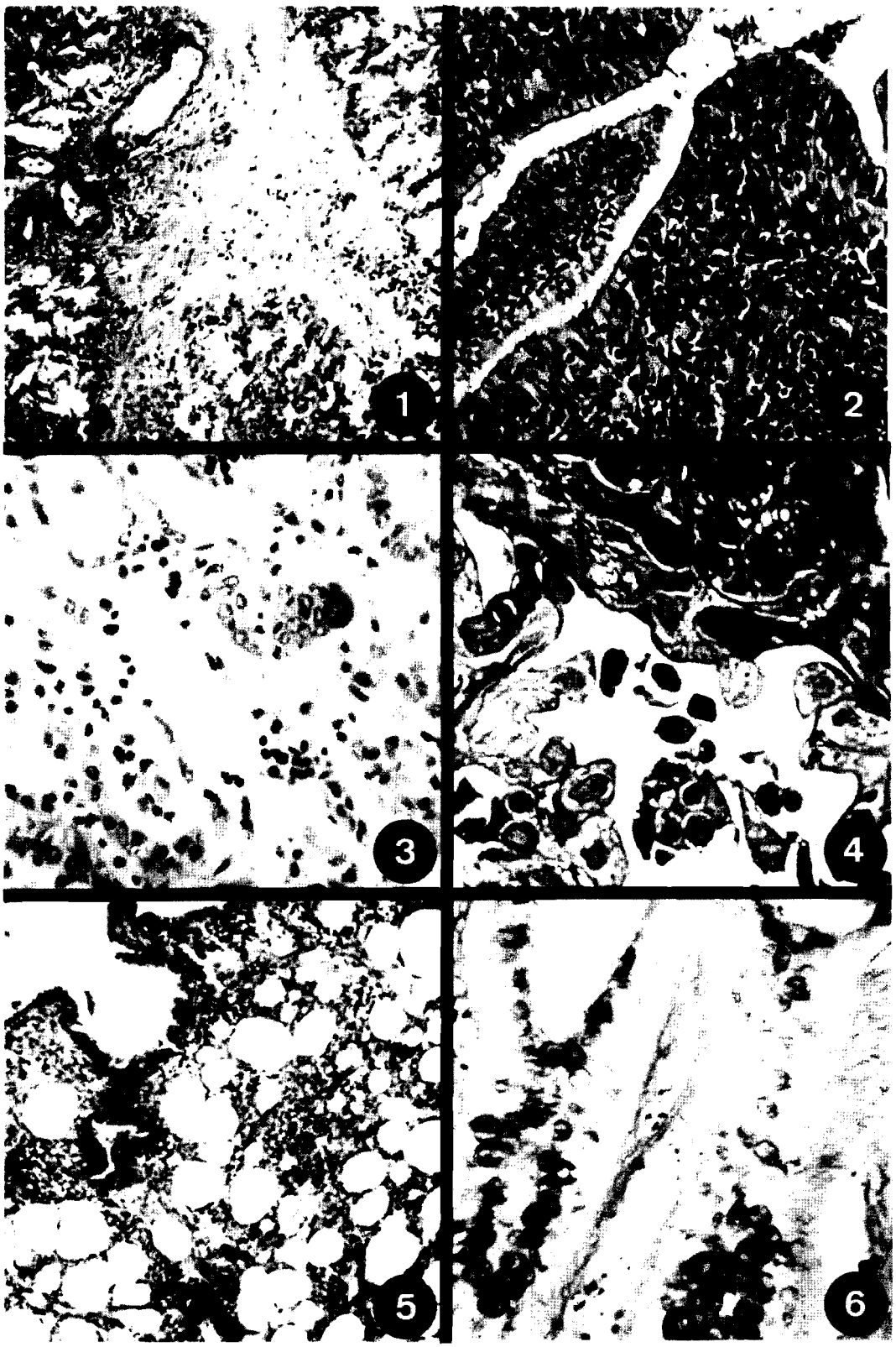

Fig. 1. PID 1. Interstitial edema. H&E. x 250

Fig. 2. PID 3. Peribronchial infiltrate. H&E. x 250

Fig. 3. PID 7. Syncytial cells. H&E. x 250

Fig. 4. PID 11. Bronchiolar lumina contained lymphocytes and macrophages. Masson's trichome. x 500

Fig. 5. PID 15. Interstitial reaction. H&E. x 250

Fig. 6. PID 3. Immunoperoxidase staining of BRSV antigen in bronchiolar epithelial cells. ABPC-haematoxylin. x 250

Ultrastructural lesions

At 1 PID, some disorganisation of cilia was observed in bronchial and bronchiolar ciliated cells. The apical cytoplasm of non-ciliated cells contained abundant granules, and a mucoprotective covering was visible over the plasma membrane.

