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**EXPERIMENTAL AIRWAY HYPERSENSITIVITY IN SHEEP:
A MODEL FOR ASTHMA**

**A thesis presented
in partial fulfilment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY IN VETERINARY PATHOLOGY
at Massey University**

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1990

ABSTRACT

This study aimed to establish an animal model for human bronchial asthma using locally bred Romney sheep. It was then planned to determine whether or not morphological and inflammatory factors in the ovine respiratory tract are associated with a predisposition to allergic bronchial hypersensitivity induced by inhaled *Ascaris suum* antigen.

The skin and airway responses to a commercial *A. suum* antigen were tested in adult Romney sheep from two local farms with and without previous exposure to pigs. Ninety percent of 101 adult sheep tested showed an immediate skin reaction, and about 70% of 43 adult sheep with positive skin reactions showed an immediate airway response, reflected as a significant increase in airway resistance and/or decreased dynamic lung compliance. Among these 43 sheep, 21 showed changes in both airway resistance and dynamic lung compliance (Group A); ten only in dynamic lung compliance (Group B) and 12 were non-responders (Group C). No significant changes were recorded when the same animals were given an aerosol of phosphate buffered saline. Although the sheep with previous exposure to pigs showed significantly greater skin reactions than those without exposure to pigs, they showed no significant differences in airway response to antigen inhalation. In addition, there was no correlation between the degree of skin reaction and the magnitude of bronchoconstriction.

Since no information was available on the respiratory tract-associated lymphoid tissue and cells in healthy sheep, study of this tissue and its associated epithelium was a prerequisite for studies of the morphological and inflammatory mechanisms involved in the development of allergic airway hypersensitivity. The ovine respiratory tract has five forms of lymphoid tissue; intra-luminal, intraepithelial, scattered forms, and dense and nodular aggregations; the dense and nodular aggregations being confined to the pharyngeal tonsil and bronchioles. Morphologically well-developed lymphoepithelium (M cells) is present only in the pharyngeal tonsil region, and absent in the lower respiratory tract. The M cell of the ovine pharyngeal tonsil is ultrastructurally and functionally similar to that in other mucosal tissues of this and other species, but its development and maturation takes place earlier than the bronchus-associated lymphoid tissue.

Mast cells in the lower respiratory tract of normal sheep are morphologically heterogeneous, and both formalin-sensitive and formalin-resistant types can be identified. The morphological and histochemical features of formalin-sensitive mast cells are similar to those from the human respiratory tract in several respects which enhances the use of the sheep model in the study of human allergic respiratory disease.

A morphometric comparison of airway structure and inflammatory components was conducted between the three groups of sheep with varying airway hypersensitivity. The epithelium of the small airways was significantly thinner and contained fewer goblet cells in the hypersensitive sheep (Groups A and B) than in non-reacting sheep (Group C). Mast cells from the hypersensitive sheep had a significantly greater volume density of secretory granules than those from non-reacting sheep. However, no morphological difference was found in the epithelial integrity of airways between hypersensitive and non-reacting sheep, and the permeability of tracheobronchial epithelium to horseradish peroxidase was of the same order in all groups. Similarly, the airway wall was not significantly thicker in hypersensitive sheep than in non-reacting sheep, and the shortening of smooth muscle required to cause complete airway closure was similar. The numerical density of mast cells, eosinophils, neutrophils and lymphocytes in the airways and lung was not significantly different between the groups.

These observations indicate that the *Ascaris*-induced airway response seen in Romney sheep is similar in several respects to that seen in human asthmatics and these sheep can therefore be used as an animal model to study human asthma. The current findings suggest that the presence of relatively low goblet cell density, thin epithelium, and high volume density of mast cell secretory granules in the small airways and lung may be important inherent factors responsible for the development of airway hypersensitivity in these sheep. It is concluded that most of the other morphological features observed in asthmatics and animal models are likely to be the result of allergic airway reactions rather than a fundamental difference between potentially allergic and non-allergic subjects.

STATEMENT

This is to certify that the work on which this thesis is based was carried out by the undersigned, and has not been accepted in whole or in part for any other degree or diploma. Assistance received is specifically recorded in the Acknowledgements section bound with this thesis.



Wangxue CHEN

(19 September 1990)

ACKNOWLEDGEMENTS

I am grateful to Professor B.W. Manktelow, Head of the Department of Veterinary Pathology and Public Health, Massey University, for providing me with the opportunity and facilities to undertake this study. I would particularly like to thank my chief supervisor Associate Professor M.R. Alley and supervisor Professor B.W. Manktelow for their continuous encouragement, valuable suggestions, constructive criticism and unforgettable friendship during all phases of this work.

I wish to express my special gratitude to Dr. R.J. Pack for his advice and guidance in the studies of respiratory physiology in sheep, Dr. D.H. Carr for his expertise and participation in the bronchial provocation tests, and to Professor R.E. Munford for his advice on statistical analysis. My thanks are also due to Associate Professors K.M. Moriarty, L.J. Holloway and W.A.G. Charleston, Professor C.R. Wilks, and Dr. R.A. Allardyce for advice in their specialised areas.

This study could not have been fully undertaken without the technical assistance of a number of people whose help I gratefully acknowledge. Skilled assistance was provided by Mrs. P.M. Slack and Mrs. P.M. Davey in preparation of histological materials and processing of electron microscopy specimens; Mr. F.K. Sharpe in sheep feeding and handling; Mr. D. Hopcroft in preparation of tissues for scanning electron microscopy; Mrs. E. Davies in haematological studies, Mr. R. Bennett and Mr. T.G. Law in printing some of the photographs; the Graphic Design Unit in drawing the diagrams in Chapters 4-7; Mrs. D.J.E. Anthony in assistance in the measurement of respiratory physiology and in drawing the diagrams in Chapter 8, Mr. P.N. Wildbore in administrative assistance, and Mrs. A.A. Scott and Mrs. S. Crawford in secretarial help in preparation of the manuscripts for publication.

I also would like to thank my colleagues in the Department of Veterinary Science, Zhejiang Agricultural University, the People's Republic of China, for supporting the extension of my leave to undertake this study.

Finally, I would like to express thanks to my friends and family for their understanding and forbearance, but most of all I would like to thank my grandparents, my wife and my son for their love, support and willingness to share in all aspects of my study.

Financially, this work was jointly supported by a grant from the Medical Research Council of New Zealand and a grant from the Palmerston North Medical Research Foundation. My personal support during this study was provided by a Postgraduate Scholarship of the University Grants Committee of New Zealand.

LIST OF PUBLICATIONS

Some of the work presented in this thesis has already been published or is to be published in the following journals:

Chen W, Alley MR & Manktelow BW (1989). Respiratory tract-associated lymphoid tissue in conventionally raised sheep. J Comp Pathol 101:327-340.

Chen W, Carr DH, Alley MR & Pack RJ (1989). The allergic hypersensitivity of New Zealand sheep to *Ascaris suum* antigen. Proceedings of 16th Annual Meeting of Physiology Society of New Zealand 9:37.

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Chen W, Alley MR, Allardyce RA & Keenan J (1990). Experimental *Ascaris*-induced airway hypersensitivity in sheep: no serological evidence of association with *Toxocara canis* infection. N Z Vet J 38:80.

Chen W, Alley MR, Manktelow BW, Hopcroft D & Bennett R (1990). The potential role of the ovine pharyngeal tonsil in respiratory tract immunity: a scanning and transmission electron microscopic study of its epithelium. J Comp Pathol (in press).

Chen W, Alley MR, Manktelow BW & Slack P (1990). Mast cells in the bovine lower respiratory tract: morphology, density and distribution. Br Vet J 146:(in press).

Chen W, Alley MR, Manktelow BW & Davey P (1990). Mast cells in the ovine lower respiratory tract: heterogeneity, morphology and density. Int Arch Allergy Appl Immunol (in press).

Chen W, Alley MR, Manktelow BW & Slack P (1990). Perinatal development of lymphoid tissue and its associated epithelium in the ovine pharyngeal tonsil: a morphological study. N Z Vet J (submitted).

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ABBREVIATIONS*

AB/PAS	alcian blue/periodic acid-Schiff
Ae	external area
Ae _r	external area in "relaxed" state
Ai	internal area
Ai _r	internal area in "relaxed" state
AM	alveolar macrophages
BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
BSM	bronchial smooth muscle
C3a, C4a and C5a	complement C3a, C4a and C5a
Cdyn	dynamic lung compliance
CTMC	connective tissue mast cells
DAB	3,3 diaminobenzidine tetrahydrochloride
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EPO	eosinophil peroxidase
f	respiratory frequency
FA	10% neutral buffered formalin
FAE	follicle-associated lymphoepithelium
GALT	gut-associated lymphoid tissue
HE	haematoxylin and eosin
HE/AB	haematoxylin and eosin/alcian blue
HRP	horseradish peroxidase
IELC	intraepithelial lymphoid cells
IFAA	isotonic formal-acetic-acid
Ig	immunoglobulin(s)
L	the length of outer layer of smooth muscle in airways
LAR	late airway response/reaction
LRT	lower respiratory tract
LT	leukotrienes
MBP	major basic protein
MC	mast cells
MMC	mucosal mast cells
NAHR	non-specific airway hyperresponsiveness
Nv	numerical density
PAF	platelet-activating factor

PAS	periodic acid-Schiff
PBS	phosphate buffered saline
Pe	external perimeter
PG	prostaglandins
Pi	internal perimeter
PMP	proportion of external perimeter of the airway occupied by muscle
PMS	the degree of muscle shortening
Ppl	intrapleural pressure
Pw	relative airway area
Raw	airway resistance
RNase	ribonucleic acidase
RTALT	respiratory tract-associated lymphoid tissue
SEM	scanning electron microscopy
SRS-A	slow-reacting substances of anaphylaxis
TEM	transmission electron microscopy
V	airflow rate
V _T	tidal volume
WA	wall area

* Abbreviations used in tables and figures are not included in this list.

INTRODUCTION

Asthma is a common respiratory disease of human beings characterised by an increased responsiveness of the trachea and bronchi to various stimuli and manifested by widespread narrowing of the airways which is totally or partially reversible either spontaneously or by appropriate treatment (Daniele, 1980; Borish, 1987; Dail, 1988; Magunssen and Nowak, 1989). Clinically, the disease is manifested by paroxysms of cough, dyspnoea and wheezing with excess sputum production (McFadden and Ingram, 1980a; Schellenberg, 1985). Physiologically, the disease is characterised by an increase of airway resistance, total lung capacity and residual volume, a decrease of lung specific conductance, airflow rates and forced expiratory volumes, pulmonary hyperinflation and an imbalance of ventilation and perfusion (McFadden and Ingram, 1980b; Tattersfield and McNicol, 1987). The pathological changes of asthma extend to all bronchi and bronchioles down to 1 mm in diameter, and feature plugging of the airway lumen with exudate, epithelial shedding, squamous metaplasia, increase in numbers of goblet cells, thickening of the mucosal basement membrane, enlargement of bronchial mucous glands and smooth muscle, vasodilatation and oedema of the airway wall, and infiltration of the mucosa and submucosa by inflammatory cells, particularly eosinophils (Spencer, 1977; Dunnill, 1982).

The prevalence of asthma varies considerably between countries (Tattersfield and McNicol, 1987). In developed countries, there is an upward trend in the mortality and hospital admission rates for childhood asthma (Mitchell, 1985; Jackson *et al.*, 1988). Its prevalence has been estimated to be of the order of 3-5% of the population in the United States, and 3-12% in Britain (Tattersfield and McNicol, 1987; Drazen *et al.*, 1987). In New Zealand, about 27% of children suffer from asthma at some time before age 9 (Jones and Sears, 1987), and the hospital admission rates and mortality in childhood asthma are higher than in most other countries (Jackson *et al.*, 1988; Sears, 1988; Mitchell *et al.*, 1990). In 1985, there were 11,038 admissions to hospital for asthma with a mean length of stay of 4.6 days, the cost of which was about 17.3 million New Zealand dollars (Mitchell, 1989).

It is difficult to properly classify the clinical types of asthma because of an incomplete understanding of its pathogenesis. The disease has been divided into two subtypes: extrinsic (allergic) asthma and intrinsic (non-allergic) asthma (Daniele, 1980; Dunnill, 1982). The allergic type is of the most common, accounting for 25-30% of all cases of asthma and probably contributing to another third (McFadden and Ingram, 1980a), and is believed to be mediated by type I hypersensitivity. This type of asthma is generally associated with a personal and/or family history of atopy, positive skin reaction, raised serum immunoglobulin E levels and positive bronchial provocation tests (McFadden and Ingram, 1980a). The disease begins at

any age, but about half of the cases develop before age 10. Most acute attacks of allergic asthma tend to be short-lived, following which the patients can clinically recover completely, although sometimes severe airway obstruction persists for days or weeks as status asthmaticus (McFadden and Ingram, 1980a).

The airway reaction which occurs in asthmatics following bronchial provocation may be one of three types: an isolated early asthmatic reaction, an early followed by a late asthmatic reaction, and an isolated late asthmatic reaction (Tattersfield and McNicol, 1987). The early asthmatic reaction usually develops within 10 minutes of provocation by inhalation, reaches its maximum within 30 minutes and is generally resolved within 1-3 hours. The late reaction usually starts after 3-4 hours, reaches its maximum over the next few hours and clears within 24 hours or more (O'Byrne *et al.*, 1987).

The pathogenesis of allergic asthma has yet to be fully clarified. Episodes of asthma can be evoked by many stimuli, such as infections, exercise, antigens, occupational stimuli, environmental causes, pharmacological stimuli and emotional stress (McFadden, 1984). Several theories have been proposed to explain the development of airway hypersensitivity and allergic asthma. These include the type I hypersensitive reaction, the β -adrenergic blockade theory, the inherent twitch of bronchial smooth muscle, and neurogenic mechanisms (Daniele, 1980; Tattersfield and McNicol, 1987). Recent studies have favoured the concept that asthma is a chronic inflammatory disease involving the interaction of many inflammatory cells (Hogg, 1982; Borish, 1987; Kay, 1987; Barnes, 1989).

Investigation of basic mechanisms in the pathogenesis of asthma has been hampered by difficulties in gaining direct access to human asthmatic airways because of ethical and safety reasons. Although the use of fibre-optic bronchoscope in asthmatics has widened the possibilities of studying asthma directly in volunteers (Flint *et al.*, 1985a,b), results from such studies have been usually compromised by a smoking history and low grade of other inflammatory diseases in the patients. Recent studies have also shown that there are great variations in the inflammatory cell counts between bronchial biopsy specimens (Azzawi *et al.*, 1990). Alternative animal models are, therefore, necessary to advance our understanding of the pathogenesis of this disease.

Several animal models, including rats, guinea pigs, dogs, monkeys, rabbits and sheep, have been used to study the pathogenesis of airway hypersensitivity and allergic asthma (Booth *et al.*, 1970; Patterson and Kelly, 1974; Hogg *et al.*, 1979; Wanner *et al.*, 1979; Kallos and Kallos, 1984; Hamel *et al.*, 1986; Murphy *et al.*, 1986; Eidelman *et al.*, 1988). Among these species, the sheep is considered to be one of the most satisfactory models (Wanner and

Abraham, 1982). Most sheep tested overseas have a natural skin reaction and an immediate airway response to *Ascaris suum* extract (Wanner *et al.*, 1979; Bosse *et al.*, 1987; Okayama *et al.*, 1989). The airway response in this species is similar in both physiological and pharmacological aspects to that seen in human asthmatics, and is more consistent than the antigen-induced bronchoconstriction in dogs and guinea pigs (Wanner and Abraham, 1982). In addition, some allergic sheep also exhibit late airway reaction (Abraham *et al.*, 1983). Both the early and late airway reactions in sheep have been demonstrated to be antigen-specific and mast cell mediator-dependent (Wanner *et al.*, 1979; Abraham *et al.*, 1983; Okayama *et al.*, 1989). Over the past decade, the sheep has been increasingly used as a model of allergic airway hypersensitivity (Wanner *et al.*, 1979; Kleeberger *et al.*, 1985; Bosse *et al.*, 1987; Okayama *et al.*, 1989). Investigations using this model have produced much useful information. However, almost all studies to date have focused on physiological and pharmacological aspects of the disease (Wanner and Abraham, 1982), and morphological and immunological studies on this model are relatively scant. Further information on these aspects will be useful for fully using this model and for a better understanding of the mechanism of the development of allergic airway hypersensitivity.

It is important to state here, that like most animals, the sheep is not a complete animal model of human asthma since sheep do not spontaneously show clinical symptoms of asthma. Also sheep do not exhibit pathological changes characteristic of asthma. However, the term "asthma model" will be used throughout this thesis since this term has already been used for the sheep model by several workers (Wanner and Abraham, 1982; Ahmed *et al.*, 1983).

In New Zealand, only very limited studies of airway hypersensitivity and asthma have been carried out using animals; the guinea pig being the main animal model available (Galland and Blackman, 1989).

Morphological studies of human asthma undertaken to date suffer from the disadvantage that most of the features described are likely to be associated with the end result of the disease process and it is not known which (if any) morphological features may predispose an individual to develop airway hypersensitivity. Recent studies by Hopp *et al.* (1990) have shown that enhanced airway reactivity usually precedes the development of asthma. It is therefore of interest to examine the possibility that certain morphological and cellular abnormalities may exist before the development of airway hypersensitivity.

The present study had two main aims. The first was to evaluate the suitability of locally bred Romney sheep as a model to study human asthma in New Zealand. Knowledge of the prevalence of natural responders in sheep in New Zealand would thus be available for future

workers in this country wishing to use the ovine model. The second aim was to use the sheep model established to determine whether or not morphological and inflammatory factors in the ovine respiratory tract could be associated with a predisposition to develop allergic airway hypersensitivity to inhaled *A. suum* antigen.

PART I
GENERAL REVIEW OF LITERATURE

CHAPTER 1

PATHOLOGY OF HUMAN ASTHMA

Knowledge of the pathology of asthma is derived largely from necropsy studies of patients dying of asthma, and from asthmatic patients dying from other causes (Dunnill *et al.*, 1969; Cutz *et al.*, 1978; James *et al.*, 1989; Holloway *et al.*, 1990), and more recently some information has become available from bronchial biopsies (Laitinen *et al.*, 1985; Beasley *et al.*, 1989; Jeffery *et al.*, 1989; Lozewicz *et al.*, 1990) and bronchoalveolar lavage fluid of patients with mild or stable asthma (Tomioka *et al.*, 1984; Flint, 1987; Kelly *et al.*, 1988, 1989; Beasley *et al.*, 1989). Thus, the pathological changes seen in most cases are usually complicated by other respiratory diseases and by therapeutic intervention, and there has been only limited opportunity to study the pulmonary pathology caused by the disease alone. In addition, little is known of the changes which are present in younger patients with mild asthma which remits at a later stage of life.

1.1 MACROSCOPIC LESIONS

Compared to many other respiratory diseases, the gross changes in asthma are characteristic and uniform. In patients dying in acute asthma the lung is over-distended and fails to collapse when the thoracic cavity is opened (Dunnill, 1982; Tattersfield and McNicol, 1987). Small foci of pulmonary collapse are found which are most pronounced along the anterior border of the upper lobes (Dunnill, 1971). On incision, many airways, especially those between 2-10 mm in diameter contain numerous grey glistening plugs (Spencer, 1977; Dunnill, 1982), and the walls of these airways are markedly thickened (Tattersfield and McNicol, 1987). Airway occlusion is one of the most striking features in asthma, although it does not appear in all cases reported (Dunnill, 1982; Sobonya, 1984; Tattersfield and McNicol, 1987). Despite the severe obstruction in airways, emphysema is rarely seen in asthmatics (Spencer, 1977).

1.2 MICROSCOPIC LESIONS

1.2.1 Changes in Airways

The most striking changes occur in the airways of 2-10 mm diameter (Spencer, 1977). In asymptomatic asthmatics however, the lesions are confined to airways with a diameter larger than 10 mm (Cutz *et al.*, 1978). Quantitative studies of airway dimensions indicate that the wall areas of both large and small airways are significantly greater in asthmatics than in controls (James *et al.*, 1989). This difference is due to a combined increase in the area of epithelium, bronchial smooth muscles and submucosa. Detailed calculations by James *et al.* (1989) concluded that the requirement of muscle shortening to cause airway narrowing was less in asthmatics than in non-asthmatics.

Occlusion of the airway lumen by exudate plugs is the most common feature seen in subjects with severe asthma. The occlusion occurs mainly in large bronchi, but may extend to terminal bronchioles (Daniele, 1980), or even to alveoli (Tattersfield and McNicol, 1987). The exudate is heterogeneous and consists of a mixture of mucous and serous components together with many shed epithelial cells, cilia, numerous eosinophils and other inflammatory cells as well as their granules (Cutz *et al.*, 1978; Dunnill, 1982). Albumin and immunoglobulins (mainly Ig G) have also been demonstrated in the plugs (Daniele, 1980). Some exudate may participate in the formation of two specialised structures frequently seen in the sputum from asthmatics; Charcot-Leyden crystals (Dolovich *et al.*, 1983) and Curschmann's spirals (Dunnill, 1971; Dail, 1988).

Changes in the bronchial mucosa vary with the severity and duration of the disease (Cutz *et al.*, 1978; Dunnill, 1982; Sobonya, 1984; Beasley *et al.*, 1989; Lozewicz *et al.*, 1990). In patients with severe asthma, the mucosa shows marked oedema and the lining epithelium in many areas becomes detached leaving behind a layer of basal cells (Dunnill, 1971; Cutz *et al.*, 1978). In the areas where the epithelium is still intact, the ciliated epithelium is usually replaced almost entirely by hyperplastic goblet cells and metaplastic squamous cells (Spencer, 1977). The remaining ciliated cells are often swollen and have a widened intercellular space (Laitinen *et al.*, 1985). Sloughed columnar cells may compact together to form clusters known as Creola bodies, which are commonly seen in the sputum from asthmatics (Dunnill, 1982).

Ultrastructural examination of bronchial biopsies from asthmatics shows that the ciliated cell is the cell type most often affected (Cutz *et al.*, 1978; Laitinen *et al.*, 1985). These cells show loss of cilia, vacuolation of their cytoplasm and formation of apical cytoplasmic protrusions and atypical cilia (Cutz *et al.*, 1978; Laitinen *et al.*, 1985). Some cilia are morphologically normal, but the alignment of central ciliary filaments is disturbed, varying from normal near-parallel alignment to an angle of about 90° from one another (Laitinen *et al.*, 1985), resulting in disturbances of coordinated stroke of cilia, which probably accounts for the defective mucociliary clearance seen in asthmatics. The significance of epithelial damage in mild asthma, however, has been questioned because this was not encountered in all studies of mild asthmatics (Lozewicz *et al.*, 1990) and was present in some control subjects (Jeffery *et al.*, 1989).

The thickening of mucosal basement membranes is probably the only morphological feature which is consistently present in the airways of asthmatics (Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Lopez-Vidriero and Reid, 1983; Sobonya, 1984; Beasley *et al.*, 1989; Jeffery *et al.*,

1989). The basement membrane becomes hyalinised and can reach more than four times normal thickness (Dunnill *et al.*, 1969). This thickening appears greater in the basement membrane of the lining epithelium than in that of the submucosal glands, and greatest in areas where epithelial damage and submucosal inflammation are prominent. The development of basement membrane thickening and its significance in asthmatics is not clear, although the demonstration of deposition of immunoglobulins including Ig E in the thickened areas by some investigators (Callera *et al.*, 1971; Gerber *et al.*, 1971) favours the hypothesis that immune complexes are involved in the formation of basement membrane thickening in airways as they are in membranous glomerulonephritis. However, other workers have either failed to confirm this (Cutz *et al.*, 1978) or consider that such Ig E staining is non-specific (McCarter and Vazquez, 1966).

Cutz *et al.* (1978) noted that the so-called "thickened basement membrane" was, in fact, a 5-10 μm thick layer of collagen. It consisted of bundles of collagen fibrils with some reticulin fibres located immediately beneath the real basement membrane which was ultrastructurally normal. This finding has been supported by a recent immunohistochemical study using a panel of monoclonal antibodies to interstitial components (Roche *et al.*, 1989). The complete absence of basement membrane components and the presence of abundant interstitial structural proteins (such as collagen III and V, and fibronectin) in the "thickened" areas indicate that the thickening is a result of fibroblast activation rather than bronchial epithelial dysfunction as suggested previously. The bronchial myofibroblasts are likely to be responsible for this characteristic subepithelial fibrosis (Brewster *et al.*, 1990).

Another characteristic change in the airways of asthmatics is mucus hypersecretion, which involves both the submucosal glands and epithelial secretory cells (Dunnill *et al.*, 1969; Dail, 1988; Aikawa *et al.*, 1990). The hyperplasia of goblet cells occurs even in distal bronchioles where goblet cells are not normally present (Dail, 1988; Aikawa *et al.*, 1990). Increase in the volume of submucosal glands is seen in almost all asthmatic patients, even in those in remission (Cutz *et al.*, 1978; Dunnill, 1982; Sobonya, 1984) and is the result of gland hypertrophy rather than hyperplasia (Sobonya, 1984). Although their density is closely related to the severity of the disease (Dunnill *et al.*, 1969; Salvato, 1980; Sobonya, 1984), asthmatics do not invariably have hypertrophy of bronchial submucosal glands or an excessive number of goblet cells (Lopez-Vidriero and Reid, 1983). Whether gland hypertrophy is a response to continued allergenic stimulation or chronic respiratory infection, or is merely the result of hypersecretion during asthmatic attacks is not clear. Cluroe *et al.* (1989) recently found that more than 70% of patients in which fatal asthma had been both clinically and morphologically confirmed had some degree of bronchial gland duct ectasia. The affected ducts were severely dilated and extended through the muscular wall of the bronchi. Sometimes there

was occlusion of the duct lumen with mucus and a periductal inflammation with predominantly eosinophil infiltration.

Bronchial secretions are also qualitatively changed in asthmatics. The mucus has a reduced affinity for acid glycoproteins and an increase in its reaction to neutral glycoproteins (Salvato, 1980).

Increase in bronchial smooth muscle (BSM) has been consistently demonstrated in patients dying in status asthmaticus (Dunnill *et al.*, 1969; Hossain, 1973; James *et al.*, 1989). When measured, it is about three times thicker in patients with status asthmaticus than in normal subjects (Dunnill *et al.*, 1969). However, whether the increased volume of BSM results from hypertrophy or hyperplasia still remains unclear. Early studies with muscle fibre counts in histological sections have revealed that this increase is mainly due to myofibre hyperplasia (Heard and Hossain, 1973; Hossain, 1973). Recent studies using unbiased 3-D morphometric technique have shown that the thickening of airway muscles in asthma is the result of cellular hypertrophy (Ebina *et al.*, 1990a). Since the increase in BSM is not present in chronic bronchitis, Dail (1988) suggested that this feature is helpful for differential diagnosis. However, this measurement should be used with caution in practice since BSM hyperplasia is not significant in some non-fatal asthmatics (Sobonya, 1984), and, for some unknown reasons, is much less prominent in female patients than in male patients (Hossain and Heard, 1970b; Hossain, 1973).

Besides hyperplasia, BSM in asthmatics also shows loss of vasoactive intestinal polypeptide (Ollerenshaw *et al.*, 1989). Although the result of this study is far from conclusive because a very small number of samples were used, it is possible that BSM in asthmatics may have some genetic and/or acquired defect in certain neurotransmitters.

Changes in the other submucosal components are neither consistent nor characteristic. Dilatation of capillaries with swelling of endothelial cells, accompanied by plasma exudation and inflammatory infiltration with eosinophils predominating is a notable feature (Salvato, 1980; Persson, 1988). The endothelial lining of most vessels in these areas has many gaps up to 1 μm in size, which are morphologically different from the fenestration seen in normal capillaries (Laitinen and Laitinen, 1987).

Infiltration of inflammatory cells into the airway lumen and walls is a prominent feature in asthma. The eosinophil is the most numerous cell type (Cutz *et al.*, 1978; Salvato, 1980; Beasley *et al.*, 1989), although some results disputing this have been published (Sobonya, 1984; Laitinen *et al.*, 1985). To better clarify the role of eosinophils in the pathogenesis of

asthma, several eosinophil mediators have been studied in lungs from asthmatics. Extracellular deposition of major basic protein and eosinophil cationic protein has been immunohistochemically demonstrated in mucus plugs, on damaged epithelial surfaces and in subepithelial areas (Filley *et al.*, 1982; Venge *et al.*, 1988; Holloway *et al.*, 1990). The staining intensity is believed to correlate well with the severity of the disease although further quantitative study is required.

Mast cell degranulation is a well established phenomenon in asthmatic patients, and is directly correlated with severity of symptoms (Salvato, 1980; Beasley *et al.*, 1989). Because of degranulation, mast cell counts are decreased in histological studies (Salvato, 1968,1980; Heard *et al.*, 1989), but normal in ultrastructural studies (Warton *et al.*, 1986a). Ultrastructural examination shows that intraepithelial mast cells contain more granules undergoing degranulation than those in the submucosa (Cutz *et al.*, 1978).

The number of neutrophils, plasma cells and lymphocytes reported in the lung of asthmatics is considerably variable, (Lopez-Vidriero and Reid, 1983; Tattersfield and McNicol, 1987), and this is probably due to the different stage of the disease and the variations between biopsy specimens. An increase of so-called "irregular" lymphocytes was found by Jeffery *et al.* (1989) to contribute most to the morphological alterations observed in their studies on the bronchial biopsies from patients with mild asthma.

1.2.2 Changes in Lung Parenchyma

Although the lung is grossly over-distended, destructive interstitial emphysema is present in only about 20% of cases of fatal asthma (Cluroe *et al.*, 1989). This emphysema is probably due to rupture of dilated bronchial gland ducts rather than to alveolar disruption. The alveoli supplied by the occluded small airways are not always collapsed as collateral ventilation keeps them aerated. Focal areas of collapse involving groups of secondary lobules may be seen (Dail, 1988), and occasionally, large areas of collapse are present (Dunnill, 1982). The alveolar parenchymal volume, mean linear intercept, and internal surface area of the lungs from patients with long-standing asthma are not quantitatively different from those in the lungs of controls (Sobonya, 1984).

1.3 CHANGES IN BRONCHOALVEOLAR LAVAGE (BAL) FLUID

The introduction of the fibre-optic bronchoscope and subsequent development of BAL has provided a new approach to the investigation of many respiratory disorders. This technique has been increasingly used to study patients with mild or stable asthma (Tomioka

et al., 1984; Flint, 1987; Kelly *et al.*, 1988,1989; Beasley *et al.*, 1989). The relationship between changes in the constituents of BAL fluid and the severity of the disease has been closely investigated. It is now generally accepted that the total cell number in lavage fluid from asthmatics does not necessarily change significantly, but the percentage of mast cells and epithelial cells and the amount of major basic protein increase significantly, in an inverse correlation with the severity of the disease (Tomioka *et al.*, 1984; Flint, 1987; Kelly *et al.*, 1988; Wardlaw *et al.*, 1988; Beasley *et al.*, 1989). However, data on the numbers of eosinophils and lymphocytes in BAL fluid in mild and stable asthma are less consistent. Although a significant increase in the proportion of eosinophils and lymphocytes was noted by Kelly *et al.* (1988,1989), the investigations of Tomioka *et al.* (1984) and Beasley *et al.* (1989) found this was not significant, and the proportion of lymphocytes was reduced in the study by Beasley *et al.* (1989).

Relatively little attention has been focused on the presence of lymphocyte subtypes in lungs of asthmatics, although a previous study on patients with an isolated acute airway response to allergen challenge showed that the T_4/T_8 lymphocyte ratio was decreased in BAL fluid (Gonzalez *et al.*, 1987b). Several recent studies have confirmed that almost all the lymphocytes in BAL fluid of asthmatics are T cells (Beasley *et al.*, 1989), and that the increase in lymphocyte percentage is confined to the T cell subgroup, especially the T_8 group. However, Kelly *et al.* (1989) have recently found that the T_4/T_8 ratio is similar in asthmatics and controls.

The demonstration of eosinophil, mast cell, neutrophil and macrophage granule markers in lavage fluid suggests the active participation of these cells in the development of asthma (Borish, 1987; Wardlaw *et al.*, 1988). Tomioka *et al.* (1984) found that mast cells from asthmatics, even in an asymptomatic stage, release more histamine when incubated with antigen or anti-Ig E. Subsequently, Flint (1985a,b) noted that the histamine content of the mast cells in the BAL fluid of asthmatics was significantly increased, and that these mast cells were mainly of the mucosal type and inherently more susceptible to the spontaneous release of histamine *in vitro* than those from controls (Flint, 1987). A recent study by Wardlaw *et al.* (1989) has also shown that the concentrations of leukotrienes C_4 and B_4 are significantly higher in BAL fluid from asthmatics than that from controls.

The metabolic activity of both neutrophils and macrophages obtained at lavage, assessed by stimulated chemiluminescence, is increased even in patients with stable asthma (Kelly *et al.*, 1988). The macrophages in BAL fluids from asthmatics also exhibit a significantly higher level of *Ricinus communis* agglutinin binding ability on their surfaces, although the proportion of macrophages is not significantly changed (Tomioka *et al.*, 1984;

Warton *et al.*, 1986b; Kelly *et al.*, 1988). Certain functions, such as phagocytosis and prostanoid release from macrophages are, however, decreased (Godard *et al.*, 1982; Aubas *et al.*, 1984).

1.4 CONCLUSION

The pathological changes in severe asthma have been extensively studied with light microscopy and to a lesser extent at the ultrastructural and immunohistochemical levels. The changes are mainly confined to airways, and include epithelial shedding, goblet cell hyperplasia and squamous metaplasia, thickening of mucosal basement membranes, enlargement of submucosal glands, ectasia of bronchial gland ducts, increase in the volume of BSM, infiltration of inflammatory cells into airway walls and lumina, and plugging of the airway lumen with exudate. However, relatively little is known of the early structural changes occurring in airways in asthma. Limited studies have shown that the changes in mild asthma vary from no apparent morphological changes to relatively severe epithelial damage.

The use of the BAL technique has widened the possibilities for studying different types of inflammatory cells during the asthma process. Among these cells, mast cells and eosinophils are thought to be especially potent in modulating pathological changes. The role of different subsets of lymphocyte in asthma remains poorly understood.

Although both morphological and BAL studies support the concept that inflammatory cells play a critical role in the development of asthma, certain discrepancies in the predominant infiltrating cell types and tissue components involved are present in the literature. This probably reflects differences in the severity, age and sex of the patients studied, and is complicated by the presence of other respiratory diseases, variation in the level of bronchi studied and differences in the BAL technique. As Mordelet-Dambrine *et al.* (1984) have found, minor differences in techniques of processing of lavage cells between laboratories may result in large differences in results. Thus knowledge of the pathology of early and mild asthma is still insufficient. Further investigations in these areas using both human material and animal models are essential for a better understanding of the pathogenesis of this important disease.

CHAPTER 2

INFLAMMATORY MECHANISMS IN THE PATHOGENESIS OF ALLERGIC ASTHMA

The importance of airway inflammation in the development of allergic asthma and non-specific airway hyperresponsiveness (NAHR), a characteristic feature of asthma, is supported by observations that airway inflammation is a common pathological feature of these conditions (Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Laitinen *et al.*, 1987; Jeffery *et al.*, 1989). It is noteworthy that any stimulus capable of inducing human NAHR may also cause airway inflammation (Borish, 1987; Magnussen and Nowak, 1989); and that potent anti-inflammatory agents, such as corticosteroids, reduce the magnitude of NAHR (Cockcroft, 1988).

The objective of this section of the literature review is to summarise the available information on the importance of airway inflammation in the development of asthma. The potential significance of respiratory tract infections and epithelial damage is also included since these factors are thought to be closely associated with airway inflammation and may be directly involved in asthmatic attacks (Nadel, 1979,1988; Barnes, 1989; Vanhoutte, 1989).

2.1 INFLAMMATORY CELLS AND ASTHMA

The most common inflammatory cells involved in asthma are mast cells, eosinophils, macrophages and neutrophils (Borish, 1987). These cells become morphologically and functionally activated during the process of the disease and release a wide range of biologically potent mediators which activate target cells in the airways to induce the pathophysiological features characteristic of asthma (Borish, 1987; Metzger *et al.*, 1987). Recent evidence has also suggested that the development of the late asthmatic reaction (LAR) is dependent on recurrent degranulation of mast cells and recruitment of other inflammatory cells to airways after initial exposure to allergens (Borish, 1987; O'Byrne *et al.*, 1987; Diaz *et al.*, 1989; Larsen, 1989).

2.1.1 Mast cells

Mast cells have long been considered to play a fundamental role in the development of immediate bronchial hypersensitivity and acute asthma (Kaliner 1985,1989; Casale *et al.*, 1987; Flint, 1987; Friedman and Kaliner, 1987). They are widely distributed in the human respiratory tract, especially in the alveolar wall and the superficial regions of airway mucosa (Brinkman, 1968; Caulfield *et al.*, 1980; Fox *et al.*, 1981; Agius *et al.*, 1986; Friedman *et al.*, 1986; Otsuka *et al.*, 1986; Warton *et al.*, 1986a; Gomez *et al.*, 1987; Shanahan *et al.*, 1987;

Heard *et al.*, 1989), and can migrate to these sites after exposure to allergens (Pipkorn and Enerback, 1986). They are therefore ideally placed to be involved in the development of allergic disorders, and according to current hypotheses, mast cells adjacent to the bronchoalveolar lumen play a critical role in the initiation of allergic bronchoconstriction (Flint, 1987).

The distribution (Pipkorn and Enerback, 1986) and density (Salvato, 1968; Goto *et al.*, 1984; Bienenstock *et al.*, 1986; Heard *et al.*, 1989) of mast cells may be altered by both physiological and pathological stimuli. The density of human lung mast cells is considerably increased in hypoxia, pulmonary hypertension, inhaled antigen challenge and vaccination, interstitial lung disease, pulmonary fibrosis, asbestosis, silicosis and sarcoidosis (Goto *et al.*, 1984; Agius *et al.*, 1985; Bienenstock *et al.*, 1986; Nadziejko *et al.*, 1989). In asthma, however, the density of mast cells in the airway and lung is either not significantly changed (Warton *et al.*, 1986a) or apparently decreased considerably due to degranulation (Salvato, 1968; Heard *et al.*, 1989). Nevertheless, mast cell numbers in the bronchoalveolar lavage (BAL) fluid are significantly increased (Tomioka *et al.*, 1984).

Over the last 5 years, increasing evidence has indicated that mast cells in the human respiratory tract are heterogeneous (Otsuka *et al.*, 1986; Flint, 1987; Shanahan *et al.*, 1987; an Overveld *et al.*, 1989). The majority of mast cells from the human lung and the lamina propria of airways and almost all mast cells in human BAL fluid are histochemically and morphologically analogous to the rat mucosal mast cell (MMC), i.e. sensitive to formalin and stained exclusively with alcian blue (Agius *et al.*, 1986; Flint, 1987; Gomez *et al.*, 1987; Shanahan *et al.*, 1987; an Overveld *et al.*, 1989). The neutral protease of mast cells obtained from human lung or obtained by BAL is mainly tryptase in contrast to combined chymases and tryptase in mast cells (analogous to the rat connective tissue mast cell, CTMC) from human skin and intestinal submucosa (Irani *et al.*, 1986). Like the rat MMC, the development of tryptase-containing mast cells in man is dependent on T lymphocyte factors (Irani *et al.*, 1987) and can be inhibited by corticosteroids (Otsuka *et al.*, 1986).

Functionally, all human lung mast cells release histamine in response to anti-immunoglobulin (Ig) E, antigens, and calcium ionophore A23187 (Church *et al.*, 1982; Agius *et al.*, 1986; Lowman *et al.*, 1988). However, mast cells from human lung are not responsive to compound 48/80, substance P, morphine, opiates, or basic polyamines (Church *et al.*, 1982; Pearce, 1986; Church and Hiroi, 1987; Lowman *et al.*, 1988), which distinguishes them from those obtained from human skin and rat serosa. The capability of mast cells from human lung to synthesise prostaglandin (PG) D₂ (60 ng/10⁶ cells) and leukotriene (LT) C₄ (50 ng/10⁶ cells) is in the same order as that of human intestinal MMC (Fox *et al.*, 1985), although mast

cells in BAL fluid are more sensitive to immunological stimulation and generate more PG D₂ when compared to those from human lung (Murray *et al.*, 1986; Flint, 1987). In addition, the response to cromoglycate differs considerably between mast cells derived from BAL fluid and those from lungs, the latter being poorly inhibited by this compound, although they show a similar response to salbutamol, nedocromil and theophylline (Flint, 1987; Fox *et al.*, 1988). Some functional differences which are important in the development of asthma are found between MMC and CTMC in human lung (an Overveld *et al.*, 1989). The MMC show a greater histamine release than CTMC to anti-Ig E challenge *in vitro*, and release of LT C₄ is only observed in MMC whereas CTMC release PG D₂.

Human mast cells are not entirely comparable to those from the rat. The clear anatomical distinction between MMC and CTMC, which exists in the rat gut, does not hold for human lung mast cells (Flint, 1987). Extrapolation of results from the rat would suggest that human skin, but not lung mast cells, should be susceptible to inhibition by sodium cromoglycate (Pearce, 1986), but in fact, the converse is true (Church, 1988). In response to stimulation with calcium ionophore A23187, mast cells of human skin and lung produce similar amounts of PG D₂ (Church, 1988).

Although many factors can activate mast cells to release mediators through either Ig E-dependent or Ig E-independent mechanisms, antigen-induced and Ig E-mediated mast cell degranulation is the principal mechanism in the development of allergic asthma (Schellenberg, 1985; Kaliner, 1989). The activated mast cells not only immediately release many preformed mediators, but also initiate a biochemical cascade ending with the generation of many newly-synthesised mediators (Table 2.1). Among these mediators, histamine is probably one of the most potent associated with asthma (Flint, 1987; Kaliner, 1989). The sources of these mediators and their manifestations in target tissues have been comprehensively reviewed by Kaliner (1985), Schellenberg (1985), Borish (1987) and Flint (1987).

Antibodies apart from Ig E may also induce mast cells to release mediators although the studies to date on man have produced some conflicting data (Bryant *et al.*, 1975; Nelson and Branch, 1977; Ricci *et al.*, 1986). Under certain circumstances, complements (C3a, C4a and C5a), lymphokines, physical agents (temperature and pressure), chemical agents (sensory neuropeptide, sulphur dioxide) and probably also psychological factors (Pavlov conditioning) (Schellenberg, 1985; Kaliner, 1989) are all capable of inducing mast cell degranulation by Ig E-independent mechanisms, although the role of these mechanisms in allergic disorders remains to be clarified. Recently, neutrophils, eosinophils, lymphocytes and other mononuclear cells have also been recognised as able to generate factors that can degranulate mast cells (Friedman and Kaliner, 1987; White *et al.*, 1989a).

Table 2.1
Mast Cell-derived Mediators[#]

=====

Preformed, rapidly eluted under physiological conditions

Histamine

Eosinophil chemotactic factors of anaphylaxis

Neutrophil chemotactic factors

Superoxide anions

Exoglycosidases (β -hexosaminidase, β -D-galactosidase,
 β -glucuronidase)

Serotonin

Kininogenase

Arylsulfatase A

Secondary or newly generated mediators

Slow-reacting substances of anaphylaxis:

Leukotrienes C₄, B₄, D₄, and E₄

Prostaglandins

Monohydroxyeicosatetraenoic acids

Hydroperoxyeicosatetraenoic acids

Hydroxyheptadecatrienoic acid

Thromboxanes

Platelet-activating factor

Prostaglandin-generating factor of anaphylaxis

Preformed granule-associated mediators

Heparin

Chymotrypsin/trypsin

Peroxidase

Superoxide dismutase

Arylsulfatase B

Inflammatory factors of anaphylaxis

=====

[#] According to Kaliner (1985), Borish (1987) and Flint (1987).

A central role for mast cells in the development of asthma has been hypothesised by Flint (1987) (Fig. 2.1). According to current theory, most pathological features of asthma can be attributed to the action of mast cell mediators (Table 2.2) (Kaliner, 1985,1989; Flint, 1987). The release of neutrophil and eosinophil chemotactic factors by mast cells and probably also other cells is thought to be responsible for the production of the inflammatory response seen during the LAR (Wenzel *et al.*, 1988).

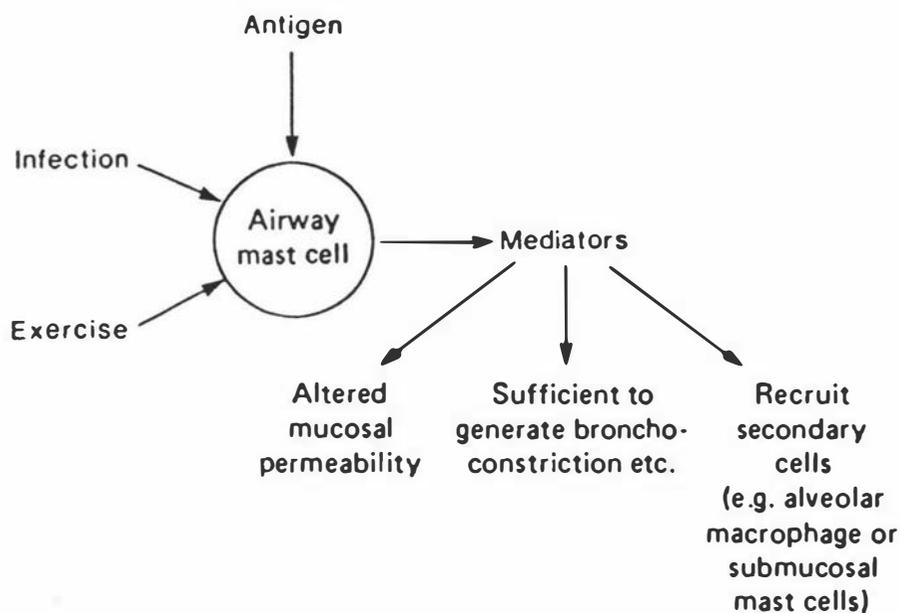


Fig. 2.1 The proposed hypothesis of the central role of mast cells in asthma (According to Flint, 1987).

Table 2.2
Pathological Changes in Asthma and the Mediators Likely to be Responsible*

Features	Proposed mediators
Bronchospasm	Histamine (H ₁ response) Slow-reacting substances of anaphylaxis (SRS-A): leukotrienes (LT) C ₄ , D ₄ , and E ₄ Prostaglandins (PG) and thromboxane A ₂ Platelet-activating factor (PAF) Acetylcholine Bradykinin
Vascular permeability and mucosal oedema	Histamine (H ₁ response) SRS-A (LT C ₄ , LT D ₄ , LT E ₄) PG E PAF Bradykinin
Cellular infiltration	Eosinophil chemotactic factors of anaphylaxis Neutrophil chemotactic factors Inflammatory factors of anaphylaxis Monohydroxyeicosatetraenoic acids (HETEs) LT B ₄
Mucus secretion	Histamine (H ₂ response) Acetylcholine α-Adrenergic agonists PGs HETEs SRS-A (LT C ₄ , LT D ₄ , LT E ₄) Macrophage mucus secretagogue Prostaglandin-generating factor of anaphylaxis
Epithelial desquamation	O ₂ ⁻ , H ₂ O ₂ , OH ⁻ Proteolytic enzymes
Basement membrane thickening	O ₂ ⁻ Proteolytic enzymes

* Modified from Kaliner (1985).

The importance of mast cells in asthma is reinforced by evidence from clinical, pathophysiological and pharmacological studies. As briefly reviewed in Chapter 1, increased numbers of mast cells have been recovered from BAL fluid of asthmatic patients, even in mild forms of the disease (Tomioka et al., 1984; Flint, 1987; Kirby et al., 1987; Wardlaw et al., 1988). The immediate response of asthmatics following provocation with allergen is associated with elevation of histamine levels in both plasma and BAL fluid (Casale et al., 1987; Metzger et al., 1987; Kaliner, 1989), and increased concentrations of PG D₂, LT C₄ and B₄ in BAL fluid (Murray et al., 1986; Wardlaw et al., 1989). Pretreatment with H₁-histamine receptor antagonists (Holgate et al., 1985) and cyclooxygenase inhibitors (Cruzen et al., 1987) can also considerably reduce this response.

Although the mast cell is recognised as a sentinel cell in the onset of acute asthma, its role in LAR is more contentious. Since pretreatment of individuals with sodium cromoglycate reduces both the early and late asthmatic reaction, mast cells have been assumed to be implicated in both reactions (Agius et al., 1986). Studies of asthmatics have shown that the development of LAR is accompanied by a second increase of histamine and neutrophil chemotactic factors levels in the peripheral blood (Durham et al., 1984; Kaliner, 1989) and in BAL fluid (Diaz et al., 1989). Mast cell mediators are found to remain at sites of degranulation several hours after initial antigen challenge and can produce a LAR (Kaliner, 1989). Studies of BAL fluid have further confirmed that mast cells continue to be activated after initial challenge (Kirby et al., 1987) although their numbers may be decreased (Diaz et al., 1989). In addition, the concentration of cell-free histamine (Casale et al., 1987) and its spontaneous release (Kirby et al., 1987) in BAL fluid are also well correlated with the degree of NAHR. All these observations have strongly suggested that mast cells also play an important role in the development of both NAHR and LAR.

Despite the above evidence, results from many recent studies using pharmacological agents argue strongly against a critical role for mast cells in LAR and NAHR (reviewed in Barnes, 1989). Although the abolishment of early and late bronchoconstriction by sodium cromoglycate can be explained by mast cell stabilisation, β_2 -agonists, a group of highly effective inhibitors of both mast cell degranulation and immediate asthmatic reaction, have little effect on the development of LAR and NAHR (Cockcroft, 1988). On the other hand, corticosteroids, which are highly effective in preventing the LAR and NAHR, have no direct action on human lung mast cells (Barnes, 1989). Therefore, the possibility that LAR and acquired NAHR may occur independent of mast cell activation and may result from the activation of other resident cells in the airway mucosa should be considered. It is now well recognised that eosinophils, respiratory epithelial cells, lymphocytes, macrophages and monocytes, and platelets all bear Ig E receptors, although these are of lower affinity than those on mast cells (Borish, 1987).

2.1.2 Eosinophils

The involvement of eosinophils in the pathogenesis of acute asthma is illustrated by the features of eosinophilia and elevation of eosinophil-associated substances in the peripheral circulation (Horn *et al.*, 1975; Dahl and Venge, 1982; Annesi *et al.*, 1988), the BAL fluid (de Monchy *et al.*, 1985; Wardlaw *et al.*, 1988; Diaz *et al.*, 1989), and sputum of asthmatics (Frigas *et al.*, 1981; Gleich *et al.*, 1988; Gibson *et al.*, 1989). The severity of peripheral eosinophilia is known to correlate well with the degree of bronchoconstriction in patients with positive skin reactions (Burrows *et al.*, 1980) and the severity of NAHR (Booij-Noord *et al.*, 1972), but is inversely correlated with forced expiratory volume in 1 s and airway conductance of asthmatics (Horn *et al.*, 1975). The predominance of eosinophil infiltration and the demonstration of eosinophil-derived substances in the airway mucosa and its lumen and in the lungs of asthmatics (Dunnill, 1982; Filley *et al.*, 1982; Holloway *et al.*, 1990) have further supported this involvement. Because of these features, some workers have proposed that asthma could be more accurately termed chronic eosinophilic bronchitis (Dail, 1988; Barnes, 1989).

Increasing evidence suggests that eosinophils are particularly involved in the development of LAR. A significant elevation of eosinophils has been demonstrated in BAL fluid and peripheral blood of patients with LAR, but not in patients with only an early asthmatic reaction or non-responders (de Monchy *et al.*, 1985). Moreover, the timing of the onset of BAL eosinophilia (6 hours after initial challenge) is later than the resolution of an immediate response but before the development of LAR (de Monchy *et al.*, 1985). This eosinophilia can persist for up to 96 hours (Metzger *et al.*, 1987). The presence of eosinophilia during LAR is associated with the elevation of eosinophil cationic protein (ECP) in BAL fluid (de Monchy *et al.*, 1985).

Circulating eosinophils can be divided into at least two populations by their density: a hypodense type and a normodense type (Fukuda and Gleich, 1989). The cell density is associated with both morphological and functional heterogeneities. The eosinophilia which occurs in asthmatics appears to be mainly due to an increase in the hypodense type (Fukuda and Gleich, 1989). These cells contain fewer cytoplasmic granules and greater numbers of membranous receptors for Ig E, Ig G and complement than normodense cells (Gleich and Adolphson, 1986). They are more easily stimulated to degranulate (Reed, 1988), which may partially explain their low density. These cells also show higher than normal oxygen consumption and potent cytotoxic activity for antibody-coated target cells (Gleich and Adolphson, 1986). Thus, the hypodense forms are activated cells and may represent partially degranulated eosinophils.

The eosinophil may play its role in the pathogenesis of asthma through the action of both granule-derived and membrane-derived mediators (Tables 2.3 and 2.4). Eosinophils contain at least four different types of granule-derived proteins: eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), major basic protein (MBP) and ECP (Gleich and Adolphson, 1986). In terms of mass, EPO is the predominant protein in human eosinophils, followed by MBP, EDN, and ECP (Gleich *et al.*, 1988). Studies of both human and animal tracheal organ cultures have shown that MBP, ECP and EPO, but not EDN, by itself or in the presence of hydrogen peroxide or halide substrates can cause dose-related damage to the mucosal epithelium, which mimics the pathological changes seen in asthma (Frigas *et al.*, 1980; Gleich *et al.*, 1988; Motojima *et al.*, 1989a). Subsequent studies of guinea pig tracheal rings indicate that MBP-induced airway hyperreactivity is associated with the inhibition of epithelial function (Flavahan *et al.*, 1988), probably through the release of epithelium-derived relaxing factor (Widdicombe, 1989), since the presence of an intact tracheal epithelium is necessary for this augmentation of airway tone. Studies of the canine trachea have revealed that MBP can also increase chloride secretion from the epithelium (Jacoby *et al.*, 1988). Changes in airway epithelial ion transport may affect epithelial water secretion, and mucociliary clearance by changing the volume and composition of respiratory tract fluid. Moreover, MBP can activate mast cells either directly or in combination with EPO (Gleich and Adolphson, 1986). In addition, eosinophils can stimulate the proliferation of fibroblasts and therefore may account for the thickening of basement membranes in asthmatics (Roche *et al.*, 1989).

Most membrane-derived mediators of eosinophils (Table 2.4) are functionally similar to those of mast cells and therefore, can induce a bronchoconstriction either directly or indirectly by inducing local inflammation and mucosal damage (Bruijnzeel, 1989).

Lysophospholipase is recognised as the substance responsible for the generation of Charcot-Leyden crystals seen in the sputum of asthmatics (Borish, 1987).

Eosinophils also contain numerous surface receptors for immunoglobulins and complement (Gleich and Adolphson, 1986; Bruijnzeel, 1989). Those important in the development of asthma are Ig E receptors, C3b receptors and a receptor for the Fc region of Ig G (Gleich and Adolphson, 1986). Activation of eosinophils through these receptors leads to the generation and release of inflammatory mediators.

Table 2.3
Human Eosinophil Crystalloid Granule-derived Mediators[#]

Mediator	Site in granule	Functional activity with respect to asthma
MBP	core	toxic to many cells, e.g., tracheal epithelial cells, pneumocytes II causes histamine release from basophils and mast cells neutralizes heparin
ECP	matrix	damages epithelial cells shortens coagulation time and alters fibrinolysis neutralizes heparin inhibits cultures of peripheral blood lymphocytes weakens RNase activity
Eosinophil-derived neurotoxin	matrix	inhibits cultures of peripheral blood lymphocytes (RNase activity)
EPO	matrix	inactivates leukotrienes causes disruption of mast cells in vitro
Lysosomal enzymes Acid phosphatase Neutral proteases Elastase Collagenase Arylsulphatase B Histaminase Phospholipase D Catalase Nonspecific esterases, vitamin B ₁₂ binding proteins, fibroblast growth factors	matrix	degrades elastin degrades collagen catabolizes proteoglycans and glycosaminoglycans degrades histamine degrades PAF
Zinc, copper, magnesium, cobalt and iron	matrix	

[#] According to Bruijnzeel (1989). MBP=major basic protein, ECP=eosinophil cationic protein, RNase=ribonucleic acidase, EPO=eosinophil peroxidase, and PAF=platelet-activating factor.

Table 2.4
Human Eosinophil Membrane-derived Mediators[#]

Mediator	Functional activity with respect to asthma
Leukotriene C ₄	bronchoconstriction, oedema, mucus production
15-Hydroxyicosatetraenoic acid (15-HETE)	chemotaxis of granulocytes, inhibition of 5-lipoxygenase activity in eosinophils
Lipoxin A	degranulation of granulocytes, inhibition of natural killer cell cytotoxicity
Thromboxane B ₂	bronchoconstriction, vasoconstriction
Prostaglandin D ₂	bronchoconstriction, vasodilatation, mucus production
Prostaglandin F _{2α}	bronchoconstriction, vasoconstriction, mucus production
Prostaglandin E ₂	bronchodilatation, vasodilatation, inhibits mucus production and mast cell degranulation
PAF	bronchoconstriction, chemotaxis of granulocytes, bronchial hyperreactivity
Lysophospholipase (Charcot-Leyden crystals)	self-protection (?)
Reactive oxygen species	tissue damage

[#] According to Bruijnzeel (1989). PAF=platelet-activating factor.

