EXPERIMENTAL PNEUMONIA INDUCED BY A **BORDETELLA PARAPERTUSSIS**-LIKE ORGANISM IN THE OVINE AND MURINE LUNG

A thesis presented in partial fulfillment (70%) of the
requirements for the degree
of Master of Philosophy
in veterinary pathology at
Massey University

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1987

ABSTRACT

Thirty-four specific pathogen-free (SPF) Swiss mice were intranasally inoculated with a suspension containing about 3 x 10^7 colony-forming units (CFU)/ml of a **B. parapertussis**-like organism isolated from pneumonic ovine lung. Eleven per cent of the animals died between 2 and 3 days of inoculation and over 90% of infected mice developed a subacute bronchopneumonia morphologically similar to early lesions of naturally-occurring ovine chronic non-progressive pneumonia (CNP). The sequential pulmonary changes were examined by light microscopy and transmission electronmicroscopy from 12 hr to 29 days after inoculation. The early stages were characterized by alveolar septal congestion and oedema, focal intra-alveolar haemorrhage, and intra-alveolar and septal infiltration by neutrophils and macrophages. Later, hyperplasia of perivascular and peribronchiolar lymphoid tissue and the deposition of collagen in the interalveolar septa were prominent. The bronchial and bronchiolar epithelium remained intact throughout the experiment, but bronchiolar lumina became occluded by inflammatory exudate at an early stage. Ultrastructural changes consisted of the degeneration of the alveolar type I and type II epithelial cells and marked degeneration of alveolar macrophages. Pure cultures of the **B. parapertussis**-like organism were consistently recovered from mouse lungs 12 hr to 6 days after inoculation. Both intact and degenerating organisms were found free in alveolar spaces and within phagocytic vacuoles of alveolar macrophages. However, replication of organisms was not observed at any stage of infection and no special association was observed between organisms and the ciliated or non-ciliated respiratory epithelium.

Injury to ovine respiratory tract was demonstrated when a similar bacterial suspension to that given to the mice was given by intratracheally to colostrum-deprived lambs. The lesions produced in the pulmonary parenchyma of the lambs were similar to those seen in both early naturally-occurring ovine CNP and the experimental infection with this organism in mice. They consisted of an acute

mild tracheobronchitis, severe alveolar collapse and acute bronchopneumonia which developed within 24 hr and was most severe at 1 to 3 days after inoculation. Ultrastructurally, the alveolar epithelium exhibited extensive degenerative changes and necrosis of individual epithelial cells. Topographical studies revealed extensive coverage of the infected tracheobronchial epithelium with a dense layer of inflammatory cells mixed with mucus, and focal extrusion of ciliated cells. Occasionally, moderate numbers of the **B. parapertussis**-like coccobacilli were seen closely associated with cilia. Inoculated lambs showed a marked elevation in the numbers of cells in bronchoalveolar lavage 24 hr after infection. Up to 93% of the cells in the lavage at 24 hr were neutrophils. However, no close interation between phagocytic cells and the organisms was detected. Many of the macrophages in the lavage exhibited cytoplasmic vacuolation from five days after inoculation onwards. Blood leucocyte and neutrophil counts in infected lambs gradually rose to reach peaks at five and three days after inoculation, respectively. The **B. parapertussis**-like organism was recovered in pure culture from the nasal cavity of lambs killed on days one, three, five and nine. The viable bacterial count in bronchoalveolar lavage fluid decreased from 24 hr to 5 days with almost complete elimination of organisms nine days after inoculation.

The retention of the **B. parapertussis**-like organism in the mouse trachea was compared to that in the mouse lung from 0 to 48 hr after intranasal inoculation. Although there was greater bacterial deposition in the trachea than the lung there was a faster clearance from the trachea. At 48 hr after instillation, almost all organisms were eliminated from the trachea but about 45% of organisms were retained in the lung.

The current investigation has shown that the **B. parapertussis**-like organism can infect SPF mice and colostrum-deprived lambs and induce a subacute bronchopneumonia. The morphological changes seen suggest that this organism has the potential to predispose the ovine respiratory tract to further infection by other microorganisms and that the organism itself may also be able to cause severe pulmonary damage. The relevance of these observations to the problem of CNP in sheep in the field has yet to be determined.

ACKNOWLEDGMENTS

I am grateful to the Department of Veterinary Pathology and Public Health, Massey University, for providing me with the opportunity to undertake this research project. I am particularly indebted to my chief supervisor Dr. M.R. Alley for his constant encouragement, and to my supervisor Professor B.W. Manktelow for his invaluable advice and helpful criticism during all phases of this work. My thanks are also due to Dr. A. Baskerville, Dr. R.B. Marshall and Dr. A. Al-Kaissi for their valuble suggestions.

This study could not have been fully undertaken without the technical assistance of a number of people whose help I gratefully acknowledge. Skilled assistance was provided by:

Mrs. P. Slack and Mrs. P. Davey for histological preparations and other technical help whenever needed;

Miss L. Cullinane in the bacteriological studies;

Mr. D. Hopcroft in the preparation of tissues for scanning electron microscopy;

Mr. P. Wildbore in technical administration;

Miss E. Davies in the haematological studies;

Mrs. J. Schrama in media preparation;

Mrs. L. Dickson and Mr. F. Sharpe for their help in arranging animals for the transmission experiments.

Special thanks are also due to Mrs. A. Scott for her skilful guidance and invaluble assistance in the preparation of the final copy of this manuscript.

Parts of tables in this thesis were typed by Mrs. E.M. Wake. The photographs were printed by the staff at the Central Photographic Unit and Mr. T. Law. Their efforts are greatly appreciated.

I also need to thank my colleagues in the Veterinary Science Department, at Zhejiang Agricultural University, People's Republic of China, for supporting my leave extension.

Finally, I would like to express my thanks to my friends and family for their understanding and forbearance, but most of all I would like to thank my wife Su Dairu for her love, support and willingness to share in all aspects of my study. This thesis is dedicated to her and my son.

Financially, this work was jointly supported by a Chinese Visiting Scholarship, the Department of Veterinary Pathology & Public Health, a Graduate Study Award from Massey University and a BNZ Postgraduate Study Bursary in Veterinary Science. I would like to express my thanks to my supervisors for their support in helping me to obtain this financial assistance.

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INTRODUCTION

Pneumonia is one of the most important infectious diseases of sheep, especially feedlot lambs, in sheep-raising countries throughout the world. As New Zealand is one of the largest exporters of lamb in the world, ovine pneumonia is of special economic importance in this country. The disease can infect animals whether they are fattened intensively indoors, are grazed extensively on pasture for all or part of the year, or are reared under nomadic conditions (Davies, 1985).

Ovine pneumonia in New Zealand is usually divided into two forms (Alley, 1975a). One is an acute fibrinous pneumonia (AFP) as has been described by Salisbury in 1957, and the other more common form is chronic non-progressive pneumonia (CNP) which was described initially by Alley (1975a) and comprehensively studied **in vivo** and **in vitro** by Alley (1975a & b) and Al-Kaissi (1986).

Fortunately, outbreaks involving mortalities from acute pneumonia in sheep are sporadic in New Zealand, so overall losses are low (Manktelow, 1984) although in some individual flocks mortality may reach up to 47% (Sorenson, 1976). The disease was second only to pregnancy toxaemia as a cause of death in a survey conducted in 1974 (Davies, 1974) and caused 1-8% mortalities of sheep 3 years and older according to surveys by Salisbury (1957) and Downey (1957). The surviving animals may lose an average of 1.4 kg body weight per animal and 0.28 kg wool per animal (Nikitin et al., 1981).

The most commmon form of ovine pneumonia in this country is CNP and up to 70 to 80% of the lambs in some flocks may be affected (Alley, 1975a). Since CNP is usually subclinical, it is difficult to estimate its real economic importance. The initial studies in this country showed that when lambs were affected at an early age, survivors were likely to take longer to reach a predetermined weaning weight (Kirton et al., 1976). More recent weight gain trials

have shown that under pastoral conditions, lambs affected by CNP had a mean liveweight gain of 1.74 kg less than controls after 30 days, and 2.19 kg after 60 days (Alley, 1987). In poor growing conditions, some affected animals even lost weight. A significant linear relationship was established between liveweight gain and the extent of the pneumonic lesions.

Another important indirect economic loss is the development of pleural adhesions, a common sequela in both AFP (Alley, 1975a) and CNP (Jones & Gilmour, 1983). Carcasses with pleural adhesions, are unacceptable in several major overseas markets such as the U.S.A., Canada, and European Economic Community (EEC), and are therefore downgraded (Brain, 1980). The pleural adhesions may sometimes account for 31.4% of the carcass defects, second only to sarcocystis (Central Districts Farmer, 1985). The annual loss attributed to pleurisy alone in the industry in New Zealand, excluding the cost of treatment or preventive measures, has been estimated at 1.8 million New Zealand dollars in 1974/1975 season (Dysart, 1976), and 26 million in 1983 (Alley, 1983).

The aetiology and details of the pathogenesis of CNP have however, not been unequivocally determined. It is currently believed that the disease is multifactorial, and that the presence of bacteria is essential for the development of the lesions (Alley & Clarke, 1979; Jones & Gilmour, 1983; Davies, 1985). A wide range of bacteria have been isolated from pneumonic ovine lungs (Steveson, 1969; Alley, 1975b; Robinson, 1983; Davies, 1985), but the aetiologic importance of many of them is doubtful. Recently, a **Bordetella parapertussis**-like organism was isolated from both pneumonic and healthy ovine lungs as well as the nasal cavities of healthy ewes (Manktelow, 1984; Alley, 1986; Cullinane et al., 1987). The organism was demonstrated to be able to attach and damage the ovine ciliary epithelium in tracheal organ culture (Al-Kaissi, 1986) and it was proposed that this organism may have a role in initiating or prolonging CNP in sheep in New Zealand (Al-Kaissi et al., 1986).

The objective of the current research was to study the possible role and pathogenesis of the **B. parapertussis**-like organism in CNP and to accumulate preliminary information on its pathogenicity. A

three-step project was designed, aimed at firstly establishing a laboratory animal model to study the pathogenesis of any disease caused by the organism. Secondly, to determine the pathogenicity of the organism for colostrum-deprived lambs and finally to investigate the persistence of the organism in the trachea and lungs of SPF mouse.

CHAPTER I

GENERAL LITERATURE REVIEW

SECTION 1

PATHOLOGY OF NATURALLY-OCCURRING OVINE PNEUMONIA

According to Winter et al. (1975), ovine pneumonias can be generally divided into three major groups. One is caused by well-recognised organisms such as parasites and fungi. These are usually sporadic and there is little if any tendency to spread from animal to animal. The second group consists of the diseases which are often referred to as progressive pneumonias, and includes Maedi. The third group may be classified as enzootic pneumonias. These occupy an important position in the sheep industry of New Zealand (Alley, 1975a & b; 1987; Davies, 1985; Al-Kaissi, 1986) and they are the main subject of this review.

The first detailed pathological description of sheep pneumonia associated with bacteria is attributed to Dungal (1931) in Iceland. Since then there have been numerous clinical and pathological accounts of pneumonia in different countries, often using different terms but describing similar pathological patterns (Salisbury, 1957; Downey, 1957; Hamdy et al., 1959; Stamp & Nisbet, 1963; Gilmour & Brotherston, 1963; Sullivan et al., 1973; Alley, 1975a).

In New Zealand, it is generally accepted that ovine pneumonia has two main morphological forms: acute fibrinous pneumonia (AFP) and chronic non-progressive pneumonia (CNP) (Alley, 1975a). The present review will follow this classification. Although the aetiology of ovine pneumonia may be relatively complicated, the disease generally shows consistent morphological features. This may be due to the limited range of response which the ovine lung has to injury (Alley, 1975a) or as has been suggested by Davies et al. (1982) because the majority of lung damage seen in any type of combined infection can be attributed to the proliferation of **P. haemolytica** rather than any initiating agent (Davies et al., 1982).

1.1 Acute Fibrinous Pneumonia (AFP)

A number of terms have been used to describe this type of pneumonia, including enzootic pneumonia (Montgomerie et al., 1938), acute necrotising or exudative pneumonia (Prince, 1985), pneumonic pasteurellosis (Gilmour, 1980a & b) and acute pneumonia (Alley, 1975a). The name "enzootic pneumonia" was first used by Montgomerie et al. in 1938 to describe an outbreak of acute pneumonia in lambs in North Wales.

Pasteurella haemolytica is the most common pathogen associated with this disease (Gilmour, 1978). Viral infections and some environmental stress factors are also considered to be possible, if not essential, predisposing factors in the initiation of the disease (St. George & Sullivan, 1973; Davies, 1985). The disease is usually of sudden onset, very short clinical course and high mortality. Animals of all ages can be affected although Salisbury (1957) believed that the disease occurred much more commonly in adult animals.

1.1.1 Macroscopic Lesions

The lung lesions in the majority of sheep with AFP are confined to the cranial lobes of the lungs (Salisbury, 1957), but sometimes both cranial and middle lobes and the cranio-ventral portion of the caudal lobes are affected. Involvement of the caudal lobes is irregular and often patchy in distribution (Alley, 1975a), but occasionally, in more severe cases lesions can be found in their dorsal aspects (Downey, 1957).

The demarcation between lesions and unaffected areas depends largely on the stage of development of the disease. In the peracute form, there is usually no clear distinction, while the lesions may be more sharply demarcated in animals which have been ill for a few days (Gilmour, 1978). In animals which die during the acute stage, the affected areas of lung are usually swollen due to congestion and

oedema and are moderately firm in consistency (Alley, 1975a; Ellis, 1984). The cut surface of consolidated areas may have a mottled appearance with greyish areas surrounding each bronchiole (Salisbury, 1957). Downey (1957) found that the cut surface of the lesions had a very characteristic appearance, showing alternating areas of grey and bluish-red consolidation. Blood-stained fluid and frothy exudate can often be expressed from the cut surfaces (Gilmour, 1978).

The suppurative changes in both lung and pleura are variable. In Downey's (1957) report, there was never any evidence of suppuration either on the pleura or in the lungs. But according to Gilmour (1978), abscesses measuring 30 to 50 mm in diameter may be present with a central necrotic core and a fibrous capsule surrounded by pale grey lung tissue, especially in the caudal lobes (Gilmour, 1980a & b). Alley (1975a) found that necrotic areas are mainly present within ventral parts of the consolidated tissue and occasionally involved whole lobes.

Acute fibrinous or serofibrinous pleuritis is a common finding in most cases of AFP. The occurrence of this feature has been reported by many workers (Salisbury, 1957; Downey, 1957; Alley, 1975a). The lesions are unilateral or bilateral, with adhesions between the parietal and visceral pleura which can easily be broken down with the fingers (Salisbury, 1957). Sometimes there is a thick layer of greenish gelatinous exudate overlying the ventral surface of the lung (Downey, 1957; Gilmour, 1978).

In addition, petechial haemorrhages associated with bacterial emboli are often present on thoracic serous surfaces and the regional thoracic lymph nodes are markedly hyperaemic and oedematous (Alley, 1975a; Ellis, 1984). The thoracic cavity frequently contains a variable amount of clear exudate containing occasional strands of fibrin (Alley, 1975a), or sometimes a yellow serous fluid (Downey, 1957), or occasionally a large volume of straw-coloured fluid with fibrin clots (Gilmour, 1980a & b).

1.1.2 Microscopic Lesions

The predominant histological feature is severe exudation of both inflammatory cells and fibrin into alveolar spaces (Alley, 1975a). Other features commonly observed include severe congestion, oedema, as well as an acute catarrhal bronchiolitis (Salisbury, 1957; Downey, 1957; Alley, 1975a; Gilmour, 1978). In some cases fibrinoid thrombi and moderate numbers of neutrophils are found within alveolar capillaries and interlobular blood vessels (Alley, 1975a). In the terminal stages, multifocal areas of necrotizing alveolitis with numerous bacterial colonies and "oat-cell" infiltrations may be present in the affected lobes (Alley, 1975a). Sero-fibrinous pleurisy is frequently present on the surface of the affected lungs (Alley, 1975a).

The presence of "oat cells" is considered to be the characteristic feature of this type of pneumonia. Gilmour (1980a) considered this feature to be pathognomonic of pasteurella pneumonia, however, this was refuted by Herceg et al. (1982). These cells have slender, dark, basophilic nuclei with a spindle- shaped outline (Downey, 1957; Herceg et al., 1982). The cells mainly appear in necrotic lung lesions associated with large numbers of **P. haemolytica** (Alley, 1975a; Herceg et al., 1982) as well as in the pleura, pleural exudate, bronchial lymph nodes and some other organs (Herceg et al., 1982). They first appear in affected areas on the second day after infection (Herceg et al., 1982) and are thought to originate from blood monocytes (Herceg et al., 1982). In calves, however, Slocombe et al. (1985) have speculated that neutrophils are involved in the origin of these streaming cells since they were identified in progression from non-degenerate neutrophils to oat-cells, and contained chloracetate esterase-positive granules. Neutrophil-depleted calves did not develop these cells. Alley (1975a) also found some evidence that neutrophils and perhaps necrotic alveolar epithelial cells may contribute to this exudate.

1.1.3 Ultrastructural Changes

The only available study using electronmicroscopy to date is that of Alley (1975a). The most prominent feature observed was extensive destruction of the alveolar type I epithelium which even occurred in mildly affected areas. The affected cells showed cytoplasmic vesiculation and blurred cell membranes, or complete sloughing and disintegration. They were often separated from the underlying basement membrane by thick proteinaceous material. Alveolar type II cells, however, showed less severe changes consisting of a vesiculated, pale-staining cytoplasm, reduction in numbers of lamellar bodies and microvilli, indistinct plasma membranes and sloughing from the basement membrane.

The integrity of the basement membrane varied with the severity of the lesions. It was often thickened and duplicated. If destruction had occurred, fibrin and platlets or even erythrocytes and neutrophils became extravasated through rupture into the interstitial space. In general, the capillary endothelium underwent a lesser degree of damage than the alveolar epithelium. Occasionally, severe vesicular change and disintergration of endothelium occurred in parallel with alveolar epithelial damage.

No detailed interaction between bacteria and alveolar cells was described in this study, although organisms were sometimes found embedded in necrotic alveolar epithelium or between the alveolar epithelium and the underlying basement membrane, either at high density or in small aggregations (Alley, 1975a).

1.2 Chronic Non-progressive Pneumonia (CNP)

The name "chronic non-progressive pneumonia" was used initially by Alley (1975a) to describe the disease of sheep in New Zealand which he and Clark (1977) considered resembled the atypical pneumonia described in Scotland by Stamp and Nisbet (1963). The term 'enzootic pneumonia' has also been used to describe subacute or

chronic pneumonic lesions seen in lambs at slaughter in this country (Thurley et al., 1977). Other terms, such as proliferative exudative pneumonia (Gilmour et al., 1979; Jones et al., 1986b), chronic pneumonia (Gilmour et al., 1979), hogget pneumonia (Ionas, 1983), summer pneumonia (St. George & Sullivan, 1973) and interstitial pneumonia (Gilmour & Brotherston, 1963) have also been used by several other authors.

The disease is characterised by both exudation and proliferation, and is often subclinical and only infrequently fatal (Alley, 1975a & b; Jones & Gilmour, 1983). It affects lambs between 3 and 10 months of age (Alley, 1975a) and its occurence and morbidity in flocks appears to be largely dependent on the type of management practised (Alley, 1975a; Jones et al., 1983).

Attempts to understand the disease fully have been generally unsuccessful. The disease has been studied extensively in both naturally and experimentally infected animals (Alley, 1975a; Alley & Clarke, 1979 & 1980; Gilmour et al., 1979 & 1982a; Jones et al., 1982a, b & c; 1986a & b), and up to date, **Mycoplasma ovipneumoniae** and **Pasteurella haemolytica** are thought to be the main aetiological agents. Chronic non-progressive pneumonia can be readily reproduced by the inoculation of conventional sheep with pneumonic lung homogenates (Alley & Clarke, 1979 & 1980) and culture suspensions which contain the above organisms with or without **M. arginini** (Gilmour et al., 1979; Jones et al., 1986b). According to Sharp et al. (1978), the combination of parainfluenza 3 (PI3) virus and **P. haemolytica** biotype A also can infrequently induce CNP. These authors suggested that CNP may represent a response to limited bacterial multiplication in an area of less severe viral damage. Another author (Ellis, 1984) has suggested that CNP may be a general response of the lung to foreign agents because it resembles milder atypical interstitial pneumonia induced by chlamydia.

Although reports of the occurrence of this disease in the literature are numerous, few workers have devoted attention to

studying the pathological aspects of respiratory lesions in any detail.

1.2.1 Macroscopic Lesions

The fundamental gross lesions of CNP consist of various degrees of consolidation of the anteroventral portions of both lungs (Alley, 1975a). Stamp & Nisbet (1963) in their first report of atypical pneumonia described sites of pneumonic consolidation which affected to a lesser or greater extent the cranial and middle lobes of the lung and occasionally the anterior borders of the caudal lobes. Alley (1975a) divided these lesions into 4 categories: type 1 (dull red consolidation), type 2 (red-grey consolidation), type 3 (grey-red consolidation) and type 4 (grey consolidation), on the basis of their gross appearance.

The consolidated areas are usually sunken below the surface of surrounding pulmonary tissue and are always well-demarcated from the surrounding unaffected lung. (Alley, 1975a). On close examination numerous grey-white granular foci can often be seen on the cut surface and on the pleural surface of the consolidated tissue (Stamp & Nisbet, 1963; Alley, 1975a; Jones & Gilmour, 1983). The consistency of the affected lung tissue is usually firm (Alley, 1975a). The consolidated areas may begin to resolve from 15 weeks post infection (w.p.i.) onwards (Gilmour et al, 1982a).

A pleurisy, characterized by the production of fibrinous "tags" or adhesions, is occasionally present (Jones & Gilmour, 1983). This lesion is the most variable factor, being observed in 27% of experimental sheep by Jones et al. (1986b). Its occurrence did not correlate on an individual basis with simple development of the extent of lesions or with isolation of **P. haemolytica** from lung tissue, but proportionately more animals inoculated endobronchially (37%) developed pleurisy compared with those (25%) injected intratracheally. It has been presumed that the development of pleurisy relates to individual susceptibility, and to the immune

"competence" of the animal (Jones et al., 1986b).

1.2.2. Microscopic Lesions

In the original description of atypical pneumonia the histological changes were divided into two types, interstitial pneumonia and lymphoid hyperplasia (Stamp & Nisbet, 1963). Later, when the histopathology of natural CNP was described in detail by Alley (1975a), 12 features were found to be common (Alley & Clark, 1977). These included five changes in bronchioles; bronchiolar epithelial hyperplasia, peribronchiolar lymphoid cuffing, peribronchiolar fibrosis, the accumulation of mucus in the lumen of bronchioles and the presence of neutrophils in their lumina, and seven in alveoli; collapse of alveolar spaces, exudation of proteinaceous fluid, accumulation of mucus, neutrophils in alveolar spaces, macrophages in alveolar spaces, type II cell hyperplasia and interstitial thickening of alveolar septa with mononuclear cells and fibroblasts.

The exudate in alveolar spaces is usually a mixture of neutrophils, macrophages and fibrin (Alley, 1975a). The numbers of neutrophils present are particularly associated with the earlier stages of the disease and vary from small to numerous. They more often infiltrate the alveolar spaces adjacent to affected bronchioles, but become disseminated in severe cases. Neutrophil exudate is widespread in alveoli and airways at three w.p.i., but soon becomes mild and focal, then restricted to bronchi, and finally disappears after 15 w.p.i. (Gilmour et al., 1982a). Macrophages are always mixed with neutrophils in moderate numbers but graduallly increase with the development of the lesions. In the later stages, alveolar spaces are filled with large macrophages, many of which are actively phagocytosing degenerating cellular debris. Marked macrophage exudate was present in alveoli at three w.p.i. to 18 w.p.i., but very mild in 24 w.p.i. (Gilmour et al., 1982a). The infiltration of macrophages into alveolar spaces is usually limited to areas adjacent to lymphoid hyperplasia (Stamp & Nisbet, 1963).

Structures similar to pulmonary microliths were found by Alley (1975a) in alveoli, alveolar ducts and bronchioles in many of his naturally-occuring cases. These stratified, spherical bodies are 30-100 µm in diameter and usually have an acidophilic matrix, containing variable numbers of concentric, basophilic outer layers. They show a positive reaction to periodic acid-Schiff (PAS) and Alcian blue staining, but are negative to calcium stains. It was hypothesised that these bodies result from the mixture of protein-rich exudate and necrotic cellular elements, but their importance and origin are not clear.

Lymphoid hyperplasia was originally considered to be a common, but late manifestation of pulmonary infection with mycoplasmas (Stamp & Nisbet, 1963; Goodwin et al., 1965; Pirie & Allan, 1975; Jones & Gilmour, 1983) but current concepts suggested that this change may be a non-specific response to a wide range of irritants (Hanichen, 1964; Jericho, 1966; Alley & Clarke, 1977). The lesions mainly develop from 2 to 28 w.p.i. but may massively enlarge within 15-24 w.p.i. (Gilmour et al., 1982a).

One of the characteristic features in the later stages of CNP is the presence of nodular scars in the walls of many of the bronchi and bronchioles. These scars are either hyaline or myxomatous in nature, projecting into the air passages causing partial obstruction, and may or may not be covered with epithelium (Stamp & Nisbet, 1963). Serial sections show no causal agent within them (Alley, 1975a), although their appearance suggests a parasitic or microbial origin or a hypersensitive reaction to their products. The majority of these hyaline scars are found between 7 to 11 w.p.i., after 15 w.p.i. their frequency diminishes and the lesions disappear after about 24 -28 w.p.i. (Gilmour et al., 1982a). Gilmour et al. (1982a) saw a possible correlation between the presence of hyaline scars, neutrophil exudate and lung colonization by **P. haemolytica**. They presumed that the scar is a consequence of inflammatory or immune responses stimulated by this bacterium.

So called "epithelialization" of alveoli is present in almost all cases of CNP and regarded as a replacement of damaged type I cells by type II cells. Alveolar epithelialization was described briefly by Stamp & Nisbet (1963) and studied by Alley & Manktelow (1971) and Alley (1975a) in more detail. The latter authors employed electronmicroscopy to determine the origin of the proliferating cells and their significance in the pneumonic lesions. Focal epithelialization of alveoli is usually present in lungs which have well-developed lesions and especially affects alveoli immediately around the involved bronchi and bronchioles, or subpleural alveoli (Alley, 1975a). The proliferating epithelium was confirmed to be type II pneumocytes by electromicroscopy and histochemistry (Alley & Manktelow, 1971).

In addition to interstitial fibrosis in the epithelialized areas (Stamp & Nisbet, 1963), hypertrophy of the muscle of the bronchioles and smaller air passages (Stamp & Nisbet, 1963; Alley, 1975a) has also been reported.

A tracheobronchitis in CNP-infected sheep was initially noted by Alley (1975a) and studied in detail by Al-Kaissi (1986). The lesions are variable in severity, mainly consisting of hyperplasia, metaplasia of epithelial cells and various degrees of ciliary degenerative change. There is often infiltration of a few macrophages and neutrophils into the lamina propria and large numbers of neutrophils between epithelial cells. The submucosal glands also exhibit very mild inflammation. In addition, small lymphoid aggregations are present in the lamina propria of bronchi and the peribronchiolar tissues in early stages of CNP and become more extensive in advanced cases. The salient changes in the bronchioles are the infiltration of neutrophils into their lumina and hyperplasia of the mucosal epithelium (Alley, 1975a; Al-Kaissi, 1986). This latter change is very frequent from 3 to 28 w.p.i. and is well-developed up to 14 w.p.i., but gradually becomes more mild after 24-28 w.p.i. (Gilmour et al., 1982a).

1.2.3 Ultrastructural Changes

As mentioned before, there are only limited ultrastructural studies on CNP: that of Alley & Manktelow (1971), Alley (1975a) and Al-Kaissi (1986). The changes seen in type I epithelial cells seem to be much milder than those seen in AFP. The cells are mildly degenerate but usually remain attached to the basement membrane. In the more advanced cases desquamation of alveolar type I epithelial cells has sometimes been observed, but is often associated with migrating neutrophils or macrophages (Alley, 1975a).

The pathological changes in type II cells vary with the development of the disease. Degeneration is one of the frequent early lesions. The degenerate cells show swollen mitochondria, mild indentations of the nuclear membrane and moderate dilation of endoplasmic cisternae. As the lesions develop, necrosis may be present. Other common degenerative changes in type II cells are thickening of the superficial plasma membrane with electron dense material, loss of microvilli and early vesiculation in the cytoplasm. The hyperplasia of type II cells is easy to identify and relatively consistent in advanced CNP. Alley & Manktelow (1971) noted that about 85% of cases examined showed evidence of type II cell proliferation as judged by the presence of three or more cells per alveolus. In about 20% of cases, the normal epithelium was completely replaced by proliferating type II cells. Sometimes these cells form nests which may contain more than five immature type II cells. The cells vary from flat to normal type II cells in appearance, and are often found in collapsed alveoli or in the niches of damaged alveoli. Immature cells are larger and less electron dense and contain numerous free ribosomes but few cytoplasmic organelles. The osmiophilic bodies, if present, are coarsely lamellated (Alley, 1975a).

The pulmonary macrophages in CNP are usually in a very active stage and have a variety of vacuolated, cytoplasmic inclusions

containing cellular debris or whorled myelin-like forms, some of which are presumably engulfed secretions of type II cells (Alley, 1975a).

The ultrastructural changes in the tracheobronchial epithelium in CNP-affected sheep have long been ingored, but a recent study by Al-Kaissi (1986) has remedied this situation. In the early stages, a large number of cilia in the tracheobronchial epithelium showed a variable degree of ciliogenesis. The ciliary carpet was covered by a layer of mixed mucus, cellular debris and organisms. Many bacteria and mycoplasmas were present between the cilia and microvillous projections. Mycoplasma organisms were often attached firmly to the cilia by means of tubular structures which penetrated the plasma membrane of the cilia, but bacteria were not seen in close contact with the external cell surfaces or within phagocytes. In more advanced lesions, large areas of epithelium exhibited severe metaplasia and the cells in others were variably damaged and contained degenerating cytoplasm and nuclei. One of the main features of the tracheal epithelium at this stage was a lack of goblet cells.

The major ultrastructural changes in the bronchiolar epithelium of both early and advanced pneumonic lesions are (1) the development of blebs in the apical cytoplasm of many ciliated cells and (2) severe degenerative changes of the superficial epithelial cell layer (Al-Kaissi, 1986).

The advent of scanning electronmicroscopy has provided an opportunity to study the tracheobronchial and alveolar surface changes in detail but the only available topographical investigation to date is that of Al-Kaissi (1986). The predominant change in the tracheobronchial mucosa is ciliary damage which includes shortening of ciliary length, loss of cilia, and ciliary degeneration. The loss of cilia occurred at all levels of airways in both early and advanced stages, but was more severe in the early lesions. Two characteristic changes were also found in affected alveoli; (1) an

increase in the thickness of the alveolar septa, and (2) an accumulation of cells within the alveolar space. With the development of the lesions, the entire alveolar space was eventially covered by hyperplastic type II pneumocytes.

SECTION 2

THE ROLE OF BACTERIA IN PNEUMONIA OF SHEEP

Investigations into the aetiology of ovine pneumonia both in New Zealand and overseas have indicated that it is not a disease attributable to a single agent (Salisbury, 1957; McGowan et al., 1957; Smith, 1957; Hamdy et al, 1959; Stamp & Nisbet, 1963; Alley et al., 1970; Alley, 1975b; Davies, et al., 1976; Gilmour, 1978; Jones & Gilmour, 1983). It is associated with a variety of infectious agents including bacteria, mycoplasmas, chlamydia, and viruses as well as environmental and physiological stress (Stevenson, 1969; Alley, 1975a; Robinson, 1983; Davies, 1985).

A large number of bacteria (Table 1.1) have been recovered from the lungs of sheep with pneumonia by various workers (Stevenson, 1969; Alley, 1975b; Davies, 1985). Most of these organisms may be regarded as secondary invaders, but some may be important in initiating the disease (Gilmour, 1980a & b), increasing the severity of the clinical and pathological signs of pneumonia (Alley & Clarke, 1979 & 1980), or causing the death of animals (St. George & Sullivan, 1973). Although most authorities agree that the aetiology of ovine pneumonia is multifactorial, the presence of bacteria is regarded as essential for the development of lesions of clinical significance (Jones & Gilmour, 1983).

Carter (1964), Stevenson (1969), Alley (1975a), Davis (1985), Al-kaissi (1986) and Jones et al. (1986b) have all written comprehensive reviews of the aetiology and pathogenesis of ovine pneumonia. In their reviews, large numbers of aetiological agents, including bacteria, have been listed and experimental infections with bacteria alone or combined with other pathogens have been described.

TABLE 1.1 BACTERIAL SPECIES
ISOLATED FROM THE PNEUMONIC OVINE RESPIRATORY TRACT

Organisms	Major Reference
Pasteurella haemolytica	Salisbury (1957), Downey (1957), Alley (1975a & b)
Pasteurella multocida	Hamdy et al. (1959), Robinson (1983)
Salmonella spp.	Robinson (1983)
Staphylococcus spp.	Hamdy et al. (1959), Alley (1975a & b)
Streptococcus spp.	Steveson (1974), Alley (1975a & b)
Escherichia coli	Alley (1975a & b)
Haemophilus spp.	Litter et al. (1980)
Neisseria catarrhalis	Alley et al. (1970)
Corynebacterium pyogenes	Gilmour et al.(1963), Alley (1975a &b)
Actinobacillus sp.	Smith (1960)
Pseudomonas sp.	Hamdy et al. (1959)
Bordetella parapertussis- like organism	Manktelow (1984), Alley (1986), Cullinane et al. (1987)

In this review the epidemiological evidence of bacterial involvment in ovine pneumonia, experimental bacterial pulmonary infection **in vivo** and **in vitro** and bacterial cytotoxin production will each be considered separately. In view of the absence of published information dealing specifically with the interaction of **Bordetella parapertussis** and the respiratory tract, this topic will be discussed in detail. Because it is generally believed that the involvement of other infectious and non-infectious agents is necessary in order to predispose the respiratory tract to the establishment of bacterial infection, some other agents will also be briefly considered at the end of this section.

2.1 Epidemiological Evidence of Bacterial Involvment

2.1.1 Pasteurella haemolytica

Of the bacteria associated with ovine pneumonia, **P. haemolytica** is thought to be most important organism (Alley, 1975a & b; Gilmour, 1978 & 1980a & b; Davies, 1985). Experimentally, the organism may induce pneumonia by combining with various viruses and mycoplasmas (Davies et al., 1977; Sharp et al., 1978; Davies et al., 1981a; Davies et al., 1982; Al-Darraji et al., 1982a, b & c; Buddle et al., 1984), however, it has also been shown to be an independent primary pathogen of the ovine lung (Gilmour et al., 1980; Gilmour et al., 1982b; Schoning & Sagartz, 1986).

Pasteurella haemolytica has two biotypes, A and T, that are related to host predilection and pathogenic potentials (Smith, 1959; Biberstein & Kirkham, 1979; Harbourne, 1979; Gilmour, 1980 a & b; Ellis, 1984). Their distinctive features are shown in Table 1.2. Attempts have also been made to classify strains of **P. haemolytica** by serological methods. The most reliable technique is the indirect haemagglutination test (IHA), which is based on the presence of a soluble surface antigen on the cells and is much more specific in its reactions than the tube agglutination test used earlier (Biberstein et. al.,1960). So far 15 serotypes have been identified,

TABLE 1.2 CHARACTERISTICS OF
PASTEURELLA HAEMOLYTICA A AND T BIOTYPES*

Characteristics	Biotype A	Biotype T
Colony size	small	large
Colony colour	grey	brown centre
Colony morphology	dome	conical
Catalase	+	-
Acid production (with 10 days) from		
L- arabinose, D-xylose	+	-
Trehalose, salicin	-	+
Lactose	+**	-
Mannose	-	+/-
Cultures	die rapidly	survive longer
Susceptibility to		
penicillin	high	low
tetracycline	slight	sensitive
Capsule serotypes	1, 2, 5 to 9 and 11 to 14, untypable strains	3, 4, 10 and 15
Somatic serotypes	A,B	C,D
Principal location in natural host	nasopharynx	tonsils and gut
Principal disease associated	pneumonia of sheep and cattle of all ages, septicamia of nursing lambs	septicemia of feeder lambs (over 3 months)
Nucleic acid homology	with type A 83-100%	with type A 8-24%
	with type T 30-50%	with type T 100%
	with **P. multocida** 0-13%	with **P. multocida** 18%
Growth inhibition		
basic fuchsin (0.2 µg/ml)	+	-
brilliant green (0.005 µg/ml)	+	-
methylene blue (3.1 µg/ml)	+	-

* According to Gilmour (1978), Biberstein (1978 & 1979) and Carter (1984).
** Serotype 2 negative.

and all the biotype A strains are recognized in New Zealand (Prince et al., 1985). Recently, Donachie et al. (1984) divided the IHA-negative strains into nine serogroups by counter current immunoelectrophoresis (CCIE), but the pathogenicity of these strains is still obscure. Two IHA-negative isolates were found to be avirulent for SPF lambs when given by aerosol and combined with PI3. Sutherland & Donachie (1986a) demonstrated **in vitro** that IHA-negative isolates were less toxic to ovine bronchoalveolar macrophages (BAM) than serotypable isolates.

The principal hosts of **P. haemolytica** are ruminants, but the organism has also been recovered from swine, poultry, horses, camels and man (Biberstein & Thompson, 1966; Perrreau & Maurice, 1968; Guerrero et al.,1973; Harbourne, 1962). Ovine pneumonia associated with **Pasteurella spp.** was first described in Iceland (Dungal, 1931) and subseqently has been shown to occur in many countries; Australia (Beveridge, 1983), Britain (Montgomerie et al., 1938), Ethiopia (Pegram et al., 1979), New Zealand (Salisbury, 1957; Alley, 1975a & b), Norway (Mohn & Utkler, 1974), Sweden (Biberstein, 1978), South Africa (Cameron, 1966), Kenya (Mwangota,et al., 1978), Somali Democratic Repbulic (Pegram, 1974) and the USA (Newsom & Cross, 1932; Gilmour & Angus, 1983). **P. haemolytica**, are believed to be associated with both AFP (Salisbury, 1957; Stevenson, 1969; Gilmour, 1978 & 1980a & b) and CNP (Alley, 1975a & b; Jones & Gilmour, 1983).

Investigations within the last decade indicate that A strains may be found more readily in the nasopharynx of adult carrier sheep and are associated with pneumonic disease, while T strains are located in high populations in the tonsils and are usually involved in more generalized forms of infection (Gilmour et al., 1974). Occasionally biotype T strains can be isolated from pneumonic lungs (Gilmour, 1978 & 1980b). It has been suggested that there might be a relationship between these sites and the routes through which the organism spreads within the body (Gilmour et al., 1974; Gilmour, 1978 & 1980b). Infection from the nasopharynx is more likely to follow the respiratory route leading to a pneumonia, whereas entry

through the tonsils may lead to a generalized septicaemia. However, there is no evidence that A and T biotypes cause distinct disease syndromes in cattle (Sutherland & Donachie, 1986a).

Since earliest times, the high recovery rate of **P. haemolytica** from the respiratory tract (Bosworth & Lovell, 1944; Smith, 1957) has attracted interest in this organism. Biberstein & Thompson (1966) investigated the nasal carriage of **P. haemolytica** in normal and pneumonic flocks, and found a relatively higher incidence and wider range of serotypes in pneumonic flocks than in normal flocks. Further studies revealed that the carrier rates of **P. haemolytica** in these flocks almost coincided with the known seasonal patterns of outbreaks of enzootic pneumonia in the region (Thurley et al., 1977; Biberstein, 1978).

Healthy flocks of sheep may carry a number of different strains of **P. haemolytica**, many of which are untypable (Biberstin et al., 1960). Animals in a diseased flock tend to harbour fewer strains of **P. haemolytica** which are often, though not invariably, common to the affected group and there is also an increase in the overall carrier rate. Once the pneumonia outbreak is over, however, there is a tendency towards widening of the spectrum and reducing the number of carriers to conform more nearly to the situation found in healthy flocks (Shreeve et al., 1972).

With regard to the ecology of **P. haemolytica**, Biberstein et al. (1960) were the first to report that certain serotypes (1,5,6,7,8,9 and 11) were commmonly isolated from the upper respiratory tract of healthy sheep whereas other serotypes (3,4,10) were never isolated from the upper respiratory tract of healthy animals.

Up to the mid 1970's, both biotypes and all 12 serotypes have been identified wherever **P. haemolytica** has been studied extensively, viz. UK (Biberstein & Thompson, 1966), Kenya (Mwangota, 1975) and the USA (Carter, 1956; Biberstein et al, 1960). However, in the Republic of South Africa no serotype 3 and 11 were detected

(Cameron & Smit, 1970) while in Australia, only biotype A strains were found (Biberstein, 1978).

Since then, numerous surveys have been conducted to determine the distribution of the different serotypes of **P. haemolytica**. In general, some serotypes are more common than others. Investigations carried out in both New Zealand and overseas have revealed that serotype A2 is probably the commonest type isolated from both pneumonic and normal sheep (Thompson et al., 1977; Gilmour, 1980a & b; Fraser et al., 1982; Hajtos et al., 1985; Ungureann & Schiunnel, 1985; Prince et al., 1985; Cameron & Bester, 1986). It may comprise a third of all isolates in cases of AFP (Thompson et al., 1977; Fraser et al., 1982), CNP and even normal sheep (Prince et al., 1985).

The frequency of other serotypes varies considerably. For instance, serotype A5 is seldom recovered from pneumonic lambs in Britain (Thompson et al., 1977; Fraser et al., 1982) and South African (Cameron & Bester, 1986), but it is relatively common in pneumonic lungs in Romania (Ungureann & Schiunnel, 1985) and in healthy sheep in New Zealand and some other countries (Mohamed & Hussein, 1981; Mogollon et al., 1983; Prince, 1985). In addition, types A5 to A9 and A12 have also been associated with both normal and "abnormal" sheep (Biberstein, 1978).

Both Biberstein (1978) and Prince (1985) found that some serotypes of **P. haemolytica** (A11 and untypable strains) did not appear in CNP lesions, although they were present in nasal isolates from affected animals. They suggested that these strains probably lack pathogenicity. However this hypothesis was disproved in a recent experiment carried out by Gilmour and his collegues (1986). They showed no clear difference in pathogenicity between the serotypes they used (A1, A2, A7 and A9). This suggests differences in frequency of isolation of these serotypes from field outbreaks may be not reflect differences in virulence. Bacteriological examination of naturally-occuring AFP indicated that the untypable

strains also seem to be associated with disease (Fraser et al., 1982) and there is a tendency for them to increase gradually during an outbreak.

Recently, antibiotic resistant strains of **P. haemolytica** were isolated from pneumonic lesions in calves in North America (Zimmerman & Hirsh, 1980) and Britain (Wray & Morrison, 1983). Their prevalence appears to be increasing (Amstutz et al., 1982). The strains are resistant to benzylpenicillin (10 µg), ampicillin (10 µg), amoxycillin (20 µg), streptomycin (10 µg), sulphonamides (50 µg) and carbenicillin (10 µg) (Wray & Morrison, 1983). The emergence of these strains in animal pneumonia is a cause for concern and whether similar strains are present in sheep remains uncertain.

2.1.2 Pasteurella multocida

Pasteurella multocida has been considered to be an important pathogen of animals for many years, but most work has been focused on the role of the organism in swine enzootic pneumonia and human infections (Carter,1967). There is only limited information about its importance in sheep pneumonia (McGowan et al., 1957; Hamdy et al., 1959).

Although the organism occurs throughout the world and has been found wherever animals have been examined (Bibertein, 1978), unlike **P. haemolytica**, it is a uncommon commensal in the ovine respiratory tract, although it can occasionally be isolated from the nasal cavity of normal animals (Guo et al.,1980; Doutre & Perreau, 1981). The only available quantitative data shows that the organism was present in the nasopharynx of 5.56% of healthy sheep in India, compared with 3.5% of cattle and 90% of cats (Smith, 1955).

Pasteurella multocida was considered by Gilmour & Angus (1983) to be only an occasional cause of sheep pneumonia in temperate climates. This is contrary to the reports of Marsh (1953) and Dohoo

et al. (1985) who considered that this organism was a frequent cause of pneumonic lesions in sheep. In Israel, this organism was also isolated from purebred East-Friesian lambs which suffered from interstitial pneumonia (Shimshony, 1983), but since this was combined with **P. haemolytica** infection its actual role in this type of pneumonia is not clear.

The pathogenesis and epidemiology of pneumonia caused by **P. multocida** do not differ significantly from that caused by **P. haemolytica** (Gilmour & Angus, 1983). However, in cattle, Schiefer et al. (1978) found that the lesions associated with **P. multocida** fulfill the criteria of a bronchopneumonia with moderate amounts of fibrin, whereas **P. haemolytica** was more likely to cause a fibrinous pleuropneumonia. These authors stated that the term "pasteurellosis" is therefore an imprecise term for this disease.

Attempts to produce pneumonia in lambs by experimental infection with **P. multocida** have generally been unsuccessful (Hamdy et al., 1959). The only exception is that of Biberstein et al. (1967) who produced a focal necrotic lesion in the lungs of two sheep with a strain of the organism obtained from calf pneumonia.

2.1.3 Other Bacteria

A variety of other bacteria are occasionally isolated from pneumonic lungs (Stevenson, 1969; Alley, 1975a & b). In most cases they are present as mixed infections, usually in combination with **P. haemolytica**, and their aetiologic significance is unclear. A few cases of experimental infection have also been reported but these will only be considered briefly in this review.

Corynebacterium spp. are only opportunist pathogens in the ovine lung. **Corynebacterium pyogenes** has been recovered from the nasal cavity of both normal and pneumonic sheep in very low numbers (Alley, 1975b) and **C. ovis**, the common cause of caseous lymphadenitis in sheep, occasionally produces pulmonary abscesses

(Seddon, 1967).

Neisseria catarrhalis is known to be a common organism in the nasal cavity of cattle (Magwood et. al., 1969), but there are only a limited number of reports in sheep. Miller (1940) may have been the first person to isolate **Neisseria** from the lungs of sheep. Thereafter, Alley et. al. (1970), St. George (1972) and Alley (1975b) recovered this organism from the nasal cavity, trachea and lungs of both healthy and pneumonic sheep in New Zealand and Australia. In a survey of normal and pneumonic sheep, the latter author found that **N. catarrhalis** was the second most common bacterium isolated and it was recovered most often in combination with **P. haemolytica.**

Haemophilus spp. have been associated with a variety of respiratory diseases in domestic animals (Poonacha & Donahue, 1984). They are common in the upper respiratory tract and oropharynx of many species, including sheep (Little et al., 1980). A **H. ovis**-like organism was isolated from sheep in Canada which died of acute bronchopneumonia (Mitchell, 1925) and from lambs which died of acute pneumonic disease in West Pakistan (Cheema et. al., 1965). Recently, Little et. al. (1980) surveyed the distribution of **Haemophilus spp.** in normal adult sheep in Britain. The results revealed that such organisms are very common commensals in the oropharynx. In their survey, the specimens from the oropharynx of 51 healthy adult sheep were cultured using a selective chocolate agar. Three X-dependant strains closely resembling **H. ovis**, two haemolytic V-dependant strains and 37 non-haemolytic V-dependant strains were isolated.

The most detailed description of ovine pneumonia caused by haemophilus organisms is that recently made by Poonacha & Donahue (1984). Infections in two week-old, mixed-breed lambs in U.S.A. were produced experimentally using **H. ovis**. Clinical signs consisted of irregular breathing, bloat and bloody discharge from the nose, mouth and anus. Necropsy revealed excess amounts of blood-tinged fluid and fibrin masses in the thoracic cavity, multiple subpleural

haemorrhages on the lung surface and bloody froth in the lower respiratory passages. Histopathologically, lung changes consisted of acute thromboembolic pneumonia characterized by multiple foci of haemorrhages, vascular thrombosis,and septic emboli. The mechanism of natural transmission of this disease, if any, is unknown (Pooncha & Donahue, 1984).

Staphylococcus aureus can also be found in the respiratory tract of the normal and CNP-affected sheep (McGowan et al., 1957; Smith, 1957; Hamdy et al., 1959; Alley, 1975b), but there is no significant difference in their recovery rate between the two groups (Alley, 1975b). Occasionally, acute pneumonia can be successfully induced by intratracheal inoculation of **S. aureus** (Biberstein et al., 1967; Alley, 1975a). Necropsy records show the organism is a common cause of pulmonary abscesses in very young lambs (Alley, 1975a).

In spite of streptococcal pneumonia (formly Diplococcal pneumonia) being a common disease of man, these organisms only occasionally infect ruminants (Steele, 1979). Streptococci have been isolated from the upper respiratory tract of both healthy and CNP-affected sheep (Alley, 1975b) as well as acute pneumonic lungs (McGowan et. al., 1957; Hamdy et al., 1959; Stevenson, 1974). The acute lesions could be successfully reproduced in sheep by inoculating **Streptococcus zooepidemicus** intratracheally (Stevenson, 1974).

Other bacteria, such as **Escherichia coli, Pseudomonas pseudomallei,** and **Salmonella spp.** are also occasionally recovered from pneumonic sheep, but they seem to be of no importance in the ovine enzootic pneumonias (Alley, 1975a; St. George, 1972; Laws & Hall, 1964; Robinson, 1983).

2.2 Experimental Bacterial Infection *in vivo*

In the majority of attempts to experimentally reproduce a bacterial pneumonia in sheep, **P. haemolytica** has been used. This is because of the high recovery rate of **P. haemolytica** at high titres from both AFP and CNP lesions and because a close association with destructive changes at the microscopic level has emphasized the importance of this organism (Davies, 1985). However, attempts at experimental infection with other organisms have also been undertaken in earlier times (Hamdy *et al.*, 1959; Biberstein *et al.*, 1967) but they will not be considered in detail in this review since it is generally agreed that these organisms are only opportunist invaders (Alley, 1975b).

Attempts to reproduce ovine pneumonia using **P. haemolytica** alone have not been uniformly successful (Dungal, 1931; Salisbury, 1957; Downey, 1957; Hamdy *et al.*, 1959, Smith, 1964). In most healthy animals the bacteria are rapidly cleared from the respiratory tract (Gilmour *et al.*, 1979; Davies, 1985). In early experiments, **P. haemolytica** was found to be capable of causing an acute necrotizing pneumonia in sheep similar to natural cases only when large doses of organism and/or special inoculation techniques were employed (Smith, 1964; Biberstein *et al.*, 1967 & 1971). However, Gilmour *et al.* (1984) recently reported that the proportion of SPF lambs experimentally affected with pneumonia did not alter when the numbers of **P. haemolytica** serotype A2 organisms in the aerosol were as low as 5.5×10^2 CFU/ml. These workers also demonstrated that **P. haemolytica** alone can occasionally produce lesions resembling CNP in SPF lambs (Gilmour *et al.*, 1979; Jones *et al.*, 1982b).

The difficulty in producing pneumonias in sheep with **P. haemolytica** alone and the fact that the organism can frequently be found in the upper respiratory tract of normal animals, has prompted a search for other organisms which may act as primary pathogens in ovine pneumonia. Infective agents that may predispose the ovine lung

to **P. haemolytica** infection include parainfluenza virus 3 (Davies et al, 1977; Sharp et al., 1978; Rushton et al., 1979; Davies et al., 1980), adenovirus (Davies et al., 1982), reovirus (Belak & Dalfi, 1974), chlamydia (Biberstein et al., 1967), mycoplasmas (Clark et al., 1974; Jones et al., 1982b & c) and respiratory syncytial virus (Al-Darraji et al. 1982a, b & c; Trigo et al., 1984). A severe and typical acute or chronic pneumonia can be produced in large proportions of animals only by inoculating some other agent in combination with **P. haemolytica**. Nevertheless, the majority of lung damage seen with any of these combinations can probably be attributed to the proliferation of **P. haemolytica** rather than the initiating agent, since these lesions are similar to those seen occasionally following inoculation of **P. haemolytica** alone (Davies et al., 1982).

The investigation undertaken by Alley and Clarke (1979) demonstrated that **P. haemolytica** is likely to have a major role in the aetiology of ovine CNP. Conventionally-reared lambs intranasally inoculated with pure **M. ovipneumoniae** cultures showed pneumonic lesions in only 20% of animals. In contrast, pneumonia was produced in 85% of the lambs infected with lung homogenate which contained both **M. ovipneumoniae** and **P. haemolytica**.

To further investigate the possible role of bacteria in the pathogenesis of pneumonia, the effect of several chemotherapeutic agents, especially penicillin, on the development of experimental lesions was studied (Alley & Clarke, 1980; Gilmour et al., 1982d). Penicillin does not prevent mycoplasmas from colonising the lung; however, only one of 10 animals treated with penicillin (10%) developed moderate gross lesions whereas sevene untreated animals (70%) showed moderate to severe CNP lesions at necropsy. These results have further proven that bacteria have an important role in the pathogenesis of CNP in lambs (Alley & Clarke, 1980).

The experiments carried by Gilmour et al. (1982a & c) and Jones et al. (1982a, b & c) comfirmed that **P. haemolytica** is capable not

only of colonizing lesions of "proliferative exudative" pneumonia produced in a proportion of cases by **M. ovipneumoniae**, but also of actively transforming less severe changes induced by the mycoplasma into CNP. The inclusion of **P. haemolytica** in the inocula increased the severity of pneumonic lesions in the lung and the incidence of "proliferative exudative" pneumonia in both conventional and SPF animals (Jones et al., 1982c). Thus **P. haemolytica** should be considered as an important exacerbating factor of the morbidity and the severity of CNP (Jones et al., 1982c). The inability to associate the organism with all natural CNP cases may be attributed partly to the fact that mycoplasmas **per se** can produce the disease in some animals and partly to the finding that **P. haemolytica** is eliminated from lung lesions more rapidly than mycoplasmas (Jones et al., 1982c).

Recently, Jones et al. (1986b) have reviewed 10 experiments in which both conventionally-raised and SPF lambs were inoculated by endobronchial and/or intratracheal routes with different preparations. Their findings have added strong support to previous evidence implicating both **P. haemolytica** and **M. ovipneumoniae** as the main aetiological agents of CNP. However, endobronchial or intratracheal routes are probably a very poor equivalent to natural modes of infection. The latter are probably better represented by the instillation of appropriately sized aerosols. If this argument is accepted then results of experiments using the endobronchial or intratracheal route must be interpreted with some caution.

2.3 Experimental Bacterial Infection in vitro

Organ cultures of animal and human tracheas provide a convenient and readily accessible model system for studying the effects of a variety of bacteria on respiratory ciliated epithelium. The most extensive studies of bacteria in tracheal organ culture have involved organisms of the genus **Bordetella**. These studies have shown that all three species, **B. parapertussis, B. pertussis** and **B. bronchiseptica** have the ability to attach to ciliated cells but not

to nonciliary portions of the respiratory epithelium (Collier et al., 1977; Muse et al., 1977; Bemis et al., 1977; Matsuyama, 1974 & 1977; Matsuyama & Takino, 1980; Al-Kaissi et al., 1986). Infection of tracheal cultures results in decrease of protein synthesis (Collier et al., 1977), ciliostasis, destruction of subcellular organelles, necrosis of ciliated cells, and finally expulsion of the cells from the epithelium (Baseman, 1977; Muse et al., 1977 & 1979; Al-Kaissi et al., 1986). These observations have confirmed the light microscopic observation of Pittaman (1970) that these organisms attach to ciliated epithelial cells in naturally-occurring respiratory infection.