The alveolar epithelium presented no evident ultrastructural alteration, although type II pneumocytes were highly active with large nulcei. Cytoplasm contained a striking amount of RER, associated with mitochondria with electrodense matrix, and a peripheral arrangement of multilayered bodies of varying shapes (Fig. 9). No changes were observed in the components of the respiratory membrane.

At 3 and 7 PID, the bronchial epithelium showed evidence of desquamation, affecting both ciliated and mucous cells (Fig. 10); cilia disorganisation continued to be observed. Airway lumina contained morphologically undefined deposits of necrotic debris (desquamated cells and mucosecretions). A certain amount of nucleoprotein material was observed in the apical cytoplasm of non-ciliated bronchiolar epithelial cells. A large number of spherical virions were visible, budding from the plasma membrane of bronchial and bronchiolar ciliated and non-ciliated cells (Fig. 11).

Viral particles observed in type II pneumocyte cytoplasm consisted of a central electrondense core and a adielectronic halo; both surrounded by a clearly-defined electrondense membrane. Alveolar lumina contained abundant mucosecretions and desquamous debris. The respiratory membrane evidenced certain degenerative changes, in the form of flaws in the plasma membrane of type I alveolar cells. Interstitial tissue showed decreased electron density, due to the presence of edema. At septum level, neutrophils, lymphocytes and alveolar macrophages were observed; macrophage cytoplasm contained virions.

At 11 PID a decrease in the number of cilia was associated with internalization of basal bodies, either separately from or together with axonemes. No virons or viral nucleoproteins were observed in epithelia.

On examination of the alveolar epithelium of animals slaughtered at 11 PID, type II pneumocytes revealed degeneration, affecting cytoplasmic proyections, in which discontinuities and desquamation were observed. Type II pneumocytes showed evident signs of activity, represented morphologically by the development of RER, the presence of a large number of mitochondria with moderately electrondense matrix. Multilayered bodies were globose. Alveolar lumina contained desquamate cells and macrophages with cytoplasmic phagosomes and residual bodies. No virions were observed in epithelial cells, although they were occasionally observed in macrophages (Fig. 12). The respiratory membrane contained alterations at epithelial cell level. The septum showed considerable electron density, due to the presence of serum proteins, as well as neutrophils and lymphoid cells.

In animals slaughtered at 15 PID, ultrastructural alterations were qualitatively similar to those observed the previous day.

The pulmonary parenchyma of control animals was apparently normal in terms of its cell components.

Discussion

The pathological findings associated with experimental BRSV infection in lambs were similar to those reported in naturally infected calves (Pirie et al., 1981; Verhoeff et al., 1984; Baker and Frey, 1985), although these natural infections were generally exacerbated by secondary infection with *Pasteurella spp.*

Focal areas of consolidation from PID 1 onwards were similar to those described in experimentally infected calves (Bryson et al., 1983), but differed considerably from the findings reported by other authors (Mohanty et al., 1975; Castleman et al., 1985b), who observed lung consolidation at 10-14 PID. Lesions were mainly located in the dorsal and declive portions of the lung, probably because of the greater ventilation in these areas (Martel and Michel, 1985).

Histopathological changes were similar to those reported in naturally and experimentally infected calves (Baker et al., 1985; Weikel, 1990), and those described in lambs (Al-Darraji et al., 1982b; Trigo et al., 1984).

The catarrhal bronchiolitis observed was associated with considerable granulocyte infiltration. Similar changes have been described in naturally infected calves (Bryson et al.,, 1979; Pirie et al., 1981; Thomas et al., 1984; Castleman et al., 1985b), and in lambs (Al-Darraji et al., 1982b).

The morphology of bronchial and bronchiolar epithelia varied considerably, showing evidence of degeneration, necrosis and epithelial hyperplasia (Bryson et al., 1979, 1983; Baker et al., 1985; Weikel, 1990).

Antigen distribution was closely correlated with lesions, and location was broadly similar to that reported in tissues from calves and sheep with naturally-occurring RSV disease (Al-Darraji et al., 1982a,b; Bryson et al., 1988).

