A COMBINED HISTOLOGICAL, HISTOCHEMICAL AND SCANNING ELECTRON MICROSCOPICAL STUDY OF THE CANINE RESPIRATORY TRACT

A thesis submitted to the Faculty of Veterinary Medicine
University of Glasgow
For the Degree of Doctor of Philosophy.

by

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University of Glasgow,
June, 1986.

Vol.1.

VOLUME I

ACKNOWLEDGMENTS

The author expresses his deep gratitude to his Supervisor Professor N.G. Wright, Head of the Department of Veterinary Anatomy University of Glasgow, for his constant advice, constructive criticism and encouragement during the course of the preparation of this Thesis.

I am also indebted to Dr. S. Solomon and all the Staff of the Department of Veterinary Anatomy for their sympathy and encouragement during the course of my stay in Glasgow.

I wish to express my thanks to Dr. I. McCandlish, Department of Veterinary Pathology, for providing the culture of Bordetella bronchiseptica used in Chapter 7.

I received much expert technical assistance in the preparation of this work and I am particularly indebted to the following people :

Dr. M. Purton for his advice on vascular perfusion methods, Mr. A.W. Minto and Miss K. Leslie for their help in the preparation and staining of material for histological and histochemical examinations, Mr. G. McMillan and Miss P. Bell for their guidance in preparing specimens for ultrastructural examination and Mr. C. Paterson for preparing the diagrams.

Shamim, my wife, and my family and friends were a constant source of inspiration and encouragement throughout my studies.

The financial support of the Government of Pakistan is gratefully acknowledged.

DECLARATION

I declare that the work presented in this Thesis was carried out by me personally. Some of the Figures, namely, Figs. 3.22, 3.89, 5.13, 5.16, 5.17, 5.21, 5.23 and 7.50 were prepared from photographs taken by Professor N.G. Wright.

ABDUL MAJID.

SUMMARY

The object of the work was twofold. First, to carry out a detailed histological, histochemical and scanning electron microscopical (SEM) study of the whole length of the canine respiratory tract and, second, to investigate how the canine respiratory surfaces respond to challenge with Bordetella bronchiseptica. The latter organism was chosen due to its prevalence in contagious respiratory disease in dogs and because it is sufficiently large to be directly visualisable with the SEM.

It was very noticeable from the review of the literature in Chapter 1 that, despite the importance of clinical respiratory disease in dogs, comparatively little information is available concerning the normal histological and ultrastructural features of the canine airways. There was no previous description of the morphological features of the whole respiratory tract from the nasal vestibule to the alveolar membrane. The high morbidity and mortality associated with contagious canine respiratory disease provided a strong stimulus to carry out a detailed histological, histochemical and SEM study of the canine airways.

In Chapter 3 of the work, 18 collie cross-bred puppies aged 14-18 weeks were used for this purpose and samples were taken from a number of sites throughout the respiratory tract viz., ventral concha, nasal septum, ethmoidal concha, nasopharynx, epiglottis, ventral larynx, trachea, extrapulmonary bronchus and lung.

Histological examination confirmed that the conducting portion of the canine respiratory tract is lined almost entirely with pseudostratified columnar ciliated epithelium. The nasal vestibule, the rostral portion of the nasal septum, the cranial region of ventral larynx, however, were covered with stratified squamous non-keratinising epithelium while olfactory epithelium clothed the ethmoidal concha and the caudal region of the nasal septum. Although widely distributed throughout the canine airways, the greatest concentration of mucus-secreting goblet cells was to be found in the extrapulmonary bronchus. With the Alcian-blue periodic acid-Schiff stain, most of these cells contained acid or mixed mucosubstances

With SEM, the degree of ciliation throughout the respiratory mucosa was more or less complete as far as the bronchiolar level. Very few non-ciliated cells were found in the ventral concha, nasal septum, trachea, extrapulmonary bronchus, large and small bronchi and large bronchioles. However, in only 7 of the 18 dogs was the nasopharynx wholly clothed with ciliated respiratory epithelium. Likewise, in the case of the ventral larynx, only five animals showed the characteristic ciliated respiratory epithelium. At the level of the alveolar membrane, no macrophages were found and alveolar pores of Kohn were rare.

In Chapter 4, a detailed analysis of the extent of respiratory epithelium covering various sites of the canine larynx was carried out. In this study, whole larynges from 11 dogs were immersed in a solution of Phloxine B and/or Alcian-blue and the areas of respiratory, compared to non-respiratory, epithelium compared. Samples were subsequently taken from the cranial epiglottis, caudal epiglottis, ventral larynx, dorsal larynx and caudal larynx and examined histologically. This investigation emphasised how little of the surface mucosa of the canine larynx is actually covered with respiratory epithelium, as well as pointing out the overlap of non-respiratory and respiratory epithelium which occurs at the level of the ventral larynx.

In Chapter 5, an opportunity was taken to carry out a joint transmission electron microscopical (TEM) and SEM study of distal airways in conjunction with a comparative fixation study (airways versus vascular perfusion). Regardless of the method of fixation used for SEM, alveolar pores of Kohn were uncommon and alveolar macrophages were never found. On the other hand TEM studies, which identified the structural details of ciliated cells, Clara cells and alveolar Type I and Type II pneumonocytes, clearly showed the presence of alveolar macrophages.

In Chapter 6, an attempt was made to identify the presence of canine alveolar macrophages using a method of lung lavage and examining the cells collected by conventional light microscopy as well as with SEM

and TEM. Smears of the pulmonary cell suspensions obtained by this method showed that the vast majority of cells (90%) had the morphological characteristics of macrophages. With SEM, the irregular surface of these cells with their occasional long slender tendrils was revealed and, with TEM, their foamy cytoplasmic nature characterised by numerous vacuoles and lysosomes was a prominent feature.

In the final Chapter, a combined histological, histochemical and SEM study of the respiratory tract of dogs exposed to an aerosol stream of Bordetella bronchiseptica was carried out. In this study, 12 dogs aged 14-18 weeks were used. Samples were taken from the ventral concha to the alveolar membrane at 4, 6, 8, 10, 20 and 28 days after infection in order to map the various surface changes taking place. Severe surface ciliary disorganisation and necrosis was found throughout the tract and, unlike the normal control dogs, numerous alveolar macrophages were identifiable with SEM. Goblet cells throughout the tract were reduced in number and stained submucosal glandular units were correspondingly decreased. By 28 days, however, the respiratory tract had returned virtually to normal.

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LIST OF ABBREVIATIONS

HE	:	Haematoxylin and eosin stain
AB-PAS	:	Alcian blue-periodic acid-Schiff stain
PAS	:	Periodic acid-Schiff stain
NBF	:	Neutral buffered formalin
TEM	:	Transmission electron microscope (or microscopy or microscopical)
SEM	:	Scanning electron microscope (or microscopy or microscopical)
PBS	:	Phosphate buffered saline

CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. GENERAL INTRODUCTION

Contagious respiratory disease is recognized to be a major clinical problem in dogs and wherever these animals are maintained in close contact for any period of time, outbreaks of respiratory disease are a significant cause of morbidity and mortality. This can effectively cause delay and disruption to research programmes as well as concern to individual dog owners and general economic losses to commercial breeders (Snow et al, 1969; Wright et al, 1974; Thompson et al, 1975; Appel and Bemis, 1978; McCandlish et al, 1978[d]).

Laboratory investigation of outbreaks of respiratory disease has led to the isolation and characterisation of a number of causative agents such as canine herpesvirus (Karpas et al, 1967; Wright and Cornwell, 1970), canine distemper virus (Bjotvedt et al, 1969; Appel, 1970; Thompson et al, 1975), canine adenovirus, sub type I, (Wright et al, 1972; Studdert and Studdert, 1972), canine adenovirus, sub type II, (Ditchfield et al, 1962; Appel et al, 1970), canine parainfluenza (SV5) virus (Binn et al, 1968; Appel and Percy, 1970) and Bordetella bronchiseptica (Appel et al, 1970; Thompson et al, 1976).

Despite the importance of respiratory disease in the dog, it is remarkable how few studies of the normal microscopic and ultrastructural features of the respiratory tract have appeared in the literature. Furthermore, such reports that are available are largely confined to histological and ultrastructural studies of the tracheobronchial tree, usually as part of an investigation of disease processes (Goco et al, 1963; Auerbach et al, 1967; Frasca et al, 1968; Spicer et al, 1971; Wheeldon and Pirie, 1974). Even fewer reports are available concerning the histological and ultrastructural appearance of the nasal fossa and nasopharynx (Okano and Sugawa, 1965; Adams and Hotchkiss, 1983).

The primary purpose of the present study was to carry out a histological and histochemical study of both conducting and respiratory

portions of the respiratory tract, i.e., from the nasal fossa to the alveolar wall and, with the aid of the scanning electron microscope, to provide for the first time in the dog normal baseline parameters for the topographical appearance of the whole of the tract.

1.2. REVIEW OF THE LITERATURE

The basic structure of the lining of the various compartments of the mammalian respiratory tract were determined by the late nineteenth century (Aeby, 1880). Since the early description of ciliated cells (Purkinje and Valentin, 1834; Sharpey, 1836) and of mucus-secreting goblet cells (Henle, 1837; Bowman, 1847; Schulze, 1872), there have been many descriptions of the microscopic anatomy of airway lining epithelium (Kolliker, 1853; Frankenhaueser, 1879; Bryant, 1916; Miller, 1932; Lucas, 1932; Schaeffer, 1932; Krahl, 1955, 1959; Hayek, 1960; Ali, 1965; Bertalanffy, 1964; Bloom and Fawcett, 1968; Pass et al, 1971; Lauweryns and Peuskens, 1971; Hage, 1973[a]; Mariassy and Plopper, 1983; Wilson et al, 1984).

1.3. TEM STUDIES

The introduction of transmission electron microscopy (TEM) in the 1950's subsequently allowed characterisation and identification of a number of additional cells populating the respiratory tract which had not hitherto been recognized by light microscopy. These include the serous cell (Jeffery and Reid, 1975), brush cell (Rhodin and Dalhamn, 1956; Meyrick and Reid, 1968; Baskerville, 1970[b]), basal and intermediate cell (Rhodin and Dalhamn, 1956; Rhodin, 1966; Jeffery and Reid, 1975), endocrine (Kultchitsky) cell (Hage, 1971; Cutz and Conen, 1972; Moosavi et al, 1973; Cutz et al, 1975), special cell (Frasca et al, 1968; Jeffery, 1983), alveolar type I and type II cells (Low, 1952; Karrer, 1956; Curry et al, 1969; Breeze and Wheeldon, 1977) and alveolar macrophages (Gail and Lenfant, 1983).

Furthermore, TEM provided further cytological details of cells already recognized by light microscopy, namely :-
ciliated and non-ciliated cells (Rhodin and Dalhamn, 1956; Rhodin, 1966; Friedmann and Bird, 1971), mucus-secreting (Goblet) cells (Rhodin and Dalhamn, 1956; Rhodin, 1959; Jeffery and Reid, 1975) and the non-ciliated bronchiolar secretory (Clara) cells (Cutz and Conen, 1971; Smith et al, 1974; Evans et al, 1978). So far, excluding the neuroepithelial bodies described by Hung and Loosli (1974), at least eleven different cell types have been identified populating the mammalian respiratory tract, although not all have been recognized in each mammalian species (Jeffery and Reid, 1975; Breeze et al, 1976; Breeze and Wheeldon, 1977; Phipps, 1981; Gail and Lenfant, 1983).

A cross-section of the ultrastructural studies carried out in different species and an indication of the areas of the respiratory tract examined is given in Table 1.1.

Arising from these studies it was considered worthwhile to provide a summary of the main morphologic features of the various types of epithelial cells populating the mammalian respiratory tract. The epithelial cells of mammalian airways are summarised on Page 8.

TABLE 1.1

TRANSMISSION ELECTRON MICROSCOPY OF THE RESPIRATORY TRACT OF VARIOUS ANIMAL SPECIES

SPECIES	SAMPLE SITE	REFERENCE
Man	Trachea	Rhodin (1959)
"	Trachea and Lung	Rhodin (1963)
"	Bronchus	Watson and Brinkman (1964)
"	Trachea	Rhodin (1966)
"	Mucous membrane of the nose	Graziadei (1970)
"	Lung: endocrine-like cells	Cutz and Conen (1972)
"	Upper respiratory tract	Friedmann and Bird (1971)
"	Small bronchiole	Rosan and Lauweryns (1972)
"	Trachea: endocrine cells	Cutz et al (1975)
"	Clara cell	Smith et al (1979)
"	Clara cell	Plopper et al (1980^{c})
"	Clara cell	Cutz and Conen (1971)
Rat	Lung	Low (1952)
"	Trachea	Rhodin and Dalhamn (1956)
"	Trachea	Rhodin (1959)
"	Lung: alveolar brush cell	Meyrick and Reid (1968)
"	Airway (trachea and lung)	Jeffery and Reid (1975)
"	Large bronchus	Jeffery and Reid (1973)
"	Bronchus: Feyrter cell	Moosavi et al (1973)
"	Extrapulmonary respiratory tract	Andrews (1974)
"	Clara cell	Kuhn et al (1974)
"	Clara cell	Smith et al (1974)
"	Clara cell	Evans et al (1978)
"	Clara cell	Smith et al (1979)
"	Trachea and bronchi	Alexander et al (1975)
"	Trachea and lung	Jeffery and Reid (1975)

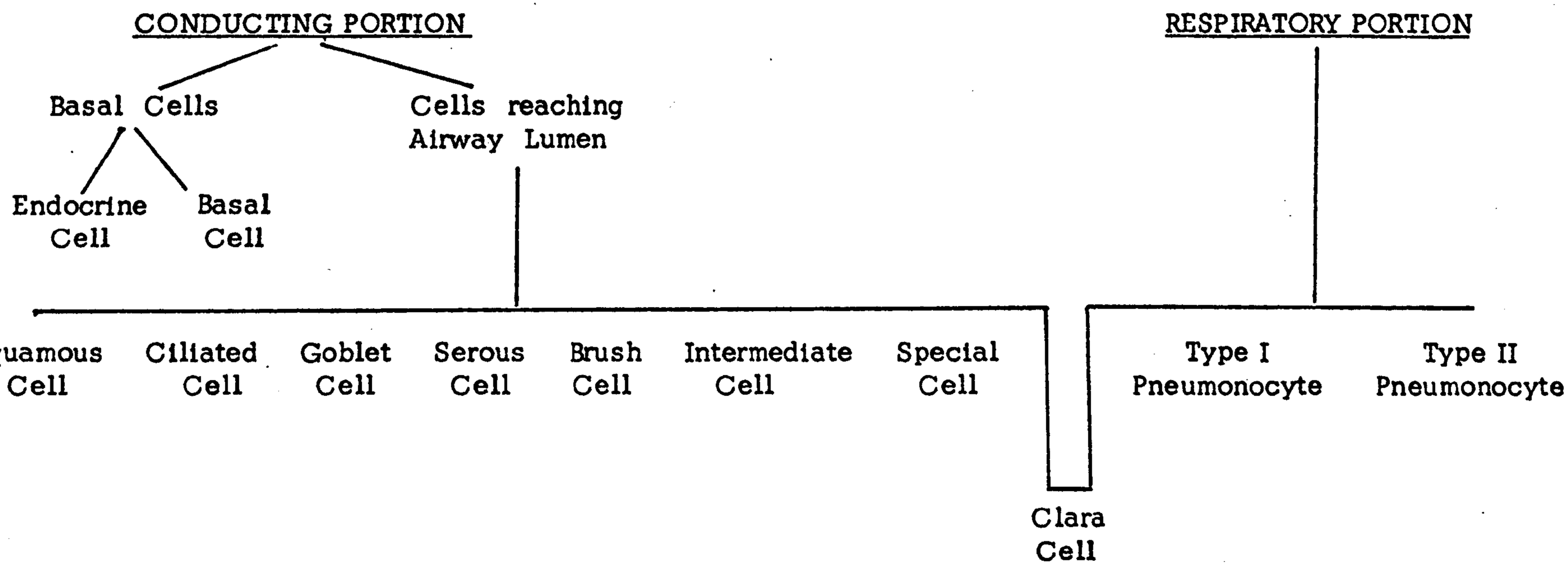
TABLE 1.1 (Cont'd)

SPECIES	SAMPLE SITE	REFERENCE
Rat	Bronchus	Yoneda (1976)
"	Alveolar macrophages	Sorokin et al (1984)
"	Nasal respiratory epithelium	Monteiro-Riviere and Popp (198[illegible] 4
Mouse	Lung	Karrer (1956)
"	Lung	Curry et al (1969)
"	Lung: neuroepithelial body	Wasano and Yamamoto (1981)
"	Trachea	Chen and Lin (1972)
"	Nasal respiratory epithelium	Matulionis and Parks (1973)
"	Bronchiole	Hung and Loosli (1974)
"	Clara cell	Stinson and Loosli (1978)
"	Clara cell	Plopper et al (1980[a])
"	Tracheobronchial epithelium	Pack et al (1981)
Guinea Pig	Trachea	Dahlgren et al (1972)
"	Clara cell	Plopper et al (1980[a])
"	Distal airway epithelium	Davis et al (1984)
Rabbit	Lung	Kisch (1958)
"	Clara cell	Plopper et al (1980[a])
"	Clara cell	Smith et al (1979)
"	Clara cell	Cutz and Conen (1971)
"	Trachea	Hilding et al (1966)
"	Endocrine cell	Cutz et al (1975)
"	Trachea	Konradova (1966)
"	Lung	Cutz and Conen (1971)
"	Bronchi	Sturgess (1977)
"	Neuroepithelial bodies	Lauweryns et al (1974)
"	Alveolar macrophages	Sorokin et al (1984)
Hamster	Tracheobronchial epithelium	Harris et al (1971)
"	Clara cell	Plopper et al (1980[a])
"	Alveolar macrophages	Sorokin et al (1984)
"	Tracheobronchial epithelium	Becci et al (1978)

TABLE 1.1 (Cont'd)

SPECIES	SAMPLE SITE	REFERENCE
Monkey	Intrapulmonary airways	Castleman et al (1975)
"	Trachea	St. George et al (1984)
"	Tracheobronchial epithelium	Wilson et al (1984)
Pig	Lung: bronchus	Baskerville (1970[a,b])
"	Clara cell	Widdicombe and Pack (1982)
"	Lung	Winkler and Cheville (1984)
Horse	Lung	Gillespie and Tyler (1967)
"	Lung	Tyler et al (1971)
"	Clara cell	Plopper et al (1980[b])
Ox	Lung	Epling (1964)
"	Clara cell	Smith et al (1979)
"	Clara cell	Plopper et al (1980[b])
"	Nasal mucosa	Bozarth and Strafuss (1974)
"	Lung: brush cell	Allan (1978)
Sheep	Tracheobronchial epithelium	Mariassy and Plopper (1984)
"	Clara cell	Plopper et al (1980[b])
Fowl	Trachea	Purcell (1971)
"	Lung	King et al (1974)
"	Larynx, Trachea, Primary Bronchi	Walsh and McLelland (1974)
"	Nasal mucosa	Reissig et al (1978)
Lamb	Trachea: endocrine cell	Cutz et al (1975)
Dog	Lung	Ortega et al (1970)
"	Lung	Kondo et al (1973)
"	Lung	Hyde et al (1978)
"	Interalveolar pores of Kohn	Parra et al (1978)
"	Nasal cavity	Edwards et al (1983)
"	Vomeronasal organ	Adams and Wiekamp (1984)
"	Clara cell	Plopper et al (1980[b])
Cat	Clara cell	Plopper et al (1980[b])

EPITHELIAL CELLS OF THE MAMMALIAN RESPIRATORY TRACT *



* Excluding the Cells of Olfactory Epithelium.

1. Mucus-Secreting (Goblet) Cells:

These cells are found throughout the respiratory tract from the nasal fossa to the bronchioles (although not usually extending to the terminal or respiratory bronchioles). In the trachea the number of goblet cells is greater in the cartilaginous than in the membranous zones of the wall (Rhodin and Dalhamn, 1956; Frasca et al, 1968). Each goblet cell has relatively electron dense cytoplasm due to their content of mucous granules and numerous ribosomes in their apical cytoplasm. The distribution of the mucous granules gives the mature cell its characteristic "goblet" shape, in which only a narrow part of the tapered basal cytoplasm touches the basement membrane. Immature or discharged goblet cells do not have this typical appearance and are more slender and columnar.

The lower part of the cell forms complex interdigitations and makes desmosomal attachments with adjacent cells. Each individual goblet cell is surrounded by an irregular intercellular space that is closed at the luminal surface by tight junctional complexes. The irregular oval nucleus, with its small nucleolus, is found towards the base of the cell along with a few mitochondria. A well developed Golgi apparatus is usually found above the nucleus. The mature cell is characterised by a dome-shaped apical portion, containing the mucous granules and which often protrudes between the adjacent ciliated cells into the lumen of the respiratory tract. A few microvilli are often to be found around the apical cell margin. Secretion of mucus is considered to be by extrusion through pits or pores in the cell surface.

In hamsters, Becci et al (1978) have described a type of small mucous granule cell in the tracheobronchial epithelium which they considered to represent developing mature mucus-secreting cells.

2. Ciliated Cells:

In all the mammalian species, ciliated and goblet cells together

make up the bulk of the respiratory epithelium, there being approximately five ciliated cells to every one goblet cell (Rhodin and Dalhamn, 1956; Frasca et al, 1968). Ciliated cells are found in airway epithelium from the nasal fossa to the small bronchioles.

There are several detailed descriptions of the appearance of respiratory tract ciliated cells and they appear to vary little in such species as the dog (Frasca et al, 1968), man (Rhodin, 1959, 1966) and rat (Rhodin and Dalhamn, 1956; Jeffery and Reid, 1975). Ciliated cells are columnar in appearance approximately 20 μm long and 7 μm wide tapering to 2 μm at their base where they attach to the basement membrane (Frasca et al, 1968). Complex interdigitations with basal, intermediate and other adjacent cells are found in the lower lateral surface of the cell. The cell cytoplasm is more electron lucent than that of most other non-ciliated epithelial cells. Rough-surfaced endoplasmic reticulum is present and a well developed Golgi apparatus is situated above the nucleus, which often contains a prominent nucleolus. Very many mitochondria are found in the upper part of the cell, just below the apical row of basal bodies, to which the cilia are attached.

There are approximately 250 cilia, each approximately 6 μm long and 0.3 μm wide, on the luminal surface of each cell, and interspersed among these are about half as many fine cytoplasmic processes or microvilli (Rhodin and Dalhamn, 1956; Frasca et al, 1968; Baskerville, 1970[a]). These microvillous projections, up to 2μm long and 0.1 μm diameter, are particularly abundant on the cell borders and fine filaments are found within their cytoplasmic core. The microvilli are particularly numerous on immature ciliated cells undergoing ciliogenesis. Only a few short cilia may project from the surface of such cells which for the most part are almost exclusively covered with microvilli. The structure of cilia, basal bodies and rootlets is basically the same for all ciliated cells in animals. Each cilium contains 11 longitudinally-oriented microtubules surrounded by an extension of the cell membrane. Two microtubules form the central core of each cilium with nine double

tubules arranged around them. The two central tubules fuse at the tip of the cilium and do not extend to the basal body as the others do. Atypical cilia characterised by fusion of a number of cilia to form giant cellular protrusions have been observed in the respiratory epithelium of man (Friedmann and Bird, 1971), hamsters (Harris et al, 1974) and dogs (Wheeldon and Pirie, 1974). In the latter animal, they are regarded as a normal feature of canine bronchial epithelium. In the tracheobronchial epithelium of hamsters, Becci et al (1978) have described an "indifferent cell" containing both mucous granules and the basal bodies of cilia.