The application of scanning electron microscopy further demonstrated that **Bordetella** organisms are closely associated with clusters of cilia, microvilli, and the lumenal border of cultured epithelial cells in the respiratory tract (Muse et al., 1979; Bemis & Kennedy, 1981; Opremcak & Rheins, 1983) as well as with ciliated cells of the oviduct (Opremcak & Rheins, 1983). Fluorescence microscopy has also shown that the principal site of bacterial growth in chicken tracheal culture is the epithelial surface (Iida & Ajiki, 1974). The adhesion of **B. pertussis** to ciliated epithelium of the respiratory tract can be prevented by serum antibody **in vitro** (Holt, 1972).

Many of the early tracheal organ culture studies indicated that attachment of **B. pertussis** to the host cell is a prerequisite of **B. pertussis** infection (Matsuyama,1977; Iida & Ajiki, 1974 & 1975; Collier et al., 1977; Muse et al., 1977 & 1979; Holt, 1972; Opremcak & Rheins, 1983) since this characteristic is unique to virulent strains. The cilia in the tacheal organ cultures infected with avirulent (phase IV) organisms showed no association with the organism and remained identical to these of uninfected control cultures (Muse et al., 1979).

The importance of attachment in the pathogenesis of **Bordetella** infections was confirmed by the observation that tracheal organ

cultures maintained in media filtered from infected organ cultures behaved similarly to uninfected cultures in terms of ciliary activity (Muse et al., 1979; Opremcak & Rheins, 1983). The initial contact between the infectious organism and mucosal surfaces is at the ciliary apex. The organisms then rapidly infiltrate through the ciliary blanket to the base of the cilia. Under TEM the organisms can be seem associated with microvilli through filamentous glycocalyx components or in direct apposition with the microvillous or ciliary membrane resulting in firm attachment (Muse et al., 1979). Thin fibres as well as small blebs were seen to extend between cilia and associated **B. pertussis** in both mouse oviduct and tracheal organ cultures (Opremcak & Rheins, 1983), but neither bacterial fimbriae (Opremcak & Rheins, 1983) nor pili (Muse et al., 1977), two major structures thought to be involved in the attachment of bacteria to mucosal surfaces, were ultrastructurally observed.

Although attachment of organism to the ciliary cell is essential for ciliostasis in certain bacterial infections, attachment does not necessarily result in ciliostasis. Bemis and Kennedy (1981) observed that heat- and formalin-treated **B. bronchiseptica** organisms could attach to cilia in the canine trachea but no ciliostasis or significant reduction of ciliary beating frequency resulted. It has been suggested that the ciliostatic effects of **Bordetella** may require close association between metabolically active organisms and cilia (Muse et al., 1979).

Application of the tracheal organ culture technique also provides the possibilility of studying different toxins derived from bacteria (Goldman et al., 1982; Manclark & Cowell, 1984). Purified tracheal cytotoxin (TCT) from **B. pertussis** in microgram amounts will cause ciliostasis and morphological changes in hamster tracheal rings compatible with those observed in infected human tissues and in tracheal rings infected with **B. pertussis** organisms (Goldman et al., 1982). A partially purified preparation of heat-labile toxin (HLT), however, did not alter the metabolic activity of hamster tracheal epithelial cells or damage hamster tracheal rings **in vitro**

(Manclark & Cowell, 1984).

Other bacteria and bacterial products which have been studied in tracheal organ cultures are **P. haemolytica** (Al-Kaissi, 1986), **Neisseria sp.** (Al-Kaissi, 1986), **E. coli** (Mastuyama, 1974), **Staphylococcus aureus** (Mastuyama, 1974), **Staphylococcal** α-toxin (Hoorn et al., 1965), **Haemophilus influenzae** (Denny, 1974; Johnson et al., 1983) and diphtheria (Baseman & Collier, 1974). Although most of these organisms were usually used as controls for the study of infections with **Bordetella**, some are also important pathogens in both human and animal respiratory diseases. In general, they have no close interaction with the respiratory epithelial cilia (Mastuyama, 1974; Al-Kaissi, 1986). Some of them cause epithelial damage in tracheal organ cultures only at high titres (Hoorn et al., 1965; Al-Kaissi, 1986); while others produced no significant changes (Al-Kaissi, 1986). For this reason, they will only be briefly considered in this section.

Use of tracheal organ cultures allowed Denny (1974) to study the action of **H. influenzae** on live ciliated respiratory tract epithelium. He grew strains of this organism in cultures of tracheas from human foetuses or from various animal species and observed cessation of ciliary action within a few days. Supernatant fluids from any of his **H. influenzae** strains, capsulated or non-capsulated, also caused ciliostasis, loss of cilia and eventually sloughing of epithelial cells when applied to rat tracheal sections. A similar effect was seen with human foetal tracheas, but it took longer to appear and was less consistent. It was suggested that the alterations of ciliary motility were due to cellular injury rather than ciliary changes because under TEM the cilia appeared to be normal, but the epithelial cells showed extensive damage and sloughing. Denny's findings were reproduced by Johnson et al. (1983) using rat tracheal organ cultures. In Johnson et al.'s study, association of the bacterium with the epithelial surface was seen infreqently under SEM and the sloughed cells were usually free of adherent bacteria, indicating that bacterial attachment was not a

necessary prerequisite for the production of tissue damage caused by **H. influenzae.**

Later, Johnson & Inzana (1986) used the same **in vitro** model to examine the ability of lipo-oligosaccharide from **H. influenzae** to damage respiratory tract mucosal tissue. Lipo-oligosaccharide produced a significant decrease in the ciliary activity of tracheal rings observed over a 3-5 day period. No loss of ciliary activity was observed with the lipid-free moiety of the lipo-oligosaccharide. This result added further weight to the idea suggested by earlier workers (Gregg et al., 1981) that lipid A is the active component in the production of tissue damage.

Although the biochemistry of diphtheria intoxication has been explained in molecular terms, the manner in which diphtheria alters host cell function in the intact animal leading to morphologic damage and possible death remains unclear. An attempt to examine the influence of diphtheria toxin on protein synthesis and the development of cytopathology was carried out with guinea pig tracheal organ cultures (Baseman & Collier, 1974). Exposure of individual tracheal rings to toxin resulted in cessation of protein synthesis as well as the development of cytopathology within a few hours. Continued incubation led to further inhibition of protein synthesis and extensive disorganisation of the epithelial layer. A similar phenomenon has been demonstrated in relation to an exotoxin produced by **Pseudomonas aeruginosa,** which has been shown to inhibit ciliary activity in rabbit tracheal organ cultures (Reimer et al., 1978).

2.4 Possible Importance of Bacterial Cytotoxins

It is well established that cytotoxin from gram-negative bacteria can induce a wide variety of reactions in the respiratory tract of sheep after infection via several routes (Demling et al., 1981; Esbenshade et al., 1982; Snella & Rylander, 1982; Snapper et al., 1983; Brogden et al., 1984 & 1986b). The cytotoxins from **P.**

haemolytica have probably received the most extensive study because of their importance in respiratory diseases of sheep and other ruminants (Kaehler et al., 1980a & b; Shewen & Wilkie, 1982 & 1985; Chang et al., 1986b). At present, it is generally recognized that **P. haemolytica** contains two types of cytotoxin: exotoxin (leucotoxin) and endotoxin (lipopolysaccharide, LPS). The cell-free supernatants of **P. haemolytica** show two different effects **in vitro**, which are firstly the impairment of phagocytosis at low concentrations and secondly cytotoxicity at higher concentrations (Markham & Wilkie, 1980). Cytotoxicity is a possible virulence factor which is common to both biotypes of **P. haemolytica** (Sutherland & Donachie, 1986a).

2.4.1 Exotoxin (leucotoxin)

This toxin is produced and released by metabolically active, pathogenic isolates of **P. haemolytica** in their culture supernatant and has toxic effects on ruminant, but not non-ruminant leucocytes (Kaehler et al., 1980b; Shewen & Wilkie, 1982 & 1985; Chang et al., 1986b). Because this exotoxin is inhibitory and cytotoxic for leucocytes, but not for other cell types, the term leucotoxin has been suggested by several authors to describe this exotoxin (Renshaw, 1984; Chang et al., 1986a).

Leucotoxin is heat labile, oxygen stable, pH stable, non-dialyzable, non-haemolytic and water soluble (Chang et al., 1986b). It has been suggested that the toxin is a protein or glycoprotein and posesses antigenic properties.

The apparent species specificity of the leucotoxic effects of living **P. haemolytica** and its sterile culture supernatant supports the view that leucotoxin is involved in determining the species-associated occurrence of **P. haemolytica**-induced pneumonia (Kaehler et al., 1980a; Shewen & Wilkie, 1982; Chang et al., 1986a). Experimental evidence from studies of the interactions of **P. haemolytica** and its culture supernatant with ruminant alveolar macrophages, peripheral blood monocytes, neutrophils (PMN), and

lymphocytes suggests that **P. haemolytica** leucotoxin is important for successful colonization and growth of the organism in pulmonary tissues (Kaehler et al., 1980a & 1980b; Shewen & Wilkie, 1982; Sutherland et al., 1983). Recent studies have shown that the cytotoxic effects of leucotoxin for leucocytes in pulmonary tissues probably contribute to subsequent pathophysiologic changes of the lungs (Chang et al., 1986b).

Initial studies of the role of **P. haemolytica** cytotoxin in the pathogenesis of animal pneumonia were focused on cattle (Shewen and Wilkie, 1982), and goats (Chang et al., 1982; Richards et al., 1982), but recently, these studies have been extended to sheep (Sutherland et al., 1983; Sutherland, 1985; Sutherland & Donachie, 1986a). The results show that leucotoxin from ovine isolates of **P. haemolytica** biotype A serotype 1 are cytotoxic for sheep bronchoalveolar macrophages, but not for guinea-pig peritoneal macrophages. The apparent specificity of **P. haemolytica** cytotoxin for ovine phagocytes suggests there may be a requirement for a specific receptor for the mediation of toxicity. Further studies have indicated that there are no significant differences in toxicity to ovine bronchoalveolar macrophages of leucotoxin from different serotypes of the organism (Sutherland & Donachie, 1986a) and weaker toxicity for the same target cells was also expressed by non-serotypable ovine isolates of **P. haemolytica**.

Additional investigations have showed that **P. haemolytica** cytotoxin is also cytotoxic for ovine peripheral blood leucocytes and lymphocytes obtained from gastric lymph (Sutherland, 1985). It therefore seems probable that all ovine leucocytes are susceptible to the cytotoxin from **P. haemolytica** A1. This further supports the suggestion that extracellular aggressins play an important role in the pathogenicity of **P. haemolytica** (Sutherland, 1985). Sensitivity of a species leucocytes to leucotoxin activity has a direct correlation to the susceptibility of that species to **P. haemolytica** - induced pneumonia (Chang et al., 1986a). Additionally, regardless of their serotype specificity, pathogenic isolates of the organism

invariably produce leucotoxin when cultured under optimal conditions, whereas untypable nonpathogenic isolates do not produce leucotoxin (Sutherland et al., 1983; Chang et al., 1986a). Taken together, these findings suggest that cytotoxin may play an important role as a virulence factor in the bacterial pneumonias of sheep.

2.4.2 Endotoxin (Lipopolysaccharide, LPS)

Bacterial LPS has been implicated as a determinant in the pathogenesis of gram-negative bacterial pneumonias (Brogden et al., 1984 & 1986b). A potential role for **P. haemolytica** LPS in the pathogenesis of bovine pneumonic pasteurellosis has been outlined by Slocombe et al. (1984). It is primarily involved in the pathogenesis of **P. haemolytica**-induced pneumonia during the middle to latter stages of the infectious process (Chang et al., 1986b). In contrast to exotoxin, endotoxin does not possess the host-species specificity or cell-type specificity (Elin & Wolff, 1973).

P. haemolytica LPS has also been implicated as a determinant in the pathogenesis of ovine pneumonic pasteurellosis (Brogden et al., 1984). Since in the lungs, LPS can react with humoral and cellular components causing the release of mediators that induce inflammation, and in the alveoli, LPS may be absorbed onto cell membranes, including those of macrophages, resulting in death of cells and activation of the complement system (Brogden et al., 1984, 1986a & b).

More recently, Brogden et al. (1986a & b) reported the **in vitro** combination of LPS from **P. haemolytica** and **P. multocida** with sheep lung surfactant. This complexing could also be formed when LPS from **Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Serratia marcescens,** and **Pseudomonas aeruginosa** were used (Borgden et al., 1986b). Since surfactant is important in reducing the surface tension at the air-alveolar interface, controlling exudation of fluid into the alveoli, reducing the effort of breathing and

protecting the lungs against collapse, these authors suggested that in pneumonic infections, LPS might not only induce mediators of inflammation, but also alter the physiological function of the lung (Borgden et al., 1986b).

Chang et al. (1986b) have suggested another possible way in which LPS may be involved in the pathogenesis of pneumonia. The endotoxin may activate the alternative complement pathway, increasing capillary permeability, neutrophil chemotaxis and causing blood clotting that may lead to extensive tissue damage and cause characteristic acute pneumonic lesions (Kim, 1977).

2.5 Conclusion

All the evidence currently available indicates that the aetiology of ovine pneumonia is likely to be complex. However, studies of both natural and experimental infections **in vivo** and **in vitro** have shown that bacteria play an important role in damaging the ovine respiratory tract and are likely to be major pathogens in most common forms of the disease. Bacteria cause disease only when predisposing factors weaken the defence mechanisms of the respiratory tract. When this occurs apparently virulent strains (or serotypes) are selected which have enhanced pathogenicity and these may spread to other animals in conditions of close contact.

Among the bacteria recovered from the ovine respiratory tract, **P. haemolytica** is likely to be the most important in initiation and development of both ovine AFP and CNP. The other organisms may be opportunist invaders and are of less significance. The potential of **B. parapertussis** as an initiator or contributor to ovine CNP requires further investigation.

Recent studies have revealed that cytotoxins produced by certain bacteria may have a significant effect on the development of pneumonia in some species. However, it is premature to conclude that this is the case in sheep although some experimental results have

proven that these substances are harmful to ovine host defences.

Section III

BIOLOGY OF BORDETELLA PARAPERTUSSIS

The clinical entity known as whooping cough has been recognized in children since 1578 and **Bordetella pertussis** is thought to be its major caustive agent (Olsen, 1975). Although **B. parapertusis** has also been known to be associated with whooping cough in children since the 1930's (Bradford & Salvin, 1937; Eldering & Kendrick, 1938), there was no evidence suggesting the importance of this bacterium in animal pneumonia until the beginning of this decade when a **B. parapertussis**-like organism was isolated from the respiratory tract of healthy and pneumonic sheep in New Zealand (Cullinane et al., 1987). Its subsequent high recovery rate from the nasal cavity and bronchoalveolar lavage fluid and its ability to produce ciliostasis of tracheal epithelium **in vitro** were suggestive of a possible role in the pathogenesis of ovine CNP (Manktelow, 1984; Alley, 1986; Al-Kaissi et al., 1986).

All organisms within the genus **Bordetella** have been recognized as important pathogens of the respiratory tract of mammals (Mallory et al., 1913; Eldering & Kendrick, 1938; Goodnow, 1980), however, most work has concentrated on **B. pertussis** and **B. bronchiseptica.** Although some research on the serological, toxic, antigenic and epidemiological characteristics of **B. parapertussis** has been reported in comparative studies of **Bordetella** organisms, little investigation has been conducted on the pathogenicity of **B. parapertussis** in humans or animals (Bradford & Salvin, 1937; Bradford & Wold, 1939; Zuelzer & Wheeler, 1946; Anderson, 1953 & 1957; Linnemann & Perry, 1977).

Since comprehensive reviews on the biology of **B. pertussis** and **B. bronchiseptica** have been published elsewhere (Olsen, 1974; Pittman, 1984b; Goodnow, 1980), the present review attempts to

summarize the information available on the possible significance of **B. parapertussis** in respiratory infection in humans and laboratory animals. Aspects of its bacteriology, epidemiology and pathology are discussed in order to provide a general view of the potential importance of this organism in ovine CNP.

3.1 General Description of Bordetella parapertussis

B. parapertussis, formly classified as **Haemophilus parapertussis**, was first isolated by Eldering and Kendrick in 1934, about thirty years after the discovery of **B. pertussis**. Three years later, Bradford and Slavin (1937) also noted the presence of some atypical **B. pertussis** strains which were later identified as **B. parapertussis**. Since 1937, the organism has been recovered from the human respiratory tract in U.S.A. (Miller et al., 1941 & 1943; Eldering & Kendrick, 1952), Mexico (Miller et al., 1941), Denmark (Miller et al., 1941), and England (Eldering & Kendrick, 1952). It is now known to occur in practically all European countries, North American, South America, Australia, and Japan (Lautrop, 1971).

The **B. parapertussis** organism is a small non-motile, non-capsulated, non-sporing, gram-negative bacillus morphologically resembling **B. pertussis**. **Bordetella parapertussis** differs from **B. pertussis** and **B. bronchiseptica** in several characteristics (Table 1.3). It is able to grow on both non-blood or non-charcoal containing media. On Bordet-Gengou medium, **B. parapertussis** is distinguished from **B. pertussis** by (1) the large size of the colonies, the surfaces of which are somewhat more dull; (2) the accompanying haemolytic zone, which on this medium is rarely seen around pertussis colonies until after 4 to 5 days incubation; (3) its rod-like shape, which on media containing horse-blood is quite definite and contrasts with the coccoid shape of most pertussis cells (Lautrop,1958); and (4) it produces a characteristic brown water-soluble pigment in peptone media (Blair, et al., 1970).

The optimal temperature for the culture of **B. parapertussis** is

TABLE 1.3
DIFFERENTIAL CHARACTERISTICS WITHIN THE GENUS **BORDETELLA***

Characteristic	B. pertussis	B. parapertussis	B. bronchiseptica
Flagella	-	-	+
Motility	-	-	+
Nitrate reduction	-	-	+
Citrate utilized	-	+	+
Urease produced	-	+ (24hr)	+ (4hr)
Oxidase produced	+	-	+
Blood-free peptone agar			
Growth	-	+	+
Browning	-	+	–
Bordet-Gengou agar			
Growth in 1-2 days	-	+	+
Growth in 3-4 days	+		
Litmus milk alkaline	+ (12 to 14 days)	+ (1 to 4 days)	+ (1 to 2 days)
Growth on MacConkey agar	-	+	+
Sensitive to cephalexin	+	++	
Sensitive mice to histamine	+	-	-
Specific heat-labile antigen			
Factor 1	+	-	-
Factor 2	-	-	+
Factor 3	-	+	-
Mol% of G + C of DNA (Tm)	67-70	66-70	68.9
Adenylate cyclase activity (intracellular)	+	-	-
Growth inhibited by unsaturated fatty acids or colloidal sulfur	+	-	-
Sensitive to sulphonamides	-	+	?
Degree of tolerance to NaCl, bile, phenol and temperature	Lowest	Intermediate	Highest

* Based on Pittman (1974 & 1984a); Eldering & Kendrick (1952); Sutcliffe & Abbott (1972) and Parker & Linnemann (1980)

35-37°C (Pittman, 1984a). During culture the organisms frequently undergo a phase variation characterized by simultaneous loss of the expression of multiple virulence factors (Pittman, 1970; Linnemann, 1979). The common manifestations of the phase change are summarized by Weiss & Hewlett (1986) as; (1) the inability of avirulent strains to produce several toxins such as pertussis toxin (PT), adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT) and haemolysin (HLY); (2) lack of expression of some factors that may have a supportive role in the disease process, such as filamentous haemagglutinin (FHA); (3) difference in colony morphology and sensitivity to antibiotics. However, lipopolysaccharide (LPS) from the avirulent phase is identical in structure and function to that from the virulent phase and tracheal cytotoxin is produced by both phases (Weiss & Hewlett., 1986). The phase variation is also recognized ***in vivo*** (Weiss & Hewlett, 1986).

The organism isolated from healthy and pneumonic ovine respiratory tract is relevant to **B. parapertussis** and **B. bronchiseptica** in some characteristics (Appendix 1, Cullinane et al. 1987). It has been temporily named as the **B. parapertussis**-like organism since it most closely resembles **B. parapertussis.**

In order to better understand the pathogenesis of infection with Bordetella, many studies of the cellular components of the organisms have been carried out (Pittaman, 1970). Besides specific agglutinogens (AGG), **B. parapertussis** is found to possess FHA, DNT, ACT, and LPS but not PT, as does **B. pertussis** (Eudoh et al., 1980; Granstrom & Askelof, 1982). The toxins tested from **B. parapertussis** are similar in their effect on animals to those from other **Bordetella** species and can be indistinguishably neutralized by **B. pertussis** antitoxic serum (Bruckner & Evans, 1939).

Recently, a new toxin, tracheal cytotoxin (TCT), was discovered to be present in **B. pertussis** (Goldman et al., 1982). This toxin is thought to be an essential substance for causing local tissue damage. Whether **B. parapertussis** also possesses a similar cellular

component is not yet clear.

The antigenic relationships of the **Bordetella** species are based on heat-labile agglutinogens (Anderson, 1953; Eldering et al., 1957). Anderson (1953) demonstrated serological differences among **Bordetella** organisms and concluded that all organisms had the same O-antigen and haemorrhagic toxin in common as well as species-specific K-antigens. Eldering et al. (1957) extended these observations into an antigenic schema involving 14 agglutinogens. A summary of antigenic factors cited from Lennette et al. (1974) and Bliar et al. (1970) is presented in Table 1.4. **B. parapertussis** shares minor antigenic factors with the other organisms in this genus. Antigen 7 is common to all members of the genus, whereas antigen 14 is species specific for **B. parapertussis** and antigens 8, 9, and 10 are shared with **B. bronchiseptica**.

Although **B. parapertussis** is microbiologically distinct in many respects from **B. pertussis**, it has recently been postulated that the two organisms do not cause two distinct syndromes (Granstrom & Askelof, 1982). **Bordetella parapertussis** may represent only a non-toxigenic strain of **B. pertussis**, the conversion to **B. parapertussis** being due to loss of a lysogenic bacteriophage. Before this hypothesis, Kumazawa & Yoshikawa in 1978 suggested that **B. parapertussis** was a mutant of **B. pertussis** probably induced by a selective pressure of antibiotics. The evidence supporting the hypothesis that **B. parapertussis** is not a distinct organism is that (1) after the introduction of vaccination against **B. pertussis**, isolation rates of **B. pertussis** and **B. parapertussis** reduced simultaneously (Lautrop, 1971); (2) the organisms of phase III **B. pertussis** are similar to **B. parapertussis** in many characteristics; (3) the occurrence of lymphocytosis in some patients infected by **B. parapertussis** has been noted, and **B. parapertussis** is considered to produce no PT (Zuelzer & Wheeler, 1946; Linnemann & Perry, 1977); and (4) DNA-hybridisation studies show a close genetic relationship among these organisms (Kloos et al., 1979).

TABLE 1.4

ANTIGENIC FACTORS OF THE **BORDETELLA** SPECIES

Species	Species-specific factor	Other factors present
B. pertussis	1	2,3,4,5,6 and 7*
B. parapertussis	14	8,9,10 and 7*
B. bronchiseptica	12	8,9,10,11 and 7*

* Factor 7 is held in common by all **Bordetella** species.

However, only partial or no cross-immunity between **B. pertussis** and **B. parapertussis** is present which does not adequately protect hosts from infection (Eldering & Kendrick, 1952; Donchev & Stoyanova, 1961; Granstrom & Askelof, 1982). Clinical investigation has also shown that the two infections are distinct, since children who develop one infection remain susceptible to infection from the other organism (Lautrop, 1958).

3.2 Epidemiology

Since the first recognition of **B. parapertussis**, it has generally been accepted that man is the only natural host (Pittman, 1984a). The organism can be recovered from the nasal passages, trachea, bronchi and lungs on a modified Bordet-Gengou medium (Blair et al., 1970).

Human respiratory disease caused by **B. parapertussis** has been reported in several areas of the world (Olson, 1975), particularly European countries (Duguid et al., 1978). A varying number of outbreaks have been reported in Bulgaria (Donchev et al., 1961), U.S.S.R. (Neimark et al., 1961), Denmark (Lautrop, 1958 & 1971), U.S.A. (Linnemann & Perry, 1977), France (Sohier & Fanchet, 1949), Yugoslavia (Skvrnova et al., 1955), Mexico (Miravete & Mora, 1966), Chile, Australia, England, Japan, Spain, Portugal, Hungary, and Finland (Lautrop, 1958).

The true incidence of **B. parapertussis** infection is not well known. Reports from different sources show a very large range of recovery rates of **B. parapertussis** from whooping cough cases, with an average of less than 5% (Linnemann & Perry, 1977), and a range of between 39% (Neimark et al., 1961) and 0.6% (Smith, 1984). In Denmark and Mexcico, **B. parapertussis** infection in clinical cases was estimated to be as common as **B.pertussis** (Lautrop, 1958; Miravete & Mora, 1966). The disease caused by **B. parapertussis** is thought to be endemic and reach epidemic proportions at intervals of a few years (Vysoka, 1958). In the densely populated areas of

Denmark, **B. parapertussis** may be recovered from almost all cases of whooping cough (Lautrop, 1971).

Two methods of study of the epidemiology of **B. parapertussis** have been employed in human medicine: (1) the results of cultures taken from children with clinical pertussis; and (2) serologic surveys for antibodies to **B. parapertussis** (Linnemann & Perry, 1977). The first method of investigation reflects only the carriage of **B. parapertussis** among cases of severe pertussis syndrome. This approach probably gives prevalence data for **B. parapertussis**, which is much higher than its true prevalence in the whole population (Linnemann & Perry, 1977). The only survey of the prevalence of **B. parapertussis** in a general population has been done in Czechoslovakia, where 1.73% of cultures from a total of 9,983 children were positive (Borska & Simkovicova, 1972).

Serologic surveys provide better information on the frequency of potential infections. In 1941, Miller and his colleagues tested 50 children in California and found that 40% of them had antibodies to **B. parapertussis**. Flosdorf et al.(1942) measured **B. parapertussis** antibody in several populations and demonstrated that 91% of urban adults showed a positive reaction. Investigations in Europe have also shown antibodies in more than half the adults tested (Vysoka, 1963). These studies clearly indicate that **B. parapertussis** infections are extremely common.

Although there are some reports indicating that **B. parapertussis** is as commonly recovered as **B. pertussis** (Donchev et al., 1961; Miravete & Mora, 1966; Lautrop, 1971), the incidence of clinical and subclinical parapertussis infection reported is usually very low; from 3 to 4%, compared to 75% for pertussis (Lennette et al., 1974). This is thought to be partly due to the clinical features of **B. parapertussis** infection which in most cases are too inconspicious to arouse the attention of parents and doctors (Lautrop 1958; Miller et al., 1941). In addition, the pathological changes, both macroscopically and microscopically, caused by **B.**

parapertussis are similar to those caused by **B. pertussis**, and furthermore the definite diagnosis depends on the identification of the bacterium, a procedure which is not available in many places (Lautrop, 1958).

Some evidence suggests the presence of a relationship between the epidemiology of **B. parapertussis** and **B. pertussis**. Lautrop (1958) found the epidemic waves of the two infections never coincided although some overlapping among them was present. High isolation rates for **B. parapertussis** were accompanied by low figures for **B. pertussis**, and **vice versa**. However, it is sometimes possible that children can contract both **B. parapertussis** and **B. pertussis** infection simultaneously (Eldering & Kendrick, 1952; Lautrop, 1958) or successively (Lautrop, 1958).

Recently it has been found that an organism similar to **B. parapertussis** can be regularly isolated from the respiratory tract of both CNP-affected and healthy lambs (Manktelow,1984; Alley, 1986; Cullinane et al., 1987). This organism was present in about 50% of ovine lungs and nearly all nasal cavities with CNP and in 30% of healthy lungs (Alley, 1986). No epidemiological relationship has yet been shown to exist between human and ovine infections (Manktelow,1984).

3.3 Pathogenesis

Early workers believed that attachment of **Bordetella** organisms to respiratory epithelium plays a key role in the initiation of the disease, and that toxin might be only a minor factor in aiding lodgement of bacilli on the surface of ciliary cells (Standfast, 1958). The clinical evidence that the symptoms are not greatly affected by the elimination of the pathogen (Pittman, 1976) together with the results of experimental infection (Goldman, 1986) have emphasised the importance of cell toxin in the development of the disease. It is now considered that pertussis is a tissue-fixed toxin-mediated disease rather than a disease caused by the direct

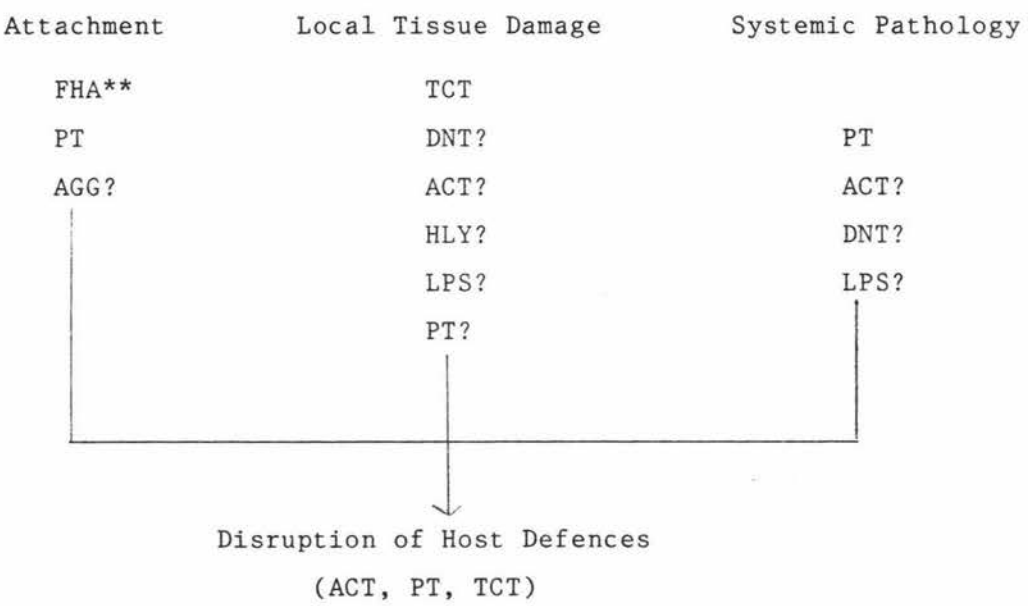
damage through bacterial colonisation of respiratory epithelium (Pittman, 1984b). A number of toxins produced by **B. pertussis** are involved in establishment of infection. These toxins are involved in bacterial attachment to the mucosal epithelium, evasion of host defences by the bacteria, local tissue damage and the occurrence of systemic disease (Figure 1.1) (Manclark & Cowell, 1984; Hewlett & Weiss, 1986; Weiss & Hewlett, 1986).

Tissue-fixed toxins may be responsible for the cough and lung tissue damage either directly or indirectly. In addition, the toxin may damage the submucosa and extend into lung tisssue. The damage is accompanied by peribronchiolar infiltration with lymphocytes and polymorphonuclear cells. The bronchioles blocked by excess mucus lead to areas of lung collapse and patches of emphysema (Duguid et al., 1978).

At present, whooping cough caused by **B. pertussis** is recognized as a two stage process: colonization of the organism and initiation of the disease (Pittman, 1984b). The colonization stage is initiated by adhesion of the bacteria to cilia of respiratory epithelial cells and their multiplication **in situ**. The symptoms of pertussis are initiated by alteration of the function of tissue cells by the toxins, secreted by localized and multiplying organisms, which attach to cells by specific receptors. The toxins enter the cells and enzymatically alter their regulatory function (Pittman, 1984b).

Although **B. parapertussis**, like **B. pertussis**, is able to adhere to ciliated respiratory epithelium and produce some types of cytotoxin causing damage of cilia and ciliostasis **in vivo** (Bradford & Wold, 1939), the exact pathogenesis of disease caused by **B. parapertussis** is still unknown. It is generally accepted that **B. parapertussis** does not produce PT (Pittman, 1984a), thus its real relationship with host respiratory epithelium may be different from that in **B. pertussis**, since it is well-recognized that PT plays an very active role in the initiation and progression of **B. pertussis** infection. In addition, whether **B. parapertussis** can also produce

FIGURE 1.1 *B. PERTUSSIS* COMPONENTS PUTATIVELY INVOLVED IN THE PATHOGENESIS OF PERTUSSIS*



* Based on Hewlett et al. (1986) & Weiss et al. (1986).

** Key refer to text.

TCT, like **B. pertussis**, has not been determined.

An **in vitro** study on the pathogenicity of the **B. parapertussis**-like organism isolated from sheep was recently carried out in the foetal ovine tracheal organ cultures (Al-Kaissi, 1986). The results of this study suggested that this organism may have a role in initiating or prolonging existing respiratory infections by interfering with lung clearance mechanisms. The organism was demonstrated to be able to attach to cilia of tracheal explants, entangling within ciliary tufts, and to induce epithelial cell extrusions. In the severely affected areas, the ciliated epithelial cells were largely denuded of cilia and the non-ciliated cells showed marked swelling and protrusion of their plasma membrane into the tracheal lumen. These effects of the organism on the tracheal epithelium were found to be dose dependent.

The pathogenesis of **B. bronchiseptica** infection has also been comprehensively studied **in vivo** and **in vitro** (Bemis et al., 1977; Bemis & Wilson, 1985). Although many **B. bronchiseptica** strains possess toxins with the potential to destroy tissue, diseases produced by this organism are usually quite mild and self-limiting. The predilection for attachment to cilia and the ability to interfere with ciliary function by virulent strains of the organism may be an important aspect of the parasitic mode of existence or pathogenicity of this organism (Bemis & Wilson, 1985). Studies of this organism indicate that a phase-I and an intermediate-phase **B. bronchiseptica** strain each caused a rapid decrease in ciliary beat frequencies of canine tracheal outgrowth cells, without causing noticeable structural damage to these cells (Bemis & Kennedy, 1981). However, a rough **B. bronchiseptica** strain had no effect on ciliary function.

It is generally assumed that the role of **B. bronchiseptica** in naturally-occurring respiratory diseases is secondary to other inciting agents such as respiratory viruses or environmental intoxicants and the organism **per se** enhances the severity of mixed

infections by elaborating toxins (Bemis & Wilson, 1985). However, the reverse situation, i.e. that **B. bronchiseptica** infection may predispose its host to infection with other agents which are largely responsible for the severity of clinical disease, has been up to now overlooked. This type of relationship is currently observed to exist between **B. bronchiseptica** and **Pasteurella multocida** in the production of atrophic rhinitis in swine (Backstrom, 1984). Two mechanisms whereby **B. bronchiseptica** might alter host susceptibility to other infections without producing overt morphological damage to tissue are (1) by the elaboration of an extracellular adenylate cyclase capable of altering phagocytosis and intracellular killing function, and (2) by the ability to cause the immobilization of respiratory tract cilia (Bemis & Wilson, 1985).

3.4 Pathology

Whooping cough is a localized respiratory infection with specific manifestations of paroxysmal coughing, lymphocytosis, and neurological symptoms (Pittman, 1970). The disease presents a number of unique symptoms and epidemiological features. After an incubation period of 10 to 16 days, there is an undifferentiated catarrhal stage with low-grade fever which usually lasts 1 to 2 weeks. Then a characteristic paroxysmal coughing is manifested, which may persist for 4 to 8 weeks and occasionally as long as 20 weeks. The infections caused by **B. parapertussis** are said to be considerably milder than the usual picture of whooping cough (Linnemann & Perry, 1977). It is not yet clear why the infection caused by **B. parapertussis** differs from that produced by **B. pertussis** in its clinical manifestations. Lautrop (1971) suggested that it might be associated with the amount of heat-labile toxin (HLT) produced by the bacteria, because **B. pertussis** is known to produce 5-10 times as much as **B. parapertussis**.

Clinical infection due to **B. parapertussis** occurs primarily in children less than 10 years of age (Olson, 1975). Less than 20% of children infected with **B. parapertussis** show significant clinical

pertussis symptoms, and 40% are asymptomatic. The remainder have mild coughs, which are easily mis-diagnosed clinically as bronchitis (Pittman, 1970). In most cases, the illness lasts less than three weeks, but 50% of patienets continue to excrete the organism for more than five weeks (Pittman, 1970). Although most of the infections are mild, a severe disease in which two children died of **B. parapertussis** pneumonia has been reported (Zuelzer & Wheeler, 1946).

3.4.1 Human Infection

The literature covering the pathological changes which occur in **B. parapertussis**-infected patients is very limited since the infection is mild and seldom causes death. The necropsies which have been reported show considerable variation in lesions (Zuelzer & Wheeler, 1946; Linnemann & Perry, 1977). It is often difficult to determine the initial pulmonary lesion caused by **B. parapertussis** because the lesions are often complicated by secondary infection (Pittman, 1970). The following summary of the pathologic changes in **B. parapertussis** infection are mainly derived from the reports of Zuelzer & Wheeler (1946) and Linnemann & Perry (1977) which are based on three necropies.

The gross changes in the lung are usually non-specific and consist of light purple discolouration in the hilar areas. Extensive areas of consolidation have been observed in both lungs, especially in the apex of the right upper lobe, the apical portion of the right lower lobe, and the left lower lobe which is often involved in its entirety. The consolidated areas are usually of medium consistency, and the cut surface is slightly raised and may be grey-brown in colour (Zuelzer & Wheeler, 1946; Linnemann & Perry, 1977).

Bronchi of all sizes contain large amounts of dense yellowish grey mucopurulent exudate. In the consolidated areas, the bronchial walls are frequently thickened. The small airways usually contain thin mucoid exudate (Zuelzer & Wheeler, 1946; Linnemann & Perry,

1977).

Mallory and Hornor (1912) were the first to describe the histological changes of whooping cough in the respiratory tract of children who died from the disease. They reported clumps of bacteria between the cilia, at the base of the cilia and within phagocytic cells.

The predominant microscopic changes are always centered on the bronchi of large and medium size. Their lumina are usually filled with a mixture of mucus and polymorphonuclear leucocytes. The epithelial mucosa and submucosa are more or less densely infiltrated by leucocytes and cilia often appear clumped, stubbed, fused, or entirely absent. The peribronchial tissue shows marked lymphoid hyperplasia and the capillary plexuses around bronchi are markedly congested. The neighbouring alveoli may be collapsed and contain a few mononuclear cells but are usually free of exudate.

In many cases, there is a bronchiolitis with bronchopneumonia which may involve all lobes of the lung. The exudate may obstruct the bronchioles, producing alveolar collapse with or without the presence of focal necrosis, haemorrhagic oedema, and hyaline membranes. In the more severely affected areas, an extensive exudate of neutrophils and macrophages fills the alveolar spaces. The bronchial epithelium is often destroyed by necrosis and ulceration. Occasionally, superficial epithelial erosions are present in the larynx and trachea.

Although the infection appears to remain localized to the respiratory tract, pathologic changes have been described in other organs such as the brain, stomach, intestine, spleen, lymph nodes, thymus and liver.

Routine post-mortem cultures of the lungs and blood are usually sterile (Linnemann & Perry, 1977). The number of bacteria in the respiratory tract is highest in the acute stage but declines rapidly

in the early stage of paroxysmal coughing and is low or nil after the second week of coughing (Pittman, 1970). In the consolidated areas, however, small numbers of organisms can often be demonstrated within phagocytic cells, but are never found within epithelial cells (Zeulzer & Wheeler, 1946).

3.4.2 Animal Models

Neither **B. pertussis** nor **B. parapertussis** are natural pathogens for animals (Pittman, 1984a). However, successful infection with **B. pertussis** in a number of species, including chimpanzees (Rich et al., 1932), monkeys (Sauer & Hambrecht, 1929; North et al., 1940), puppies (Mallory et al., 1913), rabbits (Preston et al., 1980; Ashworth et al.,1982), mice (Bradford, 1938; Burnet & Timmins, 1937), chicken embryos (Gallavan & Goodpasture, 1937) and child volunteers (MacDonald & MacDonald, 1933), has been reported. A few workers have also infected laboratory animals with atypical pertussis organisms which have some similar characteristics to **B. parapertussis** (Anderson, 1957; Bradford & Wold, 1939). These animal experiments have made invaluable contributions to the study of the pathogenesis of whooping cough and provided basic information for vaccine production (Pittman, 1984b).

Of experimental animals used, primates are the only animals known to develop the paroxysmal cough and prolific mucus production characteristic of human infection with **B. pertussis** (Weiss & Hewlett, 1986). However, the expense of primate studies has prohibited widespread use of such a model. The only other animals that cough when infected with **B. pertussis** are rats but they accumulate plasma in the lungs and do not secrete mucous as primates do (Hornibrook & Ashburn, 1939). Rabbits become infected, but do not develop systemic manifestations of disease (Ashworth et al., 1982). Infection in the mouse has been studied more extensively than other species (Pittman, 1984b), since this closely mimics human pertussis (Pittman et al., 1980), although the mouse does not cough and there is no spread of infection between mice (Pittman, 1984b).

Inoculation of mice with **B. pertussis** via the respiratory tract causes mainly an interstitial pneumonia (Burnet & Timmins, 1937). If a sublethal dose is given, the number of bacteria increases for 7 to 14 days and then gradully declines, but infection may last 3 to 4 weeks (Cooper, 1952).

Experimental respiratory infection with **B. parapertussis** in the mouse shows a very similar lesion to that produced by **B. pertussis** (Bradford & Salvin & 1937; Bradford & Wold, 1939). In each case the early lesion reveals an acute pulmonary haemorrhagic pneumonia followed by an interstitial pneumonia with mild bronchitis essentially resembling that produced by **B. pertussis**. The amount of intrabronchial exudate and the degree of invasion by neutrophils seems to be greater in the infection produced by **B. parapertussis.** In Bradford & Salvin's (1937) experiments, about 75% of inoculated mice died within 2 to 10 days. Almost pure cultures of the organism could be recovered from the lungs of animals sacrificed 14 to 22 days after inoculation. In some instances a marked leucocytosis occurred before death (Bradford & Salvin, 1937).

In other studies by Bradford and Wold (1939), 68% of mice died following intratracheal inoculation with a suspension containing 10^{11} atypical pertussis organisms per ml. In another group, 55% of mice died within 1 to 10 days following intranasal inoculation with the same dosage. Bacterial stains showed numerous clumps of gram-negative cocco-bacillary forms covering and extending between the cells of the bronchial epithelium as well as within alveolar spaces.

In contrast, Anderson (1957) found that **B. parapertussis** was only slightly pathogenic for mice. The percentage of mice showing pulmonary infection was relatively low and almost independent of the amount of infecting dose. Only one mouse infected with 3.5×10^6 organisms of a particular strain isolated from a whooping cough patient was ill 14 days after infection.

Finally, it is worth noting that there may be a species specificity present among the genus **Bordetella** in their ability to adhere to ciliated respiratory epithelial cells. Tuomanen and others (1983) investigated the adhesion by three species of this genus to ciliated respiratory epithelial cells from humans, rabbits, chickens, hamsters and mice. Their results showed that **B. pertussis** and **B. parapertussis** adhere better to human ciliated cells than to those from nonhumans. In contrast, **B. bronchiseptica** demonstrated preferential adherence to nonhuman mammalian ciliated cells. The authors suggested that these differences must reflect species differences in bacterial-ciliary interaction.

3.5 Conclusions

Although it is generally considered that **B. parapertussis** is not as important as **B. pertussis** in human respiratory infections, it has, however, been demonstrated to be prevelant at high rates in some areas of the world where it produces a whooping cough syndrome similar to pertussis. Limited experimental studies have indicated that **B. parapertussis** can attach and damage the ciliated respiratory epithelium, causing ciliostasis, but the detailed interaction between the organism and host cell has not been fully explored.

The recent hypothesis that pertussis is a toxin-mediated disease increases the importance of exploring the potential effect of **B. parapertussis** toxins on the respiratory tract of animals. In whooping cough due to **B. pertussis**, the latest data indicates that pertussis toxin is important not only as a systemic toxin, but also at other steps in the infection such as attachment and evasion of host defences. Nevertheless, it appears that some other virulence factors such as FHA, ACT and TCT may also be important contributors. An understanding of the pathogenesis of **B. parapertussis** infection will depend on a more detailed understanding of the toxins produced by this organism.

The isolation of a **B. parapertussis**-like organism from CNP-

affected ovine lungs and the demonstration that the organism may cause ciliostasis **in vitro** has raised the possibility that this organism may be important in initiating or prolonging ovine respiratory disease, since it is well recognized that other members of **Bordetella spp.** have the ability not only to initiate infection, but also to inhibit host reaction to other potential pathogens.

CHAPTER 2

INDUCTION OF PNEUMONIA IN MICE WITH A **BORDETELLA PARAPERTUSSIS**-LIKE ORGANISM

CHAPTER 2

INDUCTION OF PNEUMONIA IN MICE WITH A **BORDETELLA PARAPERTUSSIS**-LIKE ORGANISM

Bordetella parapertussis is one of the causative agents of whooping cough, a noninvasive infection of the lower respiratory tract of humans (Linnemann & Perry, 1977; Lautrop, 1971). Recently, an organism similar to **B. parapertussis** was isolated from the respiratory tract of both CNP-affected and healthy lambs (Cullinane et al., 1987). Subsequent laboratory studies showed the organism could attach to the ciliary epithelium of ovine tracheal organ culture (Al-Kaissi et al., 1986) and at high doses produce ciliostasis and damage to the respiratory epithelium. These studies raised the possibility that this organism may play an active role in the pathogenesis of ovine respiratory disease.

Although work carried out 50 years ago by Bradford & Salvin (1937) and Bradford & Wold (1939) demonstrated that human strains of **B. parapertussis** were pathogenic for mice, the pathogenicity of the ovine isolate **in vivo** remained unknown. The objective of the present study was to investigate the pulmonary reaction of mice to infection by strains of the ovine **B. parapertussis**-like organism and thus establish a laboratory animal model for the study of the role of this organism in ovine CNP.

MATERIALS AND METHODS

Animals. Sixty-seven (29 male and 38 female) three-week-old specific-pathogen-free (SPF) Swiss mice free from intercurrent respiratory disease were used. They were kindly supplied by Auckland Medical School, were random-bred, caesarian-derived, and weighed about 20 g at the beginning of the experiment.

The mice were observed for one week before the experiment. They were fed a standard diet and provided with tap water **ad libitum**.

Origin and growth of the B. parapertussis-like organism. The isolates were obtained from lungs of lambs with CNP by a bronchoalveolar lavage technique. Pneumonic lungs were collected from freshly slaughtered lambs at a local meatworks. After the lungs and trachea were removed, their external surfaces were washed in cold tap water and the lungs transported in clean plastic bags to the laboratory within one hour. There the lungs were washed again and each lung was slowly filled with about 500 ml of phosphate-buffered saline (PBS), pH 7.2 through a sterile funnel which was inserted into the upper trachea. The lungs were gently massaged to ensure adequate flushing of the airways and the washings were decanted through the funnel covered with a double layer of gauze into a sterile wide-mouthed flask. This procedure was carried out twice, and about 700 ml of fluid was recovered. Washings which were contamined with blood were discarded. The washings were then centrifuged at 3000 rpm for 10 minutes. After careful removal of the supernatant the sediment was inoculated onto blood agar (Difco) for isolation of the **B. parapertussis**-like organism.

The **B. parapertussis**-like organisms used in the inoculum were a mixture of five isolates recovered originally from bronchoalveolar washings, then established in pure culture on blood agar, identified again and subcultured on blood agar before inoculation. A pilot study showed no difference between five of these isolates in their pathogenicity for mouse lung. The suspension for inoculation was made from three day blood agar cultures. A suspension of colonies in 0.01 M PBS was prepared immediately before inoculation.

Viable counts of the suspension were estimated by serial dilution of samples in sterile PBS. This was done by spreading 0.1 ml of the appropriate dilutions onto blood agar plates which were incubated at 36°C for three days. Viable counts expressed in colony-forming units (CFU) were recorded as arithmetic means. The final titre in the inoculum contained approximately 2.7×10^7 CFU/ml.

Inoculation procedure. The technique of inoculation was similar to that used by Rushton (1978). Narcosis of the mice was induced with halothane (Fluothane, I.C.I.) inhaled from saturated cotton wool beneath the false floor of a bell jar. When the level of narcosis with halothane was correct, mice were recumbent, insensitive and breathing evenly and shallowly, leaving enough time for handling outside the jar before they recovered.

When completly anaesthetized, the mouse was removed from the jar and grasped behind its neck with the thumb and forefinger of the left hand. The inoculum was drawn into a 0-150 ul adjustable micropipette and administered by depositing it on the external nares, the head being held with the nose pointing vertically. If the mouse was correctly anaesthetized, the inoculum was rapidly drawn into the nose without bubbling. Only mice accepting the inoculation in this fashion were included in the experiment and mice were discarded if there was evidence of bubbling or loss of inoculum during inoculation.

Experimental design. The mice were divided randomly into three groups (Table 2.1). Each mouse in Group 1 was inoculated intranasally with 0.05 ml of a suspension containing approximately 2.7×10^7 CFU/ml of the **B. parapertussis**-like organism in PBS while each mouse in group 2 was given 0.05 ml of sterile PBS intranasally. The mice in group 3 were only anaesthetized.

After inoculation the mice were observed twice daily. Dead mice were removed promptly for necropsy and collection of specimens. The mice which survived were killed at predetermined intervals. Two mice each from groups 1 and 2 were killed by dislocating cervical vertebrate at the times shown in Table 2.1 or were examined when they died. The mice in group 3 were sacrified at 12 hours, 10 days and 30 days after inoculation. The lungs were removed and the lesions in the lungs were recorded and photographed. Samples were selected from one mouse lung in each group for bacteriology. The remaining portions of lung were fixed for electronmicroscopy and

TABLE 2.1 EXPERIMENTAL DESIGN: SCHEDULE OF INOCULATION AND KILLING OF SPF MICE INOCULATED WITH THE B. PARAPERTUSSIS-LIKE ORGANISM

Group No.	No. of Mice	Day and Treatment							
		0	½	1	2	3	4	5	6
1	34	BPLO*	2 killed	2 killed 3 died	2 killed	1 killed 1 died	2 killed	2 killed	2 killed
2	30	PBS**	2 killed	2 killed	2 killed	2 killed	2 killed	2 killed	2 killed
3	3	none	1 killed	-	-	-	-	-	-

* BPLO: B.parapertussis-like organism
** PBS: sterile phosphate buffered saline

Table 2.1 (continued)

Day and Treatment							
8	11	14	17	20	23	26	29
2 killed	2 killed	2 killed	2 killed	2 killed	2 killed	2 killed	2 killed
2 killed	2 killed	2 killed	2 killed	2 killed	2 killed	2 killed	2 killed
-	1 killed	-	-	-	-	-	1 killed

histological examination.

Bacterial recovery. Samples for bacterial culture were taken from experimentally infected animals and control animals at necropsy by removing a portion of affected lung with sterile instruments. Unless the distribution of lesions made modification desirable, the anterior portion of the left lung was routinely selected. The number of bacteria recovered from the lungs was semi-quantitatively recorded as large numbers (+++), small numbers (++), very small numbers (+) and none (-).

Electronmicroscopy. For transmission electronmicroscopy samples were selected from parts of the lungs showing gross lesions, if present. Several tissue blocks of 1 mm^3 in size were taken immediately at necropsy and fixed in modified Karnovsky's fixative (Appendix 2) in 0.1 M PBS (pH 7.2) at 4°C for 24 hours. After washing in two changes of cold 0.1 M PBS, the tissue samples were post-fixed for one hour in 1% osmium tetroxide, processed by standard dehydration procedures and embedded in epoxy resin. Suitable areas of tissue were selected by light microscopy of 0.5 um sections, cut with glass knifes and stained with toluidine blue (Tb) (Appendix 3). Ultrathin sections from suitable blocks were cut with a sharp glass or diamond knife and stained with uranyl acetate and lead citrate (Appendix 4). The sections were examined on a Philips EM201c transmission electronmicroscope.

Histopathology. Samples for histopathological examination were fixed in 10% formol-saline solution, and processed by standard paraffin embedding methods. Sections of 3-4 µm thickness were cut and routinely stained with Haematoxylin-Eosin (H.E.) or Gram and Geimsa methods.

RESULTS

Clinical signs

Three days after inoculation, most mice in Group 1 had a rough hairy coat, showed anorexia, were less active and had a reduced consumption of food and water. These changes were observed for about 10 days, after which their behavior returned to normal. No abnormal signs were found in the other two groups of mice.

Four mice from Group 1 died during the experiment, three at 36 hr after inoculation and one at 72 hr (Table 2.2). No deaths occurred in the two control groups.

Pathology

Gross Pathology:

Twenty-two out of 34 mice (64.7%) in the inoculated group showed gross lesions (Table 2.2). The first macroscopic lung lesions were seen in the mice killed at 12 hr after inoculation. Areas of slightly dark red consolidation were visible grossly in the lungs of most infected animals which died or were sacrified from one post-inoculation day (p.i.d.) to p.i.d. 14 (Fig. 2.1). The distribution of the lesions was random but the anterior portion of left lung and the cranial and middle lobes of right lung were the areas most consistently involved (Fig. 2.2). The most severe consolidation was found in the animals killed at p.i.d. 2. At this time the lesions usually involved almost the entire lung (Fig. 2.3). After p.i.d. 8 the lesions were pale, purple-grey in colour and occurred much less commonly than in the early stages of the disease. No lesions were seen in the lungs from inoculated animals killed at 17 p.i.d. onwards apart from one mouse killed at p.i.d. 26 which showed red-grey consolidation in the left cranial lobe.

TABLE 2.2 RESULTS OF INTRANASAL INOCULATION OF MICE WITH *B. PARAPERTUSSIS*-LIKE ORGANISM

Days post-inoculation	No. Mice sacrificed	No. Mice died	No. Mice showing lesions	Severity of lung lesions *	Recovery of organism from Lung **
½	2	0	2	+	++
1	2	0	2	++	++
2	2	3	5	+++	+++
3	1	1	2	+++	+++
4	2	0	1	++	+++
5	2	0	2	++	+
6	2	0	2	+	+
8	2	0	2	+	+
11	2	0	1	+	-
14	2	0	0	-	*Staphylococcus epidermidis* (++)
17	2	0	0	-	*Staphylococcus epidermidis* (+)
20	2	0	0	-	-
23	2	0	0	-	-
26	2	0	1	++	-
29	3	0	2	+	-

* +++ (severe consolidation, involving 50% lung surface);
++ (moderate consolidateion, involving between 30 - 50% lung surface);
\+ (mild consolidation, involving 30% lung surface);
\- (no gross lesions).

