AIRWAY HYPERSENSITIVITY AND REMODELLING INDUCED BY REPEATED EXPOSURE TO *ASCARIS SUUM* ANTIGEN: AN OVINE MODEL OF HUMAN ASTHMA

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This study is dedicated with love to my mother.

ABSTRACT

This study was an attempt to develop a model of asthma which shows all the structural changes of airways that occur in human disease by repeatedly exposing sheep to an aerosol of *Ascaris suum* antigen.

Twenty two sheep were tested for cutaneous reactivity to a commercial preparation of the antigen. For each of three experiments three sheep were selected, two skin reactive and one non-reactive. One week prior to the experiment a tracheostomy was performed according to the method described by Dueck *et al.*, (1985). In each group, the respiratory response of the two reactive sheep to the *Ascaris* antigen was augmented using 2-3 fortnightly respiratory exposures to the antigen (82,000 protein nitrogen units /ml) for twenty minutes delivered via an endotracheal tube passed through the tracheostomy. One of the reactive sheep (experimental) was then further exposed to antigen for twenty minutes daily for two weeks. The other reactive sheep (sensitized control) and the non-reactive sheep (non-sensitized control) were exposed to the saline vehicle alone daily for two weeks. Airway resistance (Raw) and dynamic lung compliance (Cdyn) were measured before the antigen/saline exposures and at five minutes intervals during the exposure. On the last day the experiment was carried out under general anesthesia. In addition to respiratory measurements, cardiac output, pulmonary arterial pressure, pulmonary wedge pressure, systemic arterial pressure and central venous pressure were obtained to calculate cardiac power output. At the end of the last exposure sheep were killed, necropsied and samples of lung and airway fixed for morphological studies.

Sixty four percent of the sheep tested showed an immediate skin reaction to the antigen. The antigen exposure caused significant changes in respiratory parameters and increased the cardiac work load of the right side of the heart in the experimental sheep. Morphological studies revealed that antigen exposure caused an increase in number of

eosinophils and goblet cells in the airways and an increase in the thickness of the 'pseudo-basement membrane' at some levels of the respiratory tract. Antigen exposure also caused an increase in the percentage smooth muscle area in the airway wall cross-sectional area in the membranous bronchioles.

Based on these observations it can be concluded that repeated daily exposure of sheep airway to *Ascaris suum* antigen can be used to reproduced morphological changes observed in human asthmatic airways.

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LIST OF ABBREVIATIONS

Ae	external area
Ai	internal area
Asm	airway smooth muscle
ASM%	percentage smooth muscle in airway wall cross-sectional area
Aw	airway wall
BAL	broncho-alveolar lavage
Cdyn	dynamic lung compliance
CO	cardiac ouput
CVP	central venous pressure
F	respiratory flow
NAHR	non-specific airway hyperresponsiveness
LT	leukotrienes
PAF	platelet activating factor
Pap	pulmonary arterial pressure
PBS	phophate buffered saline
PBM	pseudo basement membrane
Pe	external perimetre
Pi	internal perimetre
P_{IO}	intra oesophageal pressure
Pwp	pulmonary wedge pressure
Raw	airway resistance
SABP	systemic arterial blood pressure
V_T	tidal volume

CHAPTER 1

INTRODUCTION

Bronchial asthma is a complex multifactorial disease characterised by airway inflammation, increased responsiveness of the airways to various stimuli and manifested by widespread episodic airway narrowing that is reversible either spontaneously or in response to treatment (Expert Panel Report, National Asthma Education program, 1991).

Asthma affects more than 5% of the population in some industrialised countries (Barnes, 1989; Aalbers *et al.*, 1993) and the incidence of asthma has risen over the past 20 or 30 years (Beasley *et al.*, 1997; Woolcock and Peat, 1997).