3. Serous Cell:

Serous cells were first recognized in the rat by Jeffery and Reid (1975). In other mammals they have only been identified with certainty in cats, hamsters and human foetuses (Jeffery, 1983). Ultrastructurally, they are characterised by having electron dense cytoplasm with abundant rough-surfaced endoplasmic reticulum. The cell rests on the basement membrane and its apical portion, which has a small number of microvilli, reaches to the airway lumen. The irregular nucleus is basally placed and the apical regions of cytoplasm contain a variable number of secretory granules smaller and more electron dense than those of epithelial mucous cells suggesting that these cells secrete chemically different products. The function of the serous cell is not known but is believed to secrete both acid and neutral glycoproteins, lysozyme and the epithelial transfer component of IgA; serous cells are also thought to contribute to the low viscosity periciliary fluid covering bronchial epithelium (Jeffery and Reid, 1975).

4. Non-Ciliated Bronchiolar Clara Cell:

The terminal bronchiolar epithelium of the mammalian respiratory tract is composed mostly of low ciliated and taller non-ciliated cells; the latter are termed "Clara cells". They are abundant in bronchioles of all mammals (Plopper, 1983) but are also found as far proximal as the hilus

in the rat (Jeffery and Reid, 1975) and possibly in tracheal epithelium in the mouse (Hansell and Moretti, 1969; Pack et al, 1981). Recently, Plopper (1983) has presented evidence that, in rabbits, Clara cells are not confined to the distal airways but extend into the tracheobronchial tree and indeed is the predominant secretory cell. The non-ciliated bronchiolar epithelial cells were first described by Kolliker (1881) but the earliest detailed study was that of Clara (1937) after whom the cell is named. It is columnar in shape with a deeply invaginated central nucleus and, characteristically, the apical region of the cell protrudes beyond adjacent ciliated cells. With the TEM, a number of differences have been observed across the species (Widdicombe and Pack, 1982); thus, in man, pig, rat and mouse many ovoid secretory granules are present, whereas in the cow and dog, these are scanty or absent. Likewise, cytoplasmic glycogen deposits are well developed in the cow and dog but scanty or absent in man, pig and rabbit (Plopper et al, 1980[c]). The function of the Clara cell is still in dispute but it is generally accepted that its secretion contributes to the bronchiolar hypophase and, since they contain abundant cytochrome P450, are now considered to play a role in detoxifying processes at the bronchiolar level (Boyd, 1977).

5. Basal Cell:

These are ovoid in shape and situated in a single row along the basement membrane giving rise to the pseudostratified appearance to the epithelium; their apical border does not reach the airway lumen. An irregular intercellular space around each cell is often present. The cell is mostly filled with the large nucleus and the sparse cytoplasm contains a small Golgi zone, many ribosomes, tonofilaments, a few mitochondria and glycogen granules (Rhodin, 1963, 1966; Frasca et al, 1968; Baskerville, 1970[a]). Basal cells are thought to be progenitor cells for both ciliated and non-ciliated cells via the intermediate cell.

6. Intermediate Cells:

These cells occupy an ill-defined layer just above the basal cells, thus contributing to the pseudostratified appearance of respiratory epithelium. They are more spindle-shaped and their apical borders extend towards the lumen of the airway. The nucleus is large and the abundant cytoplasm contains mitochondria. Cytoplasmic projections contact other cells and some form desmosomes (Rhodin and Dalhamn, 1956; Rhodin, 1966; Baskerville, 1970[a]). They do not usually reach to the lumen in the rabbit and pig (Baskerville, 1970[a]) but this has been observed in the rat, where the apical surface of the cell was found to be furnished with a few microvilli (Jeffery and Reid, 1975).

7. Brush Cell:

This airway cell was first noted in rat tracheal epithelium by Rhodin and Dalhamn (1956). In the rat, it has been estimated that brush cells make up approximately 1% of the cells in the tracheal epithelium (Jeffery and Reid, 1975). Popp and Martin (1984) have also identified brush cells in their SEM study of rat nasal epithelium while Monteiro-Riviere and Popp (1984) in a similar TEM study have also identified these distinctive cells. They have also been found in the tracheobronchial tree of the mouse, pig, cow and guinea-pig (Hama and Nagata, 1970; Inoue and Hogg, 1974; Baskerville, 1970[b]). In addition, cells resembling brush cells and termed type III pneumonocytes, have been observed in rat alveoli (Meyrick and Reid, 1968).

Brush cells are columnar cells which rest on the basement membrane and reach the airway lumen. The nucleus is found in the lower part of the cell below the Golgi zone. The cytoplasm contains abundant free ribosomes and glycogen granules. The apical part of the cell has numerous mitochondria and many small vacuoles and vesicles. Bundles of filaments which occur throughout the cytoplasm are a characteristic feature of the cell (Rhodin and Dalhamn, 1956; Baskerville, 1970[b]).

The luminal surface of the brush cell is covered by microvilli which are taller, wider and more uniformly arranged as compared to those of goblet and ciliated cells (Andrews, 1974). They contain axial filaments some of which appear to be continuous with bundles of filaments in the apical cytoplasm. It has been suggested, however, that they may have an absorptive function; due to their resemblance to intestinal brush border cells (Jeffery and Reid, 1975). The presence of cytoplasmic filaments has also led to the suggestion that the cell may function as a stretch receptor (Meyrick and Reid, 1968). On the other hand, afferent nerve endings, observed to contact the lateral borders of the brush cell, suggest a possible chemoreceptor role (Breeze and Wheeldon, 1977).

8. Endocrine Cell:

This cell resembles the intestinal Kultchitsky cell and hence is sometimes referred to as a "K" cell. It has been found in the airway epithelium of man (Bensch et al, 1965) and a number of laboratory animals (Cutz et al, 1974; Edmondson and Lewis, 1980). The morphologic and cytochemical features of these cells have been described in detail (Breeze and Wheeldon, 1977). Because of their morphologic and cytochemical characteristics which resemble those of other endocrine cells elsewhere in the body, they are included in the APUD (amine and amine precursor uptake and decarboxylation) series of cells (Pearse, 1969). The cell has a roughly triangular shape with a round or oval nucleus. The Golgi apparatus is well developed and the cytoplasm contains some smooth endoplasmic reticulum and free ribosomes. The basal portion of the cell contains characteristic granules that have an electron dense core and clear zone or halo between it and the surrounding limiting membrane.

Endocrine cells may or may not reach the airway lumen. There are contradictory reports on this point with differences observed between the species (Moosavi et al, 1973) and even within species (Moosavi et al, 1973; Cutz et al, 1974). Interdigitations of the lower cell borders occur with neighbouring cells, but without desmosomes or terminal bars. Microvilli

may be seen in these cells reaching the lumen (Hage, 1973[b]). Pulmonary endocrine cells may occur singly or in groups of three to five cells (Hage, 1972; Cutz et al, 1975). The function of the amines or polypeptides secreted by the endocrine cell is presently unknown.

9. Neuroepithelial Body:

These structures, which are collections of endocrine cells, are located at or near the bifurcation points of respiratory airways (Edmondson and Lewis, 1980; Wasano and Yamamoto, 1981). Although, like individual endocrine cells, little is known of the function of the neuroepithelial body, there have been many recent descriptions of their distribution, structure and histological properties (reviewed by Sorokin et al, 1983). They are found throughout the entire tracheobronchial and bronchiolar airways, even within the alveolar ducts and alveoli, but they appear to be particularly abundant in the bronchioles, at least in the rabbit (Lauweryns and Goddeeris, 1975).

Each neuroepithelial body is composed of variable numbers of tall, non-ciliated cells. The lateral edges of the bronchiolar neuroepithelial bodies are sometimes covered by Clara cells (Hung and Loosli, 1974). In the human infant lung, neuroepithelial bodies extend from the basement membrane to the lumen of the airway. Beneath the basement membrane fenestrated capillaries are closely associated with each body. Foetal neuroepithelial bodies are large and contain 10 - 30 cells; in the adult they are smaller and are composed of 3 - 10 cells (Lauweryns et al, 1972). Individual cells are tall and slender with a large oval nucleus which contains a small nucleolus. A few microvilli are present on the apical surface of the cell. Desmosomes and interdigitation of lateral cell margins have been observed (Lauweryns et al, 1972). Within the cytoplasm a well-developed Golgi apparatus is present. Like the singly occurring pulmonary endocrine cells, neuroepithelial cells contain characteristic granules in their basal regions and appear to be in close association with intraepithelial nerve endings. Their function is unknown

but it has been suggested that they act as chemoreceptors, responding to changes in the airways gases by releasing vasoactive amines such as serotonin (Gail and Lenfant, 1983).

10. Special Type Cell:

This cell was first identified in the dog (Frasca et al, 1968) and has also been noted in the cat and even in man (Jeffery, 1983). These wedge-shaped cells are found very rarely and have no apparent contact with the lumen. They form interdigitations with neighbouring cells, to which they are attached by desmosomes and the cell rests on the basement membrane. The nucleus is oval and the Golgi body situated above the nucleus. In the cytoplasm electron dense membrane bound disc-like or curved inclusions containing fibrogranular material are found. It is these structures which characterise the cell. The function of the special cell is unknown.

11. Alveolar Type I Cell: (Type I Pneumonocyte)

The alveolar type I cell is a highly differentiated cell that has lost its capacity to divide and covers approximately 93% of the alveolar surface in the human lung and 97% of that of the dog (Crapo et al, 1983). They have flattened nuclei which protrude slightly into the interior of the alveoli and have a poorly developed granular endoplasmic reticulum. In some areas, they present short microvilli on their surface and are bound to neighbouring cells by desmosomes.

12. Alveolar Type II Cell: (Type II Pneumonocyte)

This alveolar epithelial cell is rounded or cuboidal in shape and bulges into the alveolar lumen between alveolar type I epithelial cells. They occur singly or in small groups of three or four cells. They possess short microvilli on their free surface and form junctional complexes with adjacent surface epithelial cells. The most distinctive feature of the cell, however, is the presence of multilamellar bodies in the cytoplasm.

These consist of thin concentric lamellae rich in phospholipid and it is thought that this is the storage site of pulmonary surfactant. The alveolar type II pneumonocyte is now recognized to be the stem cell of the alveolar epithelium. It can generate a new alveolar epithelium by proliferating and differentiation into type I cells after diffuse alveolar injury (Kauffman, 1980).

1.4 SEM STUDIES

A further important step in the understanding of the morphology of the respiratory tract was the introduction of the scanning electron microscope (SEM). Its great depth of focus, wide range of magnification and relatively great resolving power makes it a logical tool for the study of the complicated array of surfaces that comprise the respiratory tract. These advantages have allowed a three dimensional visualisation of respiratory passages not possible with conventional light microscopy nor with the TEM. One of the earliest applications of the SEM to respiratory tissue was described by Jaques et al (1965) but it wasn't until 1969 that the technology had improved sufficiently to stimulate a flurry of reports. A summary of SEM studies carried out on the respiratory tract of a variety of animal species is presented in Table 1.2.

Not all of the cells shown on Page 8 are normally visualisable with the SEM as many such as basal, intermediate and endocrine cells are hidden from view. With the SEM, the most commonly observed cell populating the respiratory tract is the ciliated cell which has been found to cover the greater proportion of the respiratory tract in man (Ebert and Terracio, 1975; Greenwood and Holland, 1975), rat (Andrews, 1974; Alexander et al, 1975), mouse (Greenwood and Holland, 1972), hamster (Becci et al, 1978), ox (Mariassy et al, 1975; Iovannitti et al, 1985), guinea pig (Davis et al, 1984), pig (Mebus and Underdahl, 1975; Williams and Gallagher, 1978), monkey (Greenwood and Holland, 1973), cat (Tandler, 1983[a,b]) and dog (Wright et al, 1983).

Domed-shaped goblet cells protruding between the cilia have also been described in most mammalian species examined such as man (Greenwood and Holland, 1975), rat (Andrews, 1974), dog (Wright et al, 1983), mouse (Greenwood and Holland, 1972), hamster (Becci et al, 1978), ox (Iovannitti et al, 1985), guinea pig (Dahlgren et al, 1972), pig (Mebus and Underdahl, 1977), monkey (Greenwood and Holland, 1973), cat (Tandler et al, 1983[a,b]) and horse (Tyler et al, 1971).

Brush cells, characterised by blunt, squat microvilli on their free luminal border, have been described in SEM studies of the mammalian respiratory tract but only in a few species such as the rat (Popp and Martin, 1984). In the bronchioles, Clara cells with their characteristic irregularly shaped blunt luminal projections devoid of cilia or microvilli have been described with SEM in man (Smith et al, 1979), rat (Kuhn and Finke, 1972), dog (Wright et al, 1983), mouse (Smith et al, 1979), hamster (Becci et al, 1978), ox (Iovannitti et al, 1985), guinea pig (Okada, 1969), rabbit (Smith et al, 1979) and monkey (Castleman et al, 1975).

Neuroepithelial bodies, located near or at bronchiolar bifurcations, have been identified in the mouse (Hung et al, 1979) and rabbit Cutz et al, 1983) while alveolar macrophages have been visualised clinging to alveolar walls of the lungs in man (Greenwood and Holland, 1975), mouse (Greenwood and Holland, 1972) and ox (Iovannitti et al, 1985). Numerous SEM observations of alveolar type I and II pneumonocytes have also been made in the dog (Kondo et al, 1973), ox (Iovannitti et al, 1985), guinea pig (Davis et al, 1984) and monkey (Castleman et al, 1975).

Apart from the squamous cells lining the extreme rostral portions of the nasal cavity and the olfactory epithelium clothing the caudal region, the only other type of cell which has been described with the SEM is the non-ciliated "microvillous" cell which appears in varying numbers in different mammalian species. Thus, they appear to be common in the tracheobronchial tree of the mouse (Greenwood and Holland, 1972) and

rat (Alexander et al, 1975) but are relatively uncommon in the lower respiratory tract of the dog (Wright et al, 1983).

1.5 RESPIRATORY TRACT OF THE DOG

As can be seen from the literature (summarized in Tables 1.1 and 1.2), references to the normal structure of the various compartments of the canine respiratory tract are extremely sparse. General descriptions of the main histological features of the tracheobronchial tree and lungs have been supplied by Engel (1958), Martin (1963), Auerbach et al (1967), Wardell et al (1970) and Adam et al (1970). These studies have established the similarity of structure between different mammalian species but have emphasised that there can be differences such as the presence of respiratory bronchioles which are poorly represented, if at all, in the ox and rodents, while several generations are represented in other mammals such as man and dog (McLaughlin et al, 1961; Tyler, 1983).

As far as histochemical studies are concerned, Goco et al (1963), Spicer et al (1971) and Wheeldon et al (1976) have characterised the nature of the mucosubstances in the surface respiratory epithelial cells and glands of the tracheobronchial tree and have pointed out that the predominant mucosubstances in the epithelial goblet cells is a sulphomucin (acid mucosubstance) while a mixture of sialomucins and sulphomucins are present in the glands of the respiratory lamina propria.

With respect to the nasal cavity and nasopharynx, very few, and for the most part brief, histological studies have appeared in the literature (Adam et al, 1970; Adams and Hotchkiss, 1983) and, as far as can be ascertained, no studies have been carried out on the type and distribution of mucosubstances in these areas.

TEM studies of the canine respiratory tract are equally sparse and largely confined to the tracheobronchial tree and lungs and are usually

TABLE 1,2

SCANNING ELECTRON MICROSCOPY OF THE RESPIRATORY TRACT OF VARIOUS ANIMAL SPECIES

SPECIES	SAMPLE SITE	REFERENCE
Man	Nasal cavity	Graziadei (1970)
"	Lung	Wang and Thurlbeck (1970)
"	Bronchial epithelium	Ebert and Terracio (1975[a])
"	Respiratory tract (lung and trachea)	Greenwood and Holland (1975)
"	Clara cell	Smith et al (1979)
Rat	Alveolus	Kuhn and Finke (1972)
"	Extrapulmonary respiratory tract	Andrews (1974)
"	Trachea and principal bronchi	Alexander et al (1975)
"	Bronchioles	Ebert and Terracio (1975[b])
"	Alveolar brush cell	Hijiya (1978[a,b])
"	Lung	Hijiya (1978[c])
"	Trachea and major bronchi	Luchtel (1978)
"	Terminal bronchiolar epithelium	Lum et al (1978)
"	Clara cell	Smith et al (1979)
Mouse	Clara cell	Okada (1969)
"	Lung	Wang and Thurlbeck (1970)
"	Nasal cavity	Adams (1972)
"	Respiratory tract surface	Greenwood and Holland (1972)
"	Alveolus	Kuhn and Finke (1972)
"	Lung	Zitnik et al (1978)
"	Lung: neuroepithelial bodies	Hung et al (1979)
"	Lung: neuroepithelial bodies	Wasano and Yamamoto (1981)
"	Clara cell	Smith et al (1979)
Rabbit	Lung	Holma (1969)
"	Lung	Sturgess (1977)
"	Clara cell	Smith et al (1979)

TABLE 1.2 (Cont'd)

SPECIES	SAMPLE SITE	REFERENCE
Rabbit	Lung: neuroepithelial body	Cutz et al (1978)
Hamster	Alveolus	Kuhn and Finke (1972)
"	Tracheobronchial epithelium	Becci et al (1978)
Monkey	Lung	Greenwood and Holland (1973)
"	Intrapulmonary airways	Castleman et al (1975)
"	Tracheobronchial epithelium	Wilson et al (1984)
Pig	Lung	Wang and Thurlbeck (1970)
"	Lung	Winkler and Cheville (1984)
"	Trachea and bronchi	Mebus and Underdahl (1977)
"	Trachea and lung	Williams and Gallagher (1978)
Horse	Lung	Tyler et al (1971)
"	Lung	Nowell and Tyler (1971)
Ox	Trachea and bronchi	Tucker (1974)
"	Lung	Mariassy et al (1975)
"	Clara cell	Smith et al (1979)
"	Lower respiratory tract	Iovannitti et al (1985)
Cat	Trachea	Tandler et al (1983[a])
"	Trachea	Tandler et al (1983[b])
Guinea Pig	Clara cell	Okada (1969)
"	Distal airway epithelium	Davis et al (1984)
"	Trachea	Dahlgren et al (1972)
Snake	Trachea and bronchi	Tucker (1974)
Koala	Trachea and bronchi	Tucker (1974)
Phalanger	Trachea and bronchi	Tucker (1974)
Dog	Orifices of bronchial glands	Wang et al (1972)
"	Lung	Groniowski et al (1972)
"	Lung	Kondo et al (1973)
"	Trachea and bronchi	Tucker (1974)
"	Lung	Hyde et al (1978)
"	Orifices of tracheal glands	Nadel (1977)

TABLE 1.2 (Cont'd)

SPECIES	SAMPLE SITE	REFERENCE
Dog	Interalveolar pores of Kohn	Parra et al (1978)
"	Nasal cavity	Edwards et al (1983)
"	Trachea and lung	Wright et al (1983)
"	Vomeronasal organ	Adams and Wiekamp (1984)
"	Bronchioles	Castleman (1985)

related to an experimentally induced disease process (Frasca et al, 1968; Ortega et al, 1970; Freeman et al, 1973; Hyde et al, 1978) or as part of a comparative study of specific cells of the lung such as the Clara cell (Plopper et al, 1980[b]). Only one early TEM investigation of the epithelium of the nasal cavity has appeared in the literature (Okano and Sugawa, 1965).

The introduction of SEM has done little to add to the overall knowledge of the canine respiratory tract. The earliest SEM studies in this animal were performed by Groniowski et al (1972) who examined the lung parenchyma of an unspecified number of dogs, Wang et al (1972) who studied the major bronchi of a single dog, Kondo et al (1973) who, in an investigation of canine lung transplants, examined the lungs of three normal dogs and Tucker (1974) as part of a comparative study of the tracheobronchial tree of a number of animal species including snakes, cows, koala bears and three dogs.

More recently, Parra et al (1978), in a comparative study of two different routes of fixation of the lungs of eight dogs, the airways route and the vascular route, examined the incidence and morphology of the interalveolar pores of Kohn. Two further reports, one associated with a study of the effects of air pollutants on the lungs of Beagle dogs in which the lungs of 12 dogs were examined with the SEM (Hyde et al, 1978) and a second concerning an investigation of the incidence of abnormal cilia in the trachea of seven healthy dogs (Wilsman et al, 1982) have appeared in the literature. Castleman (1985), in a study of experimental adenovirus respiratory disease in dogs, has employed SEM to study the bronchiolar epithelium.

The most recent and detailed SEM study of the normal canine respiratory tract was carried out by Wright et al (1983) who compared the patterns of cilia formation in the tracheobronchial tree of 25 dogs whose ages ranged from newborn (four hours) to six months. These workers showed that, at birth, the dorsal wall of the trachea was completely carpeted with cilia while the lateral and ventral walls showed only patchy

ciliation. This pattern of cilia formation persisted until five days after birth when the whole tracheal surface was found to be completely ciliated. The bronchus of newborn puppies was uniformly poorly ciliated but almost complete ciliation was also achieved by five days. Likewise, the bronchioles of newborn puppies were shown to have few cilia and, although the number of ciliated cells had increased by two days after birth, complete ciliation was never observed from then on. Regardless of the age of the animal, ciliated cells were not found in the respiratory bronchioles of any of the 25 dogs examined.

Although Adams and Wiekamp (1984) have recently carried out a detailed TEM and SEM investigation of the canine vomeronasal organ, only one SEM study of the nasal mucosa as a whole has appeared in the literature (Adams and Hotchkiss, 1983). This was a combined histological and brief SEM investigation of the nasal cavity of a total of 20 dogs of which five were examined with SEM.

It is clear from the reports in the literature that many gaps exist in the knowledge of the normal surface morphology of the canine respiratory tract. In Chapter 3, a combined histological, histochemical and SEM study was carried out in 18 dogs in order to characterise, for the first time, the normal features of the canine respiratory tract from the nasal fossa to the alveolar wall.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Litters of Collie cross-bred puppies for the most part aged 14 - 18 weeks were obtained from commercial sources. On arrival they were immunized against the common canine pathogens, viz., canine parvovirus, distemper virus, leptospira canicola, leptospira icterohaemorrhagiae and adenovirus sub-type I, using standard commercial vaccines. They were observed for at least two weeks before use for experimental purposes. During their stay they were fed on commercial dog food (Lassie Pet Food Limited, England) and reconstituted dried milk.

2.2 POSTMORTEM TECHNIQUES

Dogs were given an intramuscular injection of a neuroleptanalgesic "Immobilon SA" 0.5ml/5kg (Reckitt and Colman Pharmacautical Division, Hull, England) and subsequently euthanized by a slow intravenous injection of sodium pentobarbitone (Euthatal, May and Baker, Dagenham, England). As soon as the femoral pulse was barely discernible, exsanguination was performed by severing the axillary artery. An incision was made from xyphoid cartilage to the submandibular space and the skin reflected. The tongue, larynx, trachea, heart and lungs were removed intact and, after a brief visual inspection, samples from the nasal cavity were then obtained by firstly reflecting the skin from the cranium and from the maxillary area. The nasal cavity was then exposed by careful use of fine bone cutters.

2.3 HISTOLOGICAL AND STAINING METHODS

(a) Fixation, embedding and sectioning:-

Tissues for light microscopical examination were fixed in neutral buffered formalin (NBF) for seven days; after trimming they were subsequently post-fixed for two days in mercuric chloride formol. These

fixatives were prepared as follows :

Neutral Buffered Formalin :

Formaldehyde (40%)	200 ml
Sodium chloride	10 g
Sodium sulphate	30 g
Distilled water	1800 ml

Mercuric Chloride Formol :

Saturated aqueous mercuric chloride	900 ml
Formalin	100 ml

After fixation, tissues were dehydrated, cleared and impregnated with paraffin-wax. Paraffin-embedded sections were cut at 3 µm with a Leitz Rotary Microtome and mounted on glass slides.