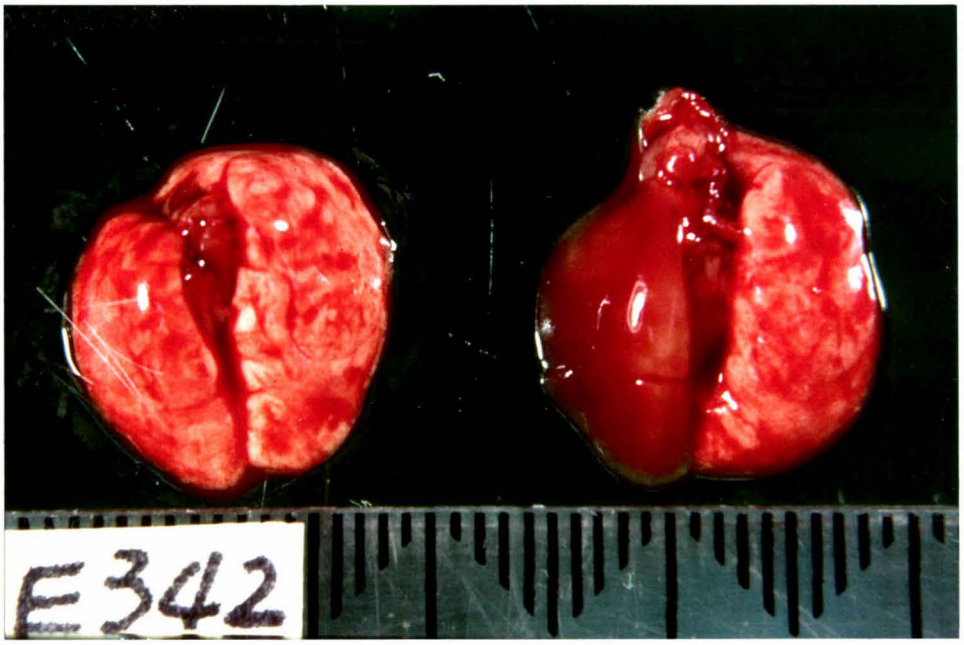
** - (none)
\+ (very small numbers)
++ (small numbers)
+++ (large numbers)

Figure 2.1

Right: Lungs from a mouse killed three days after inoculation of the **B. parapertussis**-like organism. There is dull red consolidation involving the entire left lobe of the lung. Left: Lungs from a control mouse killed at the same time.

Figure 2.2

I: Pulmonary consolidation in a mouse killed five days after inoculation of the **B. parapertussis**-like organism. The lungs are enlarged and show dull red consolidation in the anterior and middle parts of the left lobe and focal consolidation in the right lobe. C: Lungs from a control mouse killed at the same time.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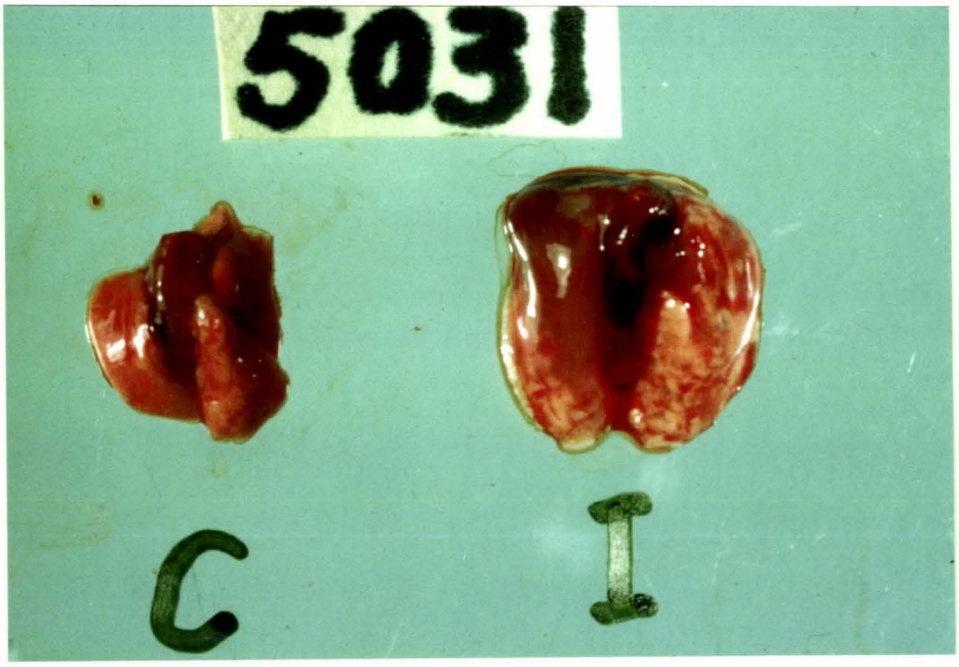
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The consolidated areas were often slight to moderately swollen and protruded above the surface of the surrounding tissue (Fig. 2.1) and usually involved one fifth to one half of the lung volume (Fig. 2.2). They were sharply delineated from the surrounding tissue and slightly firm in consistency.

The pleural surfaces of the lungs were smooth and normal in appearance. The thoracic cavity contained a normal range of clear fluid and no adhesions or exudate were found.

In the dead animals, the lungs exhibited a severe congestion and oedema throughout the entire tissue (Fig. 2.4).

In those mice which received sterile PBS and in those anaesthetised without treatment the lungs were grossly normal apart from one mouse in Group 2 which at p.i.d. 1 showed focal collapse of the dorsal aspect of the right lung.

Light microscopy:

No significant changes were found in the lungs of the mice from either Group 2 (PBS-treated controls) or Group 3 (anaesthesia only controls). More than 90 % of mice from Group 1 (inoculated) showed pathological changes.

The tissue alterations were not identical in extent in each lung or in all the animals inoculated at a given time. There was no localization in any particular lobe although the anterior parts of the lungs were consistently involved. The rate of evolution of the lesions varied considerably; when some animals were showing extensive consolidation, others only had small foci of inflammatory change.

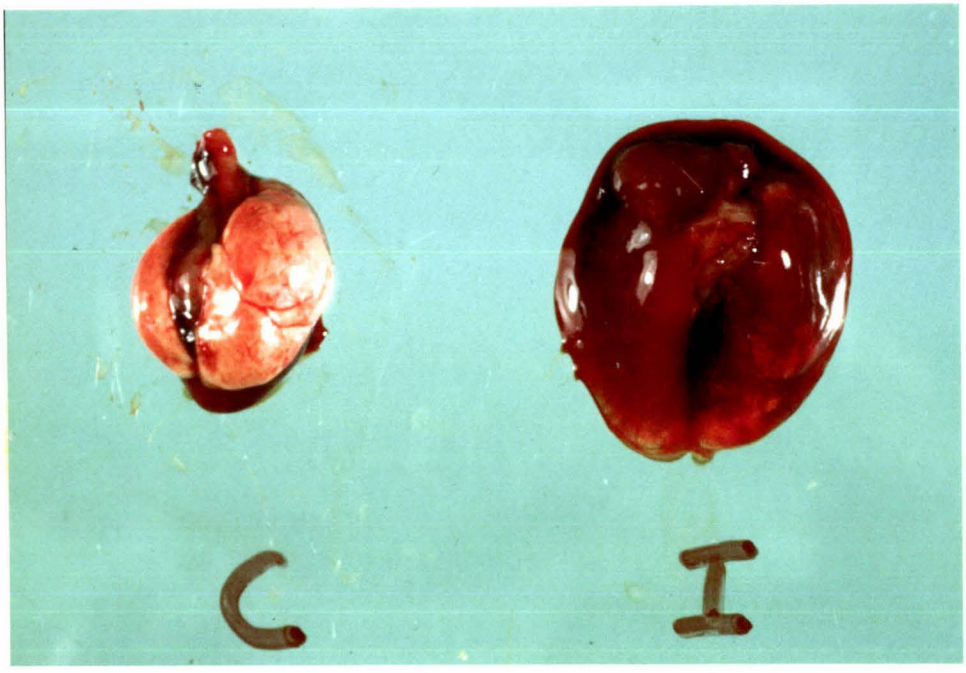
12 HOURS TO 5 DAYS: The most pronounced histological feature of the lungs from mice killed at 12 hours was small areas of pneumonia which usually involved five to ten adjacent alveoli (Fig. 2.5). They consisted of focal alveolar collapse and infiltration of

Figure 2.3

I: Gross appearance of mouse lung showing marked enlargement two days after inoculation with the **B. parapertussis**-like organism. Red areas of consolidation extend through almost the entire lungs. C: Lungs from control mouse killed at the same time.

Figure 2.4

Extensive pulmonary congestion and oedema in a mouse which died three days after inoculation of the **B.parapertussis**-like organism.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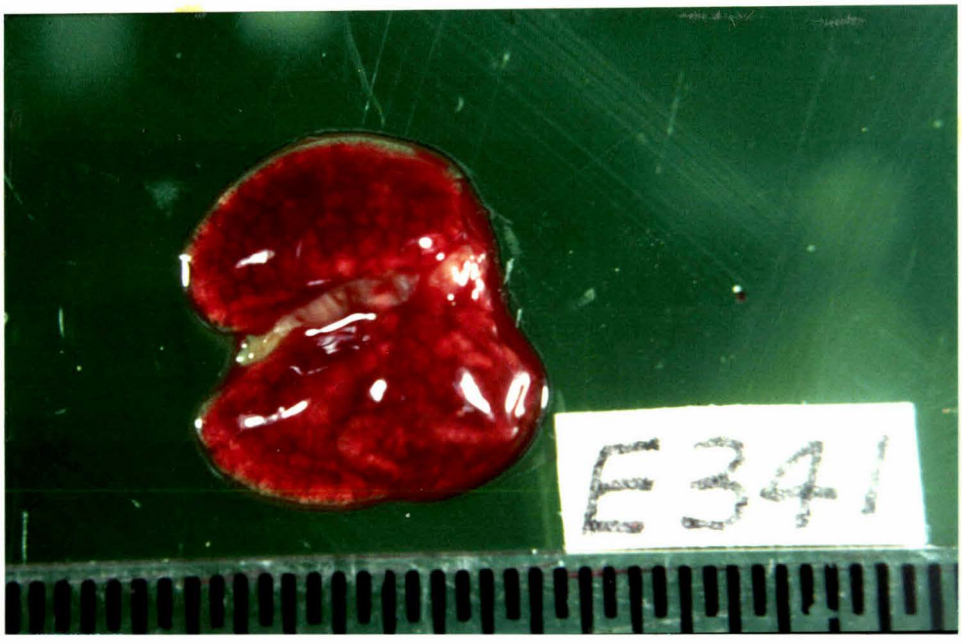
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neutrophils, with a small number of slightly enlarged alveolar interstitial cells and occasional macrophages in the interalveolar septa (Fig. 2.6). The consolidated areas were usually around terminal bronchioles. The focal lesions were most commonly disseminated throughout the lung although sometimes they were limited to one lobule. The interstitial spaces were thickened due to capillary congestion, enlargment of interstitial cells and infiltration of inflammatory cells (Fig. 2.6). In the affected areas, the lumina of small bronchioles often contained a small number of neutrophils, erythrocytes and a little proteinaceous exudate. Sometimes neutrophils were found in the peribronchial and peribronchiolar interstitial tissue (Fig. 2.7). The arterioles in these areas frequently contained fibrinous material.

After a further 12 hours, focal consolidation was more pronounced; the lesions showing increases in number, size, and content of neutrophils. In some areas, the alveoli adjacent to airways were severely collapsed and a variable number of neutrophils had infiltrated into alveolar spaces giving the pulmonary tissue a very dense appearance (Fig. 2.8). The peripheral alveoli showed slight congestion. By p.i.d. 2-3, the areas of consolidation had enlarged considerably and become confluent (Fig. 2.9). Portions of the lung were completely airless, but aerated alveoli were present in most regions. Bronchioles and proximal alveoli contained a large number of neutrophils mixed with a variable amount of fibrin and a few macrophages (Fig. 2.10). Occasionally, mild to moderate focal haemorrhage was present within the alveolar and bronchiolar lumina (Fig. 2.11).

From p.i.d. 4 onwards, neutrophils still predominated in the inflammatory exudate, but their numbers had decreased and many macrophages lay free in alveolar spaces and interalveolar septa (Fig. 2.12). Some macrophages had extensive foamy cytoplasm and were very large in size. Infrequently, small numbers of mononuclear cells were found accumulating in the peribronchiolar and perivascular areas (Fig. 2.13). Occasionally, an individual bronchiolar lumen was occluded by mucus, fibrin and mixed inflammatory cells.

Figure 2.5

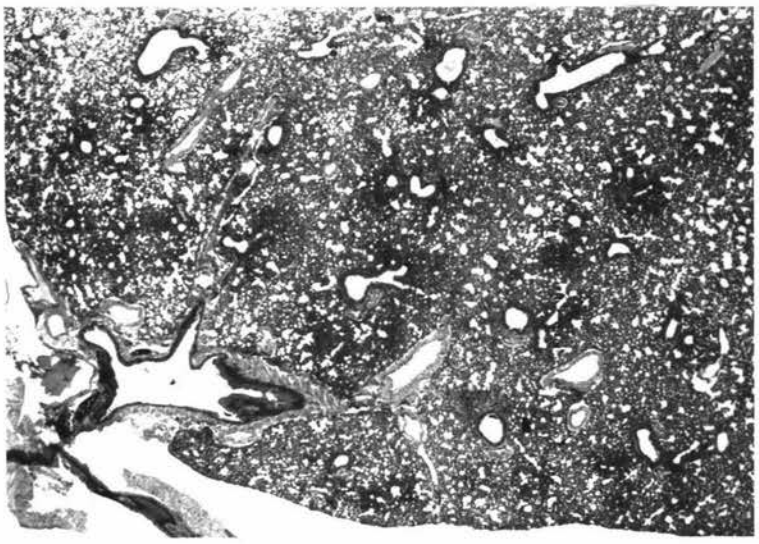
Mouse lung 12 hr after inoculation of the **B. parapertussis**-like organism showing patchy pneumonia surrounding small bronchioles. H.E. x 12.5

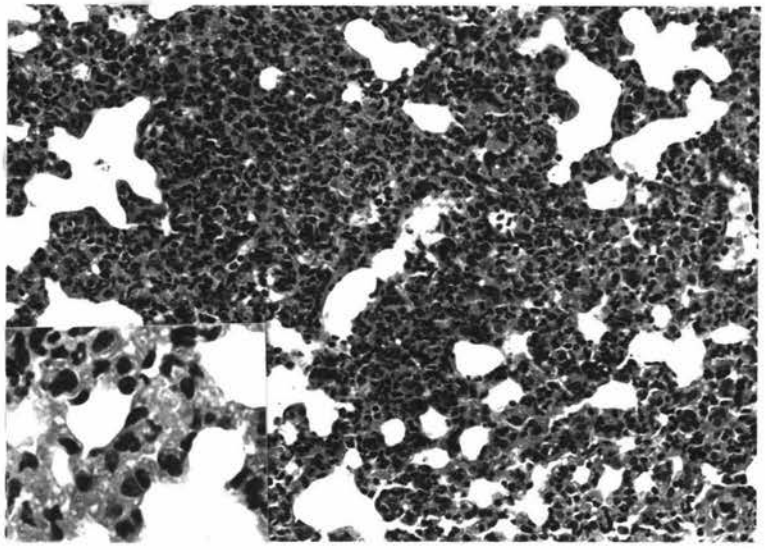
Figure 2.6

A focal area of pneumonia in mouse lung 12 hr after inoculation. There is moderate alveolar collapse and interstitial hypercellularity. H.E. x 50 Inset: Higher power photomicrograph from the same field. H.E. x 250

Figure 2.7

Peribronchiolar infiltration by neutrophils in a mouse killed 12 hr after inoculation. The surrounding alveoli are congested and also infiltrated by many neutrophils. H.E. x 125





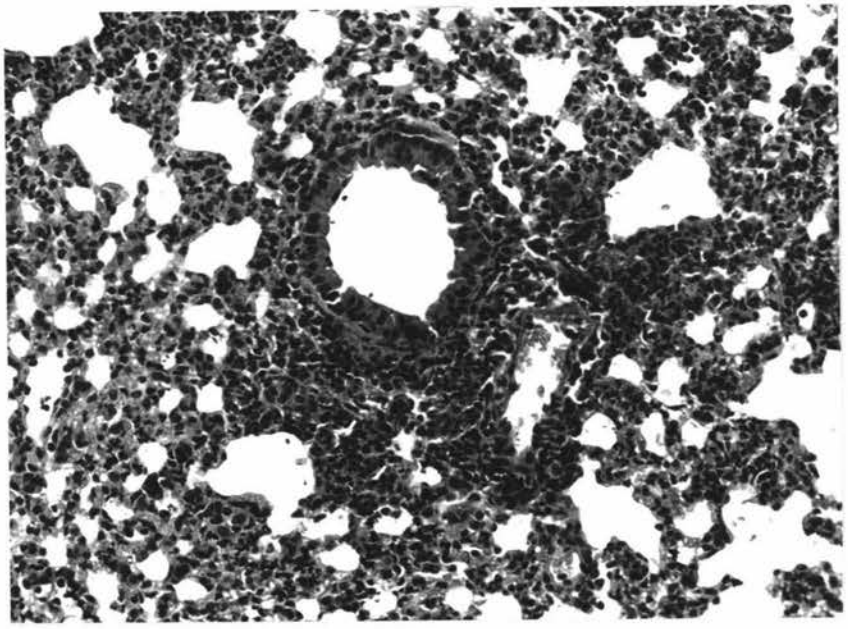


Figure 2.8

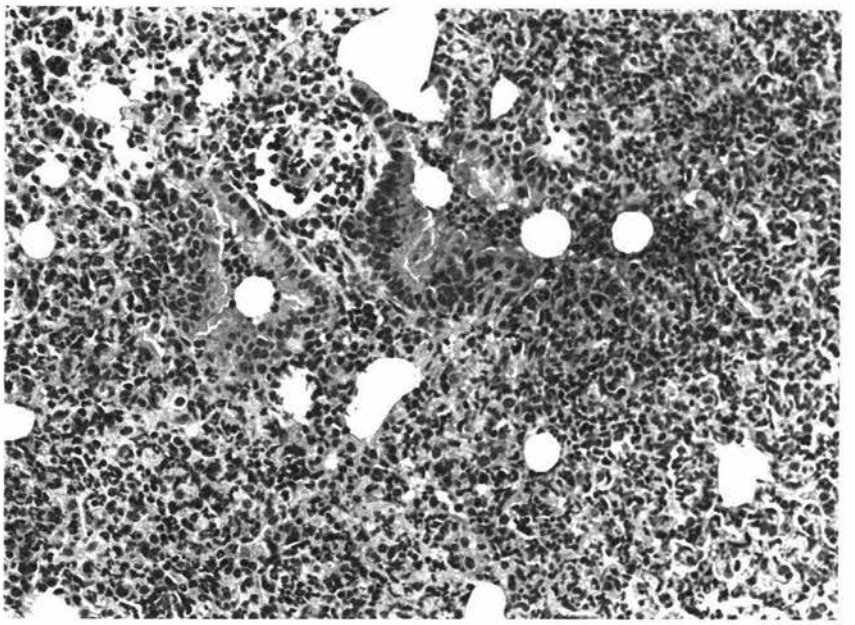
The dense homogenous appearence of pulmonary tissue in a mouse killed 24 hr after inoculation. Note the marked infiltration of neutrophils and severe alveolar collapse. H.E. x 125

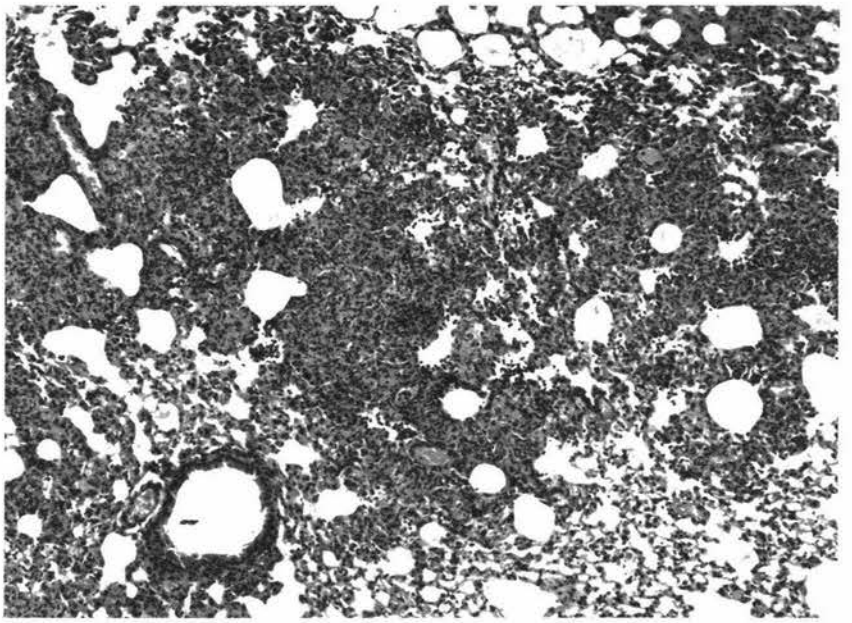
Figure 2.9

Confluence of focal pneumonia in a mouse killed two days after inoculation. H.E. x 50

Figure 2.10

An intense neutrophilic exudate has accumulated in a bronchiole and surrounding alveoli of a mouse killed two days after inoculation with the **B. parapertussis**-like organsim. H.E. x 250





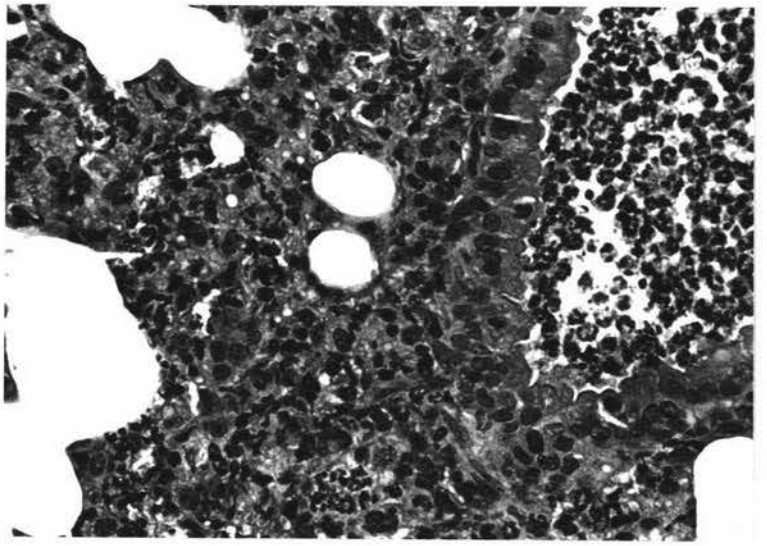


Figure 2.11

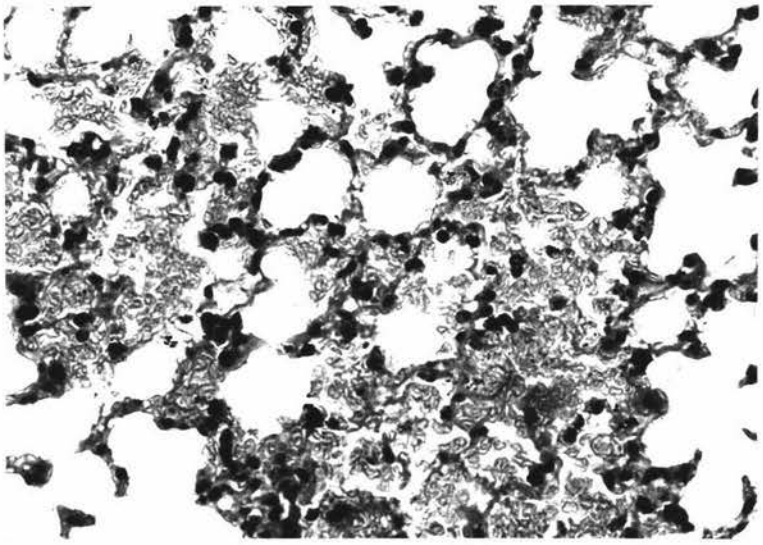
Lung from mouse killed two days after inoculation showing focal intra-alveolar haemorrhage. H.E. x 250

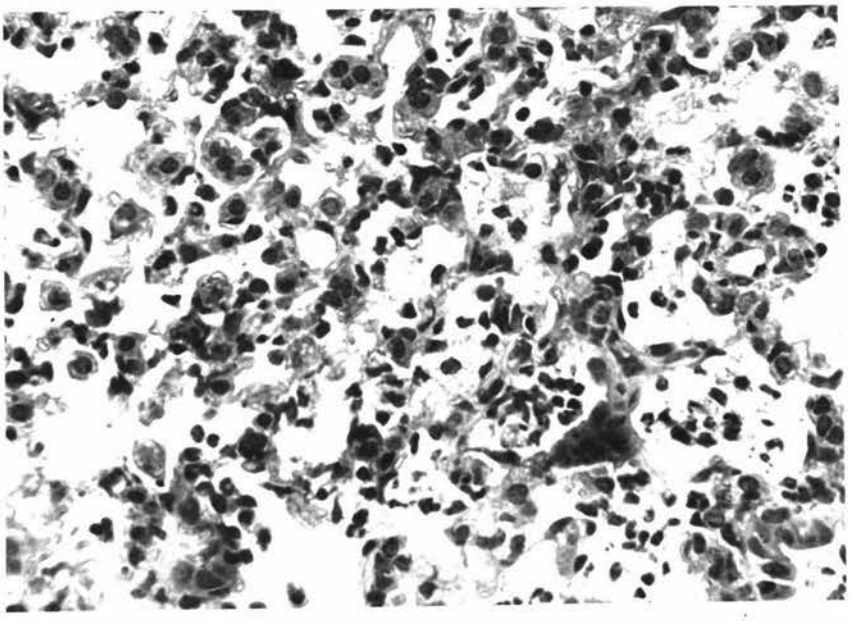
Figure 2.12

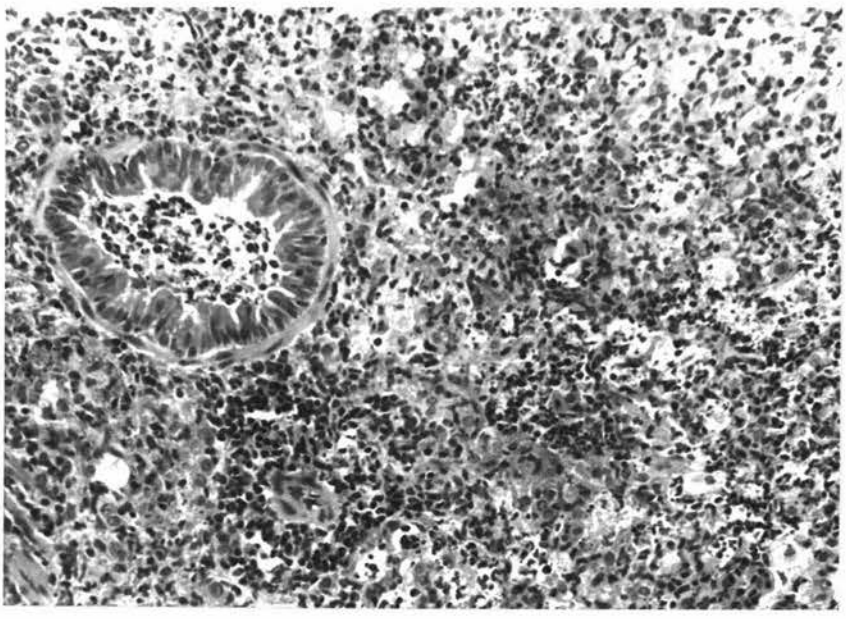
Mouse lung four days after inoculation with the **B. parapertussis**-like organism showing an increase in numbers of macrophages in some alveolar spaces. Neutrophils are still present at this time. H.E. x 250

Figure 2.13

Mouse lung five days after inoculation. Besides the marked infiltration of neutrophils and macrophages into the bronchiolar lumen and alveolar spaces, a perivascular and very mild peribronchiolar lymphoid hyperplasia is present in some areas. H.E. x 125







The mice which died between p.i.d. 2 to 3 showed a severe diffuse congestion and moderate oedema (Fig. 2.14). The interstitial lymphatics in interlobular septa and perivascular spaces were markedly dilated and flooded with pale-staining proteinaecous fluid in which a few neutrophils and occasional macrophages were seen (Fig. 2.15). The small bronchioles and surrounding alveoli were necrotic (Fig. 2.14). A few haemosiderin-laden macrophages were present in the necrotic areas. The alveoli often contained densely packed fibrinous material mixed with small numbers of inflammatory cells (Fig. 2.16).

6 DAYS TO 23 DAYS: Both exudative and proliferative changes were now present and these were usually widespread throughout the lungs. Proliferative changes gradually became predominant towards the end of this period.

From p.i.d. 6 onwards, the majority of lung tissue remained moderately congested. The lumina of bronchioles and alveoli still contained neutrophils, although their number was reduced and some were degenerate or necrotic. In contrast, the number of macrophages in alveoli was markedly elevated (Fig. 2.17). Most of these cells were very large in size and had a foamy cytoplasm (Fig. 2.18), while some showed phagocytosis of neutrophils and cellular debris. A moderate amount of free necrotic debris and erythrocytes remained in some areas. A very mild epithelial hyperplasia of bronchioles was now evident throughout the entire bronchiolar tree. By p.i.d. 8, a loose infiltration of mononuclear cells surrounded many of the arterioles and bronchioles (Fig. 2.19). This infiltration was always more extensive around blood vessels than around airways.

By p.i.d. 14, the mononuclear cell perivascular cuffs had become an outstanding feature of the infected lungs. They were on average 10 cells in thickness but measured up to 30 cells thick in some areas. This was sufficiently severe to compress the airways and narrow their lumina. Another pronounced feature at this time was the occlusion of some bronchioles by dense mucus mixed with fibrin and a

Figure 2.14

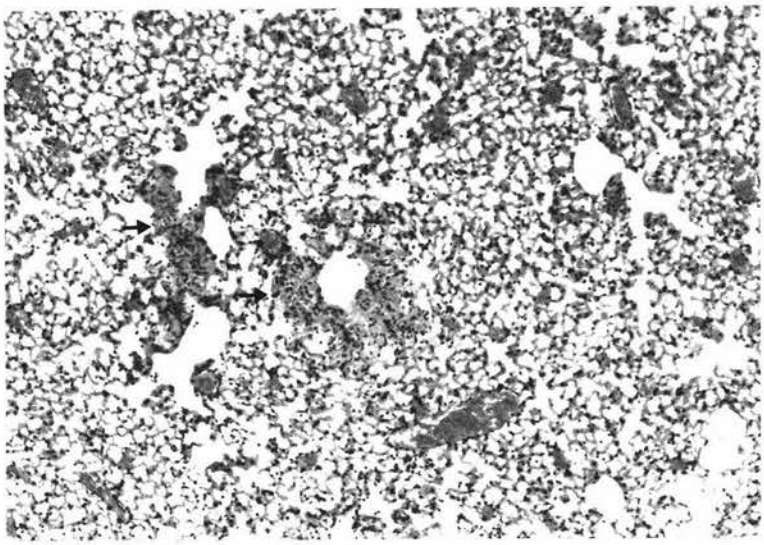
Lung from a mouse which died two days after inoculation showing extensive pulmonary congestion and moderate oedema. Many small bronchioles and their surrounding alveoli are necrotic (arrows). H.E. x 50

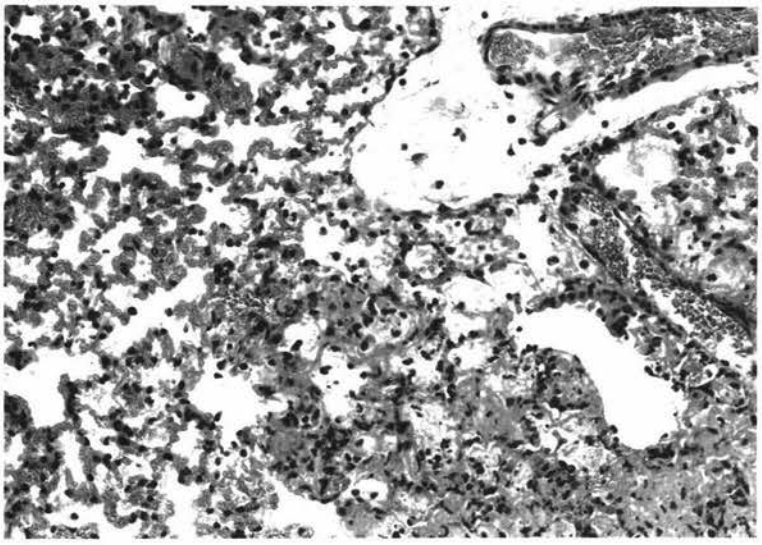
Figure 2.15

Severe extensive pulmonary congestion and oedema in a mouse which died three days after inoculation of the **B. parapertussis**-like organism. The perivascular lymphatics are markedly dilated and contained much fibrinous exudate. H.E. x 125

Figure 2.16

Many alveoli in the lungs of mice which died three days after inoculation contained densely packed fibrinous material and small numbers of mixed inflammatory cells. H.E. x 250





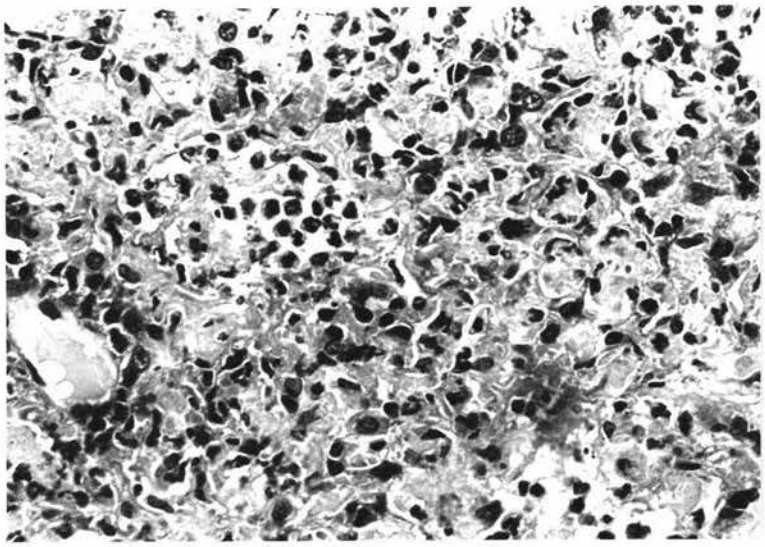


Figure 2.17

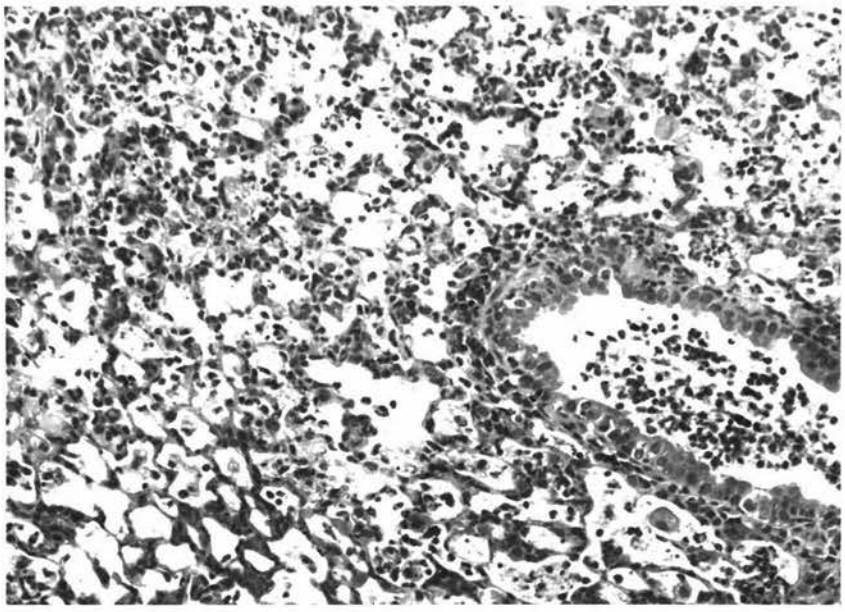
Mouse lung six days after inoculation. Neutrophil infiltration is still a prominent feature in many bronchioles. The numbers of macrophages in alveoli are significantly increased. H.E. x 125

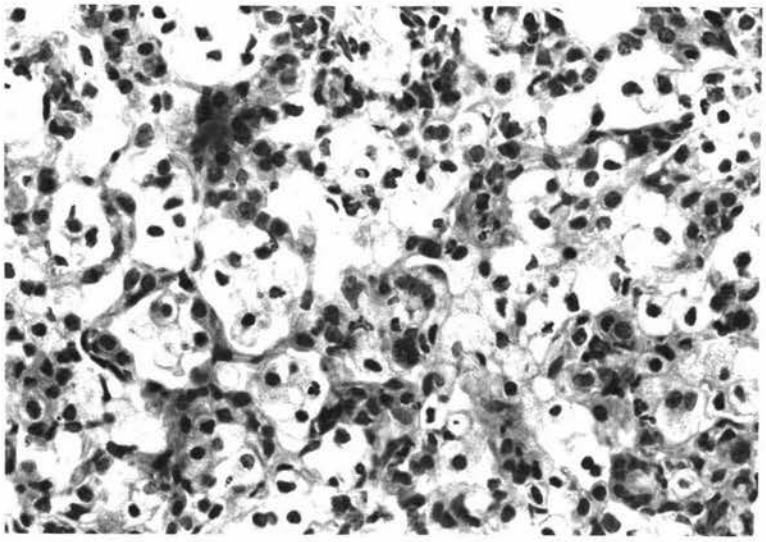
Figure 2.18

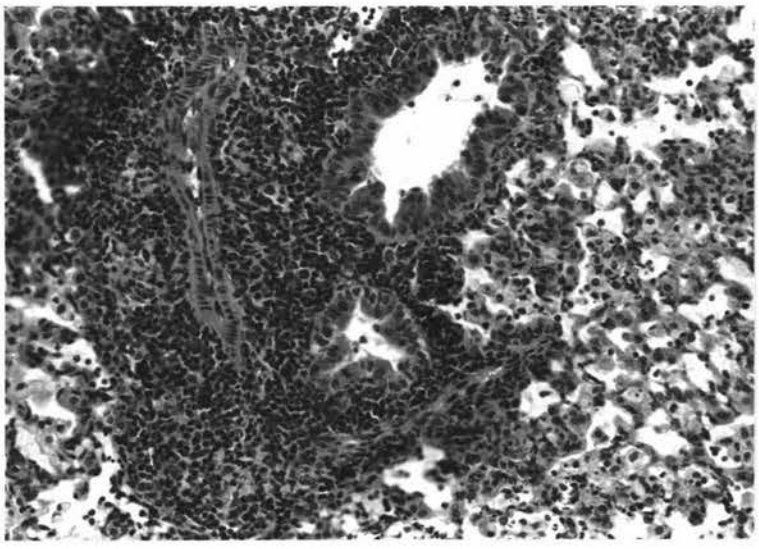
Infiltration of large numbers of macrophages into the alveolar spaces in a mouse killed eight days after inoculation with the **B. parapertussis**-like organism. These cells are very large in size and have a foamy cytoplasm and a relative small nucleus. H.E. x 250

Figure 2.19

Perivascular and peribronchiolar lymphoid aggregations. Note that lymphoid hyperplasia is more severe in areas surrounding blood vessels than around bronchioles. The adjacent alveoli contain small numbers of macrophages. Mouse killed eight days after inoculation of the **B. parapertussis**-like organism. H.E. x 125







few neutrophils (Fig. 2.20).

The alveolar septa were now more markedly thickened than previously. This was mainly due to infiltration of small mononuclear cells into the interstitium and to a lesser extent infiltration of neutrophils and lymphocytes.

A combination of these proliferative and exudative features persisted until p.i.d. 23, but at this time exudation was usually limited to a single lobule or only involved a few alveoli.

24 TO 29 DAYS: At this stage, lungs from most of the animals had entered a resolution phase. The most obvious feature of this was the prominent perivascular and peribronchiolar lymphoid cuffs. Alveolar capillaries were still slightly congested but the majority of alveoli were now fully aerated (Fig. 2.21). A few still contained small numbers of macrophages in their lumina, but exudate was no longer visible. Occasionally, a mild proliferation of type II alveolar epithelial cells was seen in isolated alveoli (Fig. 2.22). However, the alveolar interstitial cellular infiltration seen before remained in some areas until the end of the experiment.

Electron microscopy

Trachea and bronchi were not examined in the present investigation, nor was the ultrastructural morphology of the peribronchiolar lymphoid aggregates studied.

BRONCHIOLES: Ultrastructural lesions were occasionally observed in the bronchioles of the inoculated mice at all stages. At 12 hr, the bronchioles in affected areas had an intact mucosa but the ciliary density of the bronchiolar epithelium was decreased, due to loss of ciliary shafts and internalization of basal bodies (Fig. 2.23). Some ciliated epithelial cells had only one or two isolated cilia left on the luminal border. These cells showed slight swelling of mitochondria in the upper parts of the cell but were otherwise morphologically normal (Fig. 2.23). Exudative changes in bronchioles

Figure 2.20

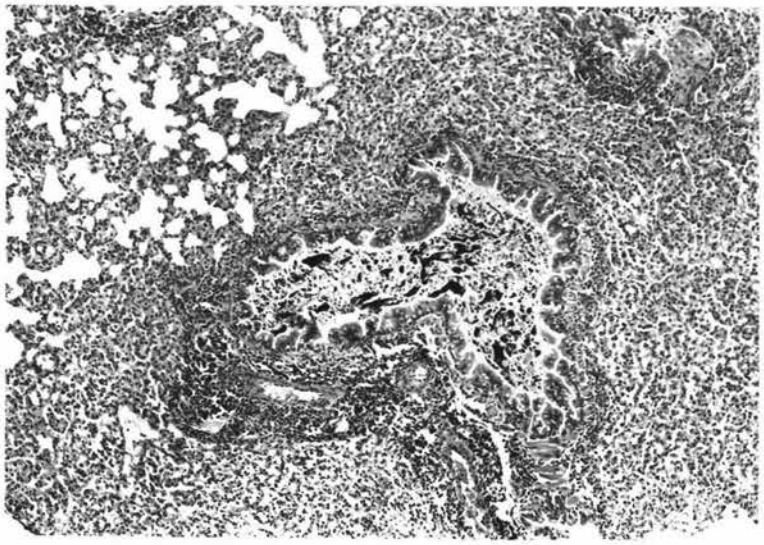
The occlusion of a bronchiolar lumen by mucus and cellular debri in a mouse killed 14 days after inoculation of the **B. parapertussis**-like organism. The bronchiolar epithelium is moderatedly hyperplastic and the surrounding alveoli are collapsed. H.E. x 50

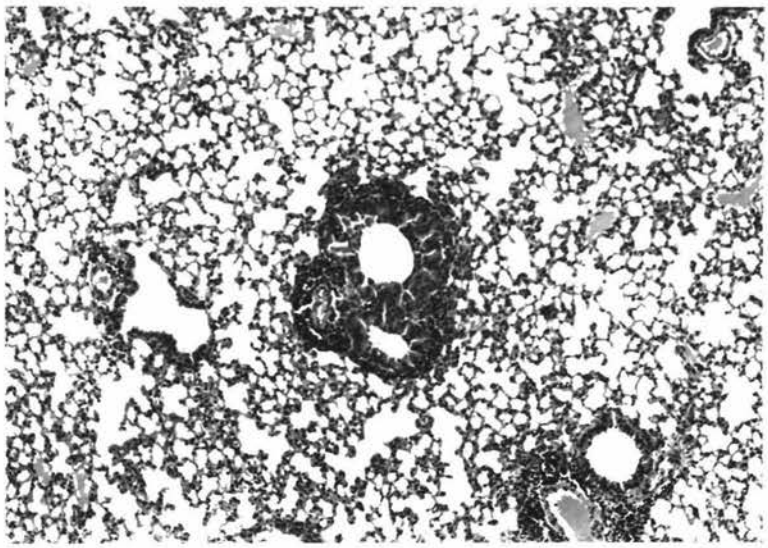
Figure 2.21

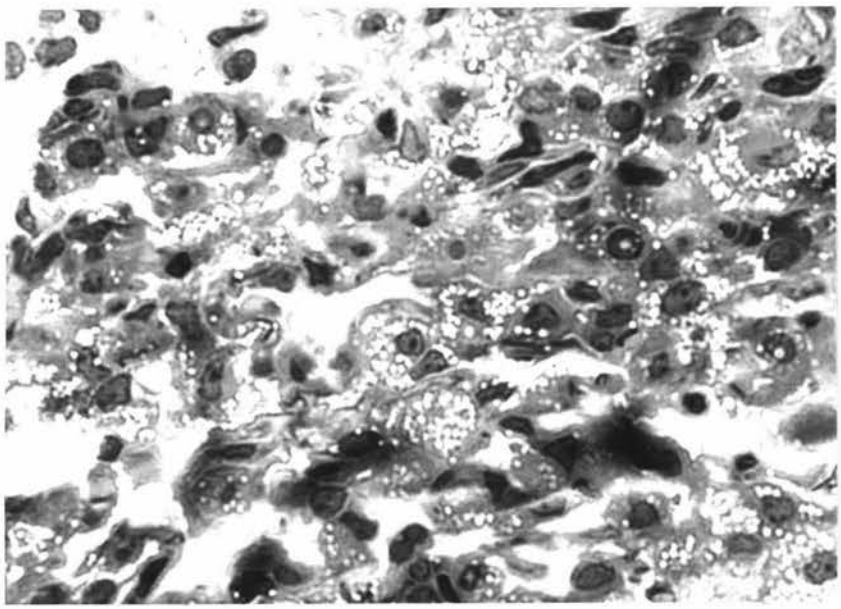
A mouse lung in the resolution stage. Apart from a few alveoli containing proteinaceous exudate, the majority of alveoli are free of exudate and re-aerated. The only remaining pneumonic feature is the presence of perivascular and peribronchiolar lymphoid cuffs. Mouse killed 24 days after inoculation. H.E. x 50

Figure 2.22

Early proliferation of alveolar type II cells can be seen in some alveoli. Mouse killed 29 days after inoculation. Epoxy embedded. Tb x 500







were predominant at p.i.d. 5 and many bronchioles contained a considerable quantity of cellular debris, amorphous electron-dense material and neutrophils as well as a few macrophages (Fig.2.24). At p.i.d. 20, only small amounts of bronchiolar exudate consisting mainly of closely-packed degenerating macrophages and amorphous material were found (Fig. 2.25).

No bacteria were observed attached to the apical surface of bronchioles at any stage of the experiment. The bronchiolar mucosa remained intact at all stages of infection although focal cytoplasmic extensions of the apex of isolated ciliated epithelial cells was seen at p.i.d. 5 (Fig. 2.26). From 12 hr to p.i.d. 8, moderate numbers of neutrophils, macrophages and lymphocytes were occasionally observed scattered between bronchiolar epithelial cells and within the slightly oedematous submucosa immediately beneath the epithelium (Fig. 2.27).

A marked increase in Clara cell numbers was sometimes observed by p.i.d. 26 (Fig. 2.28). Most of these cells were morphologically similar to the common Clara cell of mice described by Pack et al. (1981). They usually had a cytoplasm of moderate electron density. The nucleus was basal and the apical portion of the cell invariably projected into the lumen of the airways and was packed with well organized arrays of smooth endoplasmic reticulum (SER). In addition to mitochondria, mitochondria-like bodies were invariably encountered which had few or no cristae. In most cells, the apical portion contained many electron-dense "serous type" secretory vesicles. The remaining small proportions of Clara cells were similar to the vesiculated Clara cells described by the same authors. The cytoplasm of these cells was filled with vacuoles containing a faint matrix at high power but they lacked microvilli. Moderate numbers of mitochondria and mitochondria-like bodies were scattered throughout the cell. The nucleus was frequently pleomorphic.

ALVEOLI: The most pronounced ultrastructural alterations of alveoli were observed in the mice which died or were sacrified at

Figure 2.23

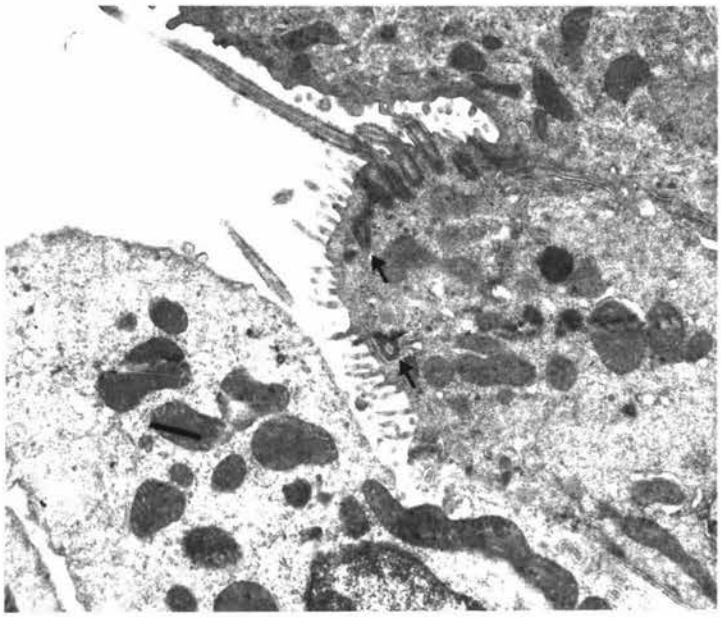
Bronchiolar mucosa from a mouse killed 12 hr after inoculation of the **B. parapertussis**-like organism. A ciliated cell shows loss of ciliary shafts and internalization of basal bodies (arrows). TEM x 7,800

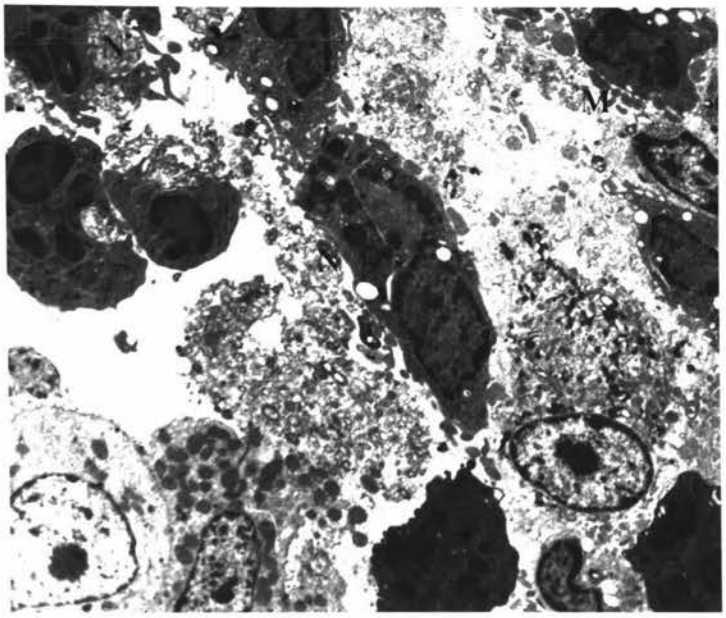
Figure 2.24

The contents of a bronchiole from a mouse killed five days after inoculation. There are large numbers of neutrophils (N), a few macrophages (M) and necrotic cellular debris. TEM x 3,400

Figure 2.25

A bronchiolar lumen densely packed with cellular debris from degenerating macrophages (M), mixed with amorphous material. From a mouse 20 days after inoculation. TEM x 5200




M

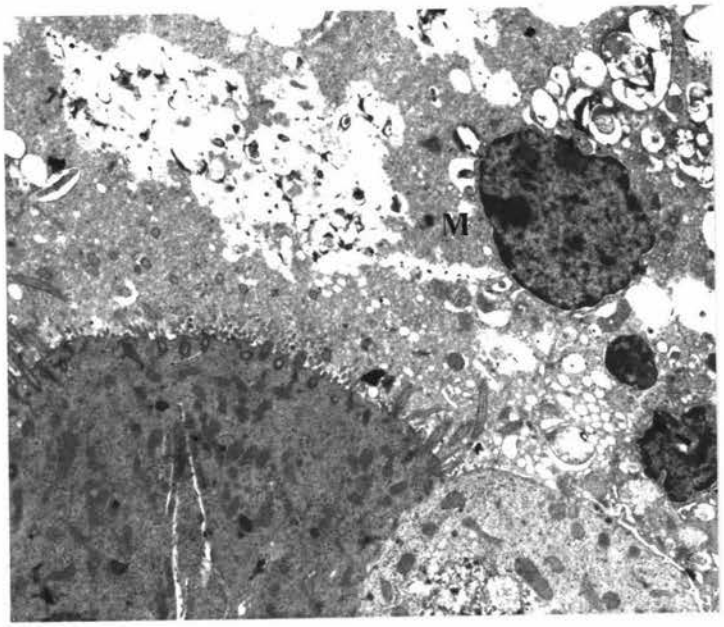

M

Figure 2.26

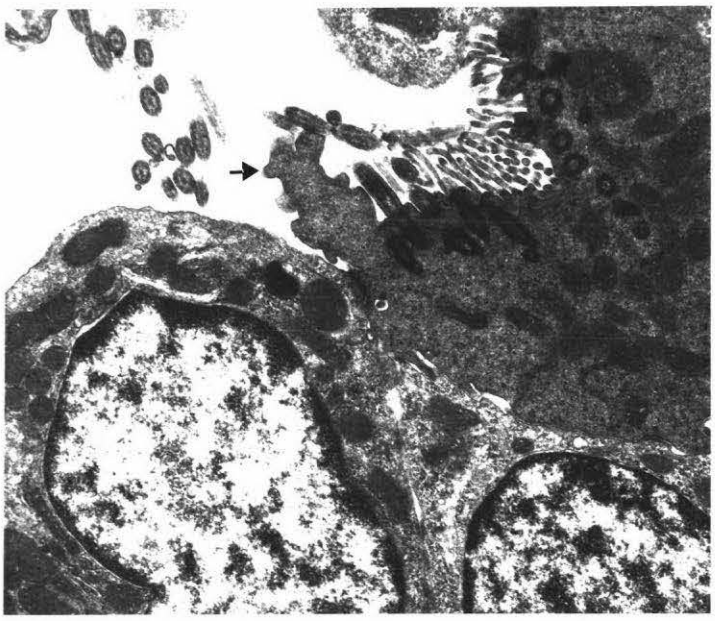
A bronchiole from the mouse killed five days after inoculation showing a focal cytoplasmic projection (arrows) on the apex of a ciliated cell. The cell itself has fewer cilia than normal. TEM x 11,200

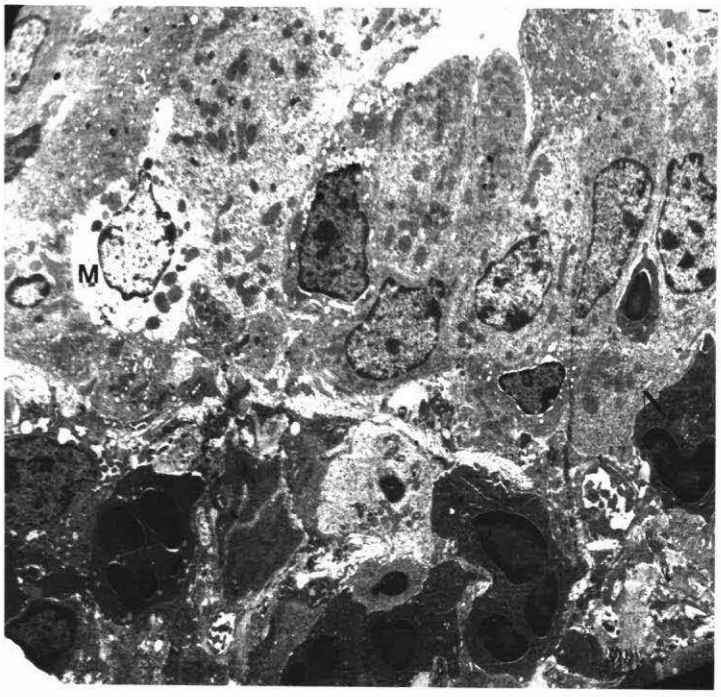
Figure 2.27

Mild infiltration of inflammatory cells into the bronchiolar mucosa in a mouse killed six days after inoculation. A neutrophil (N) and a small mononuclear cell (M) are migrating between epithelial cells and large number of neutrophils are present in the submucosa. TEM x 3,400

Figure 2.28

A bronchiole showing marked increase in numbers of Clara cells. Mouse killed 26 days after inoculation with the **B. parapertussis**-like organism. TEM x 3,400

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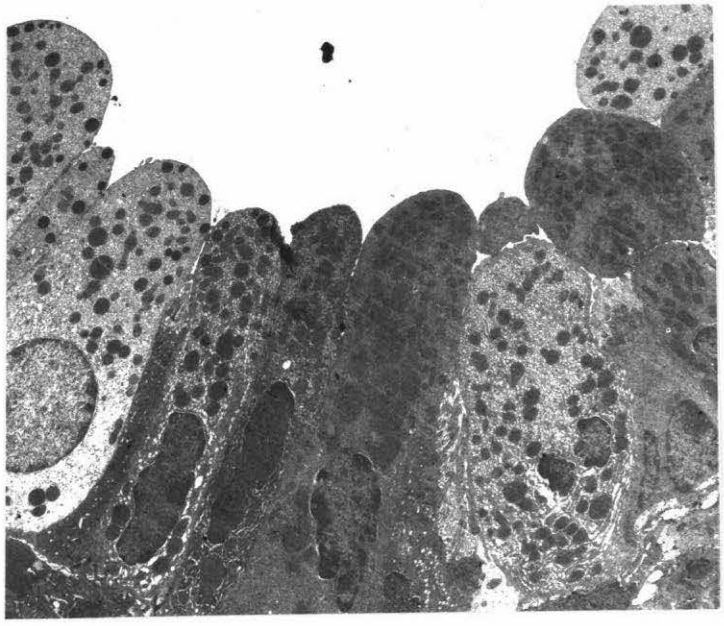


p.i.d. 3.