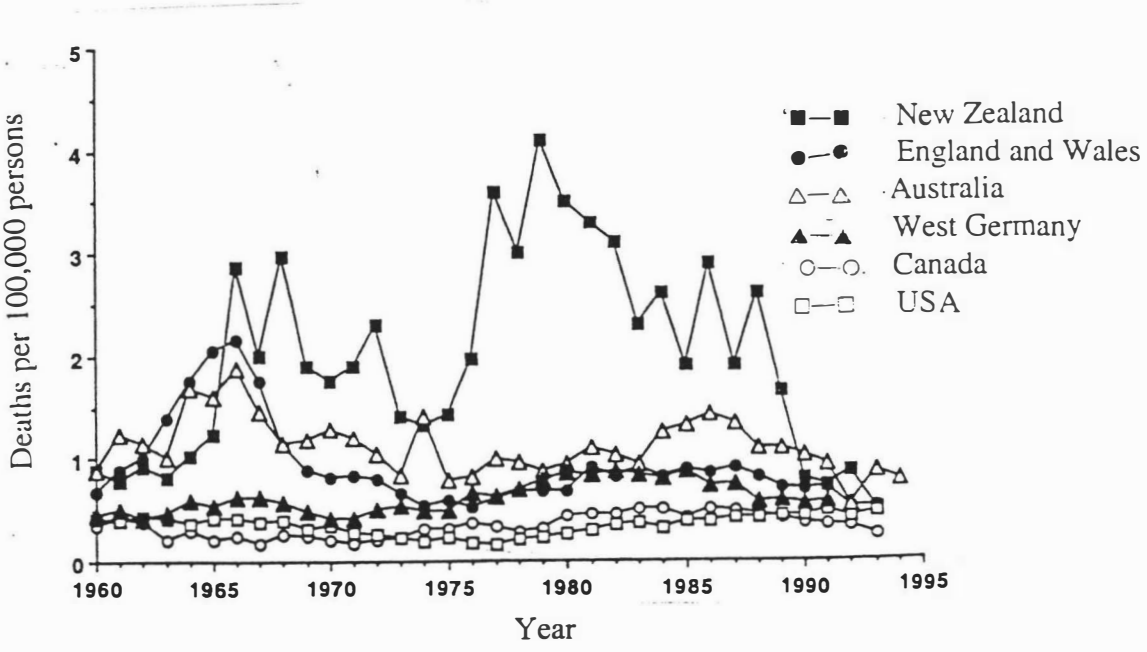


Fig. 1.1 International trends in asthma mortality (deaths per 100,000 persons) in the age group of 5-34 years during the period of 1960-1994. (Reproduced from Sears and Taylor, 1994)

Considering the prevalence, severity (Figure 1.1) and the economic cost of the disease, it has been suggested that it is important to understand the clinical importance of each

pathological change and to identify the early pathogenesis of the condition (Smith, 1989; Boushey and Fahy, 1995).

The pathological picture of asthma consists of epithelial damage, airway lumen occlusion, thickening of the sub-basement membrane connective tissues, airway smooth muscle hypertrophy/hyperplasia, mucous gland hyperplasia and infiltration of the mucosa with inflammatory cells (Dunnill, 1960; Hogg, 1984; Laitinen and Laitinen, 1994; Cho *et al.,* 1996). These changes in asthma indicate long-standing persistent airway inflammation and structural changes (Saetta *et al.*, 1989). Some of these changes could be associated with thickening of airway wall and may contribute to the excessive airway narrowing observed in an acute asthmatic episode (Saetta *et al.*, 1991; Lambert *et al.*, 1993; Carter *et al.,* 1997). It has been hypothesised that patients with long-standing disease and altered airway structure are more likely to die from asthma (Carrol *et al.,* 1996). Although the mechanisms underlying such deaths are not clear, the thickening of airway walls and amplification of airway narrowing resulting from these structural changes could be contributory factors (Carrol *et al.,* 1996).

Morphological studies in humans are restricted because of difficulties in obtaining asthmatic airways for study. Most investigations involve either studies of bronchial biopsies (Jeffery *et al.*, 1989; Saetta *et al.,* 1989) or post-mortem studies of patients who have died in the *status asthmaticus* (Dunnill *et al.,* 1969; Cutz *et al.*, 1978; Saetta *et al.,* 1989; Djukanovic *et al.,* 1990). Studies using fibre-optic bronchoscopy are limited only to the larger airways and results from such studies may be compromised by factors such as therapeutic interventions (Jeffery *et al.*, 1989). Morphological studies of tissues from

patients that died in *status asthmaticus* have the disadvantage that many of the features observed are likely to be the end result of the fatal disease (Saetta *et al.*, 1989). In post-mortem studies, it is not clear to what extent the findings reflect the varied clinical picture of the developing condition. The pathological picture seen in *status asthmaticus* could be quite different from the pathological features present between and during non-fatal asthmatic episodes (Saetta *et al.,* 1989; Black and Johnson, 1996). From these post-mortem studies it is not possible to declare which features are associated with the early pathogenesis of the disease. Therefore, experimental asthma in animal models has a vital role to play in the attempt to understand the pathogenesis of the condition.