2.1.3 Macrophages and monocytes

The alveolar macrophage (AM) is another candidate for the primary inflammatory cell involved in the initiation of asthma (Fuller 1989; Wilkinson *et al.*, 1989). Because they are widely distributed in the lower respiratory tract of both normal and asthmatic subjects, AM are likely to be one of the first potential inflammatory cells exposed to inhaled antigens (van Furth, 1985). In addition, AM possess low affinity Ig E receptors which can be activated to release mediators following Ig E-dependent challenge *in vitro* (Melewicz *et al.*, 1982). Finally, the sensitivity of AM to various therapeutic agents is in keeping with the response of asthmatic patients to those agents (Fuller, 1989).

Evidence for the involvement of AM and monocytes in the pathogenesis of asthma as primary effector cells has also been supported by many clinical and experimental studies (Annesi *et al.*, 1988; reviewed in Wilkinson *et al.*, 1989). Metzger *et al.* (1987) have demonstrated that the total numbers of AM in the BAL fluid of asthmatics are increased at 48 and 96 hours after antigen challenge. Monocytes and AM from asthmatics become functionally active as the number of cells expressing Ig E and complement receptors increases (Kay *et al.*, 1981; Melewicz *et al.*, 1981), and both monocytes and AM can release a number of mediators, such as LT B₄, LT C₄, platelet-activating factor, PG E₂, PG F_{2α} and thromboxane B₂, which are potent in the development of bronchoconstriction (Arnoux *et al.*, 1980; Holgate *et al.*, 1984; MacDermot *et al.*, 1984). Furthermore, the pro-inflammatory effects of oxygen metabolic products from these cells may also contribute to the airway inflammation observed in asthmatics (Wilkinson *et al.*, 1989). Monocytes and AM also release many well-defined factors, such as interleukin-1, tumour necrosis factor, colony-stimulating factors, eosinophil cytotoxicity-enhancing factor and histamine-releasing factors, as well as a number of yet uncharacterised substances (Wilkinson *et al.*, 1989). All these factors may be able to modulate the function of other inflammatory cells, especially mast cells, eosinophils and neutrophils (Schulman *et al.*, 1985; Tonnel *et al.*, 1987).

2.1.4 Neutrophils and lymphocytes

Compared to studies of other inflammatory cells, the role of neutrophils and lymphocytes in the development of asthma has received relatively little attention. Neutrophilia is present in BAL fluid of asthmatics before, during and after late asthmatic reactions, but not in early reactions induced by allergens (Diaz *et al.*, 1986, 1989; Metzger *et al.*, 1987). Peripheral neutrophilia is also pronounced in man with NAHR (Annesi *et al.*, 1988). Studies on the rabbit model of asthma have shown that the presence of neutrophils at the time of antigen challenge is essential for the development of LAR (Murphy *et al.*, 1986). However, numerical changes of neutrophils in BAL fluid (Tomioka *et al.*, 1984; de Monchy *et al.*, 1985) and bronchial biopsies

(Jeffery *et al.*, 1989) of asthmatics are more variable than that observed in eosinophils, although activation of this cell has been demonstrated by changes of its complement receptors, arachidonate metabolism, and the response to pro-inflammatory mediators (Borish, 1987). The release of toxic oxygen metabolites and other elastases and proteases by activated neutrophils contributes to the irreversible tissue damage characteristic of chronic asthma (O'Byrne *et al.*, 1984b). In addition, neutrophils secrete a yet poorly-defined substance which is capable of inducing mast cell degranulation (White *et al.*, 1989a).

Analysis of BAL fluid from asthmatics indicates that the number of lymphocytes present is either slightly increased (Tomioka *et al.*, 1984; Kirby *et al.*, 1987) or not changed (de Monchy *et al.*, 1985; Metzger *et al.*, 1987). A significant increase of lymphocytes in BAL fluid (Graham *et al.*, 1985; Kelly *et al.*, 1989) and peripheral blood (Diaz *et al.*, 1989) has only occasionally been reported. Apart from numerical changes, the phenotype of lymphocytes is sometimes altered also (Lam *et al.*, 1984; Diaz *et al.*, 1986; Kelly *et al.*, 1989). The increased numbers of lymphocytes in BAL fluid are reported to be confined to the T cell subgroup (Kelly *et al.*, 1989), and these remain up to 96 hours after local antigen challenge (Metzger *et al.*, 1987). In BAL fluid from patients with early asthmatic reaction only, the suppressor phenotype (CD8) and the helper phenotype (CD4) are, respectively significantly increased and decreased (Gonzalez *et al.*, 1987b), whereas in individuals with LAR, a significant and prolonged increase in CD4 cells has been reported (Metzger *et al.*, 1987). An infiltration of so-called "irregular" lymphocytes has also been demonstrated in the submucosa of bronchial biopsies taken from mild asthmatics (Jeffery *et al.*, 1989). On the other hand, the circulating CD8 cells in chronic asthma were found to be decreased in number, and their *in vitro* growth was abnormal (Corrigan *et al.*, 1988).

In some instances, the lymphocytes from asthmatics are functionally more active than normal, although the proportion of each phenotype is in the normal ranges (Kelly *et al.*, 1989). Corrigan *et al.* (1988) have found that circulating CD4 cells from patients with acute severe asthma show a significant increase of their surface proteins; interleukin-2 receptor, class II histocompatibility antigen, and "very late activation" antigen. These proteins are closely associated with T lymphocyte activation. The percentages of interleukin-2 receptor- and class II histocompatibility antigen-positive lymphocytes subside with clinical improvement after treatment. Canonica and Bagnasco (1988) further commented that the expression of some early activation molecules (e.g. 4F2 antigen) in peripheral T lymphocytes is also elevated in asthmatics. These observations have strongly suggested that lymphocytes may have an important immune-modulating role in asthma. Although details of the mechanisms by which lymphocytes influence airway hypersensitivity are not clear, they may regulate the production of Ig E, the activity of macrophages, and the recruitment of eosinophils and neutrophils into

airways by releasing a wide range of lymphokines. In addition, human T lymphocytes can release mediators which may alter histamine release of mast cells (Sedgwick *et al.*, 1981).

2.2 AIRWAY EPITHELIAL DAMAGE

Although allergic asthma has long been believed to be triggered by the interaction between allergens and airway submucosal mast cells, the most common allergens such as pollen are about 20 μm in diameter (Reed and Swanson, 1987), and it is therefore difficult for them to penetrate into the airway submucosa of normal subjects. In order to trigger such reactions, the normal barrier of airway mucosa must have therefore been altered. Airway epithelial damage is indeed a common pathological finding in both autopsy and biopsy studies of patients with asthma (Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Laitinen *et al.*, 1985; Jeffery *et al.*, 1989), and both animals and man develop NAHR after exposure to various agents known to cause airway inflammation and epithelial damage (Empey *et al.*, 1976; Gerrard *et al.*, 1980; Hogg, 1982; Murlas and Roun, 1985). Thus, damage to airway epithelium has been postulated to play an important role in inducing NAHR and asthma by altering one or more of the following functions; the mechanical barriers to antigen penetration, the secretion of mucus and fluid, the output of arachidonic acid metabolites, the release of relaxing factors, or the modulation of neuropeptide action by epithelial endopeptidases (Nadel, 1979; Borish, 1987; Flavahan *et al.*, 1988; Widdicombe, 1989).

2.2.1 Increased mucosal permeability

A great variety of factors are known to increase airway mucosal permeability. Included in these are respiratory virus infection (Laitinen *et al.*, 1976; Miura *et al.*, 1989), methacholine (Simani *et al.*, 1974; Boucher *et al.*, 1978), histamine (Boucher *et al.*, 1978), sulphur dioxide (Maurer *et al.*, 1981; Magnussen *et al.*, 1987), nitrogen dioxide (Orehek *et al.*, 1976), ozone (Golden *et al.*, 1978; Murlas and Roun, 1985), cigarette smoke (Simani *et al.*, 1974; Boucher *et al.*, 1980; Hogg, 1981) and probably also the secretions from goblet cells (Hogg, 1981). Increased airway permeability has also been demonstrated in both man (Ilowite *et al.*, 1989) and animals (Boucher *et al.*, 1977; Mukherjee *et al.*, 1986) during asthmatic attacks or following exposure to allergens.

Several studies using animal models have suggested that airway mucosal permeability may be increased by the mechanism of disrupting interepithelial tight junctions and/or elevating epithelial uptake ability (Boucher *et al.*, 1977, 1979, 1980; Schellenberg, 1985; Mukherjee *et al.*, 1986). The tracheal epithelium of sensitised rats shows significantly more intercellular penetration of lanthanum than that of control animals (Mukherjee *et al.*, 1986). However, recent studies on guinea pigs by Walker and Burns (1988) suggest that the intercellular

penetration of tracer into the airway mucosa is due to epithelial lesions in the adjacent mucosa rather than disruption of tight junctions.

Results from studies of the change in mucosal permeability in the airways of asthmatics are generally conflicting and discouraging. Although the mucosal permeability is markedly increased in bronchitics (Honda *et al.*, 1988) and in cigarette smokers who show no NAHR (Tattersfield and McNicol, 1987), it is not significantly increased in subjects with NAHR (Dolovich *et al.*, 1983) or in the early or late asthmatic reaction (Dolovich *et al.*, 1983; Elwood *et al.*, 1983; O'Byrne *et al.*, 1984a; Honda *et al.*, 1988). Studies of young smokers have also revealed that respiratory epithelial permeability is unrelated to bronchial reactivity or small airway function (Taylor *et al.*, 1988). In contrast to these observations, Ilowite *et al.* (1989) have recently reported that the permeability of bronchial mucosa in patients with stable asthma is significantly increased over that of normal subjects. Previous work which failed to demonstrate this change was explained by a failure to deliver the aerosol to the bronchi (O'Byrne *et al.*, 1984a) or to adjust for changes in mucociliary clearance in the analysis of results (Elwood *et al.*, 1983).

Disruption of tight junctions and increase in airway mucosal permeability may not only allow large molecular antigens more easy access to mast cells in the submucosa, but also enhance the direct access of inhaled or released inflammatory mediators to effector organs including bronchial smooth muscle. Several recent studies, however, have shown that NAHR evoked through epithelial damage cannot be fully explained by either increased mucosal permeability, reduced enzymatic breakdown of acetylcholine or disruption of a diffusion barrier (reviewed by Vanhoutte, 1989).

2.2.2 Epithelium-derived relaxing factor(s)

Removal of the epithelium from human, guinea pig, bovine, rabbit, porcine and canine airways *in vitro* heightens the sensitivity of bronchial smooth muscle to different bronchoconstrictors, including allergens, and reduces the relaxant potency of isoproterenol (Murlas and Roum, 1985; reviewed in Goldie *et al.*, 1988; Vanhoutte, 1989), particularly in sensitised animals (Montano *et al.*, 1988). It has been proposed, therefore, that normal bronchial tone is regulated by factors which are continuously released from the intact epithelium. Epithelial damage may reduce the local production of these substances and, consequently, contribute to the abnormal responses in bronchial tone.

The most active substance identified to date is epithelium-derived relaxant factor(s), which is analogous to endothelium-derived relaxant factor; a substance which can relax both bronchial and vascular smooth muscle (Vanhoutte, 1989). Based on the limited studies

undertaken to date, it has been postulated that this substance has a very short half-life and is inactivated by mucus (Vanhoutte, 1989). Its release is preferentially toward the underlying smooth muscle. Although this hypothesis is attractive, the identity and even existence of this compound are, at this stage, still the subject of considerable debate (Widdicombe, 1989).

Prostaglandin E₂ can also modulate smooth muscle constriction and the airway epithelium of several mammalian species including man is capable of releasing PG E₂ (Goldie *et al.*, 1988; Nadel, 1988).

2.2.3 Neutral endopeptidase

Smooth muscle tone in the normal airway is also modulated by the interaction of several peptides (such as tachykinins) stored in sensory nerves and neutral endopeptidase or enkephalinase, a membrane-bound enzyme which exists in airway epithelium and smooth muscle (Nadel, 1988). The removal or damage of airway epithelium may down-regulate the production of this enzyme, and, thereby potentiate neural inflammatory responses. Using an organ culture system, Fine *et al.* (1989) have recently demonstrated that the removal of epithelium from the guinea pig trachea demolishes membrane-bound neutral endopeptidase, and this largely accounts for the increased airway reactivity they observed.

2.3 THE ASSOCIATION BETWEEN RESPIRATORY TRACT INFECTIONS AND ASTHMA

The role of respiratory virus infection in precipitating acute asthmatic attacks has received increasing attention during the last two decades (reviewed in Busse, 1985,1989; Eggleston, 1988). Many epidemiological and clinical studies have suggested that viral infections of the respiratory tract can exacerbate acute attacks in asthmatic subjects (Table 2.5), or precipitate the first attack of asthma in persons who are predisposed to this disease (Frick *et al.*, 1979). There is also evidence that respiratory virus infections may induce a prolonged small airway obstruction (Pickens *et al.*, 1972) and cause persistent airway hyperreactivity in non-atopic subjects (Empey *et al.*, 1976; Nadel, 1979; Busse, 1988; Magnussen and Nowak, 1989). Increased bronchial responsiveness has also been demonstrated in animal models experimentally infected with respiratory virus (Lemen *et al.*, 1990). Recent studies have shown that respiratory virus infection is not only the most common factor precipitating acute asthmatic attacks in children (Carlsen *et al.*, 1984), but also commonly implicated in the exacerbation of asthma in adults, particularly in severe asthmatic attacks (Beasley *et al.*, 1988). However, there is no close association with the occurrence of chronic asthma and respiratory virus infection (<5%) (Jennings *et al.*, 1987). Neither is asymptomatic virus shedding and mild infection of importance in the exacerbation of asthma (Busse, 1985).

Table 2.5
Association of Respiratory Virus Infections and Asthmatic Attacks[#]

Study	Year	n	Age (years)	Asthmatic attacks with positive viral cultures	
				n	%
<u>Children</u>					
McIntosh et al.	1973	32	1-5	58/139	42
Minor et al.	1974	16	3-11	23/43	53
Shapiro et al.	1974	44	5-7.8	9/44	20
Minor et al.	1976	41	1-18	17/71	24
Horn et al.	1979	22	1-15	35/72	49
Carlsen et al.	1984	169	>2	73/256	29
Jennings et al.	1987	204	0-12	38/204	19
<u>Adults</u>					
Huhti et al.	1974	63	15-77	27/142	19
Minor et al.	1976	8	22-60	3/17	19
Hudgel et al.	1979	19	24-67	8/76	11
Beasley et al.	1988	31	15-56	18/178	10

[#] Modified and updated from Eggleston (1988).

The viruses commonly associated with exacerbations of asthma are listed in Table 2.6. Although studies both in New Zealand and overseas have found that respiratory syncytial virus infection is the most prevalent and likely virus to provoke asthma (McIntosh *et al.*, 1973; Busse, 1985; Jennings *et al.*, 1987), exacerbation of acute asthma is not specifically associated with any one virus (Jennings *et al.*, 1987). The organisms linked with asthmatic attacks are likely to vary with the patient's age. In children aged below 5 years, respiratory syncytial virus and parainfluenza infections are most common (McIntosh *et al.*, 1973; Carlsen *et al.*, 1984; Jennings *et al.*, 1987), whereas in older children rhinovirus and influenza A virus infections predominate (Minor *et al.*, 1974). In adults, however, all these viruses may exacerbate asthma (Beasley *et al.*, 1988).

Although the association between respiratory virus infection and acute asthma is well established, the precise mechanisms by which viruses produce change in airway reactivity are not clear. Results of studies on both human patients and animal models have suggested that the induction of airway inflammation and its associated epithelial damage (Frick, 1986; Saban *et al.*, 1987; McDonald, 1988; Nadel, 1988; Miura *et al.*, 1989) as discussed above, may be factors in the pathogenesis. In addition, a number of other mechanisms, acting either separately or together may be involved, and different viruses are likely to share similar mechanisms in provoking asthma (Busse, 1989). Possible alternative mechanisms include the enhancement of cholinergic sensitivity (Empey *et al.*, 1976), β -adrenergic dysfunction (Busse, 1977; Buckner *et al.*, 1981), attenuation of receptor and post-receptor activation of adenylate cyclase activity (Scarpace and Bender, 1989), production of virus-specific Ig E which may cross-react with certain allergens (Welliever and Orga, 1981), change of phenotype of immunoregulatory cells involved in Ig E antibody production (Frick, 1986; Holt *et al.*, 1988; Leibovitz *et al.*, 1988), increase in mast cell numbers in the bronchoalveolar lumen (Miura *et al.*, 1989), and enhancement of mediator release from basophils (Ida *et al.*, 1977; Graziano *et al.*, 1989) and mast cells (Miura *et al.*, 1989). It has also been shown that respiratory virus infections can induce mice with Ig E-isotype-specific immunological tolerance to develop high titres of antigen-specific Ig E in response to inhaled antigens (Holt *et al.*, 1988). From these investigations, it may be concluded that the effects of respiratory virus infection on the development of airway hyperreactivity and asthma are complex, and likely to involve more than one single mechanism.

An association between infection of the upper respiratory tract with certain viruses and the development of LAR has also been noted recently in human patients (Busse, 1989; Lemanske *et al.*, 1989).

Table 2.6
Viruses Associated with Exacerbations of Asthma[#]

Rhinovirus
Respiratory syncytial virus
Parainfluenza types 1,2, and 3
Influenza A
Influenza B
Coronavirus
Adenovirus
Enterovirus
Herpevirus
Coxsackie virus

[#] According to Eggleston (1988).

In contrast to viral infections, there is little evidence to implicate bacterial infections in the development or precipitation of asthma (Davies, 1981; Busse, 1985). Several studies have shown that both the number and type of respiratory bacteria isolated from asthmatics and non-asthmatics are not significantly different (Turner-Warwick, 1982). Although specific Ig E antibodies against common pulmonary bacteria have been identified in the sera of patients with asthma (Pauwels *et al.*, 1980), the titres obtained were similar to those in patients with other respiratory diseases (Turner-Warwick, 1982). Therefore, bacterial infection cannot be considered as an important factor in the exacerbation of asthma.

2.4 CONCLUSION

Accumulated evidence from studies during the last decade suggests that asthma is a chronic inflammatory disease involving many inflammatory cells (Hogg, 1982; Borish, 1987; Kay, 1987; Reed, 1988; Barnes, 1989; Bruijnzeel, 1989; Kelly *et al.*, 1989; Magnussen and Nowak, 1989; Wilkinson *et al.*, 1989). These cells interact in the disease process by releasing a wide range of biologically potent mediators which subsequently activate target cells in airways and induce the pathophysiological features characteristic of asthma (Borish, 1987).

Based on recent studies, an inflammatory theory of the development of allergic asthma can now be proposed (Fig. 2.1). It includes several steps of mediator release and accumulation of inflammatory cells after initial allergen exposure. The release of histamine and

other mediators from mast cells is the first step in the process, and this is likely to take place on the luminal surface of the airway epithelium. Mast cells and possibly other cells such as AM, located superficially on the airway mucosa or free in the bronchoalveolar lumen are activated after contact with inhaled antigens through an Ig E-dependent mechanism. The activation of these cells leads to release of at least two sets of inflammatory mediators which may be either preformed or newly synthesised. One set of these mediators, such as histamine and LTs, is able to directly induce an immediate bronchoconstriction that is usually transient and rapidly reversed. The second set of mediators are chemotactic to leucocytes, promoting an accumulation and migration of these cells (especially neutrophils and eosinophils) into the submucosal areas of airways. These cells and their mediators alone or together with mast cell mediators induce recurrent bronchoconstriction (i.e. LAR).

The increased epithelial permeability induced by inflammatory mediators and probably allergens acting either alone or together with the epithelial damage caused by factors such as viral infections, not only enhances the contact of allergens and mediators with mast cells, other inflammatory cells and afferent nerve endings in the submucosa, but also impairs the epithelium's ability to release epithelium-derived relaxant factor. This promotes bronchial smooth muscle sensitivity to released mediators and neurotransmitters and causes bronchoconstriction. Both the denudation of epithelial surfaces and inflammation of the mucosa might consequently contribute to the development of NAHR. These events constitute a vicious circle which may continuously maintain and amplify the inflammatory processes and bronchoconstriction in airways.

Although the inflammatory theory can satisfactorily explain most pathophysiological features characteristic of asthma, airway inflammation is probably only one of many contributory mechanisms. The airway inflammation is not an outstanding feature in some patients with mild asthma (Lozewicz *et al.*, 1988). Clinically, not all respiratory tract infections and airway inflammation induce asthmatic attacks or increase NAHR (Jenkins and Breslin, 1984; Halperin *et al.*, 1985). Furthermore, airway inflammation is considerably more severe in cigarette smokers and in patients with chronic bronchitis or cystic fibrosis, but these patients generally have less NAHR than asthmatics (Magnussen and Nowak, 1989). Bacterial infections of the respiratory tract are often better tolerated than viral infections in asthmatics, although the extent of airway inflammation is more pronounced in bacterial infections. Thus, further studies are needed to clarify the significance of inflammatory infiltration in the development of allergic airway hypersensitivity. It is also important to determine the mechanisms responsible for the infiltration of inflammatory cells into local tissues and their activation.

CHAPTER 3

ANIMAL MODELS OF HUMAN ALLERGIC ASTHMA

Naturally-occurring respiratory diseases similar to asthma are very rare in animals although feline asthma has been recognised in veterinary practice (Moise and Spaulding, 1981). Canine ragweed pollenosis, characterised by conjunctivitis, rhinitis, dermatitis and bronchoconstriction, may be the sole example of a spontaneous animal allergic disease which has been used to study human allergic bronchial hypersensitivity (Patterson, 1960). A clinical syndrome of dyspnoea in ponies known as heaves, related to exposure to mouldy hay, is thought to have similarities to human asthma (Snapper, 1986), but heaves may be more closely related to the farmer's lung type of hypersensitivity pneumonitis since the pathological changes of heaves are confined to the bronchioles and respiratory acini (Patterson and Kelly, 1974; Breeze, 1979).

Allergic bronchial hypersensitivity, however can be induced experimentally with certain antigens in a number of animals, including guinea pigs (Kallos and Kallos, 1984), monkeys (Weiszer *et al.*, 1968; Hamel *et al.*, 1986), dogs (Booth *et al.*, 1970), sheep (Wanner *et al.*, 1979), rats (Eidelman *et al.*, 1988), and rabbits (Murphy *et al.*, 1986). A comparison of the physiological, immunological and pharmacological aspects of the commonly used animal models with human asthma is undertaken in Tables 3.1 and 3.2. Experimental investigations of allergic airway hypersensitivity in animal models have produced much useful information which has increased understanding of the human condition.

The objective of this chapter of the literature review is to briefly describe the nature and characteristics of the various animal models commonly used for studies of asthma. Emphasis has been given to the sheep model since this has been increasingly used in the study of human asthma (Wanner and Abraham, 1982) and the work described in this thesis is based on this model. Comparison of the sheep model with both human asthma and the other commonly used animal models will also be attempted.

3.1 GUINEA PIGS

The guinea pig is a traditional animal model used for studying human asthma and other allergic airway hypersensitivities (Kallos and Kallos, 1974). According to Fugner (1985), the first use of this model can be traced back to 1910. Since then, this model has been extensively used in the study of the role of inflammatory mediators in the development of allergic bronchoconstriction and the evaluation of different types of anti-asthmatic drugs (reviewed in Fugner, 1985; Takizawa *et al.*, 1988). In addition, the contractile tissue of guinea pigs, such as

Table 3.1
Comparisons of the Characteristics Between Animal Asthma Models and Human Asthmatics*

	Human	Sheep	Monkeys	Dogs	Guinea pigs
Natural skin reaction	yes	yes	yes	yes	no
Spontaneous airway responses	yes	no	no	some	no
Late response	yes	yes	yes	no	no
Reproducibility	consistent	consistent	inconsistent	inconsistent	inconsistent
Respiratory frequency	increased	variable	increased	increased	increased
Tidal volume	decreased	variable	decreased	decreased	decreased
Airway resistance	increased	increased	increased	increased	increased
Residual volume	increased	?	unchanged	unchanged	increase(?)
Total lung capacity	increased	?	unchanged	unchanged	decreased
Functional residual capacity	increased	increased	variable	variable	increased
Lung elastic recoil	decreased	unchanged	unchanged	unchanged	unchanged
Dynamic lung compliance	decreased	decreased	decreased	decreased	decreased
Peak expiratory flow rate	decreased	?	decreased	decreased	decreased
Arterial oxygen tension	decreased	decreased	decreased	decreased	?
Arterial carbon dioxide tension	increased	no change	increased	increased	?
Immunoglobulins	Ig E	?	Ig E, Ig G	Ig E	Ig E, Ig G ₁
Circulating histamine levels	increased	increased	inconsistent	inconsistent	increased
Nonspecific airway hyperresponsiveness	have	have	variable	variable	no
Vagal reflexes	variable	no	no	yes	yes

* Based on Patterson and Kelly (1974), Pare et al. (1976), Wanner et al. (1979), Holrovde et al. (1980), Wanner and Abraham (1982), Abraham et al. (1983), Kallos and Kallos (1984) and Fugner (1985). ?=unknown.

Table 3.2
Comparison of the Effects of Pharmacological Agents on Acute Airway Hypersensitivity
in Human Asthmatics and Animal Models[#]

Drug or class of drugs	Inhibitory activity					
	Man	Sheep	Monkeys	Dogs	Guinea pigs	Rats
β -agonists	++	++	++	++	++	++
Cromolyn	++	++	+	0	++	++
Antihistamines	++	++	0	0	++	0
Antiserotonin	NK	?	?	?	0	++
Anticholinergics	?	0	?	+	+	0
FPL-55712	0	0	?	?	+	+

[#] According to Fugner (1985). ++=strong, +=partial, 0=no and ?= variable inhibition. NK=unknown.

tracheal rings and tracheal strips has been continuously used as an *in vitro* model to study the properties of airway smooth muscle (Souhrada and Dickey, 1976; Miura *et al.*, 1989). The authors of these studies have claimed that the guinea pig model bears the closest similarity to human asthma (Lichtenstein and Austen, 1977; Kallos and Kallos, 1984; Fugner, 1985).

Although allergic airway hypersensitivity does not naturally occur in guinea pigs, the animals can be actively sensitised with certain antigens and adjuvants by repeated inhalation, or intracutaneous and intraperitoneal injections (Kallos and Kallos, 1974). The antigen most commonly used for the sensitisation challenge is ovalbumin (Motojima *et al.*, 1989b), but the animals are also sensitive to *Ascaris suum* (Fugner, 1985). Airway hypersensitivity in guinea pigs can also be passively induced with administration of sera from other sensitised guinea pigs or rabbits (Fugner, 1985). When the experimentally sensitised animals are exposed to aerosols of homologous antigen, acute and late airway responses may be elicited (Karlsson *et al.*, 1989).

The occurrence of airway response in guinea pigs depends on previous contact with antigens and is strictly antigen-specific (Kallos and Kallos, 1984). The principal immunoglobulins (Ig) involved in this process are Ig G₁ and Ig E (Fugner, 1985). The induced airway hypersensitivity is retained from weeks to months, depending on the method of sensitisation and the frequency of antigen exposure (Patterson and Kelly, 1974; Kallos and Kallos, 1984).

Changes of pulmonary mechanics in the responding guinea pigs during an asthmatic reaction can be measured either under anaesthesia (Fugner, 1985) or in a conscious state (Amdur and Mead, 1958). Apart from the respiratory parameters traditionally measured, such as airway resistance and dynamic lung compliance (Amdur and Mead, 1958), it is now possible to measure forced pulmonary mechanics, such as peak expiratory flow rate, forced vital capacity, forced expiratory volume in 0.1 s and maximal mid-expiratory flow rate in this model (Holroyde *et al.*, 1980). The evaluation of these parameters allows comparison of the changes of respiratory function between human asthmatics and this animal model.

The acute airway response in guinea pigs is short in duration, with the greatest changes occurring within 2 minutes of the antigen challenge and spontaneous reversal 15 minutes later (Holroyde *et al.*, 1980). The airway constriction can also be reversed by the administration of certain anti-asthmatic drugs in a manner similar to that in human asthma (Holroyde *et al.*, 1980; Kallos and Kallos, 1984). Some animals may also develop a late reaction 4-5 hours later, which subsides at 20 hours (Karlsson *et al.*, 1989). Some guinea pigs also develop non-specific airway hyperresponsiveness (NAHR) (Motojima *et al.*, 1989b).

The guinea pig is probably the only animal model studied to date showing pathological changes identical to those seen in man who die in status asthmaticus (Pagel, 1939). The airway exudate in responders is rich in eosinophils, epithelial cells and neutrophils (Kallos and Kallos, 1984). Recent studies in this species have shown that eosinophils may also participate in the development of NAHR by damaging respiratory epithelial cells (Motojima *et al.*, 1989b).

The guinea pig model also exhibits several unique features which are not commonly seen in other animal models. Firstly, the hypersensitive animals show clinical signs characteristic of asthma, such as a cough and respiratory distress, after repeated exposure to antigens (Kallos and Kallos, 1984), and some animals may even develop so-called "learned asthma" (Noelpp-Eschenhagen and Noelpp, 1954). Secondly, the airway hypersensitivity in this model can be achieved *in utero* (Kallos and Kallos, 1984). The offspring of sensitised female guinea pigs show strong respiratory reactions to the inhaled homologous antigen at the age of 4 weeks. Finally, some evidence has suggested that airway hypersensitivity in this species is genetically controlled (Lundberg, 1979), since inbred guinea pigs, obtained through brother x sister mating of guinea pigs with "high" or "low" respiratory response, showed an uniform "high" or "low" airway response to inhaled homologous antigen.

The major disadvantage of the guinea pig model is that the airway hypersensitivity is not naturally present and has to be experimentally induced. Since airway hypersensitivity in this model usually lasts less than 12 months, re-sensitisation is necessary for animals used over a long period. In addition, the airway hypersensitivity induced by non-parasitic antigens (such as ovalbumin) is mediated predominately by Ig G₁ rather than Ig E (Fugner, 1985). A further disadvantage is the small size of the animal which limits multiple sampling of tissue and fluid and repeated measurements of physiological parameters.

3.2 MONKEYS

Although the natural occurrence of allergic airway disease has not been confirmed in monkeys, the rhesus and cynomolgus monkeys are naturally sensitive to *A. suum* antigen and may show immediate skin and airway reactions when challenged with this antigen (Weiszer *et al.*, 1968; Pare *et al.*, 1976; Gundel *et al.*, 1990). These reactions can be retained for several years (Patterson and Kelly, 1974). The trigger for the airway response has been demonstrated as specific Ig E on mast cells (Weiszer *et al.*, 1968; Hogg *et al.*, 1979). In addition, the monkey is the only animal model in which a direct correlation between skin reaction and airway response has been reported (Weiszer *et al.*, 1968). However, this has not been supported by later studies on this model (Pare *et al.*, 1976) and the number of animals used in the initial study was small (Weiszer *et al.*, 1968).

Like guinea pigs, the airway hypersensitivity in monkeys can be studied in both anaesthetised (Kelly *et al.*, 1974; Pare *et al.*, 1976) and conscious states (Hamel *et al.*, 1986). The onset and duration of the airway response after antigen challenge in this species are also similar to that in the guinea pig. The responding monkeys show an increase in airway resistance and respiratory frequency, a fall in dynamic lung compliance, peak expiratory flow rate and tidal volume with increased minute ventilation, and a prolongation of expiration, whereas the changes of total lung capacity, functional residual capacity and residual volume are minor (Patterson and Talbot, 1969; Kelly *et al.*, 1974; Pare *et al.*, 1976; Hogg *et al.*, 1979).

Some squirrel monkeys with a natural skin reaction can also develop a late asthmatic reaction, which usually begins 2-8 hours after antigen challenge and peaks between 4 and 10 hours (Hamel *et al.*, 1986). The late airway reaction in monkeys is identical to that in human asthmatics, occurring mainly in the peripheral airways (Hamel *et al.*, 1986). The late response in monkeys is usually more severe than the early response and is dose-related (Hamel *et al.*, 1986).

One of the original disadvantages of the monkey model was the limited numbers of naturally hypersensitive animals available. The prevalence of rhesus monkeys with allergic bronchoconstriction is estimated to be less than 5% in an unselected population (Patterson and Kelly, 1974). This has now been overcome by the successful sensitisation of laboratory-bred monkeys with *A. suum* antigen (Pritchard *et al.*, 1983; Johnson and Stout, 1989). The airway response induced in these animals is similar to that observed in naturally sensitised animals, and is Ig E-mediated and accompanied by a bronchoalveolar eosinophilia which was initially suspected to be due to parasite infestation rather than an allergic inflammatory reaction (Johnson and Stout, 1989). Recently, Gundel *et al.* (1990) reported that chronic antigen inhalation in monkeys could result in NAHR which is associated with a prolonged airway eosinophilia. Immediate skin and airway hypersensitivity has also been induced in passively sensitised rhesus monkeys (Patterson, 1969).

However, the development of acute allergic airway responses in some monkeys is not associated with mast cell degranulation or histamine release (Fugner, 1985). In contrast to man, guinea pigs and sheep (Kleeberger *et al.*, 1985; Heard *et al.*, 1989), responding monkeys do not show any significant changes in their pulmonary mast cell numbers after exposure to antigen, and the acute response can not be completely prevented by the administration of anti-histamine drugs (Hogg *et al.*, 1979; Fugner, 1985). It is also worth noting that the cost, availability and ethical considerations involved in working with primate models further restrict their use.

3.3 DOGS

The dog is probably the only animal in which a clinical syndrome similar to asthma occurs naturally in pollen-sensitive individuals and this has been used to study human asthma (Patterson, 1960). The clinical signs in the respiratory system of dogs with pollenosis are coughing, dyspnoea, and production of thick ropey mucus (Patterson and Kelly, 1974). The disease is Ig E-mediated and can be passively transferred to normal animals (Patterson and Kelly, 1974). Investigations using this model have in the past provided much useful information in the pathogenesis and treatment of allergic airway disease, particularly in the importance of neurogenic mechanisms in the development of allergic bronchoconstriction (reviewed in Patterson, 1969; Patterson and Kelly, 1974). However, the relative unavailability of dogs with pollenosis has prevented the wide use of this type of model.

Booth *et al.* (1970) found that almost all mongrel dogs tested had various degrees of skin reactivity to *A. suum* antigen. Many of these dogs developed an acute airway response to the initial aerosol challenge with the homologous antigen. The airway response can also be induced in the remaining dogs after repeated exposure to aerosols of *Ascaris* antigen (Patterson and Kelly, 1974). The degree of skin reaction however, does not correlate with the presence or absence of an initial respiratory response (Booth *et al.*, 1970). The presence of *Ascaris*-induced airway hypersensitivity in the dog made this species the most popular large animal asthma model throughout the late 1960's and early 1970's (Patterson and Kelly, 1974; Fugner, 1985).

The acute airway response to *Ascaris* antigen in dogs correlates well with specific Ig E levels in their sera and airway secretions (Patterson and Kelly, 1974), although other studies have suggested that this response is not immunologically mediated and is probably due to a vagal reflex (Gold *et al.*, 1972).

The changes in respiratory function seen in hypersensitive dogs can be measured either under anaesthesia or in a conscious state using a permanent tracheostomy (Dain and Gold, 1975) or non-invasive methods (Lewis *et al.*, 1982). The changes in the respiratory parameters which have been measured generally resemble observations made in the monkey (Patterson and Kelly, 1974).

The Basenji-Greyhound dog has been recently reported to be more satisfactory as an asthma model than other breeds currently used (Hirshman *et al.*, 1980, 1981; Hirshman and Downes, 1981). This breed of dog shows a greater bronchial response (9-20 fold increase in airway resistance compared to 2-3 fold changes in mongrels). The Basenji-Greyhound also

shows NAHR and responds to citric acid and methacholine (Hirshman *et al.*, 1980,1981; Hirshman and Downes, 1981), giving it a potential for use in studying some types of occupational asthma.

Naturally-hypersensitive dogs may also develop a late airway response, which usually begins 6-8 hours after initial antigen challenge and is sustained over 30 hours (Ohruai *et al.*, 1988). In contrast to human asthma, the development of the late response in this species is more closely associated with neutrophil infiltration rather than eosinophils (Ohruai *et al.*, 1988). Nevertheless, the Basenji-Greyhound may still be an excellent model to study the role of eosinophils in the pathophysiology of asthma since eosinophils in the peripheral circulation of this breed are composed of a larger percentage of hypodense eosinophils, compared to those of mongrel dogs (Brown and Hirshman, 1989).

The main disadvantages of the canine model are the inconsistent airway responses to *Ascaris* antigen (Fugner, 1985), the occurrence of sudden death in some animals during antigen challenge (Booth *et al.*, 1970), and the involvement of vagal reflex mechanisms in the response of central airways to antigen (Fugner, 1985). The role of histamine as a mediator of experimental asthma in dogs is not clear. Airway responses to *Ascaris* in dogs, like monkeys, can not be prevented by the administration of cromolyn sodium and H₁-histamine antagonists (Fugner, 1985).

3.4 SHEEP

The sheep model of asthma was originally developed in North America in 1979 (Wanner *et al.*, 1979), and is now used in several other countries including Canada (Bosse *et al.*, 1987) and Japan (Okayama *et al.*, 1989). Most sheep in North America have a natural skin reaction to *A. suum* antigen (Wanner *et al.*, 1979), presumably resulting from previous exposure to pigs infested with roundworms or due to the cross reaction of *Ascaris* antigen with antigens from other nematodes since sheep are not naturally infested with this parasite (Clark *et al.*, 1989). Animals without this reaction can be sensitised experimentally by repeated intramuscular injections of a mixture of *Ascaris* antigen and Freund's adjuvant (Wanner *et al.*, 1979) or by intratracheal infusion of the antigen (Bosse *et al.*, 1987).

Most of the sheep with a positive skin reaction to *Ascaris* antigen show an airway response to initial inhalation challenge with the same antigen (Wanner *et al.*, 1979), although the correlation between skin reactions and airway responses in sheep, as in the most of other animal models, is poor (Booth *et al.*, 1970; Pare *et al.*, 1976; Wanner *et al.*, 1979; Kleeberger *et al.*, 1985; Johnson and Stout, 1989). The airway responses in allergic sheep, as in asthmatics, can be divided into two distinct subtypes: 1) the isolated acute response (acute

response) (Wanner *et al.*, 1979) and 2) the acute response followed by a late response (dual response) (Abraham *et al.*, 1983).

3.4.1 Acute airway responses

Acute airway responses to challenge with inhaled *Ascaris* antigen in sheep usually begin 3-10 minutes after challenge, reach a maximum at 30 minutes and resolve within 2 hours (Wanner *et al.*, 1979). The responding sheep show bronchoconstriction of variable severity with consistent changes in respiratory function, including an increase in airway resistance and functional residual capacity, and a decrease in specific pulmonary conductance, dynamic lung compliance and arterial oxygen tension (Wanner *et al.*, 1979; Abraham *et al.*, 1981; Wanner and Abraham, 1982). The changes in respiratory frequency and tidal volume in this model are more variable than in other animal models (Patterson and Kelly, 1974; O'Byrne *et al.*, 1987), while changes in lung static compliance, and arterial carbon dioxide tension and arterial blood pH value are usually not significant (Wanner *et al.*, 1979). Possible changes in the subdivisions of lung volume have been suspected (Wanner *et al.*, 1979), but are yet to be proven. The changes in respiratory function indicate that acute bronchoconstriction in sheep involves both central and peripheral airways.

Failure to induce acute bronchoconstriction in the *A. suum*-responders by inhalation challenge with ragweed (Wanner *et al.*, 1979), ovalbumin (Dworski *et al.*, 1989) or phosphate buffered saline (Kleeberger *et al.*, 1985; Bosse *et al.*, 1987) suggests that the acute airway response in sheep is antigen-specific and immunologically mediated. A reagin-type antibody, demonstrated in sheep earlier by Hogarth-Scott (1969) is thought to initiate this response (Wanner *et al.*, 1979), but there is not enough evidence to date to confirm that this antibody is Ig E (Miller *et al.*, 1985). Involvement of immunological mechanisms in the development of acute airway response in sheep has also been supported indirectly by the presence of skin reactions to antigens (Wanner *et al.*, 1979), a good correlation between circulating histamine levels and the severity of bronchoconstriction (Wanner *et al.*, 1979), and the blocking of bronchoconstriction by mast cell stabilisers (Abraham *et al.*, 1981).

Mast cell-derived histamine is likely to be the main mediator of acute airway responses in sheep (Wanner *et al.*, 1979; Dworski *et al.*, 1989). Acute bronchoconstriction is accompanied by a transient but significant increase of histamine levels in both arterial plasma and bronchoalveolar lavage (BAL) fluid (Wanner *et al.*, 1979; Dworski *et al.*, 1989). Local challenge of sheep lung with *Ascaris* antigen can induce an immediate release of histamine from mast cells into BAL fluid (Dworski *et al.*, 1989). This has been supported further by numerous pharmacological studies, which have shown that acute airway responses in sheep can be prevented by blocking either the release of mast cell mediators through the administration of

mast cell stabilisers (cromolyn sodium), or by blocking H₁-histamine receptors by using H₁-histamine receptor antagonists (chlorpheniramine) (Abraham *et al.*, 1981; Weissberger *et al.*, 1981; Fugner, 1985). Pretreatment with H₂-histamine receptor antagonists, or anticholinergic agents, on the other hand fails to prevent such responses (Abraham *et al.*, 1981; Weissberger *et al.*, 1981; Fugner, 1985). This failure implies that H₂ receptors in allergic sheep are generally depressed and that the cholinergic reflex mechanisms are not involved in acute responses (Krainson *et al.*, 1981; Ahmed *et al.*, 1983; Ahmed and King, 1986). The acute airway response in sheep can also be completely prevented by pretreatment with glucocorticosteroids or terbutaline, a β_2 -receptor agonist (Abraham *et al.*, 1981; Weissberger *et al.*, 1981; Fugner, 1985).

Mast cell-derived mediators other than histamine may also contribute, to some extent, to the acute airway response in sheep. In their initial studies Wanner *et al.* (1979) noted that about 20% of acute responders showed no changes in arterial plasma histamine levels. Recently, Okayama *et al.* (1989) found that increase in airway resistance did not correlate with the elevation of BAL fluid histamine levels in sheep during either immediate or late responses.

The formation of arachidonic acid in BAL fluid promptly after allergen challenge of sheep has suggested that some products of arachidonic acid may participate in the development of acute airway responses in sheep (Dworski *et al.*, 1989). Release of biologically active cyclooxygenase products, such as prostaglandin D₂ and thromboxane A₂ into BAL fluid of sheep has been demonstrated after local challenge with *Ascaris* antigen (Dworski *et al.*, 1989). Acute responses induced by antigens in sheep can also be blocked by pretreatment with a thromboxane antagonist L-641,953 (Abraham *et al.*, 1987). However, failure to prevent such response after pretreatment with prostaglandin synthetase inhibitors (Abraham *et al.*, 1981; Weissberger *et al.*, 1981; Fugner, 1985) questions the importance of some of these mediators.

The observation that leukotriene D₄ (LT D₄) can induce a cromolyn-reversible acute airway response similar to that induced by *Ascaris* antigen in sheep suggests that some lipoxigenase products may also initiate the release of mast cell mediators (Abraham *et al.*, 1983). Results from BAL fluid analysis of *Ascaris*-responding sheep, however indicate that at least LT B₄ and LT C₄ are unlikely to be involved in the development of acute airway responses (Dworski *et al.*, 1989). The LT D₄ level in BAL fluid was not measured in Dworski *et al.*'s study. In addition, inhalation of slow-reacting substances of anaphylaxis (SRS-A) antagonists (FPL-55712 and FPL-57231) did not affect the magnitude of acute response in sheep (Abraham *et al.*, 1981; Abraham *et al.*, 1983; Lanes *et al.*, 1986), although treatment with FPL-57231 shortened the duration of the response (Lanes *et al.*, 1986). The weight of evidence therefore suggests that leukotrienes are probably not important in the development of acute bronchoconstriction in sheep.

The significance of certain other inflammatory mediators in the development of acute allergic bronchoconstriction in sheep has also been investigated. Administration of platelet-activating factor to allergic sheep, via different routes, induced an acute airway response which was completely abolished by pretreatment with a selective platelet-activating factor antagonist WEB-2086 (Fernandez *et al.*, 1989). Subsequently, Soler *et al.* (1989) found that pretreatment with WEB-2086 can also reduce the severity of acute allergen-induced airway responses, NAHR, and the recruitment of inflammatory cells to BAL fluid in a dose-related fashion or completely block these responses.

Like human asthmatics, allergic sheep show a decrease in tracheal mucus velocity after exposure to antigens (Wanner and Abraham, 1982). *In vitro* studies have further confirmed that *Ascaris* antigen can induce a mediator-dependent mucus hypersecretion and a biphasic change in ion fluxes, initially increasing net absorption and later net secretion of Cl^- and Na^+ , from the trachea of allergic sheep (Phipps *et al.*, 1983). Elevation in the net secretion of ions may cause alterations to the physical properties of mucus secreted and the depth of periciliary fluid.

Although there have been numerous physiological and pharmacological studies, morphological investigations using the sheep model have been limited. The information available indicates that repeated allergic bronchoconstriction in sheep does not produce histological lesions consistent with human asthma (Wanner and Abraham, 1982). The major changes in the pulmonary tissue of allergic sheep are a decrease of ciliated and Clara cell numbers and an increase of basal and intermediate cell numbers in the airway epithelium (Maurer *et al.*, 1981).

3.4.2 Late airway responses

Like asthmatic patients, many allergic sheep exhibit a late bronchoconstriction at about 6-8 hours after initial challenge with inhaled antigens (Abraham *et al.*, 1983; Okayama *et al.*, 1989). The late response in sheep is also antigen specific and immunologically mediated (Abraham *et al.*, 1983), and its magnitude is closely related to the severity of acute response (Wanner and Abraham, 1982; Abraham *et al.*, 1983; Lanes *et al.*, 1986). In contrast to the acute response, the late response occurs predominately in central airways (Abraham *et al.*, 1983). The late response can be pharmacologically modified by the same agents which are effective in human asthmatics (Abraham *et al.*, 1983).

Mechanisms of development in late airway responses of sheep have not been well defined. Studies using pharmacological probes and direct detection of inflammatory mediators

in responders and their BAL fluid have suggested that the late airway response in sheep is also mediator-dependent, and that SRS-A (particularly LT C₄) are the principal mediators (Delehunt *et al.*, 1984; Okayama *et al.*, 1989). The late response can be partially reversed or completely blocked by inhalation of SRS-A antagonists, but not by pretreatment with cyclooxygenase antagonists, or H₁-histamine receptor antagonists alone or with atropine (Delehunt *et al.*, 1984; Lanes *et al.*, 1986). The significance of SRS-A in the development of the late response has been supported by the recent finding that LT C₄ and LT B₄ levels in BAL fluid from sheep with a late response were significantly increased (Okayama *et al.*, 1989). It is also known that LT D₄ can induce a dual response, similar to that induced by *Ascaris* antigen (Abraham *et al.*, 1983), and that sheep with only acute airway responses can develop late responses to inhaled allergens when pretreated with cyclooxygenase inhibitors (Blinder *et al.*, 1987). On the other hand, histamine levels in BAL fluid of dual responders elevate only during the immediate response (Okayama *et al.*, 1989). These findings provide convincing evidence that leukotrienes are the main mediators contributing to the late airway response in sheep.

Mast cell-derived mediators other than leukotrienes may also contribute to the development of late airway responses. The late response can be elicited in allergic sheep after non-immunological mast cell activation by compound 48/80, a mast cell degranulator (Russi *et al.*, 1984). In addition, the late response can be blocked by inhalation with cromolyn sodium and/or glucocorticosteroid before antigen challenge, and with intravenous injection of methylprednisolone 3 hours after antigen challenge (Abraham *et al.*, 1983). Furthermore, the late response is poorly controlled by inhaled β_2 -adrenergic agents (Perruchoud *et al.*, 1983).

The development of late airway responses in sheep is accompanied by an increase in the number of inflammatory cells in BAL fluid (Abraham *et al.*, 1985). Although studies on cellular components in BAL fluid of late responders have produced some useful information, the results of these studies remain inconclusive. A recent study by Abraham *et al.* (1988) indicates that the late airway response in sheep, as in man is associated with a bronchoalveolar eosinophilia, which can be prevented by agents capable of blocking late responses. However, earlier studies in sheep at the same institute have shown that acute responders had a greater number of eosinophils, although not statistically significant, in their BAL fluid than dual responders 24 hours after initial antigen challenge, while the BAL fluid from dual responders contained a slightly higher number of neutrophils (Lanes *et al.*, 1986). More recently, Okayama *et al.* (1989) reported that allergic sheep showed a significant increase in percentage of BAL fluid neutrophils, which was closely correlated with airway resistance during the late response. On the other hand, BAL fluid eosinophils, although increased during the period of immediate response, decreased during the late response (Okayama *et al.*, 1989), a finding which conflicts with those of Abraham *et al.* (1988).

3.4.3 Chronic airway responses

Chronic airway responses, which persist at least 7 days after initial exposure to antigens, have recently been elicited from sheep in Canada (Bosse *et al.*, 1987). These responses are not naturally present in sheep, but may be induced by repeated exposure to intratracheal *Ascaris* antigen. Like acute and dual responders, these sheep show significant changes in their pulmonary resistance, dynamic lung compliance, residual volume, functional residual capacity and arterial oxygen tension. In contrast to acute and dual responses, chronic airway responses affect predominantly peripheral airways. The mechanisms of their development in sheep are still poorly understood. Histamine levels in the BAL fluid of chronic airway responders are nearly double those in antigen-exposed non-responders. These sheep also show a significant increase in the number of eosinophils and neutrophils in their BAL fluid (Bosse *et al.*, 1987).

3.4.4 Non-specific airway hyperresponsiveness

Non-specific airway hyperresponsiveness is a characteristic feature of human asthma and is thought to be one of the major contributors to the severity and duration of a patient's symptoms (Cockcroft *et al.*, 1977). Allergic sheep with acute (Wanner and Reinhart, 1978) or dual responses (Lanes *et al.*, 1986) to *Ascaris* antigen show an increase in NAHR, in a manner analogous to asthmatics, after provocation with inhaled histamine (Ahmed *et al.*, 1983), carbachol (Abraham *et al.*, 1981) or methacholine (Wanner and Reinhart, 1978). The lung parenchyma from such sheep also shows a significantly greater contraction in the presence of histamine and thromboxane A₂ analogue than that from non-allergic sheep (Wagner *et al.*, 1985).

The mechanism of the development of NAHR in sheep is not clear. Although some evidence suggests that only dual responders develop NAHR, the development of allergen-induced NAHR is probably independent of the development of a late increase in airway resistance or the severity of pulmonary inflammation (Lanes *et al.*, 1986). Results from pharmacological studies have suggested that different mechanisms are involved in the development of late airway responses and the development of NAHR in sheep, since NAHR can be prevented by both a cyclooxygenase inhibitor (indomethacin), which has no effect on late response, and a SRS-A antagonist FPL-57231, which causes a blockade of late airway responses (Lanes *et al.*, 1986). In contrast, inhalation with glucocorticosteroid, a blockade of both early and late airway response, does not prevent NAHR (Lanes *et al.*, 1986). These studies have also suggested that development of NAHR in sheep may be dependent on both cyclooxygenase and lipoxygenase products of arachidonic acid metabolism (Lanes *et al.*,

1986). The development of NAHR in sheep has also been suggested to be associated with a functional depression of dilatory H₂-histamine receptors (Kleeberger *et al.*, 1985). However, the importance of other factors, such as the parasympathetic pathway and local infiltration of inflammatory cells, in the NAHR in sheep is still not well established (Kleeberger *et al.*, 1985; Lanes *et al.*, 1986). Lanes *et al.* (1986) found that there were no significant differences in the cellular components of BAL fluid 24 hours after antigen challenge between sheep with and without NAHR.

3.5 CONCLUSION

Allergic bronchoconstriction has been extensively studied in animal models in order to elucidate the pathogenesis of human asthma and improve its treatment. The methodology used in antigen preparation and delivery, and measurements of respiratory function in these animal models has been very similar. The magnitude and time course of the response, the sites of acute airway response, and the response to β -agonists after antigen challenge has been comparable to that of human asthmatics although some notable differences are present both between the animal models and human asthmatics and between the animal models themselves (Tables 3.1 and 3.2).

Among the animal asthma models, the sheep is thought to be one of the most satisfactory, since allergic airway responses are easily reproduced and more consistent than those observed in dogs and guinea pigs (Wanner and Abraham, 1982). Allergic bronchoconstriction in sheep shares many similarities to human asthma. The changes observed in respiratory function (respiratory mechanics, lung volume and gas exchange), the presence of NAHR and the response to a variety of pharmacological agents are similar in both species. In addition, the ovine lung shows a poor collateral ventilation; a feature it has in common with the human lung. Because of the sheep's size, the measurement of multiple physiological parameters, the sampling of tissue and body fluid for analysis of inflammatory mediators at regular intervals and the use of the same animal many times over extended periods are possible. The use of the sheep is therefore preferable to laboratory animals in the study of many physiological aspects of allergic bronchoconstriction and particularly for long-term observations. However, a better knowledge of the characteristics of ovine respiratory immunity is needed to fully define the relationship between this model and allergic asthma in man.

Studies on the sheep model indicate that airway responses to inhalation challenge with *Ascaris* antigen are mediated by mast cell derived-mediators. Histamine is the major contributor in the acute response whereas leukotrienes are the key mediators involved in the late response. However, most studies using this model have so far focused on investigating the significance of inflammatory mediators in the development of allergic bronchoconstriction.

Factors responsible for any inherent differences in airway response between individual animals and the mechanisms which make one individual more responsive than another are difficult to study in man and have been generally ignored. In addition, morphological studies of the immune-associated tissue and cellular components in the ovine model have been limited. Information on these aspects would therefore be useful in order to gain a better understanding of the pathogenesis of allergic airway disease and may be relevant to the prevention and treatment of human asthma.

It is worth noting that the respiratory function tests commonly used for human asthmatics are based on forced expiratory maneuvers, such as forced vital capacity, forced expiratory volume in 1 s and peak expiratory flow rate. These are obviously effort-dependent and difficult, although not impossible, to measure in animal models (Holroyde *et al.*, 1980). The respiratory parameters measured in animal models are usually airway resistance, dynamic lung compliance and functional residual capacity which are relatively crude parameters and give only a gross indication of major changes in airway dynamics. For this reason it is difficult to compare the results of human and animal studies as such measurements are not used routinely in most studies of human asthma.

The inherent differences in airway response between different animal species outlined above are important considerations when using these models. Spontaneous and persistent bronchoconstrictions, which are typical features of human asthma, are not present in most animal models discussed, including sheep. These animals are therefore only useful models of antigen-induced bronchoconstriction and do not exhibit the full spectrum of changes in human asthma.

PURPOSE OF THE PRESENT STUDY

The present study was carried out in order to evaluate the suitability of New Zealand Romney sheep as a model to study human asthma and to determine whether or not airway morphological and inflammatory factors are associated with a predisposition to develop allergic airway hypersensitivity in this species. Special attention has been paid to the following objectives:

- * to study the distribution and histological features of lymphoid tissue in the respiratory tract of conventionally raised sheep;
- * to examine the morphology and perinatal development of the lymphoid tissue and its associated epithelium of the ovine pharyngeal tonsil, and to investigate the ability of this structure to take up intra-luminal particulate material;
- * to study the numerical density, morphological and histochemical heterogeneity of mast cells in the ovine lower respiratory tract;
- * to investigate the natural skin and immediate airway responses of locally bred Romney sheep to inhaled *Ascaris suum* antigen, and to evaluate their suitability to study allergic airway hypersensitivity;
- * to examine the inherent differences in mucosal permeability of the tracheobronchial epithelium in hypersensitive and non-reacting sheep;
- * to identify and compare quantitatively any inherent differences in airway tissue structure between hypersensitive and non-reacting sheep;
- * to measure and calculate the airway dimensions and the muscle shortening required to occlude the airway lumen in hypersensitive and non-reacting sheep;
- * to study inherent differences in the numerical profiles of inflammatory cells, particularly mast cells and eosinophils, in peripheral blood and the lower respiratory tract between hypersensitive and non-reacting sheep.

PART II
MORPHOLOGICAL STUDIES
OF IMMUNE-ASSOCIATED TISSUE AND CELLS
IN THE RESPIRATORY TRACT OF CONVENTIONALLY RAISED SHEEP

CHAPTER 4

RESPIRATORY TRACT-ASSOCIATED LYMPHOID TISSUE IN CONVENTIONALLY RAISED SHEEP

4.1 INTRODUCTION

Since Bienenstock *et al.* (1973a) called attention to the presence of subepithelial lymphoid tissue (bronchus-associated lymphoid tissue, BALT) in the respiratory tract of laboratory animals and man, morphological and functional studies of lymphoid tissue in the respiratory system have been performed by many investigators in a number of laboratory and domestic animals (Bienenstock, 1985; Anderson *et al.*, 1986; Mair *et al.*, 1987). These studies have shown that the respiratory mucosa of all the species investigated has lymphoid tissue in intraluminal, intraepithelial, and subepithelial sites. It may be diffuse or organised as dense aggregates or nodules. This respiratory tract-associated lymphoid tissue (RTALT) is now considered an integral part of the local immune system of the respiratory mucosa, involved in uptake, transport and presentation of antigen to cells of the immune system (McDermott *et al.*, 1982; Blazquez *et al.*, 1987).