The results of this study demonstrate that BRSV can replicate and induce cytopathological changes in ciliated and non-ciliated bronchial epithelial cells. Similar findings have been reported in naturally-occurring and induced BRSV disease (Wellemans, 1977; Thomas and Stott, 1981; McNulty et al., 1983; Castleman et al., 1985a,b; Bryson et al., 1988). The period during which antigen was detected by these authors coincides largely with that of this experiment. In studies of induced infection in lambs, antigen in bronchial epithelium at 10 PID, has also been detected although antigen detection started at 24 hours PI (Al-Darraji et al., 1982a,b).

There, is however, some disagreement regarding the presence of BRSV antigen in bronchial epithelium. Stott and Taylor (1985) in natural disease in calves, and

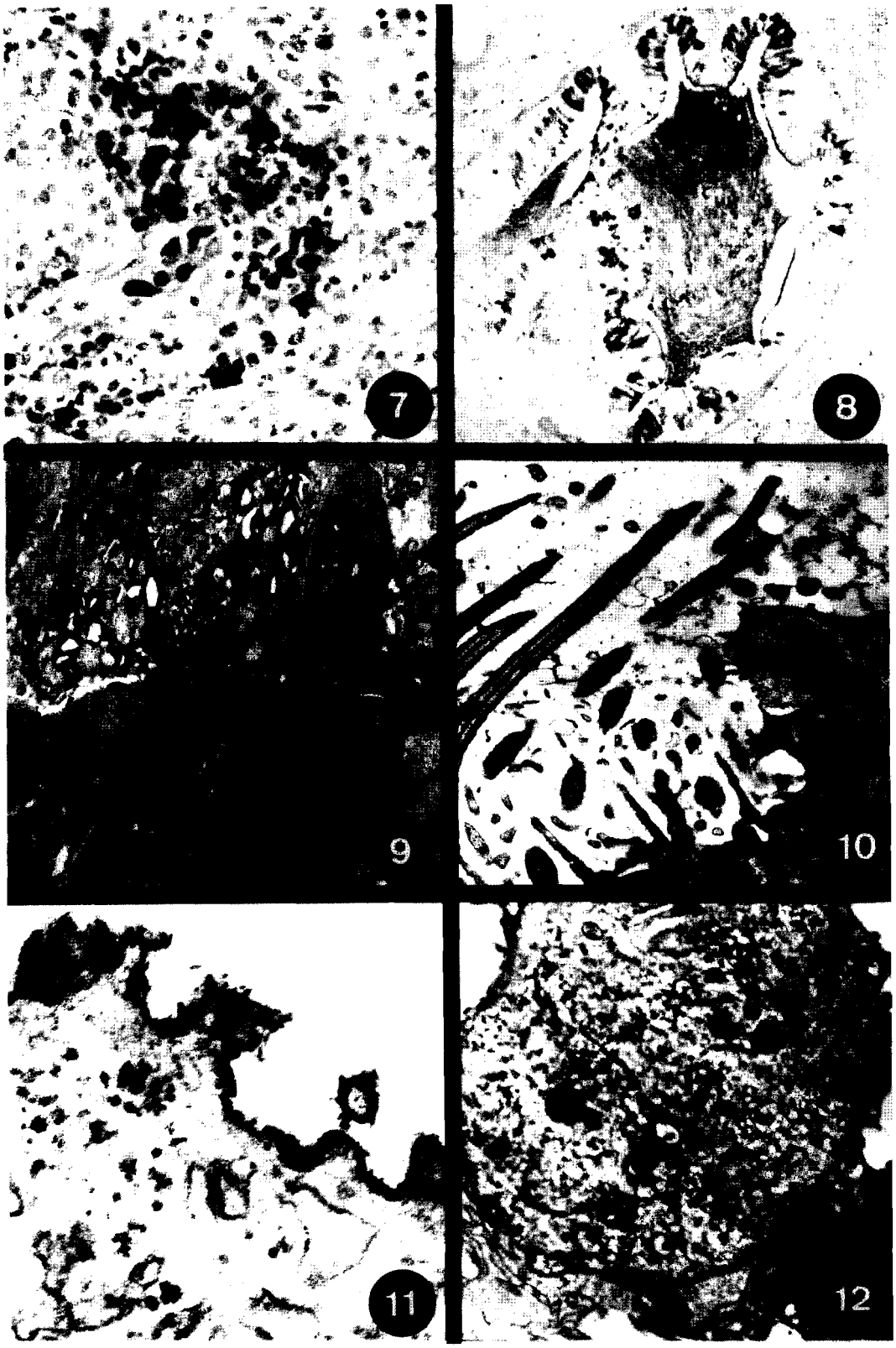

Fig. 7. PID 7. Immuno-peroxidase staining of BRSV antigen in interstitial mononuclear cells. ABPC-haematoxylin. x 250