Animal models that have been developed so far have reproduced some but not all of the features of the disease (Saetta *et al.,* 1989). Most of the studies using animal models either consider the physiological and pharmacological mechanisms of increased airway reactivity (Wanner and Reinhart, 1978; Hirshman *et al.*, 1980, Abraham *et al.*, 1981, Gold. 1986, Johnson and Stout, 1989, Buijs *et al.*, 1995, Abraham *et al.,* 1997) or mediators of acute and late responses in the disease (Long *et al.*, 1990; Abraham *et al.,* 1983; Hamel *et al.*, 1986; Abraham *et al.,* 1997).

The aim of this study was to develop an ovine model of asthma which shows all the structural changes that occur in human asthmatic airways including the thickening of sub-basement membrane connective tissue.

CHAPTER 2

PATHOLOGY OF ASTHMA

2.1 INTRODUCTION

Earlier pathological descriptions of asthma were mainly based on autopsy studies of patients who died of asthma and asthmatics that died from other causes (Huber and Koessler, 1922; Dunnill. 1960; Dunnill *et al.*, 1969; Cutz *et al.*, 1978). Over recent years, the use of fibre-optic bronchoscopy to obtain broncho-alveolar lavage and bronchial biopsies has enabled pathologists to gain insight into the changes *in situ*. However, there is a lack of precise knowledge of the pathology of moderately severe disease (Kay, 1996). It is difficult to describe the pathology of asthma because of lack of precise definition of the disorder and poor clinical documentation of some studies of asthma deaths as well as the coexistence of post-mortem changes. Nevertheless, recognition of pathological changes is important for anatomical diagnosis of asthma and to differentiate it from other allergic conditions of the respiratory system.

2.2 GROSS PATHOLOGICAL CHANGES

The lungs of an asthmatic patient at necropsy have a characteristic appearance. On opening the thoracic cage, the lungs appear acutely distended, over-aerated and pale in colour (Huber and Koessler, 1922; Dunnill, 1960; Tatersfield and McNicol, 1987; Saetta *et al.*, 1989; Hogg, 1997; Jeffery, 1998). Although there is no emphysema (Dunnill, 1960; Spencer, 1977; Saetta *et al.,* 1989), focal areas of collapse are frequently seen in both lungs along the anterior margin and the apices of the lobes (Dunnill, 1960, 1971). These areas of collapse appear as firm, dark airless areas of depression below the surface of the lung (Dunnill, 1960). On incision, the lumen of many airways is densely packed with grey glistening exudate (Dunnill, 1960; Dunnill *et al.*, 1969; Spencer, 1977).

2.3 CHANGES IN AIRWAYS

The occlusion of the airway lumen by exudate was a consistent finding in severe asthmatics and the sputum consisted of a mixture of sloughed epithelial clumps (Creola bodies), cilia, remnants of eosinophils (Charcot-Leiden crystals), mucus casts (Churchman's spirals) and other inflammatory cells and their granules (Dunnill, 1960; Hogg, 1984; Saetta *et al.*, 1989; Jeffrey, 1992; Laitinen and Laitinen, 1994).

Changes in the bronchial mucosa vary with the severity and duration of the condition (Cutz *et al.*, 1978; Jeffery, 1991, 1992; Cho *et al.*, 1996). In severe asthmatics, the airway mucosa showed a marked oedema and shedding of airway columnar epithelial cells, leaving behind a layer of reserve or basal cells on the basal lamina (Dunnill, 1960; Dunnill *et al.*, 1969; Saetta *et al.,* 1989; Jeffery, 1991, 1992; Laitinen and Laitinen, 1994, 1995). In the areas with an intact epithelium, the ciliated epithelium was usually replaced by goblet cells and squamous cells (Casale and Marom, 1983; Saetta *et al.*, 1989; Jeffery, 1991). Goblet cell hyperplasia may reflect a reaction to irritation or a cell maturation defect (Laitinen and Laitinen, 1995).