The increasing use of sheep as a model to study human allergic respiratory disease (Wanner and Abraham, 1982) requires better understanding of the local immune system in this species. The present study investigated the distribution and histological features of lymphoid tissue in the respiratory tract of normal conventionally raised sheep.

4.2 MATERIALS AND METHODS

Respiratory tracts without gross lesions of respiratory disease were collected from 20 Romney sheep, aged from 6 months to 9 years. All the sheep were healthy prior to euthanasia, and were not known to have any previous signs of respiratory disease. The animals were killed by intravenous injection of pentobarbitone and the upper respiratory tract was disconnected from the lower respiratory tract by cutting through the trachea immediately behind the larynx. The upper respiratory tract was immersed in 10% neutral buffered formalin for 24 hours fixation and the lower respiratory tract was fixed as a whole by intratracheal perfusion with the same fixative for 24 hours. Tissue blocks up to 2 cm x 1 cm x 0.5 cm in size were taken from 19 different sites in the tract (Figs. 4.1 and 4.2). They were processed routinely for histology and embedded in paraffin wax. Sections were cut 5 µm thick and stained with haematoxylin, eosin and alcian blue at pH 2.5 (HE/AB). Selected sections were further stained by periodic acid-Schiff (PAS), toluidine blue (pH 1.5), methyl green-pyronin and Giemsa (Culling *et al.*, 1985). To study carbon uptake by RTALT, an aerosol of 10 ml colloidal carbon (Pelikan c11/1431a, Gunther Wagner, Germany) prepared as described by Naukkarinen and Sorvari

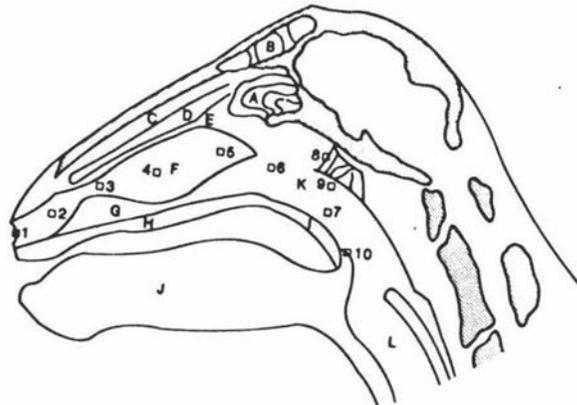


Fig. 4.1 Diagram of paramedial sagittal section through sheep's head. □ = tissue sampling sites; 1=nostril; 2=nasal vestibule; 3=cranial concha; 4=mid concha; 5=caudal concha; 6=anterior nasopharynx; 7=mid nasopharynx; 8=pharyngeal tonsil; 9=opening of auditory tube; 10=epiglottis. A=ethmoid conchae; B=frontal sinus; C=dorsal nasal meatus; D=dorsal nasal concha; E=middle nasal meatus; F=ventral nasal concha; G=ventral nasal meatus; H=hard palate; I=soft palate; J=lower jaw; K=nasopharynx; L=trachea.

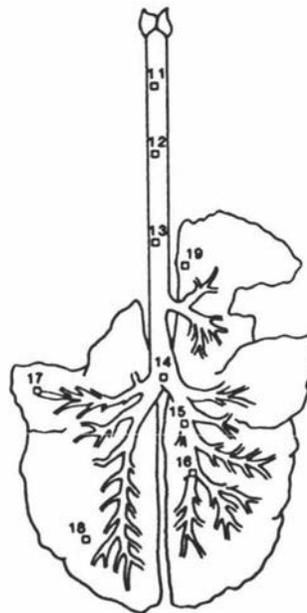


Fig. 4.2 Diagram of ovine lower respiratory tract showing the tissue sampling sites (□).
 11=upper trachea; 12=mid trachea; 13=lower trachea; 14=major bronchus; 15=lobar bronchus A; 16=lobar bronchus B; 17=medium bronchus; 18=bronchioles and lung; 19=lung and pleura.

(1984) was administered intranasally to two healthy adult sheep. The sheep were killed 30 and 60 minutes after administration of carbon and tissues were processed as described above.

4.3 RESULTS

The RTALT from all animals examined showed a similar form of distribution but varied in the amount between regions and individuals (Table 4.1). Based on its morphology, the lymphoid tissue could be divided into the following five forms:

4.3.1 Nodular aggregations of lymphoid cells

Lymphoid nodules structurally similar to those in peripheral lymph nodes and other mucosa-associated lymphoid tissues (MALT) (Racz *et al.*, 1977; Owen and Nemanic 1978; Naukkarinen and Sorvari 1984) were observed in the pharyngeal tonsil, at the opening of the auditory tube and in the mid nasopharynx. The nodules in ten sheep less than 2-years old were generally more developed than those in aged animals. On the basis of their histological characteristics, these nodules could be divided into three different areas: a) the dome area underlying the mucosal epithelium; b) the follicular area lying under the dome; and c) the parafollicular areas (Fig. 4.3).

The epithelium overlying the dome was flat, devoid of goblet cells and frequently heavily infiltrated from below with lymphocytes and occasionally other mononuclear cells (Fig. 4.4). It was easily distinguished from the adjacent respiratory epithelium, especially when the sections were stained with alcian blue and PAS. The continuity of the basement membrane of the overlying epithelium was often interrupted by lymphocytes.

In the dome and parafollicular areas, plasma cells and small to medium-sized lymphocytes were the predominant cell types. The plasma cells were particularly numerous in the dome area where high endothelial venules were occasionally present. At the base of the follicles, small to medium-sized efferent lymphatics, often occluded by small and medium-sized lymphocytes, were found.

Most lymphoid follicles underlying the dome were secondary follicles and contained a germinal centre which was more or less completely surrounded by a mantle zone consisting of densely packed small lymphocytes with dark staining nuclei (Fig. 4.3). Medium to large lymphocytes as well as macrophages were the predominant cells of the germinal centre. Small numbers of lymphoid cells showing mitotic figures and scattered macrophages containing phagocytosed cellular debris were often present.

Table 4.1
Distribution of Different Forms of Lymphoid Tissue
in the Ovine Respiratory Tract[#]

	Intraluminal lymphoid cells	Intraepithelial lymphoid cells	Scattered lymphoid cells	Dense aggregation	Nodular aggregation
Nostril	-	+	+	-	-
Nasal vestibule	-	+	+	-	-
Nasal conchae	-	±	+	-	-
Anterior nasopharynx	-	+	++	±	-
Mid nasopharynx	-	++	++	+	±
Pharyngeal tonsil	++	+++	+++	+++	+++
Auditory tube	-	++	+++	++	+
Epiglottis	-	±	±	-	-
Trachea	+	+	++	±	-
Bronchus	±	±	+	±	-
Bronchioles	±	±	++	+++	-

[#] Results from 20 sheep studied. +++=extensive, ++=moderate, +=a few, ±=rare and -=none.

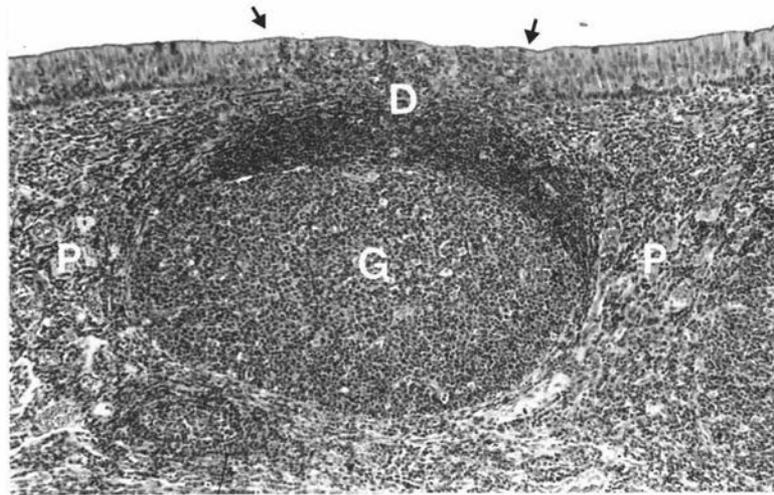


Fig. 4.3 Isolated lymphoid nodule in the pharyngeal tonsil consisting of germinal centre (G), dome area (D), and parafollicular region (P). The lymphoepithelium overlying the nodule is flattened and heavily infiltrated with lymphocytes (between arrows). HE/AB, x50.

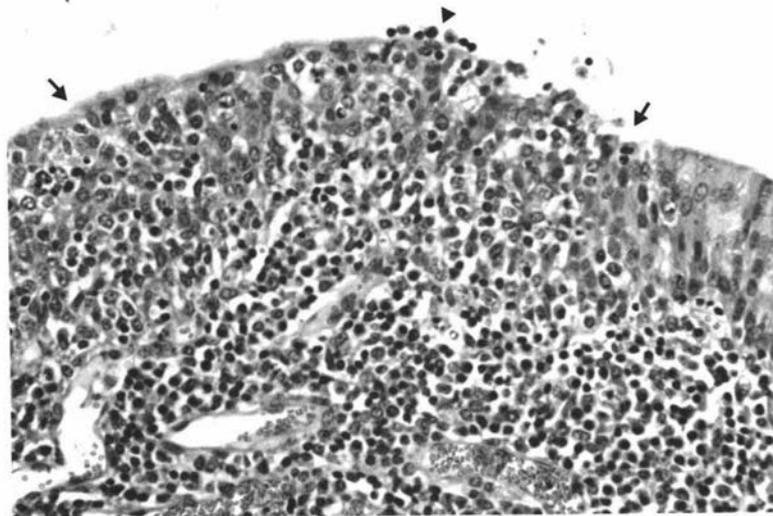


Fig. 4.4 Lymphoepithelium in the pharyngeal tonsil. The pseudostratified epithelium infiltrated with lymphocytes is devoid of goblet cells and cilia (between arrows). The basement membrane is partly disrupted by lymphocytes. Intraluminal lymphoid cells (arrowhead). HE/AB, x150.

4.3.2 Dense aggregations of lymphoid cells

These varied in size from small collections of mononuclear cells to large islands. They were present in the mid nasopharynx, pharyngeal tonsil, about the opening of the auditory tube, as well as around the bronchi and bronchioles and occasionally associated with the trachea and anterior nasopharynx. In the lower respiratory tract, they were most pronounced in the airways in which cartilaginous support was meagre or absent. In the wall of the bronchi and bronchioles the location of the aggregated lymphoid tissue was random. Aggregations were frequently encountered beneath the muscularis (Fig. 4.5) or assumed a "collar stud" appearance as described by Bienenstock *et al.* (1973a) (Fig. 4.6). The aggregated cells were predominantly mononuclear cells of the lymphocyte-plasma cell series, sometimes with a few mast cells. The epithelium overlying the aggregates was normal and unspecialised. Small lymphatics were often present in the areas adjacent to the large aggregates.

4.3.3 Scattered lymphoid cells

This form of lymphoid tissue was universally present in the lamina propria and submucosa throughout the respiratory tract. It consisted of predominantly small lymphocytes and often had an admixture of small numbers of plasma cells and mast cells. Occasionally, eosinophils and neutrophils were also present. The cells were most numerous in the lamina propria adjacent to dense lymphoid aggregates and lymphoid nodules (Fig. 4.7), in regions surrounding the submucosal glands (Fig. 4.8) and immediately beneath the mucosal epithelium (Fig. 4.9).

4.3.4 Intraepithelial lymphoid cells (IELC)

These were seen throughout the respiratory mucosa (Fig. 4.10), although they varied in number from region to region. The IELC consisted of lymphocytes and there was frequently an association between IELC and intraepithelial macrophages and mast cells. In addition, small numbers of neutrophils and eosinophils were often mixed with IELC in some animals. The IELC were most numerous in the epithelium overlying dense lymphoid aggregates and lymphoid nodules such as those of the pharyngeal tonsil, or at the opening of the auditory tube and mid nasopharynx. They were less common in the tracheobronchial epithelium and the epithelium of the epiglottis and rare in the epithelium overlying the nasal conchae. Although IELC could be located in all planes of epithelium, they were present most commonly in areas immediately above the basal cell layer. However, in the pharyngeal tonsil, in those areas where lymphoepithelium was present, many IELC were in close contact with the epithelial surface.

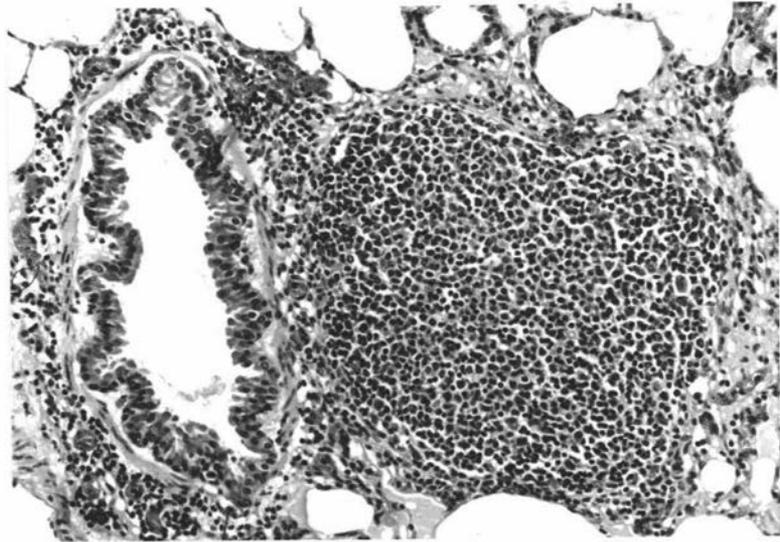


Fig. 4.5 Dense aggregation of lymphoid cells in the submuscular tissue of a bronchiole. Note that the epithelium overlying the aggregate is unspecialised HE/AB, x100.

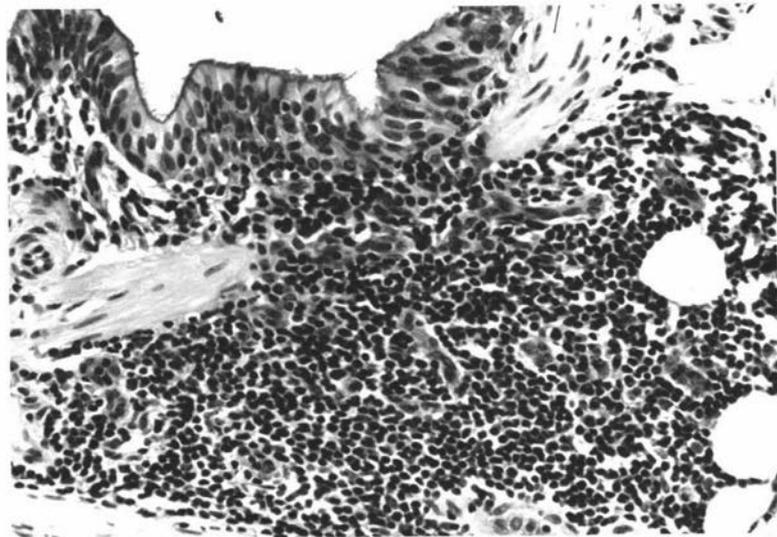


Fig. 4.6 Dense lymphoid aggregate within the lamina propria of a bronchus showing "collar stud" appearance with the major aggregate below the muscularis and a narrow neck of lymphoid cells passing between adjacent muscular bundles. HE/AB, x150.

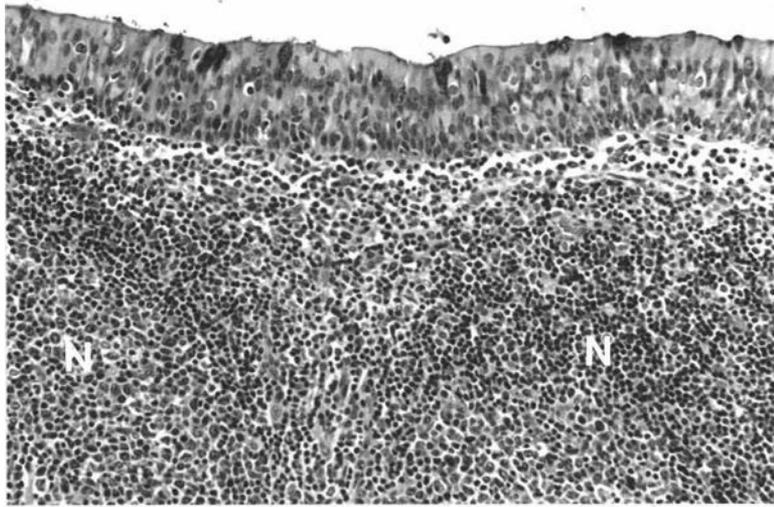


Fig. 4.7 Scattered lymphoid cells in the lamina propria adjacent to lymphoid nodules (N) in the mid nasopharynx. HE/AB, x100.

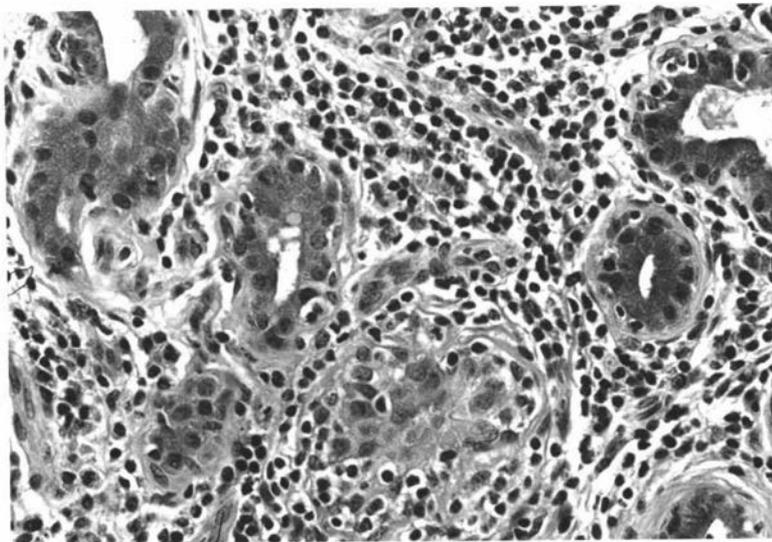


Fig. 4.8 Scattered lymphoid cells in the region surrounding the submucosal glands of the trachea. The cells are mainly lymphocyte-plasma cell series. HE/AB, x200.

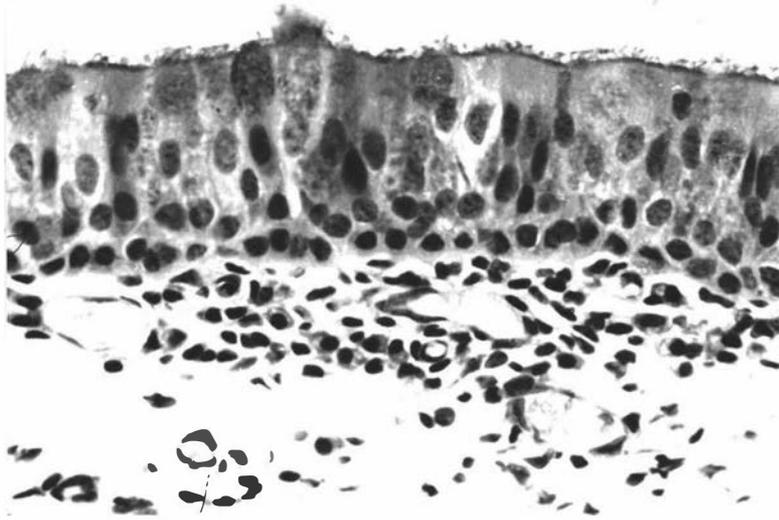


Fig. 4.9 Scattered lymphoid cells in the lamina propria of the trachea. HE/AB, x300.

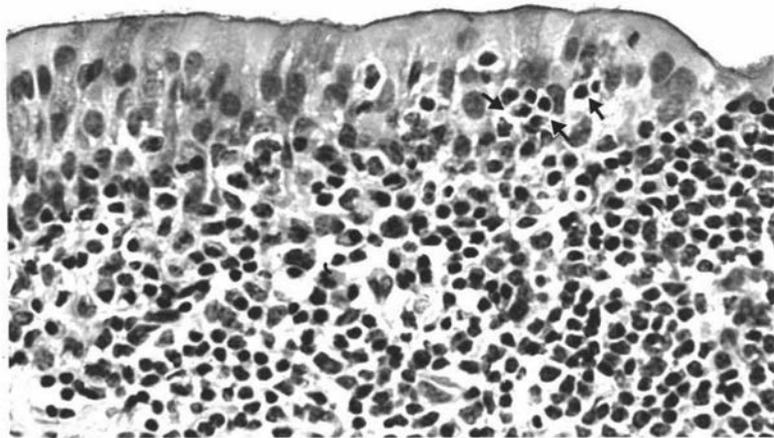


Fig. 4.10 Intraepithelial lymphoid cells (arrows) in the epithelium of the mid nasopharynx.
HE/AB, x125.

4.3.5 Intraluminal lymphoid cells

Intraluminal location of lymphoid cells was not an outstanding feature in the ovine respiratory tract. It was most prominent on the epithelial surface of the pharyngeal tonsil (Fig. 4.4) and at the opening of the auditory tube region. It was also occasionally seen in the lumen of the trachea, bronchi, and large bronchioles. The cells were mainly small lymphocytes and some showed moderately swollen cytoplasm with a wrinkled cell membrane. They were either free in the lumen or on the ciliary surface. In the pharyngeal tonsil region, the intraluminal lymphocytes were usually mixed with small numbers of neutrophils and some cellular debris.

In sheep killed 30 and 60 minutes after the administration of colloidal carbon, large amounts of carbon were found in the lumina of the small bronchi, bronchioles and alveolar spaces as well as in the cytoplasm of alveolar macrophages located either in the pulmonary interstitium or free in alveolar spaces. In addition, moderate amounts of carbon were adherent to the cilia of the airway mucosa. However, carbon uptake by the respiratory epithelium was demonstrated only in the pharyngeal tonsil where carbon particles were observed in the cytoplasm of the lymphoepithelium but not in the adjacent respiratory epithelial cells (Fig. 4.11). Very small numbers of carbon particles could also be found in the dome area.

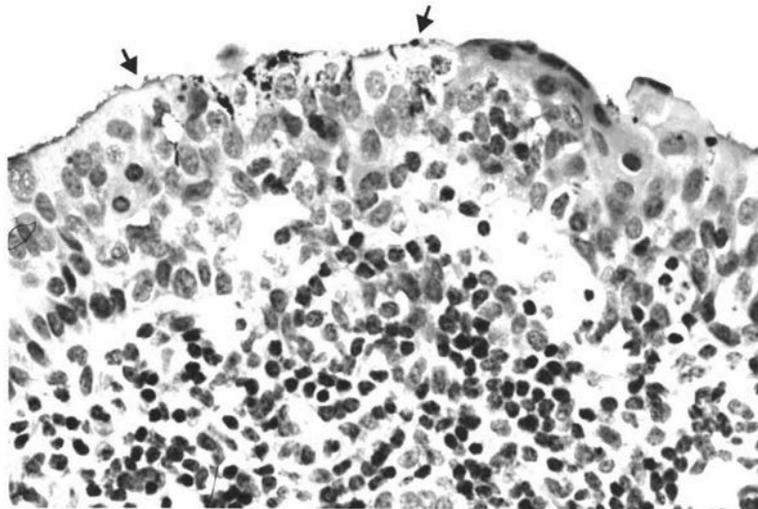


Fig. 4.11 Presence of small amounts of carbon (between arrows) in the lymphoepithelium of the pharyngeal tonsil. Sheep killed 30 minutes after carbon aerosol. HE, x250.

4.4 DISCUSSION

The distribution and morphology of RTALT in sheep has not been systematically investigated previously, although proliferation of lymphoid tissue is commonly present in ovine lungs with chronic pneumonia (Stamp and Nisbet, 1963). The present study has shown that RTALT is universally present in healthy sheep, but its distribution varies between regions of the tract and individuals. The variation observed in the current study is probably due to the use of conventional animals of varying ages. These are likely to have had a differing history of respiratory exposure to antigenic and infectious agents which have the potential to cause differing effects on the RTALT.

Although five forms of lymphoid tissue which are morphologically similar to those seen in other species (Mair *et al.*, 1987) have been identified, the most predominant form in the ovine respiratory tract is widely scattered lymphoid cells. Compared to most species (Bienenstock *et al.*, 1973a; Loo and Chin 1974; Bienenstock and Johnston 1976; Mair *et al.*, 1987), the respiratory tract of healthy sheep possesses relatively few dense lymphoid aggregates and lymphoid nodules. The few reports available indicate that domestic animals are likely to be relatively deficient in well-organised lymphoid tissue in the large airways (Joel and Chanana 1985; Anderson *et al.*, 1986; Mair *et al.*, 1987). This may reflect low antigen contact under normal circumstances. Recent work cited by Plesch *et al.* (1983) showed that if the afferent lymphatics of a lymph node were cut, the T and B cell areas became poorly defined, whereas they reappeared when antigen was injected into the node. The pilot work, conducted by Joel and Chanana (1985), also revealed that the lymphoid tissue was more organised in antigen challenged ovine lungs than in unchallenged lungs. However, dense aggregations of lymphoid tissue were frequently seen in the bronchiolar regions of ovine lungs. This is similar to the findings in both bovine and equine lungs (Anderson *et al.*, 1986; Mair *et al.*, 1987). The elevated concentrations of lymphoid tissue associated with bronchioles could be related to increased antigenic stimulation in these areas because of the paucity of a protective mucous layer and the relative deficiency of ciliated cells.

The lymphoid tissue seen in normal ovine lungs usually lacked an overlying lymphoepithelium, an observation which disagrees with the hypothesis of Bienenstock *et al.* (1973b) that lymphoepithelium is a necessary adjunct to BALT. Although the present study could not completely exclude the possibility that lymphoepithelium might be missed because of the plane of section, examination of large numbers of tissue sections from different levels of the lungs suggests this possibility unlikely. The studies of Gregson *et al.* (1979a) and Anderson *et al.* (1986) have shown that readily identifiable lymphoepithelium is not ubiquitous in the lower respiratory tract in either specific pathogen-free rats, conventional rats or conventional cattle. Their studies showed that even in adult conventional animals several

lymphoid follicles could be found covered only by normal respiratory epithelium. The lymphoid tissue described in this study may therefore represent an early stage in the developing BALT which may subsequently mature as a result of further antigenic stimulation.

Although the full functions of BALT are not yet determined, its role both in sampling luminal antigens for the initiation of local immune response and as a source of immunoglobulin A (Ig A) immunoblasts for mucosal secretory defence has been demonstrated experimentally (McDermott *et al.*, 1982; Bienenstock, 1985). Immunoglobulin A has been shown to be the major immunoglobulin in the respiratory secretions of sheep (Smith *et al.*, 1975) and Ig A-containing cells predominate in the bronchial mucosa and lung in this species (Alley *et al.*, 1980). Hence, a deficiency of BALT in the ovine respiratory tract could be expected to result in inadequate numbers of Ig A cell precursors produced in this area. This is supported by the work of Scicchitano *et al.* (1984) who found that BALT is not a major site for producing Ig A precursor cells in sheep. The possibility that a major portion of the Ig A plasma cell population in the ovine respiratory mucosa originates from other mucosal sites such as the pharyngeal tonsil should therefore be considered.

The pharyngeal tonsil was one of the major tissues in which morphologically well-developed lymphoepithelium and subepithelial lymphoid nodules were observed in the current studies. The lymphoid tissue in nasopharyngeal regions of cattle (Anderson *et al.*, 1986) and man (Surjan, 1987) are believed to be an important source for supplying Ig-producing cells to other mucosal sites including the tracheobronchial tree. Future studies should be therefore designed to investigate the association of morphological and functional alterations of this tissue when challenged with pulmonary antigen via the respiratory route.

Although much more limited in distribution, the lymphoepithelium in sheep is histologically similar to that found overlying BALT and other MALTs in a number of species including man (Bienenstock *et al.*, 1973a; Bockman and Cooper, 1973; Fournier *et al.*, 1977; Owen and Nemanic, 1978; Naukkarinen and Sorvari, 1984; Anderson *et al.*, 1986; Mair *et al.*, 1987). The lymphoepithelium has been demonstrated to be able to take up and transport particulate material in the current study and some other studies (Richardson *et al.*, 1976; Tenner-Racz *et al.*, 1979; Naukkarinen and Sorvari, 1984), and thus it provides a means of direct contact between luminal antigens and immunocytes in the subepithelial lymphoid tissue.

The presence of intraluminal lymphoid cells in some levels of the respiratory tract is not readily explained. Chu *et al.* (1979) have proposed that gut IELC are mainly migrating from the underlying lamina propria. The IELC entering the respiratory mucosa could account for the lymphocytes seen in bronchoalveolar lavage which according to Burrells (1985) are mainly T lymphocytes. Any specific immunological functions they may have remain undefined.

This work together with that of Anderson *et al.* (1986) indicates that RTALT is less abundant in cattle and sheep than in most other animals so far studied. Further observations on the ultrastructure and morphological development of RTALT in relation to age and stimulation by different types of antigen are needed to better understand its functions and to explain within species and between species variations in morphology.

4.5 SUMMARY

The distribution and morphological features of respiratory tract-associated lymphoid tissue were investigated in 20 clinically healthy sheep aged from 6 months to 9 years. Five different forms of lymphoid tissue were identified, but their distribution varied between regions of the respiratory tract and between individuals. Scattered lymphoid cells were the most predominant form. Dense lymphoid aggregations were frequently seen in the pharyngeal tonsil and bronchioles. They were less common in the mid nasopharynx and about the opening of the auditory tube, occasionally seen in the anterior nasopharynx, trachea and bronchus and rarely found in the regions of the nasal conchae, nostril, nasal vestibule and epiglottis. Nodular lymphoid aggregations with morphologically distinct lymphoepithelium were seen only in the pharyngeal tonsil and opening of the auditory tube region. Small numbers of intraepithelial lymphoid cells were always present throughout the mucosa, but intraluminal lymphocytes were observed mainly in the regions of pharyngeal tonsil, trachea, bronchi and large bronchioles. The respiratory tract-associated lymphoid tissue is less developed in sheep than in most other species studied except perhaps in cattle.

CHAPTER 5

THE POTENTIAL ROLE OF THE OVINE PHARYNGEAL TONSIL IN RESPIRATORY TRACT IMMUNITY: A SCANNING AND TRANSMISSION ELECTRON MICROSCOPIC STUDY OF ITS EPITHELIUM

5.1 INTRODUCTION

Both morphological and functional studies of gut- and respiratory tract-associated lymphoid tissues (GALT and RTALT) have shown that the palatine and pharyngeal tonsils are important in the mucosal immunity of these systems (Loo and Chin, 1974; Owen and Nemanic, 1978; Anderson *et al.*, 1986; Surjan, 1987; Chapter 4). Functionally, tonsils contain both T and B lymphocytes and produce all five classes of immunoglobulin (Ig) with Ig G and Ig A most prominent (Ishikawa *et al.*, 1972; Korsrud and Brandtzaeg, 1980; Yamanaka *et al.*, 1983). In addition, the pharyngeal tonsil also contains secretory component in its epithelium (Mogi, 1975). Human patients with adenectomy and tonsillectomy show a depression of the production of secretory Ig A antibodies to poliovirus in nasopharyngeal washings (Ogra, 1971). However, most knowledge of the ultrastructure of tonsils has come from studies of palatine tonsils (Olah and Everett, 1975; Owen and Nemanic, 1978; Howie, 1980; Korsrud and Brandtzaeg, 1980), and the results of these studies indicate that specialised structures related to their function as a part of mucosa-associated lymphoid tissues are well developed (Olah and Everett, 1975; Owen and Nemanic, 1978; Howie, 1980). Studies of the relationship between the pharyngeal tonsil and mucosal immunity have been generally neglected in both man and animals (Hiriade and Nomura, 1974; Lenz, 1974; Owen and Nemanic, 1978).

Because sheep are now being promoted as a suitable species for the study of human allergic asthma (Wanner and Abraham, 1982), knowledge of local mucosal immunity-associated tissue in this species is of considerable importance to advance our understanding of this disease. The initial studies of RTALT in healthy sheep (Chapter 4) have shown that the pharyngeal tonsil is the only site where a histologically well-developed follicle-associated lymphoepithelium (FAE) is present. It has also been demonstrated experimentally that the epithelium of the pharyngeal tonsil is capable of taking up luminal particulate material. The objective of the present study was to extend this information and look in particular at the lymphoepithelium of the pharyngeal tonsil of normal conventionally raised sheep.

5.2 MATERIALS AND METHODS

Ten healthy Romney-cross sheep, aged from 8 months to 6 years without a previous history of respiratory disease were killed by intravenous injection of pentobarbitone. The pharyngeal tonsil was carefully removed immediately and rinsed with cold 0.1 M phosphate buffered saline to remove superficial mucus and cellular debris. For scanning electron microscopy (SEM), large blocks of 0.5 cm x 0.5 cm x 0.5 cm were taken from the caudal, middle and cranial regions of the tonsil and fixed in Karnovsky's fixative at 4 °C for 48 hours (Karnovsky, 1965). They were then washed in two changes of cold phosphate buffered saline for 1 hour, dehydrated in a series of graded ethanol, critical point dried, placed on aluminium stubs, coated with gold and examined in a Cambridge 250 MK III scanning electron microscope. Additional small tissue blocks were collected for transmission electron microscopy (TEM) from the adjacent areas. These were fixed in Karnovsky's fixative at 4 °C for overnight, then processed as described previously (Chen *et al.*, 1988) and examined under a Philips EM201c transmission electron microscope. In addition, two transverse sections from the cranial and caudal parts of each pharyngeal tonsil were collected and fixed in 10% neutral buffered formalin. They were processed routinely and embedded in paraffin wax. Sections for histology were cut 4 µm thick and stained with haematoxylin and eosin (HE).

5.3 RESULTS

5.3.1 Scanning electron microscopy

The surface of the pharyngeal tonsil was crossed by deep furrows which were often occluded with varying amounts of mucus, mixed inflammatory cells and cellular debris. The epithelium overlying lymphoid follicles (FAE) and the surface furrows had a flattened and stretched appearance, making it distinct from that covering non-follicular regions (Fig. 5.1). Cells in the FAE were predominately non-ciliated but had variable numbers of microvilli or microfolds. The cells with microvilli could be generally divided into two types; tall or flattened.

The tall type of microvillous cell was cuboid or low columnar and distributed either singly or in nests. When single, the cells were usually located at the junction of ciliated and non-ciliated epithelium or were scattered among ciliated cells. The microvilli on these cells were usually tall and densely packed (Fig. 5.2). When present in nests, these cells often bulged from the external surface, forming many deep intercellular cervices between adjacent cells (Fig. 5.3). The microvilli on these cells were usually regular and thick, but less densely packed.

Flattened types of microvillous cells were also common in the FAE, and were invariably found closely knitted together. These cells were usually polygonal and had ledge-like cytoplasmic elevations around their borders. The surface of the cells was more variable,

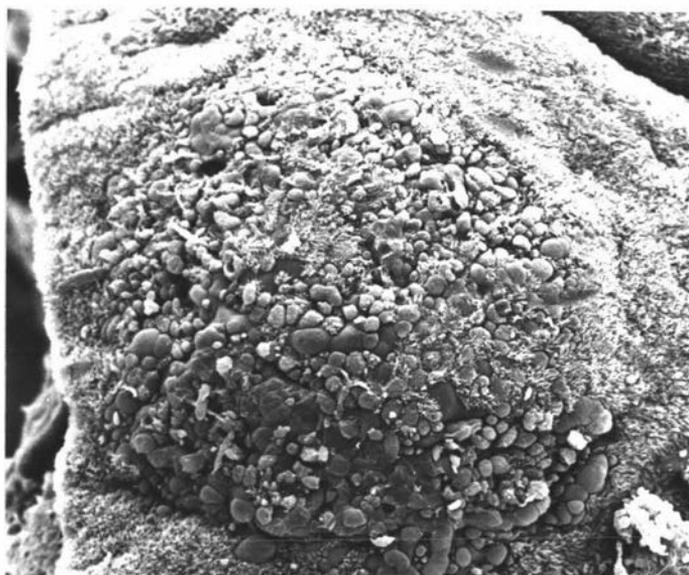


Fig. 5.1 Mucosal epithelium of the pharyngeal tonsil from an 8-month-old sheep showing an island of follicle-associated epithelium which is distinct from the adjacent ciliated epithelium. SEM, x270.

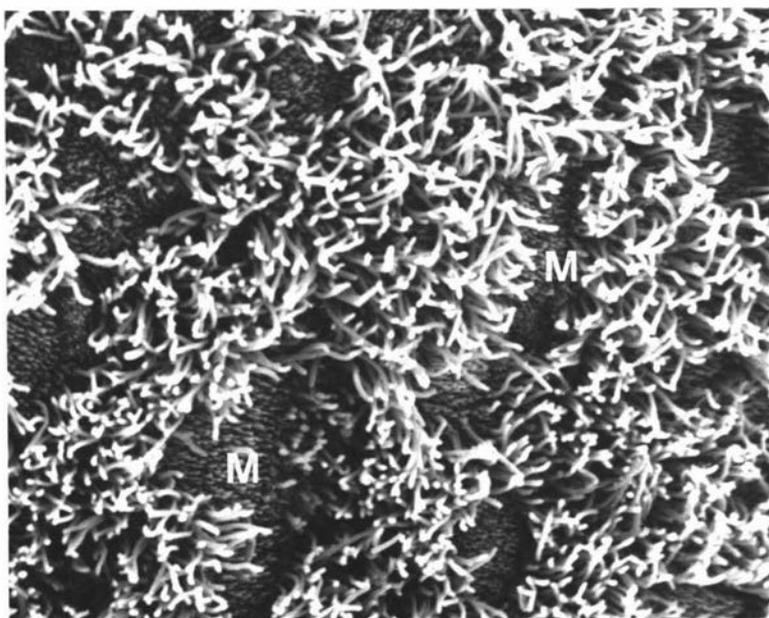


Fig. 5.2 Singly distributed microvillous cells (M) in the mucosal epithelium of the pharyngeal tonsil of an 8-month-old sheep. Microvilli on these cells are usually tall and densely packed. SEM, x3,100.

ranging in appearance from densely packed, very short microvilli (Fig. 5.4), and knob-like microvilli (Fig. 5.5), through to a cobblestone pattern of cytoplasmic elevations (Fig. 5.6) and irregular, ridge-like microplacae (Fig. 5.7). Occasionally, intermediate cells with both microvilli and scattered cilia (Fig. 5.5) were found in these areas.

Non-keratinised squamous epithelial cells were also found, and they were usually mixed with microvillous cells in the FAE or distributed in the areas containing surface furrows (Fig. 5.8). The surface of these cells had many irregular and shadow-like microfolds. The cells were usually joined together closely and often had ledge-like cytoplasmic elevations on their borders. Desquamation of superficial squamous cells was sometimes seen (Fig. 5.8). Occasional gaps filled with intact or degenerate lymphocytes and tissue debris were also found between microvillous cells, ciliated cells and squamous cells.

The epithelium overlying non-lymphoid follicle areas consisted of mainly ciliated cells interspersed by single or sometimes nests of goblet cells and microvillous cells. The ciliated cells were similar to those seen in the rest of ovine respiratory tract, and the goblet cells had a few scattered short microvilli on their surface. Sometimes the goblet cells showed accumulation of secretory material under the apical cell membrane, or exhibited prominent secretion orifices on the cell surface.

5.3.2 Transmission electron microscopy

The epithelial cells of the ovine pharyngeal tonsil were ultrastructurally heterogeneous and joined to each other with desmosomes or by tight junctions alone. The microvillous cells in the FAE contained many mitochondria and some rough endoplasmic reticulum in the apical regions while the basal region was occupied by an elongated nucleus (Fig. 5.9). Their cytoplasm varied in electron density. Some cells contained many apical vesicles, vacuoles, electron-dense granules and occasional phagolysosomes (Figs. 5.10, 5.11, 5.12 and 5.13). Other cells were flattened and contained only occasional cytoplasmic organelles (Fig. 5.14). The type of microvilli varied from sparse, thin, short and irregularly arranged to dense, thickened, tall and regularly distributed. Occasionally, finger-like cytoplasmic projections were also found (Fig. 5.13).

In addition to multiple desmosomes and tight junctions, the adjacent microvillous cells often formed intercellular digital junctions (Fig. 5.12). However, these junctions were not found between microvillous cells and adjacent lymphocytes. In some areas, adjacent microvillous cells were loosely organised, resulting in intercellular spaces of varying size (Fig. 5.15). Some microvillous cells had slender lateral cytoplasmic processes which formed disordered networks enclosing intraepithelial lymphocytes.

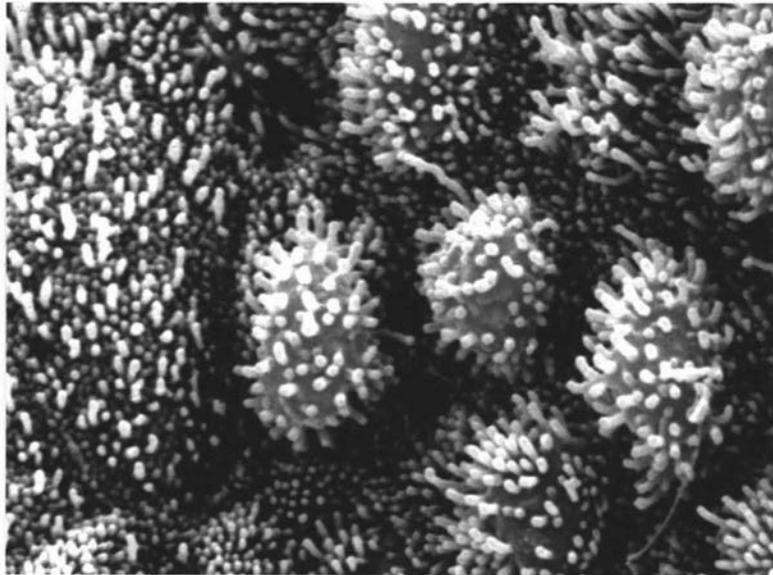


Fig. 5.3 A nest of microvillous cells in the follicle-associated epithelium of the pharyngeal tonsil from a 9-month-old sheep. Note the intercellular crevices formed by the bulging surface of the microvillous cells. The microvilli on these cells are thick but less densely packed than those of single cells. SEM, x7,700.

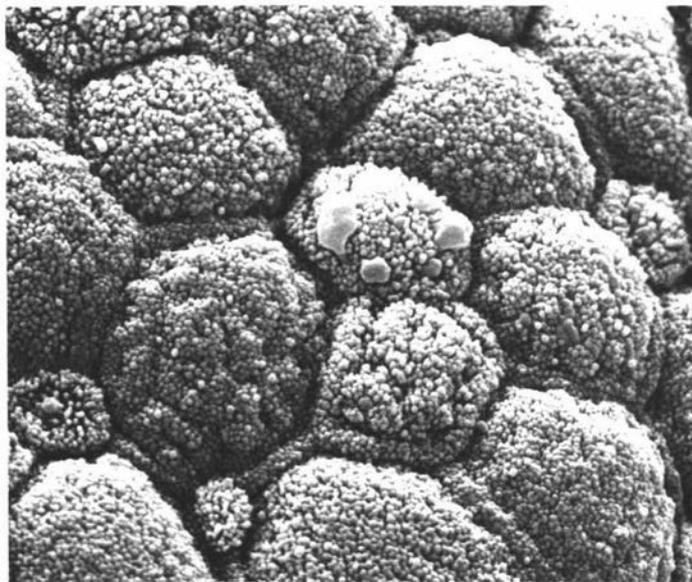


Fig. 5.4 One type of flattened follicle-associated epithelium showing microvillous cells of various shapes and sizes with a slightly bulging surface. The microvilli on these cells are short and densely packed. Pharyngeal tonsil from a 9-month-old sheep. SEM, x5,000.

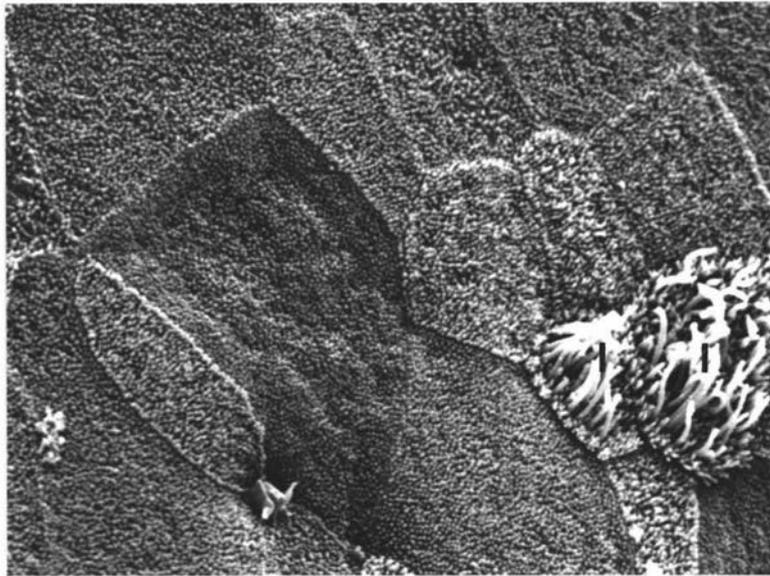


Fig. 5.5 A second type of flattened follicle-associated epithelium in the pharyngeal tonsil of an 8-month-old sheep. The cells are polygonal, closely joined to each other and have ledge-like cell borders. Numerous densely-packed knob-like microvilli are evenly distributed on the cell surface. Two intermediate cells (I) containing both cilia and microvilli are also present. SEM, x3,200.

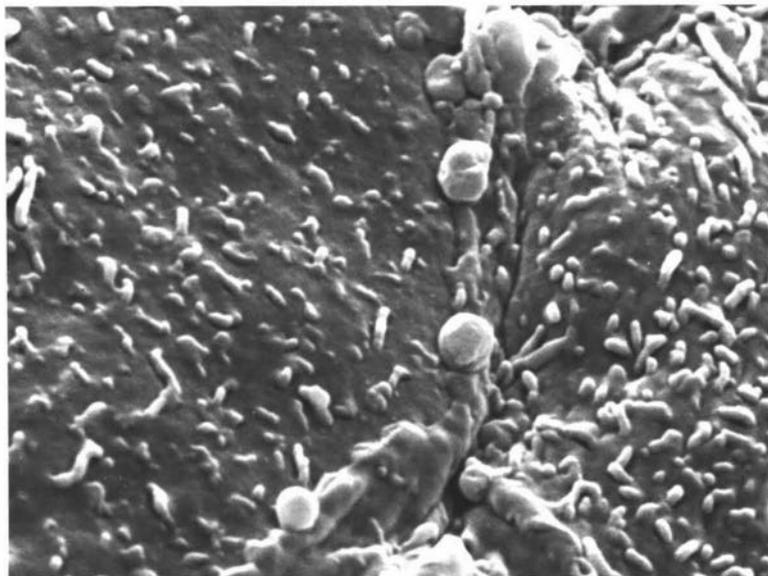


Fig. 5.6 A microvillous cell in the follicle-associated epithelium of the pharyngeal tonsil from a 2-year-old sheep showing a cobblestone pattern of cytoplasmic elevations on the surface. SEM, x8,100.

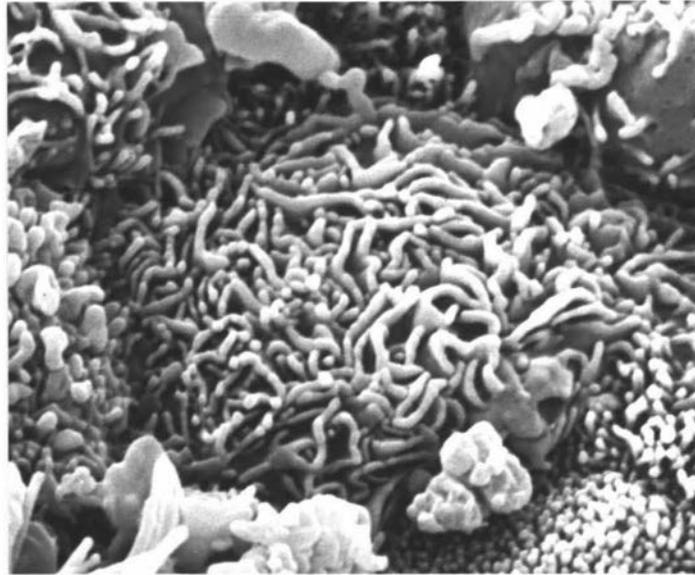


Fig. 5.7 A microvillous cell in the follicle-associated epithelium from the pharyngeal tonsil of a 2-year-old sheep showing irregular, ridge-like microplacae on its surface. SEM, x7,400.

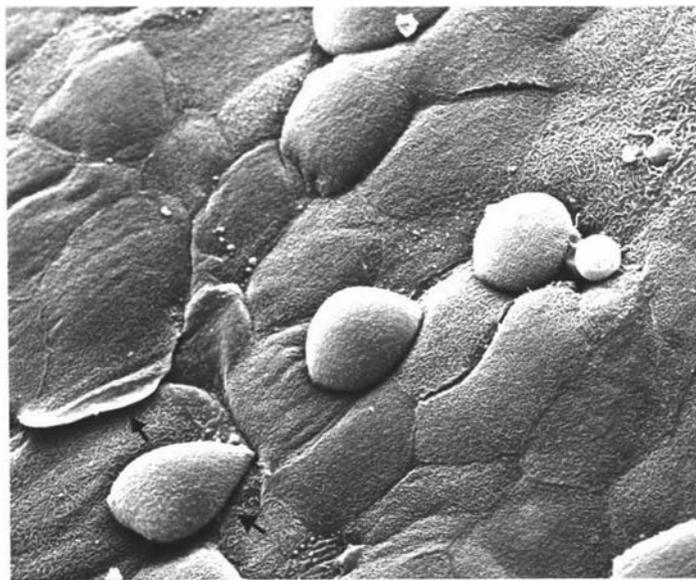


Fig. 5.8 Squamous epithelium in the pharyngeal tonsil from a 9-month-old sheep showing many irregular shadow-like microfolds on the surface and ledge-like cytoplasmic elevations at the cell borders. A few squamous epithelial cells are undergoing desquamation (arrows). SEM, x1,500.

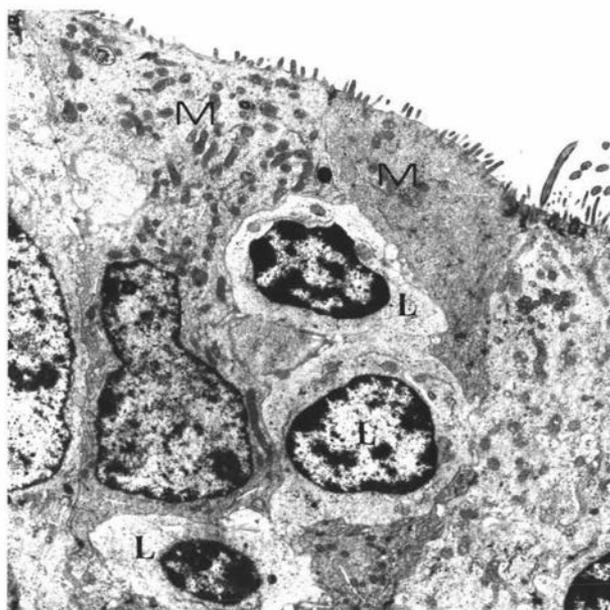


Fig. 5.9 Microvillous cells (M) in the mucosa of the pharyngeal tonsil from a 3-year-old sheep showing an absence of cilia. The apical cytoplasm is rich in the mitochondria and there is infiltration of lymphocytes (L). TEM, x4,850.

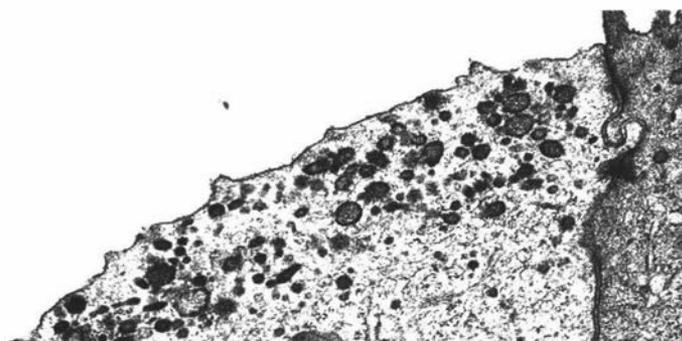


Fig. 5.10 A microvillous cell containing many vesicles in its apical cytoplasm and a few short microvilli on its surface. Pharyngeal tonsil of an 8-month-old sheep. TEM, x21,200.

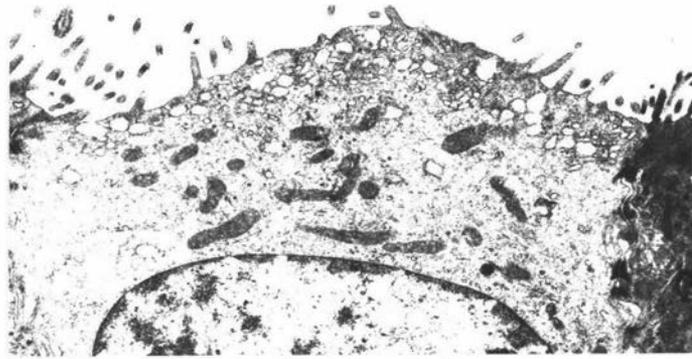


Fig. 5.11 A microvillous cell showing an abundance of vacuoles in the cytoplasm nearest the lumen. The microvilli in this cell are sparse and slender. Pharyngeal tonsil of an 8-month-old sheep. TEM, x11,200.

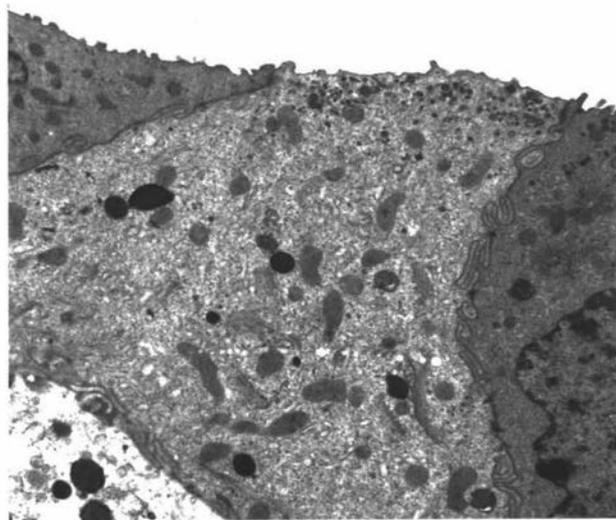


Fig. 5.12 A microvillous cell containing many electron-dense cores in its apical cytoplasm and prominent intercellular digital junctions on its lateral surface. The pharyngeal tonsil of an 8-month-old sheep. TEM, x7,800.

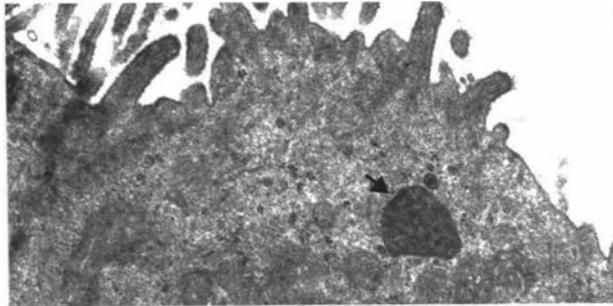


Fig. 5.13 A microvillous cell showing the presence of a phagolysosome (arrow) in its apical cytoplasm and finger-like cytoplasmic projections on its surface. Pharyngeal tonsil of a 2-year-old sheep. TEM, x11,200.

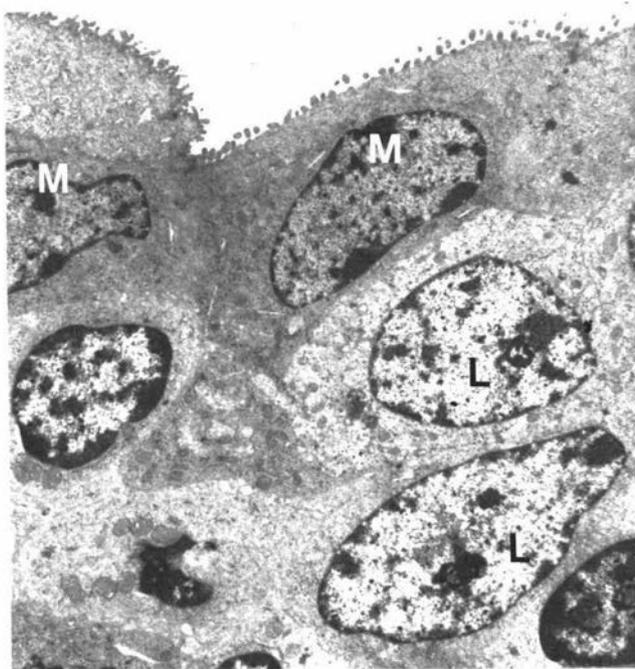


Fig. 5.14 Flattened microvillous cells (M) in the follicle-associated epithelium of the pharyngeal tonsil from a 3-year-old sheep. These cells contain densely-packed knob-like microvilli and few cytoplasmic organelles. Several lymphocytes (L) are nested beneath these microvillous cells. TEM, x5,200.