(b) Staining:-

Mounted sections were routinely stained with standard haematoxylin and eosin (HE and periodic acid-Schiff (PAS) methods. Another stain used on occasion was the Alcian blue - PAS (pH 2.5) method (AB-PAS) for acidic and neutral mucosecretory units according to a modification of the method of Mowry (1956) as detailed below :-

Solutions :

(1) 1% Alcian blue in 3% acetic acid (pH 2.5)

(2) 1% Periodic acid

(3) Schiff's reagent

Steps :

(1) Hydrate sections

(2) Solution (1) for 4 mins.

(3) Wash in distilled water

(4) Solution (2) for 2 mins.

(5) Wash in distilled water

(6) Solution (3) for 8 mins.

(7) Wash in running water for 10 mins.

(8) Mayer's haematoxylin for 4 mins.

(9) Wash in running water

(10) Differentiate in acid alcohol for 10 seconds

(11) Wash in running water

(12) Blue nuclei in Scotts tap water substitute

(13) Wash in running water

(14) Dehydrate, clean and mount

Results :

Acidic mucosubstances	-	blue
Neutral mucosubstances	-	red
Mixed mucosubstances	-	purple

2.4 SCANNING ELECTRON MICROSCOPIC METHODS

Samples of tissues, less than 2mm thick, were gently washed in 0.2M cacodylate buffer in order to remove surface blood or mucus and then immersed in Karnovsky's fixative (paraformaldehyde glutaraldehyde) for 24 hours. The specimens were then rinsed in 0.2M cacodylate buffer for 4 hours before dehydration in a series of acetones.

Specimens from the rinse solution were first passed through graded acetones during which process they were gradually dehydrated. Cold dehydration helped to decrease extraction of cell components by the organic solvent. The time interval the specimens were kept in acetone was strictly controlled to avoid shrinkage. The dehydration schedule used in the present study is given below :-

70% acetone :	4 hours
90% acetone :	2 hours
100% acetone :	2 hours

The specimens were then critical point dried in liquid CO_2 (Polaron, Watford, U.K.), orientated and mounted on aluminium stubbs and subsequently sputter-coated with gold paladium.

0.2M Cacodylate Buffer (500 ml)

0.4M Sodium cacodylate	=	250 ml
0.2M Hydrochloric acid	=	40 ml
Distilled water	=	210 ml

Karnovsky's Fixative (500 ml)

Dissolve 10g paraformaldehyde in 100 ml distilled water at 60°C together with 10 drops of NaOH and the mixture added to the stock solution.

Stock Solution :

0.2M Cacodylate buffer	=	250 ml
25% Glutaraldehyde	=	50 ml
Distilled water	=	100 ml

2.5 TRANSMISSION ELECTRON MICROSCOPIC METHODS
(Pulmonary Tissue Only)

Small portions of the right caudal lobe of the lung were minced into cubes less than 0.5mm thick in a Petri dish. These small pieces of pulmonary tissue were then transferred into Bijou bottles containing Karnovsky's fixative and left for 24 hours at 4°C. The fixative was drained and replaced by 0.1M Cacodylate buffer for one hour. The specimens were then post-fixed for one hour in 1% osmium tetroxide before washing three times in distilled water.

A graded series of acetones (as used for SEM) was used to dehydrate the specimens which were then placed in two changes of propylene oxide for 20 minutes, then in a 1:1 mixture of propylene oxide and Emscope Emix resin (Emscope, Ashford, Kent) for one hour and finally neat resin for three hours. Finally, the tissue was embedded into neat resin in plastic mounting moulds and left in an oven at 60°C to effect polymerisation.

Using glass knives prepared by an LKB 780 1A Knife Maker (LKB, Croydon, Surrey), the blocks of embedded pulmonary tissue were trimmed by means of an LKB Pyramitome and sections cut at one µm. The sections were collected from the knife edge using a pair of fine forceps and placed in a droplet of water on a glass slide. The sections were then flattened using xylene vapour, stained with toluidine blue (see below) and examined by means of a Leitz Laborlux II microscope. Suitable portions of the distal airways were then selected for ultrathin sectioning.

Each block was finally trimmed and then placed in a LKB Mk III ultramicrotome. Sections of a silver or pale yellow colour (60 - 90nm thick) were collected in a water chamber. They were then flattened using xylene vapour, picked up on Polaron 300 mesh grids, stained with uranyl acetate and lead citrate (see below) and viewed by means of an Hitachi HS8 electron microscope.

Toluidine Blue :

1% Borax (sodium tetraborate)	1 g
1% Toluidine blue	1 g
Distilled water	100 ml

Staining time : 15 seconds

Uranyl Acetate :

0.2g of uranyl acetate was dissolved in 10 ml of distilled water providing a saturated solution.

Staining time : Five minutes

Lead Citrate :

1.33g of lead citrate and 1.76g of sodium citrate were dissolved in 30 ml of distilled water and shaken for 30 minutes. 8 ml of 0.1M NaOH were added followed by distilled water to give a final volume of 50 ml.

Staining time : Five minutes

2.6 PHOTOGRAPHIC METHODS

(a) Light Microscopy :

A Leitz Laborlux 12 microscope equipped with a Wild MPS 45 Photoautomat Unit was used. For black and white photography, Agfa PAN 35mm film (12 ASA) was employed while for colour transparencies Kodachrome 25 (25 ASA) film was used. For black and white prints Agfa-Gevaert Rapitone Photographic paper P1-P4 using an Agfa-Gevaert Rapidoprint PD 3700 automatic processor was employed.

(b) Scanning Electron Microscopy :

All the specimens used in this study were examined by means of a Philips 501B SEM at an operating kilovoltage of 15 Kv and using spot sizes ranging from 200 to 1000. An automatic Rolliflex camera using Ilford FP4 120 (125 ASA) film was employed. Black and white prints were prepared as for light microscopy.

(c) Transmission Electron Microscopy :

Electron micrographs were taken using Ilford Technical EM plates (3.1/4" x 4.3/4"), developed in PQ Universal and fixed in Ilford Ilfospeed Fixer. Black and white prints were prepared as described above.

CHAPTER 3

A HISTOLOGICAL, HISTOCHEMICAL AND SEM STUDY OF THE CANINE AIRWAYS

3.1 INTRODUCTION

In the review of the literature, it was pointed out that, despite the importance of clinical respiratory disease in dogs, comparatively little information is available concerning the normal histological and ultrastructural features of the canine airways. Indeed, although some morphologic publications mentioned in the review refer to specific localised compartments of the canine respiratory tract, there is no complete description of the histological features of the whole respiratory tract, from the nasal vestibule to the alveolar membrane. Furthermore, with the exception of the tracheobronchial tree, the surface characteristics of the respiratory tract as observed by SEM are particularly poorly documented.

The purpose of this section of the work was to carry out a detailed histological, histochemical and SEM study of the canine airways.

3.2 MATERIALS AND METHODS

18 collie cross-bred dogs, whose ages ranged from 14 - 18 weeks, were used in this study. All the dogs were clinically normal and showed no evidence of overt respiratory disease. Subsequent histological examination of the respiratory system confirmed the absence of any respiratory lesions.

All dogs were sedated with the neuroleptanalgesic "Immobilon" and subsequently euthanized with sodium pentobarbitone. Exsanguination and necropsy procedures were performed as described in Chapter 2. Samples for histology and SEM were taken from the following sites :-

(1) Nasal septum; at the level of the canine tooth (Fig. 3.1)

(2) Lamellar portion of ventral concha (Fig. 3.2)

(3) Ethmoidal concha (Fig. 3.2)

(4) Ventral aspect of the caudal nasopharynx, 1-2cm from the free

edge of the soft palate (Fig. 3.2)

(5) Cranial portion of the epiglottis (Fig. 3.3)

(6) Ventral larynx, caudal to the vocal cords (Fig. 3.3)

(7) Trachea. A complete section from the middle portion of the trachea was divided into two portions to expose the dorsolateral and ventral surfaces (Fig. 3.4)

(8) Extrapulmonary bronchus (Fig. 2.4). A complete section as described for the trachea.

(9) Right caudal lobe of the lung. This was inflated, by means of a 20 ml syringe, with cold paraformaldehyde/glutaraldehyde fixative (2% paraformaldehyde/2.5% glutaraldehyde in 0.1M cacodylate buffer) via its primary bronchus (Fig. 3.4)

For SEM, thin slices of lung were washed overnight in 0.1M cacodylate buffer. They were then dehydrated in a series of acetones, dried in a Polaron critical point dryer, mounted on the stubs and coated with gold in an Emscope sputter coater before viewing in a Philips 501B SEM.

(10) The left caudal lobe was inflated by means of a 20 ml syringe filled with 10% NBF via its primary bronchus. Following immersion in fixative for seven days, thin slices of the lung were post-fixed for two days in mercuric chloride formol, dehydrated, cleared and impregnated with paraffin wax. Sections were routinely stained with HE. Sections from 15 dogs were also stained with PAS and AB-PAS stains.

Specimens from the extrapulmonary sample sites (1-8) were processed in a similar manner to the lung material for histological examination and for SEM.

3.3 RESULTS

HISTOLOGICAL FINDINGS : (See Appendix I)

(1) Ventral Concha:

The mucosa of the lamellated surface consisted of pseudostratified columnar ciliated epithelium with numerous goblet cells (Figs. 3.5 and 3.6). In some areas the mucosa was very thin (two nuclei deep); in others, a pseudostratification of up to six nuclei were found. In the rostral border and alar fold, a stratified transitional epithelium (Fig. 3.7) merged gradually with the stratified squamous cornified epithelium of the nasal vestibule (Fig. 3.8). With the AB-PAS stain (Fig. 3.9) the goblet cells were shown to contain predominantly acidic or mixed mucosubstances. Neutral-staining goblet cells, with one exception (WA 13), were relatively sparse. In the lamina propria, some poorly stained neutral or mixed glands were seen and some glands were unstained. In the former, the secretion granules were mainly found at the apex of the cell. Only three animals had some acid units (WA 4, WA 9 and WA 10).

(2) Septum:

For the most part, the epithelial surface was of the pseudostratified ciliated columnar type with many goblet cells (Fig. 3.10). The rostral portion, however, was lined by thick stratified transitional epithelium containing only an occasional goblet cell. Caudally, the septum was lined with olfactory epithelium. Many glands of the serous type were found in the lamina propria. With AB-PAS (Fig. 3.11) the surface goblet cells contained predominantly acidic or mixed mucosubstances. A few poorly stained acidic neutral or mixed glands were present, others were unstained (Fig. 3.12). The few goblet cells found in the transitional epithelium contained acidic mucosubstances.

(3) Ethmoidal Concha:

In areas adjoining the dorsal concha, the epithelium was of the pseudostratified ciliated columnar type with many goblet cells (Fig. 3.13). In the olfactory area, which comprised the greater portion of the ethmoidal concha, the epithelial surface was a much thicker pseudostratified columnar epithelium lacking in goblet cells.

The greatest concentration of glands in the nasal cavity was found in this region. These branched, serous cells (of Bowman) contained numerous alcianophilic granules (Fig. 3.14). In five dogs, however, glands containing neutral or mixed mucosubstances were observed (Fig. 3.15).

(4) Nasopharynx:

The nasopharynx was covered by pseudostratified ciliated columnar epithelium with numerous goblet cells (Fig. 3.16) and mucus-secreting glands were present in the lamina propria. Towards the free border of the soft palate, however, the epithelium was of the stratified squamous non-keratinising type (Fig. 3.17). Goblet cells were mainly acidic or mixed. There were many glands in the lamina propria and these were mainly mixed. In some cases, however, many glands were also found which had acidic and neutral units.

(5) Epiglottis:

On the cranial dorsal and ventral surfaces, the epithelium was of the stratified squamous non-keratinising type with no cilia or goblet cells (Fig. 3.18); occasional taste buds were found embedded in the epithelium. There were in the lamina propria, however, many glands which contained acid or mixed mucosubstances (Fig. 3.19). Some glands, however, were unstained with AB-PAS or contained only a few neutral granules.

(6) Ventral Larynx:

At this site, the type of epithelium varied. Only four animals had respiratory epithelium with relatively few goblet cells, which contained either acid or mixed mucosubstances (Fig. 3.20). The remaining dogs had stratified squamous non-keratinising epithelium (Fig. 3.21). Many glands were present in the lamina propria. These glands contained mainly acidic or mixed units. In one dog (WA 14), however, only a few neutral glands were observed.

(7) Trachea:

The epithelium was wholly of respiratory type, i.e., pseudostratified ciliated columnar epithelium, with goblet cells (Figs. 3.22 and 3.23). The majority of goblet cells were present in the lateral and ventral walls. With AB-PAS, these appeared to have a patchy distribution and were mainly of the acid or mixed type. Glandular units in the lamina propria varied from animal to animal with many in some while in others only a few were found. They were equally distributed throughout the dorsal, lateral and ventral walls. Some of them were unstained but most were acidic or mixed (Fig. 3.24). In four animals, however, many neutral glands were also present.

(8) Extrapulmonary Bronchus:

The lining of the epithelium was again of the pseudostratified columnar ciliated type. Goblet cells were particularly numerous and were concentrated on the lateral and ventral walls (Fig. 3.25). The greatest number of goblet cells in the whole of the canine respiratory tract were found here. The dorsal wall also contained many such cells, more so than in the dorsal wall of the trachea. With AB-PAS, the goblet cells were mainly acid or mixed although, in five dogs, many cells containing neutral mucosubstances were noted (Fig. 3.26). In general, fewer glandular units were found in the lamina propria in the

extrapulmonary bronchus than in the trachea. They contained mainly acid or mixed mucosubstances although some units were unstained or stained only for neutral mucosubstances.

(9) Bronchus:

The mucosa of both large and small bronchi was lined with pseudostratified ciliated columnar epithelium (Figs. 3.27 and 3.28). Goblet cells were still numerous but less abundant than in the extrapulmonary bronchus and, with AB-PAS staining, the type of mucosubstance was again predominantly acidic or mixed (Fig. 3.29). Only four dogs had many neutral staining goblet cells. Few stained glands were noted and these usually contained sparse neutral granules although a few acidic or mixed units were also to be found, particularly in the larger bronchi.

(10) Bronchiole:

These were characterised by the presence of low pseudostratified ciliated columnar epithelium with varying numbers of goblet cells. In one animal (WA 9), a few acidic glandular units were found. (In most dogs, a few unstained glands persisted in the lamina propria, particularly in the large bronchioles). With AB-PAS, many acidic or mixed cells were present in the large bronchioles (Fig. 3.30), whereas only a few such cells were identified in the small bronchioles.

(11) Terminal Bronchiole:

In all the dogs the epithelium was of the simple low columnar or cuboidal type; there were neither glands nor goblet cells. With HE, the lining cells were characteristically poorly-stained and bulged into the bronchiolar lumen (Fig. 3.31). With AB-PAS, no staining was observed, although with PAS staining alone the basal and, to a lesser extent, the apical portion of the lining bronchiolar epithelial (Clara) cells was PAS-positive leaving an unstained halo around the nucleus (Fig. 3.32).

(12) Respiratory Bronchiole:

The epithelium, interrupted by mural alveoli was of the simple cuboidal type similar to that of the terminal bronchioles (Fig. 3.33). Goblet cells and glands were absent. Again the Clara cells were AB negative but showed peripheral cytoplasmic staining with PAS.

(13) Alveolar Duct and Alveoli:

The epithelium was of the simple squamous type (Fig. 3.34) with no goblet cells or glands. No staining was observed with AB-PAS or PAS stains.

SEM FINDINGS :

(1) Ventral Concha:

Under low magnification, the folded lamellar portion of the ventral concha was thickly carpeted with ciliated cells. Each fold had many longitudinally oriented grooves and smaller irregular fissures. The depth of the grooves and fissures varied from area to area in each specimen. Among the cilia, goblet cells either singly or in groups were commonly observed (Fig. 3.35). The orderly densely packed ciliated surface was interrupted by these protruding dome-shaped cells. Although some of these were almost completely masked by surrounding cilia, others were found to be fully engorged with granules of mucus which could be clearly seen through the thin apical cell membrane (Fig. 3.36). A few were found to be discharging granules of mucus onto the surface (Fig. 3.37). Occasionally mucus droplets were also noted clinging to the surface of the cilia. In areas where the epithelium had been fractured in processing, the narrow base of ciliated cells extending to the basal lamina could be seen (Fig. 3.38), while the apical portions of goblet cells bulging with mucus granules were very prominent (Fig. 3.39).

Other non-ciliated cells were also found and these seemed to

differ in some respects from goblet cells. The apical cell surface was flat and did not protrude between the cilia as was the case with many of the goblet cells (Fig. 3.40). They had a few scattered microvilli on their surface but the most striking feature was extrusion of secretion droplets resembling mucus from the central part of the cell. In the vicinity of these secretory cells some of the ciliated cells had only a sparse complement of short thin cilia although many microvillous projections could be seen (Fig. 3.41).

At the cranial end of the concha near the alar fold, the number of ciliated cells decreased dramatically to be replaced by rounded cells which were covered with numerous short microvilli. This gave a "cobblestone" appearance to the epithelium. Some of these cells retained a few slender cilia on their surface (Fig. 3.42). Occasional goblet cells were still present in this region but the other flat-surfaced secretory cells were not seen.

Further cranial and extending into the alar fold and nasal vestibule, the surface cells were more squamous-like, flat surfaced with prominent cell boundaries and studded with microvillous projections and microplicae (Figs. 3.43 and 3.44). In addition, many of these cells showed circular structures on their surface, usually one per cell. These possibly represented secretory "pores" on the cell surface but in no case was secretion found. Absence of cilia allowed gland orifices of the underlying glands of the lamina propria to be visualised.

(2) Septum:

In general, the surface topography of the septum resembled that of the ventral concha. The cranial region was of the squamous type and populated by flat cells with well-defined borders. The surface of each squamous cell was covered with numerous short microvilli and microplicae (Fig. 3.45). Some desquamated cells were always to be found. These left circular or irregular depressions on the epithelial surface (Fig. 3.46).

The orifices of glands were easily identifiable (Fig. 3.47). In two animals (WA 7 and WA 18), numerous bacteria were found in the squamous region (Fig. 3.48).

At the junction with the respiratory area of the septum, the surface cells gave a "cobblestone" appearance to the mucosa. These cells were rounder than squamous cells and bulged somewhat into the nasal fossa (Fig. 3.49). Their surfaces were covered with stubby microvilli but did not show microplicae. Many had a few cilia of varying length and thickness. Further caudally, fully ciliated cells appeared until the whole septal surface was totally clothed in ciliated cells. Here, under low magnification, the septum showed numerous longitudinal folds of variable depth and the orifices of glands could occasionally be seen (Fig. 3.50).

At higher magnification many goblet cells, some discharging mucous droplets, could be seen protruding between the cilia. Occasionally, sheets or strands of mucus were found on the septal surface (Fig. 3.51). In only one dog (WA 18) were a few cells with a flat surface and extruding mucous granules from a centrally disposed apical pore to be seen in the septal mucosa (Fig. 3.52).

(3) Ethmoidal Concha:

The surface of the ethmoidal concha, together with the caudal osseous portion of the septum, was covered with olfactory epithelium. For the most part, the latter was characterised by a tangled mass of long slender cilia which was so dense that the microvillous projections of the sustentacular cells were often masked. Scattered among the cilia some olfactory vesicles, some with short thick cilia, were often to be seen (Fig. 3.53). There were no goblet cells but patches of secretory droplets, larger that the olfactory vesicles, and representing apical secretion from underlying sustentacular cells were a characteristic feature of the canine olfactory epithelium (Fig. 3.54). Large openings

among the tangled cilia were probably the orifices of the serous glands of Bowman. Junctional zones with respiratory epithelium were relatively sharp (compared to the junctional zones of respiratory and non-respiratory squamous epithelium more cranial in the nasal fossa). Here, there was a sudden transition from the tangled surface mat of olfactory cilia to the relatively orderly arrangement of respiratory cilia (Fig. 3.55).

(4) Nasopharynx:

In seven of the 18 dogs, the epithelium was wholly of the respiratory type with the irregular folded surface covered with ciliated cells (Figs. 3.56 and 3.57). Many goblet cells, some discharging granules of mucus, were present. In one animal (WA 4), the mucosal surface was almost completely covered with mucus. In all the dogs, only an occasional gland orifice was identifiable.

In a further seven animals, the nasopharyngeal epithelium was of the non-respiratory stratified squamous type similar to that observed in the cranial region of the ventral concha and nasal septum. Here, the epithelium was extremely corrugated with numerous deep irregular depressions and fissures. In some areas, numerous desquamating surface squamous cells were to be found (Fig. 3.58). At higher magnification, the cell boundaries delineating each irregular flat surfaced cell were very distinctive. Some surface cells had a characteristic mosaic appearance due to the presence of numerous microplicae (Fig. 3.59). The surface of other cells were studded with short microvillous projections (Fig. 3.60). In one of these dogs (WA 16), numerous bacteria were observed on the squamous nasopharyngeal surface.

In the remaining four dogs, the surface epithelium appeared to be a junctional zone between ciliated respiratory epithelium at the cranial end of the specimen and squamous non-ciliated epithelium at the caudal end near to the free border of the soft palate. The surface epithelial cells were rounded, the cell boundaries being less distinct than those of nearby

squamous cells (Fig. 3.61). The overall "cobblestone" appearance was interrupted here and there by isolated, or groups of, ciliated cells. The cell surface of the rounded cells was studded with microvillous processes; some also had a few slender cilia (Fig. 3.62). Occasional goblet cells and gland orifices were to be seen. Towards the free border of the soft palate, the "cobblestone" appearance gave way to epithelium of the stratified squamous type. Further cranially, respiratory epithelium predominated.

(5) Epiglottis:

Under low magnification, both dorsal and ventral surfaces of the epiglottis were covered in irregular folds separated by fissures of variable depth (Fig. 3.63). The folds were particularly noticeable in the peripheral areas of the epiglottis while the central zones had a more wrinkled appearance.

Under higher magnification, the surface epithelial cells were of the squamous type with distinct cytoplasmic borders and numerous microvillous projections and microplicae (Fig. 3.64). Occasional epithelial squames were found on the surface of the epiglottis; circular depressed areas representing sites where squamous epithelial cells had been shed were also commonly found. No ciliated cells were found and only occasionally were gland orifices seen.

(6) Ventral Larynx:

In only five specimens was the surface epithelium shown to be wholly of the respiratory type with a complete covering of ciliated cells. As elsewhere in the respiratory tract, the ciliated surface epithelium showed irregular longitudinally orientated grooves (Fig. 3.65). Goblet cells occurred singly or in patches (Fig. 3.66) but, in all animals, large areas were devoid of such cells. Glandular orifices were extremely sparse.

In six dogs, the ventral laryngeal mucosa proved to be of the non-respiratory stratified squamous type similar to that found in the epiglottis. In some areas, the surface epithelial cells were arranged in a relatively flat sheet with desquamating cells (Fig. 3.67), while in others there were many irregular folds giving the laryngeal surface a wrinkled or gnarled appearance (Fig. 3.68). The surface of these cells was covered with numerous stumpy microvillous projections. In some of these flat-surfaced cells, numerous curved microplicae were a prominent feature and the cell boundaries were particularly distinct. Occasional desquamated squames were found and the orifices of underlying glands were often to be seen.

In a further seven animals, specimens of ventral laryngeal mucosa consisted of a junctional zone between respiratory and non-respiratory squamous epithelium. Here, the surface epithelial cells were rounded with indistinct cytoplasmic borders and with a roughened apical border studded with microvillous projections (Figs. 3.69 and 3.70). Some cells were devoid of cilia while, in others, a sparse complement of slender cilia was present. Caudal regions of these specimens, extending towards the laryngo-tracheal junction, were clothed in ciliated respiratory epithelium while the cranial borders were always of the stratified squamous type.

(7) Trachea:

At low magnification, differences were not observed between the folded membranous dorsal wall and the cartilaginous lateral and ventral walls. In all areas, the surface of the trachea had closely packed longitudinally-oriented folds, shorter secondary folds or creases arose at an angle to these longitudinal folds. The depth of these furrows and creases varied from area to area (Figs. 3.71 and 3.72).