Epithelial Damage: Throughout the experiment, the alveolar epithelium exhibited a varying degree of degenerative change and occasionally showed focal necrosis. The earliest changes in alveolar type I cells were detected at p.i.d. 1, when the cytoplasmic extensions of the cells showed slight to moderate swelling. Occasionally an entire cell was diffusely swollen and the nucleus was enlarged (Fig. 2.29). Both the sacrified and dead mice at p.i.d. 3 exhibited marked degeneration and focal necrosis of type I cells. In the less severe areas, the type I cells showed marked swelling and vesiculation (Fig. 2.30) or a focal increase in density of the plasma membrane (Fig. 2.31); while in the severely damaged areas, type I cells had occasionally sloughed leaving a denuded basement membrane (Fig. 2.32) or showed focal cytoplasmic protrusion to the alveolar lumina. In the mice which died at p.i.d. 3, dissolution of alveolar type I cells and the basement membrane was seen at some sites causing discrete breaks in the alveolar septa with migration of macrophages into alveoli.

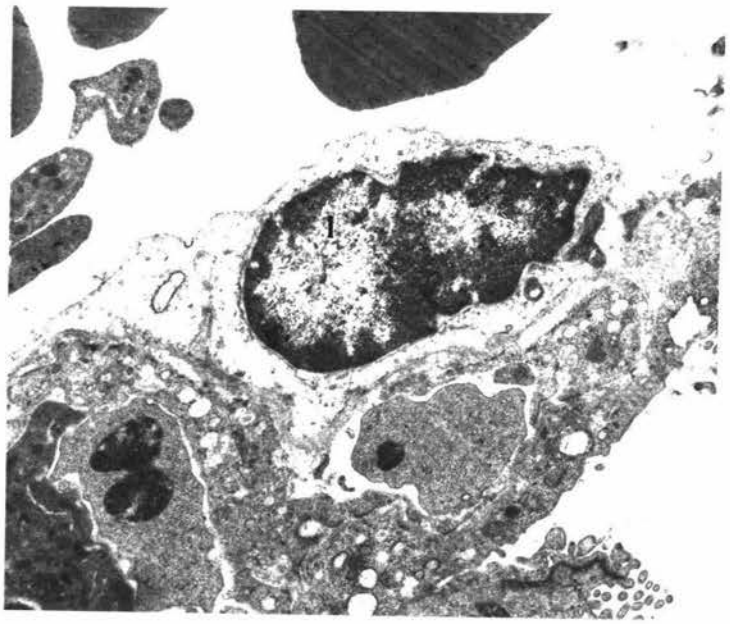
Alveolar type II epithelial cells also showed damage of varying severity, but the changes were relatively mild and occurred later than those in the type I cells. The affected type II cells first showed mild degenerative changes at p.i.d. 2 consisting of swollen mitochondria, focal formation of electron-dense plasma membranes and damage to laminated bodies. With the development of infection, the type II cells exhibited more marked swelling of mitochondria and severe distention of endoplasmic reticulum (Fig. 2.33). The number of free ribosomes in the cytoplasm was significantly increased , but the number of laminated bodies and superficial microvilli markedly decreased (Fig. 2.34). Some alveolar type II cells showed cytoplasmic projections of their apex, and the others exhibited marked thickening of the superficial plasma membrane (Fig. 2.35). Occasionally in mice which died at p.i.d. 3, type II cells became detached from the underlying basement membrane and sloughed into alveolar spaces (Fig. 2.36). No degenerative changes were detected at p.i.d. 20. At p.i.d. 23, mild focal proliferation of type II

Figure 2.29

Diffuse swelling of an alveolar type I cell (I) in a mouse killed one day after inoculation of the **B. parapertussis**-like organism. TEM x 11,200

Figure 2.30

An alveolus from the lung of a mouse which died three days after inoculation. The alveolar type I (I) and type II (E) epithelial cells show moderate degenerative changes. There is accumulation of electron-dense proteinaceous fluid and necrotic debris in the alveolar spaces (A). The alveolar capillaries (c) contain erythrocytes, neutrophils and macrophages. TEM x 5,200



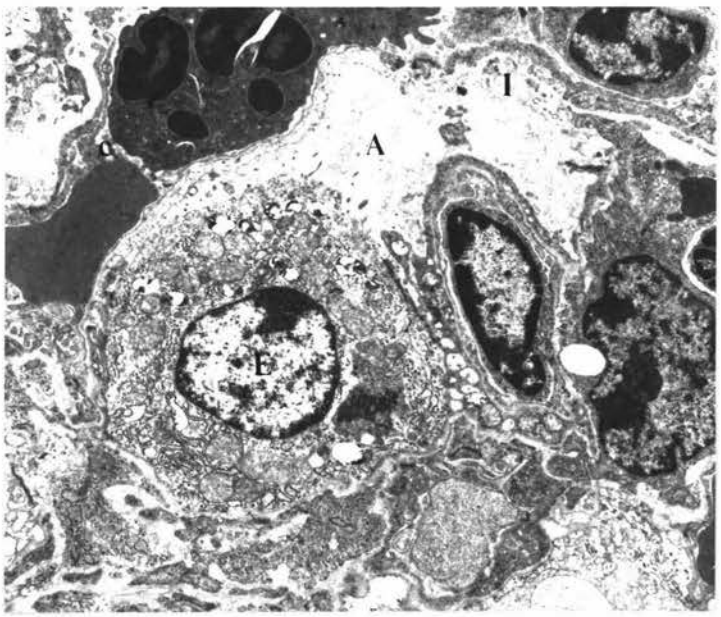
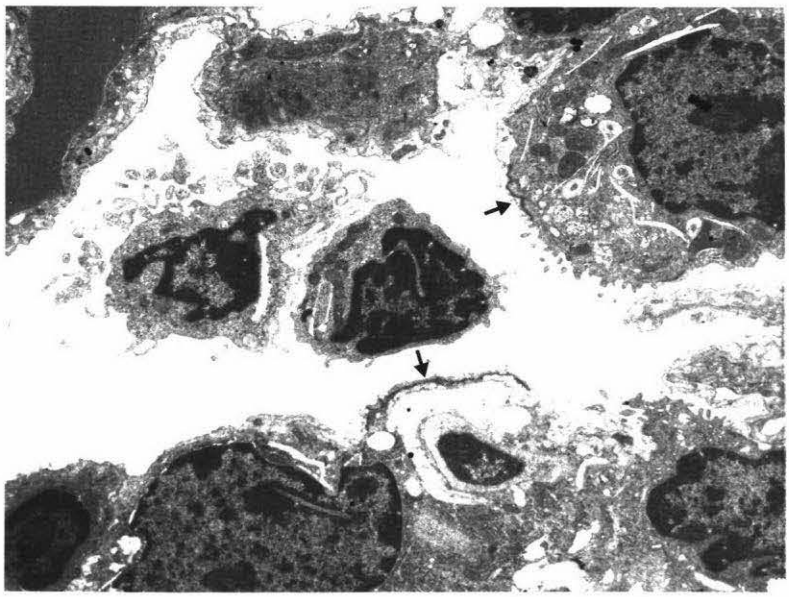

A
B

Figure 2.31

An alveolus from a mouse killed 11 days after inoculation. The alveolar epithelium shows areas of thickening of plasma membrane (arrows) in both types of cell. TEM x 5,200

Figure 2.32

Alveolar wall showing focal sloughing of a type I epithelial cell (arrow) leaving a denuded basement membrane. A macrophage (M) is closely adherent to the underlying capillary endothelium. Mouse killed three days after inoculation.TEM x 7,800



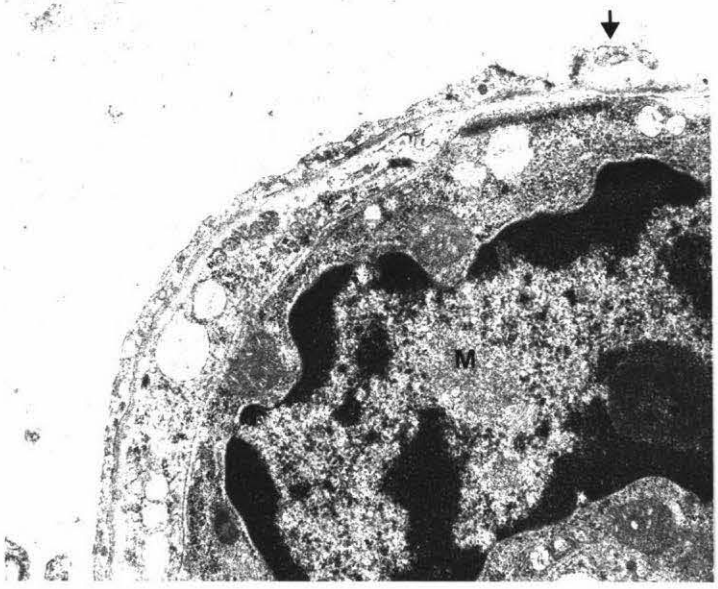
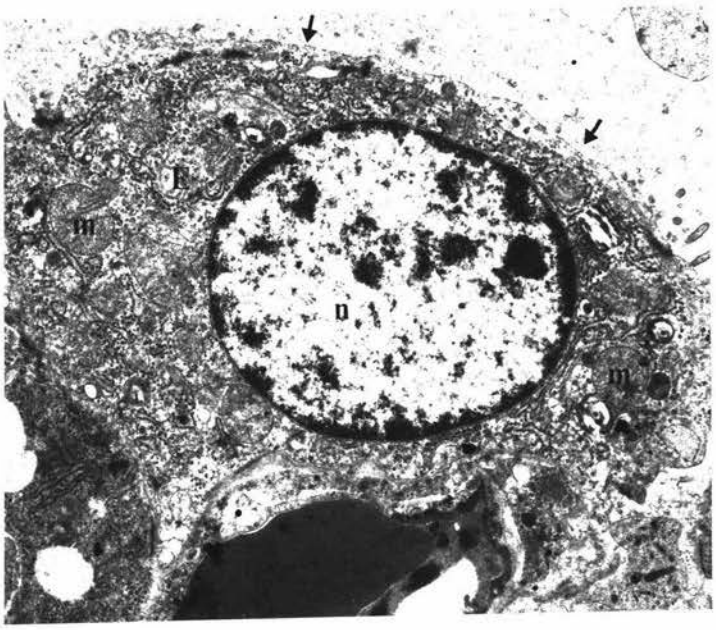

M

Figure 2.33

Degenerative changes in an alveolar type II cell three days after inoculation. The cell (E) has swollen mitochondria (m), increase in ribosomes, severe decrease in number of lamellated bodies, loss of microvilli (arrow) and migration of chromatin to the periphery of the nucleus (n). TEM x 7,800

Figure 2.34

Early degenerative changes in an alveolar type II cell consisting of severe swollen and disrupted mitochondria and distention of the rough endoplasmic reticulum (arrow). Mouse which died three days after inoculation. TEM x 15,300


E
m
n
m

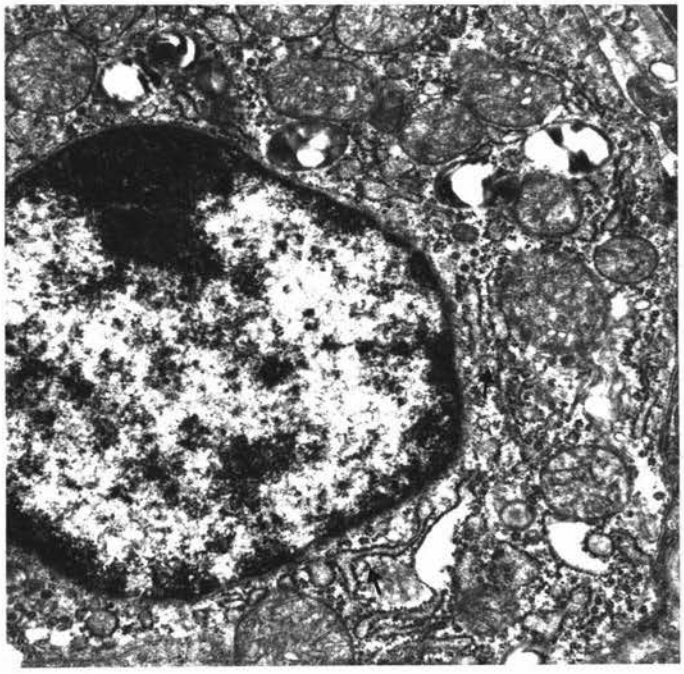
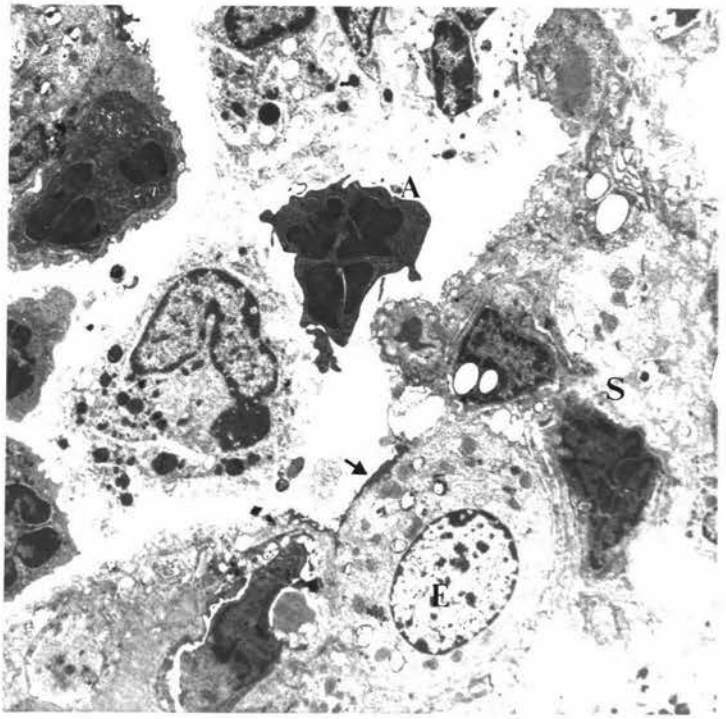


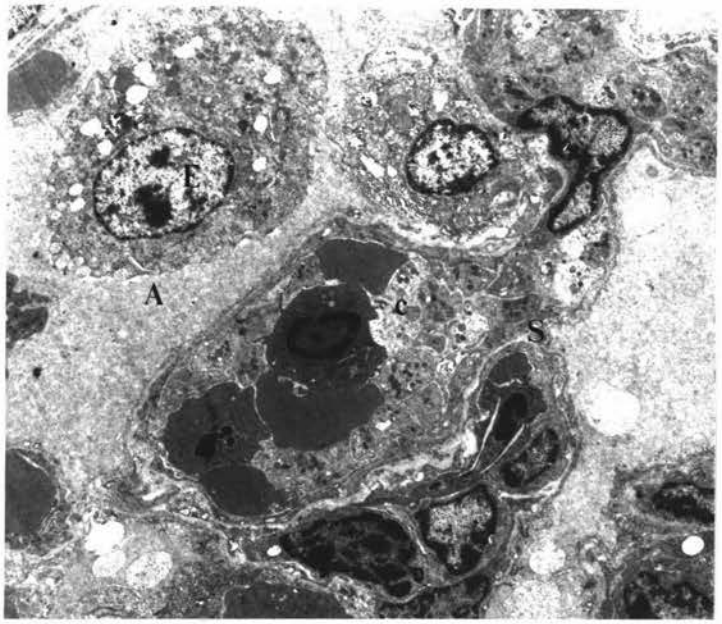
Figure 2.35

A type II alveolar epithelial cell (E) of a mouse killed five days after inoculation having an electron-dense plasma membrane (arrow) on its outer surface. The alveolar space (A) contains inflammatory exudate which is composed of many macrophages and a few neutrophils. The alveolar septum (S) shows infiltration of macrophages and neutrophils into the interstitium. TEM x 11,200

Figure 2.36

Interalveolar septum (S) and alveolar spaces (A) from a mouse which died three days after inoculation. The alveolar space contains a desquamated type II cell (E) and extensive amorphous material. There are aggregations of platelets and monocytes in a dilated capillary lumen (c). TEM x 7,800


A
S
E


E
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cells was seen in a few alveoli. The proliferating type II cells often contained many lamellated bodies in their cytoplasm and lacked microvilli on their surface.

Cellular Exudate: At all stages from p.i.d. 1 to 8, the alveoli in most regions examined were infiltrated by inflammatory cells and contained a variable quantity of proteinaecous fluid and degenerating cellular debris as well as a few erythrocytes. The intra-alveolar oedema was most extensive and severe in the mice which died at p.i.d. 3. The infiltrations tended to be more marked in the alveoli proximal to bronchioles. At p.i.d. 1 to 3, the cellular infiltrate consisted predominantly of neutrophils (Fig. 2.37). They were very actively engaged in phagocytosis, contained much amorphous material and some bacteria-like structures within digestion vacuoles (Fig. 2.38). Neutrophils persisted even in the late stages of the disease when they often had a normal appearance. However, their numbers declined in proportion to the total infiltrating cellular population from p.i.d. 4 onwards, while the proportion of macrophages increased markedly (Fig. 2.35).

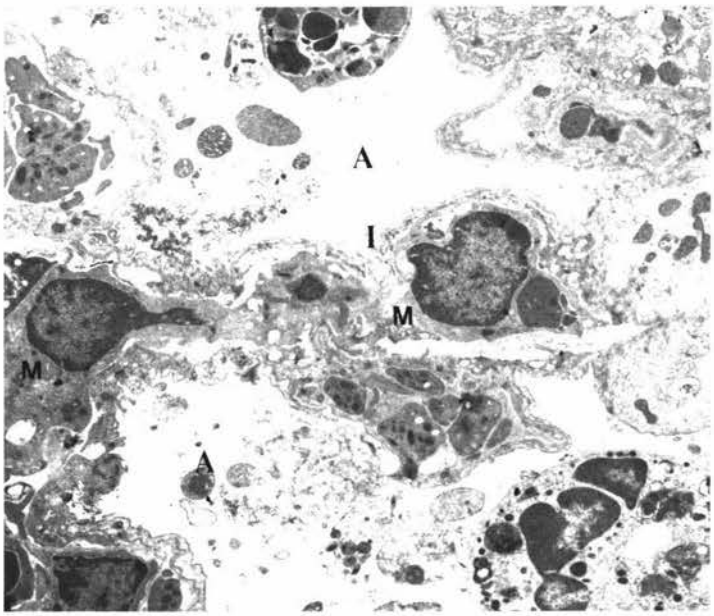
The macrophages infiltrating into alveolar spaces exhibited several distinct morphologic features at different stages of the infection. Initially, most of them had the normal appearance of pulmonary macrophages with many cytoplasmic projections, and some contained numerous vacuoles in their cytoplasm. By p.i.d. 5, they showed: (1) large numbers of empty vacuoles of varying size in the cytoplasm (Fig. 2.39); (2) phagocytic vacuoles which compressed the cell nucleus to the periphery of the cytoplasm and contained a large quantity of cellular debris (Fig. 2.40); and (3) some contained many aggregates of roughly circular electron-dense cytoplasmic particles which measured about 30 nm in diameter and resembled the β-glycogen described by Baskerville et al. (1983) (Fig. 2.41). The particles were never associated with rough endoplasmic reticulum or other membranes of cell organelles and were distributed randomly throughout the cytoplasm, either singly or in clumps of varying size. From p.i.d. 11 onwards the alveolar macrophages exhibited normal morphological features and often contained many electron-

Figure 2.37

Alveolar spaces (A) from the lung of a mouse killed one day after inoculation. Neutrophils are predominant in the intra-alveolar exudate. The alveolar type I cells (I) show extensive vesiculation of the cytoplasm. The interalveolar septa contain leucocyte-packed capillaries (c) and infiltrating macrophages (M). TEM x 5,200

Figure 2.38

Typical intra-alveolar cellular population in a mouse killed one day after inoculation. The neutrophils have numerous digestion vacuoles (v) in the cytoplasm and these contain much phagocytosed material and occasional **B. parapertussis**-like organisms (arrow). TEM x 7,800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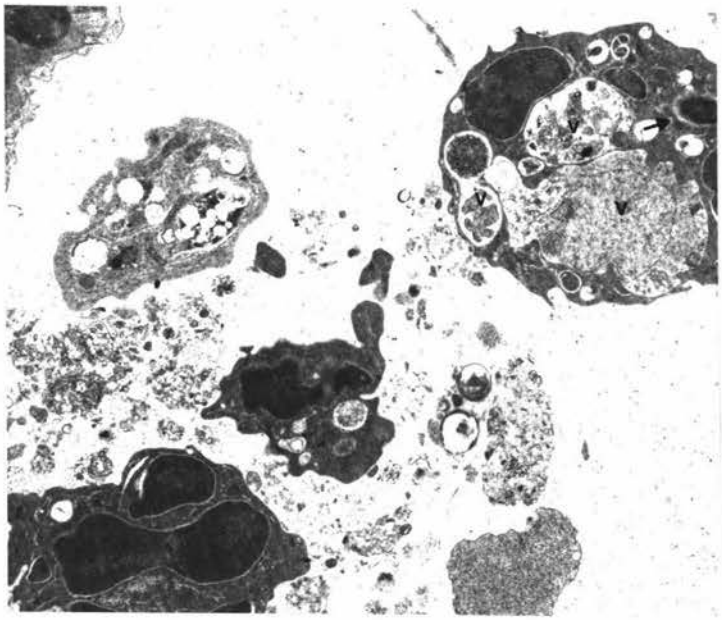

V
V
V

Figure 2.39

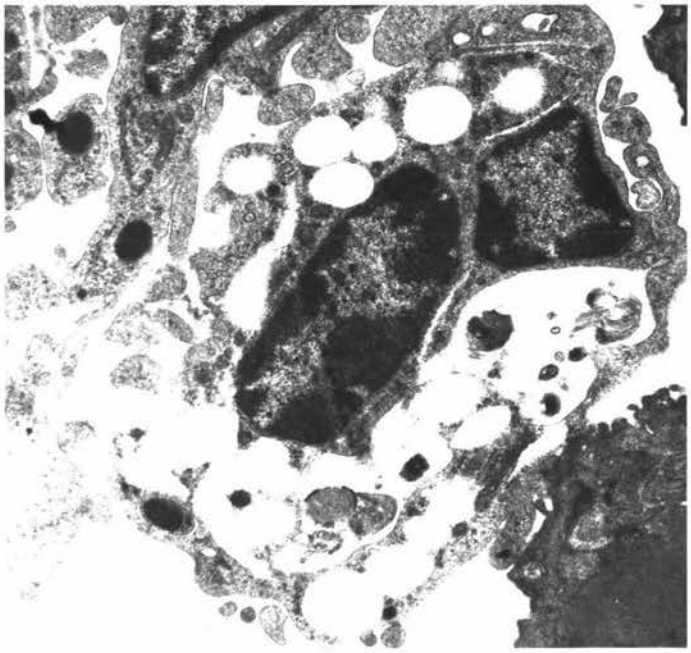
An alveolar macrophage from a mouse killed three days after inoculation showing the cytoplasm almost completely occupied by numerous empty vacuoles. TEM x 5,200

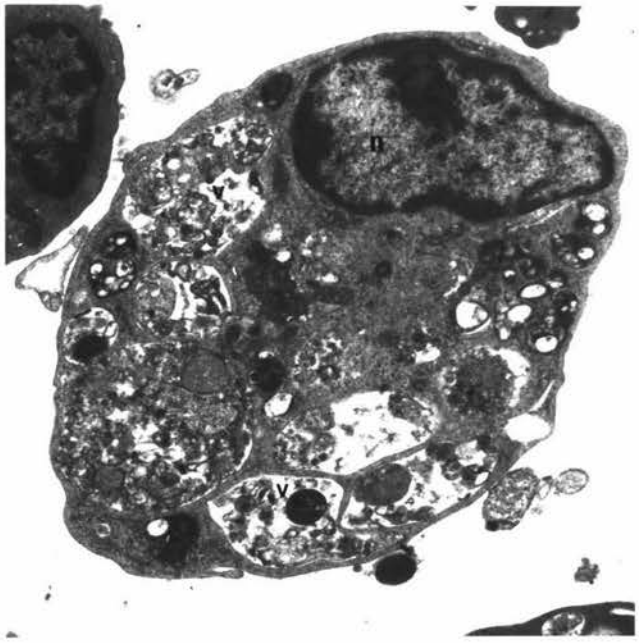
Figure 2.40

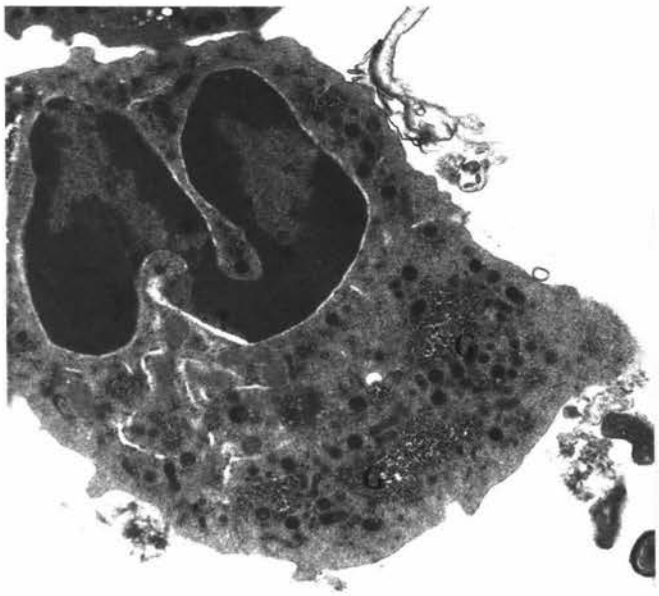
An alveolar macrophage from a mouse killed five days after inoculation. The cell has many phagocytic vacuoles (v) which contain large quantities of cellular debris. The nucleus (n) has been displaced to the periphery of the cell. TEM x 7,800

Figure 2.41

An alveolar macrophage containing cytoplasmic aggregations of glycogen-like particles (G). Mouse killed five days after inoculation of the **B. parapertussis**-like organism. TEM x 7,800




n
V
V



dense granules and occasional aggregations of proteinaecous material in the cytoplasm (Fig. 2.42).

At p.i.d. 14, the amount of alveolar exudate was markedly reduced and most alveoli and bronchioles were free of exudate and re-aerated by p.i.d. 23. At p.i.d. 29, the alveoli contained only large amounts of presumed degenerate surfactant material which was undergoing phagocytosis by macrophages (Fig. 2.43).

Interstitial Changes: The alveolar septa were often moderately thickened by the accumulation of macrophages, a few neutrophils, lymphocytes and fibrinous material in the interstitial tissue. These changes were visible as early as 12 hr after inoculation and were most severe at p.i.d. 1 to 11 (Fig. 2.44). They were usually most marked in the alveoli surrounding small bronchioles. In some areas the normal alveolar architecture was completely replaced by dense sheets of cells formed by the concurrent expansion of septa and obliteration of alveoli (Fig. 2.36). Alveolar capillaries in the thickened septa were invariably occluded by densely packed erythrocytes, neutrophils and monocytes. In the acute phase, large numbers of platelets were also present. At p.i.d. 3, there was extensive congestion and small numbers of neutrophils and macrophages were closely packed in the capillary lumina immediately beneath the alveolar epithelium.

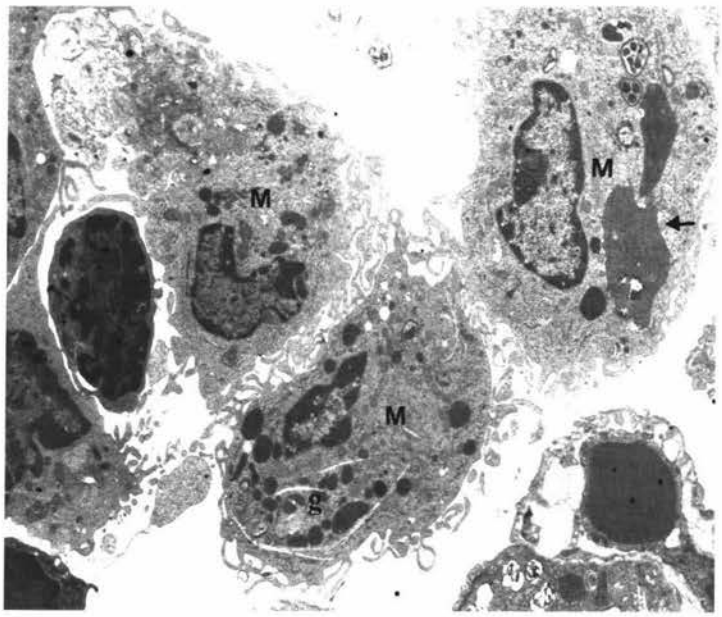
By p.i.d. 8, the interalveolar septa were more markedly thickened than previously. They contained a moderate amount of collagen and a few fibroblasts (Fig. 2.45). Sometimes, the proliferating collagen separated the basement membrane from the underlying interstitial tissue (Fig. 2.46). Occasionally, an infiltrating neutrophil was very closely associated with the overlying alveolar epithelium (Fig. 2.46). The pulmonary tissue exhibited a tight homogenous appearance due to focal alveolar collapse, combined with alveolar capillary congestion and interalveolar oedema (Fig. 2.47). At p.i.d. 26, interstitial fibrosis was the most predominant change (Fig. 2.48).

Figure 2.42

Pulmonary macrophages (M) in the alveoli of a mouse killed 11 days after inoculation. There are many electron-dense granules (g) and other electron-dense material (arrow) in the cytoplasm as well as numerous pseudopodia. TEM x 5,200

Figure 2.43

A macrophage containing large amounts of presumed degenerate pulmonary surfactant material (s) in phagocytic vacuoles. Mouse killed 29 days after inoculation. TEM x 11,200


M
M
M
g

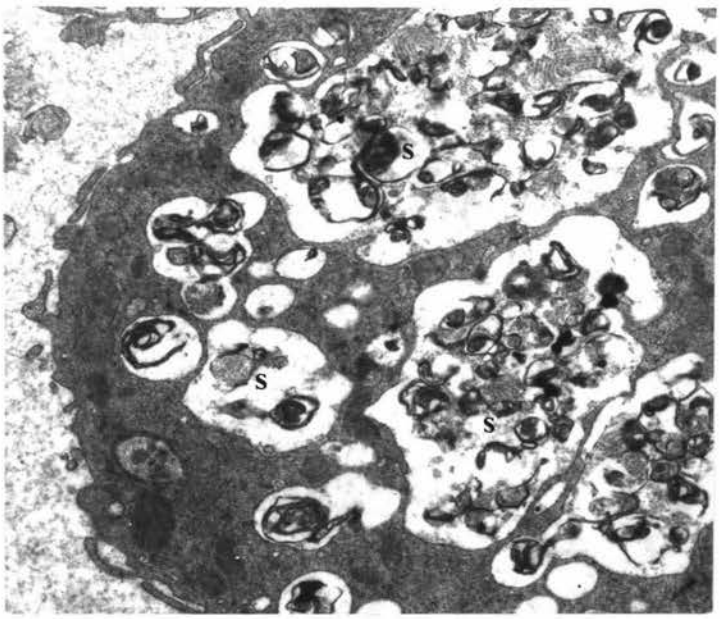

S
S
S

Figure 2.44

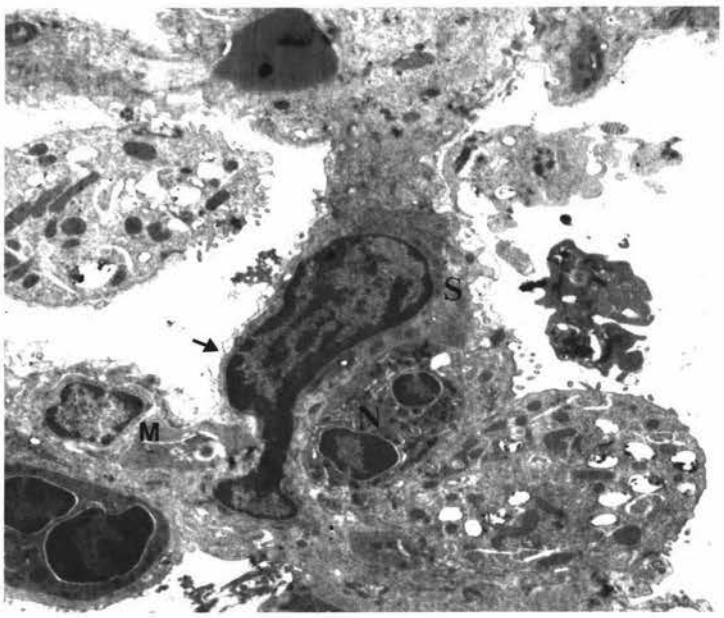
The interalveolar septum (S) of a mouse killed one day after inoculation. The alveolar wall is moderately thickened by the infiltration of neutrophils (N) and mononuclear cells (M). The alveolar epithelium shows areas of swelling (arrows). TEM x 5,200

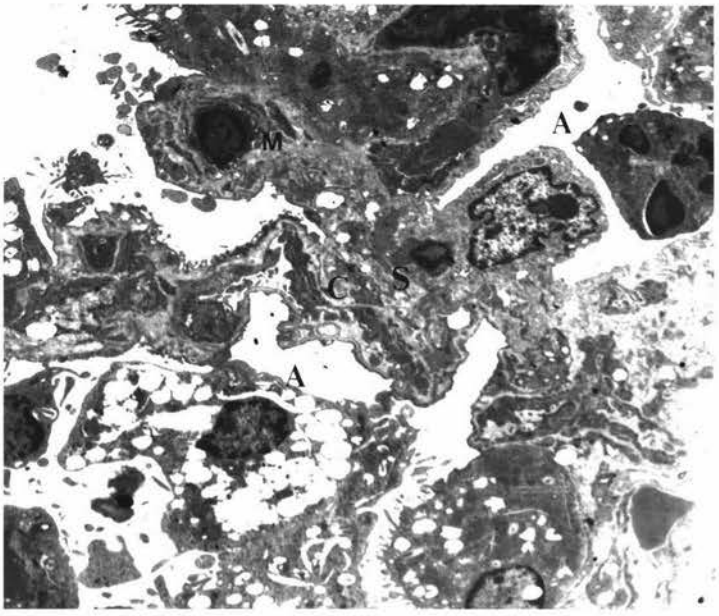
Figure 2.45

Thickening of interalveolar septa (S) in a mouse killed eight days after inoculation. There is proliferation of collagen (C) and infiltration of mononuclear cells (M). The alveoli (A) contained occasional inflammatory cells. TEM x 3,400

Figure 2.46

The alveolar basement membrane (arrows) is separated by proliferating collagen (C) from the underlying interstitial tissue. A neutrophil (N) can be seen closely associated with an alveolar type I cell (I). Mouse killed 11 days after inoculation. TEM x 5,200


S
M
N


M
A
C
S
A

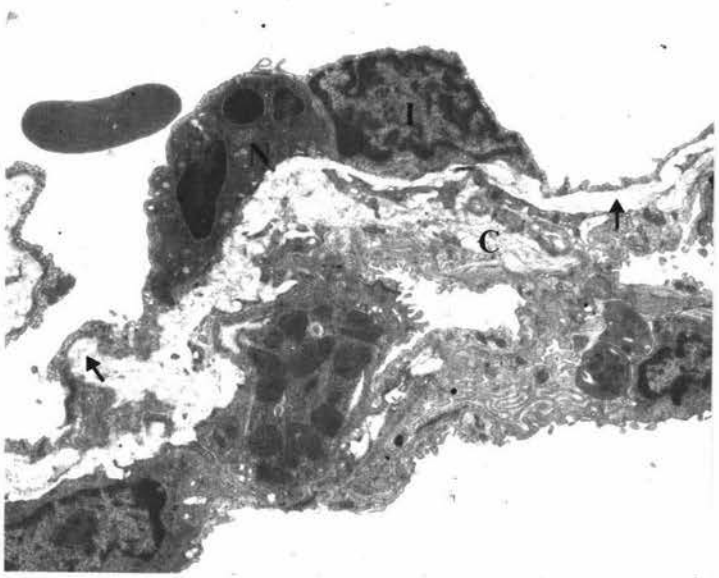
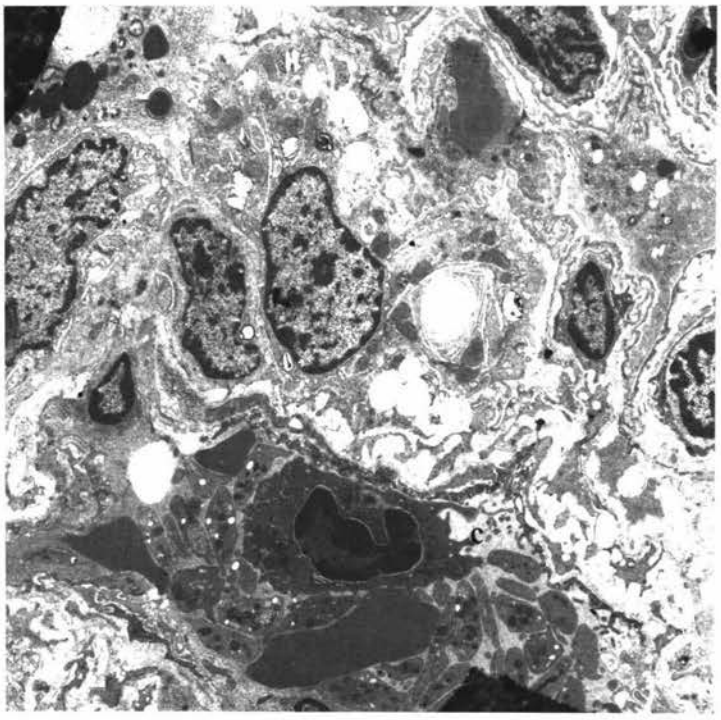

I
N
C

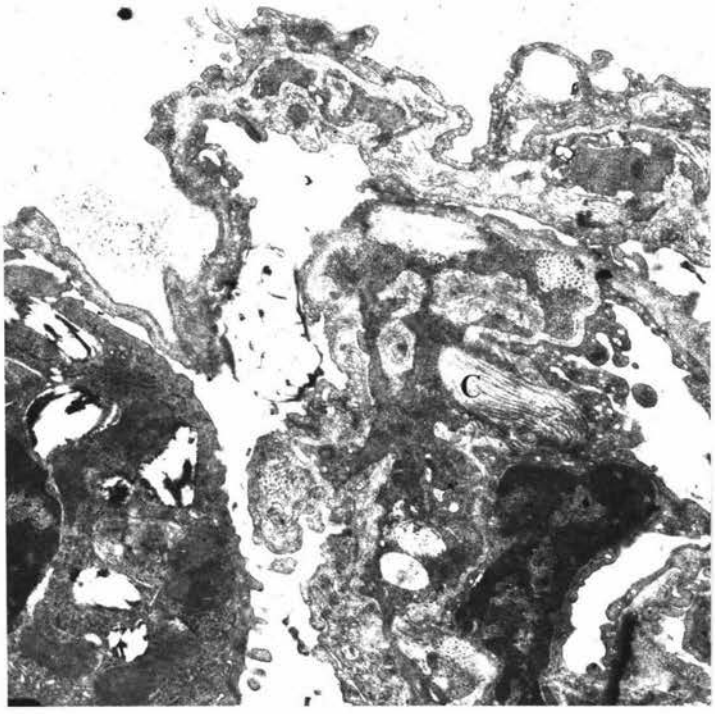
Figure 2.47

Alveoli from a mouse killed 11 days after inoculation. There is severe collapse, congestion and leucocytes in alveolar capillaries (c) as well as interstitial infiltration of inflammatory cells and early fibrosis (F). TEM x 5,200

Figure 2.48

Alveoli from a mouse killed 26 days after inoculation. The interalveolar septa are markedly thickened by the proliferation of collagen (C). The alveolar epithelium at this stage appears to be normal. TEM x 11,200





Interaction of Phagocytes and Organisms: Bordetella parapertussis-like organisms were found, in moderate numbers, free in alveoli or phagocytosed by macrophages and neutrophils at 12 hr to 5 days after inoculation, but they were never seen within alveolar epithelial cells. The organisms found in degenerating alveolar spaces were often trapped among the exudative material. They had similar ultrastructural features to those described by Al-Kaissi (1986) **in vitro**. They showed a furrowed cell wall with abundant ribosomes in their cytoplasm. The ribosomes were usually disposed in a well-demarcated zone around the nucleoplasm which was quite distinct and appeared to be whorled and rarefied (Fig. 2.49). The organisms phagocytosed by macrophages and neutrophils usually showed degenerative and necrotic changes (Fig. 2.50). These included separation of cytoplasm from the cell wall, distortion of the cell wall and clumping and disintegration of cytoplasm. No dividing organisms were observed in either phagocytes or alveoli.

Bacteriology

Cultures from the portions of lungs showing consolidation were always positive (Table 2.2). Large numbers of **B. parapertussis**-like organisms were present in pure culture at three days after inoculation and small numbers of pure organisms were recovered up to p.i.d. 6. At p.i.d. 11 and 28, very small numbers of organisms were isolated from one mouse on each occasion. At p.i.d. 14 and 17, **Staphylococcus epidermidis** was recovered from the lungs of one mouse from the infected group. Cultures from control animals were always negative for **B. parapertussis** and other organisms.

DISCUSSION

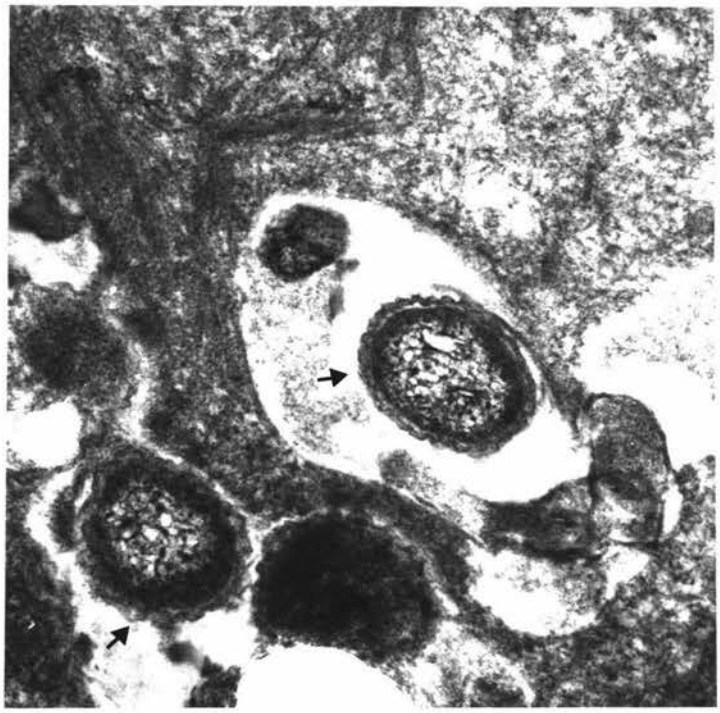
Although **B. parapertussis** is known to be one of the causes of respiratory disease in humans, particularly children, in animals, hitherto only Bradford & Wold (1939) have been able to produce interstitial pneumonia by intratracheal infection with an atypical pertussis organism from a whooping cough patient, which was later

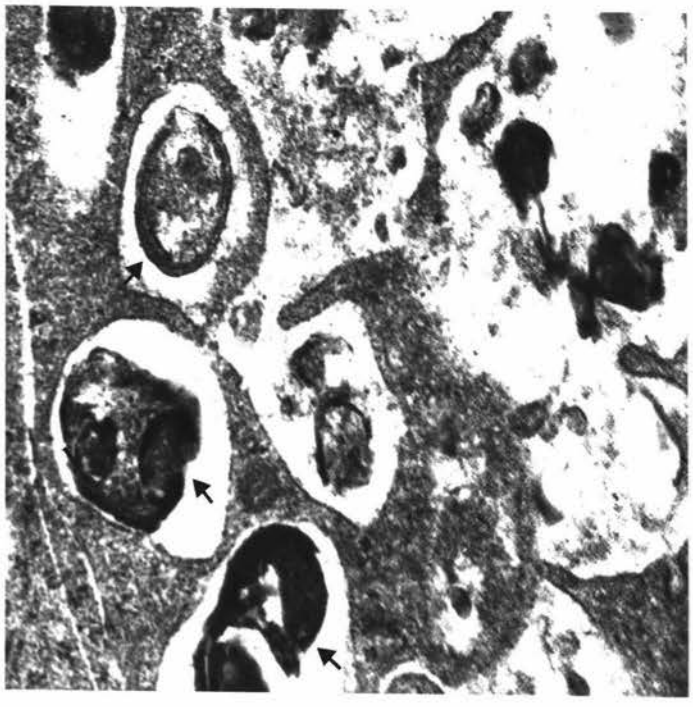
Figure 2.49

Bordetella parapertussis-like organisms in the alveolar spaces of a mouse which died two days after inoculation. Many intact organisms (arrows) are trapped in the necrotic debris and exudate in alveolar spaces. The organism has a furrowed cell wall with abundant ribosomes in its cytoplasm. The nucleoplasm of the cell is whorled and rarefied. TEM x 48,600

Figure 2.50

Bordetella parapertussis-like organisms (arrows) in the phagocytic vacuoles of alveolar macrophages showing varying degrees of degeneration. From a mouse killed six days after inoculation. TEM x 31,800





identified as **B. parapertussis**. The present work has successfully produced a subacute bronchopneumonia in mice by intranasal inoculation with a sublethal dose of a similar organism isolated from CNP-affected lambs.

The lesions in the infected mice involved chiefly the alveolar parenchyma and to a lesser degree the bronchiolar epithelium in the early stages. They were similiar in nature to other reports of **B. parapertussis** and **B. pertussis** infection in mice (Bradford & Salvin, 1937; Bradford & Wold, 1939; Bradford, 1938; Burnet & Timmins, 1937), but contrasted to the results of Anderson (1957), who found that all four strains of **B. parapertussis** isolated from human patients were only slightly pathogenic for mice.

The pathological and microbiological evidence provided by this study indicates that the mouse is highly suitable for use as an experimental model for the study of infection produced by ovine strains of the **B. parapertussis**-like organism. The pulmonary lesions appeared to be a direct response to the inoculated bacteria and/or their metabolic products as shown by the correlation between recovery of the pure organisms from the lung and the pulmonary inflammatory reaction. As far as can be determined, the experimental lesions produced in mice were similar to those seen in early CNP lesions of lambs (Alley, 1975a; Al-Kaissi, 1986). The most significant exception was the absence of obvious proliferation of alveolar type II cells and bronchiolar epithelial hyperplasia which are characteristic of ovine lesions (Alley & Manktelow, 1971; Alley, 1975a). However, light and electron microscopy demonstrated that both alveolar type I and type II epithelial cells were damaged and a large population of alveolar macrophages showed cytotoxic features at later stages of infection.

Although the light microscopic changes in the lung of infected mice were similar to those reported in mice with intranasal and intratracheal infection by human isolates of **B. parapertussis** and **B. pertussis**, some differences were noted. The relative intactness of the bronchiolar mucosa and absence of bacterial colonization of

ciliated epithelial cells, even in the areas of heavy inflammatory infiltration, seen in the present study are perhaps surprising since there is strong evidence that the establishment of **Bordetella** infection is largely dependent on the organism's adherence to the respiratory mucosa (Manclark & Cowell, 1984). Many pathological studies **in vitro** and **in vivo** have shown that **Bordetella** organisms are able to colonize the tracheobronchial airways (Sato et al., 1980; Bemis et al., 1977). **B. parapertussis** has also been proven to be closely associated with ciliated epithelium both **in vitro** (Al-Kaissi et al., 1986) and **in vivo** (Bradford & Salvin, 1937; Bradford & Wold, 1939). The apparent lack of such an association in the present study may be due to differences in species specificity of the organism with regard to adherence to ciliary epithelial cells (Tuomanen et al., 1983). A very close relationship between the natural host of the organism and its adherence to ciliated cells has been demonstrated by Tuomanen et al. (1983) **in vitro**. Human isolates of **B. parapertussis** have been found to adhere very poorly to respiratory ciliated cells in the rabbit, hamster, mouse and chicken and at least 10^8 organisms were required. However, adhesion of the organism to respiratory ciliated cells was not observed by Shibley (1934) using light microscopy on lungs from chimpanzes infected by **B. pertussis**.

The majority of degenerative changes observed in the alveolar epithelium have been described previously in pathological studies of naturally-occurring ovine CNP and in a number of pulmonary chemical intoxications (Schaefer et al., 1964; Manktelow, 1967; Adamson et al., 1970; Alley, 1975a). This suggests that alveolar epithelial injury generally follows a basic pattern of reaction which is somewhat independent of the nature of the inducing agent. It is interesting that the formation of thickened, electron-dense plasma membranes on the superficial areas of alveolar epithelial cells seen in this study have only been reported in naturally-occurring acute and chronic ovine pneumonia in New Zealand (Alley, 1975a). This implies that there may be some general factor(s) involved in the development of this change which are common to these two separate studies. Unfortunately, there is no information available on whether

or not this lesion is also present in human patients and animal models infected with **B. pertussis**. It is therefore premature to hypothesise that this change is directly associated with infection due to the **B. parapertussis**-like organism.

As is the case in human pertussis (Pittman, 1970), in experimental pertussis infection in chick embryos (Gallavan & Goodpasture, 1937) and in many other pulmonary injuries unrelated to **Bordetella** organisms, a rather generalized formation of perivascular and peribronchiolar lymphoid cuffs was a prominent feature in the lungs from mice killed in the later stages of infection (Alley, 1975a; Gilmour et al., 1982a; Jones et al., 1986b). In the past this change was considered to be an indication of the widespread effect of pertussis toxin(s) (Gallavan & Goodpasture, 1937) or of mycoplasma infection (Pirie & Allan, 1975), but it is now recognized as a general reaction to antigen rather than to any special agent (Alley & Clarke, 1977). The appearance of these cuffs was closely associated with the clearance of bacteria from the mouse lung which further confirms the assumption that the aggregations are part of a general immune response.

No obvious damage was found in alveolar capillaries by electronmicroscopy although mild interstitial oedema was a consistent feature in mice during the early stages of infection. It is possible that the oedema resulted from pathophysiological factors related to alveolar collapse such as congestion, increased pulmonary vascular resistance and capillary permeability, as suggested by Alley (1975a). Another possible factor likely to change alveolar capillary permeability is the endotoxin released by the organism (Munoz, 1961). The close similarity in the development and persistence of alveolar oedema in this study with that seen in **B. pertussis** infection suggests that the **B. parapertussis**-like organism, like **B. pertussis**, may also possess a toxin which affects capillary permeability.

The only residual features seen in the infected mouse lungs after 21 days were alveolar interstitial fibrosis and

hypercellularity. This was similar to the results of Bradford (1938) who found residual interstitial changes present three weeks after infection with **B. pertussis**. Interestingly, the presence of this finding has been shown to correlate well with protection against sequential challenge with **B. pertussis** after intranasal inoculation of a pertussis vaccine (North & Anderson, 1942). Thus it is possible that the presence of mononuclear cells in the interalveolar space, as in the peribronchiolar space, is an indication of the establishment of a host immune reaction.

The failure to demonstrate extensive phagocytosis of the organism by neutrophils and alveolar macrophages with electronmicroscopy was unexpected. Throughout the experiment, only very few organisms were found within the cytoplasm of macrophages despite examination of large numbers of tissue blocks. Resistance of **B. pertussis** to phagocytosis by neutrophils has been noted and purported to be caused by a polymorphonuclear inhibitory factor (PIF) released by **B. pertussis** (Pittman, 1984b), however, the interaction between alveolar macrophages and these organisms has not been fully explored. An **in vitro** study by Muse et al, (1979) demonstrated that only 10% of non-opsinized alveolar macrophages from guinea pigs phagocytosed **B. pertussis** whereas opsinisation of macrophages increased the rate of phagocytosis to 42%. These authors showed that the organism was able to survive within the phagocytic vacuoles of macrophages. In the present study, a large proportion of macrophages exhibited cytotoxicity of varying severity, as shown by the presence of severe cytoplasmic vacuolation and reduction in numbers of phagocytic vacuoles. Cytotoxicity of alveolar macrophages is also a feature in **B. bronchiseptica**-colonized rabbits (Zeligs et al., 1986). Since "toxic" macrophages exhibit significantly decreased cell adherence, phagocytic uptake and bacteriocidal activity (Zeligs et al., 1986), this cytotoxicity is likely to be an important factor in predisposing the lung to further infection by other pathogens.