Although the lymphocytes enclosed in the FAE were mainly small to medium-sized (Fig. 5.9) and were in intimate contact with the epithelial cells, no specialised intercellular structures were observed between the FAE cells and lymphocytes or among lymphocytes themselves. Intact and degenerate macrophages and neutrophils, and sometimes plasma cells, were frequently encountered within the FAE, although plasma cells were rarely seen in the apical areas. Lymphocytes and plasma cells were occasionally found on the mucosal surface. The basement membrane within the FAE region was often disrupted because of cell migration from the underlying lymphoid tissue. Sometimes, the surface of the FAE was disrupted and formed a channel which allowed exposure of the underlying lymphocytes to the nasopharyngeal cavity (Fig. 5.16).

Squamous cells in the FAE and other areas were stratified, and characterised by bundles of tonofilaments in their cytoplasm and numerous desmosomes on their lateral surfaces (Fig. 5.16). Intermediate cells usually had a few cilia and many tall, slender microvilli on their surface. They contained a few vacuoles, basal bodies and many mitochondria in their cytoplasm. Lymphocytes were frequently enclosed between these cells.

The ciliated cells and goblet cells in the mucosa of the pharyngeal tonsil showed similar features to those in the lower respiratory tract of sheep (Mariassy and Plopper, 1984). Atypical cilia previously described in the tracheobronchial mucosa of man (Ailsby and Ghadially, 1973) and sheep (Chen *et al.*, 1988) were occasionally encountered.

5.4 DISCUSSION

A better understanding of the pathogenesis of respiratory infections and airway hypersensitivity will require more knowledge of local immune systems. Initial histological studies of RTALT in sheep have shown that the pharyngeal tonsil is one of the major sites in which morphologically well-developed lymphoid tissue is present (Chapter 4). The present study has revealed that the mucosa of ovine pharyngeal tonsil contains a specialised epithelium, FAE, which can be topographically and ultrastructurally distinguished from the adjacent epithelium by the attenuation of its cell height, presence of microvillous cells, loss of ciliated and goblet cells and heavy infiltration of lymphocytes. Based on their morphological features and their relationship to underlying lymphoid tissue, most microvillous cells in the FAE can be considered as equivalent to the M cell described in other mucosa-associated lymphoid tissues in animals and man (Wolf and Bye, 1984; Bienenstock, 1985). Thus, the M cell, together with some squamous cells and intermediate cells forms the basic structure of FAE in the ovine pharyngeal tonsil.

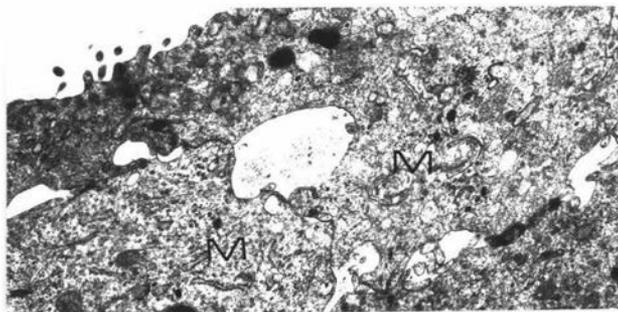


Fig. 5.15 Intercellular spaces between two adjacent microvillous cells (M). The microvillous cells also contain many vesicles in their apical part of the cytoplasm. Pharyngeal tonsil from an 8-month-old sheep. TEM, x11,200.

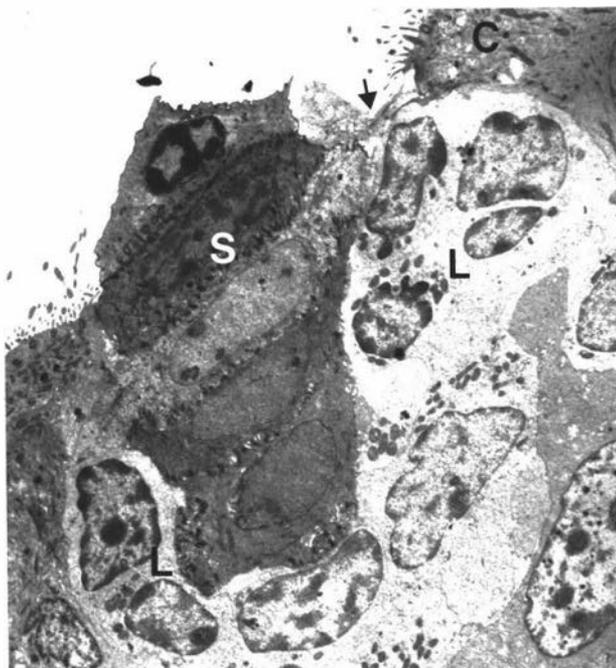


Fig. 5.16 Focal epithelial disintegration (arrow) in the mucosa of the pharyngeal tonsil from a 9-month-old sheep. Note many lymphocytes (L) of different maturities are directly exposed to the nasopharyngeal cavity. Squamous cells (S) and ciliated cells (C). TEM, x3,400.

Although the ultrastructural features of M cells in the ovine pharyngeal tonsil are similar to those seen in the most other mucosal sites (Olah and Everett, 1975; Bienenstock and Johnston, 1976; Owen and Nemanic, 1978; Howie, 1980; Liebler *et al.*, 1988; Fix and Arp, 1989), the majority of FAE cells described in rat nasal lymphoid tissue are cuboid ciliated cells and only occasional groups of non-ciliated cells have been observed (Spit *et al.*, 1989). They contain variable amounts of filaments running in the core of the microvilli or free in the cytoplasm (Spit *et al.*, 1989). The cytoplasm of M cells in the pharyngeal tonsil of sheep is generally more electron-dense than adjacent ciliated cells. This contrasts with the M cells in GALT which are usually more electron-lucent than the adjacent enteroabsorptive cells (Liebler *et al.*, 1988). The M cell in the pharyngeal tonsil also contains fewer phagolysosomes than that in GALT (Liebler *et al.*, 1988). The observed differences may reflect a different origin of M cells in GALT compared to RTALT rather than a difference in their function. It has been suggested that M cells originate from adjacent undifferentiated epithelial cells (Bye *et al.*, 1984).

The similarities between FAE in the ovine pharyngeal tonsil and most other mucosa-associated lymphoid tissues implies that they have a similar role in mucosal immunity. The presence of microvilli and pinocytotic vesicles in the M cells observed in the current study suggests that they are functionally active in vesicular transport. This is supported by our previous study which demonstrated that FAE in the ovine pharyngeal tonsil was able to take up intranasally administered colloid carbon (Chapter 4). These observations add weight to the hypothesis that the M cell is necessary for communication between lymphoid tissues and the outside microenvironment (Wolf and Bye, 1984). The presence of many deep surface furrows in the ovine pharyngeal tonsil may not only increase the concentration of lymphoid tissue in a given area, but also offer a good opportunity for antigen entrapment and attachment.

Unlike those in GALT, the M cells in RTALT including the pharyngeal tonsil observed in the current study, are difficult to identify with certainty using only topographical information (Bienenstock and Johnston, 1976; Anderson *et al.*, 1986; Spit *et al.*, 1989) since other types of cell containing microvilli but devoid of cilia are also present in the respiratory tract. Using TEM however, the cells can be easily differentiated by typical features such as the presence of cytoplasmic vesicles and vacuoles, large numbers of adjacent lymphoid cells and the formation of digital junctions between neighbouring cells. Some variation in the ultrastructure of individual M cells has been noted in both the current and previous studies (Owen and Nemanic, 1978; Bye *et al.*, 1984; Liebler *et al.*, 1988; Spit *et al.*, 1989). This is thought to reflect differences in cell maturities (Bye *et al.*, 1984), previous antigen exposure (Spit *et al.*, 1989), and artifact produced during tissue processing (Owen and Nemanic, 1978). The sheep used in this study were conventionally raised and of variable age. M cells in the tonsil would thus have had a different opportunity of exposure to antigens and could therefore reflect this in their morphology.

The M cell has been suggested as a portal for some pathogenic microorganisms entering the host (Wolf and Bye, 1984). Experimental studies on GALT have demonstrated the importance of M cells in the attachment, adherence and penetration of a range of pathogens including viruses, bacteria, and protozoa (Inman and Cantey, 1983; Wolf *et al.*, 1983; Pohlentz *et al.*, 1984), and a similar function is also expected in RTALT (Wright, 1950; Bienenstock, 1985). Although the ovine pharyngeal tonsil and the bronchus-associated lymphoid tissue from a number of animal species have been demonstrated experimentally to be capable of taking up and transporting particulate and soluble antigens (Bienenstock, 1985; Chapter 4), their ability to deal with other inhaled antigens, such as microorganisms and allergens under natural conditions is not clear. Attempts to demonstrate this have been made by several groups (Stephens *et al.*, 1983; van der Brugge-Gamelkoom *et al.*, 1985), but the results are generally disappointing.

The presence of large numbers of mature intraepithelial lymphocytes, but very few plasma cells in the FAE of ovine pharyngeal tonsil is in keeping with the studies of human pharyngeal tonsil by Yamanaka *et al.* (1983). Using immunohistochemical techniques these authors found that up to 90% of intraepithelial mononuclear cells were helper T lymphocytes. Similar findings were made in other mucosal sites such as palatine tonsils (Yamanaka *et al.*, 1983) and the gut (Owen and Nemanic, 1978). The results of these studies together with our present study suggest that Ig-producing lymphocytes mature in the submucosal areas of RTALT but may not enter the respiratory tract lumen directly through the FAE. Indeed, Burrells (1985) reported that about 60-80% of lymphocytes in ovine bronchoalveolar lavage are T lymphocytes. However, the significance of these intraepithelial T cells in local and systemic immunity is not entirely clear, although it has been suggested that these cells are concerned with immunomodulation (Bienenstock, 1985).

The functional significance of the squamous cells and intermediate cells seen in the current study is not clear. The normal human pharyngeal tonsil contains only a few islands of squamous cells, and about 70% of surface epithelial cells are ciliated (Owen and Nemanic, 1978). However, the proportion of squamous cells increases when subjects suffer from chronic infection such as tonsillar hyperplasia (Lenz, 1974; Owen and Nemanic, 1978). Thus squamous metaplasia in the pharyngeal tonsil may simply be related to an inflammatory process. The intermediate cells seen in this study are morphologically similar to those described as potential ciliated cells in rat nasal mucosa (Andrews, 1974) and human pharyngeal tonsil (Lenz, 1974). However, the close association between intermediate cells and FAE cells and their moderate infiltration by lymphoid cells suggests that they could also be the precursors of M cells.

The pharyngeal tonsil is located in a strategic position in the respiratory tract, and the morphological similarities between its FAE and that at other mucosal sites suggests that it may play a critical role in local mucosal immunity. Future studies on the ontogeny and morphological development of the pharyngeal tonsil as well as its functional aspects will be necessary to obtain more information on its significance in immunity and its role in the pathogenesis of respiratory disease.

5.5 SUMMARY

The mucosal epithelium of the pharyngeal tonsil was studied in ten conventionally raised sheep using scanning and transmission electron microscopy. The surface of the tonsil was covered by two types of topographically and ultrastructurally distinct epithelium. The epithelium overlying the lymphoid follicle region (follicle-associated epithelium) was depressed below the adjacent ciliated epithelium, and consisted of predominantly non-ciliated cells of varying height. The majority of these cells contained microvilli or microfolds of varying number, height and density on their surface. Small numbers of squamous cells and occasionally partly ciliated intermediate cells were also present in these areas. Ultrastructurally, the microvillous cells showed features similar to M cells as they occur in other mucosal sites. They contained cytoplasmic vesicles and vacuoles, were heavily infiltrated by lymphoid cells and formed intercellular digital junctions. Focal disintegration of the mucosal epithelium was occasionally seen. The epithelium covering non-follicle areas consisted of mainly ciliated cells interspersed with some goblet cells and squamous cells, morphologically resembling those elsewhere in the respiratory tract. These findings have provided morphological evidence which is consistent with the pharyngeal tonsil and its follicle-associated epithelium playing an important role in the immune function of the respiratory tract.

CHAPTER 6
PERINATAL DEVELOPMENT OF LYMPHOID TISSUE
AND ITS ASSOCIATED EPITHELIUM
IN THE OVINE PHARYNGEAL TONSIL: A MORPHOLOGICAL STUDY

6.1 INTRODUCTION

Morphological and functional studies of respiratory tract-associated lymphoid tissues have highlighted the importance of the pharyngeal tonsil in local mucosal immunity (Ishikawa *et al.*, 1972; Loo and Chin, 1974; Owen and Nemanic, 1978; Korsrud and Brandtzaeg, 1980; Yamanaka *et al.*, 1983; Anderson *et al.*, 1986; Surjan, 1987; Chapters 4 and 5). Investigations of mucosa-associated lymphoid tissues in several mucosal sites in sheep and other animals have shown that their development during the perinatal period varies with tissue site and animal species, and the differences in the prenatal development of these structures are likely to be important in the establishment of a postnatal mucosal immune response (Emery and Dinsdale, 1974; Naukkarinen *et al.*, 1978; Gregson *et al.*, 1979b; Plesch *et al.*, 1983; Reynolds and Morris, 1983; Anderson *et al.*, 1986; Asari *et al.*, 1989).

Because sheep are now promoted as a suitable species for the study of human allergic asthma (Wanner and Abraham, 1982), knowledge of local mucosal immunity-associated tissues in this species is of considerable significance to advance our understanding of this important disease. Previous studies (Chapter 5) have shown that the lymphoid tissue of the pharyngeal tonsil in adult sheep is morphologically similar to other mucosa-associated lymphoid tissues, in that it contains a specialised follicle-associated epithelium (FAE) and many secondary lymphoid follicles, but no afferent lymphatics (Chapters 4 and 5). Little information is available, however, on the morphological development of these structures during the perinatal period in this and other species.

6.2 MATERIALS AND METHODS

Twelve fetuses from healthy Romney-cross sheep were used for this study. Six fetuses were between 80 and 96 days gestation, and six between 140 days gestation and full term. The foetal ages were estimated from their crown-rump length, according to the method of Evans and Sack (1973). In addition, six 1-2 week old Romney-cross lambs without clinical abnormalities or gross lung lesions were studied. All postnatal sheep were killed by intravenous injection of pentobarbitone.

The pharyngeal tonsil was carefully removed immediately and rinsed with cold 0.1 M phosphate buffered saline to remove superficial mucus and cellular debris. Transverse sections from the cranial, middle and caudal parts of each pharyngeal tonsil were collected and fixed in 10% neutral buffered formalin. They were processed routinely and embedded in paraffin wax. Sections were cut 4 μm thick and stained with haematoxylin and eosin (HE) or alcian blue and periodic acid-Schiff at pH 2.5. For scanning electron microscopy (SEM), large blocks of 0.5 cm x 0.5 cm x 0.5 cm were taken from the caudal, middle and cranial regions of the tonsil and fixed in Karnovsky's fixative at 4 $^{\circ}\text{C}$ for 48 hours (Karnovsky, 1965). They were then washed in two changes of cold phosphate buffered saline for 1 hour, dehydrated in a series of graded ethanol, critical point dried, placed on aluminium stubs, coated with gold and examined in a Cambridge 250 MK III scanning electron microscope. Additional small tissue blocks were collected for transmission electron microscopy (TEM) from the adjacent areas. These were fixed in Karnovsky's fixative at 4 $^{\circ}\text{C}$ overnight, then processed as described previously (Chen *et al.*, 1988) and examined under a Philips EM201c transmission electron microscope.

6.3 RESULTS

6.3.1 Foetuses of 80-96 days

The mucosal epithelium consisted entirely of columnar ciliated epithelium interspersed with a few goblet cells (Fig. 6.1). In foetuses of 80-90 days, the subepithelial regions contained mainly mesenchymal cells with occasional neutrophils, but no lymphocytes or other mononuclear cells (Fig. 6.1). The tonsils from foetuses 92 days and older, however, contained small numbers of subepithelial lymphocytes and other mononuclear cells with a scattered distribution (Fig. 6.2). These cells sometimes appeared in clusters. The mucosal epithelium at this stage was morphologically similar to that seen earlier, but single intraepithelial lymphocytes were occasionally encountered in the basal plane. The ciliated and goblet cells were similar ultrastructurally to those in the rest of the respiratory tract, although the ciliated cells usually had fewer cytoplasmic organelles and contained microvilli that were more slender than normal.

6.3.2 Foetuses of 140 days to full term

At this stage, the number of subepithelial lymphocytes had increased considerably. They usually formed cellular bands or dense aggregates lying immediately beneath the mucosal epithelium (Fig. 6.3). Within these aggregates small numbers of neutrophils, mast cells and other mononuclear cells were often seen. The mucosal epithelium in most foetuses was similar to that seen previously, but contained more goblet cells. Within the epithelium varying numbers of lymphocytes, singly or in clusters were often found. In some areas, the ciliated epithelium

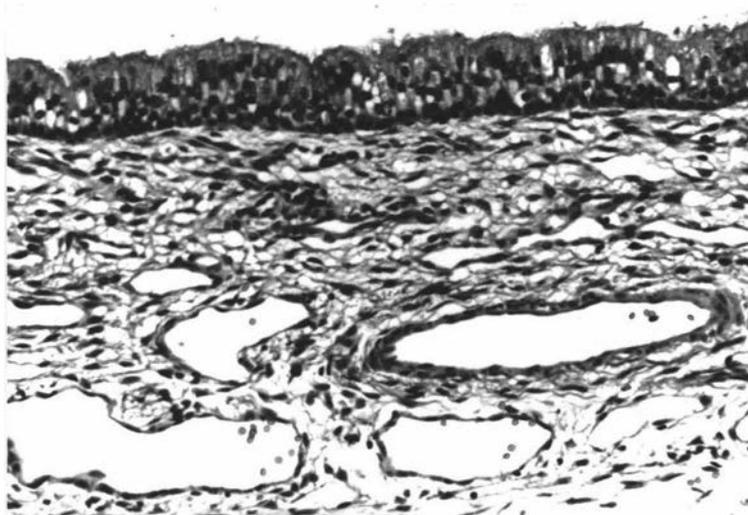


Fig. 6.1 The mucosal epithelium of the pharyngeal tonsil from an 80-day ovine foetus. It consists almost entirely of ciliated cells with a few goblet cells. Small numbers of mesenchymal cells with occasional neutrophils are present in the subepithelial areas. HE, x185.



Fig. 6.2 The pharyngeal tonsil from an ovine foetus of 96 days gestation showing subepithelial infiltration of small numbers of lymphocytes and other mononuclear cells. HE, x185.

covering dense lymphoid aggregates was partially transformed into FAE and contained lymphoepithelial cells (M cells) singly or in nests (Fig. 6.4). At this stage the M cells often showed bulging of the apical cytoplasm, in which was present many mitochondria and several vacuoles of varying size together with a few endoplasmic reticula and vesicles (Fig. 6.5). The surface of these cells was irregular and contained varying numbers of large cytoplasmic processes but few microvilli (Fig. 6.6). There were a few early interdigitating processes between the lateral borders of adjacent cells, and the mucosal surface was crossed by many shallow invaginations. In the full-term foetuses, the efferent lymphatics were congested with many small lymphocytes.

6.3.3 The 1-2 week old lambs

The subepithelial lymphoid tissue was well-organised by this stage, and could be divided into three areas; a dome area underlying the mucosal epithelium, follicular areas lying under the dome and parafollicular areas (Fig. 6.7). Most follicles were active and contained a prominent germinal centre, but the follicles were generally better developed in the caudal areas of the tonsil than in the cranial regions.

Patches of FAE were easily seen in the mucosal epithelium overlying the dome and lymphoid follicles (Fig. 6.8). These were distinct and depressed below the adjacent ciliated epithelium. They consisted of mainly non-ciliated M cells which varied in size, shape and surface structure (Fig. 6.9). A few ciliated cells were often found scattered amongst the FAE, but goblet cells were rare.

Ultrastructurally, the large bulging cytoplasmic processes seen previously in M cells had almost disappeared (Fig. 6.10). Instead, these cells had a few thick microvilli. The distribution of cytoplasmic organelles within M cells was similar to that seen earlier although many intercellular spaces had formed between M cells and adjacent cells (Fig. 6.10), in which well-developed interdigitating processes were sometimes seen. By now, the number of intraepithelial lymphocytes had increased markedly. The adjacent ciliated epithelium sometimes had small numbers of intraepithelial microcysts containing a few leucocytes and a small amount of cellular debris. Scanning electron microscopy showed the invaginations of the mucosal surface were now more distinct than at earlier stages.

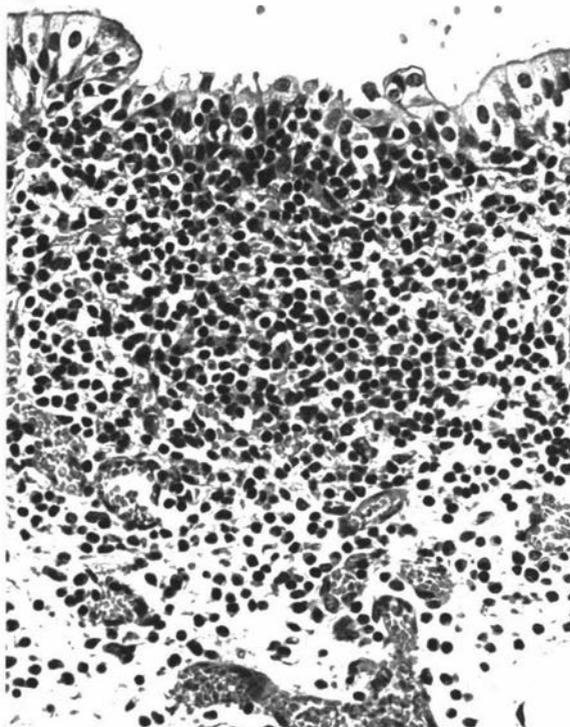


Fig. 6.3 A dense aggregation of lymphoid cells in the subepithelial region of the pharyngeal tonsil from a 140 day ovine foetus. HE, x300.

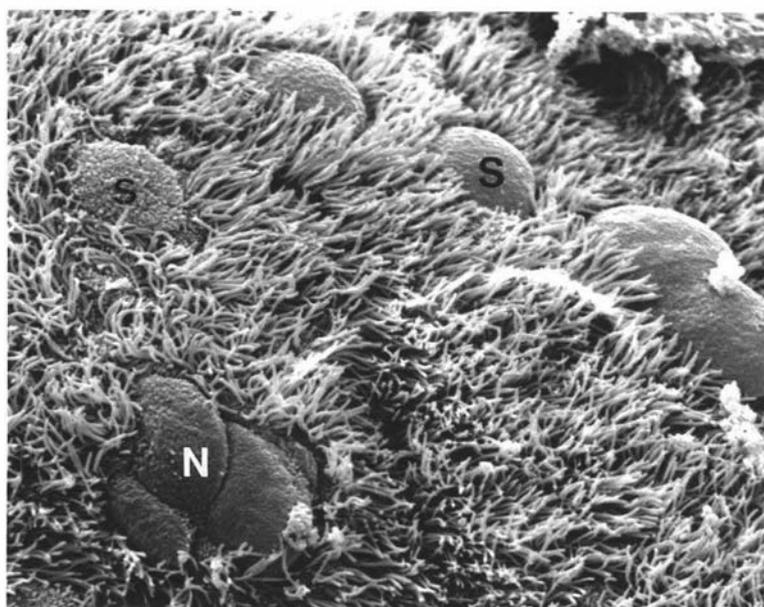


Fig. 6.4 Mucosal epithelium of the pharyngeal tonsil from a newborn lamb showing non-ciliated epithelial cells singly (S) and in nests (N). SEM, x1,500.

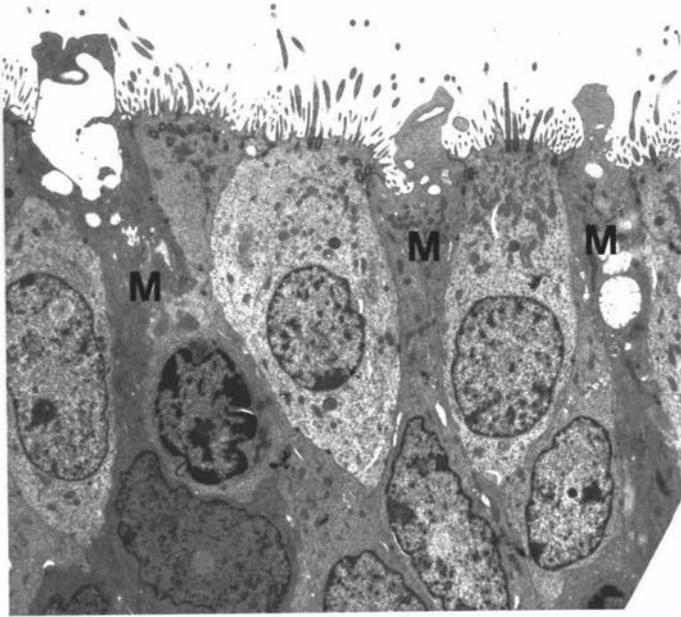


Fig. 6.5 M cells (M) in the mucosa of the pharyngeal tonsil of a newborn lamb. The cells show absence of cilia and bulging of the apical cytoplasm. TEM, x3,600.

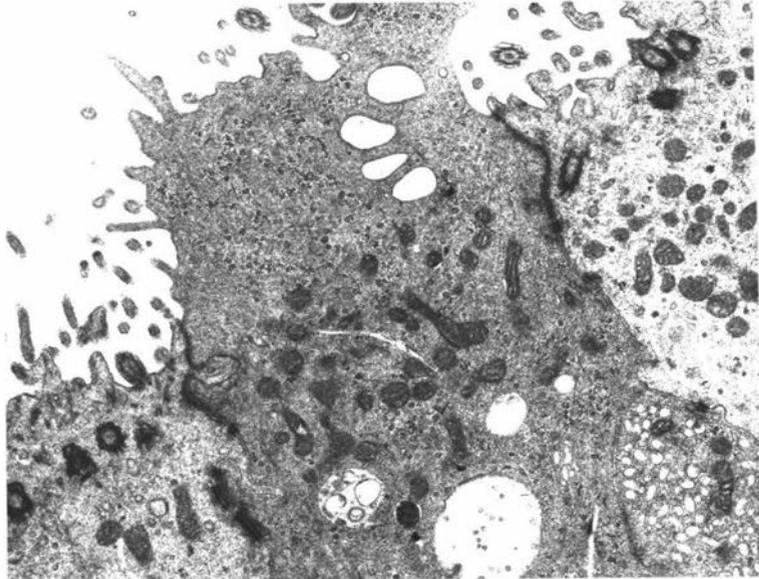


Fig. 6.6 High magnification of an M cell showing many mitochondria and several vacuoles in the apical cytoplasm. Pharyngeal tonsil from a newborn lamb. TEM, x8,300.



Fig. 6.7 Tissue of the pharyngeal tonsil of a 7-day-old lamb showing a lymphoid follicle containing a germinal centre (G), dome area (D) and parafollicular region (P). The follicle-associated epithelium is flattened and infiltrated with lymphocytes (between arrowheads). HE, x60.

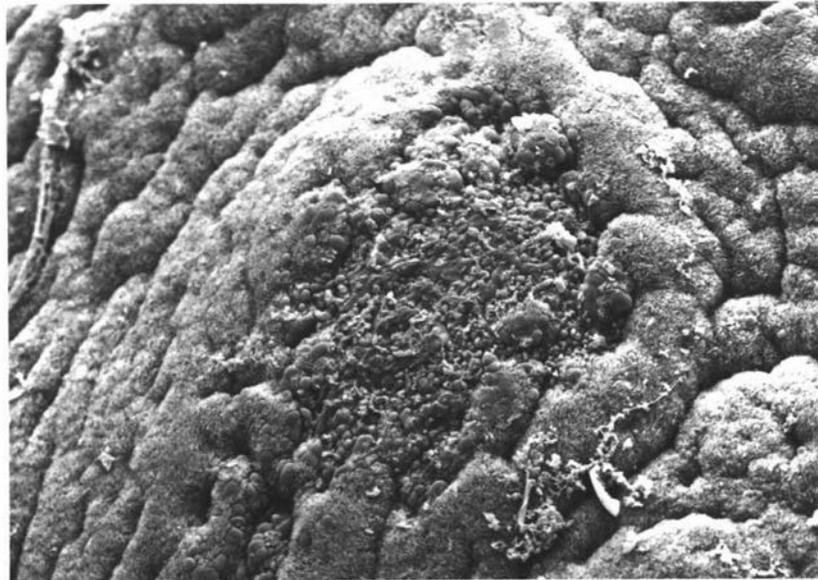


Fig. 6.8 Mucosal epithelium of the pharyngeal tonsil from a 7-day-old lamb. It has an island of follicle-associated epithelium which is depressed and distinct from the adjacent ciliated epithelium. SEM, x190.

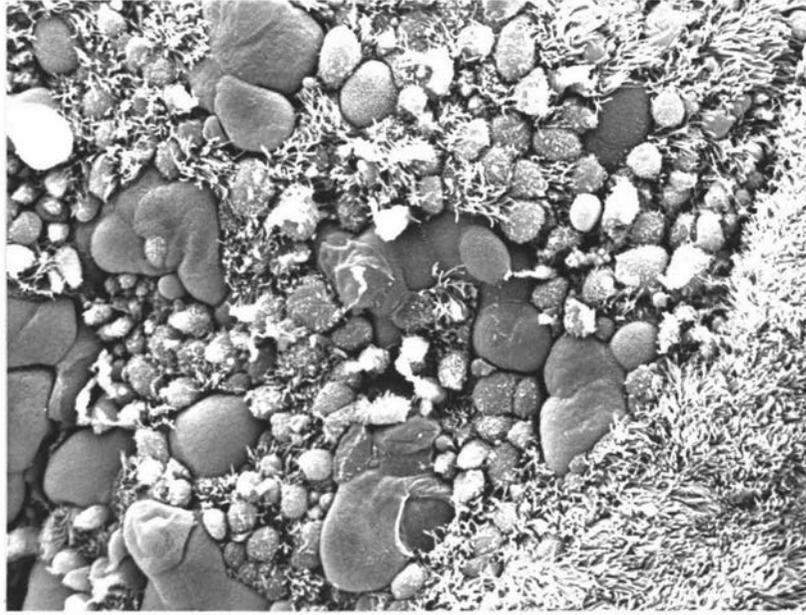


Fig. 6.9 High magnification of the follicle-associated epithelium of the pharyngeal tonsil from a 7-day-old lamb. There is considerable variation in the size and shape of M cells. SEM, x850.

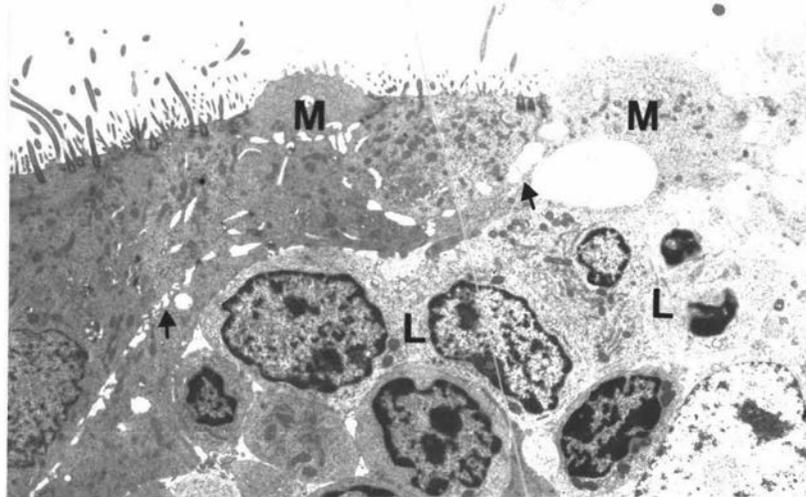


Fig. 6.10 Mucosal epithelium of the pharyngeal tonsil from a 7-day-old lamb. There is heavy infiltration of lymphocytes (L) and the M cells (M) possess a few thick microvilli. There are many intercellular spaces between M cells and adjacent cells (arrows). TEM, x3,400.

6.4 DISCUSSION

Although the morphological development of the pharyngeal tonsil has been previously studied in man (Snook, 1965), little attention has been focused on the tissue components associated with mucosal immunity, such as the FAE and lymphoid tissue. The present study has shown that morphologically distinguishable lymphoid cells first appeared in the subepithelial region of the pharyngeal tonsil of sheep at about 92 days after gestation. By birth, the ovine pharyngeal tonsil showed many dense subepithelial lymphoid aggregates and early formation of FAE together with the presence of lymphocytes in efferent lymphatics. Mature M cells and lymphoid follicles with vigorous lymphopoiesis appeared soon after birth. The FAE and lymphoid tissue in 1-2 week old lambs were morphologically similar to those seen in adult sheep (Chapters 4 and 5), although their size was smaller. The presence of lymphocytes in the efferent lymphatics in full-term fetuses has provided morphological evidence for the participation of the ovine pharyngeal tonsil in the recirculation of lymphocytes at an early stage of development.

The development of lymphoid tissue and its associated epithelium has been studied previously in several mucosal sites in a number of species (Emery and Dinsdale, 1974; Naukkarinen *et al.*, 1978; Gregson *et al.*, 1979b; Plesch *et al.*, 1983; Reynolds and Morris, 1983; Anderson *et al.*, 1986; Asari *et al.*, 1989). The results of present study have shown that their appearance and development in the pharyngeal tonsil of sheep is earlier than that in the bronchus-associated lymphoid tissue (BALT) in a number of species (Bienenstock *et al.*, 1973a,b; Emery and Dinsdale, 1974; Gregson *et al.*, 1979b; Plesch *et al.*, 1983; Anderson *et al.*, 1986), the conjunctiva-associated lymphoid tissue of turkeys (Fix and Arp, 1989), and the gut-associated lymphoid tissue (GALT) of laboratory animals (Waksman *et al.*, 1973; Roy and Varvayanis, 1987), in which lymphoid tissue and its associated epithelium do not appear until birth. However, its development is later than that in the bursa Fabricius of chicken (Naukkarinen *et al.*, 1978) and in Peyer's patches of man and most domestic animals studied to date, including sheep (Reynolds and Morris, 1983; Asari *et al.*, 1989). The FAE and lymphoid tissue in the bursa and Peyer's patches of all these species are well-developed prenatally and their development is considered to be antigen-independent. The appearance and early development of the lymphoid tissue in the ovine pharyngeal tonsil is similar to that in humans (Snook, 1965). The human pharyngeal tonsil shows the first accumulation of lymphocytes between 3 and 6 months gestation, but germinal centres are absent in the tonsils of fetuses and the newborn.

Mechanisms regulating the development of FAE are not fully understood although evidence from some studies (Bienenstock *et al.*, 1973a,b; Emery and Dinsdale, 1974; Gregson

et al., 1979b; Plesch *et al.*, 1983; Anderson *et al.*, 1986) favours the proposal that development is antigen-dependent and closely associated with the development of subepithelial lymphoid tissue. The results of this and other studies have demonstrated that the development of FAE can begin prenatally when the foetus has little exposure to foreign antigens (Reynolds and Morris, 1983; Asari *et al.*, 1989). On the other hand, FAE is absent from the mucosal epithelium of BALT in adult cattle (Anderson *et al.*, 1986), horses (Mair *et al.*, 1987) and sheep (Chapter 4). In cattle and sheep, FAE is only seen in BALT of pneumonic lungs (Anderson *et al.*, 1986; Chen, unpublished data). Therefore, it may be postulated that factors apart from or in addition to antigen stimulation and lymphocytic infiltration are required for the development of FAE. It is also possible that the development of FAE in different mucosal sites and animal species is controlled by different mechanisms. Recently, Roy and Varvayanis (1987) proposed that maternal immunoglobulin A may also play a role in the development of M cells in neonates.

The current finding that lymphoid tissue was present in the prenatal ovine pharyngeal tonsil adds support to the proposal made by Bienenstock (1985) that antigens may not be required for the appearance of lymphoid tissue. Nevertheless, antigens are likely to be of great importance in their development and maturation. Included among the changes which occurred in the ovine pharyngeal tonsil soon after birth are the amount of FAE, the morphological differentiation of epithelial cells into M cells, and the organisation, cellular content, and growth rate of the lymphoid follicles. Studies on most other mucosa-associated lymphoid tissues have shown that the amplification of lymphoid tissue is dependent on antigen stimulation, and it is well known that lymphoid proliferation is reduced when animals are maintained in germ-free conditions (Jericho, 1970; Bockman and Cooper, 1973) and increases significantly when germ-free and specific pathogen-free animals are conventionally housed (Jericho, 1970; Smith *et al.*, 1987; Fix and Arp, 1989). Amplification of lymphoid follicles following experimental challenge with several types of antigen has also been demonstrated in the BALT of pigs (Jericho *et al.*, 1971), rabbits (Racz *et al.*, 1977), rats (Gregson *et al.*, 1979c; van der Brugge-Gamelkoorn *et al.*, 1985), turkeys (van Alstine and Arp, 1988), and mink (Jericho, 1982).

Although the development of lymphoid tissue in the ovine pharyngeal tonsil is likely to follow a similar mechanism to most mucosa-associated lymphoid tissues, its development occurs much earlier than at other sites with the exception of Peyer's patches of domestic animals and man. A study on the human pharyngeal tonsil has suggested that abundant vascular supply and low physical pressure from the adjacent tissue are factors considered to favour proliferation of lymphoid tissue at this site (Snook, 1965). Other factors likely to be important are continuous exposure to a high level of antigens and the presence of intrinsic controls of which little is known at present.

The requirement of antigens for the full development of lymphoid tissue and FAE implies that the pharyngeal tonsil in sheep is not equivalent to GALT in this species. In sheep, primordial Peyer's patches first appear in the small intestine at about 60 days after gestation, and lymphoid follicles are present by 75 days with vigorous lymphopoiesis by 100 days. From 120 days after gestation until birth, the GALT lymphoid follicles are histologically mature and contain the greatest density of proliferating lymphoid cells in the body. A distinctive lymphoepithelium develops soon after the appearance of lymphocytes (Reynolds and Morris, 1983). The early appearance and rapid amplification of the lymphoid tissue and FAE in the ovine pharyngeal tonsil following antigen exposure suggests that it is somewhere between GALT and BALT in priority of development. Future studies will be necessary to clarify its position in local and systemic immunity.

6.5 SUMMARY

The perinatal development of lymphoid tissue and its associated epithelium in the pharyngeal tonsil of sheep was investigated by light and electron microscopy. The lymphoid cells first appeared in the subepithelium in a scattered form at about 92 days gestation. These cells proliferated rapidly during the last trimester of gestation, and had formed many dense aggregates at the time of parturition. At birth, the epithelium overlying the aggregates was extensively infiltrated with lymphocytes and showed early transformation of follicle-associated epithelium. The lymphoid tissue and its associated epithelium did not, however, fully develop until after birth, when well-differentiated follicle-associated epithelium and lymphoid follicles with vigorous lymphopoiesis were present. In 1-2 week old lambs, these structures were smaller but otherwise ultrastructurally similar to those seen in adult sheep. The results of this study suggest that the lymphoid tissue of the ovine pharyngeal tonsil and its associated epithelium are morphologically ready to cope with antigens in the extra-uterine environment at birth, but that their full development and maturation are probably dependent on postnatal antigenic stimulation.

CHAPTER 7

MAST CELLS IN THE OVINE LOWER RESPIRATORY TRACT: HETEROGENEITY, MORPHOLOGY AND DENSITY

7.1 INTRODUCTION

The importance of mast cells (MC) in the mediation of physiological and pathological reactions, especially allergic hypersensitivity (Bienenstock *et al.*, 1986) has stimulated extensive studies on their morphological and functional characteristics in a number of mammalian species including man (Gold *et al.*, 1977; Guerzon *et al.*, 1979; Goto *et al.*, 1984; Eady *et al.*, 1986; Gomez *et al.*, 1987; Shanahan *et al.*, 1987; Bachelet *et al.*, 1988). Results of these studies indicate that MC from different tissue sites in the same species, or even within the same tissue of each species, are morphologically, histochemically and functionally heterogeneous (Enerback, 1966a,b; Ruitenbergh *et al.*, 1982; Goto *et al.*, 1984; Gomez *et al.*, 1987; Shanahan *et al.*, 1987).

Much of the knowledge on MC heterogeneity has come from studies comparing connective tissue mast cells (CTMC) and intestinal mucosal mast cells (MMC) in rodents (Enerback, 1966a,b; Church, 1988). The technique used most commonly to study the morphological and histochemical heterogeneities of MC is the use of two types of fixative containing different concentrations of formalin since CTMC resist formalin fixation and MMC are highly sensitive to formalin. Although two histochemically distinct subtypes of MC have recently been demonstrated in the respiratory tract of rodents and man (Goto *et al.*, 1984; Gomez *et al.*, 1987; Shanahan *et al.*, 1987; Bachelet *et al.*, 1988), little is known about MC heterogeneity in the respiratory tract of other mammals.

Because sheep are now being promoted as a suitable species for the study of human allergic bronchoconstriction (Wanner and Abraham, 1982), information on MC in the ovine respiratory tract is of considerable importance. Evidence that allergic bronchoconstriction in sheep is associated with the significant elevation of arterial plasma histamine levels (Wanner *et al.*, 1979), together with recent observations that MC characteristics in one species may not necessarily parallel those from others (Enerback *et al.*, 1986) prompted the current interest in investigating the density and morphological heterogeneity of MC in the ovine respiratory tract.

7.2 MATERIALS AND METHODS

7.2.1 Tissue sources

The lower respiratory tract (LRT) was obtained from six conventionally raised adult Romney-cross sheep. No LRT collected showed gross evidence of respiratory disease. Two consecutive sets of tissue blocks were taken from each site in the upper, mid and lower trachea, left and right major bronchi, and cranial, middle and caudal lobes of the lung.

7.2.2 Fixation and staining

Immediately after sampling, one set of tissue blocks was fixed in 10% neutral buffered formalin (FA) (pH 7.0) for 24 hours, and the other set was fixed for 12 hours in isotonic formal-acetic-acid (IFAA) fixative (pH 2.9), as used for studies on rat intestinal MC (Enerback *et al.*, 1986). Tissues fixed with IFAA were further transferred to 70% ethanol for another 12-hour fixation. Tissues fixed by both methods were then processed routinely and embedded in paraffin. Sections of 4 μm thickness were cut and stained with 0.5% toluidine blue (Gurr, England) in 0.5 N hydrochloric acid solution at pH 0.5 for 30 minutes (Enerback *et al.*, 1986) or haematoxylin and eosin (HE). Sections from two sheep, together with sections of adult rat lung (control), were also stained with alcian blue and safranin O (Enerback *et al.*, 1986).

For ultrastructural studies, small tissue blocks were taken from the trachea, bronchi and lung. These were fixed in 3% glutaraldehyde in 0.1 M phosphate buffered saline overnight, and processed and examined under a transmission electron microscopy as described previously (Chen *et al.*, 1988).

7.2.3 Counting technique

The density of MC in the tissues fixed with IFAA was determined in the toluidine-blue-stained sections using a JAVA image analyser (Jandel Scientific, California) at a final magnification of 185. The tissue area of the respiratory tract was outlined with a DEXXA mouse (DEXXA International, California). From this outline, the image analyser counted automatically the number of MC and determined the area examined which was at least 6.5 mm^2 per specimen in the lung parenchyma and 4.5 mm^2 per specimen in the airways. Because the staining of MC in tissues fixed in FA was not suitable for automatic counting, MC density in the tissues fixed with FA was assessed by counting manually the number of cells in ten high power fields (x200), the equivalent of 7.386 mm^2 . The MC density in each site was calculated and recorded as MC numbers/ mm^2 . In the airways, MC were only counted in those sections which were well orientated, and had an intact mucosal wall. The ratio of the mucosa of the cartilaginous part to the mucosa of the membranous part was arbitrarily set as 3:1. The MC

located outside any cartilage were not included. In the lung, MC were counted in fields where no large airways were present, but no attempt was made to correct for the area occupied by alveolar spaces. The MC distribution at each level of the tract was assessed by recording the position of 500 cells. The tissue compartments used for the record were; the epithelium, lamina propria, submucosal peri-glandular tissue and tunica muscularis in the airways, and the subpleural and interlobular connective tissues, peribronchiolar areas, perivascular regions, and alveolar septa and spaces in the lung.

7.2.4 Data analysis

All results were expressed as mean and the standard error of means. Statistical analysis of the data was made with regard to fixative and site using computer-assisted one-way or two-way analysis of variance (Winer, 1971) as appropriate. Values of $p < 0.05$ were considered significant.

7.3 RESULTS

7.3.1 Heterogeneity, density and distribution

Mast cells were identified at all levels of the tract using both types of fixative, but their density in the tissues was entirely dependent on the type of fixation (Fig. 7.1). Significantly greater numbers of MC were found in the IFAA-fixed tissues than in the same tissues fixed in formalin ($p < 0.01$). Using IFAA fixation the density of MC increased distally from the trachea to the peripheral lung, whereas the bronchi contained the greatest number of MC in the tissues fixed with FA (Fig. 7.1).

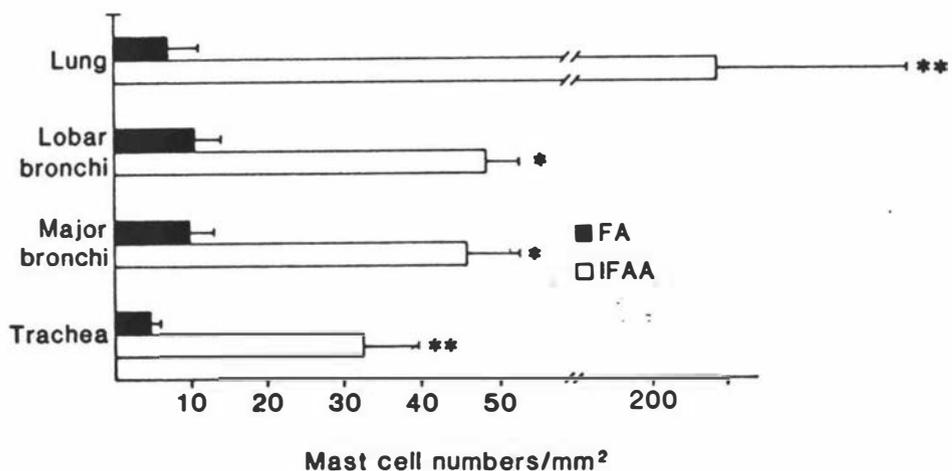


Fig. 7.1 Effects of fixation on perceived mast cell density (cells/mm²) in the ovine lower respiratory tract. FA=10% neutral buffered formalin; IFAA=isotonic formal-acetic-acid; Values are mean \pm the standard error of means ($n = 6$); *= $p < 0.01$ and **= $p < 0.001$ significantly different from mast cell numbers in the same anatomic region of FA-fixed specimens.

The MC distribution in the LRT is illustrated in Fig. 7.2. Although MC were located predominantly in the lamina propria of the airway (>90%) and in the alveolar septa (>80%) using both fixatives, the cells were abundant in the superficial lamina propria of airways using IFAA fixation, and in the tunica muscularis and deep lamina propria using FA fixation.

7.3.2 Morphology

The MC varied in shape from ovoid to elongate, depending on their location and fixation. Using FA fixation, all MC, except those located in the tunica muscularis, were ovoid (Fig. 7.3) and contained a few purple-stained, discrete granules, whereas those in the tunica muscularis were larger, spindle-shaped or elongate (Fig. 7.4) and usually contained more abundant granules. The MC in the tissues fixed with IFAA were variable in shape and size. They were generally smaller than those seen in the FA-fixed tissues, and often contained many intensely stained granules which frequently obscured the nucleus (Fig. 7.5).

With alcian blue and safranin O staining, MC in the sections from the ovine LRT, regardless of their location and fixation, showed alcian-blue-positive reactions exclusively, whereas MC in the rat lung (control) sections exhibited alcian-blue-positive, safranin-positive and mixed reactions.

Ultrastructurally, the MC exhibited small to moderate numbers of short villous processes arranged at right angles or parallel to the cell surface. The MC cytoplasm was dominated by numerous round, or ellipsoid granules of varying size, together with small numbers of mitochondria and ribosomes. The cell nucleus was either centrally or eccentrically located and was usually non-segmented, although in some cells it was deeply invaginated or even lobulated. The granules were surrounded by a perigranular membrane which was often difficult to distinguish. The granules of most MC in the superficial lamina propria of airways and the lung were relatively uniform in their structure, and could be classified into two types (Fig. 7.6). The most common type (type I) was homogeneous and electron-dense, whereas the second type (type II) was of lower electron-density with a fine granular matrix. Transitional forms between these two types were occasionally found. Sometimes MC showing almost complete degranulation were also seen (Fig. 7.7). The granules in the majority of MC lying in the deep lamina propria and tunica muscularis of airways were more heterogeneous (Fig. 7.8). Apart from the types described above, many granules were irregular or crescent-shaped, less electron-dense and contained a more granular matrix, which was well demarcated from the remaining dense, homogeneous contents (Fig. 7.9a). Some granules also had a "robe-like" appearance or showed combinations of several types (Fig. 7.9b).

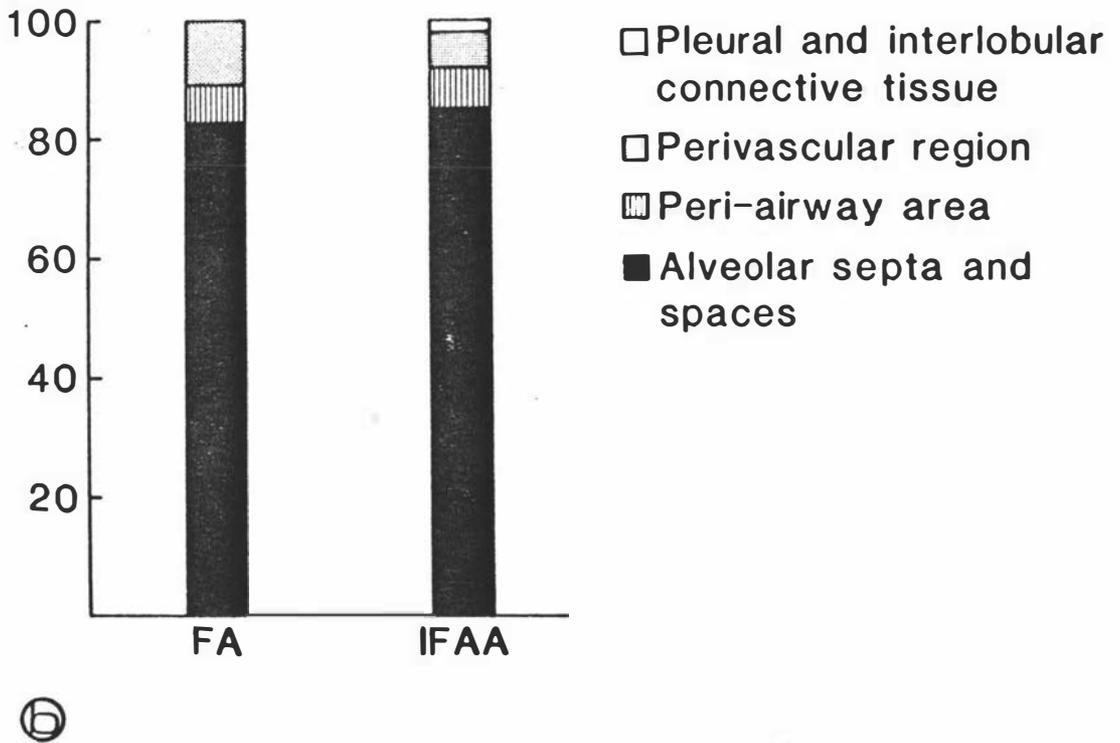
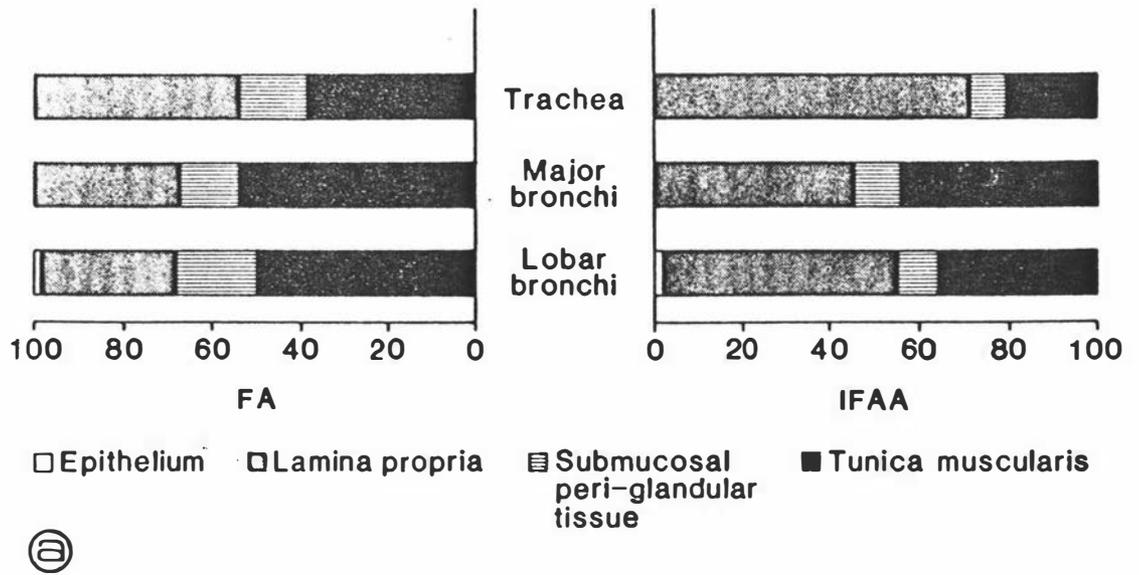


Fig. 7.2 Distribution of mast cells within tissue compartments in the ovine airway (a) and lung (b).

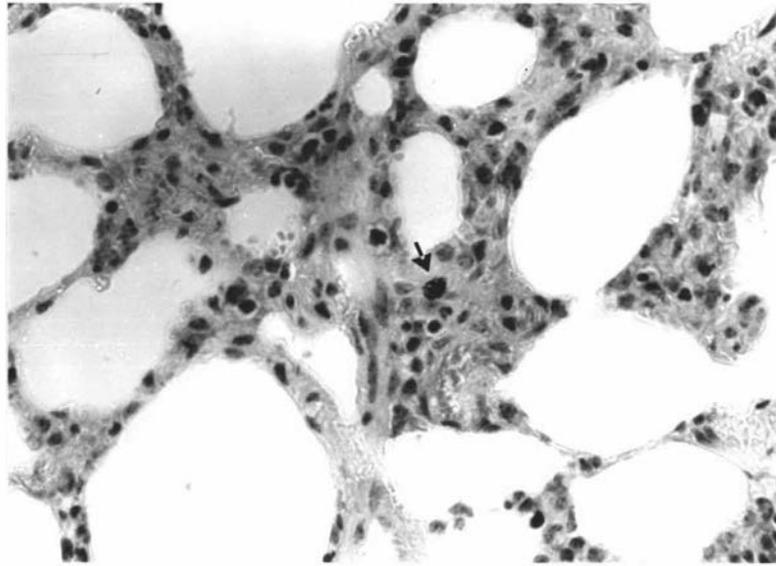


Fig. 7.3 Ovoid type of mast cell (arrow) in the ovine lung fixed in 10% neutral buffered formalin. Toluidine blue, x185.

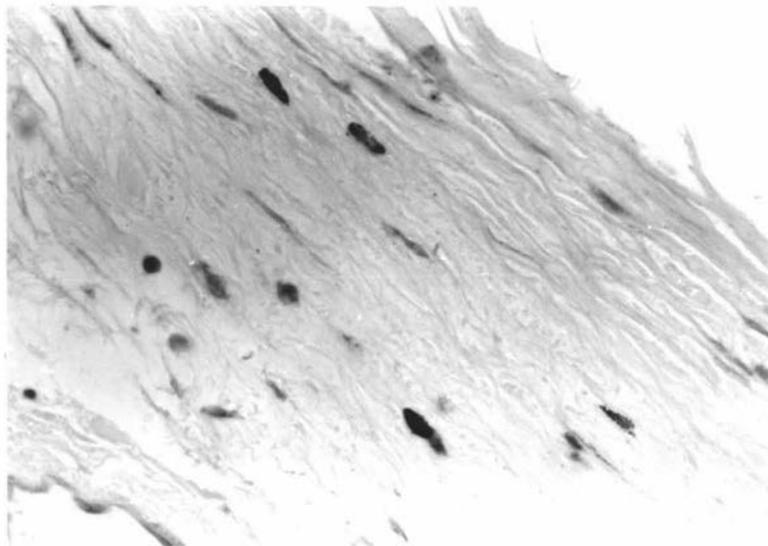


Fig. 7.4 Elongated type of mast cell in the bronchial smooth muscle of a sheep. Fixed in 10% neutral buffered formalin. Toluidine blue, x185.