At higher magnification, the whole surface of the trachea was seen to be carpeted by long slender, densely packed cilia (Fig. 3.73). The

extreme tip of some cilia was bent sharply in the direction of the wave pattern, giving rise to a hook-like appearance. In only one dog (WA 15) were occasional patches of non-ciliated cells to be seen (Fig. 3.74). In these areas, the cells were irregular in outline and their apical surface covered with stumpy microvilli. Goblet cells were seen either individually or in groups and interrupted the ciliary surface by their dome-shaped projecting apices (Fig. 3.75). They were more numerous in the lateral and ventral wall than in the dorsal wall. In these areas where the epithelium had been fractured in processing, the narrow base of the cell extending to the basal lamina could be seen, while in the apical region the circular granules of mucus were prominent and a few microvillous projections could be seen at the cell border (Fig. 3.76). Occasionally goblet cells were found discharging granules of mucus on to the ciliated surface. Some goblet cells were, however, almost completely masked by adjacent cilia and probably represented developing cells or cells which had already discharged their contents. Some mucous droplets were occasionally seen on the surface of the cilia, but large amounts of mucus were never observed. For the most part, gland orifices were masked by adjacent cilia and only occasionally were deep ciliated pits seen which probably represented the location of a gland.

(8) Extrapulmonary Bronchus:

In all respects, the extrapulmonary bronchus resembled the trachea with its longitudinal grooves and total ciliation. The whole surface was thickly carpeted with densely packed cilia. The mucus-producing goblet cells were again distributed either singly or in dense groups and, as in the trachea, many protruded through the ciliary surface. The overall number of goblet cells, however, varied from area to area. Generally, the lateral and ventral walls were thickly populated while, in the dorsal wall, goblet cells tended to be scattered at random (Fig. 3.77). As in the trachea, some were discharging mucus from their apical pore, while others were partially hidden by the adjacent cilia. In contrast to the trachea, scattered small foci of non-ciliated polyhedral cells were

present in all dogs (see Fig. 3.74, Trachea). These cells were covered with numerous microvilli which varied in length from cell to cell. Gland orifices, shrouded with cilia, were more commonly observed than in the trachea (Fig. 3.78).

(9) Bronchus:

Intrapulmonary bronchi, as identified by the presence of intramural cartilaginous plates, had well-defined longitudinal grooves covered by a thick population of ciliated cells (Figs. 3.79 and 3.80). A very few non-ciliated patches, however, were found in all the specimens. These non-ciliated cells occurred in patches of one to five cells, had well-defined borders and their surface was studded with numerous short microvillous processes (see Fig. 3.74, Trachea). The number of protruding goblet cells varied from animal to animal but, in general, were fewer than observed in extrapulmonary bronchi or trachea. Very few glandular orifices were found in the bronchial mucosa.

(10) Bronchiole:

The bronchiolar mucosa also showed longitudinally-oriented grooves, although the number of ciliated cells had decreased, particularly in the smaller bronchioles (Figs. 3.81 and 3.82). Only a very few goblet cells were observed in the larger bronchioles. The non-ciliated bronchiolar epithelial (Clara) cells were, like goblet cells, dome-shaped and protruded into the bronchiolar lumen but did not contain obvious mucous granules (Fig. 3.83). Their apical surface was, for the most part, smooth and only occasionally were surface irregularities and microvillous projections to be found. No glandular orifices were found in the bronchioles at any level.

(11) Terminal Bronchiole:

The bronchiolar epithelium was now populated almost completely by non-ciliated Clara cells; very few sparsely-ciliated cells were present

(Fig. 3.84). No goblet cells or gland orifices were observed.

(12) Respiratory Bronchiole:

These structures were characterised by intramural alveoli and intermittent patches of Clara cells (Figs. 3.85 and 3.86). No goblet or ciliated cells were found in any of the dogs nor were glandular orifices to be seen.

(13) Alveolus:

Fixation by means of perfusion of the airways allowed easy identification of alveolar ducts and access to the alveolar wall itself for critical examination (Fig. 3.87). The cell boundaries of the flat Type I pneumonocyte were not always easy to detect particularly where an underlying capillary containing red cells bulged into the alveolar lumen (Fig. 3.88). The surface of Type I cells was not completely smooth and showed many short stubby projections and irregular surface folds. Only very rarely was an alveolar pore of Kohn found (Fig. 3.89).

Type II pneumonocytes were far fewer in number than Type I cells and not all sectioned alveoli showed such cells. Type II cells were irregular in outline and bulged into the alveolar lumen (Fig. 3.90). Their surfaces were studded with microvilli particularly at the fringe of the cell where it made contact with Type I cells. The dome-shaped apical portions of Type II cells often contained irregular surface projections and some cells showed small pores which possibly represented recent release of surfactant material (Fig. 3.91). Type II cells usually occurred singly but occasionally groups of up to five cells were observed, particularly in alveolar ducts near the respiratory bronchioles (Fig. 3.92). In no case was an alveolar macrophage observed, even after a prolonged search.

3.4 DISCUSSION

It can be seen from the literature that the normal structure of the

canine respiratory tract has received relatively little attention and, for this reason, the present study was undertaken in order to achieve a more complete understanding of the respiratory tract as a whole from the rostral region of the nasal fossa and the nasal septum to the alveolar wall itself. The surface topography was difficult to define accurately with light microscopy alone and the findings of the present study indicated that surface structural information is best obtained with SEM.

The general histological features of the normal respiratory tract of the dog as detected by light microscopy in the present study were in essence similar to those described for other mammals (Schaeffer, 1932; Krahl, 1959; Bertalanffy, 1964; Ali, 1965; Bloom and Fawcett, 1968; Mariassy and Plopper, 1983). They were also in accord with previous reports of the normal microscopic appearance of canine lungs (Engel, 1958), tracheobronchial tree (Auerbach et al, 1967; Adam et al, 1970) and nasal cavity (Adam et al, 1970; Adams and Hotchkiss, 1983).

Thus, extending as far as the small bronchioles, most of the canine respiratory tract was lined by pseudostratified columnar ciliated epithelium in which, depending on the region, numerous goblet cells reside.

Apart from olfactory epithelium which occupied much of the caudal region of the nasal fossae, particularly the ethmoidal concha and the caudal region of the nasal septum, the only other areas of non-respiratory, i.e., stratified squamous non-keratinising epithelium, were to be found in the nasal vestibule, the rostral portion of the nasal septum and variable portions of the caudal region of the nasopharynx. As expected, the epiglottis was also wholly of the non-respiratory type but, in the region of the ventral larynx, only four of the 18 dogs showed ciliated respiratory epithelium while that area in the remaining dogs was covered by stratified squamous non-keratinising epithelium.

The observation by Adam et al (1970) that the dorsal wall of the canine trachea often shows a "transitional" type of epithelium was not supported by the present investigation and, in all 18 dogs, there was a

uniformly thick pseudostratified columnar respiratory epithelium lining the dorsal, lateral and ventral regions of the trachea wall.

Areas of thickly stratified non-ciliated transitional epithelium, similar to that which lines the mammalian renal pelvis, ureter and urinary bladder were, however, found in the rostral portion of the lamellar concha and alar fold and the rostral region of the nasal septum and presumably represented junctional zones between the wholly respiratory epithelium of the nasal fossae and the stratified squamous epithelium of the nasal vestibule.

A similar type of transitional epithelium has been described by Adams and Hotchkiss (1983) in their study of the topography of the nasal cavity of the dog. These workers, without giving any morphologic details, stated that a transitional epithelium 35-136 µm thick occupied a junctional zone between the stratified squamous epithelium of the nasal vestibule and the ciliated respiratory mucosa. They estimated that this type of epithelium lined 15-20% of the nasal cavity and was found most rostrally on the alar fold. In the present study no such transitional or junctional zones were found interposed between respiratory and non-respiratory (stratified squamous) epithelium of the nasopharynx and ventral wall of the larynx and, at these sites, the respiratory epithelium appeared to change relatively abruptly to stratified squamous epithelium. Bryant (1916) and Ali (1965) have, however, again without supplying morphological detail, reported the occurrence of a "transitional" epithelium in the nasopharynx of a number of different mammalian species including the rabbit, guinea pig, cat, monkey and man. As will be pointed out later, mucosal or junctional zones, intermediate between respiratory and non-respiratory (squamous) epithelium, were found with the SEM in the nasopharynx and ventral larynx as well as in the rostral region of the nasal fossa of the dog. This type of epithelium, some of whose constituent cells were ciliated, albeit sparsely, could not be identified with light microscopy.

The luminal surface of the respiratory tract, in particular the areas lined by pseudostratified ciliated columnar epithelium, i.e., the nasal fossae, nasopharynx and tracheobronchial tree is bathed in a layer of mucus which, by means of the rhythmic beating of cilia, is constantly being transported towards the pharynx where it is removed by swallowing or by means of the cough reflex. This airway mucociliary system or escalator plays a vital role in the entrapment and subsequent removal of injurious agents such as bacteria and viruses which the animal may inhale and consequently has a major defence role to play in the protection of the respiratory tract (Kilburn, 1968; Phipps, 1981).

Mucous glycoproteins are synthesized and secreted from two sources in the respiratory airways (1) the mucus secreting goblet cells resident in the pseudostratified columnar epithelium and (2) the mucous glands present in the walls of the airways. Histochemical analysis of these mucous glycoproteins or mucosubstances has shown some variation between different mammalian species (Breeze et al, 1976) and, until the present study, reports of the histochemical composition of canine respiratory mucosubstances have been confined to those of the tracheobronchial tree (Goco et al, 1963; Spicer et al, 1971; Wheeldon et al, 1976). The earliest report by Goco and co-workers, a comparative study of the mucous glands of the tracheobronchial tree of man and a number of other mammals including the dog was limited in scope since, as they used only the PAS staining method, they were only able to provide an estimation of the number of mucosecretory units and not the nature of the mucosubstances.

Spicer et al (1971), however, compared the mucosubstances of the tracheobronchial tree of man and the dog and in so doing employed a battery of histochemical methods. The most noticeable difference was the predominance of sulphomucins (acid mucosubstances) in the goblet cells of the canine tracheobronchial tree. Wheeldon et al (1976) in their histochemical study of the tracheobronchial mucosubstances in normal dogs and in dogs suffering from chronic bronchitis found that the majority of goblet cells and mucous gland cells in both groups contained

predominantly acid mucosubstances when stained with the AB-PAS method.

The present investigation confirmed and extended the findings of these previous workers and, although the study was confined to assessing the distribution of acid and neutral mucosubstances and no attempt was made to further classify the acid mucosubstances into sulphomucins and sialomucins, as has been done in man (Lamb and Reid, 1969); it was clearly established that throughout the respiratory airways of the dog goblet cells as well as the mucosecretory units of the lamina propria contained predominantly acid or mixed mucosubstances.

In the ventral concha and nasal septum, very few goblet cells contained neutral mucosubstances. Many glands were either unstained or contained neutral staining granules in the apex of the cell; others showed acidic or mixed units. The occasional goblet cell found in the stratified transitional epithelium always contained acid mucosubstances.

In the nasopharynx, epiglottis and ventral larynx, there was a particularly high concentration of glands in the lamina propria. The acinar units contained a mixture of acid and mixed mucosubstances but, particularly in the nasopharynx and ventral larynx many individual cells containing neutral granules were also observed.

The greatest concentration of goblet cells was found in the extrapulmonary bronchus although, like the trachea, these cells were concentrated mainly in the lateral and ventral walls. Although again acid or mixed mucosubstances predominated in these cells, some cells containing neutral mucosubstances could always be found, particluarly in the extrapulmonary bronchus.

At the level of the small bronchioles, very few goblet cells were to be found and these had disappeared entirely from the terminal and respiratory bronchioles. The uneven staining of the bronchiolar epithelial secretory cell (Clara cell) with the PAS method presumably indicated the presence of cytoplasmic glycogen deposits (Plopper et al, 1980[a]).

The nature of the acid mucosubstances, sialomucin or sulphomucin, which made up the majority of the mucous glycoproteins in the canine respiratory tract will have to await further histochemical analysis. It is, nevertheless, important to have established that the pattern of mucosubstances in the goblet cells and in the glands of the lamina propria varied little qualitatively but often quantitatively at different levels of the canine airways.

The SEM is increasingly becoming an effective tool in the study of the surface changes operating in the respiratory tract during diseases of the airways (Greenwood and Holland, 1975; Ebert and Terracio, 1975[a,b]; Mebus and Underdahl, 1977; Lum et al, 1978; Zitnik et al, 1978; Hijiya et al, 1978[b]; Hyde et al, 1978).

For these studies to be meaningful, the normal surface topography must be clearly established and any species differences taken into account when a particular animal is chosen for a proposed experimental study. It would appear for instance that the degree of ciliation of the normal mouse trachea (Greenwood and Holland, 1972) is less than that observed in the normal dog (Wright et al, 1983) where, in contrast to the mouse, patches of non-ciliated tracheal cells are rare. At the level of the distal airways there are also a number of species differences particularly with respect to the presence of respiratory bronchioles which are well-developed in the dog and cat but rare or absent in the rat and bovine animal (Mariassy et al, 1975; Phalen and Oldham, 1983).

Before embarking on a SEM study of experimentally-induced respiratory disease in dogs, it was therefore of necessity to have as full a knowledge of the topographical features of the normal canine airways as possible. A search of the literature (summarised in Table 1.2) quickly established that, while SEM had been applied to the respiratory tract of the dog as early as the first part of the 1970's (Wang et al, 1972; Groniowski et al, 1972; Tucker, 1974), such studies were performed when SEM technology was still in its infancy and were confined to the

tracheobronchial tree and to the lung.

Since then, a number of more detailed SEM reports have appeared all, however, dealing with an investigation of only a part of the canine airways. Thus, Parra et al (1978) carried out a combined TEM and SEM study of the interalveolar pores of Kohn. Wilsman et al (1982) also using both TEM and SEM have studied variations in morphology in the tracheal cilia of normal dogs while Adams and Hotchkiss (1983), in their study of the nasal cavity of the dog, provided an interesting but brief description of the SEM features.

So far, the most detailed SEM study of the normal canine airways is that of Wright et al (1983) who made an investigation of the patterns of cilia formation in the tracheobronchial tree of 25 normal dogs whose ages ranged from the newborn to six months. These workers showed that there were differences in the degree of surface ciliation at different levels of the tracheobronchial tree and, depending on the age of the dog, these would have to be taken into consideration in any assessment of the surface topography in respiratory disease situations.

Thus, in the newborn puppy the longitudinally-oriented folds of the dorsal (membranous) wall of the trachea were clothed in a thick unbroken carpet of cilia while the lateral and ventral (cartilaginous) walls were poorly ciliated. It was not until five days after birth that the tracheal wall at all levels was completely ciliated. Likewise, the bronchial walls were uniformly poorly ciliated in the newborn puppy but again had a complete covering by five days. Large and small bronchioles of the newborn puppy had few ciliated cells and, although the number of ciliated cells had increased by two days, complete ciliation was never observed regardless of the age of the dog. No ciliated cells were found in the respiratory bronchioles of any of the 25 dogs.

Although the study by Wright and co-workers was limited to the tracheobronchial tree and did not extend to the alveolar wall or to the

upper respiratory tract, it did provide for the first time in the dog useful information on the normal SEM characteristics of a large segment of canine respiratory tract. The present study was designed to build on the work of Wright and co-workers and extend their observations to the respiratory tract as a whole.

The general features of the ciliated respiratory segments of the canine airways, particularly of the tracheobronchial tree, were similar to those recorded by other workers in other mammalian species (Greenwood and Holland, 1972; Alexander et al, 1975; Castleman et al, 1975) and, more recently in the dog, (Wright et al, 1983). There was no difference in the length of the cilia between the dorsal (membranous) and ventral regions of the trachea as has been suggested by Mellick et al (1977) and Wilson et al (1984). Unlike the mouse (Greenwood and Holland, 1972), rat (Alexander et al, 1975) and monkey (Wilson et al, 1984) the ciliated carpet especially of the trachea was complete and it was not until the level of the smaller bronchi and bronchioles that patches of non-ciliated cells became prominent. As a result of this almost total coverage of cilia in the tracheobronchial tree of the dog, the orifices of the underlying mucous glands of the lamina propria were difficult to distinguish. On the other hand, goblet cells either singly or in groups were easily located, some discharging granules of mucus onto the luminal surface. In no case were large amounts of mucus found on the respiratory surfaces.

Brush cells found by Andrews (1974), Alexander et al (1975) Hijiya (1978[a,b]) and Popp and Martin (1984) in their SEM studies of the rat were not detected in any of the dogs. This is similar to the observations by Iovannitti et al (1985) in their SEM study of the lower respiratory tract of a series of six calves and four adult cattle although Allan (1978) had previously reported their presence in the bovine animal by means of TEM. Recently, however, Adams and Wiekamp (1984) in their combined TEM and SEM study of the canine vomeronasal origin, identified brush cells in the lateral (non-receptor) wall but only with TEM.

The neuroepithelial bodies described by Cutz et al (1978) and Hung et al (1979) in their SEM studies of the respiratory tract of foetal rabbits and mice respectively were also not detected in the present work at any level of the canine airways. Failure to detect these structures may reflect the age of the dogs studied although Wright et al (1983) also did not observe them in newborn puppies.

In the nasal cavity most of the surface of the ventral concha and nasal septum was clothed in a thick ciliated carpet whose orderly appearance was broken at irregular intervals by single, or groups of, goblet cells. The flat secretory cells with scanty surface microprocesses and extruding a secretion product resembling mucus from a central small pore were only observed in the rostral region of the ventral concha and nasal septum. These cells have not so far been described in previous SEM reports of the nasal cavity of other mammals (Greenwood and Holland, 1972; Andrews, 1974; Popp and Martin, 1984) nor in the dog (Adams and Hotchkiss, 1983).

At the cranial region of the ventral concha and nasal septum, a "cobblestone" type of epithelium whose prominent cells were covered with long microvillous processes and possessed sparse or no cilia was interposed between the ciliated respiratory epithelium and the more flattened non-ciliated transitional epithelium. This junctional zone of well-defined cuboidal cells was irregular in extent and merged with the flat non-ciliated cells which represented the stratified transitional epithelium seen with the light microscope. This type of junctional epithelium has not so far been described in the nasal cavity of mammals studied with SEM and a histologic counterpart was not detectable with light microscopy. In a recent SEM study of the nasal epithelium of the rat, however, a narrow band of non-ciliated cells referred to as "domed" cells have been described by Popp and Martin (1984). Although little detail was given, these cells were interposed between ciliated respiratory epithelium and squamous epithelium and may represent a similar junctional zone as noted in the dog.

Thus the assumption that the richly-ciliated respiratory epithelium merges with stratified non-respiratory transitional epithelium (Andrews, 1974; Adams and Hotchkiss, 1983) is not strictly true at least in the dog (and possibly also in the rat) and there appears to be in the nasal cavity, and indeed elsewhere in the respiratory tract in the nasopharynx and ventral larynx, an intervening junctional zone of cobblestone-like epithelium characterised by prominent surface microvillous projections and sparse ciliation. This zone cannot be recognised with light microscopy and must be distinguished from the stratified "transitional" epithelium which, with the latter method, appears to form the sole boundary between respiratory and stratified squamous types of epithelium.

In the present SEM study, the stratified transitional epithelium which was so noticeable with the light microscope was represented by flat irregular cells with prominent cell borders and abundant short microvillous processes; in some cells the surface was etched with numerous wavy microplicae. Such epithelium has already been described in the nasal cavity by Greenwood and Holland (1972) and termed "microvillous epithelium" by Andrews (1974) in view of the characteristic array of surface microprojections and in order to distinguish it from ciliated respiratory epithelium. A feature of many of these cells was the presence of a single large circular pore on the luminal surface. These pores were not mentioned by Greenwood and Holland (1972) or Andrews (1974) in their description of "transitional" epithelium in the nasal cavity although, in the SEM illustrations of the nasal cavity of the dog produced by Adams and Hotchkiss (1983), "transitional" cells are shown with similar pores but not commented on. Although no secretion was observed in association with these pores, it is possible that they represent the orifices of the goblet cells, seen in the transitional epithelium by light microscopy, and which were found to contain acid mucosubstances.

In the caudal region of the nasal fossa and covering the ethmoidal

concha, the ciliated respiratory epithelium was abruptly converted to olfactory epithelium; in this sample site there was no transitional epithelium or junctional zone. The general features of canine olfactory epithelium were similar to those described in other mammalian species by Adams (1972), Greenwood and Holland (1972), Andrews (1974) and Kessel and Kardon (1979). The olfactory surface consisted of a tangled mat of long slender cilia which tended to mask the olfactory vesicles and the microvillous projections of neighbouring sustentacular cells. There were also patches of globular secretory droplets representing apical secretion from underlying sustentacular cells.

In only seven of the 18 dogs used in the present study was the nasopharynx completely covered with ciliated respiratory epithelium and in this respect resembled the respiratory epithelium elsewhere in the canine airways including the presence of numerous quiescent or discharing goblet cells. In only one of these animals was sufficient surface mucus present to almost blanket the underlying ciliated epithelium.

In four other dogs, a "cobblestone" type of epithelium similar to that described to occur in the ventral concha and septum was found adjoining the ciliated respiratory surface. In a further seven dogs, the nasopharyngeal epithelium was extremely folded and corrugated and, while transitional epithelium was not observed by the light microscope in this area, the surface characteristics of these cells with their numerous short microvillous processes and microplicae was similar in many respects to the stratified transitional epithelium of the nasal cavity. As the latter type of epithelium was found adjoining the "cobblestone" type of respiratory epithelium, it probably represented the beginning of the stratified squamous epithelium of the caudal region of the nasopharynx where it merges, at the free edge of the soft palate, with the oropharynx. Indeed, desquamating cells were often to be seen in this region. In one of these animals numerous rod-shaped bacteria were found on the epithelial surface.

References in the literature to the SEM features of the nasopharynx of other mammals are few although Greenwood and Holland (1972) mentioned that the nasopharynx was predominately ciliated while Andrews (1974) stated that the nasopharynx was lined with ciliated cells interspersed with patches of microvillous cells, similar to those observed in the present study, and which became more numerous towards the junction with the oropharynx.

In all 18 dogs, the surface of the epiglottis was lined with a corrugated epithelium composed of flat microvillous cells similar to those in the caudal region of the nasopharynx and presumably represented epithelium of the stratified squamous non-keratinising type. This type of epithelial lining was also present in the ventral larynx of six of the 18 dogs. Samples from only five dogs showed ciliated respiratory epithelium. The remaining seven animals had a junctional "cobblestone" epithelium sandwiched between ciliated respiratory epithelium at the laryngo-tracheal junction and non-ciliated squamous epithelium at the cranial end of the specimen.

References in the literature to the SEM features of the larynx are also sparse although Andrews (1974) in his study of the respiratory tract of the rat stated that, apart from the epiglottis, the larynx is lined with a mixture of ciliated and microvillous cells.

At the alveolar level, Type I and Type II pneumonocytes were easily identifiable. The cell boundaries of the former, however, were not always easy to identify and their surfaces showed many stubby microvillous projections. In contrast to the work of Parra et al (1978) interalveolar pores of Kohn were rare in the present study and never more than one per alveolus was found. The latter authors who used a method of fixation and airway perfusion similar to the present investigation calculated an average of 18 pores per exposed alveolar surface which gave a moth-eaten appearance to each alveolus. Likewise, in an earlier SEM study, Groniowski et al (1972) stated that alveolar pores were common in

the dog and Kondo et al (1973) and Hyde et al (1978) have also reported their presence.

It is difficult to provide a reason why so few pores of Kohn were found in the present study since the alveoli were apparently adequately inflated with fixative and it is unlikely that residual surfactant fluid clinging to the alveoli would mask virtually all of the alveolar pores as Parra et al (1978) postulated when they found few pores when the lungs were fixed by vascular perfusion.