Aggregation of glycogen-like granules was seen in the cytoplasm of a proportion of macrophages in the present study. Glycogen has

been described in normal unstimulated peritoneal macrophages and in saline-stimulated macrophages of guinea pigs (Daems & Brederoo, 1973), but it has rarely be described in other pulmonary infections (Baskerville, 1972; Baskerville et al., 1972 & 1982; Baskerville & Wright, 1973; Alley, 1975a; Al-Kaissi & Alley, 1983; Bryson et al., 1983; Cutlip & Lehmkubl, 1986; Al-Darraji et al., 1982b), including experimental **B. pertussis** infection (Baskerville et al., 1983). However, it was a common finding in the peritoneal and splenic macrophages of guinea pigs as well as in pulmonary macrophages and neutrophils of guinea pigs, rhesus monkeys and mamosets about four days after infection with **Legionella pneumophila** (Baskerville et al., 1983). It has also been seen occasionally in Kuffper cells of grivet monkeys infected with **Francisella tularensis** (Baskerville, et al., 1978). Thus it appears that the presence of glycogen in phagocytic cells is not specifically related to infection with a particular organism. It was suspected by Baskerville et al (1981), however, that glycogen was to be associated with changes in the metabolism of phagocytes when involved in the phagocytosis of infected organisms (Baskerville et al., 1983). In the present study, the aggregations of glycogen are present only in macrophages and not neutrophils. This lends weight to Baskerville et al.'s hypothesis because in the present study only macrophages were found to phagocytose **B. parapertussis**-like organisms while in Baskerville et al.'s study organisms were found in phagocytic vacuoles of both neutrophils and macrophages.

The ultrastructural observations suggested that in the mouse, the primary targets of infection by **B. parapertussis** are the alveolar parenchyma and alveolar macrophages. In all species, infection and destruction of these cells predisposes the lung to subsequent infection by other pathogens. In addition, damage to these cells may also cause pulmonary dysfunction. The pulmonary lesions are likely to be mainly associated with bacterial cytotoxin rather than a direct injury induced by the organisms since there was no observable invasion or attachment by organisms to cell surfaces. Pulmonary lesions in the infected mice persisted and developed even when the organisms were almost completely cleared. A cytotoxic

effect induced by toxins from **Bordetella** organisms was suggested by Gallavan & Goodpasture as early as 1937. In humans, it is now well established that pertussis is a toxin-mediated disease (Pittman, 1984b). **B. pertussis** organisms can produce at least seven different types of cytotoxin which are involved in all stages of bacterial infection (Weiss et al., 1986). Among them tracheal cytotoxin (TCT) is the main toxin responsible for local tissue damage (Goldman, 1986; Hewlett & Weiss, 1986). However, whether **B. parapertussis** also possesses this substance is unknown.

A lack of close attachment of the organism to the respiratory epithelium in mice may partially explain the microbiological and pathological results of low yields of organisms from the lung, their short duration in the lung and the non-progressive course of the disease. Although it is possible to infect mouse lung with the **B. parapertussis**-like organisms, multiplication of the organisms **in situ** seems to be inefficient. During the time the pneumonia was developing, the **B. parapertussis**-like organism was apparently being rapidly removed from the lung. Although the clearance of **B. parapertussis** in mice lung was only semi-quantitatively investigated in this study, the yield of the organisms reached its peak on the second and third day after inoculation, declined dramatically over the next few days and disappeared at p.i.d. 8, indicating that the **B. parapertussis** organisms undergo very little, if any, replication in mouse lung. Using electronmicroscopy, no bacteria in division stages were observed although large numbers of sections were screened. These results contrast with the study by Bradford & Salvin (1937) in which **B. parapertussis** was found to last in the lung as long as 22 days after inoculation. With **B. pertussis** infection, replication of organisms also persists in the lung for long periods and they reach a peak at p.i.d. 14 (Sato et al., 1980).

The pneumonic lesions and mortality rate in mice after inoculation of the **B. parapertussis**-like orgnaism was relatively mild in the present study, compared to other previous studies with either **B. parapertussis** (Bradford & Salvin, 1937; Bradford & Wold, 1939) or **B. pertussis** (Bradford, 1938; Burnet & Timmins, 1937). This

may be related to the dose of infective bacteria or to differences between the strain of **B. parapertussis** used by Bradford & Wold (1939) and the **B. parapertussis**-like organism used in the current study. In the studies by Bradford & Salvin (1939), the dose of **B. parapertussis** used was about 10^4 times, which was close to lethal dosage (Pittman, 1984b) and higher than in the current experiment. If a comparable dose had been used in the present study a mortality rate of 67% could have been expected. This is close to the mortality rate observed by Bradford & Wold (1939) using a human strain of the organism. This suggests that the strains of the **B. parapertussis**-like organism used in the current investigation had a pathogenicity of the same order. Other factors may also contribute to the difference in extent of the pneumonic lesions. These include the age of animals, strain of mice and bacterial virulence. In **B. pertussis** infection, Sato et al. (1980) found 18-day-old mice were more susceptible to infection than were 28-day-old mice. Similar results were also obtained by Pittman et al. (1980). Evidence from both **in vitro** and **in vivo** studies have also indicated that different phases of **B. pertussis** show significant differences in virulence (Matsuyama, 1977; Weiss & Hewlett, 1986). Unfortunately, information on the bacterial phases used by Bradford & Wold (1939) is not available so comparison of results is difficult.

The technique used in the present study to induce respiratory infection of mice is simple and reliable. The high incidence of infected and affected lungs indicated that the method of inoculation was reasonably reproducible and that the variation in lesions between individual mice was relatively small. The use of halothane rather than ether as an anaethetic agent not only made the mouse more amenable to handling and instillation, but also gave more accurate results since there was no pulmonary reaction to anaethesia in the control mice.

The mouse model is convenient and reliable. It may therefore provide an opportunity for not only studying the potential role of the **B. parapertussis**-like organism in the initiation and development of ovine CNP, but also for further investigating the sygeristic

effect of this organism with other potential pathogens, such as **P. haemolytica** and **M. ovipneumoniae**. Using this experimental approach it may be possible to determine the relationship between the organism, host defenses and interaction of any cytotoxins produced. Investigation of the morphological changes in the upper respiratory tract of infected mice will also be necessary to fully understand the nature of infection, adherence and ciliostasis.

SUMMARY

The intranasal inoculation of SPF mice with suspensions of a **Bordetella parapertussis**-like organisms isolated from CNP-affected ovine lungs, killed about 11 percent of animals (4 out of 34) between 2 to 3 days after infection and produced a subacute pneumonia morphologically similar to early lesions of naturally-occurring CNP in over 90% of animals. Sequential changes in the lung were examined by both light and electron microscopy from the time of initial infection to 30 days after instillation. Pulmonary lesions characterized by alveolar septal congestion and oedema, focal alveolar haemorrhage, and intraalveolar and septal infiltration by neutrophils and macrophages were seen in the early stages. Later, hyperplasia of perivascular and peribronchiolar lymphoid tissue and the proliferation of collagen in the interalveolar septa were the most prominent changes. The bronchial and bronchiolar mucosa was usually intact, but bronchiolar lumina often contained an exudate of macrophages, neutrophils and fibrin, surrounded by zones of consolidation.

Electronmicroscopy revealed that the pulmonary damage caused by infection of the **B. parapertussis**-like organism consisted mainly of extensive degenerative changes in the alveolar epithelium and marked cytotoxicity of alveolar macrophages.

Pure cultures of the **B. parapertussis**-like organism were consistently recovered from infected lungs from 12 hr after inoculation to p.i.d. 6. Intact organisms and organisms showing varying degrees of degeneration were found free in alveolar spaces or phagocytosed by alveolar macrophages. No replication of organisms was detected at any stage of the infection using morphological examinations. No close specific association was observed between organisms and the ciliated or non-ciliated respiratory epithelium.

The results of this experiment show that the **B. parapertussis**-like organism can infect the mouse lung and produce pneumonic

lesions and damage to alveolar macrophages. This evidence supports the hypothesis that the **B. parapertussis**-like organism may play an important role in initiating or prolonging the respiratory infection in sheep.

CHAPTER 3

EXPERIMENTAL INFECTION OF LAMBS WITH A **BORDETELLA PARAPERTUSSIS**-LIKE ORGANISM

CHAPTER 3

EXPERIMENTAL INFECTION OF LAMBS WITH A **BORDETELLA PARAPERTUSSIS**-LIKE ORGANISM

Although **P. haemolytica** is the most common bacterial isolate obtained from pneumonic ovine lung, experimental reproduction of the disease with this organism alone has had only limited success (Smith, 1964; Biberstein et al., 1967). Predisposing factor(s) or agent(s) may therefore be necessary to enable **P. haemolytica** to colonise the lungs of sheep and cause respiratory disease. Biberstein et al. (1971) have shown experimentally that PI3 virus may have this role. In pigs, however, it is generally recognized that combined infection with **B. bronchiseptica** and **P. multocida** plays an important role in the development of atrophic rhinitis and sometimes pneumonia (Bemis & Wilson, 1985). The possibility that a bacterial synergism of this type may also occur in the ovine respiratory tract is therefore worth consideration.

The consistent isolation of a **Bordetella parapertussis**-like organism from both healthy and CNP-affected lambs has raised the possibility that this organism may have a role in initiating or prolonging ovine CNP (Manktelow, 1984; Alley, 1986; Al-Kaissi et al., 1986; Cullinane et al., 1987). There has to date, however, been little **in vivo** work to support this hypothesis. In a previous study (Chapter 2), a subacute bronchopneumonia was successfully reproduced in SPF mice by intranasal inoculation with ovine strains of the **B. parapertussis**-like organism. The pathological changes observed were similar in many respects to early lesions of naturally-occurring ovine CNP. In addition, alveolar macrophages from the infected mice showed severe degeneration. These results supported the hypothesis that the **B. parapertussis**-like organism may be a pathogen in ovine pneumonia.

The objective of the present study was to determine whether the **B. parapertussis**-like organism was capable of damaging either the ovine tracheobronchial airways or the pulmonary parenchyma. The microbiological, cytological, histological and ultrastructural changes which occurred in infected lambs were observed and the tracheobronchial epithelium was examined by scanning electron microscopy(SEM).

MATERIALS AND METHODS

Experimental animals. The twelve Romney lambs used in this experiment were hysterectomy-derived and colostrum-deprived. They were reared in an isolated building, fed cold reconstituted whole milk powder (Anlamb, N.Z. Dairy Ltd.) and allowed water **ad libitum**.

The lambs were randomly assigned to two age-matched groups. Lambs in Group 1 (n=4) received sterile PBS as a control; and those in Group 2 (n=8) received an inoculum containing the **B. parapertussis**-like organism. The lambs were 7-14 days old at the time of inoculation. After inoculation the two groups were housed in separate pens at the opposite ends of the building. They were observed daily for one wk before inoculation, and thereafter they were observed twice daily at which time their rectal temperature and clinical signs were recorded.

Inoculum. A suspension of the **B. parapertussis**-like organism was prepared by pooling five isolates of the organism grown on blood agar (Difco) as described previously (Chapter 2). The inoculum was adjusted to an oposity by spectrophotometer at 460 μm to give a titre of 2×10^6 CFU/ml and then inoculated onto blood agar to identify viable bacterial colonies. The inoculum used had a final titre of 2.3×10^6 CFU/ml.

Experimental design and inoculation technique. All inoculations were carried out by intratracheal injection using a 20 g, 1" sterile needle inserted between the rings of the trachea immediately beneath the larynx. During inoculation the animals were restrained in a vertical position. A total of eight lambs were inoculated with 5 ml of bacterial suspension. The remaining four were inoculated with the same volume of sterile PBS.

Unless death intervened, two inoculated lambs and one control lamb were euthanased at 24 hr, 3, 5 and 9 days after inoculation by rapid intravenous injection of pentobarbitone sodium and exsanguinated by incision of the brachial artery.

Microbiology. Nasal swabs for bacterial isolation were collected before inoculation and then every second day after inoculation until slaughter. The swabs were inoculated onto blood agar and cultured for three days at 36^{o}C. The number of **B. parapertussis**-like organisms in the bronchoalveolar lavage fluid obtained at necropsy was calculated by counting viable bacterial numbers. After centrifugation of the lavage fluid, a duplicate, 0.1 ml volume of serial 10-fold dilutions of the sediment were spread on the blood agar plates. Colony counts were determined after 72-96 hours incubation at 36^{o}C.

Haematology. Blood samples were collected in EDTA-vacutainers from the jugular vein of the lambs immediately before euthanasia. Leucocyte counts were determined in a Cell-Dyn 900 haemotological analyzer (Sequoia-Turner, California), and differential leucocyte counts were made from blood smears stained with modified Wright's stain (Sigma, U.S.A.).

Pathology. Gross lung lesions visible at the time of necropsy were recorded and photographed.

Portions of tissue about 1 mm^3 were excised from the trachea, bronchi, bronchioles and several areas of affected lung if lesions

were present and fixed in modified Karnovsky's fixtive (Appendix 2) for 12 hr. They were then processed and examined as described in Chapter 2.

The remainder of the lung and trachea was fixed in 10% formol saline for 24 hr, after which 6-8 different areas were selected for histological examination and immunohistochemistry. Tissues for histopathology were processed by conventional methods, cut at 4 µm and stained with H.E..

Larger blocks approximately 3 x 5 mm in size were taken from the trachea and bronchi for scanning electron microscopy. These were immediately immersed in modified Karnovsky's fixative at 4°C for 24 hr. They were then washed in two changes of cold PBS (0.1M, pH 7.2) at room temperature for one hr, dehydrated in a series of graded alcohols, critical point dried, placed on aluminium stubs, coated with gold and examined in a Cambridge 250 MK III scanning electronmicroscope.

Immunohistochemistry. The peroxidase anti-peroxidase technique (PAP) was applied to study the bacterial distribution in the lung. The sections were stained according to the method of Robinson (1982) with minor modifications.

After 24 hr formalin fixation, tissues were paraffin embedded, cut at 5 µm thickness, mounted and dried overnight at 37°C. They were then deparaffinized in two changes of xylene and hydrated through three changes of ethanol to distilled water. After 10 min., sections were incubated in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to block any endogenous peroxidase activity. Slides were then rinsed in PBS (pH 7.4) for three changes each of five min. and flooded for 30 min with 1% bovine serum albumin (BSA) (ICP, Aukland) to reduce background staining. After blotting off excess serum, the rabbit anti-**B. parapertussis** serum (Wellcome, England) was applied to the sections for three hr at various dilutions. Slides were rinsed in PBS and

flooded with the second linking antibody; goat anti-rabbit immunologlobin G (ICP, Aukland) applied in excess at a dilution of 1:20. Following 30 min. incubation at room temperature and rinsing in PBS, sections were flooded with 1:60 dilution of rabbit peroxidase-antiperoxidase complexes (Dakopatts, Denmark).

The slides were then rinsed again in PBS, drained and stained with freshly prepared 0.05% solution of diaminobenzidine tetrachloride (Sigma) with 0.01 per cent hydrogen peroxide in 0.1 M Tris hydrochloric acid buffer pH 7.6 for 10 minutes. The sections were washed in distilled water before counterstaining with Mayers haematoxylin, dehydrating, clearing and mounting in DPX.

The control staining was carried out on one of the duplicated sections by omitting each antiserum step in turn. Sections were also stained with diaminobenzidine tetrachloride only, to reveal the presence of any endogenous peroxidase.

Bronchoalveolar cytology. At each time interval after inoculation one euthanased lamb from each group was used for a cytological study of bronchoalveolar lavage fluid. The trachea was clamped and the lung was carefully removed and placed in a clean plastic bag. Lung lavage was then carried out as described previously (Chapter 2) using a total volume of 350 ml of normal saline solution. Washings from the lavages were pooled together and sampled for total and differential cell counts. Total cell numbers were counted in a haemocytometer and differential counts were made on smears fixed in methanol, air dried, and stained with Giemsa (Luna, 1968). The smears were examined by light microscopy and the percentage of neutrophils and alveolar mononuclear cells was recorded.

The remaining washings were then centrifuged at 700g for 10 min at 4^{o}C. The cell pellets were transferred to a 10 ml centrifuge tube containing PBS and centrifuged at 3000 rpm twice for five min each. After each centrifugation, the cell pellets were washed in two

changes of PBS to remove mucus. Finally, the pellets were resuspended in 1.5 ml of PBS, transferred to capillary tubes (Ternmo, Tokyo) and then centrifuged in a micro-capillary centrifuge (Model MB, IEC, U.S.A.) for five min to allow the cells to form a solid pellet. Five pellets were sampled from each lavage specimen. These were fixed in modified Karnovsky's fixative in 0.1 M PBS and processed for TEM as described Chapter 2.

RESULTS

Clinical Signs

All eight lambs from Group 2 (inoculated) showed slight depression and anorexia 12 hr after inoculation but returned to normal at 36 hr. Occasionally a single cough was noticed in individual animals between 12 to 24 hr after inoculation. The lambs in Group 1 (control) showed no abnormal clinical signs. No pyrexia was present in either inoculated or control lambs (Fig. 3.1).

Haematology

The blood concentrations of leucocytes in the infected and control lambs are shown in Fig. 3.2. Twenty-four hours after inoculation of the **B. parapertussis**-like organism there was a significant increase in leucocyte counts which lasted until p.i.d. 5 when it returned to normal. In contrast, total leucocyte counts in the control group showed no significant change throughout the experiment.

Circulating neutrophil counts increased in all lambs during 24 hr to 5 days after inoculation and returned to the normal range at p.i.d. 9 (Fig. 3.3). Twenty-four hours after inoculation of the **B. parapertussis**-like organism, neutrophil counts rose rapidly and peaked on p.i.d. 3 at $7.93 \times 10^9 \pm 1.49 \times 10^9$/L (64%) and then gradually decreased to $1.77 \times 10^9 \pm 9.90 \times 10^2$/L (26%) at p.i.d. 9.

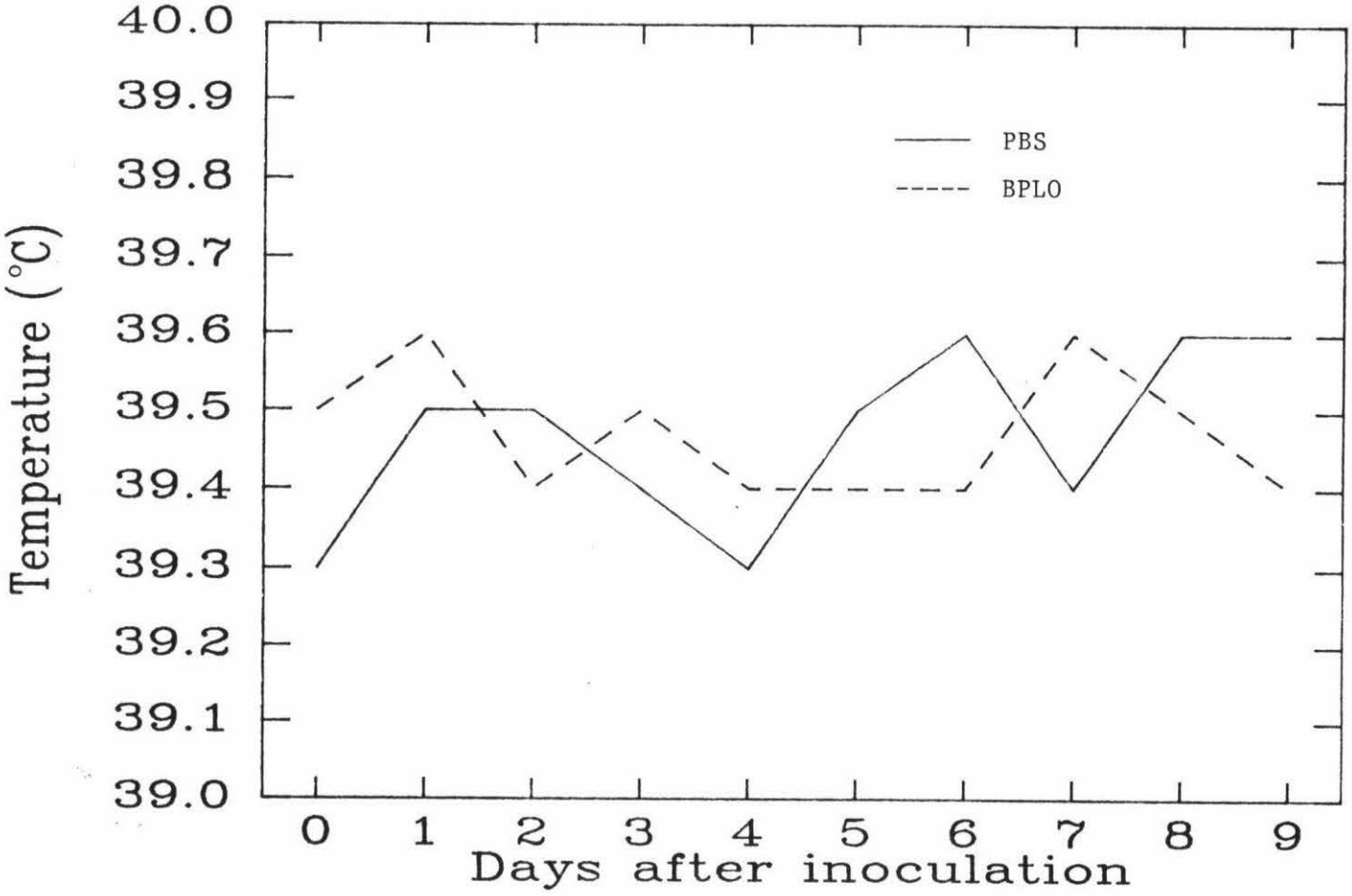


Figure 3.1 Average daily rectal temperature of lambs inoculated with either sterile PBS or the B. parapertussis-like organism (BPLO)

Figure 3.2 Comparison of total blood leucocyte counts between groups of lambs after intratracheal instillation of either sterile PBS or the B. parapertussis-like organisms (BPLO)

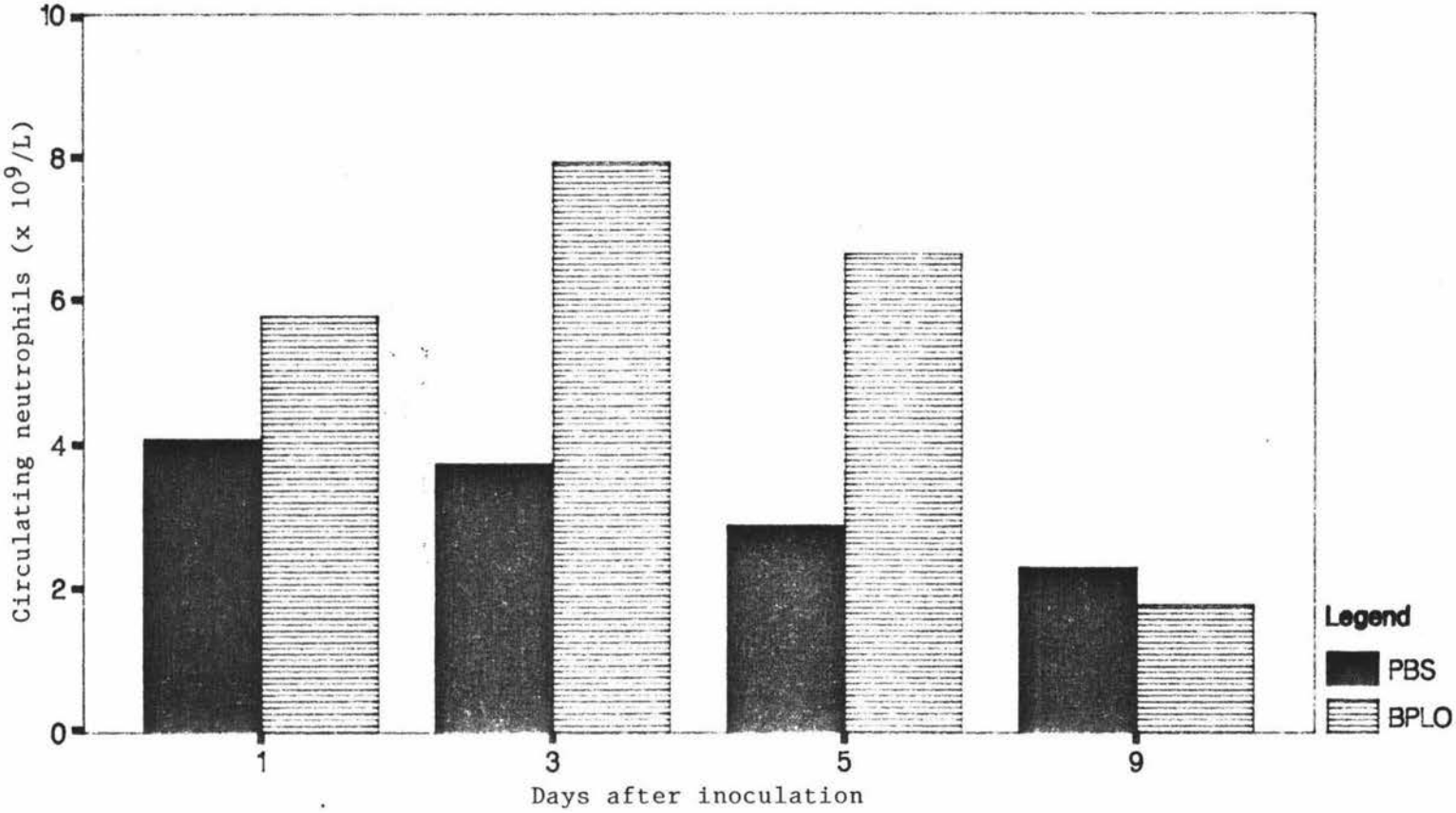


Figure 3.3 Comparison of blood neutrophil counts between groups of lambs after intratracheal instillation of either sterile PBS or the B. parapertussis-like organism (BPLO)

Immunohistochemistry

No **B. parapertussis** positive staining organisms could be demonstrated in lung tissue from lambs inoculated with **B. parapertussis**-like organisms or in tissues from the controls throughout the experiment. Smears of the cultures of the **B. parapertussis**-like organism used in the experimental inoculum and smears of cultures of the lung lavages obtained at 24 hr after infection exhibited a positive reaction.

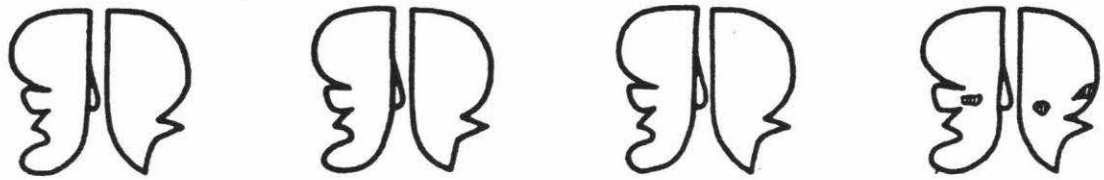
Gross Pathology

The distribution and severity of the gross lesions in both groups is summarised in Fig. 3.4. Variable gross areas of collapse and consolidation were seen in the lungs of all lambs in the inoculated group. The lungs from lambs in the control group showed no significant changes (Fig. 3.5) apart from one lamb which showed focal collapse at p.i.d. 9.

Two types of lesion were seen in the inoculated lambs: small areas of pulmonary collapse and small foci of consolidation.

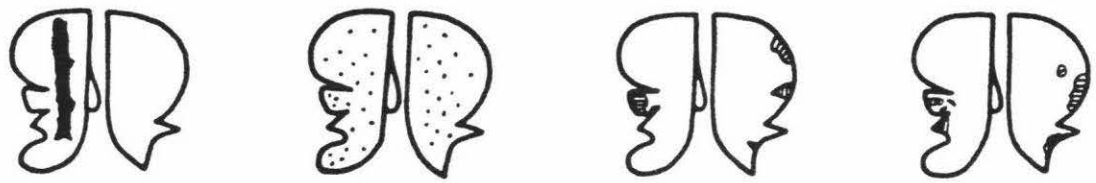
The consolidated foci were more commonly seen in the lungs of lambs sacrificed between 24 hr to 3 days after inoculation and were most severe at 24 hr after inoculation (Fig. 3.6). These areas were usually small, measuring a few mm to 1 cm in size, and dull red in colour. They were irregular, and randomly scattered throughout the pulmonary parenchyma, but sometimes more obvious in the sharp ventral margins of both lungs (Fig. 3.7). The lesions on the lung margins were always wedge-shaped, with their base on the margin. They were usually slightly sunken below the surface of the adjacent lung and were homogeneous in appearance with a smooth pleural surface. All consolidated areas were sharply delineated from surrounding normal tissue. On incision, they were found to be distributed around bronchi or bronchioles.

Group I. Intratracheal inoculation with sterile PBS



E308 E311 E316 E317

Group II. Intratracheal inoculation with the B.parapertussis-like organism



E309 E310 E312 E313

E314 E315 E318 E319

Figure 3.4 Distribution of gross lung lesions in infected and control lambs

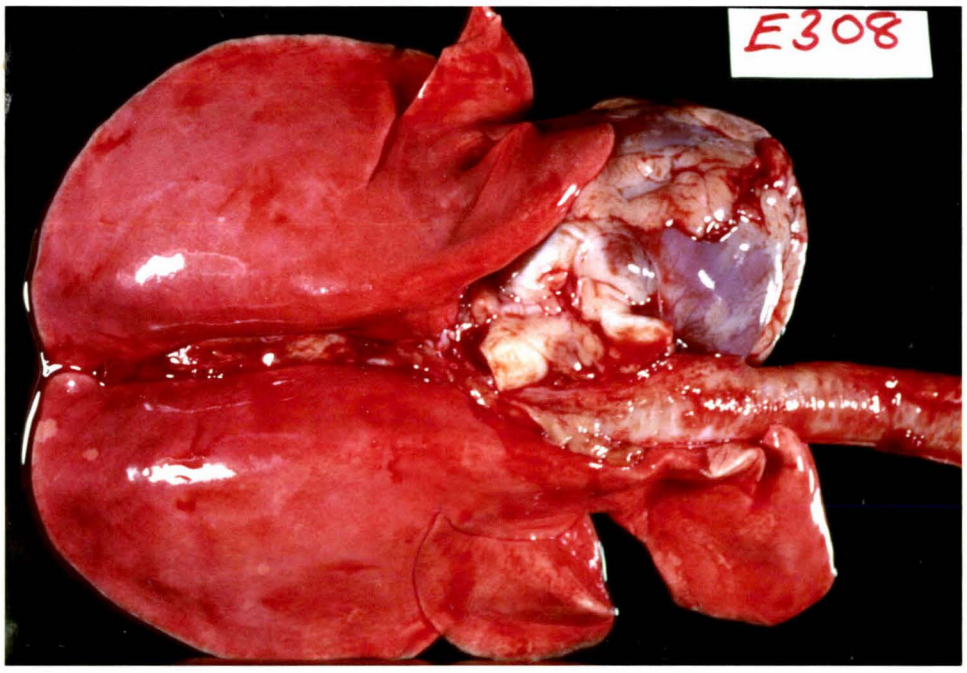
Key Consolidation Collapse

Figure 3.5

Lung from a lamb 24 hr after inoculation of sterile PBS showing a normal appearance.

Figure 3.6

Lungs from a lamb 24 hr after inoculation of the **B. parapertussis**-like organism. A thick dull red band (arrow) of consolidation is present in the right lung roughly parallel with the midline. It extends from the posterior part of the cranial lobe to the mid ventral region of the caudal lobe.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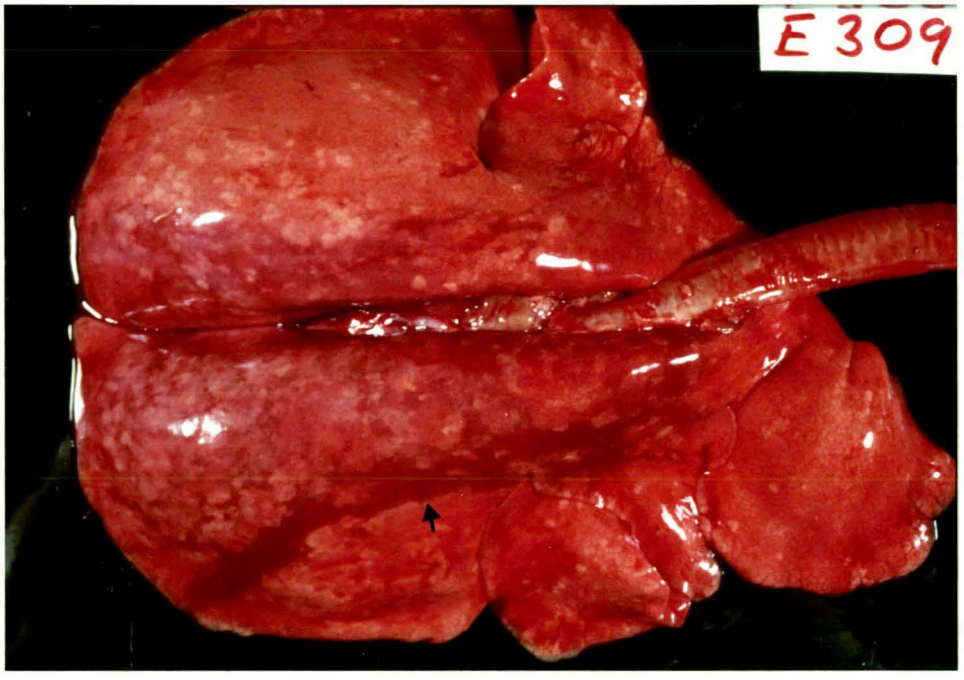

E 309

The areas of collapse had a similar appearance and distribution to the consolidation and were sometimes difficult to differentiate grossly. However, they were usually softer in consistency and more homogeneous on incision. They occurred more commonly in the lambs killed at 5 to 9 days after inoculation (Fig. 3.8).

Bronchoalveolar cytology

Bronchoalveolar washings were harvested from four control and four inoculated lambs. A total of approximately 2.5 x 10^9 bronchoalveolar cells were washed from the control lambs and there were no significant changes in cell numbers or type throughout the experiment (Fig. 3.9). Based on their morphology using a Giemsa stain, 95% of the cells were mononuclear (Fig. 3.10).

In the inoculated group, however, total lavage cell counts increased markedly 24 hr after inoculation. At this time a total of 1.7 x 10^{10}, about 8 times the numbers in the control lavage, were harvested (Fig. 3.9).

The type of cell response in inoculated lambs is illustrated in Fig. 3.9. Neutrophil numbers increased rapidly to a peak of 1.6 x 10^{10} cells/L at 24 hr after infection (Fig. 3.11) and then declined rapidly. Macrophage numbers rose slightly between 0 and 24 hr but became markedly elevated from three days after infection, maintaining these levels until the end of experiment. Five days after infection, total cell numbers were not greatly different from those in the control animals, but most of the macrophages showed varying stages of degeneration. This could be easily detected by light microscopy. The cells contained many vacuoles in their cytoplasm (Fig. 3.12).

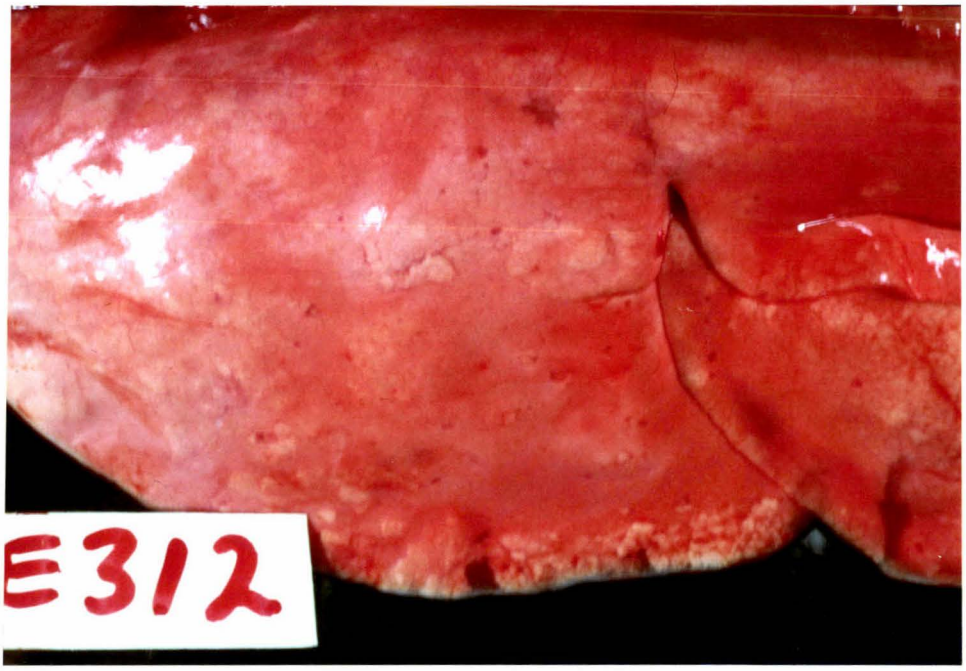
Ultrastracturally, the bronchoalveolar macrophages (BAM) could be divided into two distinct types of cell. One type was similar to the ovine BAM described elsewhere (Sutherland, 1985; Al-Kaissi &

Figure 3.7

Three days after inoculation of the **B. parapertussis**-like organism. Several small irregular foci of dull red pulmonary consolidation and patchy areas of congestion are present on the ventral margins of the right middle and caudal lobes.

Figure 3.8

Nine days after inoculation of the **B. parapertussis**-like organism. Several dull red ares of collapse of varying size persist in the ventral margin of right middle and caudal lobes of the lung.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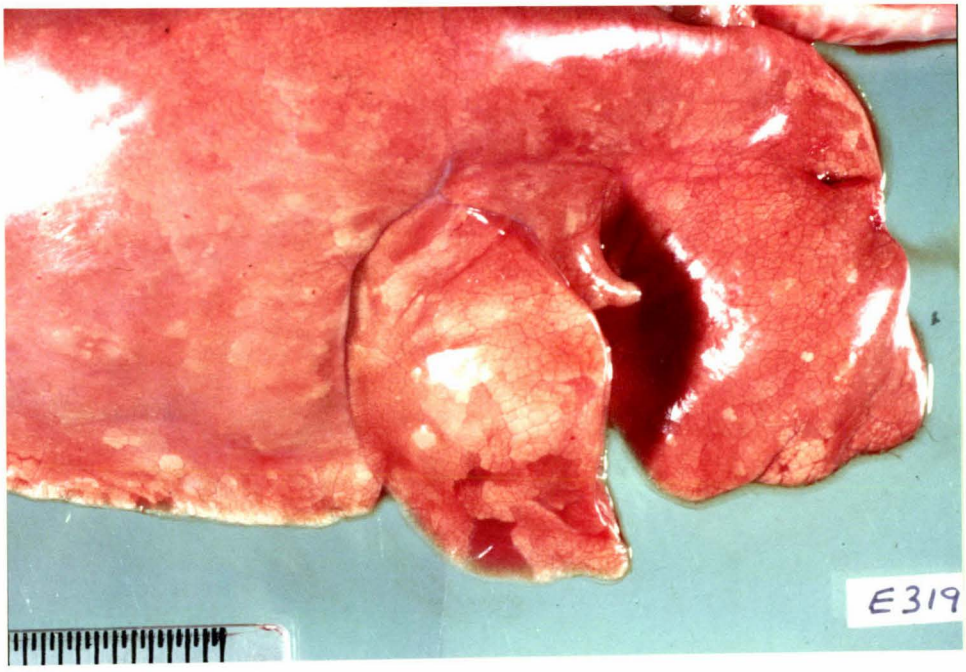
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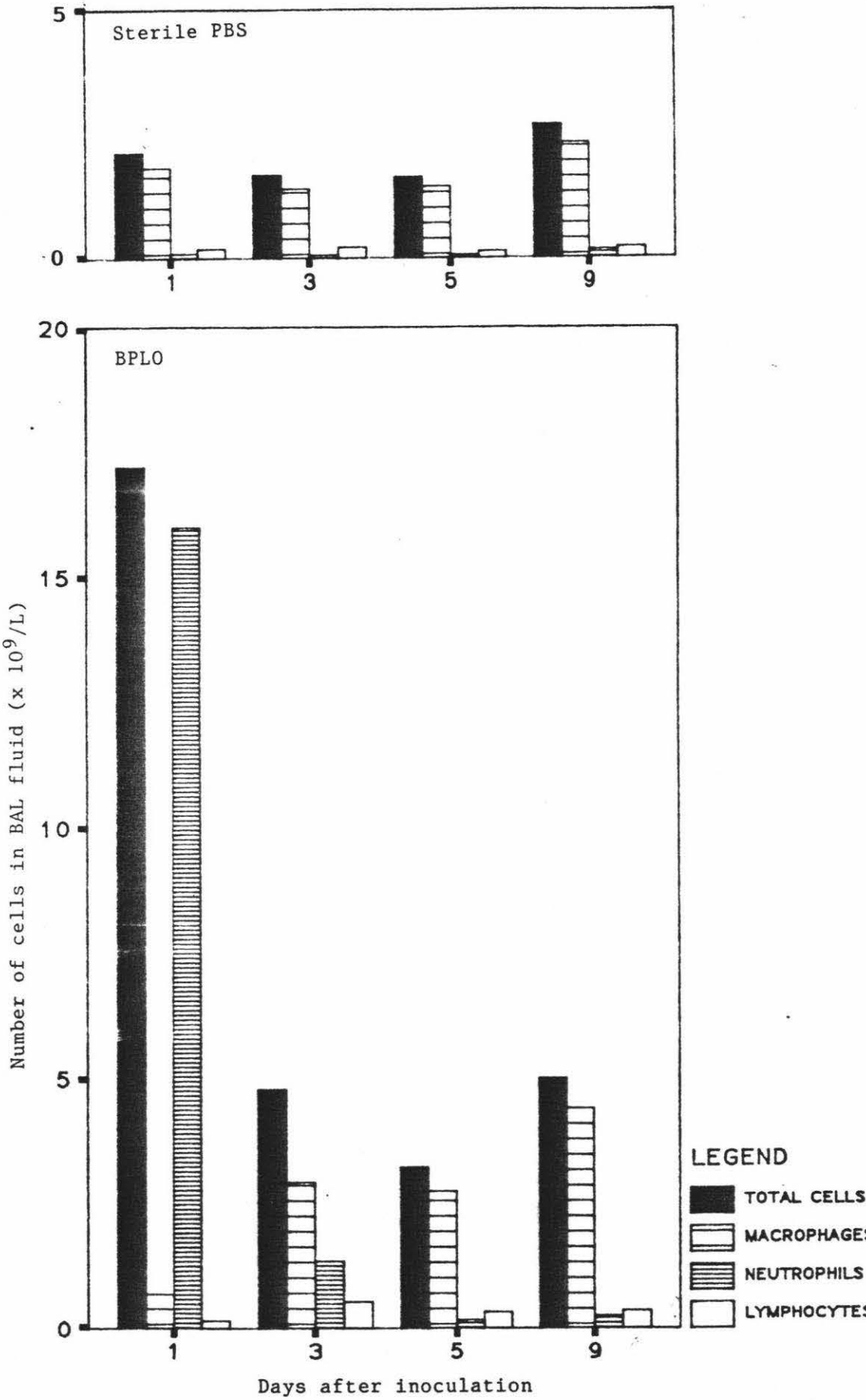


Figure 3.9 Comparison of total bronchoalveolar lavage cell counts between groups of lambs receiving either sterile PBS or the B. parapertussis-like organism (BPLO)

Figure 3.10

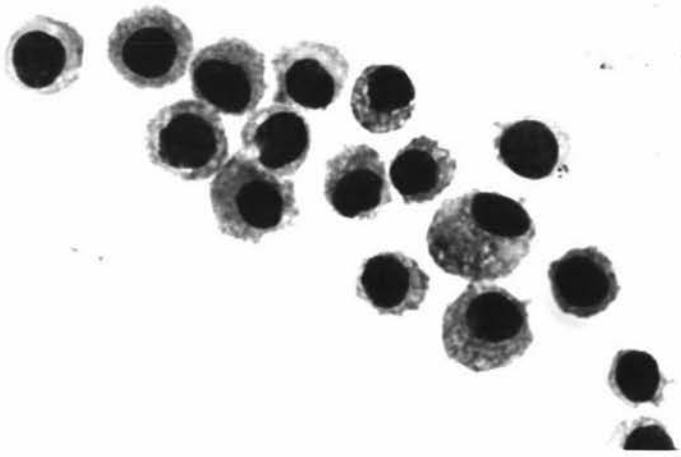
Bronchoalveolar lavage cell from a control lamb killed nine days after inoculation consisting mainly of macrophages. Giemsa stain, x 500

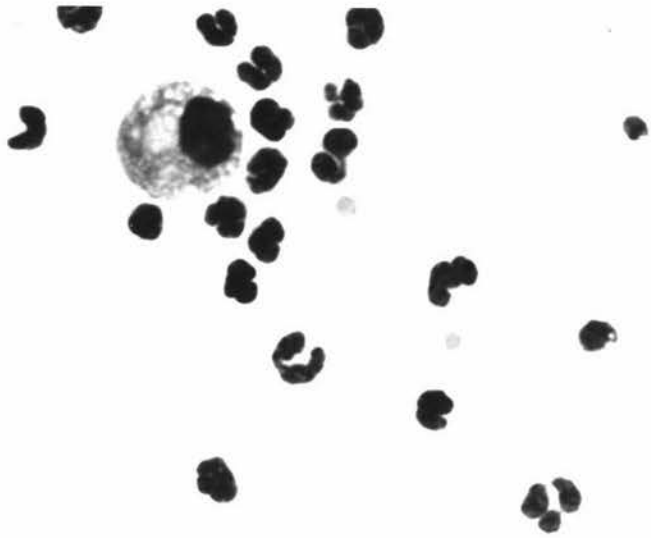
Figure 3.11

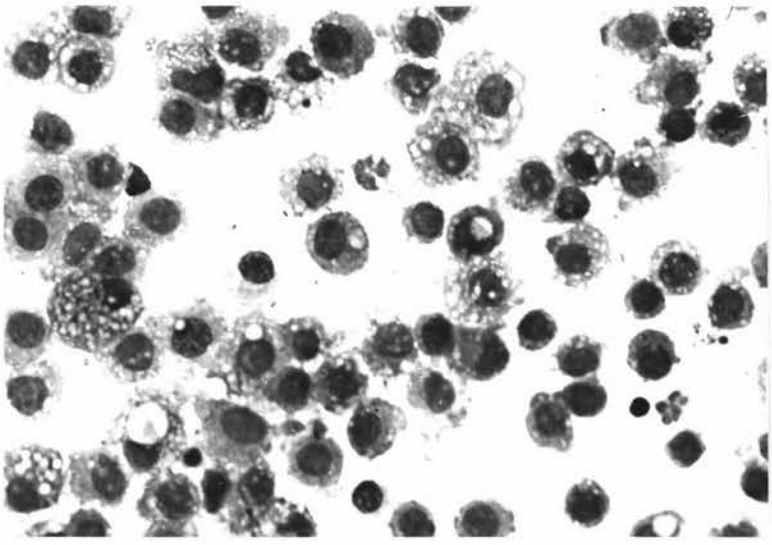
Bronchoalveolar cells from a lamb inoculated with the **B. parapertussis**-like organism and killed 24 hr later. Note that a large proportion of cells are neutrophils. Giemsa stain, x 500

Figure 3.12

Alveolar macrophages recovered from an animal killed nine days after inoculation showing extensive cytoplasmic vacuolation. Giemsa stain, x 250







Alley, 1983). They contained variable numbers of mitochondria, much rough and smooth endoplasmic reticulum and some phagocytic vacuoles. The cell nucleus was usually irregular in shape. The surface of the cells was irregular and extended into many finger-like projections (Fig. 3.13). The other cell type had a relatively electron-lucent cytoplasm containing numerous distended cell organelles. Large numbers of cytoplasmic pseudopods extended from their cell surface (Fig. 3.14). On average, there were about equal numbers of these two cell types.

In contrast, BAM from inoculated lambs showed biphasic changes. Lambs killed between 24 hr and p.i.d. 3 had BAM with the same characteristic morphology as those from control lambs. Nevertheless, the majority of macrophages at this stage were very active and contained large amounts of cellular debris, degenerate and necrotic leucocytes and phospholipid-like bodies within phagocytic vacuoles (Fig. 3.15). Occasionally, different stages of degenerate bacterial organisms resembling the **B. parapertussis**-like organism (Al-Kaissi, 1986) were observed within these enlarged phagocytic vacuoles. Between the phagocytic vacuolar membrane and the organism's cell wall there were varying amounts of moderately stained amorphous material (Fig. 3.16).

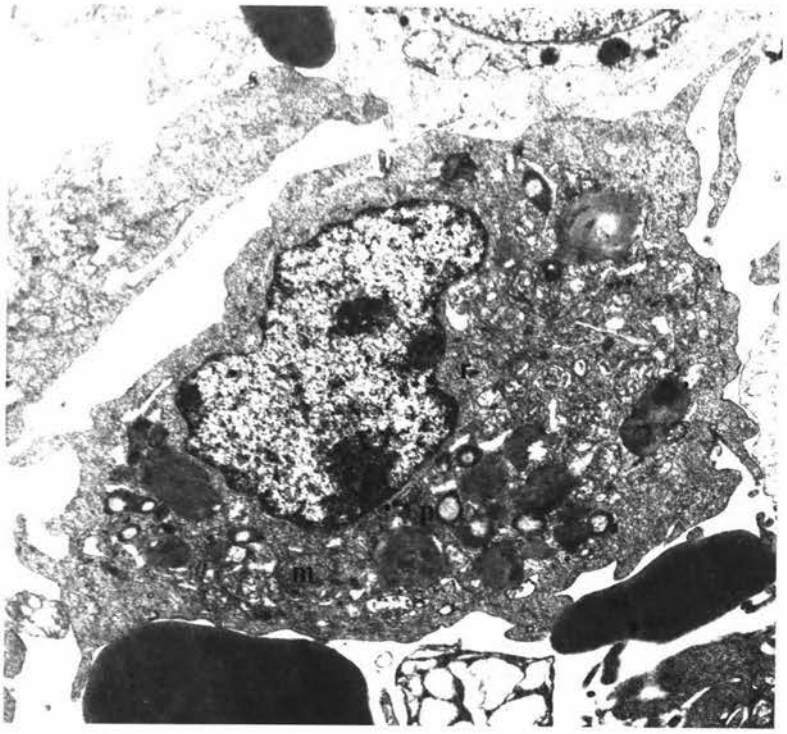
Many BAM harvested from lambs killed at p.i.d. 5 onwards possessed a round pyknotic nucleus (Fig. 3.17) with a thickened nuclear membrane (Fig. 3.17 and 3.18). The cytoplasm of these cells was less electron-dense than normal and contained many large vacuoles but few ribosomes (Fig. 3.17). Most noticeably, the cell surface lacked finger-like projections. Some BAM showed a more advanced stage of degeneration with a sparse cytoplasm containing distended endoplasmic reticulum, swollen mitochondria and decreased numbers of phagocytic vacuoles (Fig. 3.18). The less damaged cells usually contained a slightly pyknotic or almost normal nucleus and a relatively dense cytoplasm including many enlarged phagocytic vacuoles, some of which were frequently fused together. The others were markedly dilated with degenerate cellular debris.