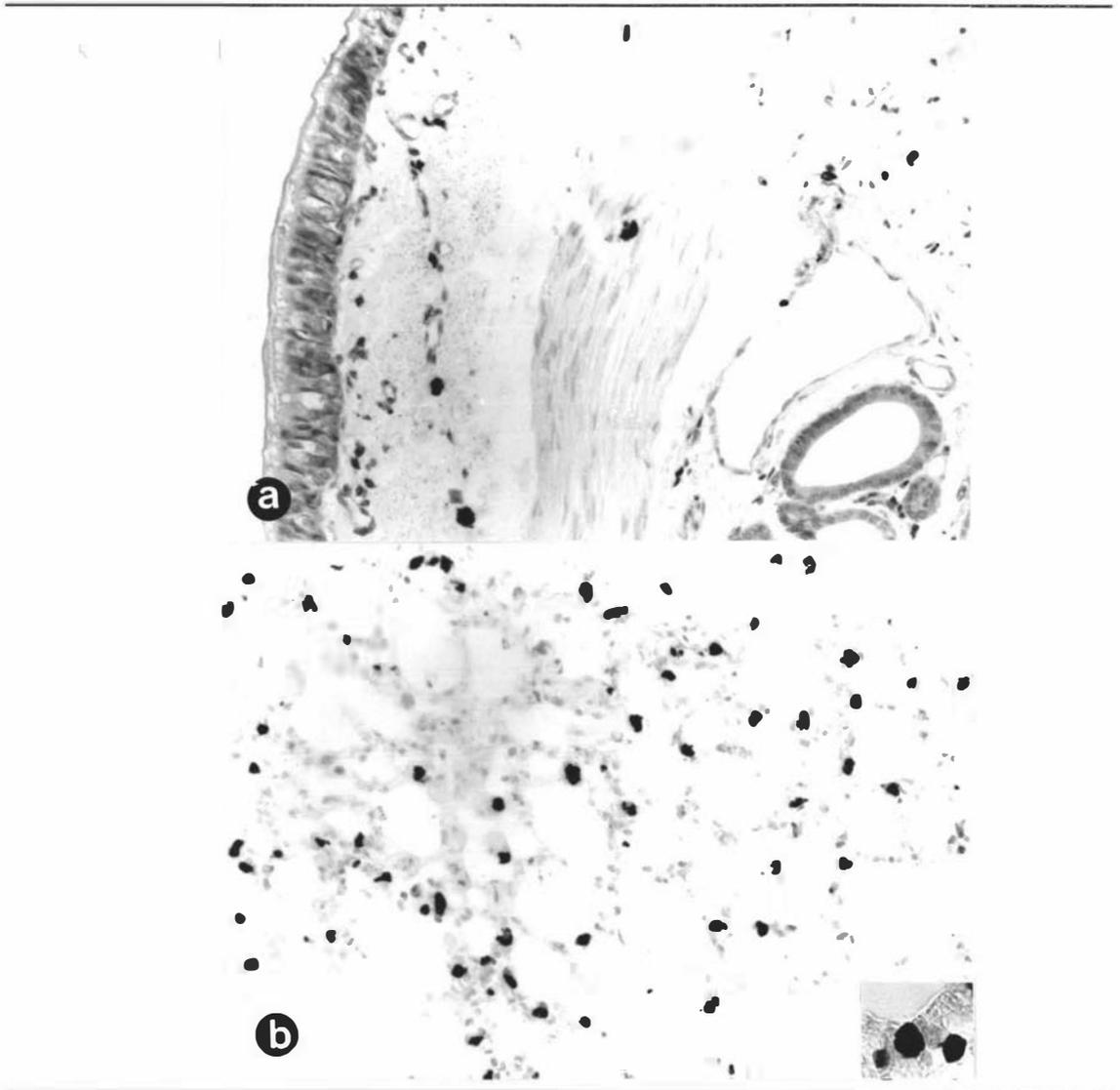


Fig. 7.5 Mast cells in the superficial lamina propria of ovine trachea (a) and lung (b) fixed in isotonic formal-acetic-acid. They are small and variable in shape. Toluidine blue, x225. Inset: High magnification of a mast cell with intensively stained intracytoplasmic granules which have obscured the nucleus. Toluidine blue, x375.

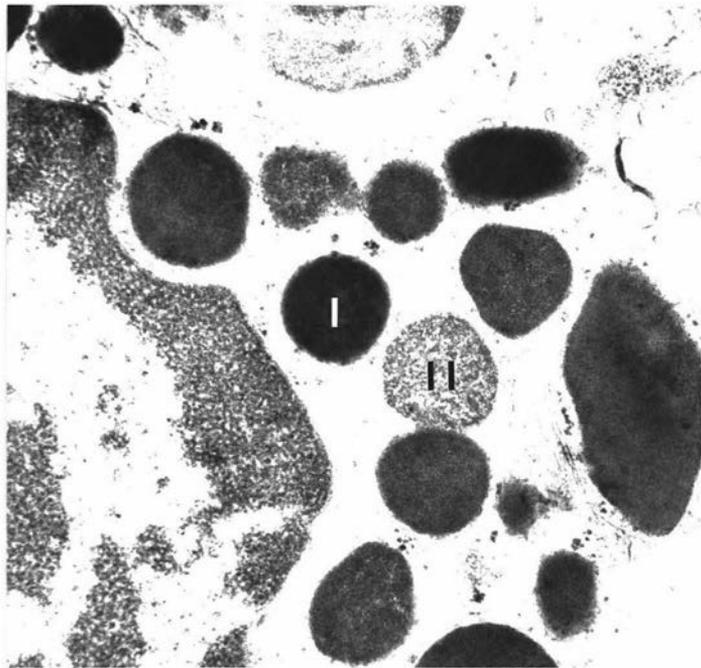


Fig. 7.6 Type I granules (I) and type II granules (II) in a mast cell located in an alveolar septum of a sheep. TEM, x31,800.

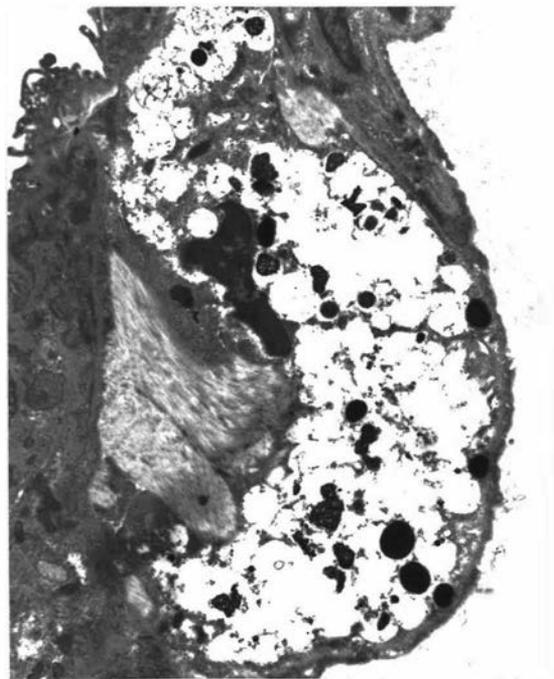


Fig. 7.7 An almost completely degranulated ovine lung mast cell containing a few remnants of the granules. TEM, x7,800.

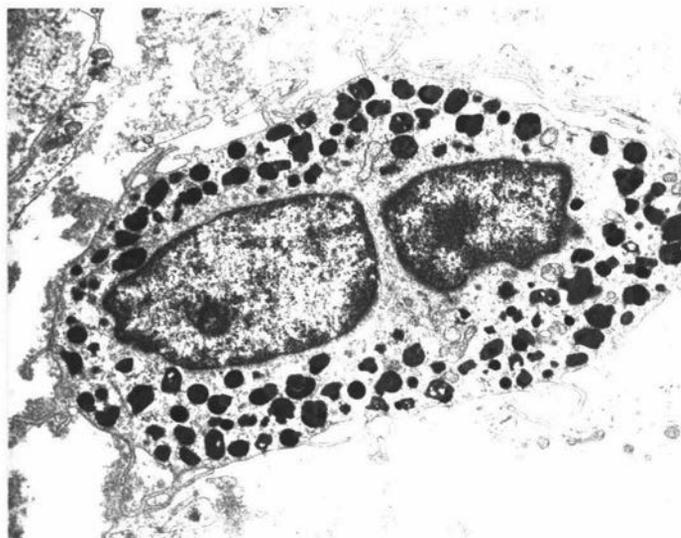


Fig. 7.8 A mast cell in the deep lamina propria of an ovine airway showing the heterogeneous nature of secretory granules. TEM, x7,800.

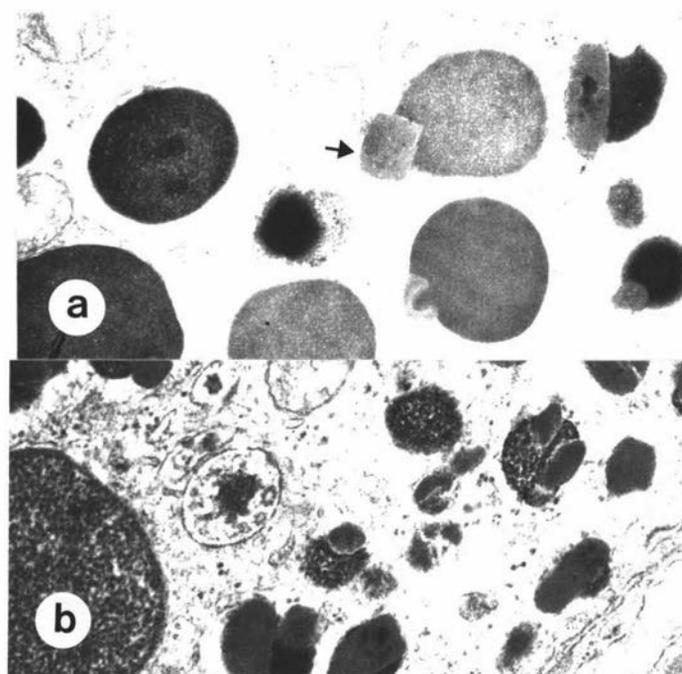


Fig. 7.9 Higher magnification of Figure 8 showing granules with crescentic electron-lucent [arrow] areas (a); and combination type of secretory granules (b). TEM, x31,800.

7.4 DISCUSSION

The ovine model of human allergic bronchial hypersensitivity is now well established in North American (Wanner and Abraham, 1982), but little is known of the mechanisms causing the similarity in human and ovine responses. Information on MC in the ovine respiratory tract is of critical importance in elucidating these mechanisms. In the current study tissues from several levels of the LRT were fixed in two types of fixative earlier shown to be essential for the differentiation of MMC and CTMC in rat intestine (Enerback, 1966a). The presence of a higher density of MC in IFAA-fixed tissues suggests that two subtypes of MC exist in this site, one histochemically analogous to the classical CTMC (formalin-resistant) and the other similar to MMC in the rat intestine (formalin-sensitive).

Histochemical and morphological heterogeneities of MC have been demonstrated previously in human lung and nasal mucosa (Gomez *et al.*, 1987; Shanahan *et al.*, 1987; Heard *et al.*, 1989), and monkey lung (Eady *et al.*, 1986), but not in the LRT of guinea pigs (Bachelet *et al.*, 1988). Studies on MC in the rat respiratory tract have produced some controversial results. Initial studies (Goto *et al.*, 1984) revealed that MC heterogeneity was only present in the tracheal mucosa of the cartilaginous part. However, this finding has been disputed by the recent report that rat lung MC are also heterogeneous (Bachelet *et al.*, 1988). As in the other species, the majority of MC in the ovine LRT are formalin-sensitive and they may contain types of glycosaminoglycan in their granules which are different from formalin-resistant MC (Enerback *et al.*, 1986). The different requirements of each MC type for fixation has also been suggested to be related to cell maturity (Flint, 1987), although a recent study on human MC suggests that development of MC subtypes follows a distinct pathway from the time granule formation begins (Craig *et al.*, 1989).

The alcian blue and safranin O double staining method was originally used to distinguish MMC and CTMC in rats (Enerback, 1966a,b). In the rat intestine, MMC stain exclusively alcian-blue-positive while CTMC are safranin-positive. The current finding that all MC in the ovine LRT are exclusively alcian-blue-positive is contrary to the results obtained in rats, but is similar to studies of MC in the lungs of man (Agius *et al.*, 1986), guinea pigs (Bachelet *et al.*, 1988) and cattle (Chen *et al.*, 1990). It has recently been suggested that results obtained by this staining method in non-rodent species should be interpreted with special caution (Enerback *et al.*, 1986). Further studies on the biochemical composition of MC in sheep will therefore be necessary to better understand the significance of these results.

There is considerable difference in the density of formalin-sensitive MC in the normal respiratory tract of different animals. The present study has shown that the density of formalin-

sensitive MC in the ovine airway is greater than that in the airways of dogs (Gold *et al.*, 1977), and horses (Mair *et al.*, 1988), but is similar to the human airway (Shanahan *et al.*, 1987). The rat airway, however contains more formalin-sensitive MC than the sheep airway (Goto *et al.*, 1984). Like rats and dogs, MC density increases distally from the trachea to the peripheral lung (Gold *et al.*, 1977; Bachelet *et al.*, 1988). The density of lung formalin-sensitive MC in sheep is greater than that in any other animals studied so far including man (Shanahan *et al.*, 1987; Bachelet *et al.*, 1988; Mair *et al.*, 1988; Heard *et al.*, 1989). In man the density of lung MC is about 50 cells/mm² (Heard *et al.*, 1989), which is about one quarter of the density of mast cells in the lung of sheep. The high density of formalin-sensitive MC in the ovine LRT may be associated with the high prevalence of parasitic infection in this species. Parasitic infection can stimulate a thymus-dependent proliferation of intestinal MMC in man, rodents and sheep (Jarrett and Miller, 1982). The demonstration of numerous MC in the ovine LRT, together with previous evidence that allergic bronchoconstriction in sheep is closely associated with the elevation of arterial plasma histamine levels (Wanner *et al.*, 1979) suggests that MC in the ovine respiratory tract are pivotal in the mediation of the allergic bronchoconstriction seen in this species.

The distribution of MC in the respiratory tract has been studied extensively in horses (Mair *et al.*, 1988), and to a lesser extent in rodents (Goto *et al.*, 1984), monkeys (Guerzon *et al.*, 1979), dogs (Gold *et al.*, 1977), cattle (Chen *et al.*, 1990) and man (Shanahan *et al.*, 1987). The abundance of MC in the lamina propria of ovine airways fixed by both methods is similar to the results of these studies, although the bovine airways contain a higher percentage of intraepithelial MC. In the lung, the MC distribution has not been studied fully in either man or animals. The available information suggests that the distribution of lung MC in sheep is similar to that seen in cattle (Chen *et al.*, 1990), but is very different from that in horses (Mair *et al.*, 1988). In horses, more than 80% of lung MC are located in the intrapulmonary connective tissue while in both cattle and sheep about 85% of lung MC are within alveolar septa. This difference may be due to the fact that horses are athletic animals and thus more likely to suffer from hypoxia which is known to significantly increase perivascular MC density (Tucker *et al.*, 1977). Ruminants on the other hand, are more commonly infected by lung parasites and microorganisms, which is likely to result in localisation of MC in the alveolar septa and bronchiolar mucosa. Cells in these positions are thought to be the major source of MC in bronchoalveolar lavage fluid (Agius *et al.*, 1986) and may play an important role in the initiation of immediate bronchial hypersensitivity to inhaled antigens.

The present studies have shown that the MC in the ovine LRT are ultrastructurally heterogeneous. Cells from areas where formalin-sensitive MC prevail contain granules which are more homogeneous than those in cells from regions where formalin-resistant MC

predominate. Considerable variation in the ultrastructure of MC granules has been noted between other species (Caulfield *et al.*, 1980; Fox *et al.*, 1981; Dvorak *et al.*, 1983; Mair *et al.*, 1988; Nishigaki, 1988; Chen *et al.*, 1990), and this finding is supported by the present study. The MC granules in the ovine LRT are generally similar to those of other animals studied so far, including mice (Dvorak *et al.*, 1983), rats (Nishigaki, 1988), horses (Mair *et al.*, 1988), and cattle (Chen *et al.*, 1990), but differ from those in man (Brinkman, 1968; Caulfield *et al.*, 1980; Fox *et al.*, 1981; Lamb and Lumsden, 1982; Warton *et al.*, 1986a). Mature MC from human lung contain granules with a variety of ultrastructural patterns, such as crystals, particles, scrolls, lattices, reticules, or combinations of these patterns (Caulfield *et al.*, 1980; Fox *et al.*, 1981; Warton *et al.*, 1986a). The difference in these ultrastructural patterns may represent stages in the process of mediator release (Caulfield *et al.*, 1980; Fox *et al.*, 1981; Nishigaki, 1988), and could perhaps reflect a heterogeneity in the biochemical constitution of their contents (Craig *et al.*, 1988).

It is of fundamental importance to determine if the morphological and histochemical heterogeneities of MC in the ovine LRT are associated with functional heterogeneity. In the rat, MMC are functionally different from CTMC in several aspects. They contain non-heparinic proteoglycan, have both surface and cytoplasmic immunoglobulin E, and are resistant to various secretagogues (Bienenstock, 1988). In particular, MMC are unresponsive to certain antiallergic compounds such as disodium cromoglycate (Church, 1988). Although the composition of MC granules in the ovine respiratory tract has not been studied in detail, studies on abomasal MMC in this species indicate that they have some similarity to MC in the human respiratory tract (Miller *et al.*, 1985).

Much of the knowledge on human MC heterogeneity has been extrapolated from studies of the rat. However, recent studies have found that rat MC do not totally reflect the histochemical and functional characteristics of MC in man and other animals (Befus *et al.*, 1986). Directly derived information on human MC heterogeneity is limited due to the difficulty in obtaining multiple tissue samples from the same individual (Barrett and Metcalfe, 1986). Alternative animal models are therefore necessary. The results of the current study indicate that MC in the ovine respiratory tract have several morphological and histochemical similarities to those in the human respiratory tract. Like man, the majority of lung MC in sheep are highly sensitive to formalin and stain exclusively with alcian blue (Agius *et al.*, 1986; Gomez *et al.*, 1987). The high density of formalin-sensitive MC in the ovine LRT may extend further the use of the sheep model in the study of human allergic hypersensitivity since ovine lung MC may be easier to isolate and purify for use in functional and pharmacological studies than those of other species.

7.5 SUMMARY

The lower respiratory tract of six adult sheep was fixed in either isotonic formal-acetic-acid or neutral buffered formalin in order to study the heterogeneity, morphology and density of mast cells. Two subtypes of mast cell were found, one similar histochemically to connective tissue mast cells (formalin-resistant) and the other similar to mucosal mast cells as found in the intestine of the rat (formalin-sensitive). Although both subtypes were present at all levels of the tract, formalin-sensitive mast cells were significantly more abundant ($p < 0.01$) at all levels, and their density increased distally from the trachea to the peripheral lung. The formalin-sensitive mast cells were located predominantly in the alveolar septa and in the superficial lamina propria of airways, and less frequently within the airway epithelium. The mast cells in the ovine lower respiratory tract were found to be morphologically heterogeneous at both the light microscopic and electron microscopic levels. These findings indicate that ovine respiratory tract mast cells have similarity to human lung mast cells, and therefore potential for use as a model for the study of human allergic disease of the respiratory system.

PART III
**AIRWAY HYPERSENSITIVITY INDUCED BY *ASCARIS SUUM* EXTRACT IN
NEW ZEALAND ROMNEY SHEEP: A MODEL FOR ASTHMA**

CHAPTER 8

AIRWAY HYPERSENSITIVITY INDUCED BY *ASCARIS SUUM* EXTRACT IN NEW ZEALAND ROMNEY SHEEP: A MODEL FOR ASTHMA

8.1 INTRODUCTION

Over the past decade sheep have been used increasingly to study the pathogenesis and pharmacology of allergic respiratory diseases (Wanner and Abraham, 1982). Most sheep tested in North America and Japan have a natural skin sensitivity to *Ascaris suum* extract (Wanner *et al.*, 1979; Okayama *et al.*, 1989), and exhibit an immediate airway hypersensitivity to challenge by inhalation with this antigen. The responding sheep show significant changes in airway resistance (Raw) and dynamic lung compliance (C_{dyn}), together with pulmonary hyperinflation and ventilation-perfusion imbalances (Wanner *et al.*, 1979).

However, the mechanisms and factors contributing to this natural allergic hypersensitivity have not been fully explored. It is not clear whether or not the previous direct or indirect contact with pigs and thereby *A. suum* is essential for this hypersensitivity. Neither is it clear whether all sheep breeds react in a similar manner. The objective of the present study was to investigate the natural skin and immediate airway responses of normally pastured Romney sheep, with and without previous exposure to domestic pigs, in New Zealand to *A. suum* and thus evaluate their suitability to study allergic airway hypersensitivity.

8.2 MATERIALS AND METHODS

8.2.1 Animals

One hundred and one Romney ewes aged 4-7 years (weighing 35-55 kg) and eight 1-month-old lambs, originating from farms in the Manawatu, were used. Fifty-two ewes were obtained from a hill country farm where there was no history of exposure to pigs (Farm I), and the remaining 49 ewes were purchased from a lowland property where they were farmed on pasture grazed by domestic pigs (Farm II). During the experiment all animals were grazed on a Massey University sheep farm.

8.2.2 Antigen

Ascaris suum extract was purchased commercially (Greer Lab, N.C.) as a stock solution containing 2×10^5 protein nitrogen units/ml. A working solution for skin and bronchial provocation tests was freshly prepared with sterile phosphate buffered saline (PBS, pH 7.3).

8.2.3 Skin tests

Skin hypersensitivity was assessed in all adult animals by intradermal injection with *A. suum* extract using the technique recommended for human patients by the Asthma and Allergic Disease Center of the United States (Chai *et al.*, 1975). Before testing, each animal was injected intravenously with 5 ml of 0.5% Evans blue (Merck Colour Index No 23860) to improve the clarity of the skin reaction. The concentrations of the antigen used were estimated from a pilot experiment on ten sheep using five 10-fold dilutions ranging from 20 to 200,000 protein nitrogen units/ml on each sheep. All sheep in the pilot experiment showed a maximum skin reaction to the antigen at a concentration of 2000 protein nitrogen units/ml and above, and this concentration was selected as standard dilution for the remaining sheep. The immediate skin reactions were read 30 minutes after injection, and late reactions were examined at 6 or 8 hours. As the resultant wheals were not always circular, both their largest and smallest diameters were measured, and the means of these used as the diameter of the reaction. The degree of skin reaction was determined by the diameter of the wheal produced by the control subtracted from that produced by the antigen. A positive response was recorded when the size of a blue wheal developed at antigen-injected site is 5 mm or more greater than that seen at control site.

8.2.4 Experimental set-up

Five sheep were used in a pilot study not described in detail here to design and test an experimental protocol for the measurement of respiratory parameters and the subsequent bronchial provocation test under anaesthesia. Results from the study indicated that: (1) sheep did not respond to a few breaths of antigen and the exposure must be maintained over several minutes; (2) animals responded to antigen whilst under halothane anaesthesia; (3) the measurement of intrapleural pressure (Ppl) by intrapleural catheter was more convenient than by intraoesophageal balloon in anaesthetised sheep; (4) passing nebulised material through a pneumotachograph head for several minutes changed the physical characteristics of the head to an extent to make airflow data analysis impossible.

A two-way valving system was then designed such that the output of the nebuliser could be directed into a closed rebreathing anaesthetic system but that the animal could be periodically connected to a pneumotachograph head for airflow measurement during the aerosol exposure (Ppl was recorded throughout the experiment). The detailed experimental set-up is illustrated in Fig. 8.1.

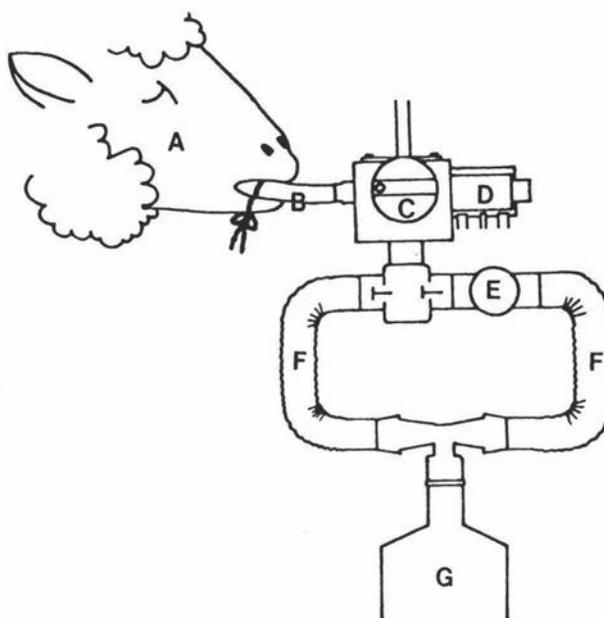


Fig. 8.1 Diagram of experimental set-up for the measurement of respiratory parameters and the bronchial provocation test in anaesthetised sheep. A=sheep, B=endotracheal tube, C=two-way valve, D=Fleisch pneumotachograph and differential pressure transducer, E=ultrasonic nebuliser, F=closed rebreathing anaesthetic system and G=oxygen and halothane supply.

8.2.5 Measurement of respiratory parameters

After halothane (6%) anaesthesia was induced using a face mask, the animals were intubated with a cuffed endotracheal tube (No.12) and positioned on their right side on a pre-heated table. The halothane was then reduced to 2.5% to maintain anaesthesia throughout the remainder of the experiment. The methods used to measure airflow rate (V), tidal volume (V_T) and Ppl were similar to those described by Colebatch and Halmagyi (1961). The Ppl was recorded relative to atmospheric pressure by inserting a 16 gauge polyethylene catheter through a hypodermic needle into the pleural cavity between the seventh and eighth intercostal space 10-15 cm above the level of the olecranon process. The proximal end of the catheter was connected to a Grass differential pressure transducer. The airflow rate was measured with a No.1 Fleisch pneumotachograph and a differential pressure transducer (Validyne DP45-16). Tidal volume was obtained by electrical integration of the flow signal using a respiratory integrator. After amplification, the Ppl, V and V_T were recorded simultaneously on a Gould four-channel chart recorder (chart speed 10 mm/s). Intrapleural pressure and V were also

displayed on an oscilloscope for "on line" estimation of changes in Raw. All the monitored variables, together with appropriate calibration signals, were recorded on a frequency modulated cassette data recorder (TEAC XR-310) for subsequent analysis.

The Raw values were obtained by displaying and analysing the taped pressure-flow records on an oscilloscope by the method of Mead and Whittenberger (1953) as modified by Nadel and Widdicombe (1962). The resistance of the endotracheal tube and valve system (0.78 cm H₂O/l/s) was subtracted from the measured Raw. The Cdyn values were obtained from analysis of the chart records from the V_T and Ppl records at points of zero flow. The respiratory frequency (f) was also calculated from the chart records.

After the breathing pattern of the animals stabilised, baseline values of V, V_T, and Ppl were recorded. Recordings were then made at 5 minutes intervals during inhalation of PBS and challenge with *Ascaris* antigen. Each value used in the analysis was the average of five consecutive breaths on each occasion. The percentage change during exposure to PBS and antigen challenge was calculated from the respective baselines.

8.2.6 Bronchial provocation test

Forty-three ewes with positive skin reactions were randomly selected from the above 101 sheep. Fourteen out of these 43 ewes were pregnant when bronchial provocation tests were conducted. In addition, eight lambs were also included in this study. An aerosol of PBS and *Ascaris* extract (10⁵ protein nitrogen units/ml) was delivered using an ultrasonic nebuliser (DeVibliss, Pennsylvania) as illustrated in Fig. 8.1. Each animal was given PBS for 20 minutes, a period of "recovery" and then followed by antigen for 20 minutes.

The changes in Raw and Cdyn were used to assess airway responses to the aerosols since these parameters have previously been demonstrated to reliably reflect the acute antigen-induced airway response in sheep and monkeys (Pare *et al.*, 1976; Wanner *et al.*, 1979). Criteria for a response was arbitrarily defined as a 50% increase in Raw, or a 50% reduction in Cdyn from their baselines.

8.2.7 Data analysis

All the statistical analysis was performed on an IBM computer based data system (Statistix, NH Analytical Software). The value of each respiratory parameter at each measurement time was computed from averaging five consecutive breaths for each sheep, with the group means determined from the animal mean value. All the data are presented as means \pm the standard error of means. The effect of *Ascaris* antigen and PBS challenge was

compared to their respective baseline values by using one-way analysis of variance with Dunnett's multiple range test (Winer, 1971). The data of sheep from different farms were examined for significance using an unpaired t test. The correlation between skin reactivity and airway response was analysed using the Spearman's correlation test. The effect of pregnancy on airway response was analysed with Mann-Whitney U test. Significance was accepted at $p < 0.05$ level.

8.3 RESULTS

8.3.1 Skin reactivity

The results of the skin tests are shown in Fig. 8.2. There was an immediate skin reaction larger than 5 mm in diameter in 90% of the sheep. A representative skin reaction from a sheep is shown in Fig. 8.3. The mean diameter of the reaction in sheep from Farm II was significantly larger than that of sheep from Farm I (11.2 ± 0.73 mm vs 13.9 ± 0.47 mm, $p < 0.01$; Fig. 8.4). No late skin reactions were observed.

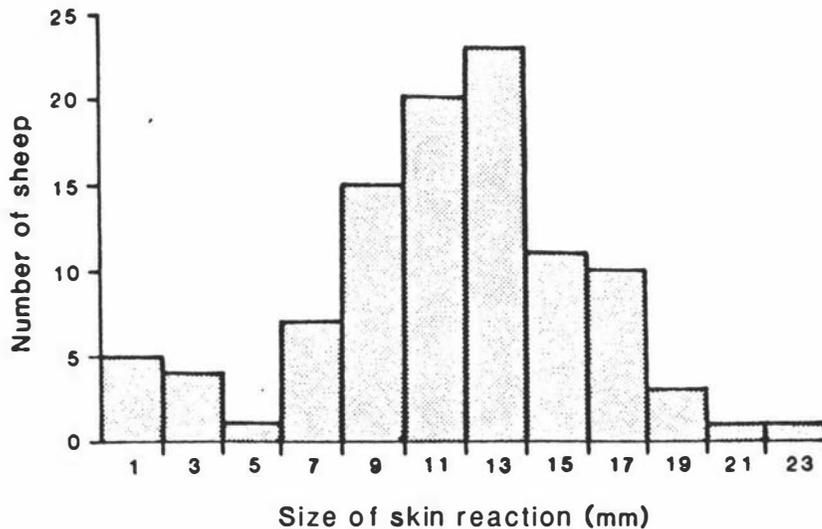


Fig. 8.2 Reactions to skin tests with *Ascaris suum* antigen in 101 adult sheep.

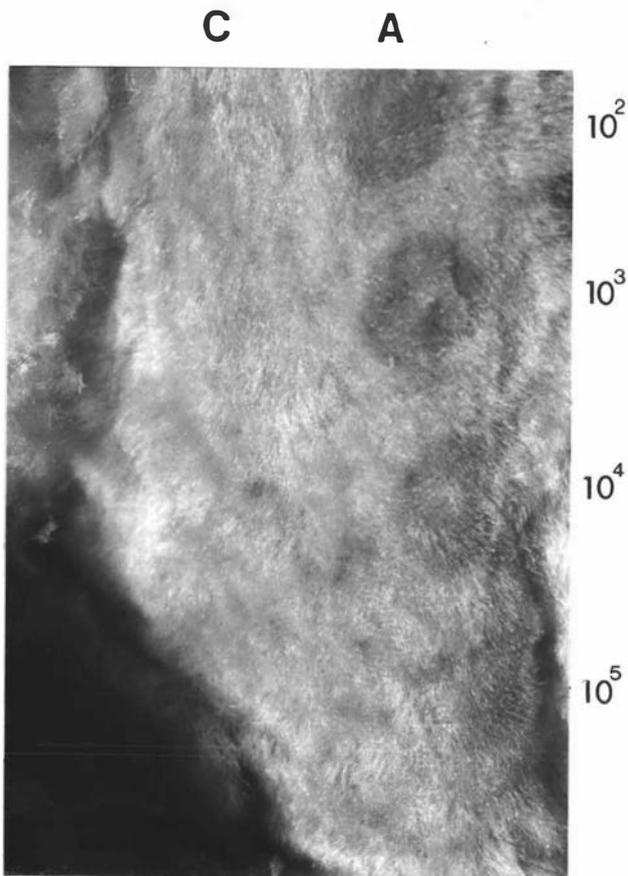


Fig. 8.3 Skin reactions to intradermal injection with *Ascaris suum* antigen (A) and phosphate buffered saline (C). The figures in the right side of the photograph are the concentrations of the antigen (protein nitrogen, units/ml).

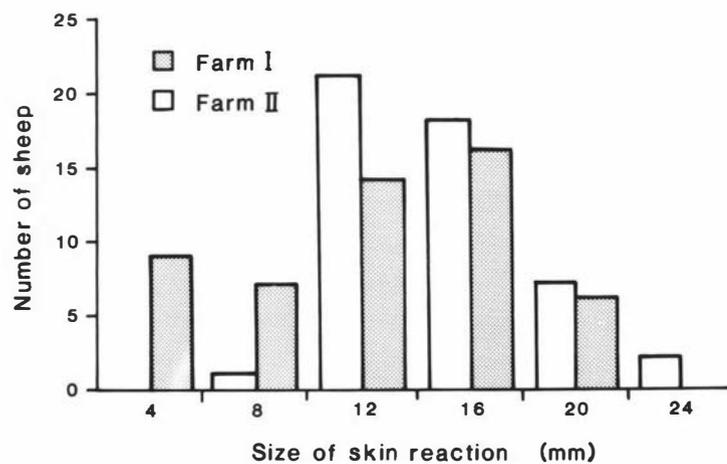


Fig. 8.4 Comparison of skin reactions to *Ascaris* antigen in sheep with (Farm II) and without (Farm I) a previous history of exposure to pigs.

8.3.2 Effect of PBS inhalation

The baseline values for Raw, Cdyn and f were 3.49 ± 0.29 cm H₂O/l/s, 57.45 ± 7.70 ml/cm H₂O and 47.37 ± 2.27 times/min for the adult sheep tested, and 2.60 ± 0.41 cm H₂O/l/s, 32.15 ± 5.48 ml/cm H₂O and 59.15 ± 6.36 times/min for lambs. The sheep showed no significant changes ($p > 0.05$) in any mean values during or after 20 minutes PBS exposure (Table 8.1). Three adult animals exhibited a large transient swing in their parameters, but these never reached their peak responses to the antigen.

8.3.3 Effect of antigen inhalation

Thirty-one out of 43 adult sheep (72%) showed an immediate bronchoconstriction. Among these responders (hypersensitive sheep), 21 sheep had significant changes in both Raw and Cdyn (Group A), while ten sheep showed significant changes only in Cdyn (Group B). The remaining 12 adult sheep (28%) had no significant changes in either Raw or Cdyn and were judged non-responders (non-reacting sheep, Group C). Similarly, all lambs showed no significant changes in the respiratory parameters to inhaled antigen. Representative records of the airway responses to the inhaled PBS and antigen in a hypersensitive sheep and a non-reacting sheep are shown in Fig. 8.5. The absolute and relative changes in the respiratory parameters between these groups are presented in Table 8.2 and Fig. 8.6. The maximum increase of Raw and the peak decrease of Cdyn in the individual hypersensitive animals were 643% and 94% of the respective baselines. In contrast, the maximum individual values for Raw and Cdyn in non-reacting animals did not change by more than 40%.

Most animals in Groups A and B developed a marked alteration in the respiratory pattern (a marked increase in Ppl and moderate increase in respiratory frequency) within 5 minutes. Clinically, these animals showed variable severity of respiratory distress. About 20% of the hypersensitive sheep also exhibited a very severe wheeze so that the inhalation challenge was terminated after 10 minutes of antigen exposure to avoid the risk of death.

The mean values of the pulmonary mechanics observed after antigen exposure were significantly changed from those found after PBS exposure at each period in Group A (Table 8.2, $p < 0.01$). In Group B, only Cdyn was significantly changed after 15 min (Table 8.2, $p < 0.05$). In Group C, neither Raw nor Cdyn was significantly changed (Table 8.2, $p > 0.05$).

No correlation was found between the degree of skin reaction and the magnitude of bronchoconstriction ($p > 0.05$). No significant differences in the changes of Raw or Cdyn were found between sheep from the two farms ($p > 0.05$).

Table 8.1
The Effect of Inhalation of Phosphate Buffered Saline (PBS) on Respiratory Parameters in Sheep*

	Base line	5	PBS inhalation (minutes)		
			10	15	20
<u>Ewes (n = 43)</u>					
Raw (cm H ₂ O/l/s)	3.49 ± 0.29	3.56 ± 0.26	3.69 ± 0.26	3.74 ± 0.26	3.62 ± 0.24
Cdyn (ml/cm H ₂ O)	57.45 ± 7.70	61.59 ± 7.86	61.59 ± 9.40	55.51 ± 5.92	60.00 ± 6.72
f (times/min)	47.37 ± 2.27	49.18 ± 2.56	49.55 ± 2.54	49.14 ± 2.47	49.72 ± 2.36
<u>Lambs (n = 8)</u>					
Raw (cm H ₂ O/l/s)	2.60 ± 0.41	2.74 ± 0.35	2.69 ± 0.37	2.59 ± 0.39	2.74 ± 0.41
Cdyn (ml/cm H ₂ O)	32.15 ± 5.48	36.06 ± 7.16	43.50 ± 9.37	37.40 ± 4.18	43.28 ± 6.74
f (times/min)	59.15 ± 6.36	57.33 ± 5.56	54.78 ± 6.74	64.23 ± 7.19	65.19 ± 6.66

* Values are mean ± the standard error of means; Values for each period of inhalation are not significantly different from respective baseline values (p>0.05). Raw=airway resistance, Cdyn=dynamic lung compliance and f=respiratory frequency.

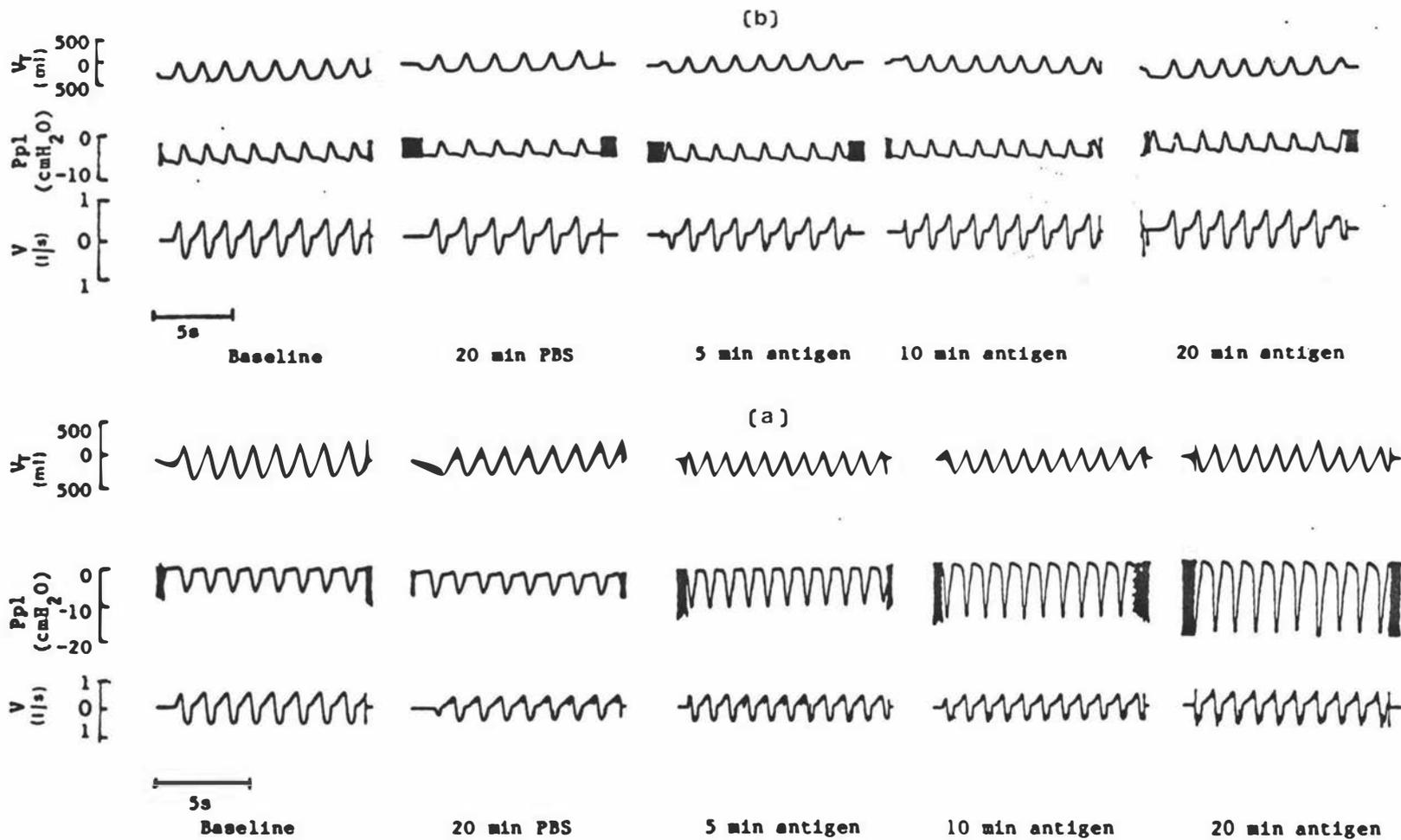


Fig. 6.5 Representative records of airway response to 20 minutes (min) phosphate buffered saline (PBS) followed by a 20 minutes aerosol of *Ascaris suum* antigen in a hypersensitive (a) and non-reacting sheep (b). V_T -tidal volume, Ppl=intrapleural pressure and V=airflow rate.

Table 8.2
Changes of Respiratory Parameters in Sheep After Inhalation of *Ascaris suum*[#]

Respiratory parameters	Base line	<i>Ascaris suum</i> (minutes)				
		5	10	15	20	
<u>Group A (n = 21)</u>						
Raw (cm H ₂ O/l/s)	2.74 ± 0.26	4.07 ± 0.52 ^b	5.52 ± 0.59 ^c	5.84 ± 0.81 ^c	6.10 ± 0.66 ^c	
Cdyn (ml/cm H ₂ O)	66.27 ± 8.19	34.34 ± 5.43 ^b	24.37 ± 4.33 ^c	24.94 ± 6.40 ^c	24.26 ± 5.63 ^c	
f (times/min)	53.32 ± 3.36	61.38 ± 3.46 ^b	70.38 ± 4.54 ^b	69.57 ± 6.37 ^b	75.08 ± 5.74 ^b	
<u>Group B (n = 10)</u>						
Raw (cm H ₂ O/l/s)	4.24 ± 0.61	4.28 ± 0.61	4.42 ± 0.67	4.74 ± 0.65 ^a	4.87 ± 0.64	
Cdyn (ml/cm H ₂ O)	73.44 ± 16.91	63.03 ± 12.63	37.77 ± 7.25 ^b	26.28 ± 4.33 ^b	29.94 ± 7.33 ^b	
f (times/min)	44.77 ± 5.77	52.08 ± 5.08	62.98 ± 7.27	65.69 ± 7.24	68.23 ± 8.17	
<u>Group C (n = 12)</u>						
Raw (cm H ₂ O/l/s)	3.85 ± 0.46	3.98 ± 0.46	3.89 ± 0.48	3.75 ± 0.34	3.68 ± 0.32	
Cdyn (ml/cm H ₂ O)	32.24 ± 5.62	30.44 ± 3.87	28.61 ± 3.85	29.23 ± 4.22	27.16 ± 3.64	
f (times/min)	39.21 ± 5.80	47.14 ± 5.12	49.98 ± 4.96	47.44 ± 6.30	48.14 ± 6.49	
<u>Lambs (n = 8)</u>						
Raw (cm H ₂ O/l/s)	2.58 ± 0.45	2.55 ± 0.43	2.45 ± 0.40	2.43 ± 0.38	2.61 ± 0.43	
Cdyn (ml/cm H ₂ O)	41.88 ± 9.92	40.85 ± 6.66	33.49 ± 4.57	40.25 ± 4.56	35.31 ± 4.44	
f (times/min)	53.16 ± 7.23	62.57 ± 6.65	64.87 ± 6.91	63.24 ± 7.58	64.37 ± 7.14	

[#] Values are means ± the standard error of means; Values for each period of inhalation are significantly different from respective baseline values by ^ap<0.05, ^bp<0.01, and ^cp<0.001. Raw=airway resistance, Cdyn=dynamic lung compliance, f=respiratory frequency.

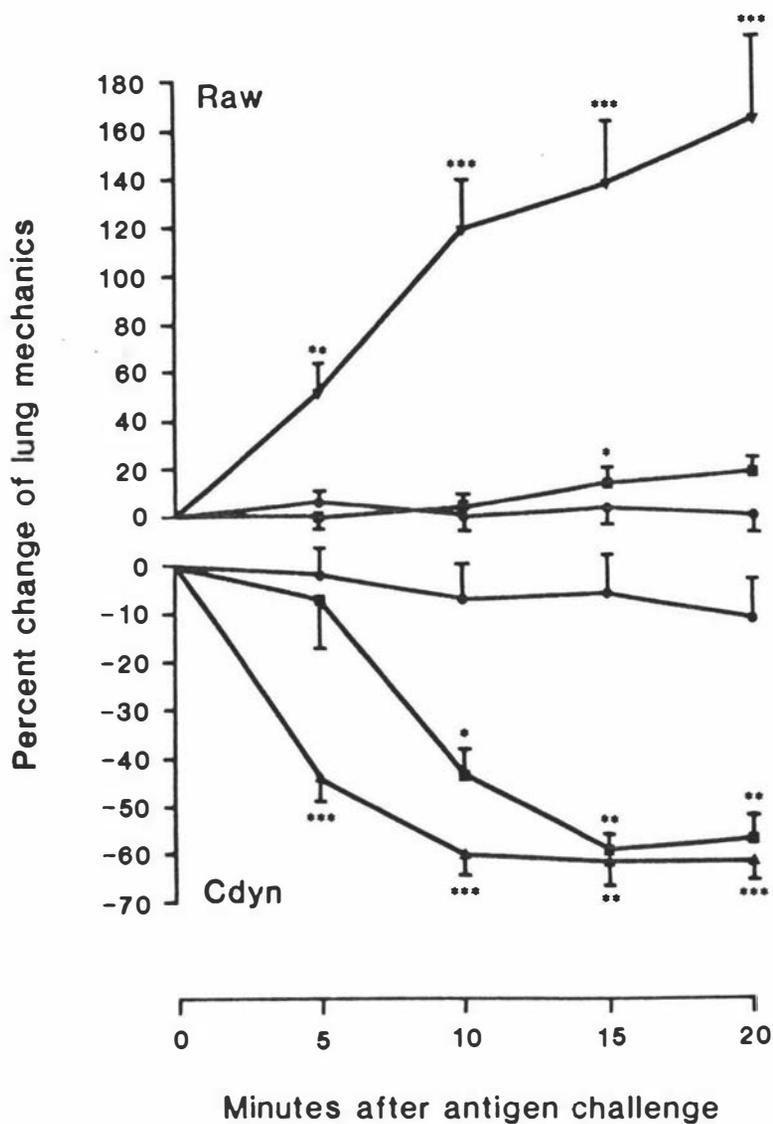


Fig. 8.6 The time course of relative changes in airway resistance (Raw) and dynamic lung compliance (Cdyn) after exposure to an aerosol of *Ascaris* antigen. ▼ =Sheep showing significant changes in both Raw and Cdyn (Group A), ■ =Sheep showing significant changes only in Cdyn (Group B) and ● =Sheep showing no significant changes in either Raw or Cdyn (Group C). Time 0 represents baseline values. Data are mean \pm the standard error of means (*= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$, with respect to the baselines).

8.3.4 Effect of pregnancy on airway response

The effect of pregnancy on airway hypersensitivity was examined by comparing the airway response in 14 pregnant sheep and 29 non-pregnant animals. Statistical analysis showed no significant difference between pregnant and non-pregnant sheep in either the mean percentage of Raw increase or the mean percentage of Cdyn decrease relative to their respective baseline ($p > 0.05$).

8.4 DISCUSSION

In the present study more than 70% of adult sheep from two farms in the Manawatu district exhibited an immediate airway response to inhalation challenge with *Ascaris* antigen at a dose similar to that used by overseas workers (Abraham *et al.*, 1981). This response usually began within 5 minutes of challenge and reached a maximum between 15 and 20 minutes; a response which is identical to that seen in allergic bronchial hypersensitivity of sheep reported overseas, and to that seen in human asthmatics (Wanner *et al.*, 1979; O'Byrne *et al.*, 1987). The results also indicate that the airway response of sheep in New Zealand is unlikely to be a non-specific irritant effect of the antigen since about 30% of adult sheep and all lambs showed no such reactivity after inhalation of a large quantity of antigen (10 ml). Other studies have shown that the immediate airway response in sheep is antigen specific and involves the release of mast cell-derived mediators including histamine (Wanner *et al.*, 1979), prostaglandin D₂ and thromboxane A₂ (Dworski *et al.*, 1989), and leukotriene C₄ (Okayama *et al.*, 1989).

The baseline values of Raw and Cdyn in the adult sheep studied were similar to the data reported previously (Wanner and Reinhart, 1978; Wanner *et al.*, 1979; Abraham *et al.*, 1983; Kleeberger *et al.*, 1985), but were respectively higher and lower than those reported by other workers (Hutchison *et al.*, 1982; Delehunt *et al.*, 1984; Okayama *et al.*, 1989). This discrepancy may be due to the different methodology used for the measurement and analysis of lung mechanics and the use of anaesthesia in the current study. The baseline values of respiratory parameters in our adult sheep did not appear to determine the subsequent response of the animals to inhalation challenge with antigen. This is in agreement with the data obtained from monkeys (Pare *et al.*, 1976) and sheep in North America (Wanner and Reinhart, 1978), but contrasts with the situation in human asthmatics who show a higher airway resistance than normal even in apparent remission (Mansell *et al.*, 1974).

Allergic airway hypersensitivity to the inhalation of *Ascaris* antigen has been documented in several species (Booth *et al.*, 1970; Pare *et al.*, 1976; Wanner *et al.*, 1979). The initial studies on sheep showed that 50% of ewes with a skin reactivity to antigen demonstrated a

mean maximum increase in pulmonary resistance of 248% and decrease in C_{dyn} of 44% after exposure to *Ascaris* at a concentration similar to that used in the present study (Wanner *et al.*, 1979; Abraham *et al.*, 1981). Our hypersensitive sheep exhibited significant changes in their Raw or/and C_{dyn}, indicating that the airway response in sheep involves both central and peripheral airways. Although the proportion of hypersensitive sheep in this study is higher than that reported by Wanner *et al.* (1979), if only those animals showing changes in both Raw and C_{dyn} are considered as responders, the figures are very similar since in the study by Wanner *et al.* (1979) C_{dyn} was not measured. In contrast, more recent overseas studies have demonstrated bronchoconstriction in almost all sheep (Kleeberger *et al.*, 1985; Okayama *et al.*, 1989).

The maximum mean airway responses observed in the present study were reflected in a 165% increase in Raw and a 61% decrease in C_{dyn} respectively. The Raw changes in our animals were generally less than those reported in most previous studies (Wanner *et al.*, 1979; Abraham *et al.*, 1983; Delehunt *et al.*, 1984; Okayama *et al.*, 1989), but were similar to those reported by one other group (Kleeberger *et al.*, 1985). The wide variation of Raw responses observed may be due to a number of factors. Individual differences in the pulmonary immunological status (Wanner *et al.*, 1979), morphology (Yanta *et al.*, 1981), and physiological condition (Nathan *et al.*, 1979) of the sheep may influence the response. The animals used in our studies were conventionally raised, aged ewes with an unknown history of antigen exposure and respiratory infection. Considerable individual variation in the airway response of animal models including sheep have been reported by other workers (Booth *et al.*, 1970; Pare *et al.*, 1976; Wanner *et al.*, 1979; Hutchison *et al.*, 1982; Abraham *et al.*, 1983). Since it has been demonstrated that the degree of airway response to inhaled mediators in dogs is closely related to the epithelial thickness and secretory cell numbers in airways (Yanta *et al.*, 1981), further studies should be designed to investigate the morphological and immunological basis of these differences.

Halothane has been reported to have a bronchodilatory effect in man and dogs (Brakensiek and Bergman, 1970; Hirshman *et al.*, 1982) and may therefore reduce the magnitude of bronchoconstriction induced by the antigen. Indeed, the magnitude of Raw change in our sheep was very similar to that in another study in which sheep were also anaesthetised with halothane (Kleeberger *et al.*, 1985). However, the effect of halothane on lung mechanics is probably species-dependent, and a recent study on newborn lambs has demonstrated that halothane anaesthesia did not reduce pulmonary resistance, but in fact induced an increase of approximately 60% (Robinson *et al.*, 1985). If this is also the case in aged sheep, it is possible that the pre-constriction induced by halothane may limit the magnitude of further response to antigen challenge in some animals.

The Cdyn change in our sheep was generally comparable or slightly higher than that previously reported (Abraham *et al.*, 1981; Delehunt *et al.*, 1984; Kleeberger *et al.*, 1985). In addition, one-third of our responders showed significant changes only in their Cdyn ($p < 0.05$) after antigen challenge, an observation which has not been emphasised previously in this model. The reasons for separation of large (Raw) and small (Cdyn) airway response is not yet known. One possibility is that the small airways of sheep are more responsive to the mediator(s) initiating airway constriction. It has been previously demonstrated that the major site of histamine action in sheep may be in the small airways (Hutchison *et al.*, 1982), and that sheep show a consistent and reproducible decrease in Cdyn with a less frequent and less reproducible increase in pulmonary resistance, when an aerosol of histamine is given. It has also been noted that the small airways are the predominant site of the chronic allergic airway response in this species (Bosse *et al.*, 1987). Another explanation for this phenomenon may be differences in the release of bronchoconstriction mediator(s), or the concentration of mediator-producing cells in different levels of the airways. A third possibility is that the use of halothane anaesthesia may attenuate changes in Raw, although it does not influence the Cdyn value in newborn lambs (Fugner, 1985; Robinson *et al.*, 1985). The separation in the changes of Raw and Cdyn has also been reported in some *Ascaris*-sensitive monkeys (Kelly *et al.*, 1974).

The results of the present study indicate that natural immediate skin and airway reactions to *Ascaris* antigen in sheep in Manawatu are generally high. The cause of these reactions is not clear and will require further investigation. Direct exposure of sheep to *A. suum* eggs is not common under New Zealand pastoral conditions and natural sensitisation of these animals may have occurred during infestation with other nematodes which antigenically cross-react with *A. suum*. Studies not presented here (Appendix 8.1) on 22 sera of those 43 adult sheep using a standardised enzyme-linked immunosorbent assay (Clemett *et al.*, 1989) have excluded the possibility of cross antigen reaction with *Toxocara canis*, a common dog nematode which has been proposed as a cause in the development of *Ascaris*-induced airway hypersensitivity in dogs (Patterson and Kelly, 1974). It must however be accepted that there may be no causal association between exposure to pigs and the increased size of skin reaction. The difference could perhaps be attributable to some other environmental factor. The fact that no significant difference in Raw or Cdyn was found in sheep with and without previous contact with *A. suum* appears not to be essential to the development of airway hypersensitivity to this antigen. The correlation between skin reactivity and airway response to *Ascaris* antigen in our sheep was poor. This is in agreement with observations in most other animals and probably also in man (Booth *et al.*, 1970; Pare *et al.*, 1976; Wanner *et al.*, 1979; Kleeberger *et al.*, 1985; Burrows *et al.*, 1989). In man aged 6-34 years, about 45% of non-asthmatic subjects

show some degree of positive skin reaction (Burrows *et al.*, 1989). The poor correlation between skin and airway response suggests that different mechanisms may be involved in skin and airway hypersensitivity. The presence of skin reaction (data not shown) but not airway response in lambs in the present study has supported this suggestion. In monkeys, it has been proposed that some factor in addition to the presence of immunoglobulin E is necessary for airway hypersensitivity (Pare *et al.*, 1976).