In asthmatics, the airway wall has areas of focal regeneration and the mitotic activity in the deeper cells in these areas is increased (Dunnill, 1960; Saetta *et al.*, 1989). The loss of epithelial cells may be a result of inflammation. The transudation of oedema fluid across the mucous membrane and increased mucus secretion may result in the detachment of ciliated cells (Dunnill, 1960). It could also be due to damage caused by the release of highly toxic and charged eosinophil granular proteins including major basic protein, eosinophilic cationic protein and eosinophil peroxidase (Jeffery, 1992; Montefort *et al.*,

1992; Cho *et al.,* 1996). In some asthmatics, epithelial columnar cells were still attached to each other at the luminal surface, but separated from the basal cells in the middle by homogenous fluid-like material (Laitinen and Laitinen, 1994). This may be related to an ongoing shedding process caused by oedema (Laitinen and Laitinen, 1994). There were vascular endothelial gaps suggesting plasma exudation (Laitinen and Laitinen, 1994, 1995). Increased epithelial fragility and weaker cellular attachment may reflect a disturbed cellular adhesion mechanism (Montefort *et al.,* 1992; Laitinen and Laitinen, 1994).

The disruption of the epithelium appeared to occur at the suprabasal-basal cell layer junction probably as a result of weaker cell adhesion (Montefort *et al.,* 1992). Destruction of intercellular tight junctions and epithelial damage seen in asthma may make it easier for irritant substances to cross the epithelial barrier thereby exposed sensory nerve endings may be stimulated by a range of stimuli causing reflex bronchospasm (Cho *et al.*, 1996).

Although not pathognomonic to asthma, changes in the epithelial basement membrane are salient features of the disease (Dunnill, 1960; Jeffery, 1992). Earlier light microscopic studies frequently refer to a thickening and hyalinisation of epithelial basement membrane (Dunnill, 1960; Dunnill *et al.,* 1969; Cutz *et al.*, 1978). The application of transmission electron microscopy to study bronchial tissues and the use of monoclonal antibodies to differentiate collagen sub-types have revealed that the 'true basement membrane' in asthma is of normal thickness (Cutz *et al.*, 1978; Roche *et al.*, 1989; Johnston and Holgate, 1991; Jeffery *et al.*, 1992; Cho *et al.,* 1996). It is the reticular lamina, the connective tissue layer just beneath the basement membrane (Hernandez and Amenta, 1983) and not the basement membrane which becomes thickened in atopic asthma (Cutz *et al.*, 1978; Jeffery

et al., 1989; Roche *et al.*, 1989; Johnston and Holgate, 1991; Laitinen and Laitinen, 1994, 1995; Wilson and Li, 1997) and this occurs even in mild stable disease (Cutz *et al.*, 1978).

Electron microscopy and immuno-histochemical studies have confirmed that the ultra-structure of the thickened 'pseudo-basement membrane' has collagen as its major component (Jeffery *et al.,* 1989; Roche *et al.*, 1989; Laitinen and Laitinen, 1995). What constitutes the stimulus for this laying down of collagen is uncertain. Formation of collagen is secondary to inflammatory and degenerative process as seen in sites of repair of damaged tissues (Jeffery *et al.,* 1989). However, this thickening of collagen is ultra-structurally different from the underlying interstitial collagen of a scar (Jeffery, 1998). Since the lamina reticularis is not seen in the foetal airway (Jeffery, 1990), its thickening with age could be a result of the normal ageing process and the increased thickening seen in asthma may be considered as an accelerated ageing process due to chronic inflammation (Jeffery, 1992). Oliveri and Foresi (1992) have observed a significantly higher number of inflammatory cells in the submucosa of asthmatics with 'thickened basement membrane' and suggested a correlation between inflammation of the submucosa and the degree of 'pseudo-basement membrane' thickening.

The term 'sub-epithelial fibrosis' has been used to describe this thickening of reticular lamina by Roche *et al.* (1989). These authors could not find any relation between the pseudo-basement membrane hypertrophy and the severity of epithelial damage, the duration of illness or the severity of the asthma. This suggested that increased collagen deposition might be due to increased fibroblast activity and not other factors. They also demonstrated the presence of collagen type IV, fibronectin and laminin in the 'true-

basement membrane', which did not differ from that of normal airways. By contrast, the sub-epithelial collagen was predominantly of sub-types III and V together with fibronectin but not laminin (Roche *et al.*, 1989; Roche, 1991; Laitinen and Laitinen, 1995) suggesting there was no contribution from products derived from epithelial cells to this thickening of reticular lamina (Roche *et al.*, 1989). In agreement with this phenomenon, a study by Brewster *et al.* (1990) revealed an expanded network of sub-epithelial myofibroblasts with contractile and collagen secreting properties. They observed a positive correlation between myofibroblast number and sub-epithelial collagen thickness suggesting a repair response secondary to chronic inflammation.