It might be argued that, in earlier studies, the number of alveolar pores had been overestimated due to inadequate technical procedures resulting in artifactual holes being blown in the alveoli on exposure to the electron beam. Nevertheless, in an earlier non-ultrastructural histological study where the lungs were fixed by airway perfusion, Martin (1963) claimed an average of 7-8 pores per alveolus.

In other mammals it seems that, in general, interalveolar pores of Kohn are readily found. Thus, Iovannitti et al (1985) in their study of the lower respiratory tract of the bovine animal have found these structures without difficulty although Mariassy et al (1975), in a previous SEM study of the ox, considered that they were rare. Tyler et al (1971), in a SEM and TEM study of the equine lung found no difficulty in detecting alveolar pores. Likewise, Nowell and Tyler (1971) supplied evidence of these structures in the hamster and the horse and Greenwood and Holland (1973, 1975) in their SEM studies of the primate respiratory tract found alveolar pores in monkeys (Macaca speciosa) and also in man.

Another observation which is at variance with the findings of other workers was the absence of alveolar macrophages in any of the 18 dogs examined, even after a prolonged search. These cells, however, have been identified by SEM in other mammalian species such as the mouse (Greenwood and Holland, 1972); horse (Tyler et al, 1971); hamster

(Nowell and Tyler, 1971) and ox (Iovannitti et al, 1985) although in the latter animal Mariassy et al (1975) considered that they were rare. Indeed, Kondo et al (1973) in a SEM study of canine lung biopsies stated that alveolar macrophages were present. Unfortunately, they did not provide any details or illustrative evidence in support. Greenwood and Holland (1973) in their SEM study of the respiratory tract of the stumptail monkey (Macaca speciosa) have also commented on the rarity of alveolar macrophages. It may be, therefore, that, in some mammals at least, the number of alveolar macrophages clinging to the alveolar wall is relatively small and most of these may be washed off when the lungs are fixed by perfusion of the airways.

It was considered worthwhile, therefore, to carry out further studies in an attempt to establish the presence or absence of alveolar macrophages in the canine lung. These investigations are reported in Chapters 5 and 6.

CHAPTER 4

A COMPARATIVE STUDY OF RESPIRATORY AND NON-RESPIRATORY EPITHELIUM IN THE CANINE LARYNX

4.1 INTRODUCTION

In Chapter 3, it became clear that the region of the ventral larynx sampled showed variation from dog to dog with regard to the presence or absence of respiratory epithelium in the sample material. On histological examination, only four of the 18 dogs had samples composed of respiratory epithelium, the remaining 14 animals having epithelium of the stratified squamous type. With the SEM, however, respiratory epithelium was found in 12 dogs (including seven animals showing junctional epithelial type). This no doubt reflected the larger surface area available for inspection.

As the overall extent of respiratory epithelium in the canine larynx is poorly documented, an opportunity was taken to carry out a more detailed histological examination of the dog larynx.

4.2 MATERIALS AND METHODS

Eleven Collie cross dogs whose ages ranged from one to three years were used in this study. Unlike the other dogs used in this dissertation, these dogs were obtained from the Glasgow Dog and Cat Home and, under the Institution's regulations, had to be destroyed *in situ*. Hence these animals were not available for SEM due to the lapse of time (up to 4 hours) between destruction and sampling of the laryngeal material. All the dogs were clinically normal and showed no evidence of overt respiratory disease. Subsequent histological examination of the larynges confirmed the absence of any respiratory lesion.

The larynx and proximal region of the trachea were removed within four hours after death and incised along their median dorsal wall. Four specimens were washed thoroughly in running tap water to remove all traces of mucus and then immersed in a 1% aqueous solution of phloxine B (BDH Chemicals Limited, Poole, England) for one minute. They were then washed and immersed in 1% solution of Alcian blue (BDH Chemicals Limited,

Poole, England) in 1% acetic acid for 5 minutes. During this procedure, the larynges were held open to allow the dyes to make contact with the entire laryngeal mucosa. The excess dye was then removed by washing the specimens in running tap water for 30 minutes. With this method, stratified squamous epithelium appeared pink while respiratory epithelium was stained blue (Watt et al, 1975). In a further seven cases, the laryngeal specimens were washed as before and stained with 1% Alcian blue alone. Areas of respiratory epithelium were stained blue while non-respiratory stratified squamous epithelium remained unstained.

After a photographic record of each larynx was made from the dorsal aspect they were fixed for seven days in NBF and post-fixed in mercuric chloride formol for two days. Samples for histological examination (HE and AB-PAS) were taken from the following Sites (Fig. 4.1):

(1) Cranial epiglottis

(2) Caudal epiglottis

(3) Ventral larynx, caudal to the vocal cords

(4) Dorsal larynx, caudal to the vocal cords

(5) Caudal larynx, at the laryngo-tracheal junction

The delay between death and sampling of the material (up to four hours after death) and subsequent staining with phloxine B and/or Alcian blue precluded examination of the larynges with SEM.

4.3 RESULTS

(1) Alcian blue staining:

There was remarkable uniformity in Alcian blue staining in the larynges of all 11 dogs. Blue staining, indicative of the presence of respiratory mucosa was not observed cranial to the vocal cords (Fig. 4.2). Caudal to the vocal cords, the lateral and ventral walls of the larynx were rather patchily stained until the level of the laryngo-tracheal junction where staining was more or less diffuse; this degree of staining continued

into the trachea. However, the angular dorsal wall of the larynx caudal to the vocal cords was uniformly deeply stained in all the dogs. Staining with phloxine B did not alter the overall distribution of Alcian blue-stained respiratory epithelium (Fig. 4.3).

(2) Histological Findings:

In all 11 animals, the mucosa of the cranial and caudal region of the epiglottis, both dorsal and ventral aspects consisted of stratified squamous non-keratinising epithelium (Fig. 4.4). This type of epithelium was also present in the ventral larynx of seven of the 11 dogs. In the remaining four animals, stratified squamous epithelium could also be seen to merge over a few millimetres, with respiratory epithelium. The latter type of epithelium covered the caudal larynx at the laryngo-tracheal junction and the dorsal wall of the larynx (caudal to the vocal cords) in all 11 dogs (Fig. 4.5).

The distribution of respiratory and non-respiratory (stratified squamous) epithelium is summarised in Table 4.1.

4.4. DISCUSSION

There was a good correlation between Alcian blue staining of the canine laryngeal mucosa and the presence of respiratory-type epithelium. It was only in the ventral larynx (sample site No. 3) that both respiratory and non-respiratory epithelium overlapped (in four of the 11 dogs). This confirmed the findings in the original 18 dogs and, as this sample site appears to be a transitional zone between the non-respiratory and respiratory regions of the ventral larynx, explains the unexpectedly high incidence of non-ciliated squamous epithelium observed with SEM.

It would appear, therefore, that the extent of respiratory epithelium in the canine larynx is somewhat less than that recorded for man (Watt et al, 1975), where most of the larynx appears to be lined by respiratory

epithelium with the exception of an area of variable width around the lateral margins and cranial third of the epiglottis and the vocal cords.

The use of SEM in this part of the work was precluded for two reasons. Firstly, most of the dogs were not available for study until up to four hours after death and the surface characterisation might have been altered by autolytic change. Secondly, the immersion of the larynges in phloxine B and/or Alcian blue made subsequent SEM study impossible.

TABLE 4.1

DISTRIBUTION OF RESPIRATORY AND NON-RESPIRATORY EPITHELIUM IN THE CANINE LARYNX ON HISTOLOGICAL EXAMINATION

Dog No	Sample Site	Respiratory	Non-Respiratory
1	1	-	+
	2	-	+
	3	-	+
	4	+	-
	5	+	-
2	1	-	+
	2	-	+
	3	-	+
	4	+	-
	5	+	-
3	1	-	+
	2	-	+
	3	+	+
	4	+	-
	5	+	-
4	1	-	+
	2	-	+
	3	-	+
	4	+	-
	5	+	-
5	1	-	+
	2	-	+
	3	-	+
	4	+	-
	5	+	-
6	1	-	+
	2	-	+
	3	+	+
	4	+	-
	5	+	-

TABLE 4.1 (Cont'd)

Dog No	Sample Site	Respiratory	Non-Respiratory
7	1	-	+
	2	-	+
	3	+	+
	4	+	-
	5	+	-
8	1	-	+
	2	-	+
	3	-	+
	4	+	-
	5	+	-
9	1	-	+
	2	-	+
	3	-	+
	4	+	-
	5	+	-
10	1	-	+
	2	-	+
	3	-	+
	4	+	-
	5	+	-
11	1	-	+
	2	-	+
	3	+	+
	4	+	-
	5	+	-

Sample Sites :

1. cranial epiglottis
2. caudal epiglottis
3. ventral larynx
4. laryngeal/tracheal junction
5. dorsal larynx

\+ present

\- absent

Respiratory = pseudostratified columnar epithelium with goblet cells

Non-Respiratory = Non-ciliated stratified squamous non-keratinising epithelium.

CHAPTER 5

A COMBINED SEM AND TEM STUDY OF THE DISTAL AIRWAYS OF THE DOG

5.1 INTRODUCTION

In Chapter 3, the surface topography of the canine airways from the nasal fossa to the alveolar wall was studied with the SEM. Two particularly interesting observations arose from this study, both related to the distal airways. First, the paucity of alveolar macrophages which, contrary to other reports in other mammals (Greenwood and Holland, 1972; Nowell and Tyler, 1971; Iovannitti et al, 1985), were not identified with any certainty. Second, the difficulty in finding pores of Kohn was in distinct contrast to other investigations (Groniowski et al, 1972; Parra et al, 1978).

As both of these observations might possibly have resulted from the method of fixation employed, i.e., perfusion by the airways, it was considered worthwhile to compare the widely used airway method of fixation as described in Chapter 3 with perfusion of fixative via the pulmonary artery.

Finally, as the review of the literature in Chapter 1 made clear that the distal canine airways from the small bronchiole to the alveolar wall have been poorly studied with both the TEM and SEM, an opportunity was taken to carry out a joint TEM and SEM study of the distal airways of the dog.

5.2 MATERIALS AND METHODS

Four Collie cross dogs aged 14 - 18 weeks were used in this experiment. All the dogs were clinically normal and showed no evidence of overt respiratory disease. Subsequent histological examination confirmed the absence of any respiratory lesion involving the distal airways.

The dogs were sedated with the neuroleptanalgesic "Immobilon" and subsequently euthanized with sodium pentobarbitone. The sedation,

exsanguination and necropsy procedures were performed as described in Chapter 2. In addition, all four dogs received an intravenous injection of Heparin (50mg/kg) (Paines and Byren Ltd., Greenford, England) prior to exsanguination.

When exsanguination was complete, the animal was laid on its right side and a vertical incision through the skin caudal to the caudal border of the left forelimb (mass of the triceps muscle) was continued through the muscles covering the thoracic wall until the rib cage was located.

A further vertical incision through the muscles of the fourth intercostal space (located at the caudal border of the forelimb) was made. Particular care was taken to penetrate the pleural membrane with a small incision initially. This introduced air into the pleural cavity, with the result that the lung collapsed into the midline mediastinum away from the thoracic wall, making the extension of the vertical incision less hazardous as far as damage to the thoracic contents was concerned. A wound retractor was inserted into the incised opening to facilitate access to the pulmonary trunk. The latter was located at the cranial border of the left side of the heart which was now exposed. A loop of cotton thread was placed around the pulmonary trunk which was then clamped off proximal to this ligature by means of artery forceps. An incision was made in the wall of the pulmonary trunk and a cannula (filled with Karnovsky's fixative; see Chapter 2) introduced and tied into place by means of the previously positioned ligature. The cannula was attached to a container of cold Karnovsky's fixative and with the latter positioned at a suitable height so that an adequate head of pressure existed for perfusing the lung, the flow clip was opened and fixation commenced. Full perfusion was allowed to continue for four minutes, at the end of which time the lung lobes were excised and sample blocks from the left and right caudal lobes were removed.

Samples for histology were then immersed in NBF whereas samples

for TEM and SEM were immersed in fresh chilled Karnovsky's fixative.

Subsequent examination techniques were as described previously in Chapter 2.

5.3 RESULTS

(1) SEM Findings:

On low power visualisation, the alveoli in general were poorly inflated and some appeared collapsed (Fig. 5.1). This was in sharp contrast to low power views of portions of lung fixed via perfusion of the airways (see Figs. 3.79 and 3.81). Furthermore, although the mucosal folds of the bronchi and larger bronchioles were prominent and the large pulmonary vessels distended (Fig. 5.2), small bronchioles, and particularly respiratory bronchioles, were less easy to identify at low magnification.

At higher magnification, there were a number of differences in all the vascular perfused lungs compared to the lung material of the dogs described in Chapter 3 and where airway perfusion was the method of fixation. In general, the airways of all four dogs were littered with small particles of surface debris and in some instances red blood cells (Fig. 5.3). There were, however, no obvious strands of mucus on the mucosal surface of the bronchi and bronchioles and the overall appearance of the ciliated carpet was similar to that of the dogs in which airway perfusion had been used (Fig. 5.4).

Despite vascular perfusion, the septal capillaries appeared to contain more red cells than those of the dogs fixed by airway perfusion and, unlike the latter, red cells were often to be found among the debris scattered over the alveolar membrane (Fig. 5.5). The surface Type I pneumonocytes appeared similar to that of the airway-perfused dogs, but contrary to the latter, Type II pneumonocytes were less conspicuous, possibly due to the distension of the septal vessels and the relatively

poor inflation of the alveoli (Fig. 5.6).

As in the alveoli fixed by airway perfusion, pores of Kohn were sparse (Fig. 5.7) and alveolar macrophages were never found.

A major difference between lung material fixed by airway perfusion as distinct to perfusion of the pulmonary artery was to be found in the distal airways of the terminal and respiratory bronchioles. As already mentioned, these structures were difficult to find on low power observation following vascular perfusion and, like the rest of the airways, were littered with granular debris. The main difference, however, following perfusion of the pulmonary artery was to be seen with regard to the Clara cells which were not so prominent and had a smoother surface compared to the findings with airway perfusion (Figs. 5.8 and 5.9).

(2) TEM Findings:

In the larger bronchioles, i.e., those respiratory airways arising directly from the small bronchi, two main types of cell were found (a) ciliated cells and (b) non-ciliated mucus-secreting goblet cells. Ciliated cells predominated; these were low columnar cells characterised, in addition to the presence of numerous surface cilia, by relatively electron lucent cytoplasm in which most of the mitochondria were concentrated near the luminal surface (Fig. 5.10). Each cilium was anchored to a dense basal body from which fine rootlets extended into the cell. Many surface microvillous projections were evident between the cilia. The other features of these cells were the complex interdigitation of the lateral cell membrane with adjacent cells, the presence of a well-developed Golgi apparatus situated above the nucleus and the occurrence of sparse rough-surfaced endoplasmic reticulum found mainly in the lower portions of the cytoplasm. Occasional basal cells with dense cytoplasm, and whose cytoplasm did not reach the lumen of the bronchiole, were found sandwiched between ciliated cells (Fig. 5.11).

Mucus-secreting goblet cells were fewer in number than the ciliated cells. The secretory granules were large and electron lucent, occupying the apical portion of the cell (Fig. 5.12). A few surface microvilli were often present and there was a well-developed Golgi apparatus adjacent to the nucleus and abundant rough-surfaced endoplasmic reticulum located in the basal region of the cell.

A further cell type identified, in small numbers, in the large bronchioles was characterised by the presence of electron dense granules in the apical region of the cell and electron dense cytoplasm (compared with surrounding ciliated cells) containing abundant rough-surfaced endoplasmic reticulum (Fig. 5.13). The cell surface showed numerous microvillous projections. This cell possibly represented an epithelial serous cell.

In the medium-sized, small and terminal bronchioles, the lining epithelium was composed almost entirely of non-ciliated Clara cells; ciliated cells were sparse and the number of cilia per cell much reduced (Fig. 5.14). No mucus-secreting goblet cells or serous cells were observed at this level. The epithelium of respiratory bronchioles, interrupted by mural alveoli, was entirely composed of Clara cells.

Regardless of their location in the distal airways, Clara cells all had a uniform morphology. They were low columnar or cuboidal cells with their rounded apical portions, containing a few stumpy microvilli, projecting into the lumen of the airway, often dwarfing a neighbouring ciliated cell (Fig. 5.15). Characteristically, their nuclei, which in the main were centrally placed, appeared to be floating in a sea of glycogen with the residual cytoplasm containing a few scattered organelles, including mitochondria, confined to the immediate perinuclear region and periphery of the cell (Figs. 5.16 and 5.17). The Clara cells were devoid of secretory granules; a few cells showed secretory granule-like structures in their apical cytoplasmic rim. Closer examination at higher magnification indicated that these were large rounded mitochondria (Fig. 5.16)

The junction between a respiratory bronchiolar epithelial cell and the alveolar membrane of a mural alveolus or alveolar duct was always sudden and always involved contact of a Clara cell with a Type II pneumonocyte (Fig. 5.18), never with a Type I pneumonocyte. The latter was a flattened cell with thin attenuated cytoplasm sprouting only a relatively few stubby microvilli (Figs. 5.19 and 5.20). The Type II pneumonocyte, on the other hand, was a much more prominent cell with many surface microvillous projections. The cytoplasm was well stocked with rough-surfaced endoplasmic reticulum but their distinguishing feature was the presence of numerous lamellar inclusion bodies (Fig. 5.21).

In contrast to the SEM findings, alveolar macrophages either in close contact with Type I and Type II pneumonocytes (Fig. 5.22) or lying free in the alveolar lumen (Fig. 5.23) were not an uncommon occurrence. These cells had irregular abundant cytoplasm, often showing numerous surface projections, and contained many lysosomes and intracytoplasmic vacuoles.

5.4 DISCUSSION

The present study has shown that, in many respects, vascular perfusion where fixation is introduced by way of the pulmonary artery, was less successful in offering a good surface view with the SEM of the distal airways than introduction of fixative directly into the airways via a large bronchus. The main disadvantages of the vascular route were poor visualisation of the alveolar surfaces due to distenstion of septal capillaries as well as of larger peribronchial and peribronchiolar blood vessels, and the presence of surface mucosal debris throughout the airways.

The method of perfusion of the pulmonary artery was essentially similar to that described by Parra et al (1978) although the latter workers used 2.5% glutaraldehyde as a fixative in contrast to the present study which employed paraformaldehyde/glutaraldehyde (Karnovsky). Also, in contrast to the work of Parra and co-workers, who found many pores

of Kohn following airways fixation (18.6 $\pm$ 8.3 per alveolus) but fewer after vascular perfusion (3.9 $\pm$ 8.3 per alveolus), the present investigation showed little difference in the number of pores, regardless of the route of fixation; in both methods, pores of Kohn were equally rare, certainly not in sufficient number to quantify. That alveolar pores exist in the dog cannot be questioned. Indeed their presence, albeit in varying numbers, have been clearly identified in many other mammalian species (Nowell and Tyler, 1971; Greenwood and Holland, 1973; Iovannitti et al, 1985). The disparity in results between the present study and the work of Parra et al (1978) is unclear and may rest simply on differences in preparative techniques or even in differences in the fixative used. It would seem that further work is needed to clarify the actual number of pores of Kohn in the dog alveolus taking more note of perfusion fixatives and pressures as well as in drying and coating methods for SEM.

As with the airway perfusion method, alveolar macrophages were not seen by SEM in any of the four dogs whose lungs were fixed by perfusion of the pulmonary artery. Thus the premise that the airway perfusion method may simply detach and flush off any such cells adhering to the alveolar wall does not seem tenable.

Fixation of the lungs by means of vascular perfusion proved very successful in preserving pulmonary tissue for examination with the TEM. This allowed a detailed examination of the internal structure of all the cell types lining the distal airways. No direct comparison was made, however, between the two methods of fixation as far as TEM studies was concerned. Of interest was the finding of a few cells resembling serous cells in the large bronchioles. These cells, characterised by electron dense granules in the apical portion of their cytoplasm, have not previously been detected in the canine airways (Jeffery, 1983). They were first described in the trachea and extrapulmonary bronchi of rats by Jeffery and Reid (1975) but have since been detected in similar sites in the cat, hamster and human foetus (Jeffery, 1983). Whether or not

the serous cell occurs throughout the canine airways awaits further studies.

Non-ciliated bronchiolar (Clara) cells have been studied in many mammalian species with the TEM (reviewed by Plopper, 1983) and it is evident that there are morphologic differences between the species. The present investigation confirmed the TEM studies of Plopper et al (1980^{b}) who, in a comparative ultrastructural study of the Clara cell in a number of mammals, including the dog, found large amounts of cytoplasmic glycogen in the latter animal. The question of the presence or absence of cytoplasmic granules in canine Clara cells remains unresolved. Although present in abundance in some species such as the mouse, rat and horse, Plopper et al (1980^{b}) suggested that they were uncommon in the dog Clara cell. In the present study, no definite granules were observed.

Of particular interest in the present TEM study of the distal airways was the presence of alveolar macrophages with their distinctive morphology in the alveolar air spaces; indeed these cells were not difficult to detect. This is in sharp contrast to the SEM findings where these cells were never seen whether or not the lungs were fixed by perfusion of the airways or by perfusion of the pulmonary artery. Bearing in mind the large alveolar surface area available for inspection with the SEM compared to that available for TEM studies, it is surprising that alveolar macrophages could only be detected by the latter method. It is also unlikely that alveolar macrophages were mistaken for Type II pneumonocytes as the surface configuration of the latter cell is quite characteristic. In order to definitely establish the presence of macrophages in the canine lungs, a method of lung lavage was employed and the result of this investigation is reported in Chapter 6.

CHAPTER 6

A COMBINED SEM AND TEM STUDY OF CANINE ALVEOLAR MACROPHAGES

6.1 INTRODUCTION

In Chapter 3, a SEM study of the lungs of 18 dogs failed to detect alveolar macrophages in the alveoli despite the fact that these cells could often be observed albeit singly or in small numbers on ordinary light microscopy. It was considered that their absence in the SEM studies may have been associated with the method of fixation of the lungs where the fixative was introduced into the right caudal lobe by way of the main bronchi thus possibly flushing out most if not all the macrophages.

A further SEM study in Chapter 5 where the lungs were fixed by perfusion of the pulmonary artery, also failed to detect any alveolar macrophages although, with TEM, macrophages were identified without difficulty.

These studies prompted a further investigation of alveolar macrophages using a method of lung lavage and examining the cells collected by conventional light microscopy as well as with SEM and TEM.

6.2 MATERIALS AND METHODS

Two Collie cross dogs aged 10 to 12 weeks were used in this experiment. Both dogs were clinically normal and showed no evidence of overt respiratory disease.

Both dogs were sedated with the neuroleptanalgesic "Immobilon" and subsequently anaesthetised with sodium pentobarbitone (see Chapter 2).

When exsanguination was complete, the thoracic cavity was opened and the upper part of the trachea was dissected free and clamped with artery forceps to avoid entrance of blood into the lungs when the trachea was transected. The trachea was cut above the point where it

was clamped and the lungs, heart and trachea were dissected out intact. The outer surface of the organs was washed with physiological saline with the trachea still closed. The heart was dissected free, carefully avoiding any injury to the lungs. The artery forceps were partially detached from the wall of the trachea leaving the lumen open. By means of a 50 ml syringe, media 199 solution (Gibco Ltd., Paisley, Scotland) was injected into the trachea until the lungs were distended. This required approximately 200 to 220 ml of fluid media. It was necessary to introduce the medium into each bronchus in order to ensure adequate distension of each lobe of the lungs. The lungs were then massaged gently and the fluid was drained into a beaker. The collected suspension was centrifuged for 20 minutes at 1200 r.p.m. The supernatant fluid was decanted and the sedimented cells were resuspended in 10 ml of media 199. The cell suspension was again centrifuged in a graduated long stem centrifuge tube for one hour at 800 r,p.m. The supernatant fluid was removed by means of a pipette and the cell sediment was resuspended in a minimal volume of media 199 from which smears were prepared on glass slides. The slides were then stained by the May-Grunwald-Giemsa technique. The remainder of the cellular pellet was subsequently prepared for SEM and TEM studies.