Figure 3.13

An alveolar macrophage recovered from a control lamb given sterile PBS intratracheally and killed 24 hr later. The cytoplasm of the cell is relatively electron-dense and contains numerous characteristic phagolysosomes (p), endoplasmic reticulum (r) and mitochondria (m). TEM x 7,800

Figure 3.14

Another type of alveolar macrophage (M) recovered from a control lamb given sterile PBS intratracheally and killed 24 hr later. The cytoplasm is less electron-dense but contains high numbers of phagolysosomes (p). Organelles are often distended, and numerous pseudopods (P) are present on the cell surface. TEM x 11,200



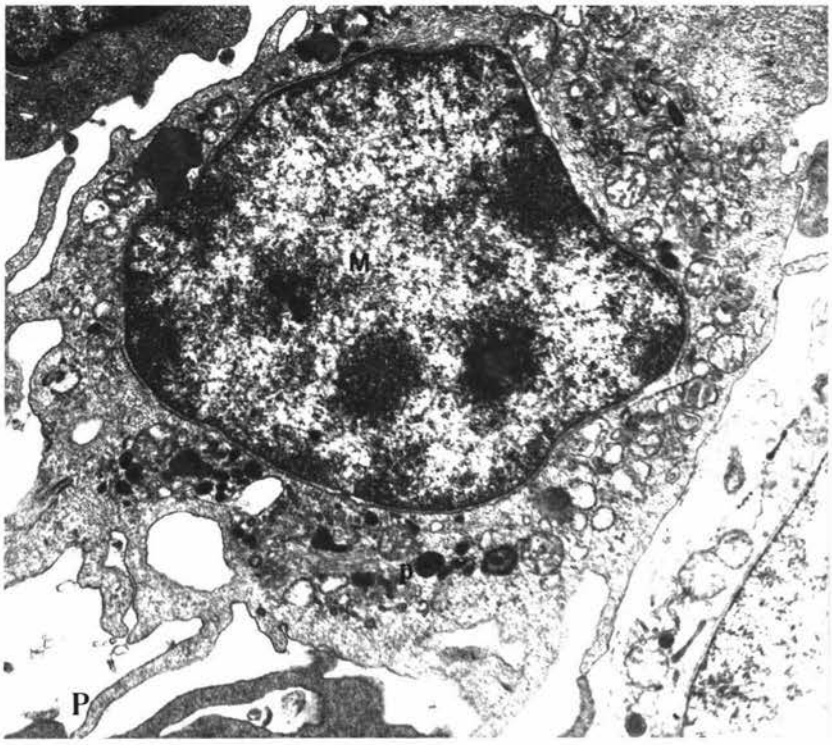
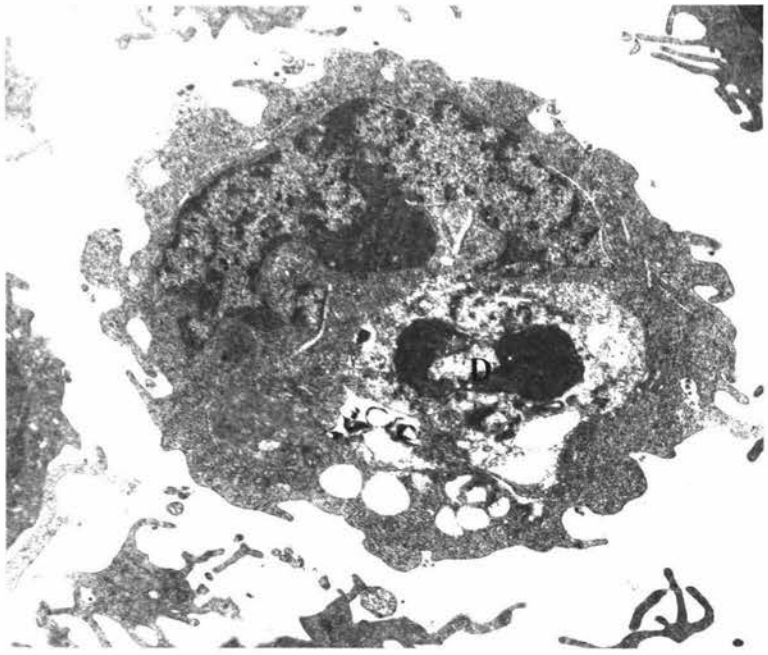

M
P

Figure 3.15

A bronchoalveolar macrophage of lamb inoculated with the **B. parapertussis**-like organism and killed 24 hr later. The cell has a similar morphology to a normal macrophage but contains a degenerate neutrophil (D) in a phagocytic vacuole. TEM x 7,800

Figure 3.16

A macrophage from bronchoalveolar lavage fluid from a lamb inoculated with the **B. parapertussis**-like organism, killed 24 hr later. Two phagocytic vacuoles containing degenerating organisms (arrows) are visible. TEM x 48,600



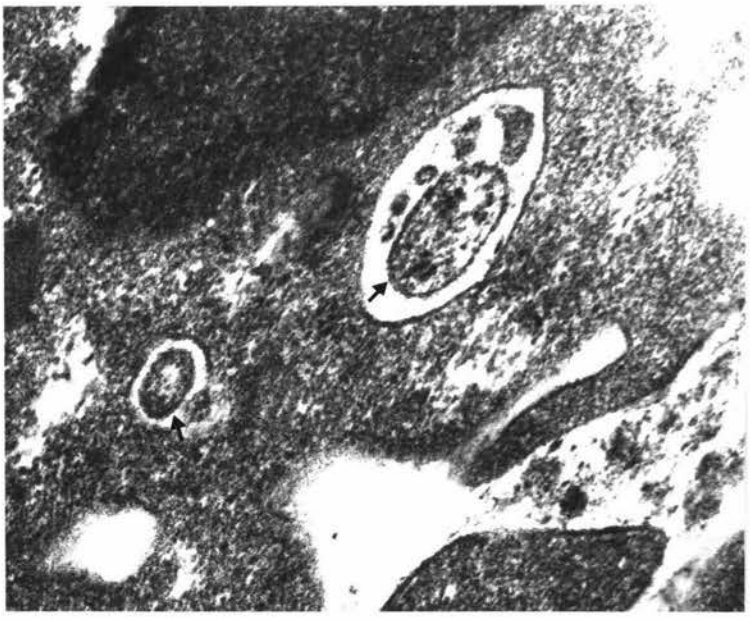
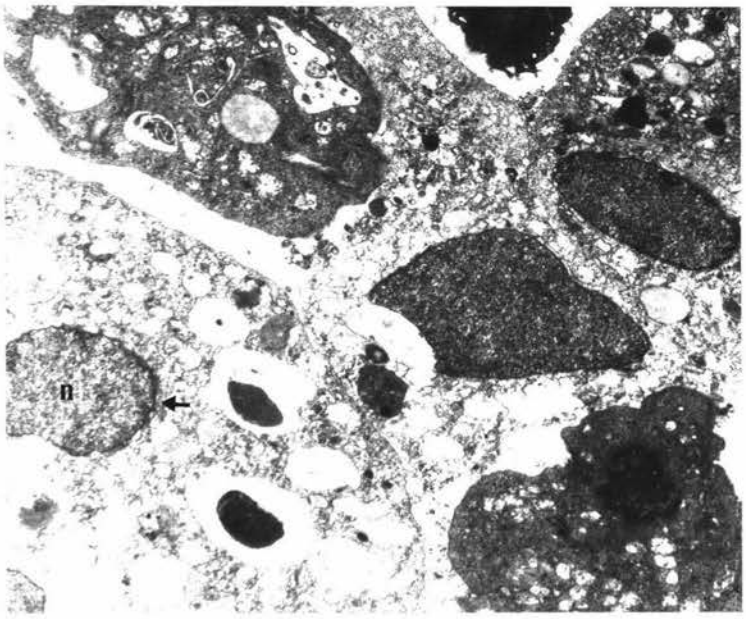


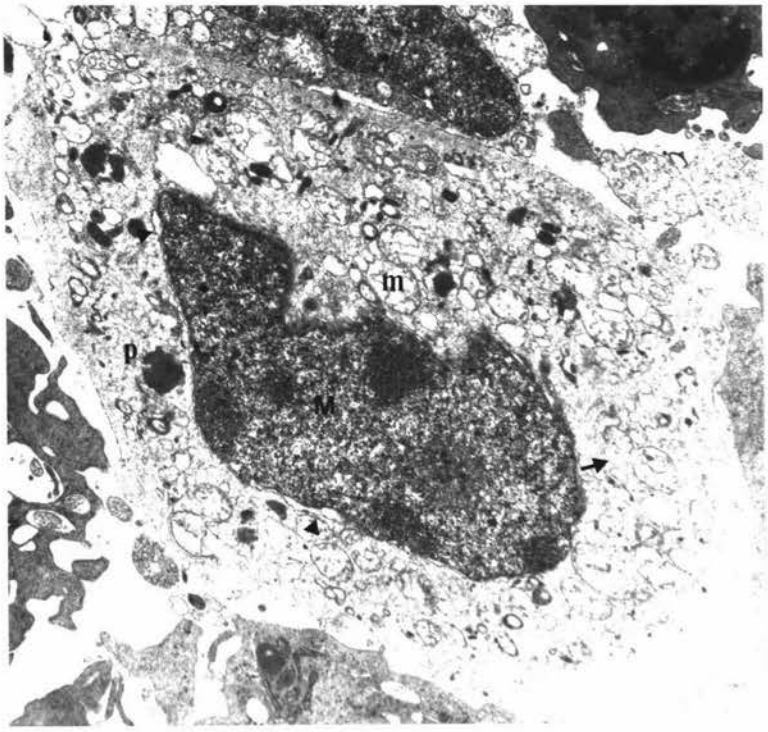
Figure 3.17

Bronchoalveolar macrophages from a lamb inoculated with the **B. parapertussis**-like organism and killed at p.i.d. 5. Most macrophages have undergone varying stages of cellular disruption. The degenerate cells contain a rounded pyknotic nucleus (n) with thickened nuclear membrane (arrow). The cytoplasm is sparse and contains many vacuoles. TEM x 5,200

Figure 3.18

A degenerate alveolar macrophage (M) from a lamb killed nine days after inoculation. The cytoplasm is electron-lucent, shows severe vacuolation of the rough endoplasmic reticulum (arrow) and swelling of mitochondria (m). It has decreased numbers of phagolysosomes (p). The nuclear membrane is separating from the nuclear contents (arrowhead) and the cell surface has lost its pseudopodia. TEM x 7,800


n


m
p
M

Light microscopy

The lesions in the lambs inoculated with the **B. parapertussis**-like organsim could be divided into two main overlapping stages. The first stage from 24 hr to 3 days after inoculation was chiefly exudative in nature, and the latter stage from 5 to 10 days consisted mainly of alveolar collapse and mild proliferative change. The severity of inflammatory reaction varied slightly between individual animals, but the overall pattern of reaction was very similar.

24 hr to 3 days: The most prominent changes observed at this stage were a mild tracheobronchitis and acute bronchopneumonia.

In the lambs killed 24 hr after inoculation, the trachea contained moderate numbers of neutrophils and a few mononuclear cells mixed with some mucus and occasional sloughed epithelial cells (Fig. 3.19). The tracheal epithelium was focally hyperplastic with a slight increase in the number of goblet cells which were secreting an excess amount of mucus. In some areas, the ciliated cells showed ciliary conglomeration (Fig. 3.19). Occasional foci of severe infiltration of neutrophils into the mucosal epithelium were found (Fig. 3.20). The lamina propria of the trachea showed slight to moderate congestion and oedema and infiltration with moderate numbers of mixed inflammatory cells in some areas (Fig. 3.20).

The majority of bronchi and almost all the bronchioles at this stage were plugged with a large number of neutrophils mixed with a few mononuclear cells, mucus and cellular debris (Fig. 3.21). The epithelium of the airways at this level was usually intact and a few inflammatory cells were adherent to the ciliary surface. Early focal lymphoid aggregations were occasionally present in the underlying lamina propria (Fig. 3.22).

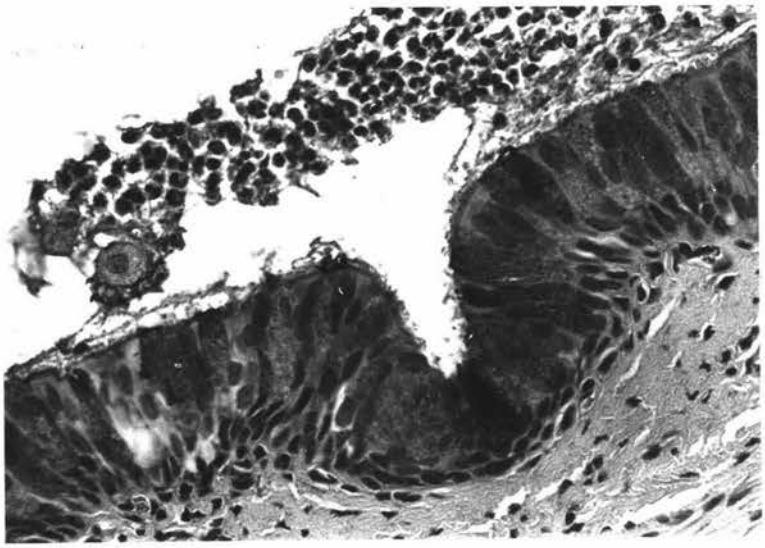
The pulmonary lesions in both lambs necropsied at 24 hr after

Figure 3.19

Exudate in the trachea of a lamb killed 24 hr after inoculation. Large numbers of neutrophils, macrophages, and sloughed epithelial cells mixed with mucus are present in the lumen. H.E. x 250

Figure 3.20

Acute focal tracheitis in a lamb killed at 24 hr after inoculation of the **B. parapertussis**-like organism. Large numbers of neutrophils are present between the epithelial cells of the tracheal mucosa. The lamina propria is infiltrated by many mononuclear cells and a few neutrophils. H.E. x 250



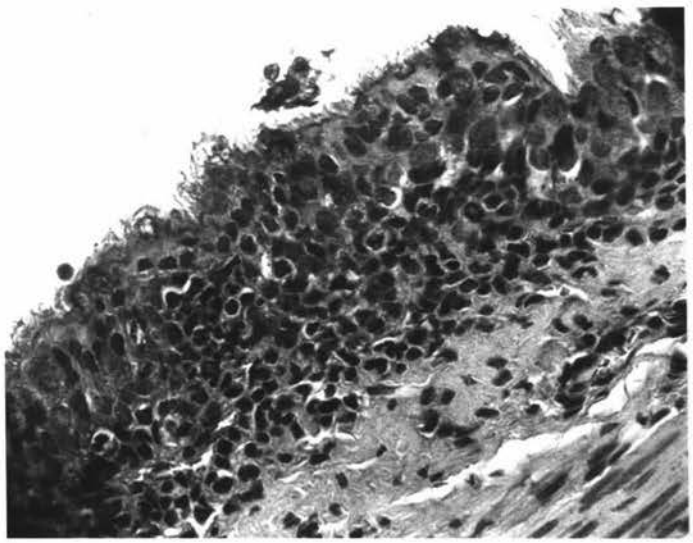
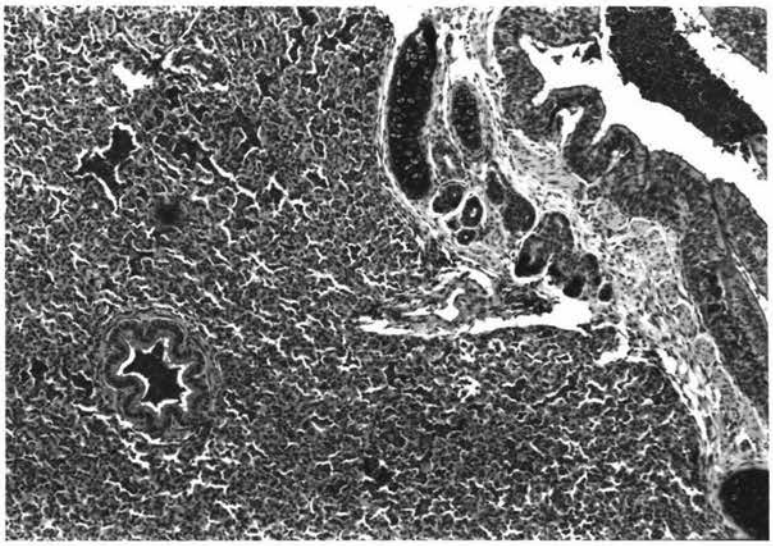


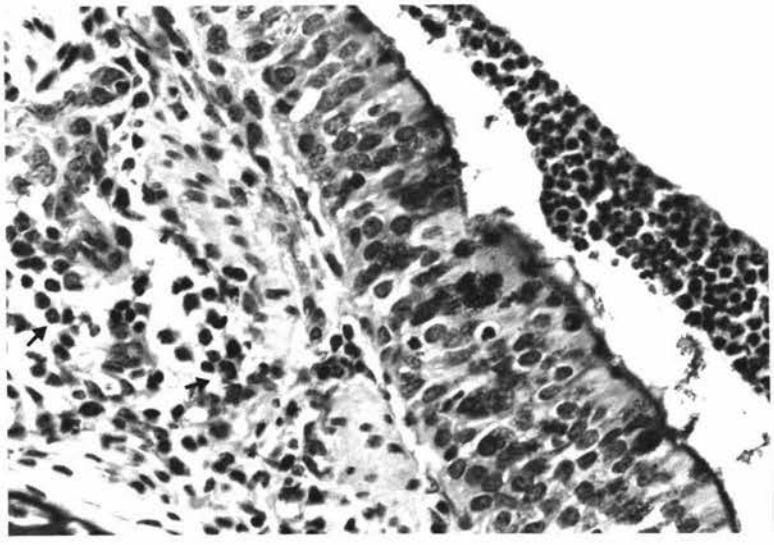
Figure 3.21

Extensive infiltration of neutrophils into bronchi, bronchioles and surrounding alveoli, many of which are collapsed. Lamb killed 24 hr after inoculation with the **B. parapertussis**-like organism. H.E. x 50

Figure 3.22

Bronchus from a lamb killed 24 hr after inoculation showing early lymphoid aggregation (arrows) in the lamina propria with large numbers of neutrophils in the bronchial lumen. H.E. x 250





inoculation were similar and consisted principally of a severe acute inflammatory reaction. The inflammatory areas were usually small and peribronchiolar. Occasionally, however they were large and involved pulmonary parenchyma without particular orientation to bronchioles. These areas were frequently well-demarcated from the surrounding tissue (Fig. 3.23).

The cellular contents of the alveoli were predominantly neutrophils with a few macrophages and lymphocytes (Fig. 3.24). In some sections, the alveoli were filled with dense fibrinous exudate which was mixed with a large number of neutrophils and macrophages (Fig. 3.25). Some macrophages had phagocytosed degenerate neutrophils and other necrotic debris. Occasionally, focal alveolar haemorrhage involving a small number of alveolar spaces was present (Fig. 3.26). The alveoli at the periphery of these consodidated areas were slightly collapsed and contained small numbers of macrophages (Fig. 3.27).

The animals killed at p.i.d. 3 showed similar, but usually milder exudative changes to those seen at p.i.d. 1. One animal showed a more pronounced acute focal tracheitis in which moderate numbers of neutrophils were seen in the submucosa and infiltrating between epithelial cells in the mucosa (Fig. 3.28). In most areas, the tracheobronchial epithelium was slightly hyperplastic but retained well-preserved cilia. Moderate numbers of lymphoid cells had aggregated in the lamina propria of both the tracheobronchial airways and the bronchioles (Fig. 3.29 and 3.30). The exudate in the tracheobronchial lumina contained only a few neutrophils, however, the lumina of many bronchioles contained a variable amount of mucus, cellular debris and inflammatory cells, which were mainly neutrophils (Fig. 3.30).

The main change seen in the lung parenchyma at this stage was severe alveolar collapse in peribronchiolar areas. Within these alveolar spaces, a few mixed macrophages and neutrophils were observed (Fig. 3.31). In some areas many clumps of neutrophils were

Figure 3.23

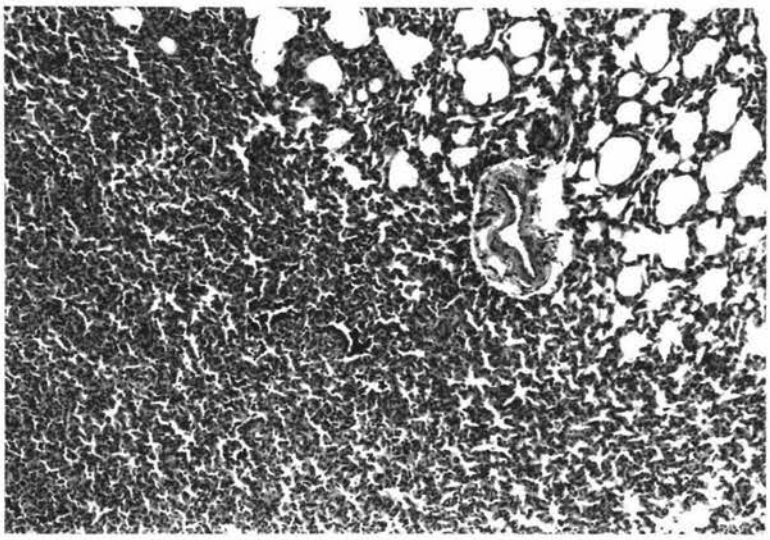
Extensive areas of alveolar collapse with the accumulation of neutrophils in terminal bronchioles. They are well-demarcated from the less affected surrounding areas. Lung from lamb killed 24 hr after inoculation. H.E. x 50

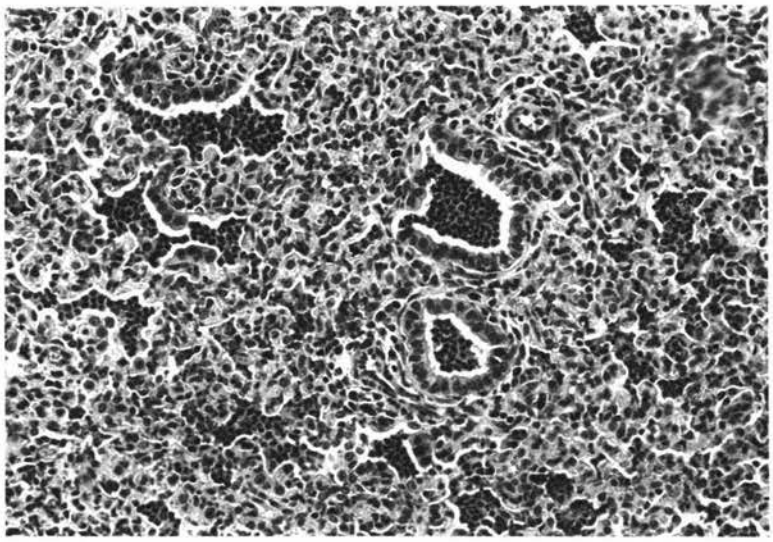
Figure 3.24

Extensive infiltration of neutrophils into terminal bronchioles and collapsed alveolar spaces in a lamb killed 24 hr after inoculation. H.E. x 125

Figure 3.25

Fibrinous exudate mixed with numerous neutrophils and a few macrophages in the alveoli of a lamb killed 24 hr after inoculation. H.E. x 125





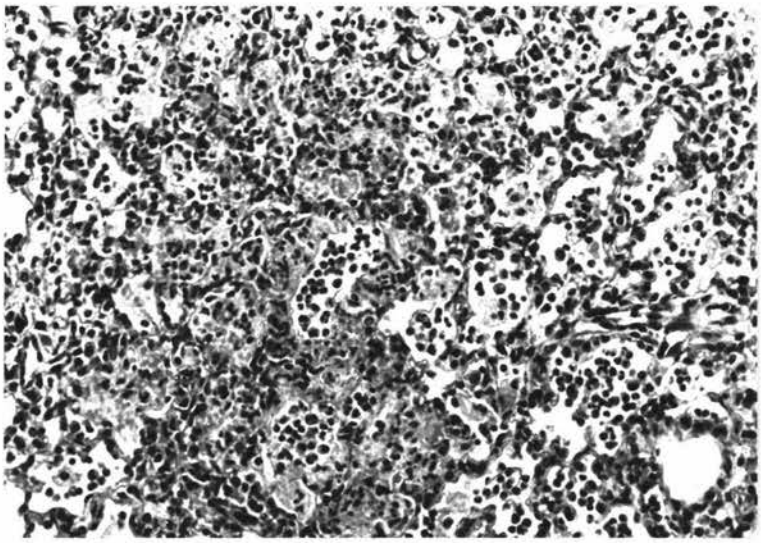
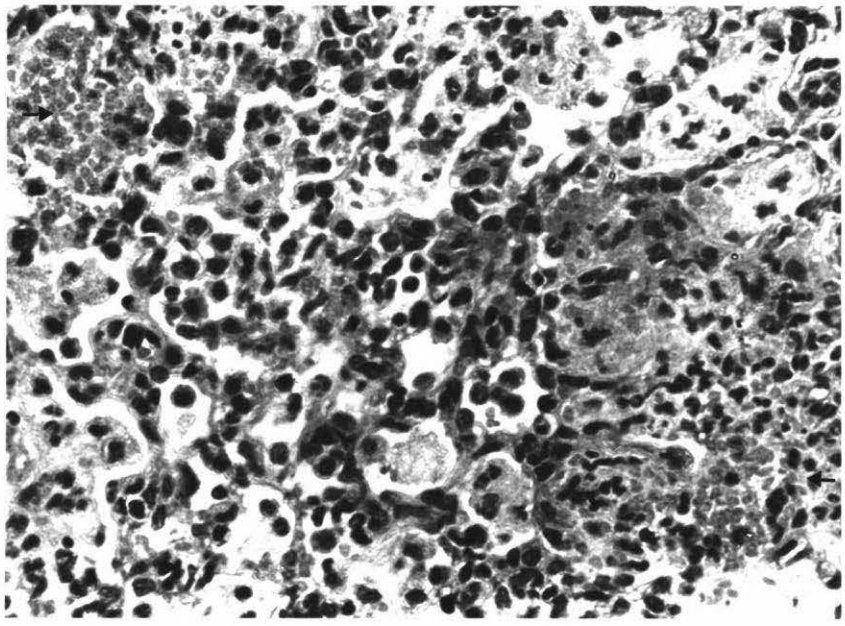


Figure 3.26

Focal intra-alveolar haemorrhage (arrows) with pronounced inflammatory exudation into alveoli of a lamb killed 24 hr after inoculation. H.E. x 250

Figure 3.27

The alveoli at the periphery of the pneumonic areas containing moderate numbers of macrophages and a few neutrophils. Lamb killed 24 hr after infection. Epoxy embedded, Tb x 250



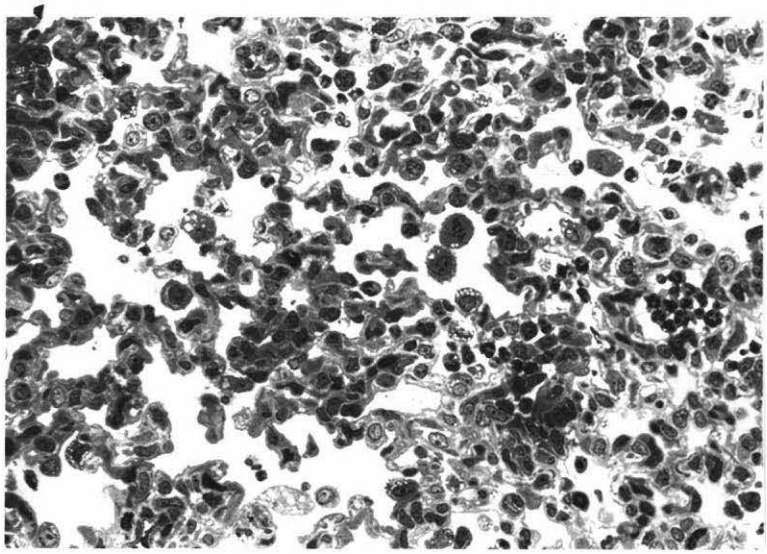


Figure 3.28

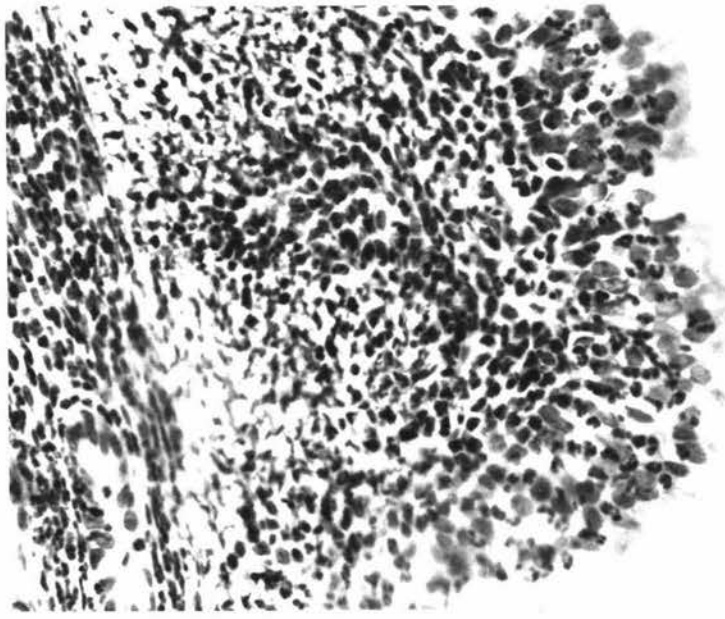
A severe tracheitis in a lamb killed three days after inoculation. Numerous neutrophils have infiltrated between the epithelial cells, and into the lamina propria and submucosa. H.E. x 250

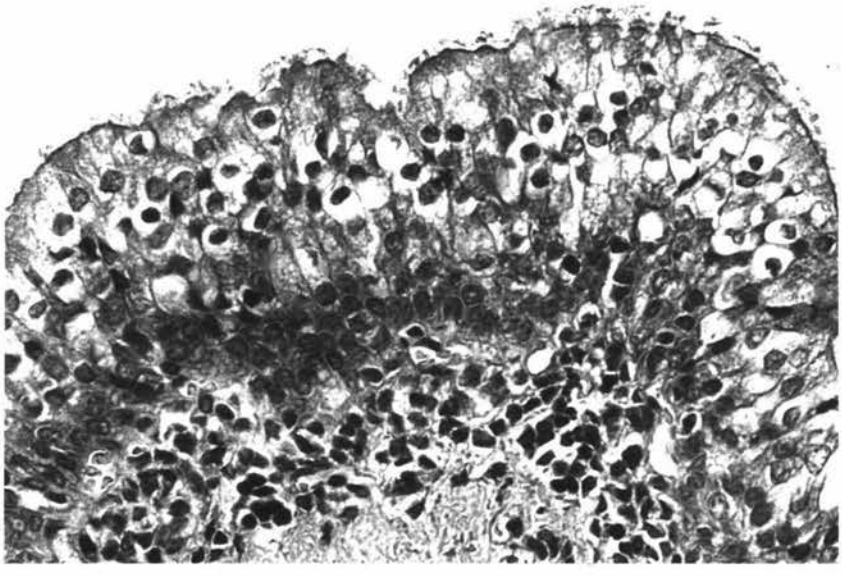
Figure 3.29

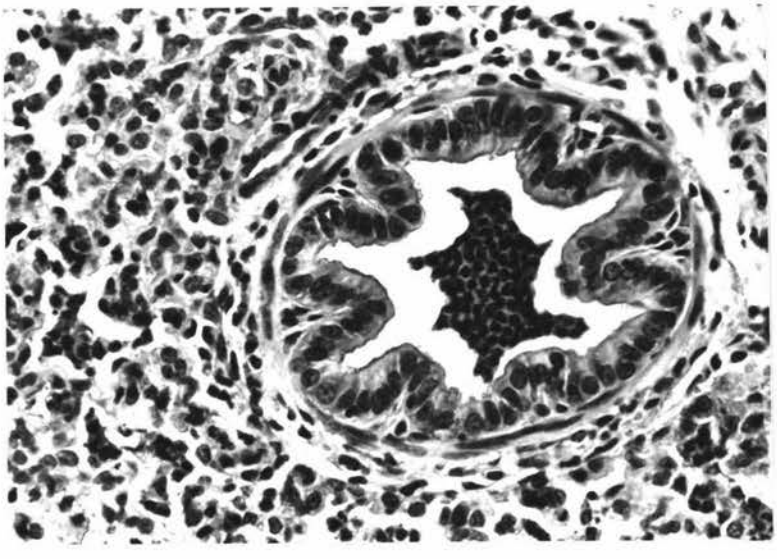
Tracheal mucosa of a lamb killed three days after inoculation. There are moderate numbers of lymphoid aggregations immediately beneath the epithelial layer. H.E. x 250

Figure 3.30

Lung from a lamb killed three days after inoculation showing accumulation of neutrophils in the lumen and neutrophilic infiltration of the peribronchiolar alveoli. H.E. x 250







present in alveolar spaces (Fig. 3.32) while in other areas a moderate congestion and severe exudation of proteinaceous material was also observed (Fig. 3.33).

Early perivascular cellular cuffing was first seen in a lamb killed at p.i.d. 1. The cuffs were mainly composed of lymphoid cells and were associated with areas of neutrophil infiltration in adjacent bronchioles (Fig. 3.34).

5 to 10 days: The predominant changes in the pulmonary tissue during this period were severe alveolar collape, together with mild lymphoid aggregations in perivascular and peribronchiolar tissue.

At p.i.d. 5, alveolar collapse was more extensive and severe than at p.i.d. 3. In the severely collapsed areas, alveolar spaces were reduced to small clefts under low power and contained small numbers of neutrophils (Fig. 3.35). The alveolar septa were slightly congested and markedly thickened due partly to an increase in the number of mononuclear cells (Fig. 3.36). In some areas, an early proliferation of type II epithelial cells could be seen. Almost every alveolar space contained a prominent type II cell and in some areas two adjacent cells were found in the same alveolus (Fig. 3.37). In the less severely affected areas, alveolar spaces contained small numbers of macrophages (Fig. 3.37). In the aerated areas, occasional foci of infiltrating neutrophils in alveolar spaces were disseminated throughout the parenchyma. Exudate within alveolar spaces was present in one animal killed at this stage, but it consisted mainly of mucus and macrophages.

By p.i.d. 9, almost all alveoli were re-aerated and only very occasional macrophages were present within the alveolar lumina (Fig. 3.38).

The control lambs to which PBS was administered showed no significant changes apart from one lamb killed at nine days after administration which showed an area of mild alveolar collapse with

Figure 3.31

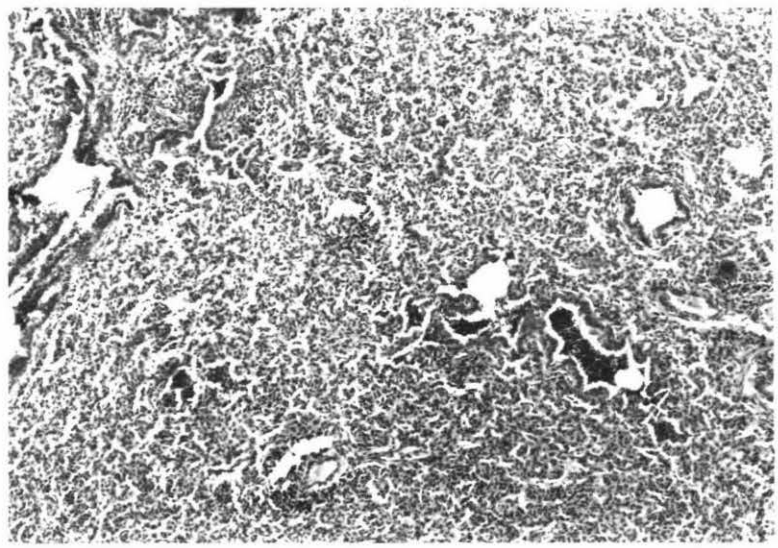
Extensive areas of alveolar collapse with the accumulation of moderate numbers of neutrophils in terminal bronchioles and alveolar spaces. Lamb killed three days after inoculation of the **B. parapertussis**-like organism. H.E. x 50

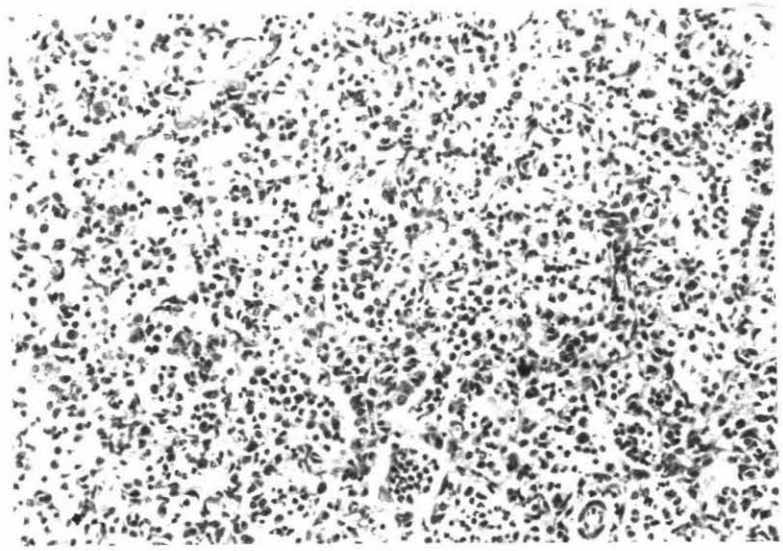
Figure 3.32

Intensive infiltration of numerous neutrophils and a few macrophages into the alveoli. Lamb killed three days after inoculation. H.E. x 125

Figure 3.33

Focal severe fibrinous exudate in the alveoli at three days after inoculation. Moderate numbers of neutrophils are embedded in the exudate. H.E. x 250





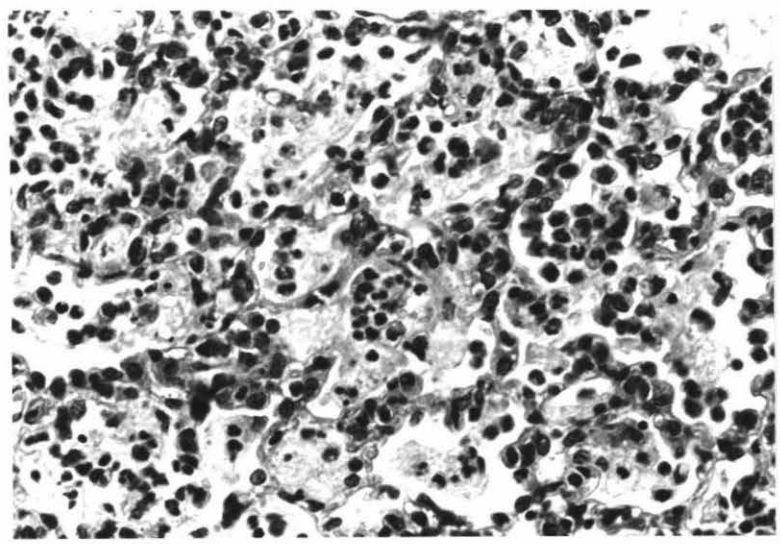
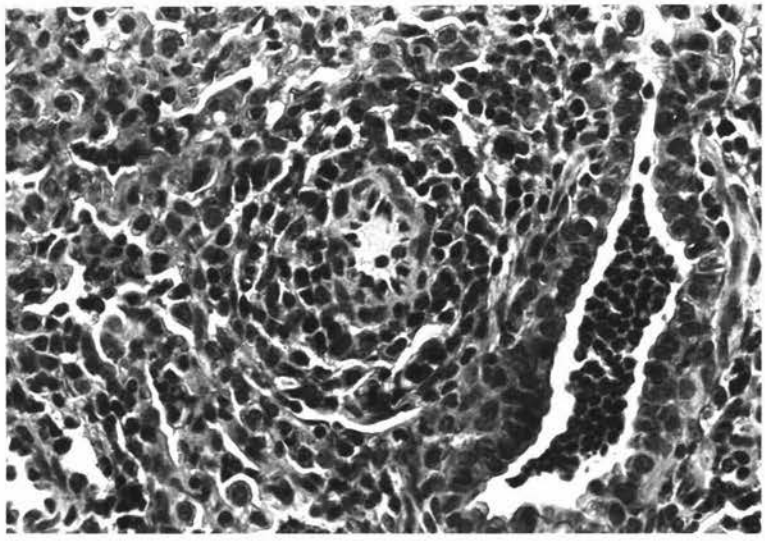


Figure 3.34

A perivascular cuff in a lamb 24 hr after inoculation composed mainly of mononuclear cells. These cuffs were usually associated with bronchioles containing inflammatory exudate. H.E. x 250

Figure 3.35

The lung from a lamb five days after inoculation with the **B. parapertussis**-like organism. The collapsed alveoli present a featureless appearance and only very mild inflammatory change can be seen at low magnification. H.E. x 125



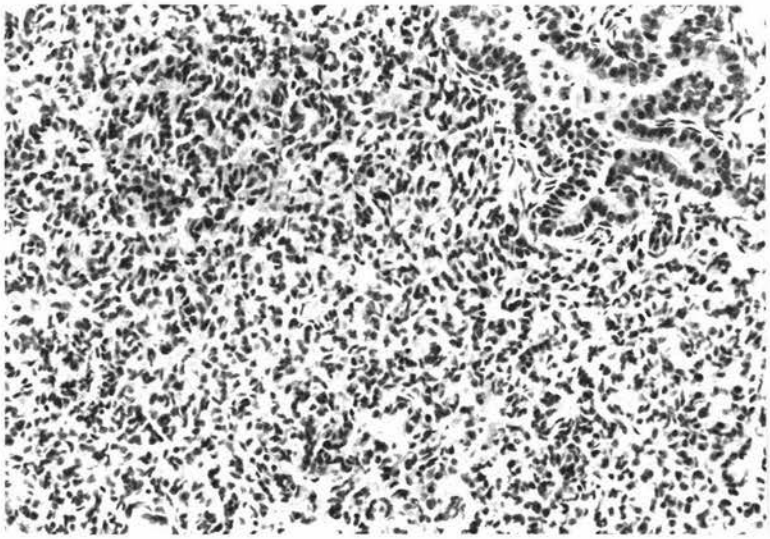


Figure 3.36

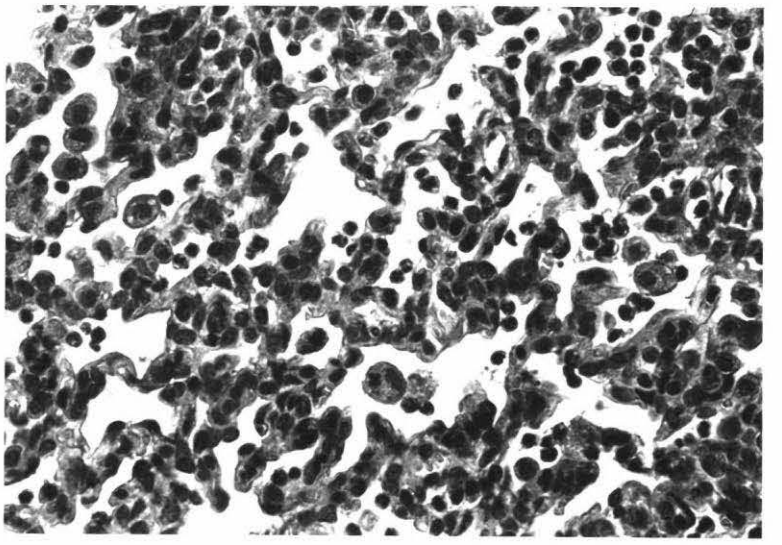
Alveoli from the lung of a lamb killed five days after inoculation. The hypercellularity of alveolar septa is mainly due to infiltrating mononuclear cells. The alveoli contain many macrophages and a few neutrophils. H.E. x 250

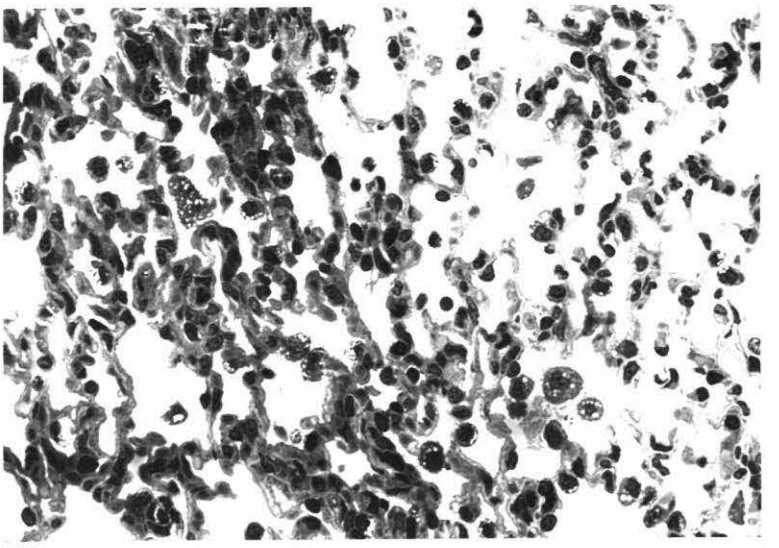
Figure 3.37

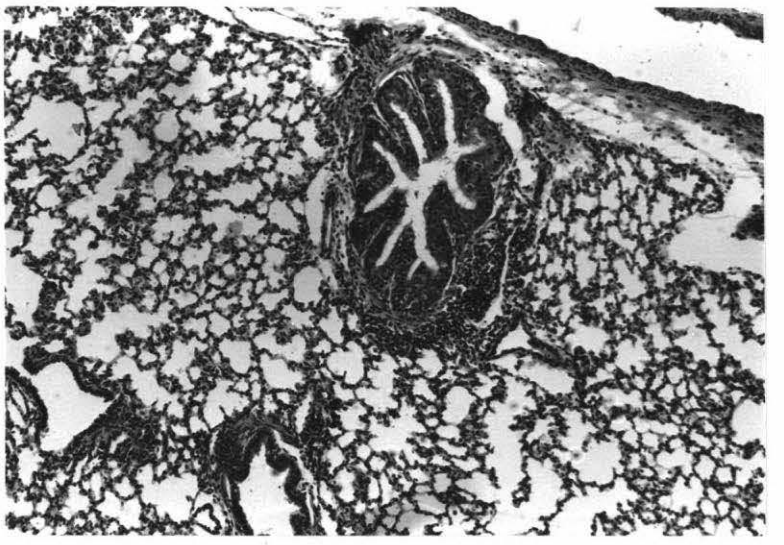
Lung from lamb killed five days after inoculation of the **B. parapertussis**-like organism showing mild proliferation of alveolar type II cells. Almost every alveolar space in the section contions a prominent type II cell. The alveoli in the less severely collapsed areas showed infiltration of small numbers of macrophages. Epoxy embedded, Tb x 250

Figure 3.38

Lung nine days after inoculation showing re-aeration of most alveoli. Mild to moderate perivascular and peribronchiolar lymphoid cuffs were invariably present. H.E. x 50







infiltration of small numbers of mixed inflammatory cells.

Topographical morphology

The surface of the trachea and bronchi from the control lambs showed a similar topographical morphology to ovine tracheobronchial airways described elsewhere (Marisaay, 1976; Al-Kaissi, 1986) although the ciliary density was greater in the present study (Fig. 3.39). Two main cell types were recognized, a common ciliated cell and a non-ciliated cell with microvilli which was interspersed between ciliated cells (Fig. 3.39 and 3.40). Due to the high density of cilia, goblet cell openings and mucus secretions from submucosal glands were not commonly observed. Most areas of the trachea and bronchi were free of mucus and cellular debris. Occasionally secretions of goblet cells could be seen fixed **in situ** (Fig. 3.41).

The topographical changes in the trachea and bronchi of inoculated lambs were similar in severity and pattern. At p.i.d. 1, the trachea and bronchi showed no significant alteration in ciliary density, however, most of the ciliated epithelial surface of the middle and lower trachea and bronchi were covered by a thick layer of adherent secretions containing numerous inflammatory cells in which neutrophils predominated (Fig. 3.42 and 3.43). In the areas free of inflammatory exudate, many cilia were clumped together (Fig. 3.44), and among them moderate numbers of coccobacilli were trapped. These bacteria were mainly located near the ciliary apex and there was no evidence of specific interaction between the organisms and cilia. Occasionally, firm attachment of the organisms to broken cilia, found within the inflammatory exudate, was observed.

Moderate numbers of ciliated cells in some areas of the trachea and bronchi were observed partially extruded from the luminal surface at p.i.d. 3 (Fig. 3.45). These extruded cells were often moderate to severely swollen and were characterized by disoriented cilia to which bacteria were adherent (Fig. 3.46). The amount of surface exudate at this stage had decreased and consisted mainly of

Figure 3.39

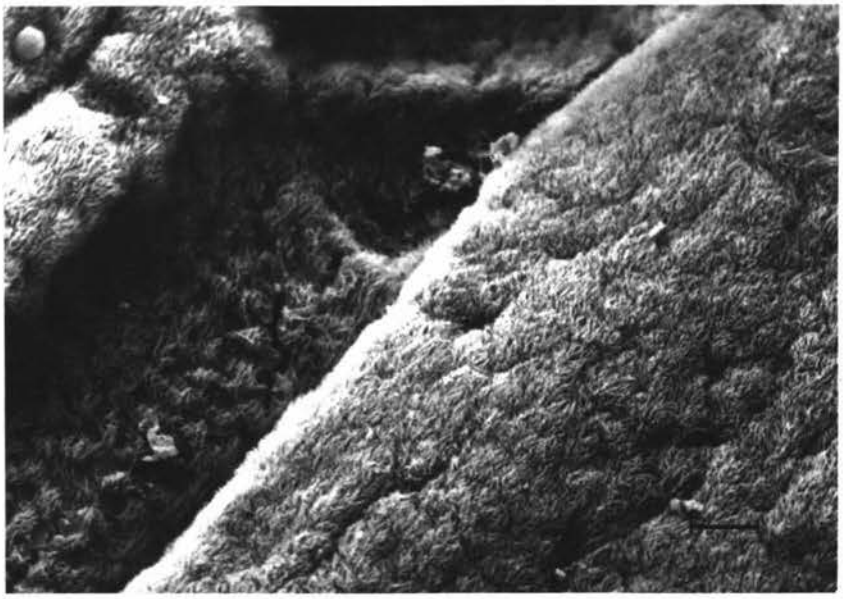
The epithelial surface of the trachea from a control lamb composed almost entirely of ciliated cells. Inset: Higher magnification showing the normal feature of cilia. SEM Bar 5 μm

Figure 3.40

The epithelial surface of a bronchus from a control lamb. Ciliated cells cover the majority of the luminal surface but many non-ciliated cells are also present in some areas. SEM Bar 10 μm

Figure 3.41

The epithelial surface of a bronchus from control lambs containing occasional non-ciliated cells, openings of goblet cells and submucosal glands. The figure illustrates the discharging secretion of a goblet cell fixed **in situ** (arrow). SEM Bar 1μm



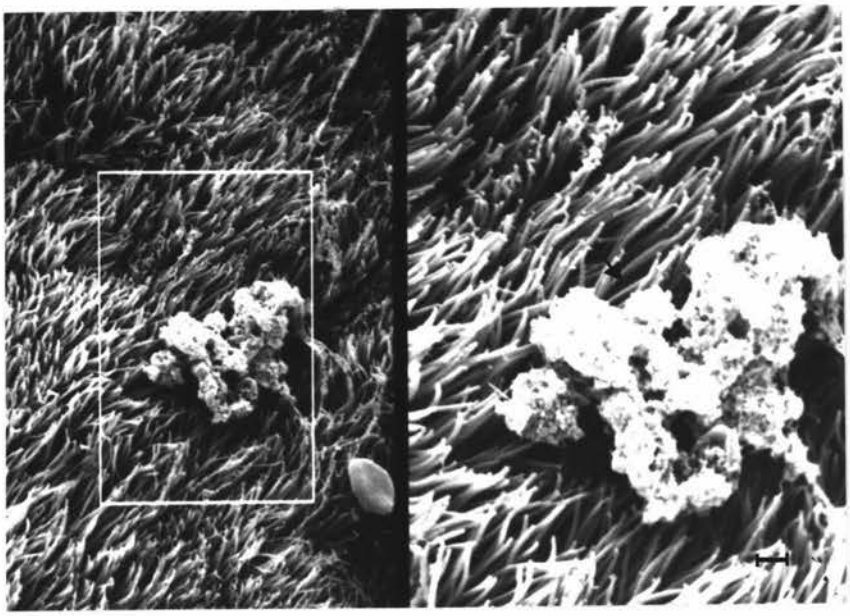
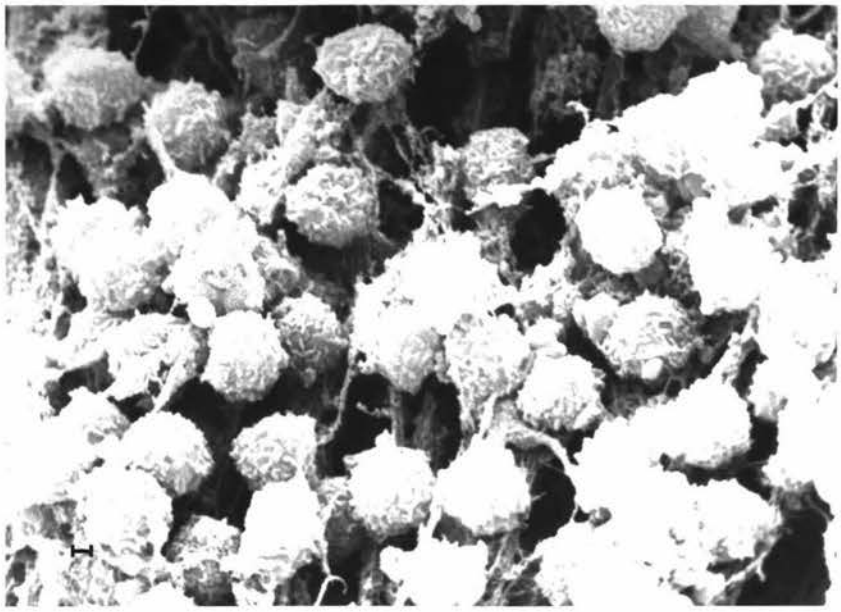


Figure 3.42

Mucosal surface of the trachea of a lamb 24 hr after inoculation of the **B. parapertussis**-like organism. The tracheal ciliated epithelium is almost completely covered by a dense layer of inflammatory cells, probably neutrophils, together with some mucous strands and granules. SEM Bar 1μm

Figure 3.43

The luminal surface of a bronchus from a lamb 24 hr after inoculation showing a thick layer of mucus mixed with neutrophils (N) and macrophages (M) covering areas of the ciliated surface. A variable number of coccobacilli (arrows) are trapped in the mucus. SEM Bar 1μm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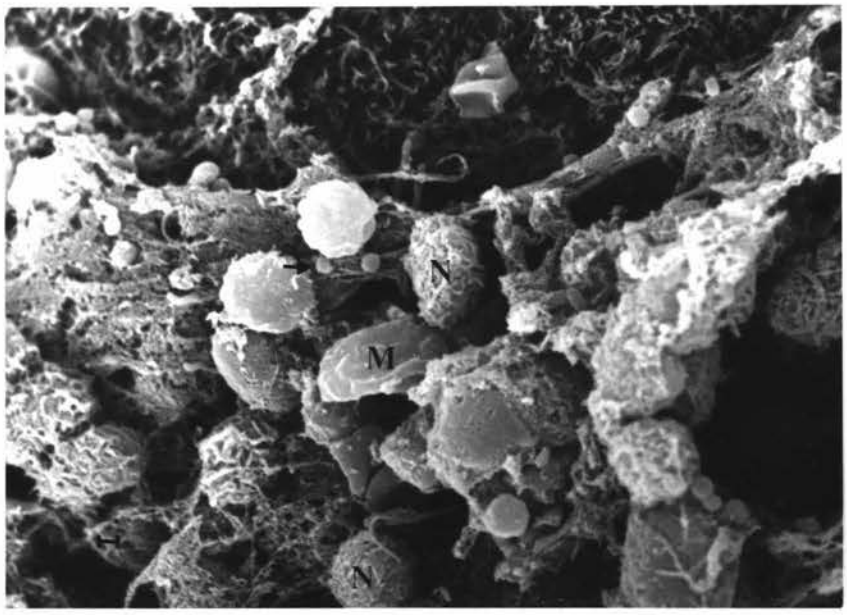
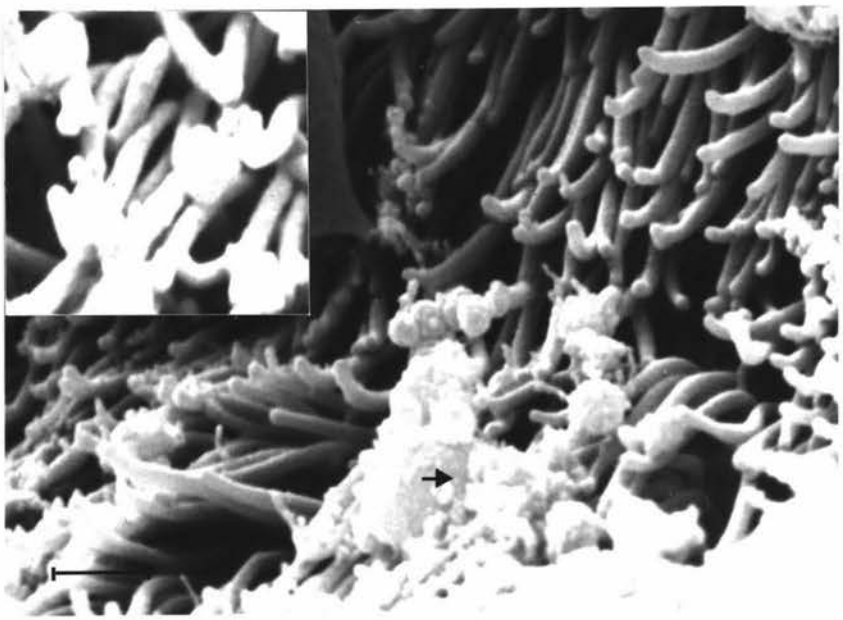

I
N
M
N

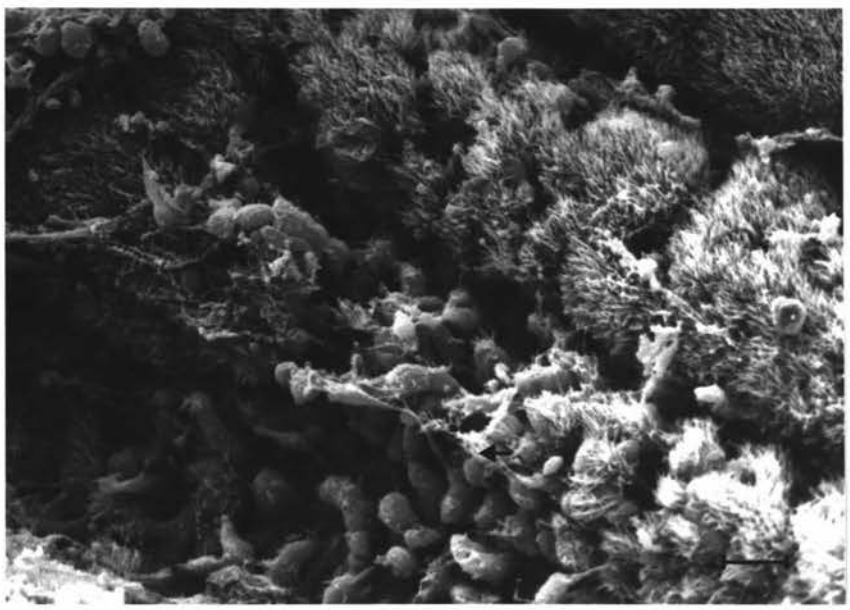
Figure 3.44

Tracheal mucosa in a lamb 24 hr after inoculation showing many cilia forming conglomerations with adjacent cilia (arrow). Inset: High magnification showing coccobacilli (arrow) on the surface of the cilia. SEM Bar 1µm

Figure 3.45

Ciliated cell damage in the bronchial mucosa three days after inoculation with the **B. parapertussis**-like organism. Severe swelling and extrusion of ciliated cells were noted (arrow). There are only few cilia retained on the surface of these disrupted cells. SEM Bar 20µm





mixed inflammatory cells and a few mucous strands (Fig. 3.47).