An increased volume of bronchial smooth muscle is a consistent finding in patients that die in *status asthmaticus* (Huber and Koessler, 1922; Dunnill *et al.*, 1969; Cutz *et al.*, 1987; Hogg, 1984; 1997; Saetta *et al.*, 1991; Carroll *et al.*, 1993; Kay, 1996; Black and Johnson, 1996; Boulet *et al.*, 1998). There is a three-fold increase in the smooth muscle volume in the asthmatic airway compared to a healthy individual (Dunnill *et al.*, 1969; Hogg, 1993). In most patients, this increase in muscle volume was seen prominently in the large bronchi (Ebina *et al.*, 1993; Vignola *et al.*, 1998). However, some patients studied had increased muscle volumes in the whole respiratory tree including bronchioles (Ebina *et al.*, 1993; Vignola *et al.*, 1998). Post-mortem studies of asthmatic patients have revealed evidence of both hyperplasia and to a lesser extent hypertrophy of the airway smooth muscle (Dunnill *et al.*, 1969; Hossain, 1973; Heard and Hossain, 1973). However, whether this increased volume is a result of smooth muscle hypertrophy or hyperplasia or both may still require further clarification (Jeffery, 1998; Boulet *et al.*, 1998).

2.4 CONCLUSION

Asthma is a clinically complex condition in which airways show classic signs of inflammation. None of the pathological changes in asthma (airway mucosal oedema, increased mucus secretion, epithelial cell sloughing, smooth muscle hypertrophy, goblet cell hyperplasia and basement membrane thickening) are pathognomonic of asthma but together they constitute its pathological picture.

Light microscopic studies frequently refer to a 'basement membrane thickening'. 'Pseudo-basement membrane thickening' is a frequent finding in asthma irrespective of age, duration and the severity of the condition (Cutz *et al.*, 1978). Morphological studies using transmission electron microscopy and monoclonal antibodies to differentiate collagen subtypes have shown that epithelial basement membrane is of normal thickness. It is the dense deposition of collagen fibrils in the reticular lamina that has mistakenly been attributed to 'basement membrane thickening' in the earlier light microscopic studies. Whether the increased presence of collagen is due to increased synthesis and secretion by epithelial cells, to reduced degradation with normal synthesis or to sub-epithelial myofibrobalst activation is not clear from the literature. Further studies are required to determine the role of this excessive collagen deposition and its role in the pathogenesis of airway obstruction.

Most of the existing knowledge of the morphological changes in asthmatic airways is based on autopsy findings. Endobronchial biopsies and broncho-alveolar lavage have enabled pathologists to gain further insight into the immunopathogenesis in asthma. However, it is unclear to what extent the often extensive post-mortem findings reflect the

varied clinical picture of living asthmatic. By endoscopy one can only study the disease process at one time point. Therefore, changes that occur over the time should be investigated by repeated studies. Whether biopsies from larger airways reflect the pathological picture throughout the bronchial tree also remains to be established. More sophisticated techniques are required to study the events in asthmatic airways *in situ.*

CHAPTER 3
ANIMAL MODELS OF ASTHMA

3.1 INTRODUCTION

Over several centuries animals have been used as models to understand normal human physiology, various disease processes and the treatment of disease. Major uses of animal models in biomedical research are to elucidate host defence mechanisms, the pathogenesis of diseases and to guide subsequent studies in humans. They are also used to screen various substances like drugs for their effectiveness and toxicity. The principal reason for using animals for biomedical research is to test hypotheses that cannot be tested in humans. Animal research has proved vital in many important discoveries in biology and medicine.

Bronchial asthma is a complex multifactorial disease characterised by airway inflammation, increased responsiveness of the airways to various stimuli and manifested by widespread episodic airway narrowing that is reversible either spontaneously or in response to treatment (Expert Panel Report, National Asthma Education Program, 1991). In developing animal models of asthma, it is necessary to establish a set criteria (Table 3.1) by which the model can be validated (Karol, 1994). An ideal animal model of asthma should exhibit responses that are not only consistent with those observed in humans but also reproducible (Turner and Watson, 1996).

Table 3.1 Parameters appropriate to study asthma in animal models (reproduced from Karol, (1994) with some modifications).