The influence of pregnancy on the severity of clinical signs of asthma remains unclear (Hiddlestone, 1964; Juniper *et al.*, 1989; White *et al.*, 1989b). Although worsening of the condition is noted in almost one fourth of asthmatic patients when they are pregnant, improvement has also been reported by slightly over one fourth of patients (Greenberger and Patterson, 1985; Juniper *et al.*, 1989). Fourteen out of 43 ewes were unexpectedly pregnant during the period of the present study, and the effect of pregnancy on allergic airway reaction in these animals were compared to the non-pregnant animals. Analysis of the results has shown that there was no significant difference in the changes of Raw or Cdyn between pregnant and non-pregnant sheep. This is in agreement with the results of most human studies which have shown that pregnancy does not seriously affect the severity of asthma (Hiddlestone, 1964; Greenberger and Patterson, 1985; White *et al.*, 1989b).

It is well recognised that many human asthmatics may develop late skin and airway reactions, which begin 4-12 hours after immediate response, are usually maximal after about 5 or 6 hours and resolve within 24 hours (Lessof, 1981; O'Byrne *et al.*, 1987). The late skin reaction has not been examined previously in the sheep model, and was not present in our sheep. Some allergic sheep do develop a reproducible late phase airway response similar to that in asthmatics (Abraham *et al.*, 1983). In the present study no attempt was made to demonstrate the late airway response. However, the results from the immediate-responding sheep suggest that some of them may be capable of developing a late airway response. Previous studies on both animal models and man have shown that the occurrence of dual airway responses are correlated with the magnitude of immediate airway response and are dose-related (Robertson *et al.*, 1974; Abraham *et al.*, 1983; Hamel *et al.*, 1986).

This study has demonstrated that the immediate skin and airway responses of New Zealand Romney sheep to *Ascaris* antigen were similar in most respects to human asthma. Since more than half the sheep tested responded to bronchial challenge with antigen, both responding and control animals are readily available. This species can therefore be used as a model to study human allergic airway hypersensitivity in this country.

8.5 SUMMARY

Sheep from local farms with and without previous exposure to pigs were tested for their skin and airway responses to a commercial *Ascaris suum* antigen. There was an immediate reaction to intradermal injection of the antigen in 90% of 101 adult sheep. A bronchial provocation test by aerosol of the same antigen was undertaken on 43 of the adult sheep with a positive skin reaction and eight lambs. About 70% of adult sheep showed an immediate airway response to the antigen as an aerosol, reflected as a significant increase in airway resistance and/or decrease of dynamic lung compliance. The mean peak airway resistance and mean lowest dynamic lung compliance were 165% above and 61% below their baselines, respectively. No significant changes were recorded when the same animals were given an aerosol of phosphate buffered saline. Similarly, no correlation was found between the degree of skin reaction and the magnitude of bronchoconstriction ($p > 0.05$). The sheep with previous exposure to pigs showed no significant differences in airway responses to antigen challenge, although they showed significantly greater skin reactions than those without exposure to pigs. There was no significant difference between the pregnant and non-pregnant sheep in their airway responses to inhaled antigen. These results indicate that the majority of Romney sheep in Manawatu have a natural skin and airway sensitivity to *A. suum* antigen and may therefore be used as an animal model to study human airway hypersensitivity. The origin of this sensitivity has yet to be determined.

PART IV
MORPHOLOGICAL AND MORPHOMETRIC COMPARISON OF
AIRWAY STRUCTURE AND INFLAMMATORY CELLS
IN HYPERSENSITIVE AND NON-REACTING SHEEP

CHAPTER 9

AIRWAY HYPERSENSITIVITY TO INHALED *ASCARIS SUUM* IN SHEEP: PERMEABILITY OF THE TRACHEOBRONCHIAL EPITHELIUM TO HORSERADISH PEROXIDASE

9.1 INTRODUCTION

The acute onset of immediate allergic airway hypersensitivity implies that a rapid interaction between allergens and airway mast cells is the initial step in the development of this reaction (Kaliner, 1989). The normal airway mucosa, however, is impermeable to large molecules (Hogg, 1981), and most common allergens, such as pollens, are about 20 μm in diameter (Reed and Swanson, 1987) and are therefore unlikely to cross the epithelial barrier. Furthermore, the vast majority of mast cells are located in the submucosa of airways (Guerzon *et al.*, 1979; Shanahan *et al.*, 1987; Mair *et al.*, 1988; Chen *et al.*, 1990; Chapter 7). Thus, the normal barrier of the airway mucosa must be altered in susceptible individuals during a hypersensitive reaction (Jansen *et al.*, 1988). Indeed, epithelial damage is a common pathological feature of both biopsy and autopsy studies of the airways of patients with asthma (Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Laitinen *et al.*, 1985; Jeffery *et al.*, 1989), and hyperpermeability of airways has been demonstrated experimentally both in asthmatics and in antigen-exposed animals (Boucher *et al.*, 1977; Mukherjee *et al.*, 1986; Jansen *et al.*, 1988; Ilowite *et al.*, 1989; Richards *et al.*, 1989).

The observations of airway permeability from human studies, however, are generally conflicting and discouraging (Elwood *et al.*, 1983; Honda *et al.*, 1988; Ilowite *et al.*, 1989; Richards *et al.*, 1989). It is not clear whether the observed differences in mucosal permeability between asthmatic responders and non-responders are inherent differences associated with the airway reactivity of individuals to inhaled antigen or if they are the consequence of pathological changes induced initially by the immediate allergic reaction and subsequently by airway inflammation. The objective of this study was to investigate the inherent differences in mucosal permeability of the tracheobronchial epithelium in sheep which exhibited a variety of airway responses to inhaled *Ascaris suum* antigen.

9.2 MATERIALS AND METHODS

Eighteen Romney-cross sheep aged from 4-8 years were used. The previous studies (Chapter 8) had shown that all animals had a positive skin reaction to intradermal injection of *A. suum* antigen and showed varying degrees of acute airway response to aerosols of *A. suum*. Six hypersensitive sheep responded with changes in both airway resistance and dynamic lung compliance (Group A), six with changes only in dynamic lung compliance (Group

B) and six non-reacting sheep (Group C). The detailed information on the skin reaction and airway response of these animals has been given in Chapter 8.

All animals were killed with intravenous injection of pentobarbitone sodium 14 days after the bronchial provocation test. The lower respiratory tract was removed *en bloc* immediately after death. Two sets of four tissue blocks were taken from the trachea and major bronchi of each sheep. Those from the trachea included tissues from both cartilaginous and non-cartilaginous regions.

To assess mucosal permeability, the tissues were reacted with horseradish peroxidase (HRP) according to the method of Walker and Bums (1988). Briefly, paired tissues were immersed in 0.01% (weight/volume) HRP in 0.1 M phosphate buffered saline (PBS, pH 7.2) or PBS only for 10 minutes, and fixed in Karnovsky's fixative in 0.1 M PBS (pH 7.2) for 4 hours at room temperature (Karnovsky, 1965). The tissues were then washed in cold PBS three times for 5 minutes each and then reacted with diaminobenzidine tetrahydrochloride (DAB) (Appendix 9.1) for 10 minutes to localise HRP. The tissues were washed in PBS again and then processed for transmission electron microscopy (TEM) as described previously (Chen *et al.*, 1988). Thin sections were cut and mounted on coded grids. They were examined first unstained under a Philips EM201c transmission electron microscope, and later stained with uranyl acetate and lead citrate for better identification of the cell types.

A preliminary study had shown that HRP penetration of the ovine tracheobronchial mucosa usually involved entire intercellular spaces rather than individual tight junctions. The airway mucosal permeability in the present study was, therefore, assessed by counting the number of HRP-containing intercellular spaces. Epithelial lesions and cells associated with the presence of HRP were also examined carefully and identified in the stained sections. The numbers of intercellular spaces and the HRP-containing spaces as well as the number of HRP-positive cells in the trachea and bronchi were recorded in each sheep. The mean percentage of HRP-positive intercellular spaces and cells in each animal was then calculated, tabulated and analysed statistically by a chi square test to find significant differences between sheep of the three different groups. Changes were considered significant if $p < 0.05$.

9.3 RESULTS

Positive HRP reaction products were found free in the luminal surface and bound to the superficial cell membrane of ciliated and secretory cells throughout the tracheobronchial tree in most sheep, regardless of their airway hypersensitivity (Fig. 9.1). Horseradish peroxidase was, however, only occasionally found in the lateral and basal areas of epithelial cells (Fig. 9.2). The tight junctions in these areas were usually penetrated by the tracer (Fig. 9.3), but intact

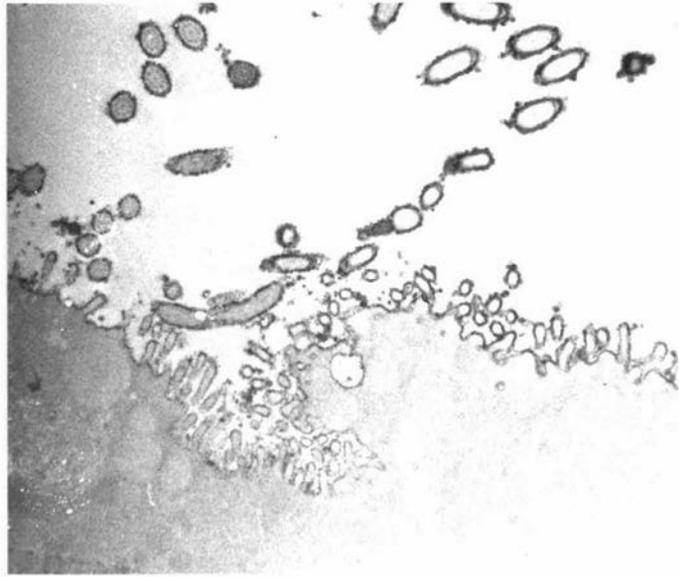


Fig. 9.1 The tracheal epithelium of a sheep with significant changes only in dynamic lung compliance (Group B) showing horseradish peroxidase reaction products free on the luminal surface and bound to the cell membrane. Unstained, TEM, x11,200.

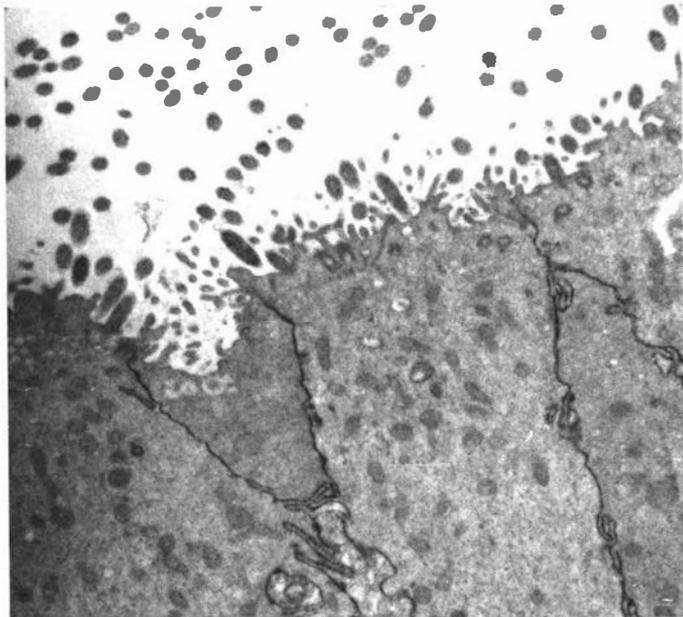


Fig. 9.2 Presence of horseradish peroxidase reaction products in the intercellular spaces of the tracheal epithelium of a sheep showing changes in both airway resistance and dynamic lung compliance (Group A). Unstained, TEM, x7,800.

junctions which were not penetrated could be occasionally found near the luminal aspect of the cells. There was no tendency for any particular epithelial cell type to surround the HRP-containing intercellular spaces. However, a very small proportion of epithelial cells showed an intense and diffuse reaction to HRP (Fig. 9.4). The cytoplasm of these cells was almost entirely involved but reaction in the nucleus was rare. Morphologically, these cells were mainly secretory cells although some degenerate epithelial cells were also involved. The cytoplasm of these cells was highly vacuolated and contained very few normal organelles. There was no epithelial damage in the sections examined although neutrophils were occasionally found between epithelial cells.

The quantitative studies (Table 9.1) showed no significant difference in the percentage of HRP-containing intercellular spaces or the percentage of intensely HRP-reacted cells in the tracheobronchial epithelium between sheep of the three different groups ($p > 0.05$), although the tracheal epithelium of sheep from Groups A and B showed a higher percentage of HRP-containing intercellular spaces than that from sheep in Group C. The sheep in Group A also had a higher proportion of intensely HRP-reacting cells in both tracheal and bronchial mucosa than those in Groups B and C. Overall, the proportion of HRP-positive epithelial cells and intercellular spaces was relatively small in both the tracheal and bronchial mucosa of all animals.

No HRP-containing intercellular spaces or HRP-positive cells were seen in the control tissues immersed in PBS.

9.4 DISCUSSION

Although hyperpermeability of the airway mucosa has been postulated as an important contributor to the development of airway hypersensitivity (Hogg, 1981), very few studies have been conducted to identify inherent differences between individuals with different airway reactivity (Boucher *et al.*, 1977; Richards *et al.*, 1989). The results of the present study have shown that the permeability of tracheobronchial epithelium to HRP was not significantly different in sheep with different airway responses to inhaled *A. suum* antigen (Table 9.1). This suggests that the observed difference in the allergic airway hypersensitivity in sheep is unlikely due to an inherent hyperpermeability of their airway mucosa. Similar results have also been reported previously in *Ascaris*-sensitive monkeys (Boucher *et al.*, 1977) and recently in the lung of patients with stable asthma (Richards *et al.*, 1989). The integrity of airway mucosa has also been ultrastructurally and morphometrically confirmed recently in the biopsies of patients with mild asthma not requiring treatment (Lozewicz *et al.*, 1990). The differences in mucosal permeability of the trachea between hypersensitive and non-reacting sheep in the present

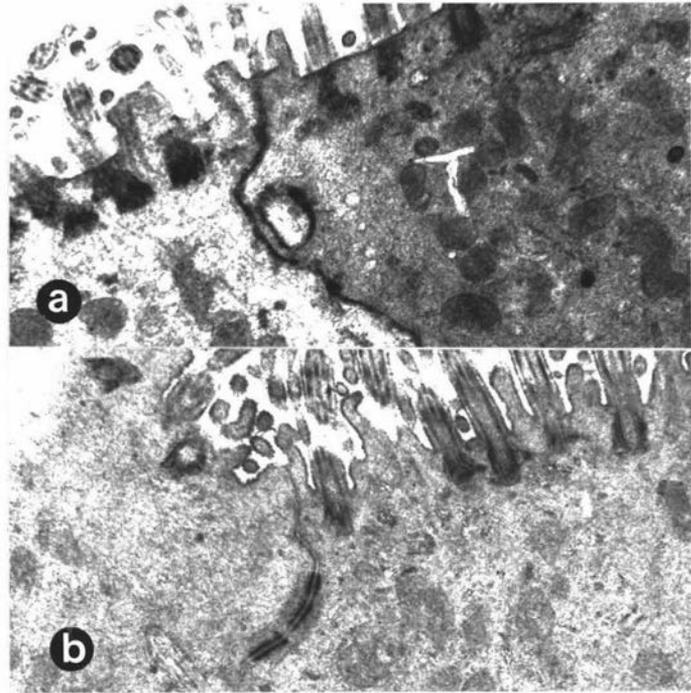


Fig. 9.3 (a) Penetration of horseradish peroxidase into the tight junctions between two ciliated cells in the bronchi of a sheep with significant changes only in dynamic lung compliance (Group B). (b) Intact tight junctions from an adjacent area of the same section. Unstained, TEM, x15,300.

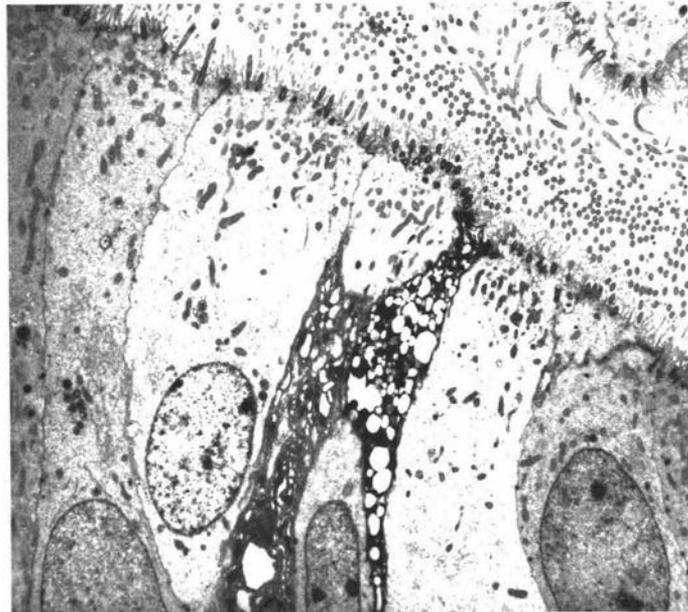


Fig. 9.4 Two intensely horseradish peroxidase-reacting cells in the bronchial epithelium of a sheep showing changes in both airway resistance and dynamic lung compliance (Group A). TEM, x3,400.

Table 9.1
Percentage of Horseradish Peroxidase (HRP)-containing Intercellular Spaces and Cells in the Tracheobronchial Epithelium of Sheep[#]

	Group A (n = 6)	Group B (n = 6)	Group C (n = 6)
<u>Trachea</u>			
Total i/c spaces counted	226	284	265
HRP-positive i/c spaces	33	54	20
Percentage of positive i/c spaces	1.36	2.28	0.76
Total epithelial cells counted	227	285	266
HRP-positive cell number	14	8	8
Percentage of positive cells	0.06	0.03	0.03
<u>Bronchi</u>			
Total i/c spaces counted	220	229	279
HRP-positive i/c spaces	4	20	20
Percentage of positive i/c spaces	0.33	0.81	0.77
Total epithelial cells counted	221	230	280
HRP-positive cell number	7	3	6
Percentage of positive cells	0.03	0.01	0.01

[#] Group A=hypersensitive sheep with changes in both airway resistance and dynamic lung compliance, Group B=hypersensitive sheep with changes only in dynamic lung compliance and Group C=non-reacting sheep. i/c=intercellular spaces. No significant difference was found in the trachea or the bronchi between sheep of different groups (all $p>0.05$).

study (Table 9.1) were nevertheless of such a degree that further investigations using techniques more sensitive than the HRP method may be needed.

In view of the present results, it therefore seems likely that the observed airway hyperpermeability in hypersensitive individuals may result from local allergic hypersensitivity reactions and subsequent inflammatory infiltration and mediator release rather than any inherent differences (Boucher *et al.*, 1977; Elwood *et al.*, 1983; Mukherjee *et al.*, 1986; Richards *et al.*, 1989). Having examined the effects of histamine on airway permeability in normal and asthmatic subjects, Rees *et al.* (1985) concluded that there was no direct relationship between bronchoconstriction and airway permeability. Studies on smokers by Kennedy *et al.* (1984) and Taylor *et al.* (1988) have shown that changes in airway permeability are not related to bronchial reactivity and small airway function. In the studies of Honda *et al.* (1988) airway permeability was increased in bronchitics but not in asthmatics although airway hyperreactivity between these two groups of subjects was similar. On the other hand, it has been well demonstrated that inhalation challenge with antigens and histamine in monkeys can induce an immediate increase in airway mucosal permeability which is not inherently abnormal

(Boucher *et al.*, 1977). Furthermore, the permeability of the respiratory epithelium in asthmatic patients became normal with the improvement of airway hyperresponsiveness which occurred after treatment (Jansen *et al.*, 1988). The results of these studies together with those of the present study have therefore raised the possibility that observed discrepancies in the mucosal permeability of asthmatics, either from the airway lumen to the bloodstream or *vice versa* (Elwood *et al.*, 1983; Honda *et al.*, 1988; Ilowite *et al.*, 1989) may, in fact, represent different stages of the disease.

The mechanisms by which the tracing material gains access into the lateral and basal areas of the airway epithelial cells are not clear. The opening of tight junctions is one of the most likely mechanisms (Boucher *et al.*, 1980), and has been experimentally confirmed *in vitro* in the trachea of sensitised rats (Mukherjee *et al.*, 1986). Inoue and Hogg (1977) found that the tight junctions between mucous cells in the tracheal epithelium of normal guinea pigs are more loosely organised than those between non-secretory cells. However, this proposal has been disputed in several recent studies on other laboratory animals (Christensen and Janeczek, 1985; Walker and Burns, 1988). Secretory cells and unidentified non-ciliated cells from the normal trachea of guinea pigs, hamsters and gerbils have been found to be able to incorporate HRP by pinocytosis and transport it to lateral and basal areas adjacent to the cells (Richardson *et al.*, 1976; Christensen and Janeczek, 1985). Walker and Burns (1988) have suggested that the tracer enters intercellular spaces by crossing disrupted secretory cells and then diffusing laterally without obvious penetration of tight junctions. Furthermore, these authors suggested that the penetration of tracer between airway epithelial cells is associated with adjacent epithelial lesions not included in the particular plane of section rather than a breach of individual tight junctions. They demonstrated that the presence of HRP in the tracheal epithelium of smoke-exposed and sham-exposed guinea pigs was similar and that the tracer was present only in those sections where there was epithelial disruption and regeneration. The small proportion of epithelial cells involved in the present study and the presence of the tracer below tight junctions adds further support to this proposal.

The intensely HRP-reacting cells seen in the present study have been described previously in a variety of HRP-exposed tissues including the rodent trachea (Owen, 1977; von Rosen *et al.*, 1981; Christensen and Janeczek, 1985). These cells are probably degenerate epithelial cells (Lewis, 1981), but may be cells intoxicated by HRP (Christensen and Janeczek, 1985). The latter possibility is unlikely, since in both the present and other studies (Christensen and Janeczek, 1985) only a minor proportion of epithelial cells (usually <2%) showed this reaction. Ultrastructural examinations of stained sections from the present study confirmed that affected cells are mainly secretory cells with small numbers of ciliated cells. The numbers of intensely HRP-reacting cells have been reported to increase greatly in the larynx of tobacco-

exposed rats (Lewis, 1981), but were not significantly increased in guinea pigs during allergic bronchoconstriction despite the fact that a significant number of intercellular spaces contained HRP (Ranga *et al.*, 1983). Although the intensely reacting cells comprised only small percentage of the total epithelial cells in the present study it is still possible that they may be of significance as a route for rapid access of antigens to subepithelial regions. Further studies are needed, therefore, to clarify the significance of these cells in allergic hypersensitivity.

Horseradish peroxidase has been used as a marker for studying the permeability of mucosal epithelium *in vivo* and *in vitro* in several animal species (Richardson *et al.*, 1976; Christensen and Janeczek, 1985; Walker and Burns, 1988). The adoption of an *in vitro* system was necessary in this study because of the relatively large volume of the ovine respiratory tract and the expense of the reagent. Results from this type of *in vitro* study must be interpreted with caution since the normal blood supply and mucociliary activity are absent. Nevertheless, the results obtained from this type of *in vitro* study have the advantage that they are not affected by other factors such as the renal clearance rate of the tracer or variations in the region of tracer deposition in the airway, which are believed to be important contributors to the discrepancies in the *in vivo* studies of mucosal permeability (Boucher *et al.*, 1977; Honda *et al.*, 1988). The pilot work carried out for the present study showed that HRP penetration usually involved the whole intercellular space rather than single tight junctions. It was therefore possible to use intercellular space counting in this experiment, instead of the tight junction counting method used by Walker and Burns (1988), thus greatly improving the speed of counting.

From the current ultrastructural observations and *in vitro* HRP permeability studies, it may be concluded that the intercellular junctions linking the tracheobronchial epithelium in sheep, like those in most other animals, are almost completely impermeable to foreign proteins under normal circumstances. Pinocytotic transport, whether through airway epithelium or through lymphoepithelium, as an avenue of antigen transport is too slow to account for the speed of mast cell discharge in response to inhaled antigens (Richardson *et al.*, 1976; Christensen and Janeczek, 1985). In addition, the lymphoepithelium is not well developed in the lower respiratory tract of conventionally raised sheep (Chapter 4), therefore, hyperpermeability of the airway mucosa is unlikely to be an essential contributor to the initial development of allergic airway hypersensitivity in some populations of this species. However, it cannot be precluded that hyperpermeability may be important in the smaller airways, where a high density of mast cells are present and where hypersensitivity reactions can have a profound constrictive effect. These have not been included in the present study because of the technical difficulties involved in this type of investigation.

9.5 SUMMARY

The permeability of the tracheobronchial mucosa to horseradish peroxidase (HRP) was examined *in vitro* in 18 aged Romney-cross sheep by comparing percentage of HRP-containing intercellular spaces and the percentage of epithelial cells showing HRP-positive reactions. Previous studies on these sheep had shown that six responded to inhalation challenge with *Ascaris suum* antigen with significant changes in both airway resistance and dynamic lung compliance (Group A), six had changes only in dynamic lung compliance (Group B) and six were non-responders (Group C). The results demonstrated that the tracheobronchial epithelium in sheep was generally impermeable to HRP and less than 2% of intercellular spaces and about 0.03% of the epithelial cells showed a positive reaction. Both the percentage of HRP-containing intercellular spaces and the percentage of HRP-positive epithelial cells were not significantly different in sheep of the three different groups although the tracheal epithelium of sheep in Groups A and B had a higher percentage of HRP-containing intercellular spaces than those in Group C. Intensely HRP-reacting cells were mainly secretory cells with some degenerate epithelial cells also involved. From these results it was concluded that the airway hyperpermeability seen in airway-hypersensitive individuals is probably a consequence of a local hypersensitivity reaction rather than an inherent factor contributing to the development of airway hypersensitivity.

CHAPTER 10

AIRWAY HYPERSENSITIVITY TO INHALED *ASCARIS SUUM* IN SHEEP: MORPHOLOGICAL AND MORPHOMETRIC STUDIES OF THE AIRWAYS

10.1 INTRODUCTION

Morphological studies of autopsy and biopsy specimens from patients with asthma of variable severity have consistently shown that the disease is characterised by airway inflammation and mucosal damage (Salvato, 1968; Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Laitinen *et al.*, 1985; Beasley *et al.*, 1989; Jeffery *et al.*, 1989). It has been suggested that these changes in the airway mucosa and wall are the major contributors to the development of airway hypersensitivity and hyperresponsiveness in asthmatics and several mechanisms for this have been proposed (Nadel, 1979; Hogg, 1982; Kaliner, 1985; Flavahan *et al.*, 1988). However, it can be assumed that most morphological features described in asthmatic airways are the result of the disease process and it is not known which (if any) morphological features could predispose an individual to develop the disease.

In Chapters 8, it was demonstrated that the acute bronchoconstriction seen in response to inhaled *Ascaris summ* antigen in New Zealand Romney sheep is similar in several respects to that seen in human asthma and that the sheep is a useful model to study the pathogenesis of the disease. The present study was undertaken to identify and compare quantitatively any inherent differences in airway tissue structure between hypersensitive and non-reacting sheep.

10.2 MATERIALS AND METHODS

10.2.1 Animals

Forty-three Romney-cross sheep aged from 4-8 years were used. Previous studies had shown that all animals had a positive skin reaction to intradermal injection of *A. summ* antigen and showed varying degrees of acute airway response to aerosols of *A. summ* antigen. Twenty-one sheep responded with changes in both airway resistance and dynamic lung compliance (Group A), ten with changes only in dynamic lung compliance (Group B) and 12 were non-reacting sheep (Group C). The details of the skin reaction and airway response have already been described in Chapter 8.

All animals were killed with intravenous injection of sodium pentobarbitone 14 days after completion of the bronchial provocation test. The lower respiratory tract was removed *en bloc* immediately after death. Any gross abnormalities were recorded on pre-designed diagrams (Appendix 10.1).

10.2.2 Tissue sampling and processing

Tissues for histological studies were taken from seven airway levels of all animals (Fig. 10.1, Table 10.1). They were fixed by immersion in 10% neutral buffered formalin for 24 hours and processed routinely (Chapter 4). Each tracheal ring was cut into two pieces, but the remaining airways were embedded as a whole. Sections of 4 μm thickness were cut and stained with haematoxylin and eosin (HE), as well as alcian blue and periodic acid-Schiff (AB/PAS) at pH 2.5 (Culling *et al.*, 1985). To avoid observer's bias, all slides were coded and examined blindly.

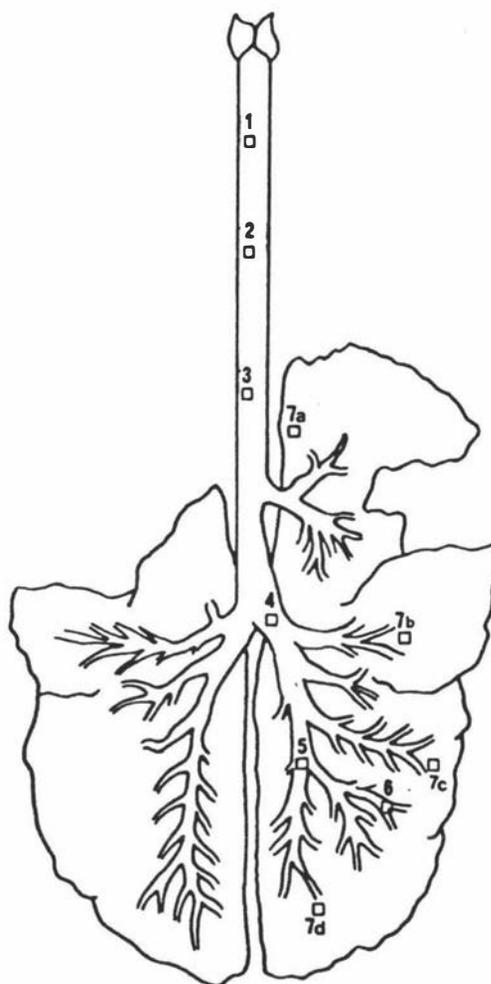


Fig. 10.1 Diagram of the ovine lower respiratory tract showing tissue sampling sites (□).

1=upper trachea; 2=mid trachea; 3=lower trachea; 4=major bronchi; 5=lobar bronchi; 6=medium bronchi; 7a,7b,7c,7d=bronchioles and lungs.

Table 10.1
Sites and Tissues Sampled

Airway level	Numbers of tissue samples
Upper trachea	1
Mid trachea	1
Lower trachea	1
Major bronchi	2
Lobar bronchi	3
Medium bronchi	4
Bronchioles	4

10.2.3 Morphological and morphometric studies

The histological sections of airways from all 43 animals were used for general morphological observations. The amount of luminal mucus and cellular occlusion, epithelial sloughing and squamous metaplasia were semi-quantitatively assessed in ten sheep from each group. A score from 0 (normal) to 4 (most severe) was assigned to each pathological feature. The trachea were excluded from the study of luminal occlusion because of the method of embedding used.

Six sheep from each group were used in the morphometric studies. All the morphometric parameters unless otherwise mentioned were measured using a JAVA image analyser (Jandel Scientific, California) and a Dexxa mouse (Dexxa International, California). With this system, the outline of an area of interest could be traced and the size of the area and distance between any two points were automatically determined by the computer and recorded for further analysis.

Epithelial thickness: Epithelial thickness was determined by measuring the distance from the basement membrane to the apical border of the epithelial cells at a magnification of x400. In order to obtain representative results, ten places were randomly selected for measurement in each airway. Places where the epithelium was stripped, folded or heaped were avoided.

Density of goblet cells: The density of goblet cells in the airway epithelium was evaluated in AB/PAS (pH 2.5) stained sections. In the trachea, major bronchi and lobar bronchi, ten fields at a magnification of x400, representing a total length of 4.85 mm of epithelium, were examined in each airway. The precise field within an airway at which measurements were made was randomly determined using eyepiece graticule markings. The result was expressed

as cells per millimetre of epithelium. The density of goblet cells in the medium bronchi and bronchioles was estimated using a semi-quantitative scoring system (0 to 4) at the same magnification since the epithelium in these airways was irregularly folded and its length was, therefore, difficult to measure accurately.

Differential counts of goblet cells were also undertaken from these sections. The cells were categorised into three groups, PAS-positive, AB-positive and mixed, based on their affinity for AB/PAS (pH 2.5) stain.

Submucosal glands: Only the trachea, major bronchi and lobar bronchi were included in this study. Six submucosal glands at each level of the airways were randomly selected from each animal for analysis. Any group of three or more acini was counted as an individual gland.

The parameters used to evaluate the dimensions of each gland included the total gland area, total acinar area, total non-acinar area, the number of acini per gland, the mean area of a single acinus and the ratio of gland to acinus areas. The area of each gland was measured in sections stained with AB/PAS at pH 2.5 using the image analyser. Simultaneously, the numbers of acini in the gland were manually counted "on-line" using the same image. The total acinar area and the areas occupied by inter-acinar tissue and acinar lumina in each gland were estimated by a point-counting method using a square lattice grid imposed over the retrieved images on a video monitor connected to a projection microscope (Weibel, 1979). Since the absolute area of a given gland and its acinar number were already known, total acinar area, non-acinar area, the mean area of a single acinus and the ratio of gland to acinus area could be calculated.

The proportion of different types of mucosubstances (acidic, neutral and mixed) in the glands was evaluated using the point-counting technique of Weibel (1979). A 100-point grid was superimposed onto the airway wall, and the number of points per grid falling on each area of interest was counted, as were the total points falling on the stained area in each gland. Thus the percentage of each type of mucosubstance could be calculated.

10.2.4 Data analysis

All the data collected from the measurements of each morphological parameter of each sheep were averaged and used for the group mean calculation. Group means were expressed as mean and the standard error of means for all the parameters except luminal occlusion. For this parameter data was presented as a median and range. Statistical analysis was performed on a microcomputer with a statistical software package (Statistix, NH Analytical Software, MN). Differences in the observations made between the different groups of sheep were analysed using a one way analysis of variance or Kruskal-Wallis test. A logarithmic or arcsin transformation was applied to some data, when appropriate, before analysis. If a statistical difference was found, a further analysis was made using Dunnett's test, which allows multiple comparisons to a single control group (Winer, 1971). The difference was considered significant if $p < 0.05$.

The coefficient of variation for the reproducibility of all morphometric parameters was assessed by measuring ten times in four different airways. It was 3% for epithelial thickness, 5% for the density of goblet cells and airway wall areas, and 11% for the submucosal gland dimensions.

10.3 RESULTS

10.3.1 Gross findings

The trachea and major bronchi of most sheep contained small amounts of foamy fluid in their lumina. The lungs of the three groups of sheep were similar and contained small to moderate numbers of dark-red to greyish foci of consolidation in the dorsal aspects of the caudal and middle lobes. The lesions were consistent with moderate *Muellerius capillaris* infestation and showed no group-preference. No pleural adhesions or pleurisy were found in the area where the intrapleural catheter had been inserted.

10.3.2 Morphological and morphometric studies

Airway luminal occlusion by exudate: Accumulation of small amounts of mucus and a few cells was common in most of the airways examined, regardless of group. Complete luminal obstruction however, was only very occasionally seen in the small airways. Alcian blue/PAS (pH 2.5) staining showed that the accumulated mucus was usually a mixture of acidic and neutral mucosubstances with the acidic products predominant. The cellular components were mainly epithelial with occasional neutrophils. Scores for the luminal occlusion at each airway level are compared in Table 10.2. There was no significant difference between the three groups in the extent of accumulation of mucus and cells in airway lumina at any of the levels examined (all $p > 0.05$).

Table 10.2
Scores of Luminal Exudative Occlusion in Airways[#]

	Mucus	Cells
<u>Major bronchi</u>		
Group A	1 (0-1)	0.5 (0-1)
Group B	1 (1-1)	0.5 (0-2)
Group C	1 (0-1)	1 (0-1)
<u>Lobar bronchi</u>		
Group A	1 (1-1)	0 (0-1)
Group B	1 (0-1)	0.5 (0-1)
Group C	1 (1-2)	0 (0-3)
<u>Medium bronchi</u>		
Group A	0 (0-2)	0 (0-1)
Group B	1 (0-2)	0 (0-2)
Group C	1.5 (0-2)	0.5 (0-1)
<u>Bronchioles</u>		
Group A	0 (0-3)	0 (0-1)
Group B	0 (0-1)	0 (0-3)
Group C	1 (0-3)	1 (0-1)

[#] Data are presented as median values, with ranges in parentheses. Group A=hypersensitive sheep with significant changes in both airway resistance and dynamic lung compliance, Group B=hypersensitive sheep with significant changes only in dynamic lung compliance, and Group C=non-reacting sheep. The difference between the three groups of sheep is not significant at any airway level (all $p > 0.05$).

Epithelial sloughing and squamous metaplasia: The data for epithelial sloughing and squamous metaplasia are not reproduced here in detail since these lesions were seen only rarely. Squamous metaplasia was only seen in the tracheobronchial epithelium of four animals (two in Group B and two in Group C), whereas epithelial sloughing was noted in the tracheal epithelium of one animal in Group B and in the bronchial epithelium of another animal from the same group.

Epithelial thickness: Quantitative comparison of epithelial thickness at different levels of the airways in the three groups of sheep is shown in Fig. 10.2. The mucosal epithelium at all levels examined apart from the lobar bronchi was thinner in hypersensitive sheep (Groups A and B) than in non-reacting animals (Group C). This difference was only significant, however, in the medium bronchi and bronchioles ($p < 0.05$).

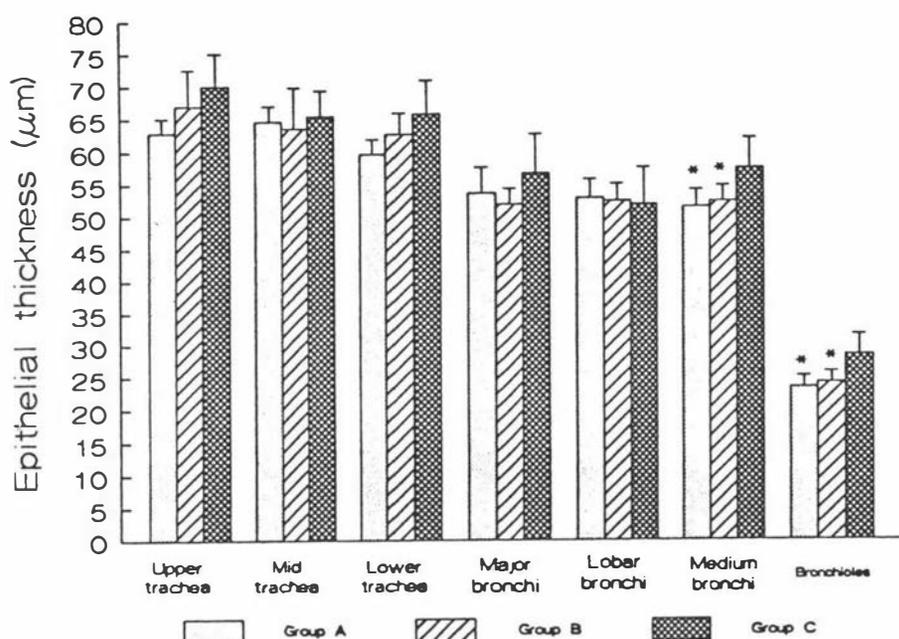


Fig. 10.2 Comparison of the epithelial thickness at different levels of airways in the three groups of sheep. Group A=hypersensitive sheep with significant changes in both airway resistance and dynamic lung compliance, Group B=hypersensitive sheep with significant changes only in dynamic lung compliance, and Group C=non-reacting sheep. * $p < 0.05$ compared to Group C.

Goblet cell density: The density of goblet cells varied considerably between individual airways. The most striking feature seen in the airway epithelium of some animals in Groups A and B was a decrease in the number of goblet cells showing affinity for AB/PAS (pH 2.5) stain (Fig. 10.3). In addition, many other goblet cells had scant secretory granules at their apex (Fig. 10.3). Results of quantitative studies on goblet cell density are presented in Fig. 10.4. Although the number of goblet cells in the trachea and small airways was generally greater in non-reacting sheep (Group C) than hypersensitive sheep (Groups A and B), this difference was only significant in bronchioles (Groups A and B, all $p < 0.05$). In the major and lobar bronchi, the number of goblet cells was more variable than at other airway levels.

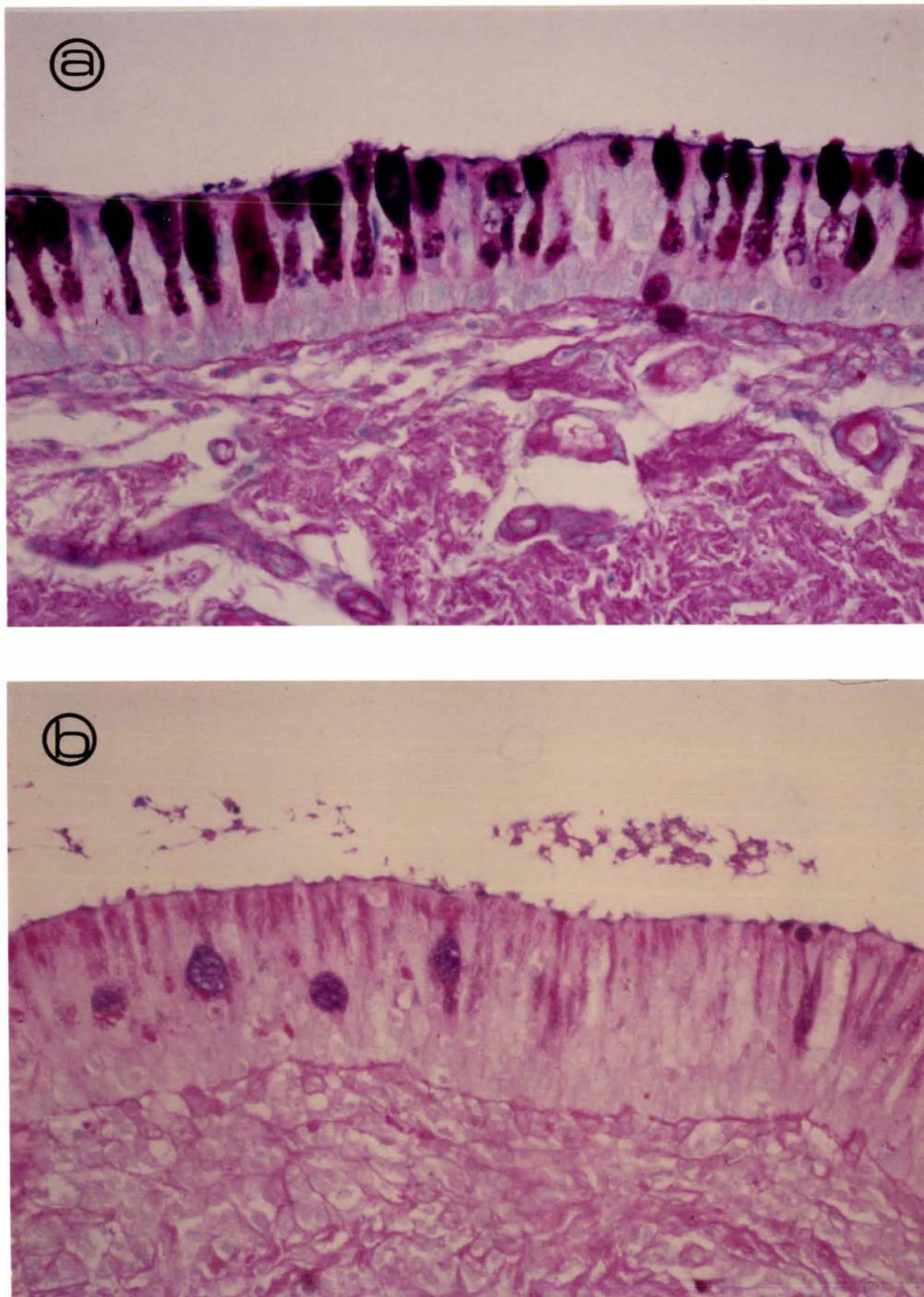


Fig. 10.3 (a) Epithelial goblet cells showing affinity for alcian blue and periodic acid-Schiff (pH 2.5) in the tracheal mucosa of a sheep without airway hypersensitivity; (b) Tracheal mucosa from a hypersensitive sheep showing goblet cells with scanty mucus granules compared to those in (a). AB/PAS, pH 2.5, x250.

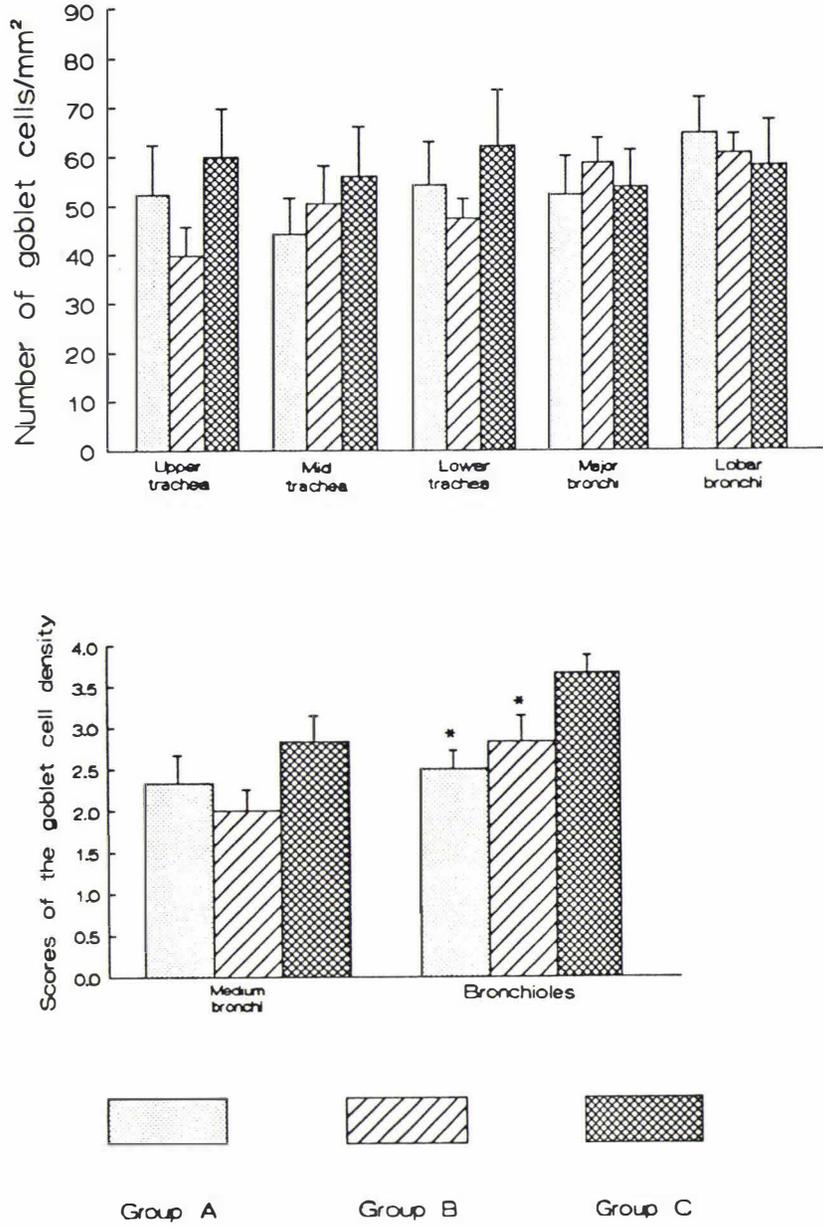


Fig. 10.4 Comparison of the density of goblet cells at the different levels of airways in the three groups of sheep. Refer Fig. 10.2 for the legend explanation. * = p < 0.05 compared to Group C.

The secretory granules of most goblet cells in the trachea, major and lobar bronchi stained magenta to purple with AB/PAS stains, indicating that their granules consisted of both periodate-reactive and alcianophilic mucosubstances. Occasionally, single cells stained exclusively red or blue, suggesting that their granules comprised mainly neutral or acidic mucosubstances, respectively. A degree of variability in carbohydrate composition of granules was observed in some cells, as a discontinuous gradient of red (neutral), to purple (acidic and neutral combined) and to blue (acidic) hues of granule staining. In the smaller airways the number of cells containing acidic mucosubstances increased considerably. The proportion of cells containing different types of mucosubstance at different airway levels of the three groups of sheep is compared in Fig. 10.5. There was no significant difference between the groups at all airway levels except for the lower trachea where, compared to non-reacting sheep, goblet cells in the sheep in Group A contained a significantly higher proportion of neutral mucosubstance ($p < 0.05$).

Submucosal glands: The dimensions of submucosal glands at the different airway levels are summarised in Fig. 10.6. Morphometric results showed that the total gland area, total acinar area and total non-acinar area of each gland at all airway levels were generally greater in sheep from Groups A and B than those from Group C, but this was only statistically significant in the lobar bronchi where differences between sheep from Group A and Group C were demonstrated (all $p < 0.01$).

In addition, the area occupied by single acinus in the upper trachea and major bronchi was significantly greater in hypersensitive sheep than that in non-reacting sheep ($p < 0.05$). There was no statistical difference at any levels of airways between the three groups of sheep in the mean acinar numbers per gland or the ratio of gland to acinus area ($p > 0.05$).

Certain variations in the staining reaction of mucous cells to AB/PAS (pH 2.5) were present between submucosal glands, airway levels and individuals. Most mucus cells in the glands examined showed an affinity to blue and mixed stains and these comprised about 80% of cells with a positive AB/PAS (pH 2.5) stain. In general, only very few cells stained red exclusively. The comparative composition of secretory components in goblet cells at different airway levels in the three groups of sheep is illustrated in Fig. 10.7. Overall, there was no significant difference between groups in the proportion of cells containing different types of mucosubstance at all airway levels apart from the major bronchi. At this level the glands of sheep from Group B contained a significantly higher proportion of mixed reaction products than those of the animals in Group C ($p < 0.05$).

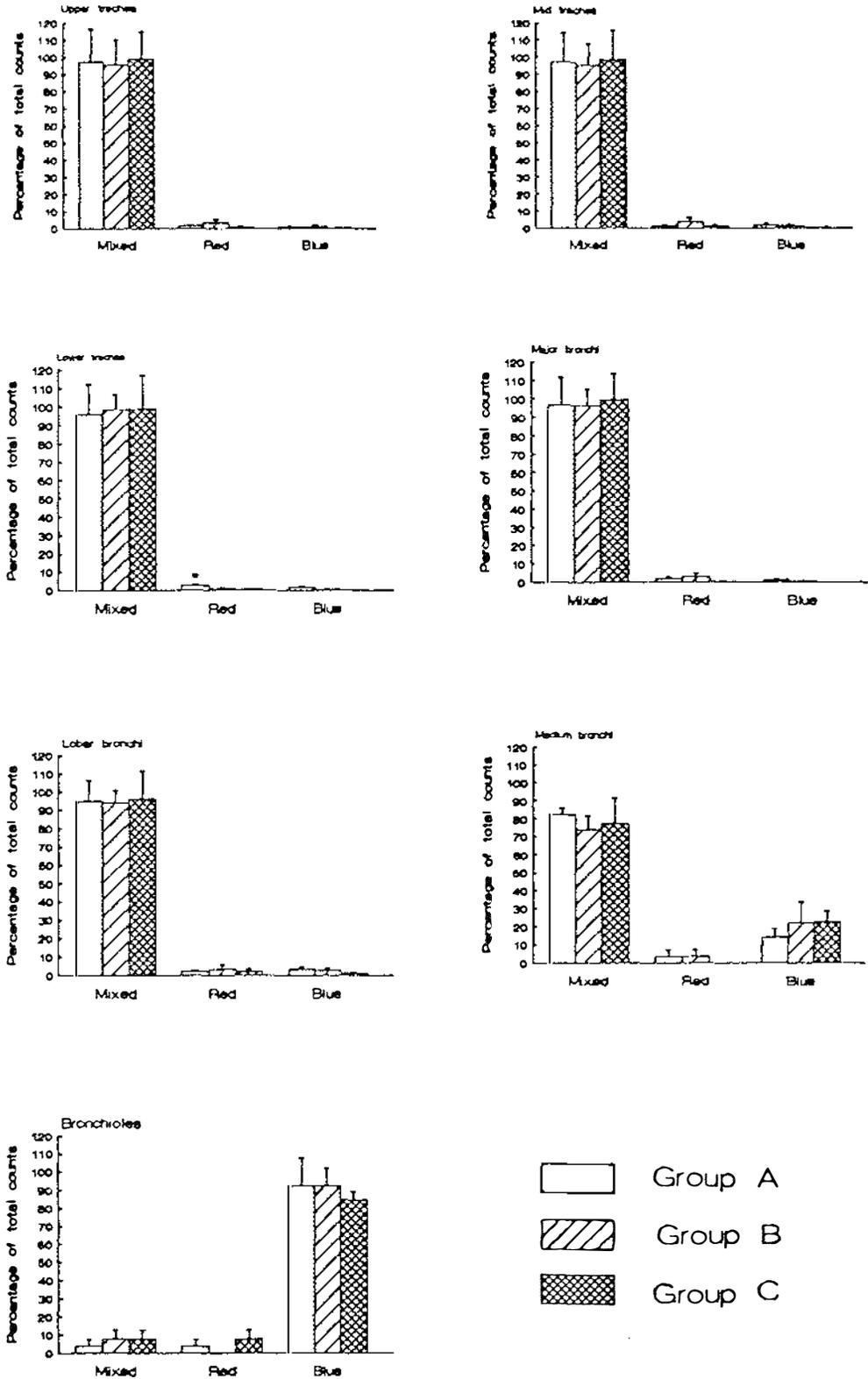


Fig. 10.5 Comparison of the affinity of goblet cells to alcian blue and periodic acid-Schiff staining (pH 2.5) at different levels of airway in the three groups of sheep. Refer Fig. 10.2 for the legend explanation. * = p < 0.05 compared to Group C.

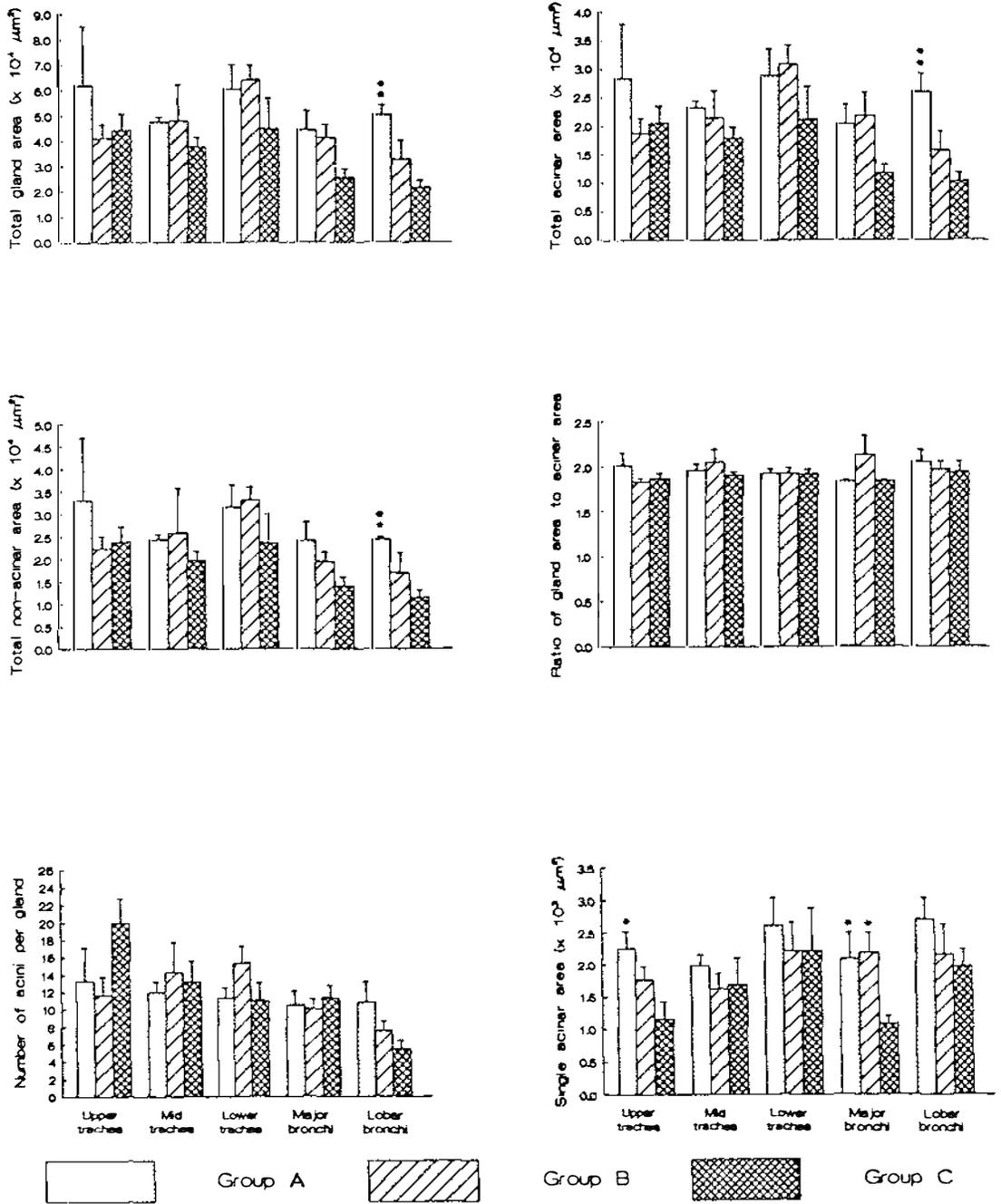


Fig. 10.6 Comparison of the dimensions of submucosal glands at different levels of airways in the three groups of sheep. Refer Fig. 10.2 for the legend explanation. * = p < 0.05 and ** = p < 0.01 compared to Group C.