Fig. 8. PID 7. Immuno-peroxidase staining of BRSV antigen in bronchiolar exudate. ABPC-haematoxylin. x 250

Fig. 9. PID 1. Multilayered bodies in type II pneumo-cytes. TEM. x 5,000

Fig. 10. PID 3. Bronchial epithelium showing desquamation affecting both ciliated and mucous cells. TEM. x 5,000

Fig. 11. PID 3. Virus budding from the plasma membrane of bronchiolar cells. TEM. x 5,000

Fig. 12. PID 11. virions observed in macrophages in contact with alveolar wall. TEM. x 7,000

Lehmkuhl and Cutlip (1979a,b) and Trigo et al. (1984) in naturally-occurring and induced disease in sheep, failed to detect antigen in bronchial epithelia.

With regard to bronchial syncytia, our findings agree broadly with those of Thomas et al. (1984) and Bryson et al. (1988) in natural and experimentally induced RSV in calves. These authors report viral antigen detection at 4-10 PID using IFAT and ABPC. In the present experiment, BRSV was detected in bronchial syncytia from 3 to 15 PID using immunological techniques. This is consistent with findings from Castleman et al. (1985a,b).

BRSV antigen was primarily located in ciliated and non-ciliated bronchiolar epithelial cells, and maximum antigen detection took place around 3 and 7 PID. This agrees with previous reports (McNulty et al., 1983). There is, however, some degree of discrepancy with other authors: Potgieter and Aldridge (1977) detected antigen with IFAT at 24 hours p.i., while some authors have detected no fluorescence at all (Thomas and Stott, 1981, 1984; Stott and Taylor, 1985).

TEM revealed the presence of the virus is ciliated and non-ciliated airway epithelial cells at 3 and 7 PID, as reported for cattle (Castleman et al., 1985a) and sheep (Al-Darraji et al., 1982a).

In those areas of plasma membrane where the virus was emerging, viral nucleocapsids and nucleoproteins bound to the membrane itself, causing membrane thickening (Cutlip and Lehmkuhl, 1979; Bryson et al., 1991a,b). The small amount of nucleocapsids and nucleoproteins observed by TEM agrees with reports from other authors in lamb lungs (Al-Darraji et al., 1982a).

The presence of BRSV in ciliated bronchial and bronchiolar epithelial cells gave rise to cytopathological changes, including the disorganisation and shortening of cilia, similar to that reported in calves (Castleman et al., 1985a). Viral action against airway mucous cells led to the destruction of the protective mucous apparatus (Castleman et al., 1985a). This, together with epithelial cell necrosis (Al-Darraji et al., 1982a,b; Castleman et al., 1985a), might be expected to decrease the efficiency of mucociliary clearance, favouring the development of secondary bacterial bronchopneumonia (Al-Darraji et al., 1982b; Castleman et al., 1985b; Redondo et al., 1990).

Our results suggest a reduction in BRSV tropism for alveolar epithelia compared to bronchial and bronchiolar epithelia. The alveolar reaction consisted in the thickening of the alveolar wall, due to hypertrophy of its constituent cells (Baker and Frey, 1985). Alveolar macro-phages showed evident signs of cell activity (Castleman et al., 1985a; Bryson et al., 1991a,b); the virions observed in the cytoplasm of these cells might lead to depressed phagocytic activity (Toth and Hesse, 1983). Monocytes and alveolar macrophages, attracted by viral antigen, would undergo local division, giving rise to the formation of syncytia (Bryson et al., 1983; Trigo et al., 1984; Weikel, 1990).

TEM confirmed that virus replication takes place primarily in type II alveolar epithelial cells (Bryson et al., 1991a,b); other authors, however, report virions only in the alveolar lumina of experimentally infected lambs (Cutlip and Lehmkuhl, 1979; Al-Darraji et al., 1982a,b).

Histopathological examination of the interstitium revealed focal areas of interstitial pneumonia (Cutlip et al., 1979; Bryson et al., 1983).

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