Parameter
Airway hyperresponsiveness
Sub-epithelial fibrosis or 'thickened basement membrane'
Airway smooth muscle hypertrophy
Elevated immunoglobulin E level
Pulmonary eosinophilia
Mast cell and/or eosinophil derived products in the bronchial mucosa or bronchoalveolar lavage
Microvascular leakage of protein
Decreased dynamic lung compliance

Naturally occurring respiratory conditions similar to asthma are rare in animals. Feline asthma, canine ragweed pollenosis and heaves in ponies are the naturally occurring allergic respiratory disease that have been recognised as having similarities to human allergic bronchial hyperreactivity (Snapper, 1986; Wanner *et al.*, 1990; Chen, 1990; Karol, 1994). However, heaves may be more related to human hypersensitive pneumonitis than to asthma (Smith, 1989). However, allergic bronchial hypersensitivity can be experimentally induced in animals using antigens such as *Ascaris suum* (Smith, 1989; Wanner *et al.*, 1979; Lanes *et al.*,1986; Bosse *et al.*, 1987; Chen *et al.*, 1990), *Toxocara canis* (Buijs *et al.*, 1994, 1995) and ovalbumin (Itoh *et al.*, 1996; Wills-Karp and Ewart, 1997). Several animal species have been used as models of human asthma including rodents, dogs, ponies, sheep and non-human primates (Hirshman *et al.*, 1980; Smith, 1989; Wanner *et al.*, 1990; Karol, 1994; Buijs *et al.*, 1994, 1995; Itoh *et al.*, 1996). There are inter-species differences in the neural control and the pharmacological responsiveness of the airways (Wanner and Abraham, 1982; Turner and Watson, 1996). The autonomic innervation of the airway smooth muscle and distribution of receptors for mediators of anaphylaxis are important determinants of airway hyperresponsiveness (Wanner and Abraham, 1982; Turner and Watson, 1996). The physiological, pharmacological and immunological aspects of commonly used animal models of human asthma are compared in Table 3.2.

Table 3.2 Comparison of the characteristics of human asthmatics and some animal models (re-produced from Chen (1990) with some modifications).

Characteristic	Human	Sheep	Monkey	Dog	Guinea pig
Natural skin reaction	Yes	yes	yes	Yes	No
Natural airway allergic disease similar to asthma	Yes	no	no	Some	No
Late response	Yes	yes	yes	No	Yes
Reproducibility	Consistent	consistent	inconsistent	Inconsistent	Inconsistent
Respiratory frequency	↑	variable	↑	↑	↑
Tidal volume	↓	variable	↑	↓	↓
Airway resistance	↑	↑	↑	↑	↑
Residual volume	↑	?	unchanged	Unchanged	↑ (?)
Total lung capacity	↑	?	unchanged	Unchanged	↓
Functional residual capacity	↑	↑	variable	Variable	↑
Dynamic lung compliance	↓	↓	↓	↓	↓
Arterial O_2 tension	↓	↓	↓	↓	?
Arterial CO_2 tension	↑	?	↑	↑	?
Immunoglobulins	IgE	?	IgE, IgG	IgE	IgE, IgG_1
Circulating histamine levels	↑	↑	inconsistent	Inconsistent	↑
Non-specific airway hyperresponsiveness	Present	present	variable	Variable	Absent

↑ = increased, ↓ = decreased, ? = unknown

3.2. THE COMMON ANIMAL MODELS OF ASTHMA

3.2.1 Guinea pigs

Despite the wide differences from the human, antigen-induced bronchoconstriction in the sensitised guinea pig is probably the most commonly used model for testing anti-allergic agents (Pretolani and Vargaftig, 1993; Karol, 1994). The first use of this model can be traced as far back as 1910 (Auer, 1910). Since then guinea pigs have been used extensively in the studies of the role of inflammatory mediators in the development of allergic bronchoconstriction (Desquand *et al.*, 1989; Wegner *et al.*, 1993; Itoh *et al.*, 1996), the types and the distribution of receptors involved in bronchial hyperresponsiveness (Jian-Ying and She, 1989) and the evaluation of various types of anti-asthmatic agents (Fugner,

1985; Hedman and Andersson, 1982; Dougall *et al.*, 1991; Kompa *et al.*, 1995). In addition, isolated tissues from this species such as tracheal strips, rings, lung extracts and atrial preparations have been used as *in vitro* models to study the properties of the tissues and to evaluate various drugs (Hedman and Andersson, 1982; Bryan-Lluka and O'Donnell, 1991; Dougall *et al.*, 1991; Cheng *et al.*, 1995; Kompa *et al.*, 1995).