By p.i.d. 5, only a little exudate remained and there were only small numbers of bacteria scattered between the tops of cilia (Fig. 3.48). The ciliary density was slightly decreased at this time but this change was more marked at p.i.d. 9 when the luminal surface was irregular and contained many non-ciliated cells. The surface of these non-ciliated cells had a few microvilli at the periphery of the cell and contained large pits or pores. There was no association between the bacteria and these cells although the remaining cilia were colonized by small numbers of orgamisms.

Electron microscopy

CONTROL ANIMALS:

The cells lining the trachea, bronchi, bronchioles and alveoli showed normal ultrastructural features similar to those described by Kikkawa et al. (1965), Alley (1975a) and Al-Kaissi (1986).

INOCULATED ANIMALS:

Trachea and Bronchi At 24 hr after inoculation there were no detectable ultrastructural changes in the tracheobronchial epithelium. By p.i.d. 3, a few ciliated epithelial cells showed a severe reduction in ciliary density. The cytoplasm of these cells often contained swollen mitochondria and the endoplasmic reticulum was severely dilated and contained vacuolated cisterni (Fig. 3.49). These degenerative changes were more severe at p.i.d. 9 when the cells were markedly swollen and had a reduced electron density due to the presence of large amounts of cystic endoplasmic reticulum (Fig. 3.50).

Atypical cilia were occasionally observed in the tracheal epithelium of some animals killed at p.i.d. 9. Two, three or more true cilia were fused to form giant, bizarre structures which were

Figure 3.46

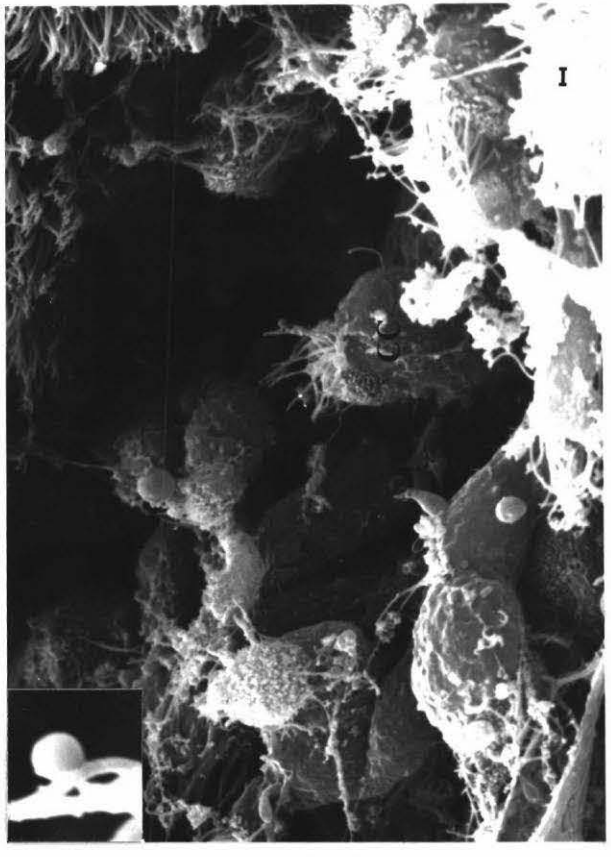
Bronchial epithelial surface of a lamb three days after inoculation showing partial exfoliation of the affected ciliated epithelium. A ciliated columnar epithelial cell (CC) with an attached organism is shown extruding from the luminal surface. Inset: Higher magnification to show an organism attached to a cilium of the extruded cell. SEM Bar 1µm

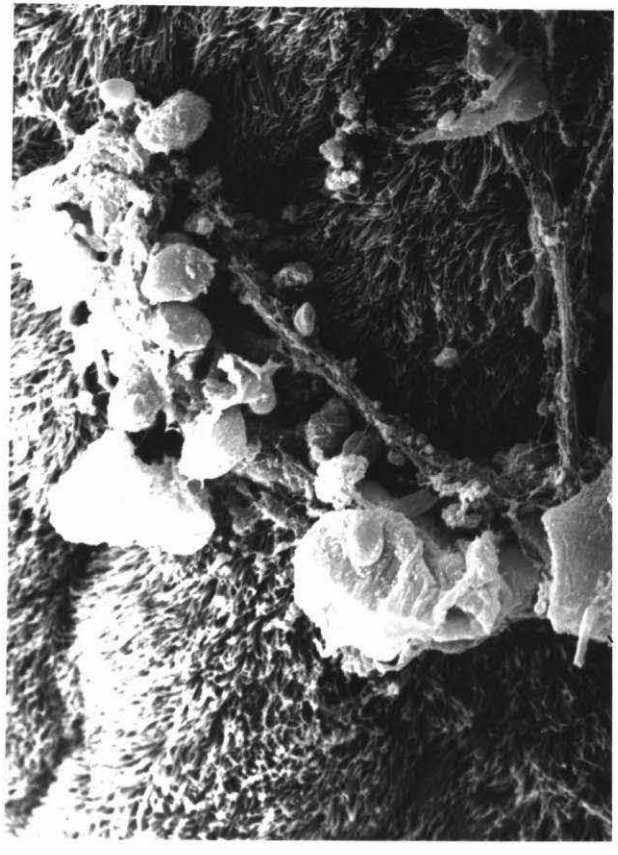
Figure 3.47

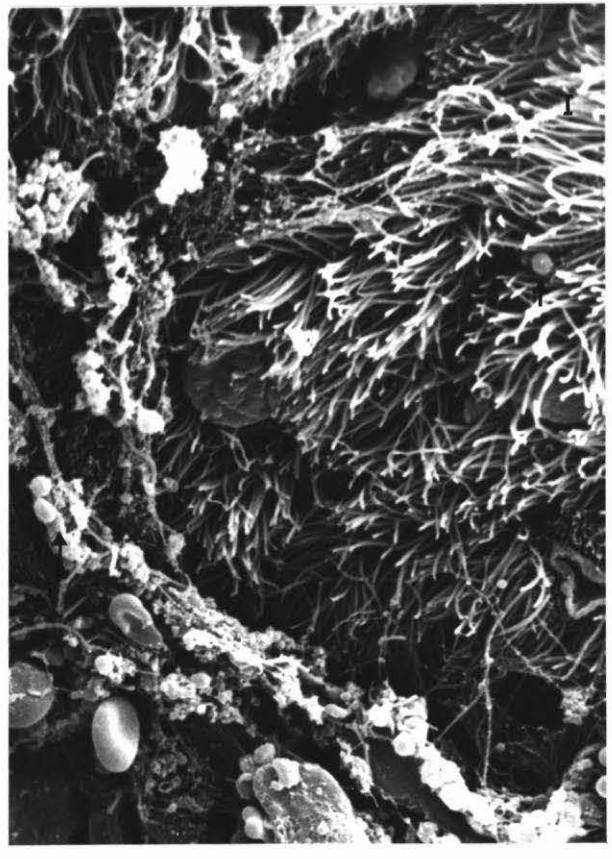
Tracheal mucosal surface three days after inoculation showing a decrease in inflammatory cell exudate and the amount of mucus. SEM Bar 10µm

Figure 3.48

The bronchial mucosa at five days after inoculation. It is covered with a little exudate and very small numbers of bacteria (arrows) randomly scattered over the tops of cilia. The ciliary density has decreased slightly. SEM Bar 1µm







enveloped by the outer cell membrane (Fig. 3.51). They often occupied about 3 to 5 normal ciliary spaces and were similar in length to the adjacent normal cilia. There were no significant changes found in the cytoplasm of either affected cells or surrounding epithelial cells.

The morphological changes in the bronchi were very mild and limited in distribution. Occasionally, a few bronchial epithelial cells showed vesiculation of their endoplasmic reticulum 24 hr after inoculation (Fig. 3.52).

Bronchioles A variety of cytopathological changes were seen in bronchioles examined at p.i.d. 1 to 5. At p.i.d. 1, the most striking feature seen was the infiltration by small numbers of neutrophils into the epithelium (Fig. 3.53). Fewer cilia than normal were seen on the luminal epithelial surface although the numbers of basal bodies appeared to be within the normal range at this stage (Fig. 3.54). The microvilli in these ciliated cells were often very thick, long and densely distributed (Fig. 3.54) The submucosa of the bronchioles was usually moderately oedematous.

The lumina of bronchioles contained a considerable amount of cellular debris mixed with neutrophils and a few macrophages (Fig. 3.53). Some of the degenerate inflammatory cells and cellular debris were undergoing phagocytosis by neutrophils. Bacteria were not seen either in intracellular or extracellular areas. Some neutrophils contained cytoplasmic vacuoles, but were otherwise normal.

The most salient feature observed in the bronchiolar epithelium 3 to 5 days after inoculation was the development of large projections of cytoplasm from the apex of ciliated cells (Fig. 3.55). The blebs were usually isolated in distribution but became very dense in some sections. Only one bleb was present on each cell and they were never seen on non-ciliated cells. The protrusions frequently occupied up to half the width of the cell apex and were one to two times the length of the cilia. They enclosed a few cilia

Figure 3.49

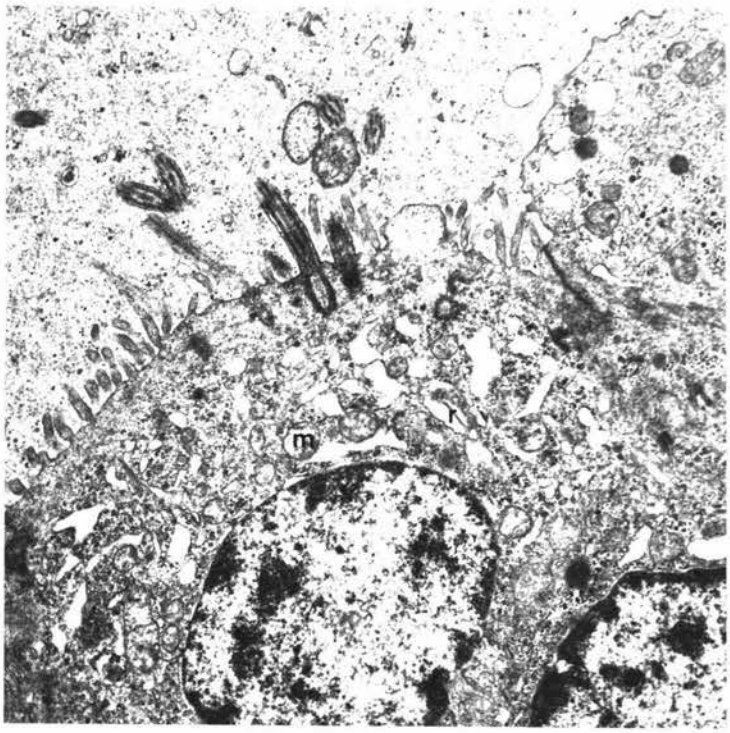
Tracheal epithelial cells from a lamb three days after inoculation with the **B. parapertussis**-like organism. The ciliated cells show severe reduction in ciliary density. The cytoplasm contains swollen mitochondria (m) and enlarged endoplasmic reticulum (r) which has markedly dilated cisterni. TEM x 11,200

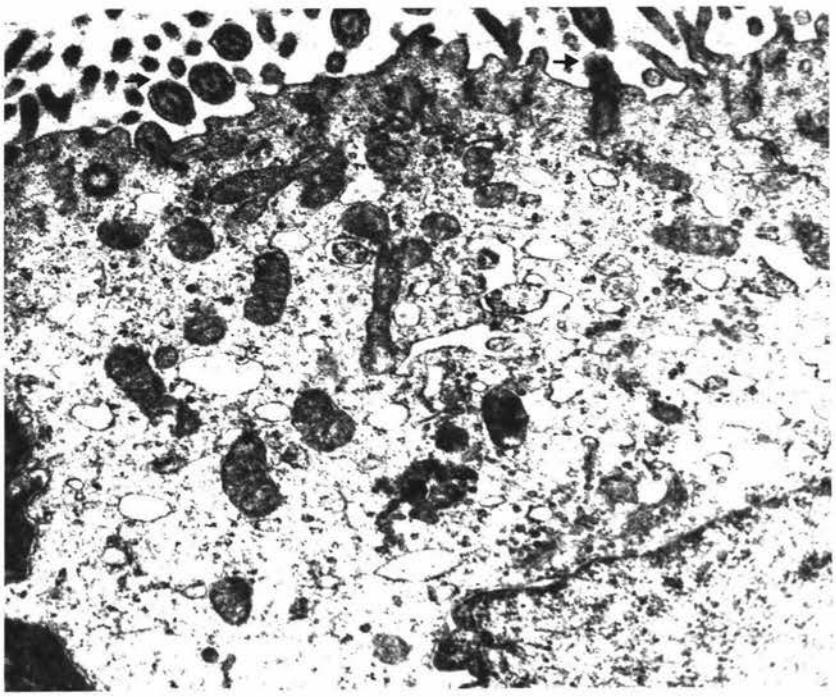
Figure 3.50

The tracheal epithelium of a lamb killed nine days after inoculation showing only a few retained cilia (arrows) and marked distention of cellular organelles. TEM x 15,300

Figure 3.51

An atypical cilial structure (arrow) observed in the tracheal epithelium of a lamb nine days after inoculation. Multiple true cilia have fused to form a giant, bizarre structure which is enveloped by the outer cell membrane. TEM x 11,200


m



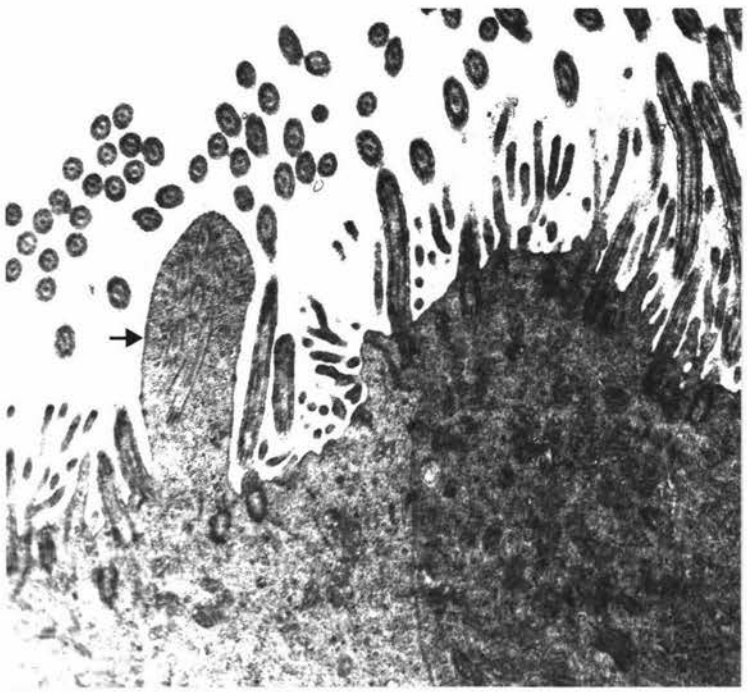
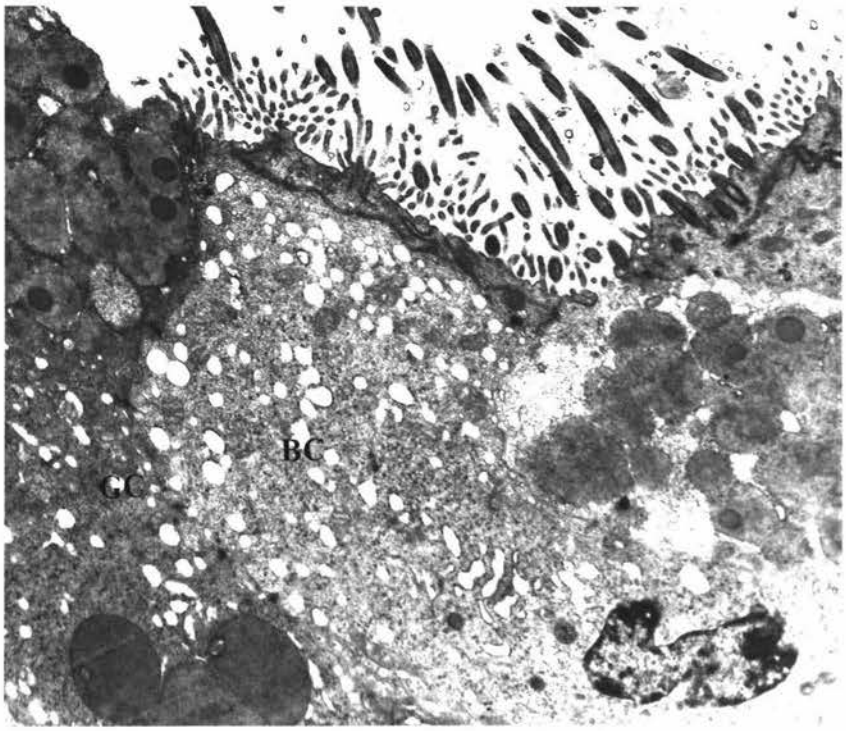


Figure 3.52

Cytoplasmic vacuolation in bronchi of a lamb killed 24 hr after inoculation. Note the vacuoles present in the cytoplasm of goblet cells (GC) and a brush cell (BC). TEM x 7,800

Figure 3.53

A bronchiole from a lamb killed 24 hr after inoculation of the **B. parapertussis**-like organism. There are several neutrophils, and some cellular debris in the lumen. Note a neutrophil (N) infiltrating between the epithelial cells. The submucosa is oedematous. TEM x 3,400


BC
GC

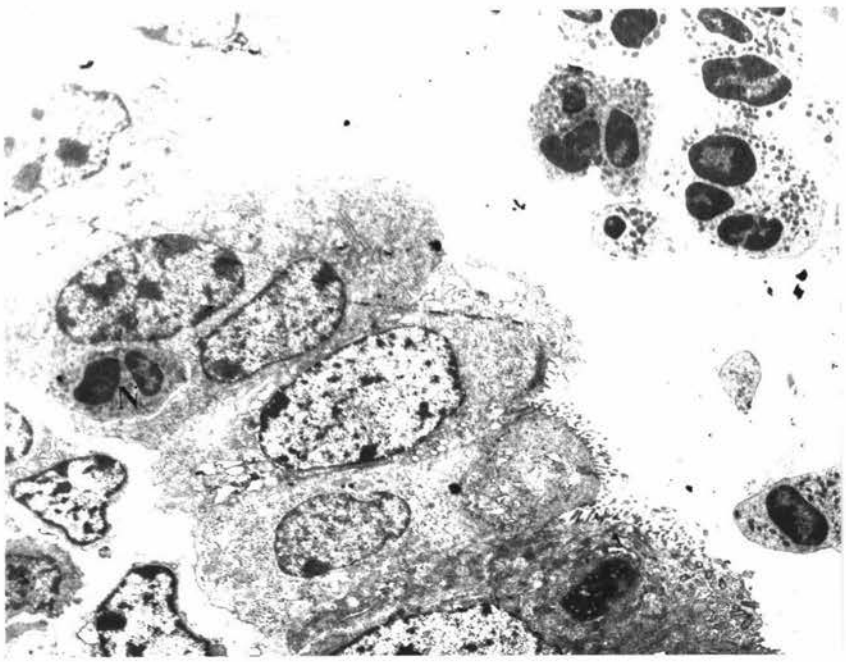
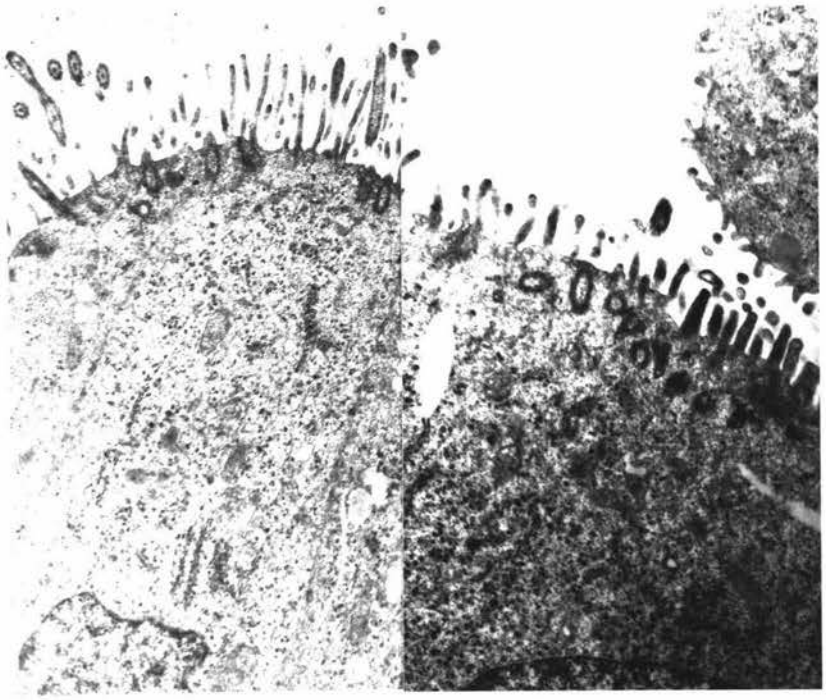

N

Figure 3.54

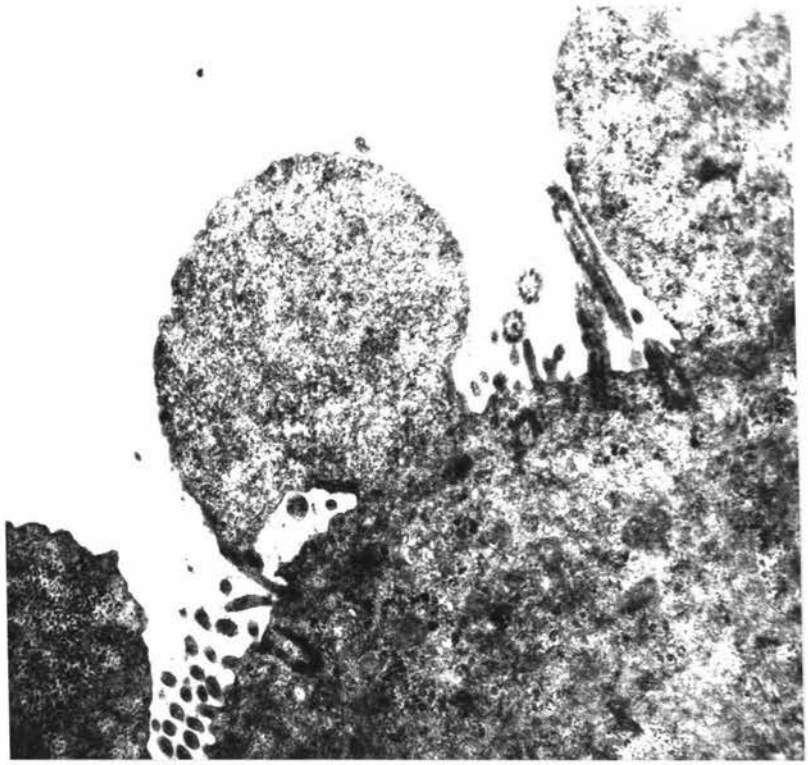
The bronchiole of lamb killed 24 hr after inoculation showing marked decrease in numbers of cilia. Instead, the microvilli on the luminal surface are higher (a) and thicker (b) than normal, and densely distributed. TEM, x 11,200

Figure 3.55

Bronchiolar epithelium three days after inoculation of the **B. parapertussis**-like organism showing cytoplasmic projections from the ciliated cells. TEM x 15,300



(a) (b)



and contained basal bodies in their cytoplasm but no other organelles. The remainder of the apex of affected cells had few cilia but the adjacent ciliated cells showed no significant changes.

Alveoli At p.i.d. 1, extensive mild to moderate damage to both type I and type II alveolar epithelial cells, inflammatory exudate in the alveolar spaces and oedema of the alveolar interstitium were the main ultrastructural features. The type I cells in most areas showed an increase in density and shrinking of their cytoplasmic extensions (Fig. 3.56). Occasionally, focal sloughing of type I cells was seen, exposing the underlying basement membrane (Fig. 3.57). Swelling and vesiculation of type I cells were seen at p.i.d. 3 (Fig. 3.58). These lesions were usually extensive and associated with the formation of small cytoplasmic blebs or ridges. Diffuse swelling of type I cells continued to be encountered in all lungs until p.i.d. 9.

The degree of damage suffered by type II alveolar epithelial cells was relatively less severe than that seen in type I cells. At p.i.d. 1, the cells exhibited mild degenerative changes consisting of cytoplasmic swelling, swelling of mitochondria, distention of the endoplasmic reticulum, loss of microvilli (Fig. 3.59) and occasional desquamation from the basement membrane (Fig. 3.60). The desquamated cells were often degenerating, but they were easily recognized by their characteristic remaining laminated bodies and microvilli (Fig. 3.61). By p.i.d. 3, further degenerative changes were seen in the cytoplasm, and disintergation of the plasma membrane was apparent in many cells (Fig. 3.62). The nuclei of the severely damaged cells exhibited migration of chromatin (Fig. 3.62) and distention of the nuclear envelope (Fig. 3.63). At p.i.d. 9, besides the universal moderate degenerative changes, some type II cells showed more significant changes than previously. They included pyknosis, disappearance of the nuclear membrane and marked thickness of large segments of plasma membrane (Fig. 3.64). Almost all the organelles in these cells were extremely distended.

Figure 3.56

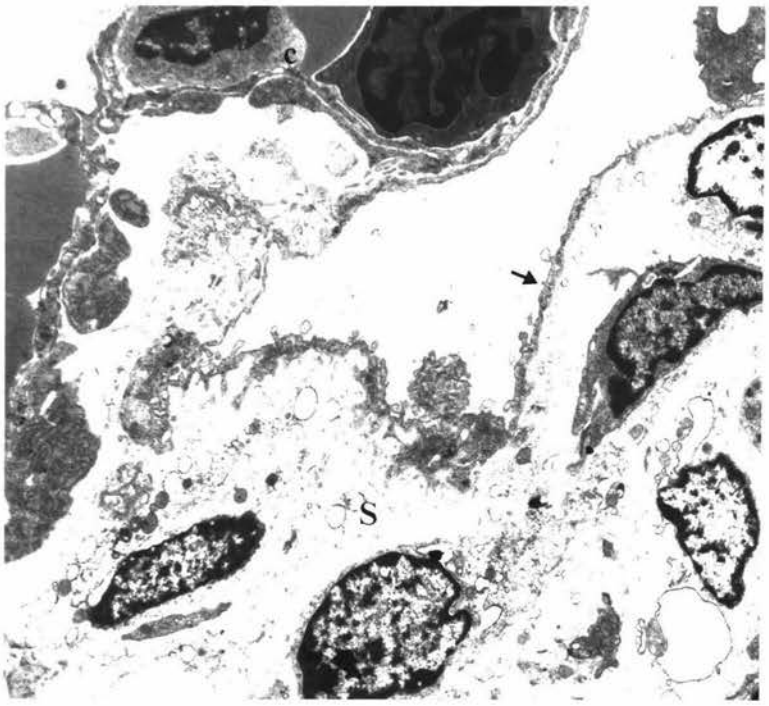
An alveolus of a lamb killed 24 hr after inoculation of the **B. parapertussis**-like organism. The type I cells show an increase in density of cytoplasmic extensions (arrow). The alveolar capillary (c) is distended by erythrocytes and leucocytes. There is marked oedema in the interalveolar septa (S). TEM x 5,200

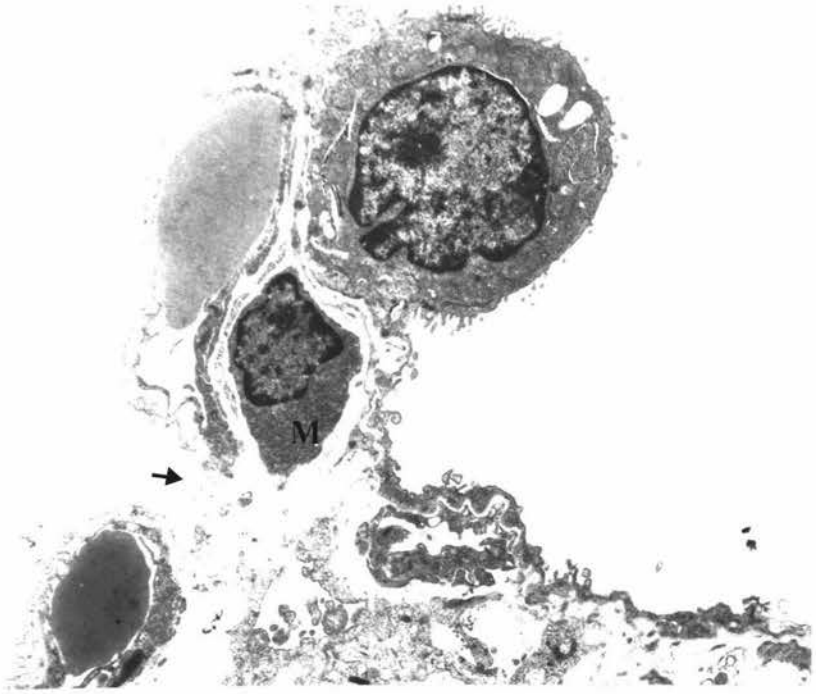
Figure 3.57

Alveoli from a lamb killed 24 hr after inoculation. Part of a type I cell has sloughed (arrow) exposing the underlying basement membrane. Elsewhere the type I cells have abnormally dense cytoplasm. A mononuclear cell (M) has infiltrated into the moderately oedematous interstitium. TEM x 5,200

Figure 3.58

Degenerative changes in alveolar type I cells three days after inoculation. The cells show diffuse swelling of the cytoplasmic extensions lining the alveolar space. The underlying capillary is packed with erythrocytes. TEM x 7,800


C
S


M

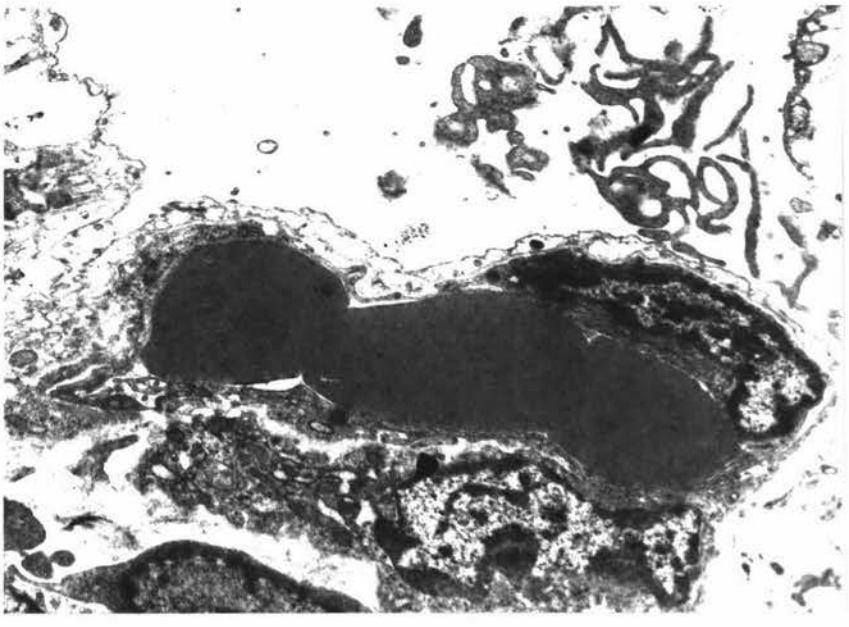


Figure 3.59

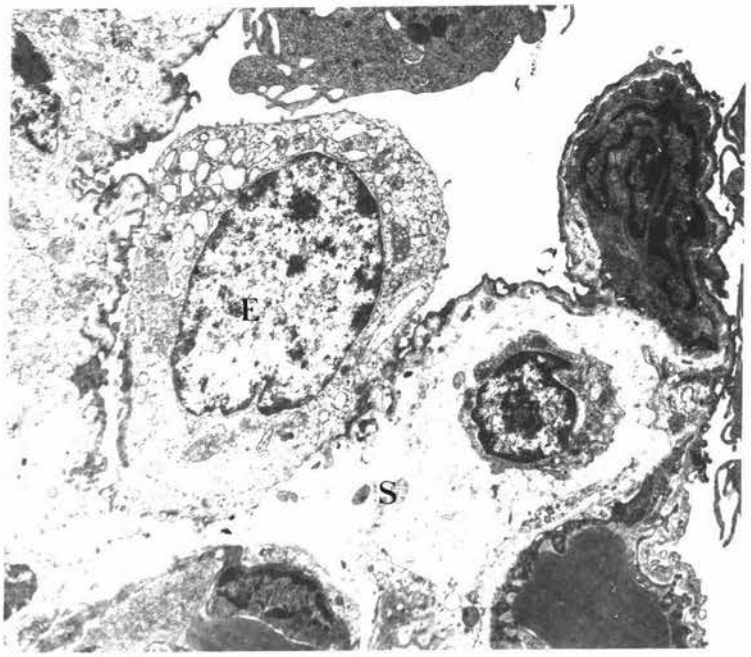
Early degeneration of a type II cell (E). The degenerative cell shows generalised enlargement, swelling of mitochondria, distention of endoplasmic reticulum and loss of microvilli. The alveolar interstitium (S) is moderately thickened by proteinaceous and cellular exudate. Lamb killed 24 hr after inoculation. TEM x 5,200

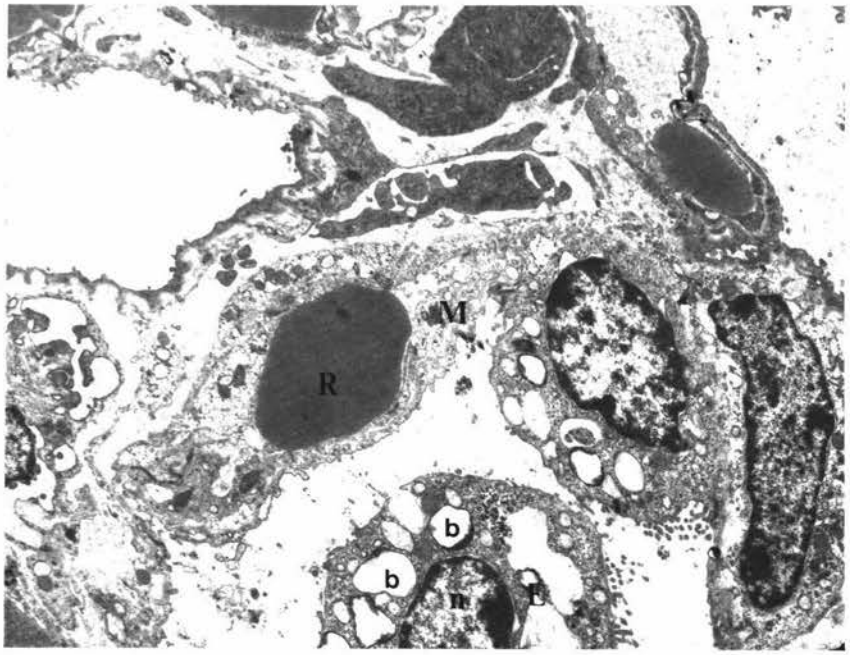
Figure 3.60

Desquamation of type II cell (E) in a lamb killed 24 hr after infection. The desquamating cell exhibits early degenerative change consisting of mild pyknosis (n) and enlargment of lamellated bodies (b). The alveolar capillaries are severely congested. An erythrocyte (R) has been phagocytosed by a macrophage (M) which is closely associated with the alveolar epithelium. TEM x 5,200

Figure 3.61

Desquamation of alveolar type II cell(E) in a lamb three days after inoculation of the **B. parapertussis**-like organism. The sloughed cell shows advanced degeneration with karyolysis (n). The alveolar space (A) contains proteinaceous exudate and underlying capillaries (c) are densely packed with erythrocytes. TEM x 7,800


E
S


M
R
b
b
n

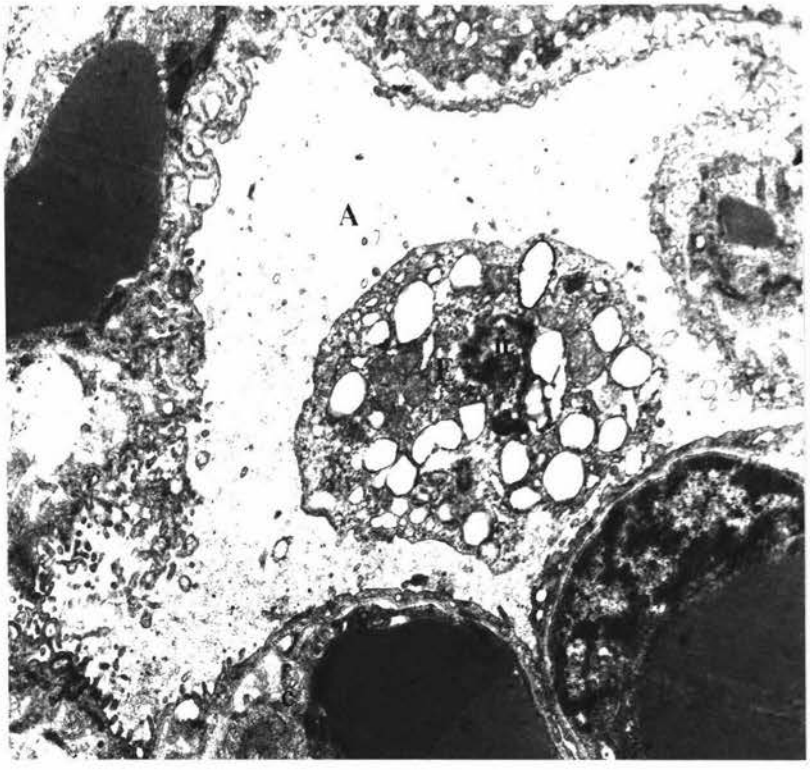
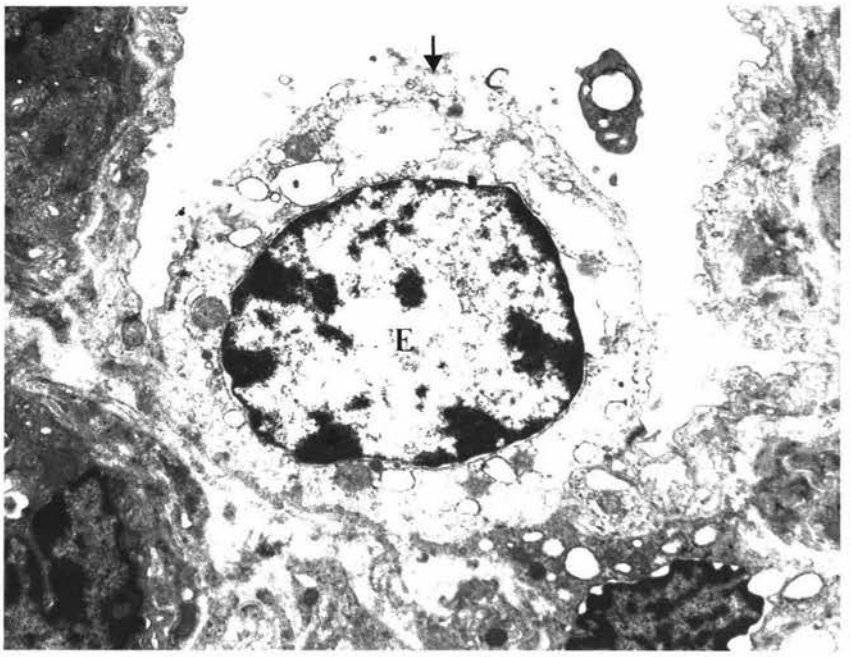

A
n

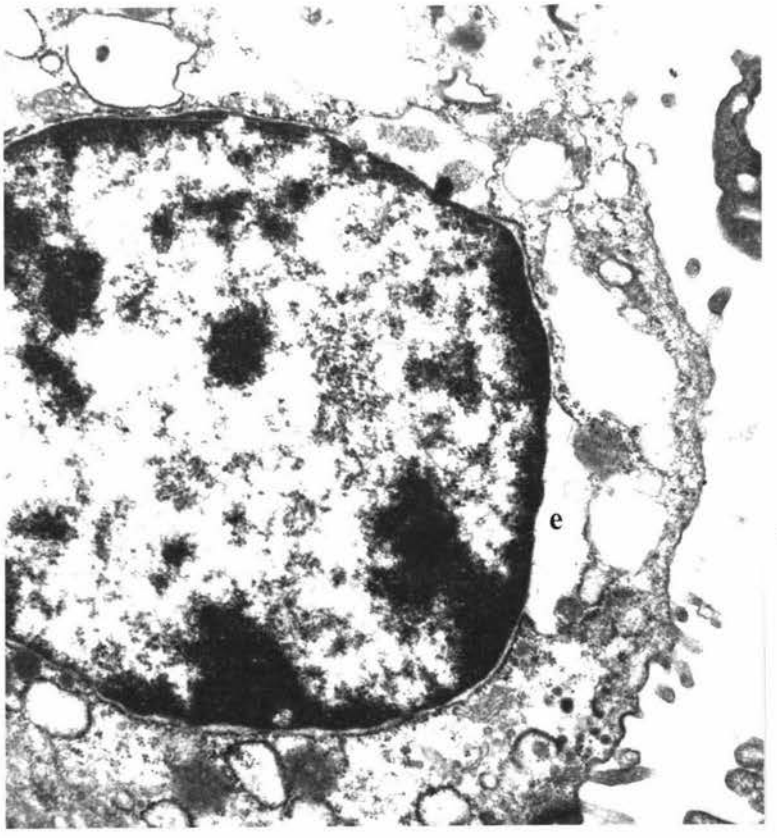
Figure 3.62

Advanced degeneration of a type II cell (E) in a lamb killed three days after inoculation. The cell has few microvilli and shows severe dilation of endoplasmic reticulum with migration of chromatin in the nucleus. The cellular membrane has disintergated (arrow). TEM x 7,800

Figure 3.63

High magnification of degenerate type II cell in an alveolus. The cell shows swollen mitochondria, distended endoplasmic reticulum, distended nuclear envelope (e) and reduced numbers of microvilli. Lamb killed three days after receiving the **B. parapertussis**-like organism. TEM x 15,300


C
E


e

Early focal proliferation of type II cells was occasionally observed as early as 24 hr after inoculation (Fig.3.65) and became common at p.i.d. 3 when many alveolar spaces contained a prominent type II cell and in some cases two type II cells were found adjacent to one another in the corner of an alveolus. The proliferating cells had slightly enlarged nuclei, their cytoplasmic contents were less electron-dense than normal and their lamellated bodies were large and numerous (Fig. 3.65). These cells usually contained no or very few microvilli.

The exudate in alveolar spaces was most severe at p.i.d. 1 and gradually reduced as the experiment advanced. Initially, the alveoli adjacent to the affected bronchioles contained large numbers of neutrophils and isolated macrophages as well as much cellular debris and proteinaceous fluid. These phagocytes contained a large amount of unidentified granular material and phospholipid-like material in digestion vacuoles. Occasionally a macrophage was encountered which had engulfed an entire neutrophil and this was retained within a large phagoctytic vacuole. At p.i.d. 3, the exudative material in alveoli was reduced to very small numbers of mixed inflammatory cells and moderate amounts of proteinaceous material. By p.i.d. 5, the majority of alveoli were free of exudate, however, in many areas they remained moderate to severely collapsed.

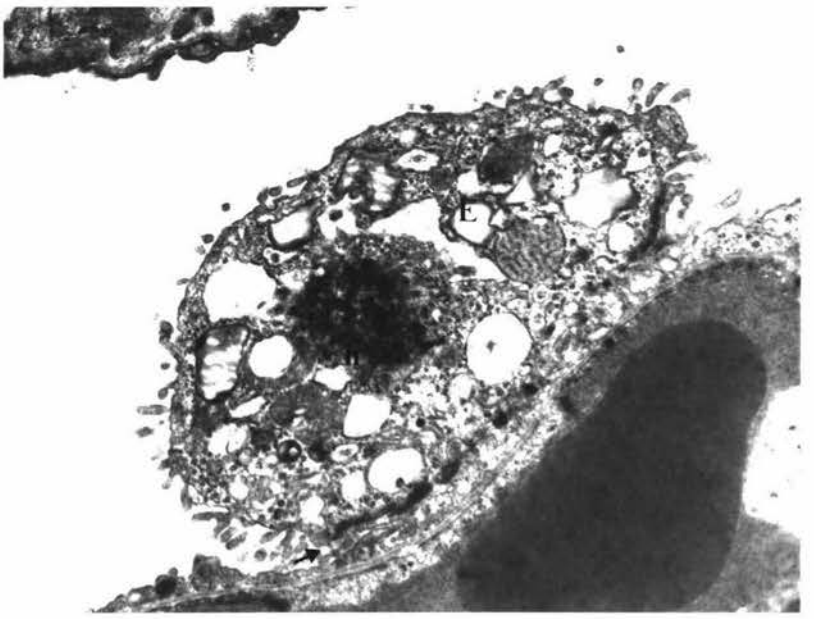
Thickening of interalveolar septa was seen at all stages of the experiment. In the early stages, the majority of interalveolar septa showed low electron density due to interstitial oedema (Fig. 3.56). Small numbers of mononuclear cells were seen infiltrating into the alveolar interstitium (Fig. 3.57), and sometimes a considerable quantity of cellular debris was present. Neutrophil infiltration was rarely seen. The lumina of alveolar capillaries throughout the affected areas were dilated and filled with tightly packed erythrocytes and leucocytes (Fig. 3.66) as well as moderately electron-dense material, presumbly serum protein (Fig. 3.60). Infrequently, small veins surrounding the terminal bronchioles contained numerous platelets mixed with tightly packed erythrocytes

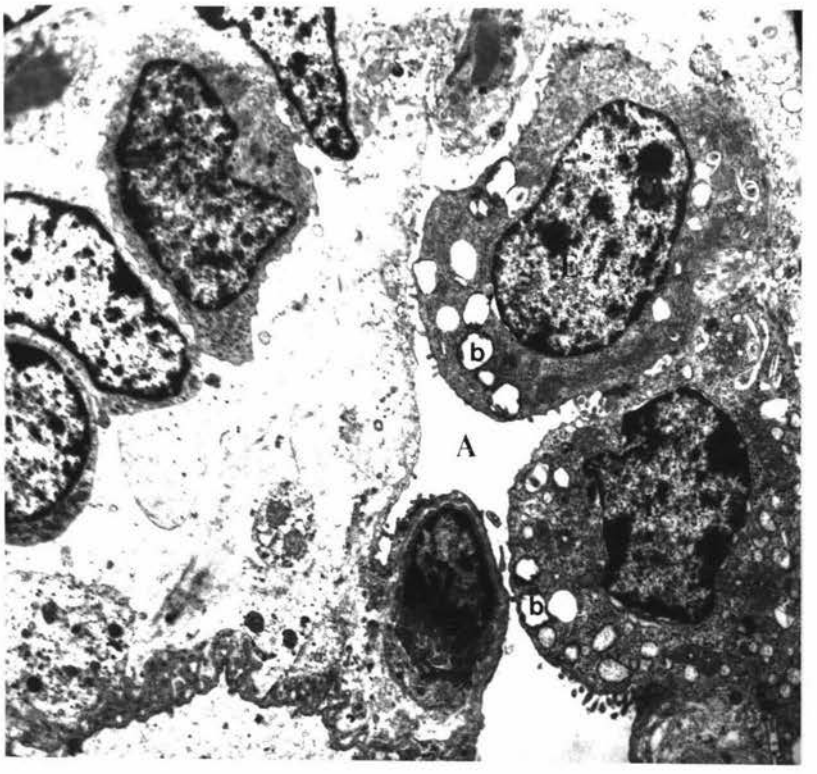
Figure 3.64

A severely degenerating type II cell (E). The cell shows severe distension of most cellular organelles as well as karyorrhexis (n). Large segments of the plasma membrane show severe thickening (arrow). TEM x 11,200

Figure 3.65

Early proliferation of alveolar type II cells (E) in the corner of an alveolar space (A). The cells contain large lamellated bodies (b) and have relatively few microvilli. Lamb killed 24 hr after inoculation. TEM x 5,200


E


b
A
b

(Fig. 3.67).

At later stages of the infection (p.i.d. 9), interalveolar thickening was mainly the result of the infiltration of moderate numbers of mononuclear cells (Fig. 3.68) and early collagen proliferation (Fig. 3.69). Although congestion of alveolar capillaries remained, the numbers of migrating leucocytes were significantly reduced. Very occasionally, migration of isolated neutrophils in capillaries could still be found. Occasionally the cytoplasm of alveolar capillary endothelial cells was swollen and contained vacuoles which were much larger than the pinocytotic vesicles normally present.

Bordetella parapertussis-like organisms were only occasionally observed in the tracheobronchial and pulmonary tissue of infected lambs at various times during the experiment.

Bacteriology

There were no **B. parapertussis**-like organisms or **Pasteurella spp.** cultured from the nasal swabs of all 12 lambs one day before inoculation. A pure culture of the **B. parapertussis**-like organism was recovered from the nasal cavity of seven out of eight lambs killed on days 1, 3, 5 and 9 after inoculation with the organism. The nasal cavity of one of the four control lambs became infected with the **B. parapertussis**-like organism at p.i.d. 5.

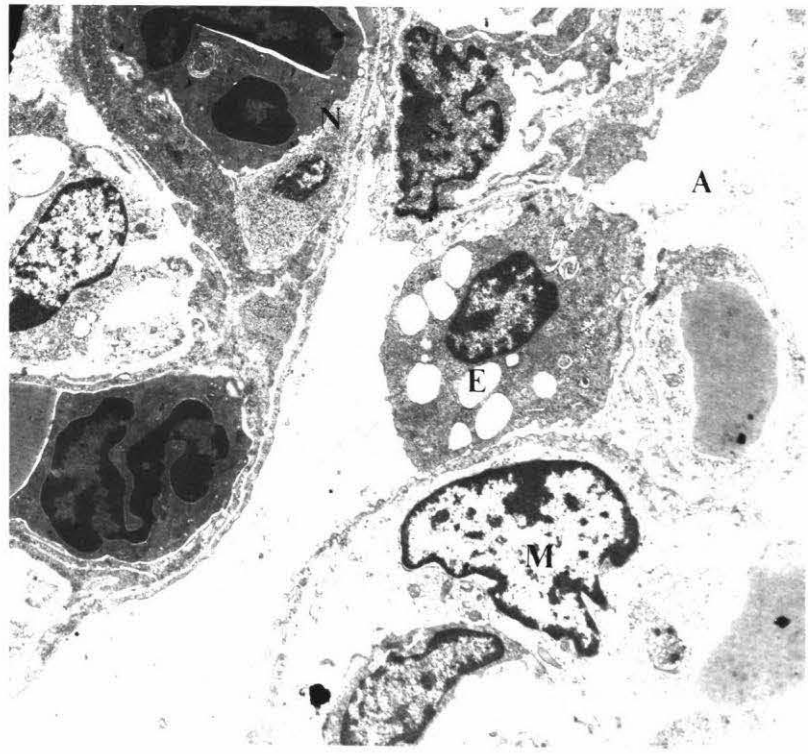
Viable bacterial counts of bronchoalveolar lung washings showed that the majority of the organisms were cleared from the lower respiratory tract within 24 hr after inoculation. Subsequently, the viable numbers of bacteria continued to decrease until at the end of the experiment only very small numbers of organisms were present in the lavage fluid (Fig. 3.70). There was no growth from the washings of control lambs.