MAY-GRUNWALD-GIEMSA TECHNIQUE

Solutions :

(a) May-Grunwald Stock Solution :

0.3g of powdered May-Grunwald dye was ground in a little methanol; the latter was decanted and the process repeated until the dye was in solution. This was made up to a final volume of 100 ml.

(b) Working Solution :

20 parts of May-Grunwald solution was diluted with 30 parts

of phosphate buffer (pH 6.8).

(c) Giemsa Stock Solution :

7.36g of Giemsa dry stain was mixed in 500 ml of glycerol which was heated to 50°C in a water bath. The mixture was left for 30 minutes at 50°C with periodic mixing. After cooling, 500 ml of methanol was added and the mixture filtered.

(d) Working Solution :

10 parts Giemsa solution was mixed with 40 parts of phosphate buffer (pH 6.8).

Technique :

(1) Formalin fixed smears were stained with the diluted May-Grunwald solution for 10 minutes

(2) Rinsed in tap water

(3) Stained in the diluted Giemsa solution for 30 minutes

(4) Washed and differentiated in buffer for 5 to 20 minutes until the desired colour balance was achieved

(5) The slide was allowed to dry and then mounted

Results :

Nuclei	purple
Cell cytoplasm	blue to mauve
Red blood cells	pink

Examination of Smears of Lung Washings by SEM:

1.0cm diameter glass coverslips were kept for two hours in 0.1% poly-l-lysine solution for "cationic" coating. A drop of resuspended cell sediment was then smeared onto coverslips and left

to dry for one hour. The latter were then processed for SEM in the manner as described previously and detailed in Chapter 2, except that air drying was used instead of critical point drying.

Examination of the Cell Sediment by TEM:

A portion of the cell sediment from each dog was re-suspended in Karnovsky's fixative for two hours. The suspension, in coarse lumps, was then centrifuged at 1500 r.p.m. for 5 minutes and the pellet re-suspended, again in coarse lumps, in 0.1m cacodylate buffer for one hour. Following centrifugation, the cells were then transferred to a specimen bottle and processed for TEM as described in Chapter 2.

6.3 RESULTS

Smears of the cell suspension obtained by lung washing showed a mixture of cells when stained with the May-Grunwald-Giemsa method. Although lymphocytes, neutrophils and even a few mast cells were identified, the vast majority of cells (> 90 per cent) appeared to be macrophages. The latter varied in size from 10 μm to 25 μm, often had eccentrically placed indented nuclei and many showed a distinctly foamy cytoplasm (Fig. 6.1). The morphology of these cells, however, was much clearer when 1 μm plastic embedded sections were stained with toluidine blue prior to the preparation of thin sections for TEM and, in these preparations, the foamy nature of the cytoplasm was particularly prominent (Fig. 6.2).

As with light microscopy of smears and 1 μm sections of plastic embedded specimens, SEM studies of the lung washings showed a number of cells of varying size, although all had an irregularly folded cell membrane with occasional long surface microvillous projections (Fig. 6.3). Closer inspection emphasised the irregular surface of these cells (Fig. 6.4).

It was not appreciated until the lung washings were processed for TEM how remarkable the surface configurations of the alveolar macrophages were. In Fig. 6.5, the foamy cytoplasmic nature of these cells is shown to be due to the presence of intracytoplasmic vacuoles, presumably phagolysosomes, indicating their active role in phagocytosis.

On higher magnification, the irregularly indented nucleus is shown with more clarity (Fig. 6.6).

6.4 DISCUSSION

It cannot be disputed that alveolar macrophages do exist in the canine lung. The TEM studies described in Chapter 5 clearly established their existence and the present, albeit limited, study has shown that, although no quantitative or qualitative assessment was performed, alveolar macrophages comprised the major cell type in lung washings. What remains unanswered is the question why these cells could not be identified with SEM. It seems difficult to conceive that, despite the greater surface area available for inspection with SEM compared to the relatively small specimens used in the TEM studies (Chapter 5) alveolar macrophages could only be identified with certainty in the latter.

The clear intention of the present section of the work was to firmly establish their presence in the canine lung. Having done so, their absence in the SEM preparations, whether by airway perfusion of fixative or vascular perfusion, indicates that during the preparation of specimens for SEM, their numbers are substantially reduced to a level where they cannot be visualised. As will become evident in the next Chapter, where alveolar macrophages were present in large numbers, they were easy to identify with SEM. Thus their absence in normal dog alveoli when viewed with the SEM cannot be adduced to confusion with other cells at this level, particularly the Type II pneumonocyte.

CHAPTER 7

A COMBINED HISTOLOGICAL, HISTOCHEMICAL AND SEM STUDY OF EXPERIMENTAL *Bordetella bronchiseptica* INFECTION IN THE DOG

7.1 INTRODUCTION

So far, this dissertation has focussed attention on the surface features of the various types of cells which populate the canine airways. It was considered important to establish these morphologic parameters in order to ensure that any changes occurring in pathological processes can be properly assessed.

In Chapter 1, it has already been stressed that contagious respiratory disease is an important clinical problem in dogs and it has now been established that among the many infectious agents that reside in and damage the canine airways, Bordetella bronchiseptica is of particular importance not only as a secondary invader but also as a primary pathogen (Wright et al, 1973; Thompson et al, 1976). As none of the canine respiratory pathogens have so far been studied with the SEM, it was considered worthwhile to choose the bacterium Bordetella bronchiseptica as a model for study as, unlike other respiratory pathogens, it is sufficiently large to be visualisable with the SEM as well as being an important primary agent in the pathogenesis of contagious canine respiratory disease. Thus, its presence and its pathologic effects on the surface respiratory epithelial cell population could be directly assessed with the SEM,

The object of Chapter 7 was to study the effects on the canine respiratory tract of an aerosol spray of Bordetella bronchiseptica. It was considered of particular importance to assess the surface morphologic alterations that this important canine pathogen causes in the canine airways and to relate these changes to the normal features described in Chapter 3.

Since the early studies of Ferry (1910, 1911, 1912), McGowan (1911) and Torrey and Rahe (1912) who, before the understanding of virological knowledge had reached its current state, claimed that the most important respiratory disease of dogs of the time, canine distemper,

was due to a bacterium known as Bordetella bronchiseptica, the fortunes of this organism have waxed and waned..

Once Carre (1905) and Laidlaw and Dunkin (1926) had shown that the causal agent of distemper was in fact a filterable virus, Bordetella bronchiseptica was relegated to a secondary role and, although the organism was frequently isolated from the respiratory tissues of dogs suffering from respiratory disease (Singh and Parnaik, 1965; Snow et al, 1969; Appel and Percy, 1970; Bemis et al, 1977[a,c]; Wilkins and Helland, 1973; Tischler and Hill, 1977; McCandlish et al, 1978[d]; Roudebush and Fales, 1981) it was not until 1973 that Wright et al, showed that Bordetella bronchiseptica, provided it was introduced by an aerosol route, was indeed a primary pathogen for the canine respiratory tract in its own right and was an important cause of contagious respiratory disease of dogs, commonly referred to as "kennel cough".

Bordetella bronchiseptica is a gram-negative pleomorphic coccobacillary bacterium ranging in size from 3.0 x 0.5 µm to 0.4 x 0.72 µm. Unlike Bordetella pertussis, the causal agent of whooping cough in children, Bordetella bronchiseptica is motile, motility being provided by peritrichous flagella (Goodnow, 1980). In addition to its role in respiratory disease in dogs, the organism has been implicated in respiratory problems in a variety of animals, particularly in the pig where it is the causal agent of atrophic rhinitis (Winsser, 1960; Goodnow, 1980). Akin to other Bordetella species, Bordetella bronchiseptica contains at least one lipopolysaccharide toxin but there is still doubt as to how the organism induces its pathologic effects on respiratory epithelial cells. It is, however, known to attach to the cilia of respiratory epithelial cells and cause ciliostasis (Bemis and Kennedy, 1981). Nevertheless, the importance of Bordetella bronchiseptica in canine respiratory disease has now been established in a number of reviews such as those by Thompson et al (1975), Bemis et al (1977[a]), Appel and Bemis (1978) and Goodnow and Shade (1980). On the other hand, other workers have failed to incriminate the organism as an important agent in the pathogenesis of

canine respiratory disease (Smith, 1961; Clapper and Meade, 1963; Binn et al, 1968). In the last two decades, however, Bordetella bronchiseptica has been firmly established as a primary pathogen in the induction of contagious respiratory disease (Bemis et al, 1977[a]; Shelton et al, 1977; Appel and Bemis, 1978; Goodnow, 1980; Wagener et al, 1984).

Recently, too, there has been a series of experimental studies to investigate the role of Bordetella bronchiseptica in the induction of contagious respiratory disease in dogs. Thus, Thompson et al (1976) infected two groups of dogs, aged six weeks and twelve weeks respectively, with an aerosol spray of Bordetella bronchiseptica isolated from a dog with bronchopneumonia. The clinical disease induced resembled that found in whooping cough (pertussis) in man and also in naturally-occurring "kennel cough" in dogs. This definitive study showed for the first time the capability of Bordetella bronchiseptica alone, in the absence of virological agents, to induce clinical respiratory disease with subsequent spread to in-contact animals. Subsequently, Bemis et al (1977[b]) produced a tracheobronchitis resembling "kennel cough" following aerosol administration of Bordetella bronchiseptica to specific pathogen free dogs and, more recently, Goodnow et al (1983) compared two isolates of Bordetella bronchiseptica, one avirulent and one virulent; only the latter induced clinical bordetellosis.

Once the realisation had dawned that Bordetella bronchiseptica was after all an important cause of naturally-occurring "kennel cough", it made a marked impact on the veterinary profession and also on commercial enterprises. Since then, a number of experimental studies have been initiated and many reports have appeared in the literature, the objective being to produce suitable chemotherapeutic agents (Bemis and Appel, 1977[a,b]; Shelton et al, 1977; McCandlish and Thompson, 1979) with varying degrees of success.

In a similar fashion, a number of immunisation protocols have been

studied (McCandlish et al, 1976; Shelton et al, 1977; McCandlish et al, 1978[a,b,c]; Goodnow and Shade, 1979, 1980; Shade and Goodnow, 1979; Chladek et al, 1981; Shade and Rapp, 1982) and the success of this work is now reflected in that some of the vaccine products employed are now incorporated along with other canine respiratory pathogens in commercially available vaccines.

Although much has now been learned about canine bordetellosis, one area which has received little attention has been the investigation of the overall topographical features of the respiratory tract during an episode of canine respiratory disease where Bordetella bronchiseptica is the primary pathogen. The object of the present part of the work was to carry out a combined histological and SEM study of the canine airways subjected to an aerosolised spray of a virulent strain of Bordetella bronchiseptica.

The only other available SEM studies involving Bordetella bronchiseptica are those by Yokomizo and Shimizu (1979) who studied the adherence of the organism to cultured swine nasal epithelial cells, Matsuyama and Takino (1980) who observed the adherence of Bordetella bronchiseptica to rabbit tracheal mucosa, Muse et al (1977) who investigated the association of Bordetella pertussis to hamster tracheal cultures and Matsuyama (1977) who carried out a SEM study of rabbit trachea inoculated with Bordetella pertussis.

To date, there has been no systematic study of the pathologic effects of Bordetella bronchiseptica on the entire canine airways, an omission of importance considering the now widely accepted primary role that the organism undoubtedly plays in the induction of canine respiratory disease.

7.2 MATERIALS AND METHODS

Twelve collie cross dogs, ranging in age from 14 - 18 weeks,

were used in this study. All the dogs were clinically normal and showed no evidence of overt respiratory disease. They had, prior to the experiment, been immunised against the common canine pathogens, as previously described in Chapter 2.

The dogs were sedated by an intramuscular injection of 0.5 ml of the neuroleptanalgesic "Immobilon" (Reckitt and Colman) and exposed to an aerosolised suspension of Bordetella bronchiseptica, using a "Wright"-type nebulising chamber (Aerosol Products, Colchester, U.K.). The inoculum was a 10 ml suspension containing 4×10^9 colony forming units/ml. The dogs were exposed to an aerosol spray for 12 mins. with a net minimum chamber airflow pressure of 10 lbs./sq. in. The dogs were then removed from the chamber and given an equal volume of "Revivon" (Reckitt and Colman) to reverse the effect of "Immobilon".

At four, six, eight, ten, 20 and 28 days, two dogs were sedated with the neuroleptanalgesic "Immobilon" and subsequently euthanized with sodium pentobarbitone. The exsanguination and necropsy procedures were performed as described in Chapter 2. Samples for histology and SEM were taken from all regions of the respiratory tract, as described in Chapter 3. For purposes of control, a further six dogs were aerosolised with PBS for 12 mins. and sacrificed at four, eight (two), ten and 20 (two) days respectively. They were subjected to the same necropsy, histological and ultrastructural procedures as were the test animals aerosolised with Bordetella bronchiseptica.

Sections were routinely stained with HE and AB-PAS. In addition, the Gram-Twort technique (Twort, 1924; Ollett, 1947) was employed for the examination of the presence of Bordetella bronchiseptica in smears and paraffin sections respectively. The above methods used are detailed as follows :-

Preparation of smears of bacteria for histological examination :

The culture of Bordetella bronchiseptica was placed in a centrifuge at 4°C for 20 minutes at 3000 rev/min. After centrifugation, the supernatant was removed and the cells suspended in 1 ml of the supernatant liquid. Using a fine pipette, one drop of the solution containing the Bordetella bronchiseptica organisms was placed on a clean dry slide. By means of the edge of a microscope slide, the culture was evenly spread over the slide and allowed to dry. The slide was then stained by the Gram-Twort technique for bacteria (Twort, 1924; Ollett, 1947).

After staining, a coverslip was placed over the smear to preserve and retain the detail of the stained preparation for future photographic record.

Materials used in the preparation of Gram's method for bacterial smears :

1. Crystal violet solution :

Crystal violet, 2g
95% alcohol, 20 ml
Ammonium oxalate, 0.8g
Distilled water, 80 ml

2. Gram's iodine :

Iodine crystals, 1g
Potassium iodide, 2g
Distilled water, 300 ml

3. Aniline-xylene :

Aniline, 2 parts
Xylene, 1 part

Technique :

(1) The slide was stained with crystal violet for 2-3 mins.

(2) The stain was washed off with Gram's iodine; then the slide was flooded with Gram's iodine and left for 2-3 mins.

(3) The smear was decolourised in absolute alcohol or acetone.

(4) Then the slide was stained for 2-3 mins., direct from the absolute alcohol, in a 1% aqueous solution of neutral red.

(5) The slide was then rinsed rapidly in water.

(6) After blotting dry and mounting in a synthetic resin medium, the slide was examined with the light microscope.

Results :

Gram-positive bacteria	-	blue black (Fig. 7.1)
Gram-negative bacteria	-	red

Gram-Twort technique for tissue sections : (Twort, 1924; Ollett, 1947)

Solutions :

(1) Crystal violet solution

(2) Gram's iodine solution

(3) 0.2% acetic acid in ethanol

Stock neutral red - fast green stain :

0.2% neutral red in ethanol, 90 ml

0.2% fast green FCF in ethanol, 10 ml

Before use, 1 part stock solution was diluted with 3 parts distilled water.

Technique :

(1) Paraffin sections taken to water

(2) Sections stained with the crystal violet solution for 3 mins.

(3) Sections washed in tap water

(4) Sections stained with the Gram's iodine solution for 3 mins.

(5) Sections washed in tap water and blot dried

(6) Sections differentiated in pre-heated acetic alcohol at $56^{o}C$ until a dirty straw colour

(7) Sections then washed briefly in distilled water

(8) Sections counterstained in neutral red - fast green stain solution for 5 mins.

(9) Sections washed in distilled water and differentiated in acetic alcohol at room temperature for approximately 10 seconds until no more red colour diffused out

(10) Finally, the sections were rinsed in alcohol, cleared and mounted and examined with the light microscope.

Results :

Gram-negative organisms and cell nuclei - red
Cell cytoplasm, collagen, red blood corpuscles - green

Examination of smears of Bordetella bronchiseptica by SEM :

1.0 cm diameter glass coverslips were kept for two hours in 0.1% poly-L-lysine solution for "Cationic" coating. A drop of Bordetella bronchiseptica containing 1×10^{9} to 1×10^{10} colony forming units/ml was then smeared on the coverslip and left to dry for one hour. The smear was subsequently processed for SEM in the manner as described previously in the general methods and materials section of Chapter 2, except that air drying was used instead of critical point drying (Fig. 7.2).

7.3 RESULTS

Throughout the experiment, all the dogs remained bright and no spontaneous coughing occurred. The two dogs killed at ten days

after aerosolisation, however, showed a copious seropurulent nasal discharge which was first noticeable at eight days. A soft dry cough, however, could be elicited by pinching the trachea of three dogs from seven days onwards (Dog Nos. WA 24, WA 38 and WA 38a).

Necropsy Findings:

Dog No. WA 19, killed at four days after aerosol administration of Bordetella bronchiseptica, showed no macroscopic changes; Dog No. WA 20, killed at the same time, showed strands of yellowish purulent exudate in the nasopharynx. At six days, both animals killed at this time (Dog Nos. WA 21 and WA 22) had abundant purulent exudate in the nasopharynx and trachea, and the tracheobronchial lymph nodes were enlarged and hyperaemic. At eight days following aerosolisation of Bordetella bronchiseptica, one of the two dogs killed at this time (Dog No. WA 24) had a copious purulent exudate in the trachea and nasopharynx and the retropharyngeal and tracheobronchial lymph nodes were enlarged, moist and hyperaemic. The other dog sacrificed at this time showed only enlargement and hyperaemia of the tracheobronchial lymph nodes.

The two animals killed at ten days (Dog Nos. WA 38 and WA 38a) showed the most severe macroscopic lesions with the presence of a sticky yellowish purulent exudate in the nasal cavity, nasopharynx and trachea. In addition to enlargement and hyperaemia of the retropharyngeal and tracheobronchial lymph nodes, all lobes of the lungs showed multiple small haemorrhagic foci. Both dogs had a copious seropurulent nasal discharge.

At day 20, both dogs showed a small amount of purulent exudate in the nasopharynx and trachea while, in the lungs, a few small greyish foci were found scattered evenly throughout all the lobes. The tracheobronchial lymph nodes were normal in appearance. The two animals sacrificed at 28 days showed no macroscopic features of note

at necropsy.

Histological Findings: (See Appendix II and Table 7.1)

Four days post aerosol infection :

Polymorphonuclear leucocytes and macrophages were found in small numbers on the ciliated surface and migrating through the pseudostratified epithelium of the ventral concha, septum, nasopharynx, trachea, extrapulmonary bronchus (Fig. 7.3) and larger bronchi. In these regions, focal areas of epithelial loosening and even frank necrosis were also found (Fig. 7.4); where the surface epithelial cells were detaching from the underlying basement membrane, there appeared to be loss of surface cilia. The only histological event of note in the lung parenchyma was the presence of small collections of macrophages and neutrophils occupying a few alveolar spaces (Fig. 7.5).

With the Gram-Twort staining method (and indeed also with the conventional HE staining method), clusters of bacteria were found intermingled with the cilia of the nasopharynx (Fig. 7.6), trachea, extrapulmonary bronchus and large bronchi in one of the two animals killed at this time (Dog No. WA 20). With AB-PAS, the number of goblet cells throughout the respiratory tract of both dogs was less than that found in the control animals or in the normal dogs described in Chapter 3. There was, however, no change in the type of mucosubstances and, like the controls, acid and mixed cells predominated (Figs. 7.7 and 7.8). There did seem, too, with the exception of Bowman's glands of the ethmoidal concha, to be an overall reduction of stained glands throughout the tract.

Six days post aerosol infection :

By six days, the numbers of neutrophils on the surface of the respiratory mucosa as well as those infiltrating through the

TABLE 7.1

EXPERIMENTAL Bordetella bronchiseptica INFECTION : HISTOLOGICAL FINDINGS

Dog No	Day Killed	Neutrophil Infiltration	Mucosal Necrosis	Squamous Metaplasia	Purulent Bronchiolitis	Presence of Bacteria (Gram-Twort Staining)
WA 19	4	1+	1+	-	-	-
WA 20	4	1+	1+	-	-	1+
WA 21	6	3+	3+	-	1+	3+
WA 22	6	3+	2+	-	1+	2+
WA 23	8	4+	2+	1+	3+	3+
WA 24	8	4+	3+	1+	3+	3+
WA 38	10	4+	4+	2+	4+	1+
WA 38a	10	3+	3+	2+	4+	1+
WA 39	20	2+	1+	-	-	1+
WA 41	20	3+	-	-	-	3+
WA 42	28	1+	-	-	-	1+
WA 43	28	1+	-	1+	1+	3+

NB. 1. Lesions and presence of bacteria were judged 1+ to 4+ according to severity.

2. - = No lesions (or bacteria).

epithelial lining had markedly increased. This was particularly noticeable in the ventral concha, septum, nasopharynx, trachea and extrapulmonary bronchus and large bronchi. Loss of surface epithelial cells had also markedly increased (Fig. 7.9) and the lamina propria was heavily infiltrated by numerous macrophages, lymphocytes and plasma cells (Fig. 7.10). With the Gram-Twort stain, many bacteria were found among the respiratory cilia throughout the tract, up to and including the small bronchi (Fig. 7.11).

In the lungs there were focal suppurative lesions characterised by flooding of alveoli with neutrophils and macrophages; some bronchioles were denuded of their epithelium (Fig. 7.12). Purulent bronchiolitis was found in a few instances.

With the AB-PAS staining method, there was an even greater reduction in the number of stained surface epithelial goblet cells (Fig. 7.13) and in the number of stained glandular units in the submucosa (Fig. 7.14). In contrast to the controls, no stained glands were distinguished distal to the ventral larynx.

Eight days post aerosol infection :

The lesions observed at eight days were essentially similar to those of six days although the density of infiltrating cells in the lamina propria of the ventral concha, septum, trachea, nasopharynx, extrapulmonary bronchus and large bronchi had markedly increased (Fig. 7.15). Likewise, superficial epithelial necrosis was more noticeable, particularly in the trachea and nasopharynx. In both dogs killed at this time suppurative bronchiolitis was evident with some bronchioles containing many macrophages, neutrophils and desquamated epithelial cells (Fig. 7.16); occasional patchy areas of squamous metaplasia were found in a few bronchioles.

In Dog No. WA 23, bacteria were found only in the ventral

concha, while, in Dog No. WA 24, numerous organisms were lodged in the cilia of the ventral concha, septum, trachea, extrapulmonary bronchus and large bronchi.

As at six days, the number of goblet cells stained with AB-PAS was much less than that of the controls (Fig. 7.17) and, apart from Dog No. WA 23, where a few neutral glands were found in the tracheal wall, no stained glands were observed distal to the ventral larynx.

Ten days post aerosol infection :

At this stage, neutrophilic infiltration into the respiratory mucosa was well established (Fig. 7.18) and focal loosening, desquamation of surface mucosal cells and frank necrosis was particularly marked in the ventral concha, trachea, extrapulmonary bronchus and large bronchi. Necrotising bronchiolitis was commonly observed with flooding of nearby alveoli with neutrophils, red cells and macrophages (Fig. 7.19). Occasional areas of flattened squamous-like epithelium was found in the ventral concha, trachea and extrapulmonary bronchus and large bronchi (Fig. 7.20). In one dog (Dog No. WA 38a) killed at this time, marked perivascular oedema was found in the lungs.