Although allergic airway hyperresponsiveness is not naturally found in guinea pigs, it can be experimentally induced with a range of antigens such as ovalbumin (Hedman and Andersson, 1983; Wegner *et al.*, 1993), *Toxocara canis* (Buijs *et al.*, 1995) and *Ascaris suum* (Kallos and Kallos, 1984; Fugner, 1985; Karol, 1994) by inhalation or subcutaneous/intraperitoneal injection. Use of small doses of antigens mixed with adjuvants such as aluminium hydroxide and *Bordetella pertussis* have been shown to favour the production of IgE type antibodies which is the main type of immunoglobulin found in human asthmatics (Milne *et al.*,1996). The occurrence of airway response in guinea pigs is antigen specific and depends on the previous exposure to the antigen (Kallos and Kallos, 1984). The guinea pig model develops both acute and late airway response (Itoh *et al.*, 1996; Milne *et al.*, 1996). The duration of retention of the induced hypersensitivity depends on the method of sensitisation and the frequency of antigen exposure (Patterson and Kelly, 1974; Kallos and Kallos, 1984; Pretolani and Vargaftig, 1993). The anaphylactic bronchoconstriction is mainly mediated by histamine in the guinea pig. It can be suppressed by small doses of antihistamine. These have no major effects in human asthma (Kallos and Kallos, 1984; Pretolani and Vargaftig, 1993).

The guinea pig model of asthma also exhibits some clinical signs and characteristics of human asthma such as cough and respiratory distress (Kallos and Kallos, 1984). Airway hypersensitivity in this species can be achieved *in utero* (Kallos and Kallos, 1984). In

addition, this model also shows some of the pathological changes similar to those found in humans that died in *status asthmaticus* (Pagel, 1939).

3.2.2 Dogs

The dog represents a model in which a clinical syndrome similar to asthma occurs naturally in pollen-sensitive individuals (Patterson and Kelly, 1974; Snapper, 1986) and animals sensitive to *Ascaris* antigen (Karol 1994). In both cases, the condition is IgE mediated (Patterson and Kelly, 1974). Pollen-sensitive dogs show clinical signs normally associated with asthma such as coughing, dyspnoea and excessive mucus production (Patterson and Kelly 1974). Use of this model has provided useful information on involvement of neurogenic mechanisms in the development of allergic bronchoconstriction (Patterson and Kelly, 1974; Snapper, 1986; Smith, 1989; Karol, 1994). Some dogs including mongrels (Booth *et al.*, 1970) and offspring of Basenji-Greyhound have been found to be extraordinarily sensitive to *Ascaris suum* antigen (Smith, 1989; Wanner *et al.*, 1990; Karol, 1994). Many of the mongrels tested developed an acute airway response to the initial aerosol challenge with the homologous antigen (Hirhsman *et al.*, 1980; Smith, 1989; Wanner *et al.*, 1990) and the response in remaining dogs was induced by repeated exposure to the *Ascaris* antigen (Booth *et al.*, 1970; Patterson and Kelly, 1974; Hirhsman *et al.*, 1980; Smith, 1989). Continuous cross-breeding of Basenji-Greyhound off-spring that are sensitive to inhaled *Ascaris suum* antigen has resulted in a line of Basenji-Greyhounds with airway hyperresponsiveness (Booth *et al.*, 1970; Wanner *et al.*, 1990). Basenji-Greyhounds have developed persistent bronchial hyperreactivity (Booth *et al.*, 1970; Hirhsman *et al.*, 1980; Smith, 1989, Wanner *et al.*, 1990; Karol, 1994). This breed of dogs was more sensitive to methacholine than mongrels (Smith, 1989; Karol, 1994). In

contrast to human asthmatics, in dogs there was an increased infiltration of neutrophils into the lungs rather than eosinophils (Smith, 1989).