Figure 3.66

Alveolus of a lamb 24 hr after inoculation of the **B. parapertussis**-like organism. The alveolar capillaries are congested and contain neutrophils (N). A macrophage (M) can be seen in the interstitium. The alveolar space (A) contains some proteinaceous material and a type II cell (E) shows loss of microvilli and abnormally dense cytoplasm. TEM x 5,200

Figure 3.67

A small bronchiolar vein (V) in a lamb killed five days after inoculation of the **B. parapertussis**-like organism. There are numerous aggregated platelets and erythrocytes occluding the lumen. TEM x 5,200


N
A
E
M

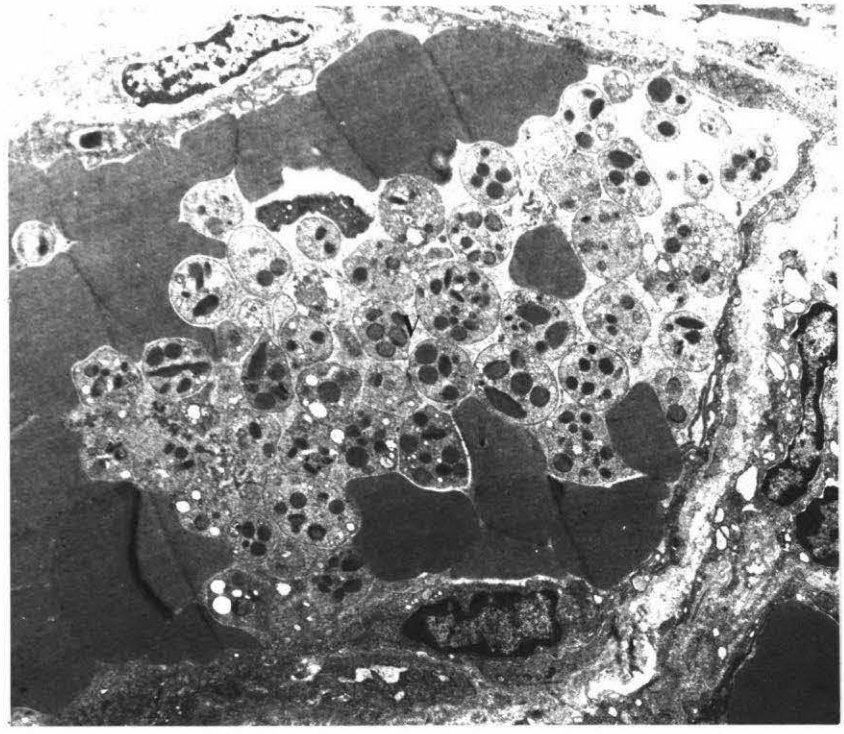
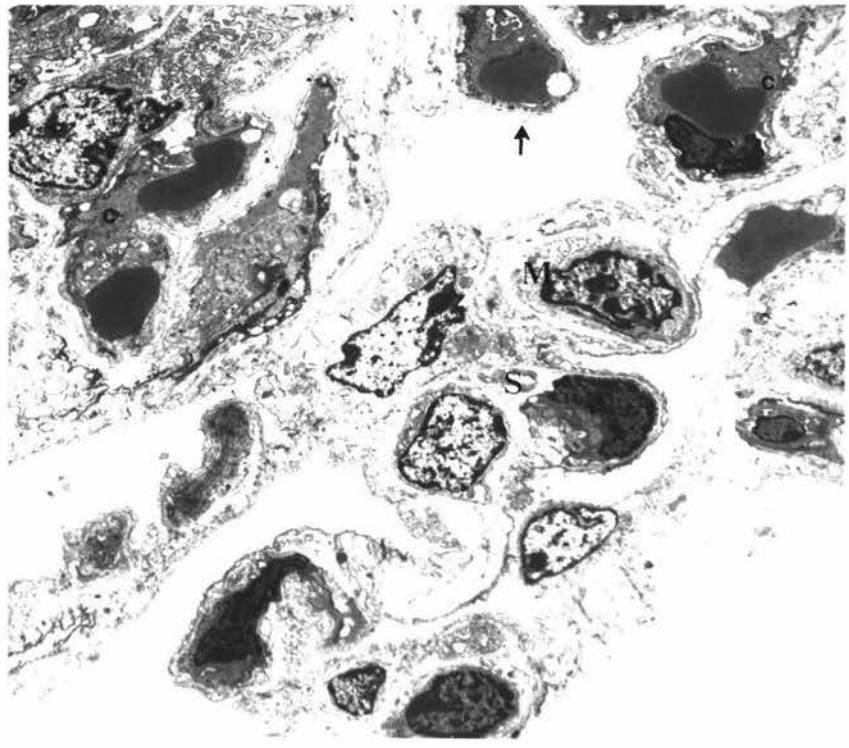

V

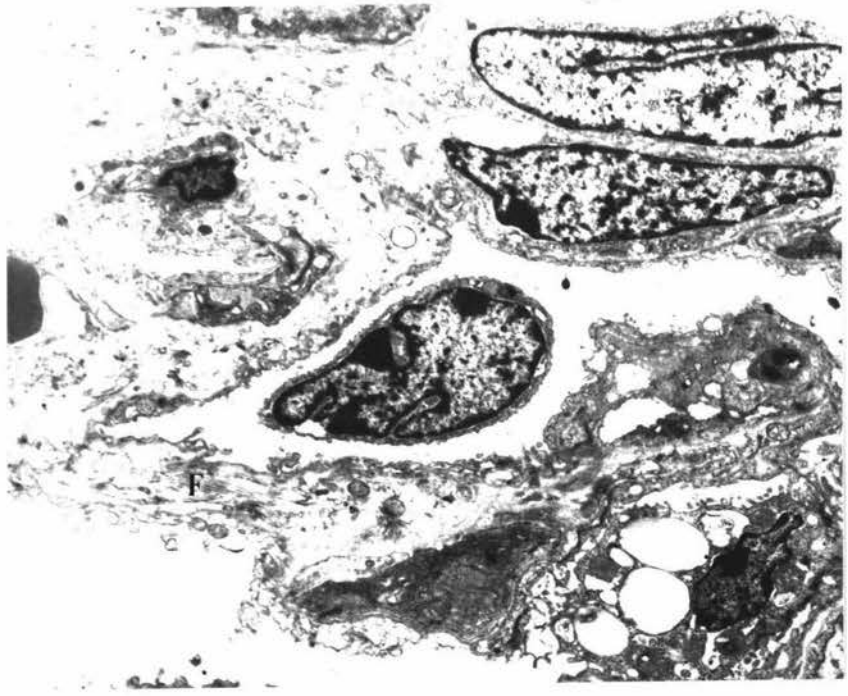
Figure 3.68

Moderately severe alveolar collapse in a lamb killed nine days after inoculation. The alveolar interstitium (S) is moderatedly thickened and contains several migrating mononuclear cells (M). The alveolar capillaries (c) are congested. The cytoplasmic extensions of alveolar type I cells are swollen in some areas (arrow). TEM x 3,400

Figure 3.69

Early interalveolar fibrosis (F) in a lamb killed nine days after inoculation. TEM x 5,200


C
C
M
S


F

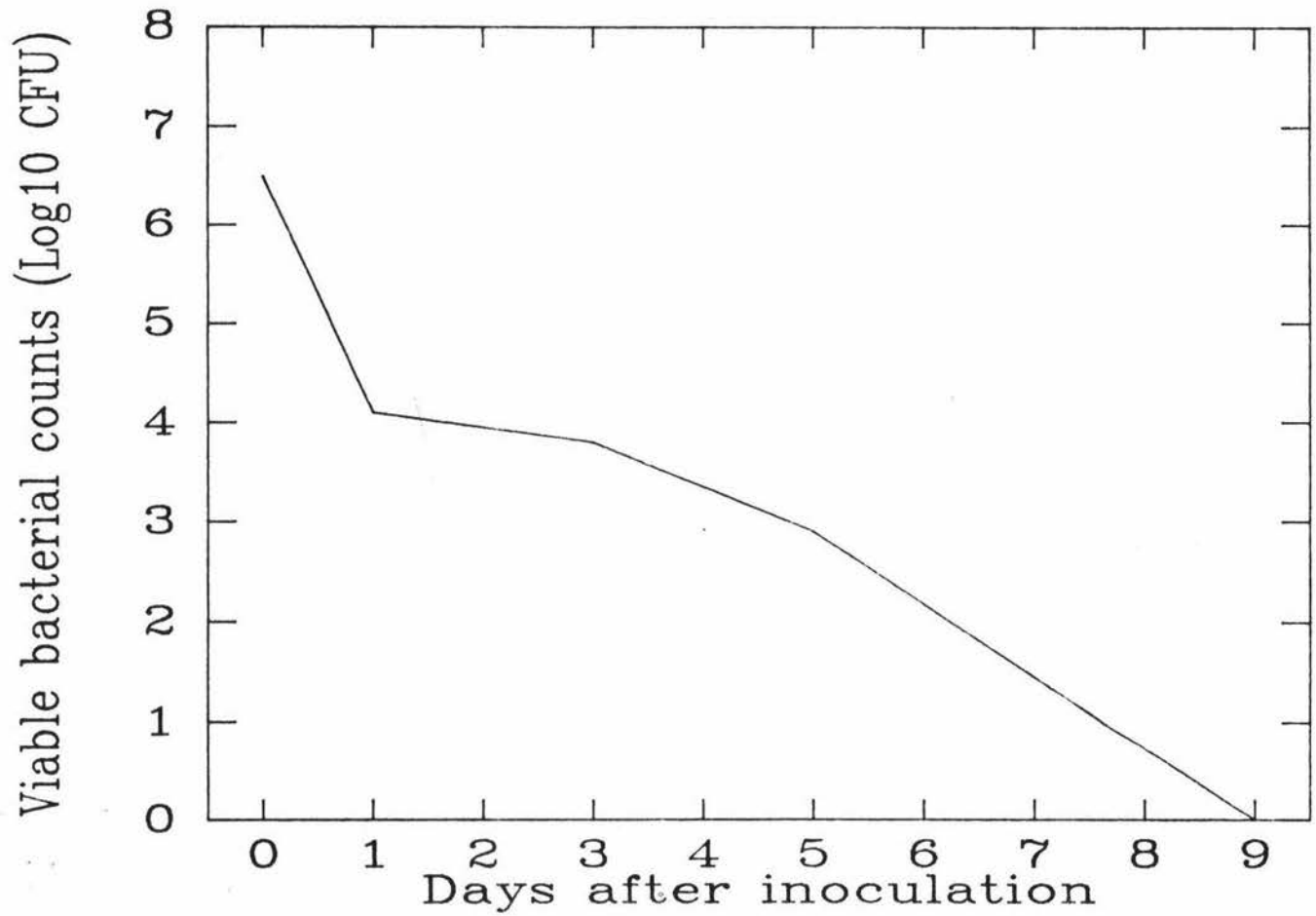


Figure 3.70 Number of viable B. parapertussis-like organisms in the bronchoalveolar lavage fluid of infected lambs

DISCUSSION

The acute tracheobronchitis, acute bronchopneumonia and severe areas of alveolar collapse induced by administration of the **B. parapertussis**-like organism closely resembled those of early naturally-occurring ovine CNP (Alley, 1975a; Pfeffer, 1981; Pfeffer et al., 1983; Al-Kaissi, 1986). Evidence of disease was found in all lambs inoculated and pure cultures of the **B. parapertussis**-like organism were recovered from nasal swabs of all inoculated animals and bronchoalveolar lavage of seven out of eight infected animals. In the present study, however, the effect of the **B. parapertussis**-like organism on the ovine respiratory tract appeared to reach its maximum about 24 hr after inoculation and then waned until nine days at which point lesions were minimal. This suggests that although this organism may be a pathogen responsible for initiating respiratory infection, other organisms may also be necessary to exacerbate and prolong the pneumonia.

The importance of the tracheobronchial tract in influencing the course of CNP has been noted by Alley (1975a) and comprehensively studied by Al-Kaissi (1986). These authors found that the tracheobronchial epithelium was invariably involved in early CNP and suggested that CNP might begin as a tracheobrochitis before extending to involve more peripheral airways and the alveolar parenchyma. Although the changes produced by the **B. parapertussis**-like organism were mild they are likely to result in sufficient disturbance to pulmonary clearance mechanisms to allow invasion of the lower respiratory tract by more destructive pathogens which are normally present in the upper respiratory tract.

This relatively mild injury contrasted with that described in ovine tracheal organ cultures infected with the **B. parapertussis**-like organism (Al-Kaissi et al., 1986) and with ***in vitro*** infections by other **Bordetella** organisms (Muse et al., 1979; Bemis & Kennedy, 1981). In ovine tracheal organ cultures infected with various concentrations of the **B. parapertussis**-like organism, it was found

that the infected ciliated epithelium was largely denuded of cilia and the non-ciliated cells showed swelling, wrinkling and protrusion of cells into the lumen. However, only very little change of this type was detected in the present study.

There are obvious differences in the tracheobronchial damage seen in infected tracheal organ cultures (Muse et al., 1979) and those seen **in vivo** by Mallory & Hornor (1912) and Mallory et al. (1913) in human natural cases of **B. pertussis** infection. There are several possible explanations for the relatively mild reaction seen **in vivo**. Aside from the possible difference in the toxin-producing ability of the organisms used, a lack of blood supply and immunological reaction **in vitro** may cause greater tissue damage. The difference may also be partly attributed to the lower numbers of viable bacteria present in pulmonary tissue **in vivo**. Al-Kaissi (1986) showed that damage to ovine tracheal explants and inhibition of ciliary activity were directly dose-dependent. A dose-dependent effect was also demonstrated by Muse et al. (1979) in hamster tracheal organ cultures infected with **B. pertussis**. According to these workers severe morphological damage needs at least 10^5 organisms/ml in the culture. However, a considerably smaller dose of organisms was used in the present study.

The atypical cilia observed in the tracheal epithelium in the present study were similar to those described in man (Clarke et al., 1981), healthy dogs (Wilsman et al., 1982), normal young sheep (Al-Kaissi, 1986) and hamsters exposed to respiratory carcinogens (Harris et al., 1974). It has been suggested that atypical cilia represent the precocious regeneration of cilia (Friedmann & Bird, 1971). It has also been speculated that they are a feature of a bronchial epithelium with impaired clearance (Ailsby & Ghadially, 1972), or represent a response to noxious environmental agents (Trump & Jones, 1978). However, they are also regarded as a normal feature of the canine bronchial epithelium (Wheeldon, 1974) and ovine tracheal epithelium (Al-Kaissi, 1986) and this view is supported, to some extent, by the present study, in which these

structures were found in the airways of both control and inoculated lambs. Although no quantitative measurement was applied it was concluded that atypical cilia were more numerous in inoculated lambs than in controls. In addition, these structures were found to be present in both types of ciliated epithelial cell, an observation which differs from the findings of Al-Kaissi (1986) who noted that they occurred mainly in Al-Kaissi's second type of ciliated cell. Thus further detailed morphometric studies of the frequency and distribution of these atypical structures may be necessary for determining their importance.

Although the cilia in the present study showed no severe morphological damage, it is nevertheless possible that they were functionally damaged. This has also been suggested by Baskerville (1972) in the context of swine pneumonia caused by mycoplasma. Studies on **B. bronchiseptica** have indicated that some strains may cause a rapid decrease in ciliary beating frequency of canine tracheal outgrowth cells without causing noticeable structural damage to these cells (Bemis & Kennedy, 1981). The plugs of mucus in the airways seen in the present study might indirectly reflect functional damage of tracheobronchial mucociliary activity and reduction of pulmonary clearance since there was a lack of any obvious hyperplasia of the secretory cells. Future experimental work should, therefore, be designed to investigate the alteration of ciliary activitity in the animals affected by the **B. parapertussis**-like organism.

One of the most striking changes which occurred in the bronchiolar epithelium was the formation of large blebs in the apical cytoplasm of some ciliated cells. Cytoplasmic projections are not a feature of normal ciliated bronchiolar cells in sheep (Al-Kaissi, 1986), nor were they found in acute ovine pneumonia (Alley, 1975a). However, the formation of similar blebs has been described in ciliated bronchial epithelium of dogs following prolonged inhalation of tobacco smoke (Frasca et al., 1969), in ciliated bronchiolar cells of pigs infected by mycoplasma (Baskerville,

1972; Baskerville & Wright, 1973) as well as in bronchiolar ciliated cells of lambs naturally affected by CNP (Alley, 1975a; Al-Kaissi, 1986). The mechanism of the formation of cytoplasmic blebs is unclear although they are thought to be associated with chronic irritation or the action of peroxide (Baskerville & Wright, 1973). Since the projections seen in this study were much longer than adjacent cilia and were present on many cells in some areas, it seems probable that they would impede normal ciliary activity and consequently interfere with ciliary clearance mechanisms.

The majority of degenerative changes seen in the alveolar epithelium in this study were described previously in pathological studies of naturally-occurring ovine CNP (Alley, 1975a) as well as in early experimental pneumonia induced in lambs by various infectious and non-infectious agents (Boidin et al., 1958; Dungworth & Cordy, 1962a & b; Alley, 1975a). The diffuse swelling seen in type I epithelial cells was not a common feature in natural ovine CNP (Alley, 1975a), although it was often seen in alveolar injury caused by certain chemicals (Alley, 1975a; Greenberg et al., 1971). This change might represent early injury of the alveolar epithelium caused by noxious agents.

Possibly one of the most important changes encountered in the alveoli affected by the **B. parapertussis**-like organism were the degenerative changes in type II cells. These ranged from mild swelling to pyknosis and karyolysis. It is likely that these changes were non-specific responses since they have been observed in a series of pulmonary injuries caused by other factors (Greenberg et al., 1971; Manktelow, 1967; Alley, 1975a). However, it is worth noting that formation of thickened, electron-dense plasma membranes in type II cells was also a prominent feature in acute fibrinous ovine pneumonia, CNP (Alley, 1975a), and mouse subacute pneumonia caused by the same strains of the **B. parapertussis**-like organism used in this study (Chapter 2). These changes have not been reported in a large range of other experimental respiratory infections (Al-Darraji et al., 1982b; Cultip & Lehmkuhl, 1986). It is thus possible

that this change may be directly associated with the involvement of the **B. parapertussis**-like organism, although the mechanism by which it occurs and its potential importance is not yet clear.

The extensive damage to type II cells is likely to be a contributing factor to the severe and prolonged alveolar collapse seen in the current study since these cells are responsible for producing pulmonary surfactant. It was postulated by Sutnick & Soloff as early as 1964 that the pulmonary collapse observed in pneumonia might be associated with a pulmonary surfactant deficiency. In addition, it has recently been demonstrated that some bacterial products may be responsible for altering the surface tension of pulmonary surfactant (Brogden et al., 1986a). Recently, Brogden et al. (1986b) demonstrated that lipopolysaccharide (LPS) from a number of gram-negative bacteria, such as **Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Serratia marcescens** or **Pseudomonas aeruginosa** could bind with ovine pulmonary surfactant resulting in a complex with higher density than that of surfactant alone. This may alter the physiological properties of surfactant and contribute to the pathophysiological changes observed in some types of pneumonia.

Alley (1975a) attributed the mild degree of epithelial proliferation produced by dilute nitric acid instillation to the lack of the continuous presence of pathogen(s) in the alveolar space. If this is the case, the rapid disappearnce of the **B. parapertussis**-like organism from the lung which was observed in this study supports this hypothesis. The possibility that the observed mild proliferation of type II cells was the normal type II cell growth in the lung of young animals (Evans et al., 1978) seems remote. A much more likely explanation is that it was a direct response to the inoculated bacteria and/or their toxin(s). Further quantitative studies are required before the exact cause of the proliferation can be assessed.

The alveolar type II cell is considered to be the progenitor of

type I cells (Dunnill, 1982). The mild proliferation of alveolar type II cells seen at p.i.d. 1 was of doubtful significance. The time when proliferation of type II pneumocytes was first observed was earlier than previously reported in the majority of natural and experimental pneumonias in sheep, swine and other species (Alley & Manktelow, 1971; Alley, 1975a; Baskerville & Wright, 1973) but was similar to that seen in studies of mouse **Proteus** infection by Pine et al. (1973) and chemical instillation in sheep and rat lungs (Alley, 1975a; Evans et al., 1978).

Alveolar collapse was a prominent feature seen early in the response to the **B. parapertussis**-like organism. Later, the areas of collapse became more confluent and extensive. Peribronchiolar alveolar collapse is a characteristic pathologic feature in early naturally-occurring CNP (Alley, 1975b; Kirton et al., 1976; McGowan et al., 1957; Stamp & Nisbet, 1963; Pfeffer et al., 1983). This lesion is usually considered to be associated with compression or airway obstruction (Jubb and Kennedy, 1970). In this study, the alveolar collapse was likely to be due to obstruction of airways by large amounts of inflammatory exudate. In the ovine lung, collateral ventilation is severely limited due to the paucity of Kohn's pores (Al-Kaissi, 1986) and collapse may be easier to induce than in most other species. In addition, the extensive alveolar collapse may also be associated with the severe destruction of type II cells with consequent deficiency of pulmonary surfactant, as mentioned above.

The rapid disappearance of the **B. parapertussis**-like organism from ovine lungs is suprising since the strains from humans have been reported to last in mouse lung as long as 22 days (Bradford & Salvin, 1937). It is unlikely that the rapid clearance was caused by phagocytosis by alveolar macrophages, at least after 24 hr, because from that time onwards very limited interaction between organisms and BAM were observed in lavage preparations or lung tissue. In this experiment, it was not possible to determine the exact clearance mechanism of the **B. parapertussis**-like organism from the respiratory tract. It is generally accepted that physical transportation of

bacteria by the mucociliary escalator is likely to be the main defence mechanism of the tracheal mucosa (Green et al., 1977), but bacteria can also be destroyed **in situ** by a potent bactericidal system present in tracheal mucus (Pijoan, 1978).

The failure to demonstrate by TEM, SEM and PAP techniques any specific bacterial adherence onto the respiratory mucosal surfaces implies that a direct association between organisms and epithelial cells is not necessary for damage to occur. The damage demonstrated in this study may therefore have resulted from the action of substances released from bacteria or enzymes released from neutrophils during degranulation processes rather than from the direct effect of the organism. These findings differ from those of Al-Kaissi (1986) who found adherence of the **B. parapertussis**-like organsim in ovine tracheal organ culture. Similarly, workers using **B. pertussis** and **B. bronchiseptica** have demonstrated adherence with these organisms in a number of animal tracheal organ cultures (Muse et al., 1979; Bemis & Kennedy, 1981). In the case of **in vitro** infection with **H. influenzae** and **N. gonorrhoeae**, adherence to ciliary epithelium was also considered to be necessary for virulence, however, Denny (1974), Johnson et al. (1983) and Melly et al. (1981) found that sterile supernatant from the infected organ cultures contained an endotoxin that caused loss of ciliary activity when transferred to fresh organ cultures.

It is also possible that the strains of organism used in the current study were weak in their interaction with cilia **in vivo**. Other **in vivo** studies have shown that cilia-associated strains of **B. pertussis** and **B. bronchiseptica** require 3 to 14 weeks to be cleared from the trachea of infected animals (Bradford, 1938; Bemis et al., 1977; Sato et al., 1980). In most experimental models of pulmonary clearance, however, the majority of non-ciliary associated bacteria are killed or removed within 24 hr of infection (Meyer, 1950; Smith et al., 1957; Jakab & Green, 1972; Goldstein et al., 1974; Toews et al., 1979). In sheep infected with **P. haemolytica**, complete clearance was achieved at about 48 hr after infection (Davies et

al., 1981c). Although Al-Kaissi (1986) demonstrated that similar isolates of the **B. parapertussis**-like organism were closely associated with ciliated cells **in vitro** (Al-Kaissi et al., 1986), the difference in the bacterial concentrations present and the antibacterial mechanisms operating **in vitro** and **in vivo** may account for differences in these results.

The size, route and technique of inoculation are also important factors affecting the clearance of bacteria from pulmonary tissue (Toews et al., 1979). Unlike aerosols where fine particles are produced (Jakab & Green, 1972), a large proportion of the organisms inoculated intratracheally are likely to be physically removed before reaching the alveoli (Davies et al., 1981c). In addition, some types of bacteria, particularly gram-negative organisms, are likely to form conglomerates due to the chemical composition of their bacterial cell wall and capsule (Lillie & Thomson, 1972). Once these particles are deposited in the mucus, clearance by normally functioning mucociliary transport is highly efficient. Most inhaled particles are removed from central airways with in a few hours, and even from distal airways within 24 hours (Dungworth, 1985).

The phagocytic cells obtained by bronchoalveolar lavage of control lambs were similar to those reported in studies of conventionally-raised sheep by Chanana et al. (1981) and Burrells (1985) but total cell counts were slightly higher than the results of Rola-Pleszczynski et al. (1981). Macrophages were the most prominent phagocytic cells in normal young sheep and the ratio of neutrophils to macrophages was approximately 1 to 9. Inoculation of the **B. parapertussis**-like organism markedly reversed the ratio within 24 hr. Similar changes in the neutrophil/macrophage ratio have also been reported in the lungs of laboratory animals exposed to live gram-negative bacteria (Pierce et al. 1977) or bacterial LPS (Hudson et al., 1977), in bovine and ovine lungs inoculated with **P. haemolytica** (Davies et al., 1981c; Lopez & Yong, 1986), and in bovine and ovine lungs following the adiministration of endotoxins extracted from **P. haemolytica** and **E. coli** (Brogden et al., 1984).

The influx of neutrophils into airways, as measured by the neutrophil/macrophage ratio, was reflected in the histopathological findings in which purulent exudate was found plugging numerous bronchi and bronchioles.

The direct correlation obtained between bacterial clearance and numbers of neutrophils in lavage fluid may suggest that these cells were involved in the destruction of the **B. parapertussis**-like organism. However, it was difficult to demonstrate phagocytic activity in these cells using both TEM and PAP techniques. The inability of neutrophils to phagocytose **B. parapertussis** organisms is in accordance with similar observations of **B. pertussis** (Confer & Eaton, 1982). Certain substances produced by this organism inhibit neutrophil chemotaxis and particle ingestion, inducing a profound bacteriacidal defect in human neutrophils. Hence, the neutrophilic response may be largely due to the bacterial cellular components, as suggested by Martire-Burnes et al. (1985), rather than the live organism itself. An identical neutrophil response has also been achieved by administration of pure LPS (Lumsden et al., 1974; Hudson et al., 1977). Future studies should therefore attempt to follow the retention and clearance of the **B. parapertussis**-like organism in the respiratory tract in more detail.

A biphasic morphological change was observed in alveolar macrophages in the **B. parapertussis** infected animals. At first, the macrophages showed very active phagocytosis but from p.i.d. 5 onwards, many macrophages exhibited degenerative changes of varying degree. Several reports of infections with **Bordetella** organisms and occasionally other pneumonic pathogens have recorded a severe degeneration as occurring in the bovine, ovine and rabbit alveolar macrophages (Markham & Wilkie, 1982; Sutherland et al., 1983; Zeligs et al., 1986) and the current investigation has confirmed that this is also the case in the **B. parapertussis**-like organism infection. It seems likely that these changes would reduce the phagocytic and bacteriocidal ability of macrophages and predispose the respiratory tract to infection by other organisms. A further study of the

phagocytic activity of alveolar macrophages and neutrophils both ***in vitro*** and ***in vivo*** using the current experimental model could provide useful information on this aspect.

A leucocytosis was seen in the lambs inoculated with **B. parapertussis**, similar to that commonly encountered in humans (Olson, 1975) and mice (Bradford, 1938) infected by **B. pertussis**. However, the most marked and significant haematologic change in the present study was the increase in circulating neutrophils which was in contrast to the results of Bradford (1938) and Olson (1975) who found increased circulating lymphocytes or mononuclear cells in **B. pertussis**-infected patients and mice. This difference may be explained by the presence of lymphocytic promoting factor, a toxin associated with **B. pertussis** organisms, but not **B. parapertussis** (Pittman, 1984b). Nevertheless, it is uncertain if the neutrophilia observed in the present study was endotoxin-mediated or an active response to an acute pulmonary bacterial infection.

The consistent recovery of the **B. parapertussis**-like organism from sheep indicates the possible involvement of this organism as a pathogenic agent in the respiratory tract of this species. In the present study, it has been proven that the organsim is able to experimentally infect colostrum-deprived lambs and cause moderate inflammation of the airways and pulmonary parenchyma. The organism induces a mild pulmonary infection similar to early natural ovine CNP. These results suggest that in combination with other agents or under centain environmental conditions, the **B. parapertussis**-like organism could have an important role in initiating and prolonging ovine CNP. The establishment of this experimental model may provide a means for studying further the pathogenesis of the **B. parapertussis**-like organism in ovine CNP, and investigating its relationship with other respiratory commensals, such as **P. haemolytica**, and **M. ovipneumoniae**.

SUMMARY

Intratracheal inoculation of a **B. parapertussis**-like organism isolated from natrually infected ovine lungs into hysterectomy-derived, colostrum-deprived lambs produced lesions which were morphologically identical to early naturally-occurring ovine CNP. They consisted of an acute mild tracheobronchitis, severe alveolar collapse and acute bronchopneumonia, which developed as early as 24 hr and was most severe at 1 to 3 days after inoculation. Ultrastructurally, the alveolar epithelium showed extensive degeneration and individual epithelial cell necrosis. A large amount of inflammatory exudate accumulated in the airway lumina and SEM revealed that the tracheobronchial epithelium showed focal extrusion of ciliated cells. Occasionally, moderate numbers of the **B. parapertussis**-like organisms were seen closely associated with cilia.

Pure cultures of the **B. parapertussis**-like organisms were consistently recovered from nasal swabs of infected lambs throughout the study. Viable bacterial counts of the bronchoalveolar lavage fluid showed a rapid elimination of this organism from the lower respiratory tract 24 hr to 5 days after inoculation.

Lambs given the **B. parapertussis**-like organism exhibited a marked haematologic response. The leucocyte counts gradually rose and reached the peak at 5 days. Neutrophil counts also slowly increased after inoculation with the **B. parapertussis**-like organism and were highest at 3 days. Body temperature remained normal.

A marked increase in total bronchoalveolar lavage cell counts was observed 24 hr after infection. Up to 93% of the lavage cells were neutrophils. However, there was an absence of close interaction between these phagocytic cells and the infective organisms. Many macrophages showed significant cytoplasmic vacuolation from 5 days after instillation.

The results of this study have shown that the **B. paraperussis**-like organism can infect colostrum-deprived lambs and cause marked cytopathological changes in respiratory epithelial cells and alveolar macrophages, leading to an inflammatory reaction and pneumonia.

CHAPTER 4

TRACHEAL VERSUS PULMONARY CLEARANCE OF A **BORDETELLA PARAPERTUSSIS**-LIKE ORGANISM IN MICE

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TRACHEAL VERSUS PULMONARY CLEARANCE OF A **BORDETELLA PARAPERTUSSIS**-LIKE ORGANISM IN MICE

In the previous studies (Chapter 2 and 3), a subacute pneumonia was experimentally produced in SPF mice and colostrum-deprived lambs by intranasal or intratracheal inoculation of a **B. parapertussis**-like organism. These studies showed that the majority of bacteria were cleared in the first few days after infection and damage to tracheobronchial airways was relatively mild. In contrast, human isolates of **B. parapertussis** are capable of persisting and colonising mouse pulmonary tissue for as long as 22 days (Bradford & Wold, 1939). These differences highlighted a need for more detailed information on the clearance of the **B. parapertussis**-like organism from the lower respiratory tract of the experimental mouse model, since it has now been established in both laboratory animals and human patients that the bacterial colonization of **Bordetella** organisms is essential for tissue damage (Weiss & Hewlett, 1986).

The following experiment was therefore designed to measure the rate of clearance of the **B. parapertussis**-like organism from the mouse respiratory tract and provide a comparative study of tracheal versus pulmonary deposition and clearance.

MATERIALS AND METHODS

Experimental animals: The thirty-six Swiss SPF mice used were from the same source and kept under similar conditions to those in the previous experiment (Chapter 2). Since it was found earlier that mice at about 20 g weight were relatively susceptible to infection, 35 day-old mice, weighing 22 g were used in this investigation.

Experimental procedure: Five strains of the **B. parapertussis**-like organism were pooled for the inoculum used. They were originally isolated from bronchoalveolar lavage fluid of CNP-affected lambs then lyophilized and stored at -70°C. The inoculum was prepared and administrated by the technique described previously (Chapter 2). The final titre of the inoculum was approximately 2 x 10^6 CFU/ml.

After inoculation, the mice were arbitarily divided into six groups of six mice each. One group was killed immediately after exposure and the other five groups were killed at 4, 8, 12, 24, 48 and 72 hr post-inoculation. The mice were sacrificed by dislocating their cervical vertebrae. The thorax was then opened aseptically and the lung and trachea removed by transection of the larynx. The excised trachea and lung were rinsed in 10 ml of sterile PBS to remove external blood and the trachea was separated from the lungs by cutting the base of the main bronchi. Lungs and trachea were put in separate Petri dishes and subsequently ground in 3 ml of PBS in a glass grinder for 3 min. Lung and tracheal suspensions were made up to 5 ml volume with sterile PBS and viable counts of the **B. parapertussis**-like organism were made by dropping 50 µl of the suspension on blood agar plates (Difco). A sample from each of six mice was then inoculated onto three plates.

The plates were incubated at 36°C for 2-3 days, at which time viable counts expressed in colony-forming units (cfu) were recorded. The number of bacteria per gram of mouse lung and trachea was calculated according to the formula described by Lopez et al. (1982). The transformation factors were adjusted for the lighter weight of mice used as shown below:

$$\text{No. of bacteria per gram of mouse lung} = \frac{\text{No. of colonies} \times 5.0 \times 6}{0.05}$$

or

$$\text{No. of bacteria per gram of mouse trachea} = \frac{\text{No. of colonies} \times 5.0 \times 40}{0.05}$$

Where:

No. of bacteria = Number of **B. parapertussis**-like organisms present in mouse lungs/trachea at a given time.

No. of colonies = Total number of colonies present on the plate.

5.0 = Volume of PBS added to the mouse lung/trachea for grinding purposes.

6 = Transformation factor for 1 g of mouse lung (the average lung weight for a 22 g mouse is about 170 mg, therefore 6 x 170 mg = 1 g of mouse lung).

40 = Transformation factor for 1 g of mouse trachea (the average tracheal weight for a 22 g mouse is about 25 mg, therefore 40 x 25 mg = 1 g of mouse trachea).

0.05 = Volume of the lung/trachea homogenate plated on the media.

The mean number of bacteria in 1 g of mouse lung or trachea was deterimined for each group of 6 mice. The mean values of each group were then compared and tested for statistical significance using the simple t test (Statistext, IBM software).

RESULTS

The mean number of **B. parapertussis**-like organisms recovered from the mouse lungs and trachea at various time intervals is shown

in Table 4.1 and Fig. 4.1. The initial deposition of bacteria was higher in the trachea than the lung (Table 4.1). A mean $17.4 \times 10^5 \pm 8.69 \times 10^5$ viable organisms per gram were deposited in the trachea (range 7.6×10^5 - 27.2×10^5), and $7.31 \times 10^5 \pm 1.13 \times 10^5$ viable organisms per gram in the lung (range 5.40×10^5 - 8.46×10^5). Between 0 to 12 hr the organisms were rapidly cleared from both trachea and lungs but thereafter clearance was faster in the trachea than in the lungs (Fig. 4.1; Table 4.1). Although there was a significant difference ($P<0.05$) between bacterial retention in the trachea at the various time intervals, this difference was not significant in the lung ($P>0.05$).

TABLE 4.1 COLONY FORMING UNITS OF **B. PARAPERTUSSIS**-LIKE ORGANISM IN THE LUNGS AND TRACHEA OF MICE AT VARIOUS TIMES AFTER INOCULATION ($x \pm SD$)*

Hours	No. **B. parapertussis**-like organisms	
	Trachea	Lung
0	$17.4 \times 10^5 \pm 8.69 \times 10^5$	$7.31 \times 10^5 \pm 1.13 \times 10^5$
6	$5.2 \times 10^5 \pm 2.22 \times 10^5$	$4.44 \times 10^5 \pm 1.79 \times 10^5$
12	$4.03 \times 10^5 \pm 3.62 \times 10^5$	$2.41 \times 10^5 \pm 1.63 \times 10^5$
18	$3.93 \times 10^5 \pm 1.37 \times 10^5$	$4.02 \times 10^5 \pm 5.52 \times 10^4$
24	$3.41 \times 10^5 \pm 1.62 \times 10^5$	$3.64 \times 10^5 \pm 2.60 \times 10^4$
48	$4.67 \times 10^4 \pm 3.50 \times 10^4$	$3.38 \times 10^5 \pm 1.32 \times 10^5$

* Mean value of CFU at each time calculated from 6 mice

DISCUSSION

The results of present study showed that the deposition of the **B. parapertussis**-like organism was greater in the trachea than in the lung and clearance of the organism was faster from the trachea

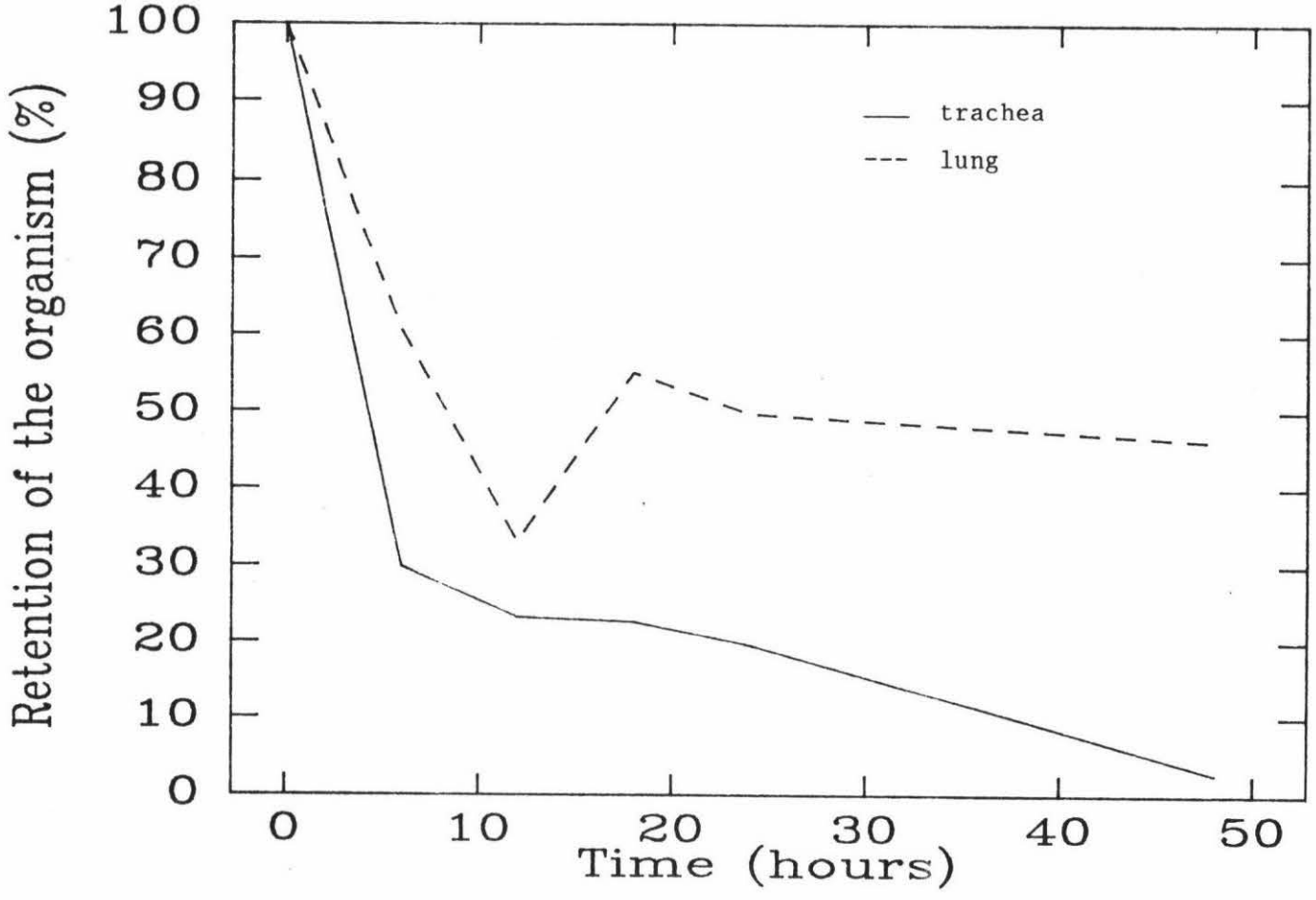

Figure 4.1 Pulmonary and tracheal retention of the B. parapertussis-like organism in mice sacrificed at different time intervals after inoculation

than the lung. The higher initial deposition of bacteria in the trachea may be simply due to the intranasal route of inoculation which could favour tracheal rather than alveolar deposition. In addition, the faster clearance of the organisms from the trachea might also relate to differences between pulmonary and tracheal mucosal surface areas (Green et al., 1977).

It should be emphasized that the initial dose of bacterial deposition does not influence clearance rate since this is calculated as the ratio between deposition and retention. The faster clearance in the trachea seen in this study indicates that the clearance mechanisms for bacteria are likely to be more effective in the trachea than in the lung. However, both a relatively weak adhesive ability and lack of multiplication **in situ** may also account for the short retention of the organism in the trachea.

Compared to other virulent **Bordetella** organisms, clearance of the **B. parapertussis**-like organism was relatively rapid. This implied that the ability of the organism to adhere to ciliated respiratory epithelium was relatively weaker than other well-identified virulent **Bordetella** organisms, but stronger than avirulent **Bordetella** organisms and non-adhering organisms, such as **P. haemolytica**. In experimentally infected mice, Sato et al. (1980) found that **B. pertussis** could multiply in 18-day-old ICR mouse lung and persist for at least 21 days. The result of Sato et al. (1980) has further confirmed earlier work (Dolby et al., 1961). Similarly, Bemis et al. (1977) demonstrated that **B. bronchiseptica** required 14 wk to be cleared from the trachea of infected dogs. In contrast, avirulent **B. pertussis** organisms were no longer visible in rabbit bronchi four hr post-inoculation (Tuomanen, 1985). **P. haemolytica** was completely cleared from the mouse trachea and lung at 8 and 24 hr, respectively (Rodriguez et al., 1985).

The absence or weakness of bacterial adhesion may partly reflect the inability of the organism to produce certain toxic substances. In the case of **B. pertussis** infection, filamentous

haemagglutinin and pertussis toxin are critical to the process of adherence (Tuomanen et al., 1985).

Many workers have demonstrated that the ability to adhere is characteristic of virulent organisms and a critical step in the establishment of infection in the respiratory tract (Yokomizo & Shimizu, 1979; Tuomanen et al., 1985). The results of the present study are in keeping with this concept. The experimental infection in SPF mice and colostrum-deprived lambs (Chapter 2 and 3) showed that damage in the respiratory tract induced by the **B. parapertussis**-like organism was more severe in pulmonary tissue than in the airways. The different rates of bacterial retention demonstrated in this experiment help explain this observation. Further studies could therefore be profitably aimed at understanding the damage to pulmonary defence mechanisms induced by this organism, particularly at the alveolar level. In addition, differences in the retention of different strains of the organism in the respiratory tract also requires investigation although preliminary studies have shown no notable differences between five different strains and a mixture of these strains (unpublished data).

The lack of multiplication of the organism in the mouse respiratory tract might also be related to the inoculation dose since it has been demonstrated that the growth rate of **B. pertussis** in the mouse lung over the first four days is dependant on the size of the inoculum (Dolby et al., 1961). Small inocula grew quickly whereas an inoculum at the level of 10^8 resulted in no growth. Similar growth differences were also found in the growth of the **B. parapertussis**-like organism **in vitro** (Al-Kaissi, 1986). There is no doubt that under natural conditions small infective doses would be involved. It seems reasonable to assume that under the influence of the many stress factors encountered in the field and in the presence of the other bacterial flora, proliferation in the respiratory tract will occur.

An interesting finding was the significant increase ($P<0.05$) in

numbers of **B. parapertussis**-like organisms in the lungs between 12 and 18 hr after inoculation. This "atypical pattern" of bacterial clearance has not been well-documented previously although a similar phenomenon was encountered by Rodriguez et al. (1985) in experimental infection of mice with **P. haemolytica**. A second study using greater numbers of mice is necessary before the finding can be regarded as valid.

SUMMARY

A comparative study of the deposition and retention of a **B. parapertussis**-like organism in the lungs and trachea of intranasally inoculated mice was undertaken.The numbers of bacteria were deterimined in the lungs and tracheae of 36 SPF mice sacrified at 0, 6, 12, 18, 24 and 48 hr after inoculation. The results indicate that the deposition of the **B. parapertussis**-like organism was greater in the trachea than in the lung, and the clearance of the organism was faster from the trachea than the lung. Up to 48 hr post-inoculation, almost all organisms were cleared from the trachea but about 45% of organisms were retained in the lung. This result correlates well with the morphological changes seen previously in the pulmonary parenchyma and airways of the infected mice and lambs.

CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5

GENERAL DISCUSSION

The main features of the lesions produced by experimental infection with the **B. parapertussis**-like organism in SPF mice and colostrum-deprived lambs were a mild acute tracheobronchitis, extensive moderate degeneration of alveolar epithelial cells, severe peribronchiolar alveolar collapse and marked degeneration of alveolar macrophages. All these changes are similar to the lesions seen in early cases of naturally-occurring ovine CNP (Alley, 1975a; Pfeffer, 1981; Pfeffer et al., 1983; Al-Kaissi, 1986). This suggests that the **B. parapertussis**-like organism may have a role in initiating or augmenting naturally-occurring CNP in lambs. This possibility is consistent with the relationship observed between **B. bronchiseptica** infection and **Pasteurella multocida** in respiratory disease of swine (Bemis & Wilson, 1985). Similarly, in humans, **B. pertussis**-infected patients show increased susceptibility to other secondary bacterial infections (Olsen, 1975). There are two mechanisms by which **B. bronchiseptica** and **B. pertussis** might alter host susceptibility to other infections without producing overt morphological damage to tissue. The first is the elaboration of adenylate cyclase which is capable of altering such cellular functions as phagocytosis and intracellular killing. Secondly, the organisms possess the ability to immobilize respiratory tract cilia (Confer & Eaton, 1982; Bemis & Wilson, 1985). The results from the present study directly and indirectly comfirm that these two mechanisms are also likely to be involved in experimental pneumonia induced by the **B. parapertussis**-like organism.

Although the damage to the tracheobronchial epithelium of lambs infected with the **B. parapertussis**-like organism was relatively mild, it was severe enough to cause dysfunction of the mucociliary

apparatus as indicated by the excess accumulation of mucus and inflammatory exudate in airways without obvious hyperplasia of secretory cells. The role of the mucociliary apparatus in defence of the respiratory tract against infectious diseases is well known. Any dysfunction of ciliary clearance could be expected to decrease markedly the resistance of the respiratory tract to pathogenic agents and severely depress the removal of inflammatory material and mucus. These changes together with the marked degenerative changes seen in alveolar macrophages may be responsible for an increased susceptability of the respiratory tract to infections.

The pulmonary parenchyma is one of the major sites of injury in infection with the **B. parapertussis**-like organism in both mice and lambs. Extensive degeneration of the alveolar epithelium was consistently seen in ultrastructural studies and the importance of these epithelial cells in maintaining expansion and the structural integrity of alveoli is well-established (Dunnill, 1982). The degeneration and loss of alveolar type I cells was likely to be directly responsible for the exudation into alveolar spaces. The damage to alveolar type II cells would result in loss of pulmonary surfactant synthesis and this together with blockage of airways may have been a major factor in the extensive alveolar collapse observed. It is interesting to note that alveolar type II cells from both infected mice and lambs exhibited an extensive increase in thickness of the plasma membrane, a feature seen in naturally-occurring ovine CNP (Alley, 1975a) but not reported elsewhere. The possibility that this degenerative change may be directly associated with the **B. parapertussis**-like organism and/or its products should therefore be considered.

There may be several reasons why the lesions in the experimentally infected mice were more severe than in the lambs. Since the ovine lung is relatively poor in collateral ventilation due to a paucity of Kohn's pores (Al-Kaissi, 1986) this may limit the spread of the inoculated organisms. The instillation technique used in the lambs did not involve anaesthesia and the coughing which occurred probably resulted in considerable variation in the amount

and distribution of inoculum reaching the lungs. In addition, the anaesthesia in the mice could depress the clearance rate of inoculated bacteria (Green et al., 1964; Dunnill, 1982) which would amplify the degree of pulmonary injury.

It has been suggested by many authors that alveolar collapse may predispose the lung to further infection (Jubb & Kennedy, 1970; Pfeffer, 1981). In addition, it might represent an early stage of infection and could progress directly to form larger consolidated lesions (Pfeffer, 1981). McGowan et al. (1957) suggested that the small lesions they observed might lead to a more widespread secondary pneumonia. The collapsed areas usually have poor oxygenation which may provide a favourable microenvironment for the multiplication of other microorganisms and deprive alveolar macrophages of oxygen.

Severe ultrastructural changes were seen in alveolar macrophages in the animals infected with the **B. parapertussis**-like organism. Although it is premature to conclude that these morphological alterations may indicate corresponding functional impairment, the evidence from studies of other **Bordetella** organisms has shown that similar ultrastructural changes correlate with dysfunctions in oxidative metabolism, significant decreases in cell adherence, phagocytic uptake and bactericidal activity (Hoidal et al., 1978; Sherman & Lehrer, 1984; Zeligs et al., 1986). The mechanisms for these dysfunctions are not yet clear, but the endotoxin and adenylate cyclase released from the organisms are suspected to be responsible (Davis et al., 1980; Confer & Eaton, 1982). A recent study by Eudoh et al. (1980) showed that **B. parapertussis** could also produce a high activity of adenylate cyclase in culture fluid during the logarithmic growth phase. It is therefore, possible that the cytotoxic changes seen in alveolar macrophages in the current study are also related to this substance. This possibility combined with the relative paucity of alveolar macrophages in the normal ovine respiratory tract (Al-Kaissi, 1986) may cause significant damage to pulmonary defence against infectious agents.

The importance of alveolar macrophage degeneration in predisposing sheep to secondary bacterial infection is nevertheless open to doubt. **Pasteurella haemolytica** is the commonest bacterial isolate from the lower respiratory tract of CNP-affected sheep (Alley, 1975b), and neutrophils have been found to be more important than macrophages in controlling the growth and multiplication of this organism (Davies et al., 1981c). Davies et al. (1986) have also recently noted that there was no difference in the number of vacuolated alveolar macrophages between lambs which controlled **P. haemolytica** infection and those which did not. However, it has been well established that alveolar macrophages are responsible for secreting interferon (Dungworth, 1985), for killing some ingested bacteria (Dungworth, 1985) and phagocytosing **M. ovipneumoniae**, another common isolate from the lower respiratory tract of CNP-affected sheep (Al-Kaissi & Alley, 1983). Therefore, degeneration of these phagocytes may allow **M. ovipneumoniae** to replicate in the lung.

Many important questions concerning the interaction of the **B. parapertussis**-like organism and the respiratory epithelium in the pathogenesis of ovine CNP remain unanswered. Although the **in vitro** studies of Al-Kaissi (1986) showed a close association between the **B. parapertussis**-like organism and cilia of tracheal explants, the present investigation revealed that this interaction was weak **in vivo** (Chapter 2 and 3). It is likely that this organism is able to induce mild inflammatory change in tracheobronchial ciliated epithelium without obvious attachment. This is analogous to the results of Magar et al. (1985) who found that in some cases mild rhinitis could be induced in suckling mice after infection with **B. bronchiseptica** strains which had lost their adhesive abilities. In naturally-occurring CNP, a close relationship between bacteria and ciliated epithelium was rarely observed regardless of whether early or advanced lesions were examined (Alley, 1975a; Al-Kaissi, 1986). This implies that factors other than adhesive ability play an role in inducing the disease process. Further studies are, therefore, needed to give a better understanding of this problem.

The current findings indicate that the **B. parapertussis**-like organism, when inoculated at doses of approximately 10^7, was not able to multiply in the lungs of colostrum-deprived lambs and SPF mice, and was eliminated at a rapid and continuous rate. The short duration of the **B. parapertussis**-like organisms infection in mouse and ovine lungs may be attributed to lack of pertussis toxin. Infection of **B. pertussis** is mediated by the combination of adenylate cyclase toxin in the acute stage followed by pertussis toxin in the later stages of infection (Weiss & Hewlett, 1986). Even though no multiplication of the **B. parapertussis**-like organism occurred, the dose used in the current studies was capable of causing pulmonary destruction. These effects presumably resulted from the elaboration of one or more toxins by the organism **in situ** (DeMaria et al., 1978).

The close similarity of the pathological changes between SPF mice infected with **B. parapertussis**-like organism and early naturally-occurring ovine CNP has shown that mice can be a satisfactory laboratory animal model for studying infection with the **B. parapertussis**-like organism in sheep. The major pathological differences observed were only in the severity and distribution of lesions. The development of a laboratory animal model for the **B. parapertussis**-like organism will be valuable for investigating the pathogenesis of this organism in ovine pneumonia, since the high prevalence of this organism in conventionally-raised sheep and the expense of SPF lamb production are obvious limiting factors to future research.

Possible changes in the nasal cavity of animals infected by the **B. parapertussis**-like organism were neglected in the present study. The organism has been found to colonize a high percentage of nasal cavities in both experimentally infected lambs and lambs with naturally-occurring CNP (Chapter 1 and 3).

Future studies on the pathogenesis of the **B. parapertussis**-like organism in ovine CNP may be profitably directed towards

epidemiological investigations of the seasonal and age incidence of this organism in the respiratory tract of healthy and CNP-affected lambs. Its relationship with other potential pathogens, such as **P. haemolytica** and **M. ovipneumoniae** is also uncertain. There is also need for a laboratory test to measure the phagocytic activity of alveolar macrophages infected with the **B. parapertussis**-like organism both **in vitro** and **in vivo**. The present study suggests that substances released by the organism may play a role in inducing morphological damage to the ovine and murine respiratory epithelium and alveolar macrophages. Information on the toxins produced or released by this organism and their roles in injuring the ovine respiratory tract is therefore required.

Although the present work strongly suggests that the **B. parapertussis**-like organism could have a role in ovine respiratory infection, the exact nature of its contribution to the pathogenesis of ovine CNP in New Zealand will remain unclear until some of the above questions have been answered.

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APPENDIX

APPENDIX 1

THE MAIN CHARACTERISTICS OF THE **BORDETELLA PARAPERTUSSIS**-LIKE ORGANISM (BPLO) ISOLATED FROM OVINE RESPIRATORY TRACT*

Chracteristics	BPLO**	BPARA	BPER	BBRO
Motility	-	-	-	+
Brown pigment production in peptone medium	-	+	-	-
Urease reaction	5-30 min	24 hr	-	4 hr
Oxidase reaction	+/-	-	+	+

* According to Cullinane et al. (1987).

** **B. parapertussis**-like organism (BPLO), **B. parapertussis** (BPARA), **B. pertussis** (BPER), **B. bronchiseptica** (BBRO).

The organism was identified by the National Health Institute (Wellington) as **B. parapertussis**.

Further work on the identification of this organism including DNA analysis and bacterial restriction enzyme analysis is in progress.

APPENDIX 2

MODIFIED KARNOVSKY'S FIXATIVE

Paraformaldehyde	2.0	gm
Gluteraldehyde	12.0	ml
$Na_2HPO_4.12H_2O$	2.51	gm
KH_2PO_4	0.41	gm

METHOD

1. Heat the paraformaldehyde (2.0 g in 80 ml distilled water) to about 60 to 80°C.
2. Slowly add 1.0 N NaOH, dropwise until the solution clears.
3. Then add $Na_2HPO_4.12H_2O$ and gluteraldehyde.
4. Make up the solution to 100 ml at 4C.

APPENDIX 3

METHOD FOR
STAINING "THICK" EPOXY EMBEDDED HISTOLOGICAL SECTIONS

1. Cut sections at 0.5 to 1.0 µm and float off in a water bath, and pick up with a Pasteur pipette.

2. Transfer sections to a glass slide on which a drop of distilled water is prepared, and then heat-dry on a hot plate at 90°C for about 1 minute.

3. While at this temperature, cover sections completely with a few drops of 1% toluidine blue (prepared in 0.01M PBS, pH 7.4) and leave on hot plate until the stain begins to evaporate at the edges.

4. Rinse under running water until the excess stain is washed off and remove the left over water with filter paper.

5. Return the slide to the hot plate to completely dry it then mount in D.P.X.

APPENDIX 4

STAINING SOLUTION AND TECHNIQUE FOR TEM SECTION

SOLUTIONS

Uranyl acetate

The stain was made by adding uranyl acetate crystals to 50% ethanol until saturation point was reached, then shaken intermittently for 5 to 15 minutes.

Lead citrate

$Pb(NO_3)_2$	1.33 gm
Trisodium citrate	1.76 gm
Distilled water	50.0 ml

1. Add lead nitrate and sodium citrate to 30 ml of distilled water and shake vigorously for 1 min. A heavy white precipitate will be formed.
2. Shake the solution every five minute over a 30 minutes period to facilitate conversion of lead nitrate to lead citrate.
3. Add 8 ml of 1N NaOH with agitation and dilute to 50 ml with distilled water. The white precipitate of lead citrate will dissolve during this step.
4. Filter the stain and keep at room temperature.

STAINING METHOD

1. Using a Pasteur pipette, place one drop of the uranyl acetate and lead citrate solutions separately on a piece of Parafilm (American Can Company) for each grid.
2. Put the grid into the drop of uranyl acetate solution for 8 min. and make sure that the grid is completely covered by the stain.
3. Rinse the grid with 50% alcohol and then distilled water thoroughly and gently, and dry the grid with filter paper.
4. Immerse the grid into the drop of lead citrate solution for 8 min. and then rinse the grid with distilled water, dry and store in the grid box for EM examination.