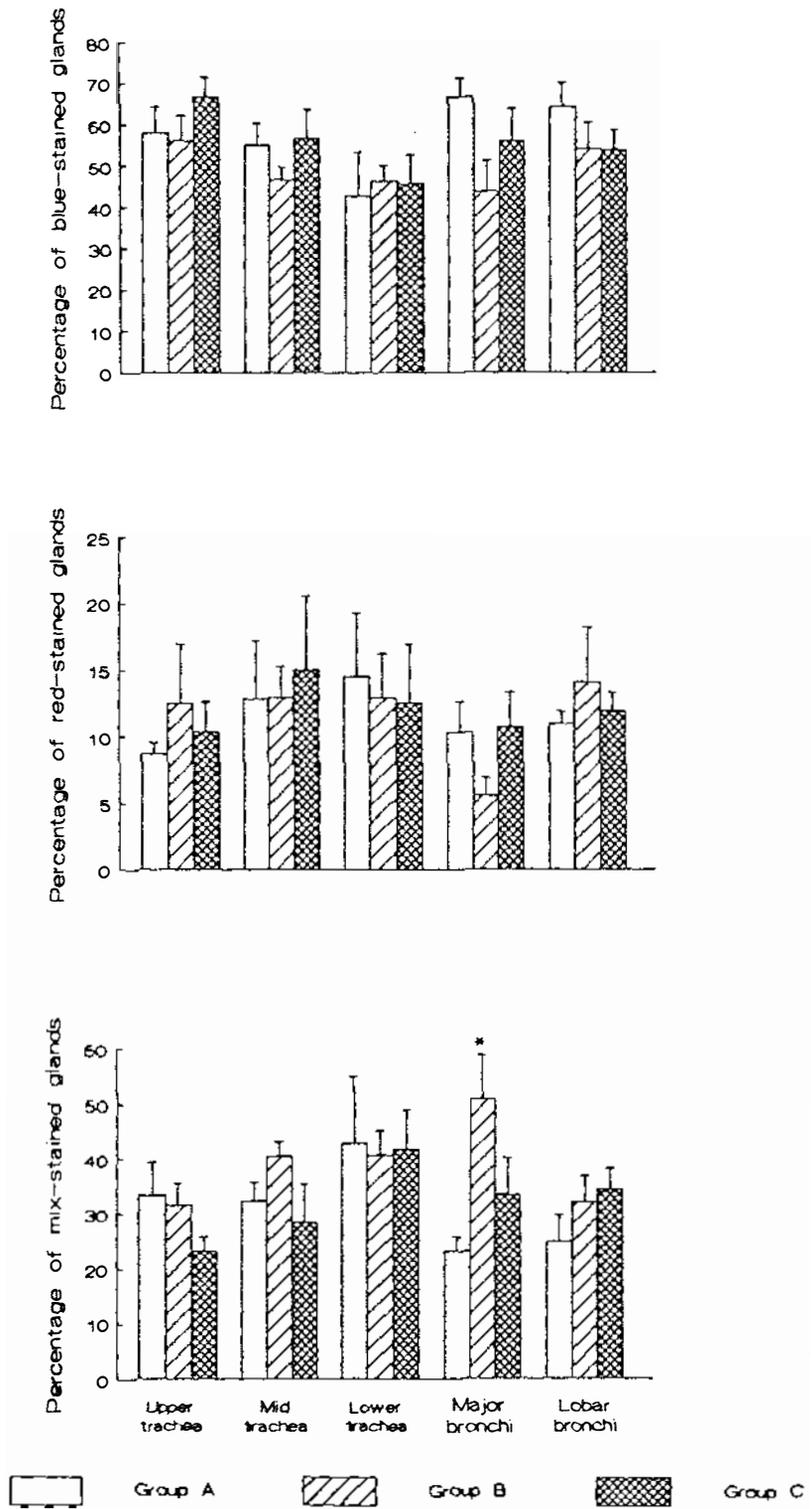


Fig. 10.7 Comparison of the affinity of mucous cells to alcian blue and periodic acid-Schiff staining (pH 2.5) in the airway submucosal glands in the three groups of sheep. Refer Fig. 10.2 for the legend explanation. * = p < 0.05 compared to Group C.

10.4 DISCUSSION

All the morphological features examined in the present study have been previously described as prominent pathological changes both in asthmatics and in subjects with airway hyperresponsiveness (Salvato, 1968; Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Dunnill, 1982; Laitinen *et al.*, 1985; Beasley *et al.*, 1989; Jeffery *et al.*, 1989), and are likely to contribute to the development of airway hyperresponsiveness singly and/or in combination. The results obtained have revealed that the hypersensitive sheep (Groups A and B) had a thinner epithelium, fewer goblet cells showing affinity for AB/PAS (pH 2.5) stain, but a greater gland volume at most airway levels than those of non-reacting sheep (Group C). However, the difference in epithelial thickness was significant only in the medium bronchi and bronchioles and the differences in the density of goblet cells and the submucosal gland area was of significance only in bronchioles and lobar bronchi, respectively. In addition, significant differences were found in the area of single acinus and the proportion of different types of mucosubstances in mucus cells of the submucosal glands, but these features varied considerably between individuals. No significant differences were found in the degree of luminal occlusion, epithelial sloughing or squamous metaplasia.

The present study has shown that the airway epithelium of hypersensitive sheep was thinner than that of non-reacting animals at most airway levels. Furthermore, in the medium bronchi and bronchioles this difference was statistically significant ($p < 0.05$), suggesting a possible relationship between the propensity to develop allergic airway hypersensitivity and airway epithelial thickness. A significantly thinner epithelium has also been reported previously in the lobar bronchi of dogs with airway hyperresponsiveness (Yanta *et al.*, 1981). Variations in airway responsiveness to inhaled allergens within a population may reflect variations in the thickness of the epithelial barrier that antigens must pass through before the airway smooth muscle can be activated. However, this hypothesis is based purely on the physical thickness of the barrier and not on its permeability and clearly both factors will be important in determining the allergic airway response. Further studies to examine directly the mucosal permeability to molecules of a similar size to those of allergens, at these airway levels will therefore be required.

Studies of epithelial thickness in human asthmatics are surprisingly scant. This is probably due to the fact that the airway epithelium in patients dying of severe asthma is likely to be partially or completely stripped and that bronchial biopsy specimens from patients with mild asthma are usually too small for unbiased morphometric studies. The available information on asthmatic airways indicates that a reduction in epithelial thickness is not a common feature of patients with mild asthma (Lozewicz *et al.*, 1990) although it has been reported (Jeffery *et al.*,

1989). The current findings indicate that more emphasis should be placed on this feature in future studies.

Goblet cell hyperplasia has been described previously in the airways of patients with asthma of varying severity (Salvato, 1968; Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Aikawa *et al.*, 1990), and in the lobar bronchi of dogs with airway hyperresponsiveness (Yanta *et al.*, 1981). However, this has not been quantitatively confirmed in bronchial biopsies from patients with mild asthma (Lozewicz *et al.*, 1990). The hypersensitive sheep in the present study contained fewer, rather than more epithelial secretory cells in their airways when compared with the non-reacting sheep. In the bronchioles, a significant decrease of goblet cells showing affinity for AB/PAS (pH 2.5) was noted in sheep with airway hypersensitivity. The decrease of goblet cell numbers seen in the present study is likely to be due to the discharge of secretory material rather than the reduction of absolute cell numbers since scant secretory granules were seen in the apex of many cells (Fig. 10.3). A decrease of goblet cell counts through the discharge of secretory material has been previously reported in a number of animals and man after exposure to cigarette smoke (Hulbert *et al.*, 1981), ozone (Murlas and Roum, 1985) or antigen challenge (Ranga *et al.*, 1983).

The significance of low goblet cell counts in the airway epithelium of hypersensitive animals demonstrated in the present studies is not clear. If, as has been suggested, goblet cells represent a highly permeable pathway for mediators (Yanta *et al.*, 1981; Christensen and Janeczek, 1985), the hypersensitive animals would be expected to have higher cell counts than the non-reacting individuals. However, this suggestion has been disputed by findings from studies of canine experimental bronchitis induced by chronic exposure to sulphur dioxide (Spicer *et al.*, 1971). In these animals, the airway responsiveness decreased at the same time as goblet cell counts increased. On the other hand, a decrease in goblet cell counts in the tracheal epithelium of guinea pigs after exposure to ozone correlated well with airway hyperresponsiveness (Murlas and Roum, 1985). These workers found that the best pathological correlate to acute airway hyperreactivity in ozone-exposed guinea pigs was a substantial decrease in goblet cells. Further studies are needed for a better understanding of the association of goblet cells with the airway hypersensitivity.

Submucosal gland enlargement was seen in most airway levels in hypersensitive sheep in the present study but it was significant only in the lobar bronchi. Enlargement of the airway submucosal glands has been quantitatively demonstrated in patients dying of status asthmaticus (Dunnill *et al.*, 1969), but studies of patients with mild and long-standing asthma (Sobonya, 1984) have shown that gland volume was not increased significantly. In experimental dogs, both gland density and acinar size was not correlated with airway hyperresponsiveness (Yanta *et al.*, 1981).

Previous studies of asthmatic airways only measured the relative area of the airway wall occupied by the glands (Dunnill *et al.*, 1969), and it was not clear which type of glandular tissue was responsible for the enlargement. The present study examined not only the degree of enlargement but also the type of glandular tissue involved. The results indicate that both acinar and non-acinar components were involved in the gland enlargement that was seen in hypersensitive sheep.

Up until now little attention has been paid to alterations in the composition of these submucosal glands in either asthmatics or animal models. Salvato (1968) found that goblet cells in bronchial biopsies from asthmatics contain more neutral mucosubstances and less acidic products than those from control subjects. The present study has shown that there was no significant difference in the proportion of different types of mucosubstance in the epithelial goblet cells of most airways although the sheep in Group A contained a significantly higher proportion of neutral products in the lower trachea. This finding is similar to the results of the only study on asthmatic submucosal gland composition which has been reported to date (Salvato, 1968). The results suggest that there is no inherent difference in the composition of mucosubstances in epithelial goblet cells and submucosal mucus cells between hypersensitive and non-reacting individuals and although mucus hypersecretion commonly occurs, the composition of mucus does not change markedly during allergic reactions. It is not clear however, whether the physical property of airway mucus is inherently different between sheep with and without airway hypersensitivity. The absence of mucus and cellular accumulation and occlusion in airway lumina in hypersensitive sheep suggests that this possibility is unlikely. Recent studies of asymptomatic asthmatic men and *Ascaris*-sensitised dogs have shown that the respiratory mucus from these subjects is considerably changed in its rigidity, hydration and spinnability (Rubin *et al.*, 1990). Studies of the sheep model have also revealed that aerosols of antigen can decrease the velocity of the tracheal mucus significantly (Wanner and Abraham, 1982).

Damage to and loss of airway epithelial cells are the characteristic pathological features in all patients with severe asthma and some patients with mild asthma (Dunnill *et al.*, 1969; Dunnill, 1982; Laitinen *et al.*, 1985; Beasley *et al.*, 1989; Jeffery *et al.*, 1989), and several mechanisms have been proposed by which airway epithelial damage can increase the airway response to antigens. Thus, it is reasonable to postulate that hypersensitive sheep may have more epithelial loss than less hypersensitive or non-reacting animals. However, the results of the present study have shown that epithelial sloughing and squamous metaplasia are not common features in the sheep examined, regardless of their degree of airway hypersensitivity. This finding is in agreement with previous studies of airway functional permeability in subjects

with stable asthma (Elwood *et al.*, 1983) or in individuals with airway hyperresponsiveness (O'Byrne *et al.*, 1984a). It is also similar to the results of studies in *Ascaris*-sensitive monkeys (Boucher *et al.*, 1979) and ovalbumin-sensitive rats (Lebargy *et al.*, 1987). The previous work described in Chapter 9 has also shown that the permeability of tracheobronchial epithelium of sheep with airway hypersensitivity to *A. suum* was not significantly higher than that of non-reacting animals.

The report of severe epithelial damage in the airways of patients with mild asthma has highlighted the importance of epithelial damage in the development of airway hyperresponsiveness. However, These findings have been challenged by the results of several recent studies. Firstly, ultrastructural and morphometric studies have shown that epithelial sloughing and squamous metaplasia are not unique features in patients with mild asthma, and they are also present in some control subjects (Jeffery *et al.*, 1989; Lozewicz *et al.*, 1990). Secondly, epithelial integrity is not significantly altered in asthmatic patients after challenge with methacholine (Lozewicz *et al.*, 1990). Finally, no apparent correlation has been found between the extent of epithelial damage and airway reactivity (Laitinen *et al.*, 1985; Lozewicz *et al.*, 1990). Thus, it may be concluded that much of the epithelial damage previously seen in the subjects with airway hypersensitivity may be the result of the allergic reaction rather than a fundamental difference in morphology between hypersensitive and non-reacting subjects.

Thickness of the basement membrane was not measured in the present study since this feature is rarely seen in sheep (Maurer *et al.*, 1981) or in other animal models with a variety of airway diseases including allergic reactions (Patterson and Kelly, 1974; Wanner and Abraham, 1982; Kallos and Kallos, 1984). The terminal airways and alveoli were not included in the current investigation because they are not the main sites contributing to the production of airway resistance (Snapper, 1986; Du *et al.*, 1989) and changes in these structures are not seen in asthma (Sobonya, 1984; Ebina *et al.*, 1990a,b). Alterations of ciliary structure and vacuolation of ciliated cells have been described in the bronchial biopsies from patients with mild asthma and have been proposed as important contributors to airway hyperresponsiveness (Laitinen *et al.*, 1985). These factors were also not included in the present study because they are not consistent features of mild asthma and are found in healthy subjects (Lozewicz *et al.*, 1990).

In conclusion, quantitative analysis of morphological changes in the airway structures have shown no significant differences between hypersensitive and non-reacting sheep in most of the tissue components examined, although hypersensitive animals had relatively fewer epithelial goblet cells, and a thinner airway epithelium in the medium bronchi and bronchioles. These findings indicate that although there was an association between epithelial thickness and

goblet cell density in small airways, the development of allergic airway hypersensitivity in sheep may occur in the absence of major morphological changes in the airway epithelium. Further studies are necessary to investigate the relationship between airway hypersensitivity, the relatively thin airway epithelium and the low density of goblet cells observed in this model.

10.5 SUMMARY

The airways of 12 sheep with naturally-occurring allergic airway hypersensitivity, six of which had changes in both airway resistance and dynamic lung compliance (Group A) and six of which had changes in only dynamic lung compliance (Group B), were compared morphologically with six non-reacting sheep (Group C) in order to examine the relationship between airway hypersensitivity and various morphological features thought to be related to airway responsiveness. Airways from seven levels of the lower respiratory tract were examined morphologically and morphometrically for evidence of luminal occlusion by mucus and cells, epithelial sloughing and squamous metaplasia, epithelial thickness, changes in goblet cell density, differences in submucosal gland dimensions and the proportion of different types of mucosubstances in goblet cells and glandular mucus cells. Compared to the non-reacting sheep (Group C), the sheep in Groups A and B had a thinner epithelium, fewer goblet cells and greater gland area at most airway levels, but the epithelial thickness and the goblet cell density were significant in only the medium bronchi and bronchioles and in the bronchioles, respectively. The differences of the gland dimensions and the types of mucosubstance between hypersensitive and non-reacting animals were more variable. No significant differences between the three groups were noted with regard to luminal occlusion or epithelial sloughing and metaplasia. These findings indicate that although there was an association between epithelial thickness and goblet cell density in the small airways, the development of allergic airway hypersensitivity in sheep may occur in the absence of major morphological changes in the airway epithelium.

CHAPTER 11

AIRWAY HYPERSENSITIVITY TO INHALED *ASCARIS SUUM* IN SHEEP: AIRWAY DIMENSIONS

11.1 INTRODUCTION

The majority of sheep that have been studied in both New Zealand (Chapter 8) and overseas (Wanner *et al.*, 1979; Kleeberger *et al.*, 1985; Bosse *et al.*, 1987; Okayama *et al.*, 1989) have shown a significant increase in airway resistance after exposure to aerosols of *Ascaris suum* antigen. The mechanisms of the development of airway hypersensitivity observed in both these animals and in asthmatics are not clear, but may be due to either functional abnormality of airway smooth muscle (Boushey, 1980), thickening of airway wall (Moreno *et al.*, 1986), or the loss of factors that relax smooth muscle (Nadel, 1988; Vanhoutte, 1989). The results of both *in vivo* and *in vitro* studies on the function of airway muscle are contradictory (Vincenc *et al.*, 1983; Armour *et al.*, 1984; Downes *et al.*, 1986; Cerrina *et al.*, 1986; Bai, 1990), and the presence of epithelium-derived relaxing factors is still in the putative stage (Vanhoutte, 1989). Although the thickening of airway walls is evident in most morphological studies of human asthma (Salvato, 1968; Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Hogg *et al.*, 1987; Persson, 1988), and has been morphometrically confirmed recently in patients with severe asthma (James *et al.*, 1989), it is not clear whether or not this factor is a fundamental contributor to the development of the asthmatic reaction in these subjects. The objective of the present study was to test the hypothesis that the allergic airway hypersensitivity seen in sheep is related to an inherent difference of airway dimensions, particularly thickening of airway walls.

11.2 MATERIALS AND METHODS

11.2.1 Animals

Eighteen Romney-cross sheep aged from 4-8 years were used. Previous studies had shown that all animals had a positive skin reaction to intradermal injection of *A. summ* antigen and showed varying degrees of acute airway response to aerosols of *A. summ* antigen. Six hypersensitive sheep responded with changes in both airway resistance and dynamic lung compliance (Group A), six with changes only in dynamic lung compliance (Group B) and six were non-reacting sheep (Group C). The details of the skin reaction and airway response have already been described in Chapter 8.

11.2.2 Tissue sampling and processing

The source of tissues and collection technique used in this study were the same as described previously in Chapter 10, but only the major bronchi, lobar bronchi, medium bronchi and bronchioles were analysed. Since the trachea was embedded in two separate blocks it was not suitable for the measurement and reconstruction of airway dimensions.

11.2.3 Airway dimension measurements

Only airways stained with haematoxylin-eosin and cut in transverse section with no bifurcation were used. The method for the measurement and reconstruction was similar to that of James *et al.* (1989). All the parameters were measured with a computerised image analyser (JAVA, Jandel Scientific, California) and a DEXXA mouse (DEXXA International, California). The measurement of large airways (the major and lobar bronchi) which could not be viewed in entirety under the microscope were made using the images grabbed from a video camera (JVC, Japan) with a slide holding extension on the lens. The measurement of small airways (medium bronchi and bronchioles) were made on images obtained from a light microscope at appropriate magnifications.

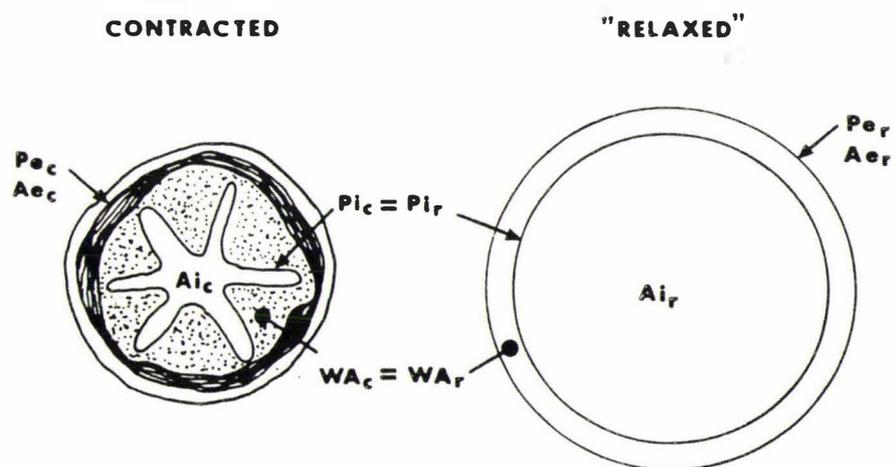
The dimensions measured in each airway included (1) the internal airway perimeter (P_i) and internal airway area (A_i) defined by the luminal surface of the epithelium, (2) the external airway perimeter (P_e) and external airway area (A_e) defined by the outer border of the smooth muscle layer, (3) the length of the smooth muscle (L) when it did not completely surround the airway (Fig. 11.1). In large airways where the muscle was present only in portions of the airway perimeter, an interpolation was made at the border between the dense and loose connective tissue on the internal side of the cartilage. In small airways where the smooth muscle was discontinuous, the external perimeter was interpolated between the ends of the adjacent portions of muscle. From these measurements, airway wall area (WA) and the proportion of the external airway perimeter occupied by smooth muscle (PMP) could be obtained by the formula: $WA = A_e - A_i$ and $PMP = L/P_e$.

11.2.4 Calculation of airway dimensions in the reconstructed airways

The dimensions of airways in the relaxed and fully dilated state could be then mathematically reconstructed from the measured parameters using the observations and assumptions of James *et al.* (1987, 1988). Firstly, it was assumed that the internal lumen of an airway in the "relaxed and dilated" state is circular, i.e. $P_i = 2R$ where R is the radius of the internal lumen. Secondly, it was assumed that the internal perimeter (P_i) and wall area (WA) are not altered by smooth muscle constriction or by lung inflation. Thus, the "relaxed and



a



b

Fig. 11.1 A photomicrograph (a) and schematic drawing (b, according to James *et al.*, 1989) of a small airway illustrating the measurements of airway dimensions. P_i =internal perimeter, A_i =internal area, P_e =external perimeter, and A_e =external area. WA =wall area, c =contracted, and r =relaxed.

dilated" internal area (A_{i_r}) could be given by $P_i^2/4\Pi$ and the "relaxed and dilated" external area (A_{e_r}) was given by $P_i^2/4\Pi + WA$. The wall area measured using the morphometric technique as a proportion of the reconstructed external area (P_w) could be then calculated for each airway by the formula: $P_w = WA/A_{e_r}$. The calculated P_w values normalised the wall area and allowed comparisons of the airways from animals with different degrees of airway constriction.

The degree of muscle shortening present in each airway could be estimated from the change in muscle length from the reconstructed and relaxed dimension ($P_{e_r} = \sqrt{4\Pi A_{e_r}}$) to that measured in the tissue specimen (P_e). The change from P_{e_r} to the calculated P_e when the lumen area (A_i) is zero gives the muscle shortening required to cause complete airway closure.

To examine the potential effect of thickening of the airway wall on airway responsiveness, the increase in airway resistance that would occur with varying degrees of shortening of smooth muscle was calculated based on the computer model developed by Moreno *et al.* (1986). Using this model, the relationship between changes of airway resistance (%Raw) and the degree of muscle shortening (PMS) at different thicknesses of the airways (P_w) could be obtained from the formula:

$$\%Raw = \left(\frac{\sqrt{(1 - PMS \times PMP)^2 - P_w}}{\sqrt{1 - P_w}} \right)^{-4}$$

11.2.5 Measurement of airway wall components

The relative areas of the airway wall occupied by epithelium, submucosa and smooth muscle were estimated using a point-counting technique (Weibel, 1979). A 100-point grid was superimposed onto the airway wall, and the number of points falling on each area of interest per grid was counted. The entire wall of each large airway was examined by superimposing the grid on successive non-overlapping microscopic fields. By knowing the relative areas of the wall components and the absolute wall area, the absolute area of each component of the wall could be calculated.

11.2.6 Data analysis

The statistical analysis of data was performed on a microcomputer using a statistical software package (Statistix, NH Analytical Software, MN). Data were presented as mean \pm the standard error of mean. The homogeneity of sizes of airways examined among groups was examined using a chi square test. Differences in the observations made between the different

groups of sheep were examined using one way analysis of variance. Some data were transformed to the logarithmic or arcsin form before analysis. If a statistical difference was found, a further analysis was made using Dunnett's test, which allows for multiple comparisons to a single control group (Winer, 1971). The difference was considered significant if $p < 0.05$.

11.3 RESULTS

The airways at all levels of the lungs showed a varying degree of infiltration with inflammatory cells and occasionally there was accumulation and occlusion of the lumina by mucus and cellular components. Folding of the epithelium was seen in some airways, especially in those with a prominent circular layer of muscle, regardless of the experimental group.

The data collected from the medium bronchi and bronchioles were divided into two subgroups because of large variations in their P_i . The frequency distribution in size of P_i in each airway group examined was not significantly different between the three groups of experimental sheep ($p > 0.05$), indicating that airways of a similar size were examined in each group. The coefficient of variation for the repeated measures was less than 5% for all measurements. The airway dimensions of each group of experimental sheep are compared in Table 11.1. In bronchioles less than 2 mm in internal perimeter, the sheep from Group A had a significantly greater wall area than those of Group C ($p < 0.05$). In the remaining airways, however, there was no statistical difference between groups in the size of this and all other airway dimension parameters (Table 11.1, all $p > 0.05$). Similarly, no significant differences were found between groups in the measured wall area relative to the reconstructed, relaxed external area (P_w , Fig. 11.2) or the other calculated parameters of reconstructed airways (Table 11.2), although in the major bronchi, P_w was slightly greater in sheep of Groups A and B than in Group C.

The relative areas of epithelium, submucosa and smooth muscle in most airways were similar in the sheep of different groups, but varied with the airway size. In large airways, the submucosa and smooth muscle were the predominant tissue components of the airway wall, and the proportion of epithelium gradually increased with the decrease in airway size. Comparison of absolute areas of each wall component at different levels of the airways are presented in Fig. 11.3. Compared with the sheep in Group C, the sheep in Group A had a significantly greater area of smooth muscle in the bronchioles ($p < 0.001$) while sheep in Group B showed a significantly higher area of muscle in the lobar bronchi ($p < 0.05$) and significantly lower area of epithelium in the medium bronchi ($p < 0.05$). However, the calculated muscle shortening required to cause complete airway closure was statistically similar at all airway levels in the three groups of sheep (Table 11.3).

Table 11.1
Measured Dimensions of Airways from Sheep with Different Airway Hypersensitivity*

	n	Pi, mm	Ai, mm ²	Pe, mm	Ae, mm ²	L, mm	WA, mm ²	PMP
<u>Major bronchi</u>								
Group A	12	25.37 ± 1.29	59.88 ± 5.71	29.95 ± 1.12	83.94 ± 5.80	28.15 ± 1.05	23.06 ± 1.54	0.94
Group B	12	26.47 ± 0.83	66.02 ± 3.94	31.43 ± 0.99	94.24 ± 5.66	29.54 ± 0.93	28.22 ± 2.28	0.94
Group C	12	27.35 ± 1.41	61.31 ± 3.06	31.41 ± 1.34	83.88 ± 3.84	29.53 ± 1.26	22.56 ± 1.79	0.94
<u>Lobar bronchi</u>								
Group A	6	17.33 ± 0.96	19.15 ± 1.50	19.86 ± 0.79	30.06 ± 1.74	19.06 ± 0.76	10.91 ± 1.19	0.96
Group B	6	19.61 ± 1.38	28.38 ± 4.06	22.07 ± 1.21	41.55 ± 5.89	21.19 ± 1.16	13.17 ± 1.90	0.96
Group C	6	16.56 ± 1.39	20.55 ± 2.82	18.28 ± 0.93	29.09 ± 2.96	17.55 ± 2.20	8.54 ± 0.88	0.96
<u>Medium bronchi (Pi ≥ 5 mm)</u>								
Group A	14	6.30 ± 0.30	0.23 ± 0.03	3.54 ± 0.14	0.79 ± 0.05	3.54 ± 0.14	0.56 ± 0.03	1.00
Group B	7	5.86 ± 0.23	0.18 ± 0.03	3.20 ± 0.21	0.64 ± 0.07	3.20 ± 0.21	0.46 ± 0.04	1.00
Group C	11	6.19 ± 0.36	0.28 ± 0.04	3.82 ± 0.18	0.81 ± 0.06	3.82 ± 0.18	0.53 ± 0.03	1.00
<u>Medium bronchi (Pi < 5 mm)</u>								
Group A	4	3.73 ± 0.54	0.12 ± 0.04	2.22 ± 0.21	0.37 ± 0.06	2.32 ± 0.21	0.25 ± 0.02	1.00
Group B	11	3.79 ± 0.14	0.12 ± 0.02	2.55 ± 0.14	0.42 ± 0.04	2.55 ± 0.14	0.30 ± 0.03	1.00
Group C	7	4.16 ± 0.15	0.14 ± 0.01	2.93 ± 0.14	0.52 ± 0.02	2.93 ± 0.14	0.37 ± 0.03	1.00
<u>Bronchioles (Pi ≥ 2 mm)</u>								
Group A	12	2.473 ± 0.159	0.034 ± 0.004	1.482 ± 0.075	0.149 ± 0.014	1.482 ± 0.075	0.115 ± 0.011	1.00
Group B	14	2.453 ± 0.060	0.030 ± 0.002	1.397 ± 0.032	0.128 ± 0.006	1.397 ± 0.032	0.098 ± 0.005	1.00
Group C	9	2.429 ± 0.134	0.042 ± 0.005	1.461 ± 0.048	0.152 ± 0.009	1.461 ± 0.048	0.109 ± 0.008	1.00
<u>Bronchioles (Pi < 2 mm)</u>								
Group A	12	1.678 ± 0.069	0.028 ± 0.005	1.272 ± 0.049	0.111 ± 0.008	1.272 ± 0.049	0.082 ± 0.005*	1.00
Group B	10	1.619 ± 0.085	0.030 ± 0.006	1.175 ± 0.047	0.093 ± 0.007	1.175 ± 0.047	0.063 ± 0.005	1.00
Group C	15	1.480 ± 0.071	0.047 ± 0.006	1.287 ± 0.047	0.114 ± 0.008	1.287 ± 0.047	0.066 ± 0.004	1.00

* Values are means ± the standard error of means. Pi=internal perimeter; Ai=internal area; Pe=external perimeter; Ae=external area; L=the length of outer layer of smooth muscle; WA=wall area; PMP=proportion of Pe occupied by muscle; Group A=hypersensitive sheep with significant changes in both airway resistance and dynamic lung compliance; Group B=hypersensitive sheep with significant changes only in dynamic lung compliance; Group C=non-reacting sheep. *p<0.05 compared to Group C.

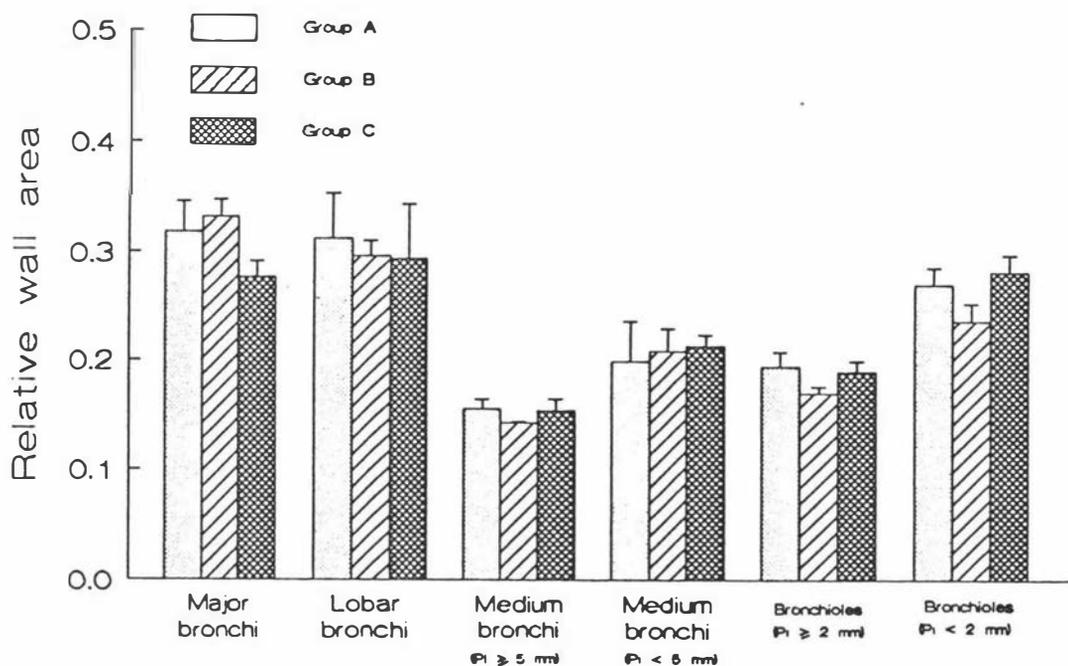


Fig. 11.2 The relative wall areas at the different levels of airways of sheep with varying degrees of airway hypersensitivity. Group A=hypersensitive sheep with significant changes in both airway resistance and dynamic lung compliance; Group B=hypersensitive sheep with significant changes only in dynamic lung compliance; Group C=non-reacting sheep. The difference between three groups of sheep is not significant at any airway levels (all $p > 0.05$).

Table 11.2
Calculated Dimensions of Reconstructed Airways of Sheep with Different Airway Hypersensitivity*

	n	Pi_r, mm	Ai_r, mm^2	Pe_r, mm	Ae_r, mm^2	Pw, mm^2	MS, mm	MS_0, mm
Major bronchi								
Group A	12	25.37 ± 1.29	52.66 ± 5.23	30.64 ± 1.09	75.72 ± 5.25	0.318 ± 0.028	0.69 ± 0.14	13.72 ± 1.12
Group B	12	26.47 ± 0.83	56.35 ± 3.55	32.42 ± 1.02	84.56 ± 5.24	0.332 ± 0.015	0.99 ± 0.10	13.77 ± 0.53
Group C	12	27.35 ± 1.41	61.28 ± 6.94	32.10 ± 1.45	83.84 ± 8.11	0.276 ± 0.015	0.69 ± 0.17	15.40 ± 1.06
Lobar bronchi								
Group A	6	17.33 ± 0.96	24.26 ± 2.71	20.96 ± 0.71	35.17 ± 2.45	0.317 ± 0.041	1.10 ± 0.18	9.35 ± 0.96
Group B	6	19.61 ± 1.38	31.37 ± 4.64	23.38 ± 1.62	44.54 ± 6.42	0.296 ± 0.014	1.31 ± 0.53	10.68 ± 0.80
Group C	6	16.56 ± 1.39	22.60 ± 3.48	19.62 ± 1.11	31.13 ± 3.44	0.293 ± 0.050	1.35 ± 0.34	9.34 ± 1.21
Medium bronchi ($Pi > 5 mm$)								
Group A	14	6.30 ± 0.30	3.25 ± 0.31	6.84 ± 0.29	3.81 ± 0.33	0.155 ± 0.009	3.30 ± 0.24	4.19 ± 0.25
Group B	7	5.86 ± 0.23	2.75 ± 0.22	6.32 ± 0.23	3.21 ± 0.24	0.143 ± 0.011	3.12 ± 0.18	3.95 ± 0.19
Group C	11	6.19 ± 0.36	3.15 ± 0.39	6.71 ± 0.35	3.68 ± 0.41	0.154 ± 0.013	2.88 ± 0.32	4.14 ± 0.31
Medium bronchi ($Pi < 5 mm$)								
Group A	4	3.73 ± 0.54	1.18 ± 0.31	4.13 ± 0.52	1.42 ± 0.33	0.199 ± 0.037	1.82 ± 0.39	2.37 ± 0.44
Group B	11	3.79 ± 0.14	1.16 ± 0.09	4.26 ± 0.14	1.46 ± 0.09	0.208 ± 0.021	1.72 ± 0.12	2.35 ± 0.13
Group C	7	4.16 ± 0.15	1.39 ± 0.10	4.69 ± 0.16	1.76 ± 0.12	0.213 ± 0.010	1.76 ± 0.14	2.53 ± 0.11
Bronchioles ($Pi > 2 mm$)								
Group A	12	2.473 ± 0.159	0.509 ± 0.073	2.749 ± 0.161	0.624 ± 0.081	0.194 ± 0.013	1.267 ± 0.124	1.560 ± 0.126
Group B	14	2.453 ± 0.060	0.483 ± 0.023	2.692 ± 0.063	0.581 ± 0.027	0.170 ± 0.006	1.294 ± 0.052	1.586 ± 0.045
Group C	9	2.429 ± 0.134	0.481 ± 0.055	2.694 ± 0.136	0.589 ± 0.062	0.189 ± 0.010	1.233 ± 0.114	1.532 ± 0.102
Bronchioles ($Pi < 2 mm$)								
Group A	12	1.678 ± 0.069	0.228 ± 0.018	1.962 ± 0.069	0.311 ± 0.021	0.270 ± 0.016	0.690 ± 0.059	0.952 ± 0.053
Group B	10	1.619 ± 0.085	0.214 ± 0.021	1.848 ± 0.083	0.277 ± 0.023	0.236 ± 0.016	0.672 ± 0.095	0.964 ± 0.067
Group C	15	1.480 ± 0.071	0.180 ± 0.017	1.740 ± 0.071	0.247 ± 0.020	0.281 ± 0.016	0.453 ± 0.072	0.831 ± 0.052

* Values are means ± standard error of means. Pi_r -internal perimeter; Ai_r -internal area; Pe_r -external perimeter; Ae_r -external area of "relaxed and dilated" airways; Pw -proportion of measured wall area in Ae_r ; MS-the degree of muscle shortening; MS_0 -the muscle shortening requiring to cause airway complete closure (when $Ai = 0$). Refer Table 11.1 for explanation of abbreviations. The difference between three groups of sheep is not significant at any airway level (all $p > 0.05$).

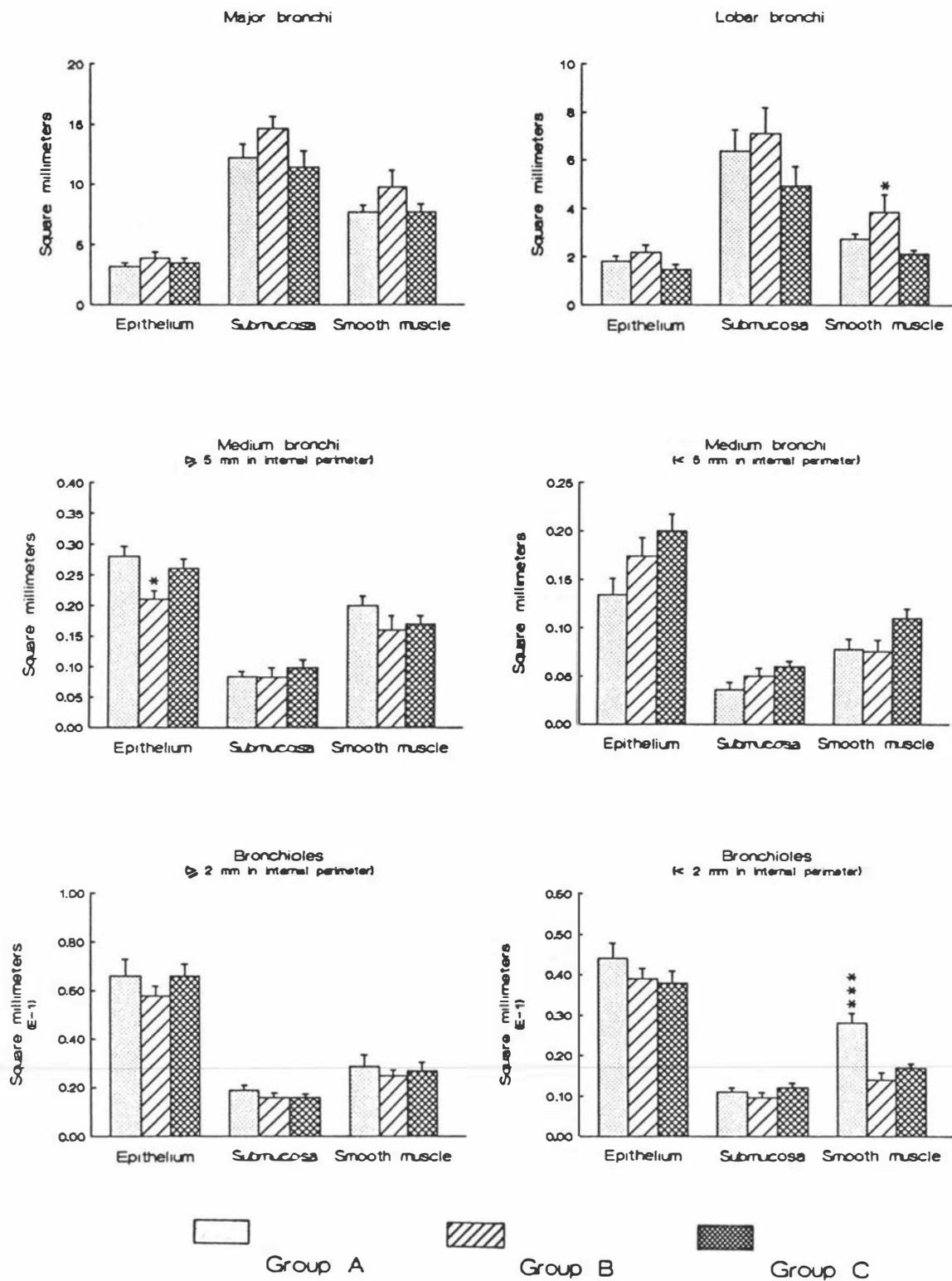


Fig. 11.3 Comparison of the areas of airway wall components between sheep with varying degrees of airway hypersensitivity. Refer Fig. 11.2 for the legend explanation.

* = $p < 0.05$ and *** = $p < 0.001$ compared to Group C.

Table 11.3
The Percentage of Muscle Shortening Required to Cause Complete Airway Closure in Hypersensitive and Non-reacting Sheep*

Airway level	Muscle shortening (%)		
	Group A	Group B	Group C
Major bronchi	44.2 ± 2.4	42.6 ± 1.3	47.7 ± 1.4
Lobar bronchi	44.3 ± 3.7	45.7 ± 1.3	46.7 ± 4.3
Medium bronchi (Pi≥5 mm)	60.8 ± 1.1	62.4 ± 1.4	61.1 ± 1.5
Medium bronchi (Pi<5 mm)	55.9 ± 4.0	54.9 ± 2.1	54.0 ± 1.1
Bronchioles (Pi≥2 mm)	56.2 ± 1.5	58.8 ± 1.0	56.6 ± 1.1
Bronchioles (Pi<2 mm)	48.3 ± 1.5	51.7 ± 1.8	47.2 ± 1.4

* Values are means ± the standard error of means. Refer Table 11.1 for explanation of abbreviations. The difference between the groups of sheep is not significant at any airway level (all $p > 0.05$).

The changes in relative airway resistance that are calculated to occur as a result of smooth muscle shortening (PMS) from 0% to 50% in medium bronchi less than 5 mm in internal perimeter are illustrated in Fig. 11.4. The calculations are based on the assumptions made by James *et al.* (1989) for resistance changes in asthmatic airways. The flow in a single airway completely surrounded by muscle (PMP = 1) is laminar and the baseline of airway resistance in non-reacting sheep (Group C) was arbitrarily set as 1.0. Although the results showed that airway resistance increased considerably when muscle shortening exceeded 35%, there was no significant difference in the magnitude of the increase in the hypersensitive animals. The results of calculations of resistance for airways of other sizes were similar (data not shown).

11.4 DISCUSSION

The pathogenesis of airway hypersensitivity in both human asthmatics and animal models remains obscure. Based on a computer model, Moreno *et al.* (1986) have recently proposed that airway narrowing resulting from a given degree of smooth muscle shortening (PMS) is greater in airways with a thick wall than in those with a thin wall. The relationship between the relative wall area (Pw) in reconstructed and relaxed airways, the proportion of muscle in the airway perimeter (PMP), and the change in airway resistance (% Raw) which occurs for a given degree of muscle shortening (PMS) can be mathematically estimated (Moreno *et al.*, 1986).

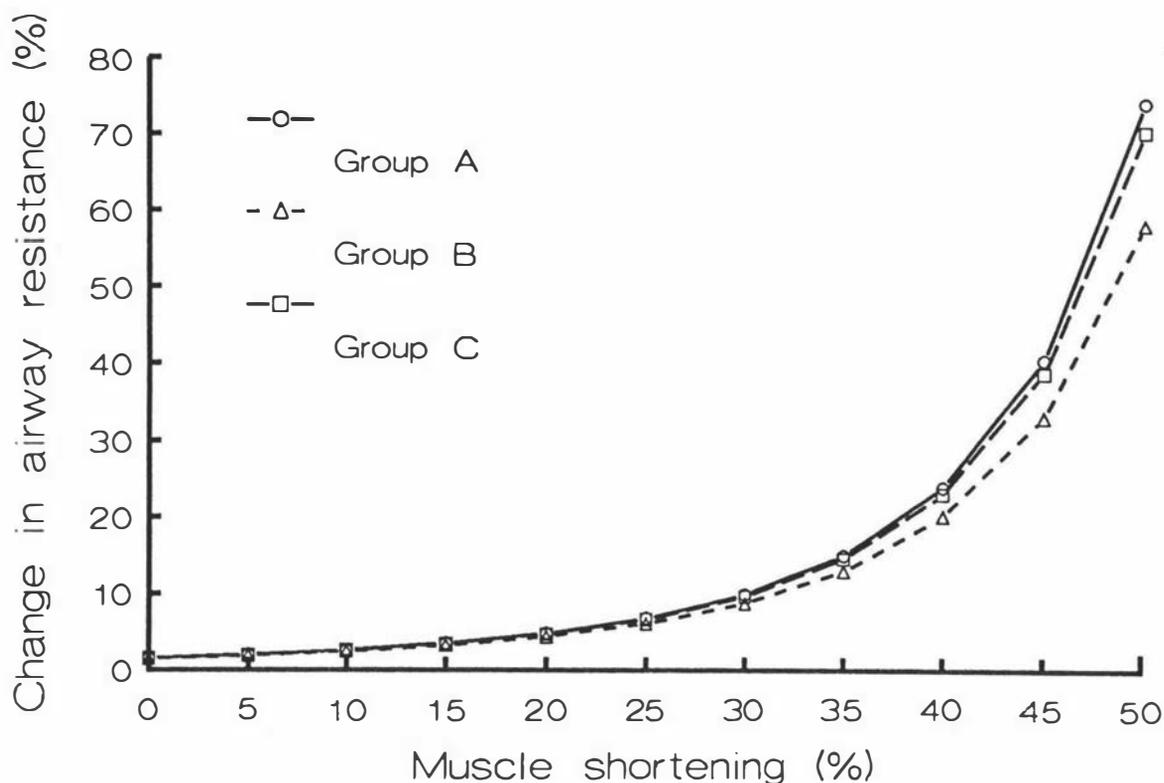


Fig. 11.4 The relative changes in airway resistance of bronchioles calculated from the mean airway dimensions of sheep with varying degrees of airway hypersensitivity. The calculation was based on the assumption that the proportion of external perimeter of airway wall occupied by smooth muscle is 1 and the baseline resistance of the non-reacting sheep (Group C) is arbitrarily set at 1.0. Refer Fig. 11.2 for the legend explanation. The difference between three groups of sheep is not significant ($p > 0.05$).

Despite extensive studies of the morphological changes of the airways of asthmatics and animal models with airway hypersensitivity, quantitative comparisons of airway dimensions are only recently becoming possible. This is because of difficulties in matching airways from different subjects which have variable smooth muscle tone and lung inflation. Studies of guinea pigs *in vivo* and the tracheal rings of pigs *in vitro* have shown that despite substantial changes in the luminal area, the internal perimeter and wall area of an airway remain constant both at different lung volumes (James *et al.*, 1988) or with different degrees of shortening of airway smooth muscle (James *et al.*, 1987). When the airway size is matched according to P_i , it becomes possible to compare the airway dimensions in individuals with different degrees of airway hypersensitivity.

An increase in thickness of the airway wall has been previously described in both asthmatics and animals with allergic airway hypersensitivity (Huber and Koessler, 1922; Salvato, 1968; Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Lebargy *et al.*, 1987; Hogg *et al.*, 1987), and has been quantitatively confirmed recently by James *et al.* (1989). When human airways were reconstructed to relaxed states, these authors found that the wall areas were significantly greater in asthmatics than non-asthmatic subjects in both large and small airways and that the asthmatic airway lumina are likely to be occluded with only minimal muscle shortening. The increase in airway wall area in these asthmatic patients, however, was most likely due to chronic airway inflammation since the majority of patients in the study had a history of recurrent wheeze and shortness of breath. It is, therefore, not clear whether the thickened airway wall is a confounding factor in determining the susceptibility of individuals to the development of allergic airway hypersensitivity.

The present study has applied Moreno *et al.*'s model to examine the inherent differences in airway dimensions and the shortening of smooth muscle required to cause complete airway closure in sheep with varying degrees of airway hypersensitivity to *Ascaris* antigen and compared them to sheep with normal airway responses. The results have shown that the measured thickness of airway walls in hypersensitive and non-reacting sheep is not inherently different at most airway levels although bronchioles less than 2 mm in internal perimeter in sheep from Group A had a significantly greater wall area than those in Group C. This difference, however, was not significant when the airways were reconstructed to a relaxed and fully dilated state. Similarly, there was no statistical difference between the three groups of sheep in the calculated muscle shortening required to cause complete airway closure or a relative increase of airway resistance following the shortening of airway muscle.

Failure to demonstrate inherent differences in airway dimensions or the relative increase of airway resistance to a given shortening of airway muscle has raised the possibility that thickening of the airway wall is not the major factor contributing to the development of allergic airway reactions in sheep. This possibility concurs with several recent morphological studies of patients with mild asthma (Sobonya, 1984; Lozewicz *et al.*, 1990). The lack of a significant increase in thickness of the airway wall in patients with mild or long-standing asthma has also been functionally demonstrated using bronchial provocation tests (Woolcock *et al.*, 1984). Studies of guinea pigs have shown that airway hyperresponsiveness induced by cigarette smoke is not associated with thickening of the airway wall (James *et al.*, 1988).

The lack of differences in airway wall thickness between hypersensitive sheep and non-reacting sheep may indicate different mechanisms are involved in the development of airway

constriction in the sheep model than are involved in human asthma. The increase of airway resistance in sheep after allergen challenge is probably due to transient muscle shortening which is reversed soon after withdrawal of the antigen challenge or perhaps during tissue processing as suggested by James *et al.* (1988). Using Poiseuille's law, James *et al.* (1988) calculated that smooth muscle constriction alone could lead to doubling of the airway resistance by reducing the area of internal lumen. In addition, it is also possible that functional characteristics of the airway muscle is different between hypersensitive and non-reacting individuals. Recent studies of asthmatic airway smooth muscle have suggested that there are intrinsic abnormalities in muscle contractility which may contribute to the clinical expression of asthma (Bai, 1990).

Morphometric studies by James *et al.* (1989) have shown that the airway epithelium, submucosa and smooth muscle are all involved in the thickening of airway walls seen in asthmatics. The present study has shown that the thickening of airway walls seen in the bronchioles of hypersensitive sheep is mainly due to an increase in smooth muscle which can induce more severe airway narrowing, for a given degree of shortening of airway muscle (Moreno *et al.*, 1986). Increase of airway smooth muscle has been well documented previously in asthmatics dying in status asthmaticus (Dunnill *et al.*, 1969; Takizawa and Thurlbeck, 1971; Ebina *et al.*, 1990a,b). However, it is not clear to what extent the increase in small airway muscle contributes to airway constriction seen in the present study since in asthmatics the large airways are the main sites of airway resistance (Hogg *et al.*, 1968) and the regions where hyperreactivity occurs (Ebina *et al.*, 1990b). Morphometric studies by other workers indicate that bronchial smooth muscle is not significantly increased in subjects with mild (Jeffery *et al.*, 1989; Lozewicz *et al.*, 1990) or long-standing asthma (Sobonya, 1984), in dogs with airway hyperresponsiveness (Yanta *et al.*, 1981), or in sheep after challenge with inhaled *Ascaris* antigen (Maurer *et al.*, 1981). Thus, it may be concluded that variations in the amount of airway smooth muscle, as seen in status asthmaticus (Heard and Hossain, 1973) or chronic bronchitis (Hossain and Heard, 1970a), cannot fully explain the inherent differences in allergic airway hypersensitivity and airway hyperresponsiveness seen in both man and animals.

The methods used for morphometric analysis of airway wall components in this study are different from most previous studies of smooth muscle (Dunnill, 1969; Heard and Hossain, 1973; Sobonya, 1984). In early studies, the airways in variously constricted or dilated states were not normalised. The major shortcoming of the present study was that all the tissue specimens were fixed by immersion because of the requirement of multiple samples from each animal for different types of morphological and histochemical study. However, the lack of significant differences between groups of sheep in most of the parameters measured is unlikely to be due to fixation. James *et al.* (1987) found that these parameters were not

significantly changed in pig tracheal rings after fixation by immersion. In addition, the present study compared morphometric differences of airway dimensions in three groups of tissues fixed and processed under identical conditions. Therefore, any artifacts induced by fixation would be universally distributed.

In conclusion, the morphometric analysis of airway dimensions has identified no significant differences between normal sheep and sheep with allergic airway hypersensitivity at most airway levels. Neither were any differences in the wall area and the shortening of airway smooth muscle required to cause complete airway closure calculated from reconstructed airways found. These results suggest that allergic airway hypersensitivity in sheep is not related to any inherent abnormalities of airway dimensions.

11.5 SUMMARY

The airway dimensions at the different levels of the lung were measured in three groups of sheep with different airway responses to inhaled *Ascaris suum* antigen (six animals in each group) to examine the inherent difference of the thickening of airway wall and shortening of smooth muscle required to cause complete airway closure in these animals. Previous studies have shown that sheep in Group A had significant changes in both airway resistance and dynamic lung compliance, sheep in Group B had changes in only dynamic lung compliance and sheep in Group C were non-responders. The methods used for the measurement and calculation of the airway dimensions before and after reconstruction were based on those used for human asthmatics and were made using a computerised image analyser. The results have shown that before airway reconstruction the sheep from Group A had a significantly greater wall area than those of Group C in bronchioles less than 2 mm internal perimeter ($p < 0.05$), and an increased wall area was mainly due to the increase of smooth muscle area ($p < 0.001$). However, when the airway was mathematically reconstructed to its "relaxed and fully dilated" state, there was no significant differences in any calculated parameters of the airway dimensions between the three groups of animals. Neither was there a significant difference in the calculated shortening of muscle required to occlude the airway lumen. These findings suggest that allergic airway hypersensitivity in sheep is not related to inherent abnormalities of airway dimensions.

CHAPTER 12

AIRWAY HYPERSENSITIVITY TO INHALED *ASCARIS SUUM* IN SHEEP: AIRWAY INFLAMMATION

12.1 INTRODUCTION

Of all the factors implicated in the pathogenesis of asthma and allergic airway hypersensitivity, airway inflammation is now considered the most important (Borish, 1987; Barnes, 1989). The mast cell (MC) is known to play a pivotal role in the development of allergic airway responses (Kaliner, 1985), but infiltration of other inflammatory cells, such as eosinophils, lymphocytes and neutrophils, into the airway epithelium and airway wall is also common in patients with severe asthma (Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Laitinen *et al.*, 1987; Beasley *et al.*, 1989; Holloway *et al.*, 1990). During the last decade, a series of clinical and experimental studies have been conducted in both human asthmatics and animal models aimed at understanding the inflammatory mechanisms involved in the development of allergic airway diseases (reviewed in Borish, 1987). The results of these studies contain certain discrepancies, particularly with regard to the role of eosinophils and neutrophils. The reason why airway hypersensitivity develops in some but not all allergen-exposed subjects is still not completely clear.

Studies both in New Zealand (Chapter 8) and overseas (Wanner *et al.*, 1979) have shown that some sheep develop an acute airway response to inhaled *Ascaris suum* antigen, and this response is associated with an elevation of most types of inflammatory cell in bronchoalveolar lavage (BAL) fluid (Rubin *et al.*, 1987). The objective of the current investigation was to use this model to define further the mechanisms involved in the development of allergic airway hypersensitivity by identifying inherent differences between hypersensitive and non-reacting sheep in the numerical profiles of inflammatory cells in peripheral blood and the lower respiratory tract (LRT).

12.2 MATERIALS AND METHODS

12.2.1 Animals

Forty-three Romney-cross sheep aged from 4-8 years were used. Previous studies had shown that all animals had a positive skin reaction to intradermal injection of *A. suum* antigen and showed varying degrees of acute airway response to aerosols of *A. suum* antigen. Twenty-one sheep were responders with changes in both airway resistance and dynamic lung compliance (Group A), ten showed changes only in dynamic lung compliance (Group B) and 12 were non-responders (Group C). Details of the skin reaction and airway response in these animals have been described earlier in Chapter 8.

12.2.2 Haematology

Blood samples were collected from the jugular vein of 24 sheep, 14 days after the bronchial provocation test. Total leucocyte counts were determined in a Cell-Dyn 900 haematological analyser (Sequoia-Turner, California), and differential leucocyte counts were made from blood smears stained with a modified Wright's stain (Sigma, U.S.A.).