With the Gram-Twort stain, bacteria were found intermingled with the cilia of the trachea, extrapulmonary bronchus and large bronchi; elsewhere, organisms could not be detected.

At ten days, the number of goblet cells and glands stained with AB-PAS continued to be sparse and, as in the earlier stages of the infection, no stained glandular units were found distal to the ventral larynx. in one dog killed at this time (WA 38a). The remaining animals, however, showed a few stained glands extending as far as the large bronchi.

20 days post aerosol infection :

At this time, the histological changes were less marked compared to those noted at earlier stages of the infection. Neither animal showed mucosal necrosis or squamous metaplasia at any level of the respiratory tract and suppurative bronchiolitis was absent. In both dogs, however, neutrophilic infiltration into the respiratory mucosa continued to be a prominent feature and the lamina propria, particularly of the ventral concha, septum and large bronchi, was infiltrated by a mixture of neutrophils, lymphocytes and plasma cells (Fig. 7.21).

With the Gram-Twort staining method, a few scattered clumps of bacteria were found in the trachea, extrapulmonary bronchus and large bronchi in one dog (Dog No. WA 39) while, in Dog No. WA 41, large numbers of bacteria were detected among the cilia of the ventral larynx, trachea, extrapulmonary bronchus, bronchi and larger bronchioles.

With the AB-PAS stain, there did seem to be an increase in the number of goblet cells and glands compared to earlier in the infection, although many goblet cells contained only a few sparse granules of mucosubstances (Fig. 7.22).

28 days post aerosol infection :

In the upper respiratory tract, i.e., the ventral concha, septum, nasopharynx and ventral larynx, the respiratory mucosa had more or less returned to normal with only a few neutrophils to be found infiltrating the epithelium and only a minimal infiltration of lymphocytes, plasma cells and macrophages into the lamina propria. At this level, bacteria were confined to the ventral laryngeal epithelium of one dog (Dog No. WA 43) (Fig. 7.23). In the tracheobronchial tree, bacteria were found as far as the large bronchi in one dog (Dog No. WA 43) but only as far as the trachea in the

remaining animal killed at this time. In the latter animal (Dog No. WA 42), the tracheobronchial epithelium appeared normal with only a few neutrophils infiltrating the mucosa and little or no infiltration of the lamina propria. There were, however, a few small lymphoid deposits in the submucosa.

In Dog No. WA 43, however, focal areas of squamous metaplasia were found in the extrapulmonary bronchus and the lamina propria of the large and small bronchi were lightly infiltrated with a few lymphocytes, plasma cells and macrophages. One large focus of necrotising bronchiolitis with flooding of surrounding alveoli with neutrophils and macrophages was also found in this dog. Small lymphoid deposits were also found in the submucosa of the smaller bronchi of this animal.

With the AB-PAS staining method, the number of mucus producing cells continued to increase compared to earlier stages of the disease, particularly in the tracheobronchial tree (Fig. 7.24). Even so, many cells contained relatively few granules of mucosubstances compared to the densely-stained goblet cells of the control dogs. In general, the number of stained glands had increased to the level of the control animals.

SEM Findings:

In all dogs, no obvious changes were found in the dorsal epiglottis nor in the ethmoidal concha.

Four days post aerosol infection :

The main feature at this time was the presence of patches of mucus on the surface of the ventral concha, septum, nasopharynx and extrapulmonary bronchus (Fig. 7.25). In the latter area, a small amount of granular debris containing a few desquamated cells and presumably representing an inflammatory exudate as distinct to mucus

was found in both dogs killed at this time.

Goblet cells, particularly discharging cells, were sparse and the few polymorphonuclear leucocytes found on the respiratory surfaces were partially masked by the surrounding cilia. For the most part the respiratory cilia throughout the tract appeared normal and only a few bacteria were identified among the cilia of the septum, ventral larynx and trachea (Fig. 7.26). In contrast to the findings with the Gram-Twort staining method for bacteria, no organisms were detected by the SEM in the nasopharynx, extrapulmonary bronchus or large bronchi.

In some areas, particularly in the ventral concha, septum, nasopharynx, trachea and extrapulmonary bronchus, patches of cilia appeared ragged and disorganised (Fig. 7.27). In the nasopharynx of one dog (Dog No. WA 19), areas of ciliary necrosis were observed. Similar lesions were also noted in the trachea and the extrapulmonary bronchus of both dogs killed at this time (Fig. 7.28).

At the alveolar level, the only feature of note was the presence of a few large irregular cells suggestive of alveolar macrophages lying on the surface of the Type I pneumonocytes (Fig. 7.29). No bacteria were seen in the alveoli.

Six days post aerosol infection :

At this time, strands or sheets of mucus continued to be prominent on the respiratory tract surface but, as at four days, goblet cells were reduced in number. Many more bacteria were found than at four days but only as far as the larger bronchi (Fig. 7.30). In some instances, fine filaments were seen to extend from the bacterial wall to neighbouring cilia. Although bacteria were recognisable in the smaller bronchi with the Gram-Twort staining method, they were not found at this site with the SEM.

As at four days after aerosolisation, there were areas of ciliary disorganisation and necrosis in the ventral concha, septum, nasopharynx, ventral larynx, trachea, extrapulmonary bronchus and in the large and small bronchi (Fig. 7.31). Within the ciliary carpet, examples of very slender or extremely thick cilia were evident; the tips of other cilia often had a hooked appearance; others had an expanded bulbous end. In the ventral larynx, trachea and extrapulmonary bronchus, patches of flat non-ciliated cells were found; the surface of these cells was studded with numerous microvillous processes (Fig. 7.32). Other cells were sparsely ciliated and showed only a few short irregular cilia (Fig. 7.33). Again, as at four days, only a few polymorphonuclear leucocytes were observed among the cilia (Fig. 7.34).

Neither of the two dogs killed at this time showed an inflammatory exudate in the trachea or extrapulmonary bronchus. In both dogs, however, some bronchioles had lost their lining epithelium and the lumen was filled with cellular debris. Adjacent alveoli contained only a few alveolar macrophages; the focal suppurative lesions observed by light microscopy were not identified with SEM.

Eight days post aerosol infection :

Areas of ciliary necrosis with total or patchy loss of cilia were found at all levels of the respiratory tract. This was particularly severe in the ventral concha, septum, nasopharynx, ventral larynx, trachea and extrapulmonary bronchus (Fig. 7.35). Groups of flat, non-ciliated microvillous cells were found among residual apparently unaltered ciliated epithelium at all levels of the respiratory tract from the ventral concha to the small bronchi.

As was the case earlier in the infection, irregular strands or sheets of mucus were found throughout the respiratory airways although goblet cells were sparse. Likewise, contrary to the

histological findings at this time, the number of polymorphonuclear leucocytes on the respiratory mucosal surface was few. Clumps of a cellular inflammatory exudate were found adhering to the walls of the trachea, extrapulmonary bronchus and the larger bronchi of both dogs (Fig. 7.36).

In the lungs, some bronchioles were filled with necrotic cell debris (Fig. 7.37). As at six days post infection, relatively few alveolar macrophages were found clinging to the alveolar membrane, fewer than anticipated following light microscopy (Fig. 7.38).

Recognisable bacteria were only present in the trachea of one dog (Dog No. WA 24) and in the extrapulmonary bronchus of both animals killed at this time. This again was contrary to the findings with the Gram-Twort staining method where many bacteria were found, particularly in Dog No. WA 24.

Ten days post aerosol infection :

At this time, patches of ciliary necrosis were found at all levels of the respiratory tract. In other areas, the ciliated surface was normal or the cilia were sparse, long and spindly or tangled in appearance (Fig. 7.39). There were also extensive areas, particularly in the ventral larynx, trachea, extrapulmonary bronchus and large bronchi, where the cilia had a short "close-cropped" appearance (Fig. 7.40). Patches of mucus were plentiful although very few discharging goblet cells were to be found. In addition to strands of mucus, large islands of a cellular inflammatory exudate were noted, particularly in the ventral concha, septum, trachea and extrapulmonary bronchus.

Although necrotising bronchiolitis was found in only one dog (Dog No. WA 38a), alveolar macrophages were plentiful in both

dogs killed at this time, and the alveolar septa appeared thickened (Fig. 7.41). A striking feature at this stage of the infection was the presence of patches of flattened non-ciliated squamous-like epithelium. These areas which often showed a sharp transition from adjacent ciliated epithelium, were particularly prominent in the trachea (Fig. 7.42) and extrapulmonary bronchus. The surface of these squamous-like cells sported numerous short microvilli and microplicae (Fig. 7.43).

Groups of bacteria were found entangled in the cilia of the nasopharynx (Fig. 7.44), ventral larynx, trachea, extrapulmonary bronchus and larger bronchi, particularly in Dog No. WA 38a.

20 days post aerosol infection :

At this stage of the infection the surface changes were essentially similar to those observed at ten days. Throughout the respiratory tract, however, normal ciliated epithelium interspersed with patches of "close-cropped" ciliated cells or occasional non-ciliated microvillous cells predominated; ciliary necrosis was rarely found (Fig. 7.45). Although patches of squamous-like epithelium persisted in the trachea and extrapulmonary bronchus (Fig. 7.46) strands of mucus or patches of inflammatory exudate were uncommon and, while there was no evidence of necrotising bronchiolitis, occasional macrophages were observed clinging to the alveolar walls and the alveolar septa often appeared thickened (Fig. 7.47). Goblet cells were more prominent than earlier in the infection (Fig. 7.48).

In general, bacteria were difficult to locate in both dogs killed at this time and were confined to the trachea and extrapulmonary bronchi (Fig. 7.49).

28 days post aerosol infection :

By this stage, bacteria had vanished from the mostly normal

ciliated respiratory surface. Patches of mucus or inflammatory exudate were extremely rare and goblet cells, either occurring singly or in clumps, were plentiful. There were, however, isolated non-ciliated microvillous cells persisting throughout the tract (Fig. 7.50). Although only a very few small areas of squamous-like epithelium remained in the trachea of one dog (Dog No. WA 42). Necrotising bronchiolitis was absent and alveolar macrophages were only rarely found.

Control Dogs :

At necropsy, all six control animals remained clinically normal and showed no macroscopic changes in the respiratory tract. Likewise, histological examination at all levels of the respiratory tract showed no lesions. With the AB-PAS staining method, the results were similar to those recorded for the normal dogs described in Chapter 3 (Fig. 7.51). (Compare Appendices I and II). SEM findings were also similar to those normal dogs.

7.4 DISCUSSION

In clinical terms, all 12 dogs in this study showed only a mild respiratory infection in which spontaneous coughing was absent and, in only three animals, could a soft dry cough be induced by pinching the trachea. This is in contrast to the reports of experimentally induced bordetellosis in dogs by Thompson et al (1976), Bemis and Appel (1977[b]) and Goodnow and Shade (1979) who recorded a spontaneous hacking, sometimes productive, cough in many of their animals. Two dogs (Dog Nos. WA 38 and WA 38a), however, both killed at ten days after aerosolisation of Bordetella bronchiseptica did show a seropurulent nasal discharge, while at necropsy several animals had a yellowish purulent exudate in the nasopharynx or trachea or both. Overt lung lesions were confined to days 10 and 20; at the former time, multiple small red foci were found scattered

throughout all lobes of the lungs while at day 20 a few small greyish foci were the only evidence of a pulmonary lesion.

The histological lesions were in the main similar to those described by Wright et al (1973), Thompson et al (1976), Bemis et al (1977[b]) and Goodnow et al (1983) in their reports of primary experimentally-induced Bordetella bronchiseptica infection in dogs, and by Tischler and Hill (1977), Shelton et al (1977) and McCandlish et al (1978[d]) in their reports of spontaneous cases of canine bordetellosis. The initial lesion at four days appeared to be infiltration of the respiratory mucosa by polymorphonuclear leucocytes from the ventral concha to the larger bronchi. This was rapidly followed by surface epithelial necrosis which reached a peak six to ten days after aerosolisation of bacteria. At the level of the large and small bronchioles, necrotising bronchiolitis was found as early as six days and persisted, in one dog, up to 28 days post aerosolisation of Bordetella bronchiseptica.

There did not appear to be any advantage in using the Gram-Twort staining method for the identification of bacteria entrapped on the mucosal surface; there appeared to be just as good a correlation with conventional HE staining. Bordetella bronchiseptica organisms were first detected clinging to the cilia of the nasopharynx and lower respiratory tract as early as four days and persisted as late as 28 days after aerosolisation, despite the fact that at this time the inflammatory changes in the respiratory mucosa had reduced in intensity and the respiratory tract appeared to be returning to normal. It was quite evident, however, that many more bacteria were found with HE and Gram-Twort staining methods than with the SEM. This was considered to be due to the fact that many bacteria were entrapped at the base of the cilia and thus not available for inspection with the SEM. The fact that Bordetella bronchiseptica has a particular predilection for cilia has been clearly shown by

Bemis and Kennedy (1981) who, in an investigation of the effects of the organism on ciliated cells in tracheal explant cultures, established that, while attachment to cilia occurred as early as five minutes and complete ciliostasis developed by three hours after inoculation, no attachment was observed to the microvilli in the same cell and there was little or no adherence to neighbouring non-ciliated cells. A similar observation was made by Yokomizo and Shimizu (1979) in their study of the adherence of Bordetella bronchiseptica to cultured swine nasal epithelial cells.

There were a number of striking differences in the AB-PAS staining compared to the control animals as well as the normal dogs described in Chapter 3. The overall number of goblet cells was reduced in all the Bordetella-infected dogs, particularly in the tracheobronchial tree and the amount of mucosubstances present in individual cells was often markedly reduced (compare Appendices I and II). There was, however, no obvious difference in the prevalence of a particular type of mucosubstance between the two groups.

In addition, there was also a marked reduction in the number of stained glandular units throughout the respiratory tract (with the exception of the ethmoidal concha) compared to the normal dogs. This was also particularly noticeable in the glands of the tracheobronchial tree which, while recognisable, were for the most part unstained. It was only on days 20 and 28 after aerosolisation that the number of goblet cells seemed to be increasing in number although, even at this stage of the infection, many of the identifiable goblet cells had a sparse complement of mucosubstance granules in their cytoplasm.

The SEM studies highlighted the striking surface changes attributed to Bordetella bronchiseptica operating in the canine respiratory tract, particularly with regard to ciliary disorganisation and necrosis.

Moreover, SEM studies emphasised the remarkable reparative properties of the canine respiratory mucosa to bacterial attack. As already mentioned, there was a relatively poor correlation between the numbers of bacteria recognisable with conventional staining and with the SEM and that this could be attributable to the fact that the organisms were deeply entrapped in the cilia and thus not readily visualisable with the SEM. Nevertheless, the SEM studies gave a much clearer indication of the damage to the respiratory surfaces than could be appreciated with light microscopy alone.

In the present study, there was in general, however, a good correlation between the overall changes observed with light microscopy compared to those detected with the SEM. The areas of squamous metaplasia so noticeable with the light microscope were readily identified with the SEM. Likewise, the presence of alveolar macrophages surrounding necrosed bronchioles was confirmed with the SEM, thus indicating that if these cells are present in any number the SEM will detect them.

The major advantage in the use of the SEM was the clarity of the effects of Bordetella bronchiseptica on the respiratory cilia. The early disorganisation with the attendant appearance of abnormal thick or slender cilia followed by frank ciliary necrosis appeared to result in ciliated cells characterised by short stubby cilia, as distinct to the appearance of non-ciliated microvillous cells. The former were considered to be regenerating ciliated cells and perhaps a TEM study, in parallel, may well have helped to substantiate this premise.

The reappearance of discharging goblet cells in the later stages of the infection (20 - 28 days) confirmed the results of the AB-PAS staining method and suggested that the respiratory mucosa was returning to a normal state.

CHAPTER 8

SUMMARY AND CONCLUSIONS

The present study was aimed at answering two important questions. Firstly, it was apparent from the literature that the detailed knowledge of the surface characteristics of the mammalian respiratory tract were largely limited to the tracheobronchial tree; no one mammal had been studied from the nasal vestibule to the alveolar wall with SEM technology. The initial question to be answered was, therefore; What is the basic surface morphology of the total length of the mammalian respiratory tract?

Secondly, the fact that the dog suffers from a number of well recognised respiratory infections indicated that a thorough study of the normal canine respiratory tract was essential in order to allow a proper assessment of respiratory pathology. By choosing the dog as a mammalian model, both of these questions were tackled. Having established the important surface features of the normal canine respiratory tract, a third question was posed , namely - How do the respiratory surfaces of the dog respond to challenge with Bordetella bronchiseptica? The latter organism was chosen for study not only because it is one of the most common canine respiratory pathogens but also because it is large enough to be directly visualisable with the SEM.

The present study has emphasised the importance of SEM in the study of the surface topographical features of the canine respiratory tract. By virtue of the relatively large areas available for inspection, compared to conventional light microscopy and TEM, a much clearer picture of the overall topography of the respiratory tract was obtained.

The main objectives of the work, i.e., the establishment of the normal topographical features of the canine respiratory tract with the SEM together with an analysis of the reaction of the tract to challenge with Bordetella bronchiseptica were achieved. This is the first time that such detailed morphologic studies have been employed

in the dog, despite the large number of reports in other mammals. Furthermore, notwithstanding the importance of Bordetella bronchiseptica in the pathogenesis of respiratory disease in dogs and other mammals, this was the first detailed SEM study of experimental bordetellosis.

The SEM is now established as an important investigative tool in the study, not only of the normal respiratory tract of the dog, but also of the effects of the various infectious agents which can inhabit and damage the tract.

In the review of the literature it was clear that numerous structural studies had previously been carried out in many different mammalian species, but most investigators had confined their attention to a segment of the tract or even to a particular cell type; very few workers had studied the whole tract. It emerged, too, that the dog had received comparatively little interest compared to other mammals such as the mouse and rat and that SEM studies were particularly sparse. With this in view, the object of Chapter 3 was to carry out a SEM study of the whole of the canine respiratory tract from the nasal fossa to the alveolar membrane. Moreover, as little information was available concerning the distribution and nature of the mucosecretory units in the canine respiratory tract it was considered worthwhile to carry out, in conjunction with SEM studies, a histochemical analysis of the epithelial mucus cells (goblet cells) and submucosal glandular units at all levels of the tract. For this purpose the PAS and AB-PAS staining methods were employed.

For both SEM and histochemical studies samples were taken from a number of sites throughout the respiratory tract, viz., ventral concha, nasal septum, ethmoidal concha, nasopharynx, epiglottis, ventral larynx, trachea, extrapulmonary bronchus and lungs. In the latter situation, the caudal lobes were fixed with NBF for conventional light microscopy or with Karnovsky's fixative for SEM studies, by

perfusion of the airways. In this manner it was possible to carry out a thorough histological, histochemical and SEM study of the whole of the canine respiratory tract.

Histological examination confirmed that, in common with other mammals, the canine respiratory tract is lined almost entirely with pseudostratified columnar ciliated epithelium. Stratified squamous non-keratinising epithelium was confined to the nasal vestibule, the rostral portion of the nasal septum, caudal region of the nasopharynx, epiglottis and, in most dogs, the ventral laryngeal site. In addition to the olfactory epithelium covering the ethmoidal concha and caudal region of the nasal septum, a further type of epithelium which appeared to be an intermediate type located between respiratory and squamous epithelium was found in the rostral portions of the ventral concha and nasal septum, but not, surprisingly, in the nasopharynx or ventral larynx. This type of stratified epithelium was strikingly similar to the transitional epithelium lining the urinary tract.

The present histochemical study of the canine respiratory tract emphasised the abundance of mucus-secreting goblet cells throughout the conducting portions of the tract and established the preponderance of acid or mixed mucosubstances not only in the goblet cells but also in the submucosal glands. In all the dogs studied, however, the greatest concentration of goblet cells was to be found in the extrapulmonary bronchus and it was only in this site, together with the larger bronchi, that a large number of cells producing neutral mucosubstances could usually be found. At the level of the small bronchioles, however, few goblet cells (containing mainly acid or mixed mucosubstances) were observed.

With the SEM, the degree of ciliation throughout the respiratory mucosa was more or less complete. Very few non-ciliated cells were found in the ventral concha, nasal septum, trachea,

extrapulmonary bronchus, large and small bronchi and larger bronchioles. It was only at the level of the smaller bronchioles that ciliated cells became less common. No ciliated cells were found in the respiratory bronchioles.

Neither brush cells nor neuroepithelial bodies (described in other mammals by Andrews (1974) and Cutz et al (1978) respectively) were identified in the present study.

A feature of note in the present SEM study was the presence of a sparsely ciliated "cobblestone" type of epithelium in the rostral regions of the ventral concha and nasal septum which apparently did not correspond to the transitional epithelium noticed on light microscopy but presumably represented an intervening junctional zone between the true ciliated epithelium and the squamous epithelium of the nasal vestibule. A further feature in this area, so far not recorded in the literature, was the presence of scattered flat non-ciliated cells with scanty surface microprocesses and possessing a centrally-disposed secretory pore, presumably exuding a form of mucus onto the mucosal surface. The transitional epithelium found by the light microscope was represented with the SEM by relatively flat cells with surface microvillous projections or microplicae.

In only seven of the 18 dogs used in this study was the nasopharynx clothed with ciliated respiratory epithelium. In four other animals, an irregular "cobblestone" epithelium similar to that found in the ventral concha and nasal septum was observed. In the remaining animals the nasopharyngeal epithelium consisted of a corrugated epithelium whose cells were characterised by many microvillous projections or microplicae. This type of epithelium presumably represented the stratified non-keratinising squamous epithelium noted on light microscopy.

In the ventral larynx, the latter type of epithelium was recorded in seven of the 18 dogs while only five animals showed the characteristic ciliated respiratory epithelium. The remaining dogs had a "cobblestone" type of epithelium located between the ciliated epithelium of the laryngo-tracheal junction and the non-ciliated squamous epithelium of the cranial portion of the specimen.

At the level of the alveolar membrane two points are of interest as far as the SEM is concerned. First, the absence of identifiable alveolar macrophages which were presumably washed off the alveolar membrane in the preparative processes and, second, the paucity of alveolar pores of Kohn. In order to investigate these two features further, separate experiments were mounted in Chapters 5 and 6.

Chapter 4 was devoted to the investigation, histologically as well as macroscopically of the presence of respiratory (i.e. ciliated) epithelium at various sites of the canine larynx. This study was stimulated by the variation shown in Chapter 3 with regard to the presence (or absence) of respiratory epithelium in the ventral larynx (four dogs with conventional light microscopy compared to 12 animals with SEM). In this study, the larynges of 11 dogs were immersed in a solution of Phloxine B and Alcian blue (four dogs) or in Alcian blue alone (seven dogs) and the proportion of respiratory mucosa estimated according to the method of Watt et al (1975). Samples from five sites in each larynx, viz., the cranial epiglottis, caudal epiglottis, ventral larynx, dorsal larynx and caudal larynx, were then subjected to histological examination. This investigation emphasised the overlap of non-respiratory and respiratory epithelium which occurred at the level of the ventral larynx and accounted for the disparity of results achieved with conventional light microscopy and SEM.

In Chapter 5 an attempt was made to discover why alveolar

macrophages could not be identified with SEM in the dog and why alveolar pores of Kohn were rare compared with other mammals. To this end, it was considered worthwhile to carry out a study of the distal airways of the dog where the lungs had been fixed by vascular perfusion, and to compare the results with those recorded in Chapter 3. Furthermore, as the distal airways had previously been particularly poorly studied in the dog, it was thought worthwhile to carry out a TEM study of this region. For this study the lungs of four dogs were fixed by vascular perfusion and, as with the airway perfusion method, alveolar macrophages were not identifiable with the SEM, thus excluding the suggestion that when the lungs are fixed by the airways, the alveolar macrophages are in some way washed off from the alveolar membrane. Likewise, contrary to the findings of Parra et al (1978), pores of Kohn were difficult to visualise.

On the other hand, TEM studies which identified clearly the structural details of ciliated cells, Clara cells and alveolar Type I and Type II pneumonocytes, clearly showed the presence of alveolar macrophages clinging to the surface of the alveolar membrane but did not help to elucidate the presence or absence of alveolar pores of Kohn.

A major feature of the TEM studies was the establishment, for the first time in the dog, of the major structural features of the Clara cell. This was found to be a low columnar or cuboidal cell with a projecting apex containing a few microvilli. The major feature of the cell, however, was the absence of secretory granules and the abundant cytoplasmic glycogen granules, the latter isolating the nucleus in a centrally or apically disposed cytoplasmic island. That the Clara cell of the dog differs substantially from that of other mammals such as man, mouse and rat (Plopper, 1983) has now been clearly established.

The absence of alveolar macrophages with SEM when both airway and vascular perfusion methods of fixation were used was puzzling. Other workers had clearly identified these cells in other mammalian species (Sorokin et al, 1984). With this in mind, the cells washed from the lungs of two dogs were examined by conventional light microscopy and with both SEM and TEM. This was done in order to establish the presence of alveolar macrophages although their number was not quantified. It was clearly shown by the above methods that alveolar macrophages comprised the major cell type in these lung washings and it was concluded that their absence in the SEM studies of Chapters 3 and 5 was related in some way to the preparative methods. It was interesting to note that with the TEM on the other hand alveolar macrophages were not difficult to identify despite the relatively smaller areas of alveolar membrane available for inspection.

The final chapter of the investigative work (Chapter 7) was devoted to a sequential histological, histochemical and SEM study of the respiratory tract of dogs exposed to an aerosol stream of Bordetella bronchiseptica. The primary aim of this part of the work was to map the various surface changes taking place from four to 28 days after aerosolisation. Although, clinically the resultant infection was mild, severe surface ciliary disorganisation and necrosis was found throughout the tract. This was noticed as early as four days, peaked around eight to ten days and was still evident, to a lesser extent, at 20 days. SEM studies, however, clearly demonstrated the remarkable reparative properties of the ciliated epithelium to bacterial challenge, as by 28 days the respiratory tract had returned to almost normal appearance. In general, fewer organisms were found with the SEM than was predicted following use of the Gram-Twort staining method and it was concluded that the reason for this was that many of the bacteria were entrapped at the base of the cilia and, therefore, perhaps not

easily visualisable with the SEM.

Necrotising bronchiolitis was a feature of the disease and, unlike the normal dogs, alveolar macrophages were easily identifiable with the SEM clinging to the alveolar membrane. Of interest too, was the reduction in the number of goblet cells throughout the tract and it was only in the latter stages of the infection that these cells were returning to normal numbers and content of mucosubstances. There was also a corresponding decrease in the number of stained glandular units.

In conclusion, the present work has shown the usefulness of SEM, not only in the study of the normal surface features of the canine respiratory tract, but also in the investigation of respiratory infections. The employment of this technology to other important canine respiratory diseases such as distemper virus and adenovirus infections will undoubtedly shed more light on the surface changes operating in the respiratory tract in these important diseases.

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APPENDICES

APPENDIX I : CHAPTER 3

Distribution and semi-quantitative assessment of mucosubstances in the respiratory tract of the dog as detected by the Alcian blue-PAS (pH 2.5) method.

EXPLANATION OF TERMS

Acid	=	Cells stained blue
Neutral	=	Cells stained red
Mixed	=	Cells stained blue and red/or purple
1+	=	Very few cells
2+	=	Few cells
3+	=	Many cells
4+	=	Very many cells
ND	=	Not done, or not enough respiratory epithelium available for assessment
-	=	Negative
NG	=	No glands present
EP	=	Extrapulmonary (bronchus)
L	=	Large (bronchus, bronchiole)
S	=	Small (bronchus)
T.R.	=	Terminal and respiratory (bronchiole)

Dog No.
WA 1

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	-	1+	NG	NG	NG
Septum	ND	ND	ND	ND	ND	ND
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	3+	-	-	3+	2+	4+
Epiglottis	-	-	-	3+	-	2+
Ventral Larynx	3+	1+	1+	3+	2+	3+
Trachea	3+	1+	3+	3+	1+	3+
E.P. Bronchus	3+	2+	4+	1+	-	1+
L. Bronchus	3+	2+	4+	1+	-	-
S. Bronchus	2+	2+	3+	-	-	-
L. Bronchiole	1+	1+	1+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 2

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	2+	-	-	2+
Septum	2+	-	2+	-	-	2+
Ethmoidal Concha	-	-	-	1+	1+	2+
Nasopharynx	1+	-	2+	1+	2+	4+
Epiglottis	-	-	-	1+	2+	3+
Ventral Larynx	ND	ND	ND	ND	ND	ND
Trachea	2+	1+	3+	-	-	1+
E.P. Bronchus	4+	1+	3+	2+	1+	1+
L. Bronchus	3+	-	1+	-	-	-
S. Bronchus	3+	-	1+	-	-	-
L. Bronchiole	1+	-	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 3

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	-	1+	-	3+	1+
Septum	ND	ND	ND	ND	ND	ND
Ethmoidal Concha	-	-	-	-	3+	-
Nasopharynx	ND	ND	ND	1+	1+	4+
Epiglottis	-	-	-	2+	1+	3+
Ventral Larynx	ND	ND	ND	ND	ND	ND
Trachea	2+	2+	4+	-	1+	-
E.P. Bronchus	2+	2+	4+	2+	2+	2+
L. Bronchus	3+	2+	3+	-	2+	-
S. Bronchus	3+	1+	3+	-	-	-
L. Bronchiole	2+	-	1+	NG	NG	NG
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 4

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	2+	-	3+	3+	2+	2+
Septum	ND	ND	ND	ND	ND	ND
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	ND	ND	ND	2+	1+	3+
Epiglottis	ND	ND	ND	ND	ND	ND
Ventral Larynx	ND	ND	ND	3+	1+	2+
Trachea	3+	1+	3+	3+	1+	3+
E.P. Bronchus	4+	1+	3+	-	-	-
L. Bronchus	3+	1+	4+	-	1+	-
S. Bronchus	3+	-	1+	-	-	-
L. Bronchiole	2+	-	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.

WA 5

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	3+	-	2+	3+
Septum	2+	-	-	-	-	2+
Ethmoidal Concha	ND	ND	ND	ND	ND	ND
Nasopharynx	2+	-	1+	3+	1+	4+
Epiglottis	-	-	-	3+	1+	4+
Ventral Larynx	ND	ND	ND	3+	1+	3+
Trachea	3+	1+	3+	4+	1+	3+
E.P. Bronchus	3+	-	3+	2+	-	-
L. Bronchus	3+	1+	2+	-	-	-
S. Bronchus	3+	1+	2+	-	-	-
L. Bronchiole	2+	1+	1+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 6

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	-	2+	-	2+	3+
Septum	ND	ND	ND	1+	-	-
Ethmoidal Concha	-	-	-	4+	-	-
Nasopharynx	ND	ND	ND	4+	3+	1+
Epiglottis	-	-	-	2+	2+	3+
Ventral Larynx	ND	ND	ND	2+	2+	2+
Trachea	3+	1+	2+	2+	2+	2+
E.P. Bronchus	4+	1+	3+	-	-	-
L. Bronchus	4+	1+	4+	1+	1+	1+
S. Bronchus	3+	1+	2+	-	-	-
L. Bronchiole	3+	1+	3+	NG	NG	NG
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 7

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	2+	-	3+	1+
Septum	2+	-	-	3+	1+	3+
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	3+	1+	-	4+	2+	4+
Epiglottis	-	-	-	3+	1+	2+
Ventral Larynx	ND	ND	ND	3+	1+	2+
Trachea	3+	2+	3+	3+	1+	3+
E.P. Bronchus	3+	1+	3+	3+	1+	2+
L. Bronchus	2+	1+	3+	1+	2+	1+
S. Bronchus	2+	2+	3+	1+	1+	1+
L. Bronchiole	3+	1+	3+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 8

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	3+	-	3+	-
Septum	3+	1+	1+	-	2+	3+
Ethmoidal Concha	-	-	-	4+	-	-
Nasopharynx	2+	2+	3+	4+	1+	4+
Epiglottis	-	-	-	3+	-	-
Ventral Larynx	ND	ND	ND	3+	2+	-
Trachea	2+	2+	3+	3+	3+	-
E.P. Bronchus	1+	4+	2+	-	2+	3+
L. Bronchus	2+	1+	4+	1+	3+	1+
S. Bronchus	3+	-	3+	1+	1+	1+
L. Bronchiole	3+	1+	3+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

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(15)

Dog No.
WA 9

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	2+	1+	3+	2+
Septum	ND	ND	ND	-	3+	3+
Ethmoidal Concha	ND	ND	ND	ND	ND	ND
Nasopharynx	ND	ND	ND	2+	2+	4+
Epiglottis	-	-	-	3+	-	-
Ventral Larynx	ND	ND	ND	3+	2+	3+
Trachea	3+	-	2+	3+	-	-
E.P. Bronchus	3+	1+	1+	1+	1+	-
L. Bronchus	3+	1+	3+	1+	2+	1+
S. Bronchus	3+	1+	3+	-	1+	1+
L. Bronchiole	3+	-	1+	1+	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 10

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	3+	2+	2+	1+
Septum	3+	1+	3+	2+	3+	1+
Ethmoidal Concha	-	-	-	3+	2+	-
Nasopharynx	3+	-	-	4+	3+	4+
Epiglottis	-	-	-	2+	-	-
Ventral Larynx	ND	ND	ND	3+	2+	3+
Trachea	3+	1+	3+	1+	3+	3+
E.P. Bronchus	3+	3+	3+	2+	2+	2+
L. Bronchus	2+	3+	2+	-	1+	-
S. Bronchus	2+	3+	2+	-	1+	-
L. Bronchiole	1+	2+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 11

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	3+	-	3+	1+
Septum	3+	1+	3+	1+	1+	-
Ethmoidal Concha	-	-	-	4+	-	-
Nasopharynx	ND	ND	ND	3+	3+	4+
Epiglottis	-	-	-	3+	1+	3+
Ventral Larynx	1+	2+	-	3+	3+	4+
Trachea	2+	1+	3+	1+	2+	3+
E.P. Bronchus	2+	3+	3+	2+	1+	2+
L. Bronchus	1+	3+	2+	-	1+	-
S. Bronchus	1+	3+	1+	-	1+	-
L. Bronchiole	-	3+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 12

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	3+	-	3+	3+
Septum	ND	ND	ND	ND	ND	ND
Ethmoidal Concha	-	-	-	4+	-	-
Nasopharynx	2+	-	-	3+	3+	4+
Epiglottis	-	-	-	3+	1+	2+
Ventral Larynx	2+	1+	2+	3+	2+	-
Trachea	3+	2+	4+	3+	1+	3+
E.P. Bronchus	3+	3+	4+	3+	1+	3+
L. Bronchus	2+	3+	2+	-	-	-
S. Bronchus	2+	3+	-	-	-	-
L. Bronchiole	1+	1+	1+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 13

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	2+	3+	3+	-	3+	-
Septum	2+	1+	3+	-	3+	2+
Ethmoidal Concha	-	-	-	4+	2+	-
Nasopharynx	3+	1+	3+	3+	2+	4+
Epiglottis	-	-	-	3+	2+	3+
Ventral Larynx	ND	ND	ND	3+	3+	3+
Trachea	3+	1+	3+	3+	3+	2+
E.P. Bronchus	2+	1+	4+	-	2+	2+
L. Bronchus	3+	2+	3+	-	1+	-
S. Bronchus	2+	2+	3+	-	-	-
L. Bronchiole	2+	2+	2+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

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Dog No.
WA 14

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	3+	-	3+	-
Septum	3+	1+	3+	-	3+	-
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	2+	-	1+	1+	1+	4+
Epiglottis	-	-	-	1+	1+	3+
Ventral Larynx	ND	ND	ND	-	1+	-
Trachea	3+	1+	3+	1+	1+	3+
E.P. Bronchus	3+	1+	3+	-	1+	-
L. Bronchus	2+	1+	3+	-	-	-
S. Bronchus	2+	1+	3+	-	-	-
L. Bronchiole	3+	2+	3+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

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Dog No.
WA 15

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	3+	-	3+	-
Septum	3+	-	3+	-	3+	-
Ethmoidal Concha	-	-	-	3+	1+	3+
Nasopharynx	2+	-	2+	1+	-	3+
Epiglottis	-	-	-	2+	2+	3+
Ventral Larynx	ND	ND	ND	2+	2+	3+
Trachea	2+	2+	3+	1+	3+	3+
E.P. Bronchus	1+	3+	3+	-	2+	-
L. Bronchus	1+	3+	2+	1+	1+	-
S. Bronchus	2+	3+	2+	-	-	-
L. Bronchiole	1+	2+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

APPENDIX II : CHAPTER 7

EXPERIMENTAL Bordetella bronchiseptica INFECTION

Distribution and semi-quantitative assessment of mucosubstances in the respiratory tract of dogs experimentally exposed to an aerosol spray of Bordetella bronchiseptica.

FOR EXPLANATION OF TERMS :

See Appendix I.

Dog No.
WA 19

Four Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	1+	-	1+	-
Septum	2+	-	1+	-	1+	-
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	1+	-	-	1+	1+	3+
Epiglottis	-	-	-	2+	1+	2+
Ventral Larynx	1+	-	-	1+	3+	1+
Trachea	2+	1+	2+	1+	1+	2+
E.P. Bronchus	-	-	1+	1+	1+	1+
L. Bronchus	2+	2+	2+	-	1+	-
S. Bronchus	1+	-	1+	-	-	-
L. Bronchiole	1+	1+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 20

Four Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	2+	1+	3+	-	1+	-
Septum	1+	-	-	-	1+	-
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	1+	-	1+	4+	1+	2+
Epiglottis	-	-	-	1+	-	-
Ventral Larynx	ND	ND	ND	ND	ND	ND
Trachea	1+	1+	1+	-	-	1+
E.P. Bronchus	1+	1+	1+	1+	-	1+
L. Bronchus	1+	3+	1+	-	1+	-
S. Bronchus	1+	2+	2+	-	-	-
L. Bronchiole	1+	2+	1+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 21

Six Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	1+	-	-	-	1+	-
Septum	1+	-	1+	-	1+	-
Ethmoidal Concha	-	-	-	2+	-	-
Nasopharynx	1+	-	-	4+	-	-
Epiglottis	-	-	-	2+	-	-
Ventral Concha	ND	ND	ND	2+	-	-
Trachea	1+	-	-	-	-	-
E.P. Bronchus	1+	-	-	-	-	-
L. Bronchus	1+	-	1+	-	-	-
S. Bronchus	1+	-	-	-	-	-
L. Bronchiole	1+	-	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 22

Six Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	1+	-	-	-	-	-
Septum	1+	-	-	-	1+	-
Ethmoidal Concha	-	-	-	1+	-	-
Nasopharynx	ND	ND	ND	2+	-	-
Epiglottis	-	-	-	2+	-	1+
Ventral Larynx	1+	-	-	-	1+	1+
Trachea	1+	-	-	-	-	-
E.P. Bronchus	1+	-	1+	-	-	-
L. Bronchus	2+	-	-	-	-	-
S. Bronchus	1+	-	-	-	-	-
L. Bronchiole	1+	1+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 23

Eight Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	1+	1+	1+	-	1+	-
Septum	1+	-	1+	-	2+	-
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	ND	ND	ND	3+	2+	3+
Epiglottis	-	-	-	2+	-	2+
Ventral Larynx	1+	-	1+	2+	2+	2+
Trachea	1+	-	1+	-	1+	-
E.P. Bronchus	1+	-	-	-	-	-
L. Bronchus	1+	-	1+	-	-	-
S. Bronchus	1+	-	-	-	-	-
L. Bronchiole	1+	-	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 24

Eight Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	1+	-	-	-	1+	-
Septum	1+	-	-	-	1+	-
Ethmoidal Concha	-	-	-	2+	-	-
Nasopharynx	1+	-	-	2+	1+	2+
Epiglottis	-	-	-	1+	1+	-
Ventral Larynx	ND	ND	ND	-	-	-
Trachea	1+	-	-	-	-	-
E.P. Bronchus	2+	-	1+	-	-	-
L. Bronchus	2+	1+	2+	-	-	-
S. Bronchus	1+	-	1+	-	-	-
L. Bronchiole	1+	-	-	-	-	-
T,R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 38

Ten Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	2+	-	1+	-	1+	-
Septum	1+	-	-	1+	-	-
Ethmoidal Concha	-	-	-	3+	-	2+
Nasopharynx	-	-	1+	3+	4+	1+
Epiglottis	-	-	-	1+	-	1+
Ventral Larynx	-	-	-	1+	3+	1+
Trachea	1+	-	-	1+	1+	1+
E.P. Bronchus	1+	-	1+	1+	1+	1+
L. Bronchus	1+	1+	2+	-	1+	-
S. Bronchus	1+	1+	1+	-	-	-
L. Bronchiole	1+	1+	1+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 38a

Ten Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	1+	-	-	-	1+	-
Septum	1+	-	-	-	1+	-
Ethmoidal Concha	-	-	-	2+	-	-
Nasopharynx	ND	ND	ND	ND	ND	ND
Epiglottis	-	-	-	1+	-	2+
Ventral Larynx	1+	-	-	1+	-	1+
Trachea	1+	1+	-	-	-	-
E.P. Bronchus	1+	1+	1+	-	-	-
L. Bronchus	1+	1+	1+	-	-	-
S. Bronchus	1+	1+	-	-	-	-
L. Bronchiole	-	1+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 39

20 Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	2+	1+	3+	-	1+	-
Septum	1+	-	3+	-	1+	1+
Ethmoidal Concha	-	-	-	3+	1+	-
Nasopharynx	-	-	-	2+	2+	3+
Epiglottis	-	-	-	-	-	-
Ventral Larynx	3+	-	2+	1+	-	1+
Trachea	1+	-	1+	1+	-	1+
E.P. Bronchus	2+	1+	1+	1+	1+	1+
L. Bronchus	3+	1+	2+	-	1+	-
S. Bronchus	2+	1+	1+	-	1+	-
L. Bronchiole	2+	1+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 41

20 Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	2+	1+	1+	-	1+	-
Septum	1+	-	1+	-	1+	-
Ethmoidal Concha	-	-	-	3+	1+	-
Nasopharynx	1+	-	-	3+	1+	1+
Epiglottis	-	-	-	3+	1+	1+
Ventral Larynx	1+	-	-	3+	1+	2+
Trachea	1+	1+	1+	2+	1+	2+
E.P. Bronchus	1+	1+	1+	2+	1+	1+
L. Bronchus	2+	2+	2+	-	-	-
S. Bronchus	2+	1+	1+	-	-	-
L. Bronchiole	1+	1+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 42

28 Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	1+	1+	1+	-	1+	-
Septum	2+	1+	3+	-	1+	-
Ethmoidal Concha	-	-	-	3+	3+	1+
Nasopharynx	ND	ND	ND	3+	-	4+
Epiglottis	-	-	-	2+	-	-
Ventral Larynx	1+	-	1+	2+	1+	3+
Trachea	3+	1+	1+	1+	2+	-
E.P. Bronchus	3+	2+	2+	-	1+	-
L. Bronchus	3+	2+	2+	-	1+	-
S. Bronchus	3+	1+	2+	-	-	-
L. Bronchiole	1+	-	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 43

28 Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	1+	1+	3+	-	1+	-
Septum	1+	-	2+	-	1+	-
Ethmoidal Concha	-	-	-	ND	ND	ND
Nasopharynx	1+	-	-	2+	1+	2+
Epiglottis	-	-	-	2+	1+	1+
Ventral Larynx	1+	-	1+	4+	1+	2+
Trachea	2+	-	-	2+	1+	-
E.P. Bronchus	2+	1+	2+	1+	-	-
L. Bronchus	3+	2+	2+	-	1+	-
S. Bronchus	2+	1+	2+	-	-	-
L. Bronchiole	1+	1+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 25 (Control)

Four Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	4+	1+	2+	-	2+	-
Septum	1+	1+	1+	-	1+	-
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	1+	-	1+	4+	3+	4+
Epiglottis	-	-	-	2+	1+	3+
Ventral Larynx	ND	ND	ND	1+	3+	2+
Trachea	3+	3+	3+	1+	1+	2+
E.P. Bronchus	2+	3+	3+	1+	1+	1+
L. Bronchus	1+	1+	3+	-	1+	-
S. Bronchus	1+	1+	1+	-	-	-
L. Bronchiole	1+	1+	1+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 26 (Control)

Eight Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	3+	-	2+	-
Septum	1+	-	1+	-	2+	-
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	1+	-	2+	4+	3+	4+
Epiglottis	-	-	-	2+	1+	3+
Ventral Larynx	1+	-	1+	2+	-	2+
Trachea	2+	1+	3+	2+	1+	2+
E.P. Bronchus	3+	1+	3+	-	1+	1+
L. Bronchus	2+	3+	2+	-	1+	-
S. Bronchus	2+	1+	1+	-	-	-
L. Bronchiole	1+	1+	1+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 29 (Control)

Eight Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	4+	1+	1+	NG	NG	NG
Septum	2+	-	2+	-	3+	-
Ethmoidal Concha	ND	ND	ND	ND	ND	ND
Nasopharynx	3+	-	2+	3+	3+	4+
Epiglottis	-	-	-	NG	NG	NG
Ventral Larynx	2+	1+	1+	1+	3+	3+
Trachea	1+	3+	3+	1+	3+	1+
E.P. Bronchus	1+	1+	3+	-	1+	-
L. Bronchus	1+	2+	3+	-	1+	-
S. Bronchus	1+	1+	3+	-	1+	-
L. Bronchiole	1+	1+	1+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 30 (Control)

Ten Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	1+	1+	3+	1+	2+	-
Septum	1+	-	3+	-	2+	-
Ethmoidal Concha	-	-	-	-	1+	-
Nasopharynx	ND	ND	ND	3+	2+	3+
Epiglottis	-	-	-	-	1+	1+
Ventral Larynx	2+	-	1+	-	2+	1+
Trachea	1+	1+	3+	-	2+	1+
E.P. Bronchus	1+	3+	3+	-	1+	1+
L. Bronchus	1+	3+	3+	-	-	-
S. Bronchus	1+	1+	3+	-	-	-
L. Bronchiole	1+	-	2+	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

Dog No.
WA 34 (Control)

20 Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	3+	1+	2+	-	1+	-
Septum	2+	-	2+	-	2+	-
Ethmoidal Concha	-	-	-	3+	-	-
Nasopharynx	3+	-	1+	2+	3+	4+
Epiglottis	-	-	-	3+	1+	2+
Ventral Larynx	ND	ND	ND	1+	-	1+
Trachea	3+	-	2+	3+	1+	3+
E.P. Bronchus	2+	1+	2+	2+	1+	1+
L. Bronchus	1+	3+	1+	-	1+	-
S. Bronchus	1+	1+	-	-	-	-
L. Bronchiole	-	1+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG

(170)

Dog No.
WA 37 (Control)

20 Days Post Aerosolisation

	EPITHELIUM			GLANDS		
	Acid	Neutral	Mixed	Acid	Neutral	Mixed
Ventral Concha	2+	-	2+	-	1+	-
Septum	1+	-	2+	-	-	-
Ethmoidal Concha	-	-	-	2+	1+	-
Nasopharynx	ND	ND	ND	3+	2+	-
Epiglottis	-	-	-	1+	1+	-
Ventral Larynx	1+	1+	-	3+	2+	1+
Trachea	2+	-	2+	1+	-	-
E.P. Bronchus	2+	-	1+	1+	1+	1+
L. Bronchus	1+	3+	1+	-	1+	-
S. Bronchus	1+	2+	-	-	-	-
L. Bronchiole	-	1+	-	-	-	-
T.R. Bronchiole	-	-	-	NG	NG	NG