12.2.3 Tissue sampling and processing

Eighteen sheep, six from each group, were used for a numerical study of tissue inflammatory cells. All animals were killed with intravenous injection of sodium pentobarbitone 14 days after the bronchial provocation test. Two consecutive sets of tissue blocks were taken from sites in the upper, mid and lower trachea, the major, lobar and medium bronchi, and the cranial, middle and caudal lobes of the lung (Fig. 10.1). Immediately after sampling, one set of tissue blocks was fixed in 10% neutral buffered formalin, and the other set was fixed in isotonic formal-acetic-acid fixative. The formalin-fixed tissues were stained with haematoxylin and eosin, or Luna's stain for eosinophilic granules (Luna, 1968), whereas the tissues fixed with isotonic formal-acetic-acid were stained with toluidine blue. Details of the fixation and processing techniques for these tissues were described earlier in Chapter 7.

For ultrastructural studies, small tissue blocks were taken from areas adjacent to those used for light microscopy. These were fixed in 3% glutaraldehyde in 0.1 M phosphate buffered saline overnight. They were processed routinely and examined under a Philips EM201c transmission electron microscope as described previously (Chen *et al.*, 1988).

12.2.4 Quantitative studies of tissue inflammatory cells

Mast cells: The numbers of MC were counted in the sections stained with toluidine blue using a JAVA image analyser (Jandel Scientific, California) at a final magnification of $\times 185$, as described in Chapter 7. All the cells stained with toluidine blue were counted as MC. The numerical density of MC at each site was calculated and recorded as MC numbers/mm².

To estimate the number, diameter and volume density of secretory granules in MC, electron micrographs of MC from the ovine lung were examined at a final magnification of 5,800 to 7,800. Twenty MC from at least three different tissue blocks of lung from each sheep were randomly selected and photographed. Their granules were counted and measured using the image analyser. With this system, the image of each cell was grabbed from the electron micrographs through a JVC video camera (Tokyo, Japan) and the outline of the cell was then traced with a mouse. After the image analyser was calibrated with a standard image, the

number of granules in each MC and the size of each granule could be read directly from the analyser, and stored in the computer for further analysis.

The numerical density (N_v) of the MC granules was calculated using the formula $N_v = N_A/D$, where N_A is the number of granules per MC and D the mean diameter of the granules. D was calculated from the relationship $D = 4d/\pi$, where d is the average diameter of the granules in the population of MC examined (Weibel, 1979; Warton *et al.*, 1986a).

The proportion of different types of granule was counted manually from the electron micrographs. The granules were classified into two morphological categories; solid and empty. An empty granule represented one in which there was the discharge of at least 50% of its contents.

Eosinophils: The morphology of eosinophils in the ovine LRT was studied in the histological sections stained with Luna's stain for eosinophilic granules (Luna, 1968) and with transmission electron microscopy. Pilot work showed that sections stained with Luna's stain were not suitable for automatic counting of tissue eosinophils using the image analyser since erythrocytes and plasma material also stained with this method. An alternative staining method was, therefore, needed. It was found that sections stained with naphthalene black and chromotrope showed a clear distinction between intensively blue-black-stained eosinophil granules and a slightly pink-stained tissue background (Appendix 12.1). Under the appropriate magnification and light intensity, the number of eosinophils in a given tissue could be automatically counted with this method using the image analyser in a similar manner to that used for MC counting. Thus, the numerical density of eosinophils was determined in naphthalene black-stained paraffin sections using an image analyser.

Neutrophils and lymphocytes: The degree of neutrophil and lymphocyte infiltration in the LRT was assessed in haematoxylin-eosin stained sections. Since the number of infiltrating neutrophils in the airway was minimal and lymphocytes were often presented in clusters or aggregates, their number was only semi-quantitatively assessed using the following variables: (1) the degree of neutrophil infiltration within the airway epithelium; (2) the degree of neutrophil infiltration within airway walls, (3) intraepithelial infiltration of lymphocytes and (4) lymphoid aggregations. A score from 0 (absent or very few) to 4 (numerous or intensive) was assigned for each variable.

12.2.5 Data analysis

Results from the quantitative studies of MC and eosinophils were expressed as mean and the standard error of means, and the remaining data were presented as a median value and

range. Statistical analysis of the data was made using computer-assisted one-way analysis of variance or Kruskal-Wallis test, as appropriate (Statistix, NH Analytical Software, MN). A logarithmic or arcsin transformation was applied to some data before analysis. If a statistical difference was found, a further analysis was made using Dunnett's test, which allows for multiple comparisons to a single control group (Winer, 1971). The difference was considered significant if $p < 0.05$.

12.3 RESULTS

12.3.1 Haematology

The total counts of circulating leucocytes were $9.41 \times 10^9 \pm 0.84 \times 10^9/l$, $12.00 \times 10^9 \pm 1.29 \times 10^9/l$ and $10.57 \times 10^9 \pm 0.73 \times 10^9/l$ in Group A, Group B and Group C, respectively, and there was no significant difference between groups. A comparison of the differential leucocyte counts of the three groups of sheep is presented in Fig. 12.1. Compared to non-reacting sheep (Group C), the hypersensitive sheep (Groups A and B) contained a higher proportion of circulating lymphocytes and a lower percentage of neutrophils. This difference was, however, only statistically significant between the sheep of Group B and Group C (both $p < 0.05$). The proportion of eosinophils was less than 10% in all groups, and basophils were absent from the sheep of Group B and constituted less than 1.5% of leucocytes in the animals of Groups A and C. There was no significant difference in the proportion of eosinophils, monocytes or basophils between the three groups of sheep.

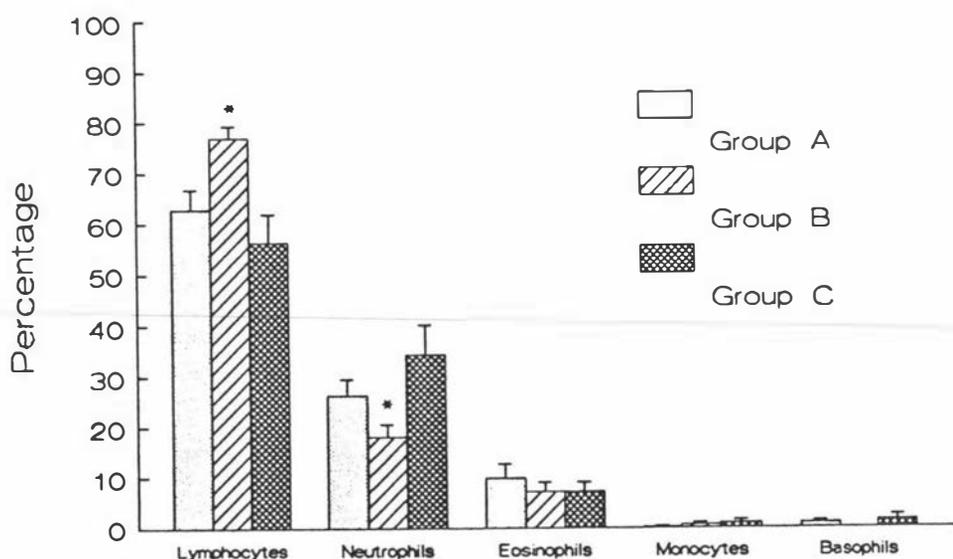


Fig. 12.1 A comparison of differential circulating leucocyte counts between hypersensitive and non-reacting sheep. Group A=hypersensitive sheep with significant changes in both airway resistance and dynamic lung compliance, Group B=hypersensitive sheep with significant changes only in dynamic lung compliance, and Group C=non-reacting sheep. * $p < 0.05$ compared to Group C.

12.3.2 Quantitative studies of tissue inflammatory cells

Mast cells: A comparison of the numerical density of MC at the different levels of LRT is made in Fig. 12.2. There was no significant difference in density between the three groups of sheep. Most MC seen in the airways of both hypersensitive and non-reacting sheep were located in the lamina propria, as previously observed in normal sheep, and intraepithelial MC were rare in all the sheep, regardless of group.

The morphometric studies showed that the average number of secretory granules per single MC was greater in the lungs of hypersensitive sheep (48 ± 2 in Group A and 52 ± 2 in Group B) than in non-reacting animals (43 ± 1 in Group C) although this was only significant between Group B and Group C (Fig. 12.3, $p < 0.05$). The diameter of individual secretory granules was 323.1 ± 6.5 nm in Group A, 321.5 ± 10.5 nm in Group B and 349.8 ± 9.1 nm in Group C. The volume density of secretory granules in the three groups of sheep was $13.01 \pm 0.88 \mu\text{m}^3$, $14.06 \pm 1.31 \mu\text{m}^3$, and $9.94 \pm 0.66 \mu\text{m}^3$, respectively. Statistical analysis revealed that the MC from hypersensitive sheep (Groups A and B) contained a significantly higher volume density of secretory granules than those from non-reacting sheep (Group C, $p < 0.01$). However, the diameter of the individual granules in MC of Groups A and B was smaller than that of MC in Group C ($p < 0.05$). The proportion of "empty" granules was not significantly different between MC from the different groups ($p > 0.05$), and about 90% of granules were of the "solid" type (Fig. 12.3).

Eosinophils: Eosinophils were identified at all levels of the LRT in all groups, but their number varied greatly within the tissue levels and between individuals. In the airways, eosinophils were located predominantly in the lamina propria, and less frequently within the mucosal epithelium and areas surrounding the submucosal glands. The cells within the epithelium were mainly present in the basal part of the epithelium. Eosinophils were rarely seen in the interstitial tissue between smooth muscle bundles in either the trachea or major bronchi, but they were sometimes present in this area in the small airways. The eosinophils in the trachea and bronchi were mainly scattered in their distribution although occasional cell nests or islands were seen. In bronchioles, their distribution varied greatly between individual airways. In some areas, large numbers of eosinophils were present immediately beneath the epithelium or within the airway lumina, whereas in other areas small numbers of eosinophils were seen only outside the circular muscle layer. In the lung, eosinophils were mainly distributed in a scattered form throughout the alveolar septa and in perivascular and peribronchiolar regions. Occasionally, numerous eosinophils were seen within and adjacent to old parasitic granulomas.

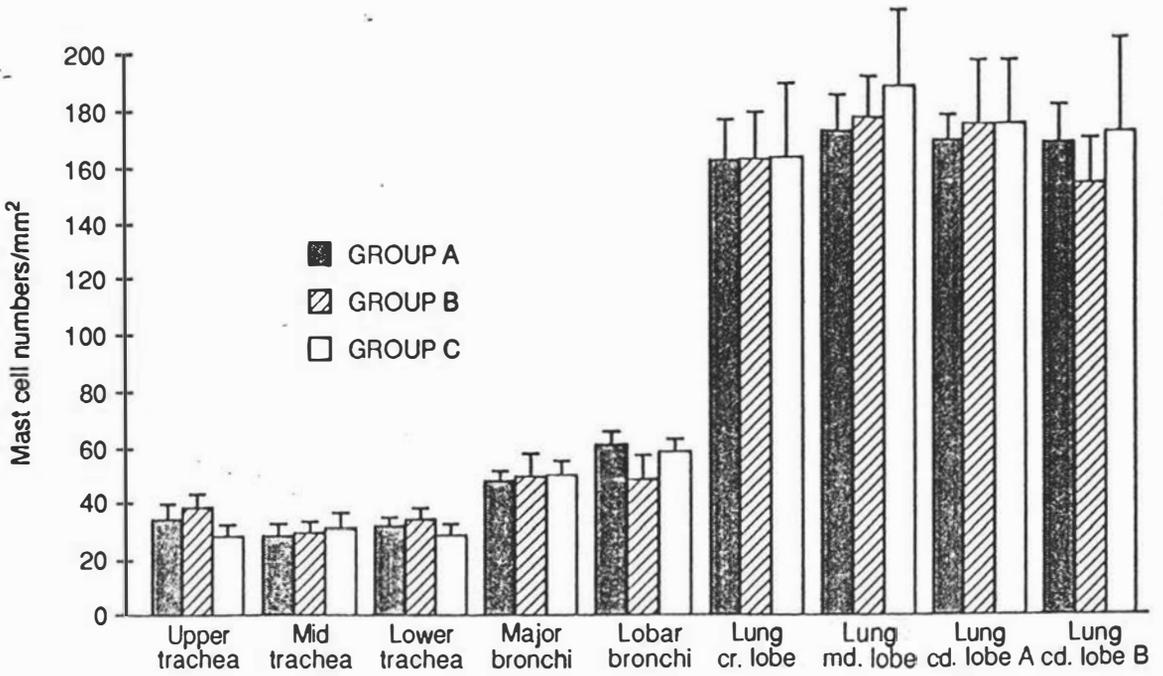


Fig. 12.2 Results of mast cell counts at different levels of the lower respiratory tract of hypersensitive and non-reacting sheep. cr.=cranial, md.=middle, cd.=caudal. Refer Fig. 12.1 for the legend explanation. The difference between three groups of sheep is not significant at any level (all $p > 0.05$).

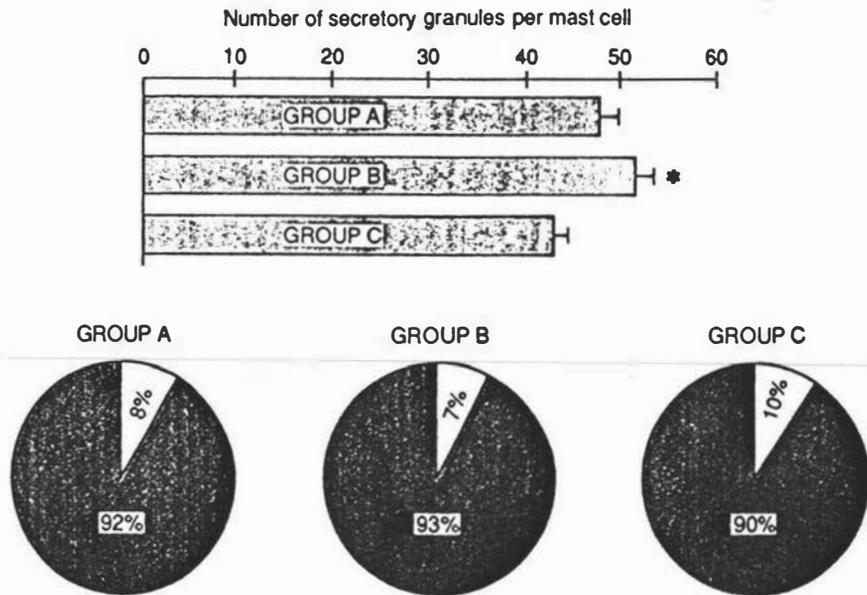


Fig. 12.3 Results of morphometric profiles of mast cell granules in the lower respiratory tract of hypersensitive and non-reacting sheep. Refer Fig. 12.1 for the legend explanation. In the pie chart, □ =empty granules and ■ =solid granules. * = $p < 0.05$ compared to Group C.

Ultrastructurally, eosinophils were characterised by their composite cytoplasmic granules (Fig. 12.4). Most of these granules were ovoid and covered with a limiting membrane. They usually contained one or several electron-dense crystal cores, which were of various irregular shapes and embedded in a fine granular matrix. The cytoplasm of some eosinophils also contained one or several lamellar bodies which were arranged eccentrically by a fine myelin-like material (Fig. 12.5). Activation of eosinophils was sometimes evident morphologically by the loss of the crystal core from the granules and the presence of extracellular granules in adjacent areas, but there was no preference for this change in the hypersensitive sheep.

Quantitative studies of the numerical density of eosinophils showed that they were more numerous in the peripheral regions than in central regions of the LRT in all groups (Table 12.1). Statistical analysis showed that there was no significant difference in the numerical density of eosinophils between the three groups of sheep at any level of the LRT ($p>0.05$).

Neutrophils and lymphocytes: The infiltration of neutrophils and lymphocytes into the airways varied considerably between animals and between the airway levels. Focal infiltrations of neutrophils were observed both within the epithelium and in subepithelial regions of large airways, but were relatively rare in small airways. Statistical analysis showed that there was no significant difference in the severity of either intraepithelial or subepithelial infiltration of neutrophils at any level of the LRT between the three groups of sheep (Table 12.2).

The presence of lymphocytes and lymphoid tissue was common throughout the LRT of all animals. The major forms of lymphoid tissue were the intraepithelial and scattered types. Dense and nodular forms of lymphoid aggregation were only found in the trachea of two sheep from Group B (data not shown). In addition, small numbers of plasma cells and macrophages were also commonly seen among these aggregates and in adjacent areas. Follicle-associated lymphoepithelium was not seen, however, in any of the sheep examined. A comparison of the degree of lymphocyte infiltration and the form of lymphoid aggregation was made between the different groups of sheep and this showed no significant differences at any level of the LRT ($p>0.05$).

12.4 DISCUSSION

The finding that both acute and chronic airway response to *A. suum* in hypersensitive sheep is associated with a transient increase in histamine levels in blood plasma (Wanner *et al.*, 1979; Bosse *et al.*, 1987) and BAL fluid (Dworski *et al.*, 1989) and is accompanied by a significant decrease in pulmonary MC (Kleeberger *et al.*, 1985) suggests that MC may play an important role in the development of airway hypersensitivity of this model. Since

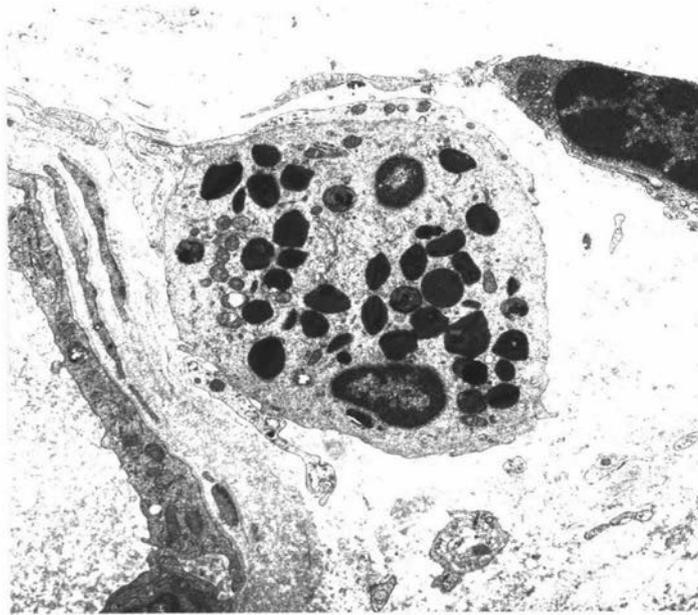


Fig. 12.4 An eosinophil with typical granules and nucleus in the perivascular area of the lung from a hypersensitive sheep. TEM, x7,800.

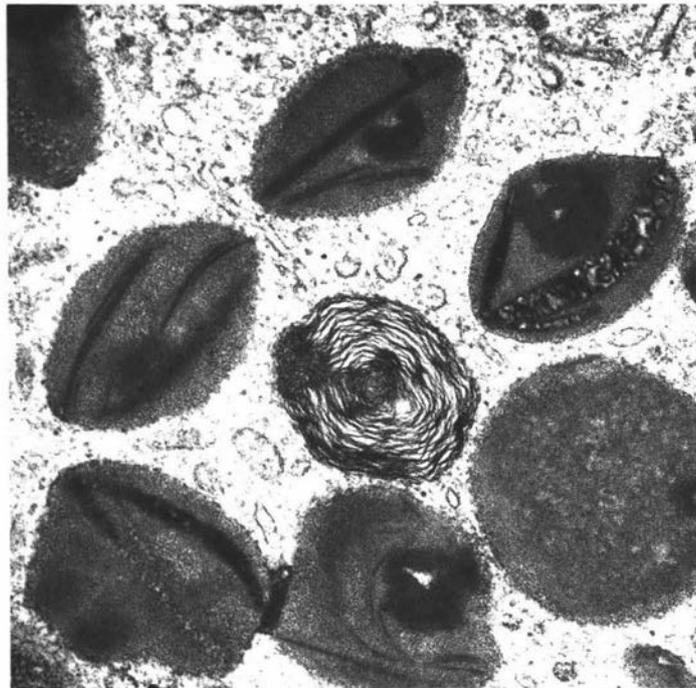


Fig. 12.5 Higher magnification of Figure 12.4 showing several granules with electron-dense crystals, and a lamellar body composed of eccentrically arranged fine myelin-like material. TEM, x48,600.

Table 12.1
Results of Eosinophils Counts in the Lower Respiratory Tract from Hypersensitive and Non-reacting Sheep[#]

	Upper trachea	Mid trachea	Lower trachea	Major bronchi	Lobar bronchi	Medium bronchi	Lung
<u>Intra-epithelium (cells/mm)</u>							
Group A	1.2 ± 0.2	1.0 ± 0.2	1.3 ± 0.4	1.2 ± 0.3	1.2 ± 0.4	0.6 ± 0.1	
Group B	1.3 ± 0.4	1.2 ± 0.4	1.3 ± 0.3	1.2 ± 0.4	1.2 ± 0.2	0.7 ± 0.1	
Group C	1.2 ± 0.4	1.4 ± 0.3	1.4 ± 0.4	1.2 ± 0.2	1.2 ± 0.2	0.8 ± 0.2	
<u>Subepithelial regions (cells/mm²)</u>							
Group A	11.0 ± 1.3	9.7 ± 2.2	10.8 ± 1.5	17.0 ± 2.8	19.5 ± 2.4	22.9 ± 3.2	80.4 ± 18.0
Group B	9.9 ± 1.8	10.5 ± 2.0	10.8 ± 1.5	16.4 ± 1.3	16.4 ± 1.6	17.3 ± 4.2	68.5 ± 26.2
Group C	10.5 ± 2.0	11.0 ± 1.3	11.4 ± 1.9	17.3 ± 6.4	18.4 ± 2.3	16.4 ± 1.3	75.8 ± 15.0

[#] Data are presented as mean and the standard error of means. Group A=hypersensitive sheep with significant changes in both airway resistance and dynamic lung compliance, Group B=hypersensitive sheep with significant changes only in dynamic lung compliance, and Group C=non-reacting sheep. The difference between the three groups of sheep is not significant at any level (all p>0.05).

Table 12.2
Severity of Neutrophil and Lymphoid Cell Infiltrations
in the Airway Epithelium and Airway Wall*

	Airway epithelium		Airway wall	
	Neutrophils	Lymphocytes	Neutrophils	Lymphocytes
<u>Upper trachea</u>				
Group A	0 (0-2)	0 (0-2)	0 (0-1)	1 (0-2)
Group B	1 (0-3)	1 (0-3)	0 (0-3)	1 (1-4)
Group C	0 (0-2)	1 (0-2)	0 (0-0)	2 (1-4)
<u>Mid trachea</u>				
Group A	0 (0-2)	1 (0-2)	0 (0-2)	2 (1-4)
Group B	0.5 (0-3)	1 (0-4)	0 (0-3)	1.5 (1-3)
Group C	0 (0-2)	1 (0-2)	0 (0-2)	2 (1-4)
<u>Lower trachea</u>				
Group A	0 (0-1)	1 (0-2)	0 (0-2)	1 (1-3)
Group B	1 (0-2)	1 (0-2)	0 (0-2)	1 (1-4)
Group C	0 (0-2)	1 (0-2)	0 (0-0)	1.5 (1-3)
<u>Major bronchi</u>				
Group A	0 (0-1)	0.5 (0-2)	0 (0-4)	1 (1-2)
Group B	0 (0-1)	1 (0-1)	0 (0-1)	1.5 (0-3)
Group C	0 (0-2)	0.5 (0-3)	0 (0-1)	1.5 (1-4)
<u>Lobar bronchi</u>				
Group A	0 (0-0)	0.5 (0-1)	0 (0-0)	1 (1-2)
Group B	0 (0-2)	0 (0-2)	0 (0-3)	1.5 (0-4)
Group C	0 (0-2)	0 (0-2)	0 (0-1)	1 (1-3)
<u>Medium bronchi</u>				
Group A	0 (0-1)	0.5 (0-1)	0 (0-0)	1 (1-2)
Group B	0 (0-1)	0.5 (0-2)	0.5 (0-2)	1.5 (1-4)
Group C	0 (0-1)	0 (0-1)	0 (0-0)	1.5 (1-3)
<u>Bronchioles</u>				
Group A	0 (0-0)	1 (1-1)	0 (0-3)	1.5 (1-3)
Group B	0 (0-0)	0.5 (0-1)	0.5 (0-1)	1 (1-2)
Group C	0 (0-0)	0 (0-2)	0 (0-3)	1.5 (1-4)

* Data are presented as median value, with range in parentheses. Refer Table 12.1 for the abbreviation explanation. The difference between three groups of sheep is not significant at any airway levels (all $p > 0.05$). Scores: 0=none, 1=mild, 2=moderate, 3=severe and 4=very severe.

activation of MC within the airway and alveolar walls is the most likely initiating event in an allergic airway reaction, it is reasonable to postulate that MC are likely to be more numerous and contain more and larger granules in hypersensitive sheep than those in non-reacting animals.

The results of the present study have shown that the baseline value of MC numbers in the LRT is not significantly different between hypersensitive and non-reacting sheep, indicating that the numerical density of MC is not a major determinant of susceptibility. Studies in patients with severe asthma have shown that MC numbers in both the airways and lung are generally decreased as a result of degranulation (Salvato, 1968; Heard *et al.*, 1989), but in bronchial biopsies from patients with stable asthma and in BAL fluid of asthmatics the number and proportion of MC are generally higher than in control subjects (Tomioka *et al.*, 1984; Flint *et al.*, 1985a,b). Studies both in patients with mild asthma and in several animal models have revealed that the number of MC and the degree of degranulation are not significantly different between normal and allergic individuals (Hogg *et al.*, 1979; Fox *et al.*, 1981; Warton *et al.*, 1986a; Beasley *et al.*, 1989; Jeffery *et al.*, 1989), and are even not significantly altered after antigen challenge (Hogg *et al.*, 1979; Beasley *et al.*, 1989).

Although the ovine LRT contains more MC than most other animal species studied so far, including man (Gold *et al.*, 1977; Shanahan *et al.*, 1987; Bachelet *et al.*, 1988; Mair *et al.*, 1988; Heard *et al.*, 1989; Chen *et al.*, 1990), the number and diameter of secretory granules as well as the volume density of these granules are similar to those in human lungs (Fox *et al.*, 1981; Warton *et al.*, 1986a). The average number of secretory granules per MC in the human lung is in the range of 40 to 55 (Fox *et al.*, 1981; Warton *et al.*, 1986a) with some extremes of 300 (Church *et al.*, 1982). The diameter of individual granules in human lung MC is about 340 nm and the volume density $6 \mu\text{m}^3$ (Warton *et al.*, 1986a). These morphometric profiles are similar in healthy and asthmatic lungs (Fox *et al.*, 1981; Warton *et al.*, 1986a).

In the hypersensitive sheep, the volume density of secretory granules in lung MC was significantly greater compared to those in the non-reacting sheep although the mean diameter of individual granules was smaller. Although it is premature to conclude that the high volume density of MC granules seen in the present study is a major contributor to the natural occurrence of allergic airway hypersensitivity in sheep, it is very likely that MC with a high volume density contain more inflammatory mediators than those with a lower volume density.

The ultrastructure of MC granules in the ovine lung is less complex than that in man. Only two categories of granules, empty and solid, were recognised in the present study. It was initially postulated that MC granules in the hypersensitive sheep may be more vulnerable to

antigen stimulation than in non-reacting animals and, for this reason, a higher proportion of empty granules was expected in MC from hypersensitive animals. However, the results showed that the overall proportion of empty granules in MC was small and very similar between the three groups of sheep. This observation is similar to the findings of Fox *et al.* (1981) in the human lungs with varying diseases and of Jeffery *et al.* (1989) in the lobar bronchi of patients with mild asthma. Studies by Kleeberger *et al.* (1985) demonstrated that the degree of degranulation of lung MC in sheep is not significantly changed after exposure to *A. suum* antigen. In contrast to these findings, Kawanami *et al.* (1979), Warton *et al.* (1986a) and Beasley *et al.* (1989) have shown that degranulation of mast cells is more common in asthmatic lungs and lungs with other diseases than in control lungs. The presence of about 10% of empty granules in the lung MC of all sheep in the present study suggests that a consistent level of degranulation occurs in healthy individuals in normal circumstances.

Although eosinophil infiltration is a characteristic feature of asthmatic airways (Filley *et al.*, 1982; Holloway *et al.*, 1990), the present study suggests that eosinophils may not be directly involved in the initial development of allergic airway hypersensitivity since neither the circulating eosinophil counts nor the tissue eosinophil numbers were significantly different between hypersensitive and non-reacting sheep. Studies of this phenomenon in both patients with mild asthma and in animal models have to date produced contradictory results. Although tissue eosinophilia has been considered both a predisposition for and a result of the intense immediate response to inhaled antigen in monkeys (Wegner *et al.*, 1987), aerosol challenge with antigens does not significantly alter the number and heterogeneity of eosinophils in BAL fluid and bronchial biopsies from hypersensitive sheep or in mild asthmatic patients (Rubin *et al.*, 1987; Lozewicz *et al.*, 1988; Beasley *et al.*, 1989). The number of eosinophils was not significantly increased in bronchial biopsies from patients with mild (Jeffery *et al.*, 1989) or severe asthma (Chanez *et al.*, 1989).

There is increasing evidence suggesting that the eosinophil plays a critical role in the pathogenesis of allergic airway disease by disrupting the airway epithelium (Ohashi *et al.*, 1989; Bruijnzeel, 1989; Gleich, 1990). In the present model, however, there was little evidence of epithelial damage to either hypersensitive or non-reacting sheep (Chapter 10) despite the presence of eosinophils in the airway wall. A recent study in the *Ascaris*-hypersensitive monkey has also shown that there is no epithelial damage or denudation of airways in which large numbers of eosinophils and free granules were present in the lamina propria (Gundel *et al.*, 1990). The reason for this is not clear although it is possible that eosinophils from different species may contain different types of granule components. Preliminary work has shown that the major basic protein of ovine lung eosinophils is antigenically heterogeneous to that of human lung eosinophils (unpublished observations). The present study has also shown that

ovine lung eosinophils are morphologically different to human eosinophils in several respects. Firstly, eosinophils are very common in the LRT, especially in the region of small airways in clinically healthy sheep, whereas in man, eosinophils are found mainly in lungs of allergic subjects and are almost absent in the nasal mucosa (Okuda and Zheng, 1989) and lung (Beasley *et al.*, 1989) of normal subjects. Secondly, the crystal cores of human lung eosinophil granules are more uniform in both shape and size than those of ovine lung eosinophils (Gleich and Adolphson, 1986; Gonzalez *et al.*, 1987a).

It is worth noting that only the quantitative differences between eosinophils of hypersensitive and non-reacting sheep were investigated in this study, and it is not known whether or not these cells differ in their subtypes. It is now well recognised that eosinophils isolated from asthmatic airways contain a high proportion of hypodense types, which are believed to represent activated eosinophils (Fukuda and Gleich, 1989). However, the ultrastructural observations made in this study have shown little difference in morphological activation of eosinophils between the three groups of sheep.

The lack of statistically significant differences in numerical density of other airway inflammatory cells (neutrophils and lymphocytes) between hypersensitive and non-reacting sheep contrasts with the degree of tissue inflammation which is characteristic of overt asthma (Salvato *et al.*, 1968; Dunnill *et al.*, 1969; Cutz *et al.*, 1978; Power *et al.*, 1989), but is in agreement with recent studies in human patients with mild asthma (Lozewicz *et al.*, 1988; Beasley *et al.*, 1989; Jeffery *et al.*, 1989). It has also been found recently that the number of inflammatory cells in the airway wall does not correlate significantly with epithelial damage or airway hyperreactivity in subjects with mild asthma (Jeffery *et al.*, 1989).

The significance of changes in the circulating lymphocyte and neutrophil proportions in the hypersensitive sheep in the present study is not clear, since data from other studies of circulating leucocytes in both asthmatics and animal models are contradictory. Grad *et al.* (1987) reported that total leucocyte counts and differential counts in peripheral blood were similar in *Ascaris*-hypersensitive and control Beagle dogs. However, studies by Annesi *et al.* (1988) have shown that total leucocyte counts in peripheral blood are significantly higher than normal in subjects with airway hyperresponsiveness and this increase involved almost every type of leucocyte, but particularly neutrophils. Further studies therefore will be necessary to further clarify any correlation between the numerical profiles of circulating leucocytes and allergic airway hypersensitivity.

The application of a computerised image analyser enabled not only the examination of a reasonably large area of tissue (between 4.5 to 6.5 mm² in each section in the present study),

but also accurate and rapid measurement of MC and eosinophil numbers in a tissue which was very irregular in shape. The method developed for the automatic counting of the numerical density of tissue eosinophils may have an application in both clinical and experimental studies. The reagents are simple and the time required for staining is short, particularly if a counterstain is not used. The main shortcoming of this method is that the pale-stained background makes interpretation of other histological features difficult.

12.5 SUMMARY

In order to investigate the relationship between airway inflammation and the development of naturally-occurring allergic airway hypersensitivity, peripheral blood samples and the lower respiratory tract were collected from three groups of sheep 14 days after challenge with inhaled *Ascaris suum* antigen. Previous studies have shown that sheep in Group A had significant changes in both airway resistance and dynamic lung compliance, sheep in Group B had changes in only dynamic lung compliance and sheep in Group C were non-responders. Compared to the non-responding sheep (Group C), the sheep in Groups A and B showed a higher proportion of lymphocytes and a lower percentage of neutrophils in their peripheral blood, although this difference was only statistically significant between Group B and Group C ($p < 0.05$). The volume density of secretory granules in lung mast cells was greater in hypersensitive sheep than in non-reacting sheep ($p < 0.01$). This high volume density was due to a high numerical density of granules in the mast cells. There was however, no significant difference between groups in levels of total circulating leucocytes, numerical density of mast cells and eosinophils, and the degree of degranulation of mast cells. Although lymphocytes were commonly seen within the airway epithelium and subepithelial regions, the infiltration of neutrophils or aggregation of dense and nodular lymphoid tissues were not common in the airways of any sheep. There was no significant difference in the infiltration of neutrophils or the aggregation of lymphocytes and lymphoid tissue in airways between the three groups. These findings indicate that there is an inherent difference in the volume density of lung mast cell granules between hypersensitive and non-reacting sheep and that other inflammatory cells are not directly involved in the initial development of allergic airway hypersensitivity in this species.

PART V
GENERAL DISCUSSION

CHAPTER 13

GENERAL DISCUSSION

To advance our knowledge of human allergic asthma, animal models have been used increasingly in studies of the pathogenesis, pathophysiology, and pharmacology of the disease (Wanner and Abraham, 1982). Although asthma is highly prevalent in New Zealand (Jackson *et al.*, 1988; Sears, 1988; Mitchell *et al.*, 1990), experimental studies using animal models have been very limited, and the guinea pig has been the main animal model used (Galland and Blackman, 1989). The inherent advantages and disadvantages of the guinea pig model have been discussed in Chapter 3, and the development of the ovine model will provide a further tool for research workers in asthma in this country. Because of its advantages as an experimental model the sheep has now been used in several countries for studying asthma. In this country, the animal has the additional advantages of being reasonably cheap and easy to obtain and care for.

The first aim of the work described in this thesis was to establish an animal model for human allergic asthma in New Zealand using locally bred Romney sheep. Before commencing this study no information was available which might suggest that Romney sheep, a different breed to that used overseas for asthma research, would respond in a similar manner to that reported for sheep in North America and Japan. Human allergic asthma is believed to be associated with genetic factors (Kallos and Kallos, 1974; McFadden, 1984). Furthermore, because *Ascaris suum* is not known to be a common parasite in the New Zealand pastoral environment it seemed quite possible that sheep in New Zealand would have less tendency to show any immunologically-based reaction to *A. suum* antigen. The studies presented in Chapter 8 have, in fact, shown that about 70% of adult sheep with a positive skin reaction showed an acute airway response to inhaled *A. suum* antigen. The response in these sheep involved either both large and small airways (Group A) or small airways only (Group B). The time course of airway response and the changes of pulmonary mechanics seen in these animals were similar in several respects to those seen in human asthmatics (McFadden and Ingram, 1980b), indicating that sheep in this country are suitable for use in studying human allergic asthma. The airway response seen in the hypersensitive sheep was not due to previous exposure to domestic pigs (Chapter 8). Neither was it associated with serum antibody against excretory and secretory antigen of *Toxocara canis* (Appendix 8.1), a nematode which is responsible for *Ascaris*-induced airway hypersensitivity in dogs (Patterson and Kelly, 1974) and probably also in monkeys (Hogg *et al.*, 1979). The other likely explanation for the natural sensitisation seen in these animals is infestation with other nematodes such as lungworms or gastrointestinal parasites which antigenically cross-react with *A. suum*. However, parasitic infestation of the gut is present in almost all sheep in New Zealand and lungworm infestation

was common in the sheep used in the present study and showed no preference for hypersensitive or non-reacting sheep.

The late airway response, an important feature of asthmatics, has not been examined in the present study because of the limitations of equipment and instrumentation. Future studies of the occurrence of late airway response in local Romney sheep using a fibre-optic bronchoscope should be carried out in order to make full use of this model since overseas studies have shown that a high proportion of sheep with acute airway response also develop late response 6-8 hours later (Abraham *et al.*, 1983; Okayama *et al.*, 1989).

Although the studies of respiratory tract-associated lymphoid tissue in healthy sheep (Chapters 4-6) are not directly relevant to the studies of the asthma model in sheep, knowledge of immune-associated tissue components in the respiratory tract of normal sheep is a prerequisite for studies of the morphological and inflammatory mechanisms involved in the development of allergic airway hypersensitivity. The results have shown that the organisation of lymphoid tissue in the ovine respiratory tract is generally similar to that in other mammalian species including man (Bienenstock *et al.*, 1973a; Anderson *et al.*, 1986; Mair *et al.*, 1987), with the exception that dense and nodular lymphoid aggregation is relatively sparse in sheep and confined to the pharyngeal tonsil and bronchioles. The morphologically well-developed lymphoepithelium (M cells), a possible pathway for antigen uptake, is present mainly in the pharyngeal tonsil, and is absent in the lower respiratory tract of healthy sheep. The ultrastructure and morphological development of the lymphoepithelium in the pharyngeal tonsil have not been studied in detail in either animals or man. Nevertheless, the M cell in the ovine pharyngeal tonsil is ultrastructurally and functionally similar to that in other mucosal tissues of most animal species studied to date, including man, but its development in the sheep is earlier than the bronchus-associated lymphoid tissue of almost all mammalian species (Bienenstock *et al.*, 1973a; Olah and Everett, 1975; Racz *et al.*, 1977; Owen and Nemanic, 1978; Gregson *et al.*, 1979b; Howie, 1980; Plesch *et al.*, 1983; Anderson *et al.*, 1986).

Some allergic gut disorders are thought to be initiated by antigen uptake via the gastrointestinal follicle-associated lymphoepithelium, leading to sensitisation and a subsequent immune or inflammatory response that is damaging to the host (Wolf and Bye, 1984). It can therefore be hypothesised that the lymphoepithelium in the respiratory tract may have a similar role in bronchial sensitisation. However, this hypothesis has not been supported by the current investigation, since well-developed lymphoepithelium was absent from the ovine lower respiratory tract and the sheep developed an airway response to *Ascaris* antigen delivered by an endotracheal route which bypassed the main site of ovine respiratory lymphoepithelium.

The second aim of the present study was to use the sheep model established to determine, by means of morphological and morphometric approaches, whether or not morphological and inflammatory factors presented in the ovine airways are associated with a predisposition to develop allergic airway hypersensitivity to inhaled *A. suum* antigen. This investigation was undertaken because there is little information available on the fundamental differences in airway structure and inflammatory components between allergic and normal subjects. Most previous morphological studies have been conducted in subjects with asthma of varying severity or in animal models following antigen challenge (Dunnill *et al.*, 1969; Maurer *et al.*, 1981; Laitinen *et al.*, 1985; Leborgy *et al.*, 1987; Holloway *et al.*, 1990), and the data obtained are likely to be the result of the allergic reaction rather than a contributing cause.

Comparison of the morphological features commonly presented in asthma in the three groups of sheep which showed varying airway hypersensitivity revealed that the epithelium of small airways was significantly thinner and contained fewer goblet cells in hypersensitive sheep (Groups A and B) than in non-reacting sheep (Group C). Morphometric studies on changes of airway epithelial thickness and density of goblet cells in asthmatics and animal models are limited. Although the epithelium in asthmatic airways is generally considered to be thickened due to epithelial hyperplasia (Dunnill, 1982), a thinner epithelium has been demonstrated recently in a patient with mild asthma (Jeffery *et al.*, 1989) and previously in dogs with non-specific airway hyperresponsiveness (Yanta *et al.*, 1981). Interestingly, the tracheal epithelium of guinea pigs exposed to ozone is also thinner and has fewer goblet cells than that of control animals (Murlas and Roum, 1985). Studies of the permeability of tracheobronchial epithelium in sheep (Chapter 9) indicated that the small numbers of cells showing an intense reaction to horseradish peroxidase were mainly degenerative goblet cells. Thus, a decrease of airway epithelial thickness and goblet cell numbers may represent an early stage of epithelial damage and may be responsible for the development of airway hypersensitivity in affected animals since antigens are likely to pass more easily through the thinner epithelium to contact underlying mast cells and smooth muscle. Further functional studies of mucosal permeability to antigen-sized markers in this region will be necessary before a definitive conclusion can be made with regard to this phenomenon.

The lack of significant differences between hypersensitive and non-reacting sheep in the epithelial integrity of airways assessed by both morphometric and ultrastructural approaches (Chapters 9 and 10) contrasts with most previous morphological studies in patients with mild or severe asthma (Cutz *et al.*, 1978; Dunnill *et al.*, 1982; Laitinen *et al.*, 1985; Beasley *et al.*, 1989; Jeffery *et al.*, 1989). However, most patients with mild asthma in which marked epithelial damage is present require medication to some extent (Laitinen *et al.*, 1985; Beasley *et al.*, 1989; Jeffery *et al.*, 1989). In bronchial biopsies from mild asthmatics not requiring medication

examined by Lozewicz *et al.* (1988,1990) the epithelial damage was not confirmed quantitatively or ultrastructurally. Some of the ultrastructural findings described in mild asthmatics as the evidence of epithelial damage (Laitinen *et al.*, 1985) are now believed to be artifacts occurring during the biopsy procedure and fixation process since these lesions are also present in some control subjects (Jeffery *et al.*, 1989; Lozewicz *et al.*, 1990). The morphological integrity of airway epithelium seen in the hypersensitive sheep and in mild asthmatics is supported by functional studies of epithelial permeability in patients with stable asthma or non-specific airway hyperresponsiveness (Elwood *et al.*, 1983; O'Byrne *et al.*, 1984a), which have shown that the airway permeability in these patients is not significantly greater than normal. In addition, the permeability studies of hypersensitive sheep have shown no significant difference in the presence of horseradish peroxidase tracer in the intercellular spaces and epithelial cells (Chapter 9). Based on the current findings and those of earlier studies, it may be concluded that the epithelial damage observed in severe asthmatics is likely to be the result of the inflammatory process of the disease itself, and is unlikely to be a contributor to the initial development of allergic airway hypersensitivity.

The hypothesis of Moreno *et al.* (1986) that a small increase in airway wall thickness can cause a marked increase in airway responsiveness and airway resistance has been recently confirmed by James *et al.* (1989) in patients with severe asthma or patients who died in status asthmaticus. However, morphometric analysis of airway dimensions in both "constricted" and "relaxed" states (Chapter 11) indicated that the airway wall was not significantly thicker in hypersensitive sheep, and that the shortening of smooth muscle required to cause complete airway closure was similar in all three groups of sheep. This finding is in keeping with the results of the physiological studies on these animals (Chapter 8), in which there was no significant difference observed in the baseline values of pulmonary mechanics between hypersensitive and non-reacting sheep.

Studies of airway inflammatory cells in the present study have focused on mast cells and eosinophils since these cells are likely to be more important in the initiation of allergic airway response than other inflammatory cells (Kaliner, 1985; Borish, 1987; Flint, 1987; Bruijnzeel, 1989; Fukuda and Gleich, 1989). Mast cells are commonly present in the ovine lower respiratory tract (Chapter 7), and are morphologically and histochemically similar to those in the human respiratory tract (Brinkman, 1968; Fox *et al.*, 1981; Warton *et al.*, 1986a; Shanahan *et al.*, 1987). Mast cells from both species are sensitive to formalin fixation, stain exclusively with alcian blue, and contain a similar number and size of secretory granules. However, the secretory granules of human mast cells are ultrastructurally more complicated than those of sheep (Warton *et al.*, 1986a). These findings may provide an opportunity to extend further the use of the sheep model in the study of human allergic airway disease.

The major finding which emerged from the quantitative studies of inflammatory components in the airways was that the volume density of secretory granules in mast cells from hypersensitive sheep was significantly higher than that from non-reacting sheep. Previous studies of mast cells in asthmatics and animal models have been confined to their numerical density since studies of volume density of secretory granules require both ultrastructural examination and morphometric analysis, which are time-consuming and expensive. The only study of volume density of mast cell granules to date was carried out in patients with symptomatic asthma, and showed no significant differences between asthmatics and control subjects (Warton *et al.*, 1986a). The data from such a study may not however, reflect the baseline values of mast cells in asthmatics since the degranulation of mast cells in asthmatics was more severe than that in control subjects. Although it is premature to conclude that the high volume density of mast cell granules seen in the present study is one of the major factors responsible for the natural occurrence of allergic airway hypersensitivity in the sheep, it is very likely that mast cells in hypersensitive sheep may contain and release more bronchoconstricting mediators after antigen stimulation than those with a lower volume density.

The lack of statistical differences between hypersensitive and non-reacting sheep in the numerical density of different types of inflammatory cells in this study is not surprising for two reasons. Firstly, in mild asthma, the numbers of most types of inflammatory cell in airways are not significantly different from normal (Lozewicz *et al.*, 1988; Beasley *et al.*, 1989; Jeffery *et al.*, 1989), while in patients with severe asthma and in animal models challenged with antigens, the numerical density of inflammatory cells in the airways, peripheral blood and bronchoalveolar fluid varies considerably between individuals (Chapters 1 and 2). There is also considerable discrepancy between bronchial biopsy and bronchoalveolar fluid cell numbers from the same patients (Jeffery *et al.*, 1989). Jeffery *et al.* (1989) recently proposed that it is not the number of inflammatory cells but the cell type that is important in determining the development of airway hyperresponsiveness in individuals. Secondly, according to the calculation cited by Agius *et al.* (1986), an immediate 2-3 fold increase in plasma histamine level in asthmatics after antigen challenge is equivalent only to total degranulation of 2.5% of all lung mast cells, or 21% of those cells superficial to the bronchial basement membrane. If this calculation is also true for other inflammatory cells, there should be enough inflammatory cells already present in the airway mucosa of sheep to initiate an allergic airway constriction.

Although the differences in most structural and inflammatory parameters examined in the present study were not significant between hypersensitive and non-reacting sheep, these results did not excluded the possibility that there are functional differences which are not severe enough to cause morphological changes detectable by the methods used in this study.

Indeed, the response of mast cells to specific and non-specific antigen stimuli is greater in patients with allergic rhinitis and with asthma than in non-atopic subjects. This difference has been demonstrated both functionally and morphologically (Warton *et al.*, 1986a; Casale *et al.*, 1987; Flint, 1987; Bleecker *et al.*, 1988; Wanner *et al.*, 1990), but has not been supported by studies in animal models (Sommerhoff *et al.*, 1989).

The morphological and morphometric studies presented here indicate that most differences between hypersensitive and non-reacting sheep in airway structure and inflammatory components are confined to the small airways. The contribution of these differences to airway response to inhaled allergens is not clear since studies in both man and animals indicate that the increase in airway resistance in disease is mainly due to the constriction of large airways (Snapper, 1986; Du *et al.*, 1989). However, inflammatory reactions in the small airways have been demonstrated as the anatomical abnormalities largely responsible for the increase of airway resistance in both smokers (Hogg *et al.*, 1968; Cosio *et al.*, 1977) and asthmatics (Wagner *et al.*, 1988; James *et al.*, 1989). Furthermore, experimental studies in sheep indicate that histamine-induced airway response is mainly limited to small airways (Hutchison *et al.*, 1982), and the physiological studies (Chapter 8) have identified a subgroup of hypersensitive animals (Group B) in which the airway response is predominantly in the small airways.

One of the initial methodological concerns in the present study was that prolonged intubation under anaesthesia may alter the degree of the epithelial integrity and cause airway inflammation. To minimise the effect of tracheal intubation as well as any changes induced by antigen challenge, the morphological and morphometric studies of airways in sheep were carried out 14 days after the bronchial provocation tests. The results have shown that such an effect was minimal at the time the animals were killed since overall the inflammatory changes and epithelial damage in airways were mild. In monkeys, experimental airway epithelial damage produced by intubation with an oversized endotracheal tube repairs almost completely at 6 days post-intubation (Way and Sooy, 1965).

There were two major shortcomings in the present study. The first was that the physiological studies of airway response to inhalation challenge were conducted under anaesthesia, which may have altered the respiratory response of these animals. Future studies of this type in sheep should therefore be conducted in conscious animals with the aid of a fibre-optic bronchoscope. The second shortcoming was that tissues used for morphometric and semi-quantitative studies were fixed by immersion rather than airway inflation because of the requirement for multiple samples for different purposes. Since all sheep underwent similar experimental conditions, and any alterations caused by tissue fixation would have been similar in all groups, and the results of analysis of differences should be valid.

In conclusion, the work presented here has demonstrated that Romney sheep can be used to study human asthma and other allergic diseases in New Zealand. The establishment of this model will enable the conduct of experiments which are difficult or not possible to undertake in man. The use of the sheep model has confirmed that it has several advantages over other animal models (Chapter 3). Results from the current study have successfully demonstrated an association between inherent airway hypersensitivity, and relatively low goblet cell density, thinner than normal small airway epithelium, and high volume density of secretory granules in lung mast cells. The results question the importance of epithelial disruption in the initial development of airway hypersensitivity in sheep and confirm that most of the pathological changes described in both asthmatics and animal models are the consequences of the allergic reaction process rather than a fundamental difference between hypersensitive and normal individuals.

PART VI
REFERENCES AND APPENDICES

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APPENDIX 8.1

80

NEW ZEALAND VETERINARY JOURNAL

1990

CORRESPONDENCE

Experimental *Ascaris*-induced airway hypersensitivity in sheep: no serological evidence of association with *Toxocara canis* infection

New Zealand Veterinary Journal 38, 80, 1990

The finding that 70% of sheep from the Manawatu district were hypersensitive to challenge by inhalation of *Ascaris suum* antigen, regardless of their previous history of exposure to pigs⁽¹⁾, has raised the possibility that these sheep had been sensitised by exposure to the common dog nematode *Toxocara canis*.

In order to test this hypothesis, 22 sera, 8 from strong responders (Group A), 6 from weak responders (Group B) and 8 from non-responders (Group C), were tested for *T. canis* antibody. A standardised enzyme-linked immunosorbent assay (ELISA) was used for detecting and quantitating serum antibodies directed against excretory and secretory antigen of *T. canis*⁽²⁾. The excretory and secretory antigen was characterised using a Western blot analysis⁽³⁾.

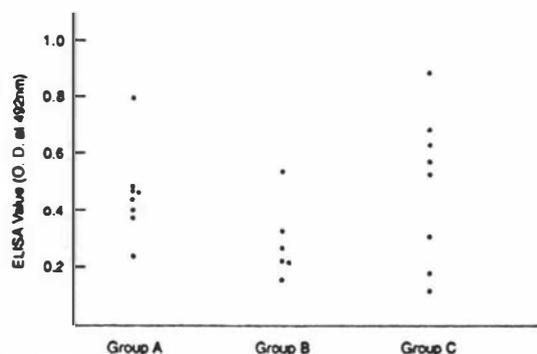


Figure 1. ELISA absorbance readings to *Toxocara canis* in 22 sheep. O.D. = optical density. Group A were strong respiratory responders (n = 8), Group B were weak respiratory responders (n = 6) and Group C were non-responders (n = 8).

The results shown in the figure indicate that there was no significant difference in the ELISA titre to *T. canis* between the three groups of sheep tested ($p > 0.05$). A positive control ovine serum gave a reading of 0.70 optical density at 492 nm. In addition, no correlation was found between the ELISA titres and the severity of increase in airway resistance ($r = 0.14$, $p > 0.05$) or dynamic lung compliance ($r = 0.16$, $p > 0.05$).

From these results it can be concluded that exposure to the common nematode *T. canis* is unlikely to be a causative factor in the development of *Ascaris*-induced airway hypersensitivity of sheep in New Zealand. Further work is therefore needed to explain the natural occurrence of hypersensitivity found in some sheep in this country.

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APPENDIX 9.1

PREPARATION OF DIAMINOBENZIDINE TETRAHYDROCHLORIDE (DAB) SUBSTRATE SOLUTION

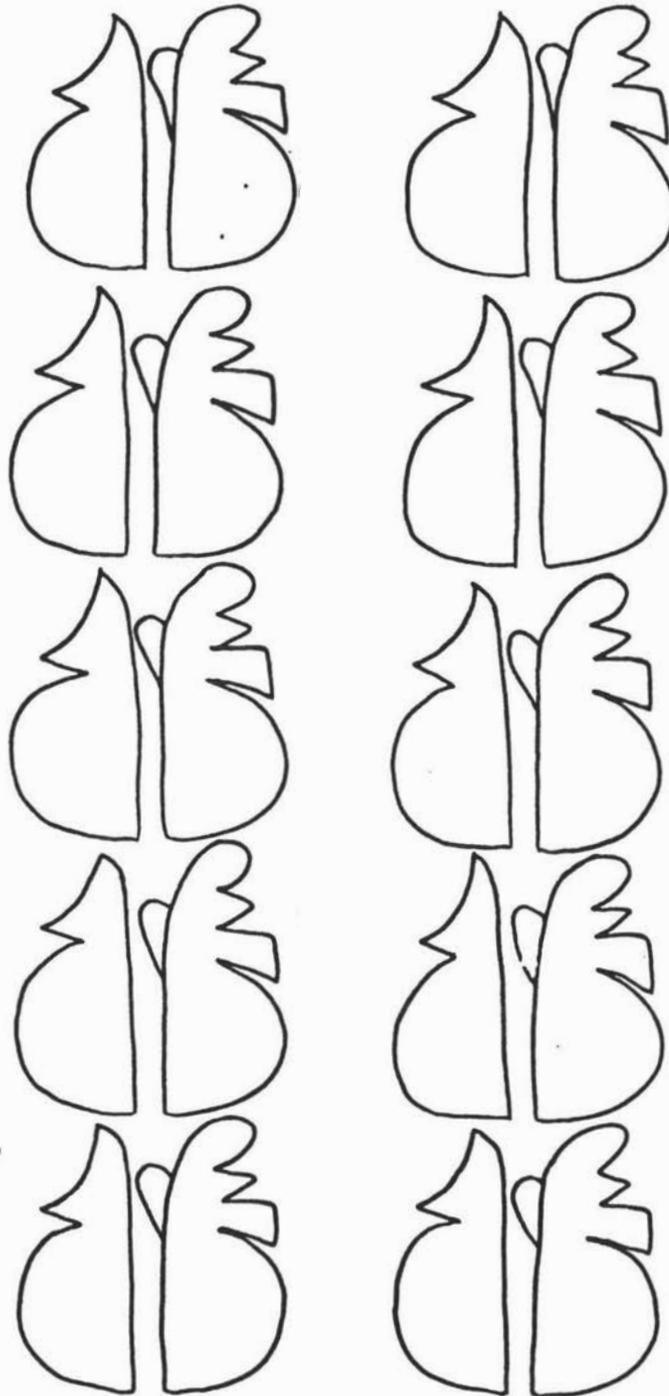
(Based on Bourne, 1983)

This solution should be prepared immediately before use. Care should be taken to avoid inhalation of the powder or contact with the skin.

1. Dissolve 6 mg of 3,3 diaminobenzidine tetrahydrochloride in 10 ml of 0.05 M Tris buffer, pH 7.6.
2. Add 0.1 ml of 3% hydrogen peroxide.
3. Place 3 ml onto each specimen bottle and incubate 30 minutes at room temperature.
4. Rinse off with water.

APPENDIX 10.1

PRE-DESIGNED DIAGRAM FOR THE RECORD OF GROSS LUNG LESION



APPENDIX 12.1**STAINING METHOD FOR EOSINOPHILS**

(Based on Ball and Hay, 1990)

1. Preparation of naphthalene black solution

Naphthalene black 12B (CI 20470)	0.5 g
Distilled water	99.5 ml
Glacial acetic acid	5 drops
Filtered	

2. Preparation of Lendrum's chromotrope solution

Chromotrope 2R (CI 16570)	0.5 g
Phenol	1.0 g
Distilled water	98.5 ml

3. Staining procedure

- (1). Dewax and take to water;
- (2). Stain with 0.5% acidified aqueous naphthalene black 12B for 30 minutes;
- (3). Wash in tap water;
- (4). Differentiate in 0.5% aqueous lithium carbonate solution for 30 seconds to 2 minutes;
- (5). Counterstain with 0.5% Lendrum's chromotrope solution for 2 minutes;
- (6). Dehydrate in grades of alcohol, clear in xylene and mount with D.P.X. mountant (BDH).