3.2.3. Monkeys

Being naturally sensitive to *Ascaris* antigen, Rhesus and Cynomolgus monkeys also provide a model of acute allergic airway response (Pritchard *et al.,* 1983; Karol, 1994; Turner and Watson, 1996). The sensitivity to *Ascaris* antigen can be retained by these animals for several years (Patterson and Kelly, 1974; Turner and Watson, 1996). Some monkeys with a natural skin reaction to *Ascaris* antigen showed an immediate and late phase airway response (Smith, 1989; Karol, 1994; Turner and Watson, 1996). The late response was dose dependent and severe in some monkeys (Hamel *et al.,* 1986). Although all the mast cell derived mediators of human asthma have not yet been identified in the monkey model, mediators associated with bronchoconstriction (leukotriene C_4, leukotriene D_4, prostaglandin D_2 and histamine), chemotaxis (leukotriene B_4) and permeability changes (leukotriene D_4) have also been observed in monkeys (Turner and Watson, 1996). One disadvantage of this model is the limited availability of naturally sensitive animals (Paterson and Kelly, 1974). However, laboratory-bred monkeys can be experimentally sensitised with *Ascaris* antigen (Pritchard *et al.,* 1983; Johnson and Stout, 1989; Turner and Watson, 1996). This model has many similarities to human asthma (Karol, 1994; Turner and Watson, 1996). Some similarities to human IgE-mediated allergic conditions (Table 3.3) have been identified in a population of monkeys with allergic conditions (Karol, 1994).

Table 3.3 Similarities of allergic conditions of monkeys to human IgE mediated allergic conditions (Karol, 1994).

Allergic condition	Similarity to human IgE –mediated allergies
Persistent and consistent IgE-mediated cutaneous and airway responses to *Ascaris* antigen.	Analogous to humans with IgE triggered asthma.
Cutaneous and airway reactivity to *Ascaris* antigen but in which the airway reactivity subsides and may disappear.	Analogous to spontaneous remission of human asthma.
Cutaneous reactivity to *Ascaris* antigen but no asthma.	Analogous to humans with IgE antibody and no asthma.

3.2.4 Horses

Horses and ponies exhibit spontaneous recurrent airway obstruction (Snapper, 1986; Smith, 1989; Wanner *et al.*, 1990; Karol, 1994). Heaves is a naturally occurring respiratory condition of horses and ponies precipitated in affected animals mainly by exposure to hay containing mould spores (Smith, 1989; Wanner *et al.*, 1990; Karol, 1994). Severely affected animals show diffuse bronchoconstriction, mucus hypersecretion, hypoxemia, goblet cell metaplasia and smooth muscle hypertrophy (Wanner *et al.*, 1990; Smith, 1989; Karol, 1994). However, heaves may be more related to human hypersensitivity pneumonitis than to asthma (Smith, 1989).

3.3 THE OVINE MODEL OF ASTHMA

For centuries sheep have supplied man with food and fibre (Kitchen, 1977). During last several decades they have also served as useful animal models for the study of a variety of biological phenomena including certain human diseases (Kitchen, 1977; Wanner and Abraham, 1982; Chen, 1990; Karol, 1994; Pack *et al.,* 1994; Abraham, 1996). They are reasonably cheap in New Zealand, economical to maintain and can be raised in variety of environments. Because of the size of the sheep, the measurement of multiple physiological parameters (respiratory, cardiovascular, etc.), sampling of tissue and body fluids (for analysis of inflammatory mediators and histopathological examination) at regular intervals,

use of invasive techniques (biopsies, bronchoscopy, cardiac catheterisation) and the use of the same animal repeatedly are possible (Wanner *et al.,* 1979; Wanner and Abraham, 1982; Kleeberger *et al.*, 1985). Conscious sheep can tolerate naso-tracheal intubation for several hours (Wanner and Reinhart, 1978; Kleeberger *et al.,* 1985; Soler *et al.*, 1991; Abraham *et al.*, 1994; O'Riordan *et al.*, 1997) and the manipulation of the upper respiratory tract is possible under local anaesthesia without producing excessive aerophagia and bloating (Long *et al.*, 1988; Soler *et al.*, 1991; Abraham *et al.*, 1994, 1997).

3.3.1. The allergic sheep

The sheep model of asthma was originally developed in North America (Wanner *et al.,*1979; Wanner and Abraham, 1982; Abraham, 1996) and is now being used in several other countries such as Canada (Bosse *et al.,* 1987), Japan (Okayama *et al.,* 1989) and New Zealand (Chen *et al.,*1990, 1991; Pack *et al.*, 1994). Wanner *et al.,* (1979) detected that most of the sheep used in their experiments had a positive skin reaction prior to airway challenge with *Ascaris suum* antigen probably resulting from previous exposure to pigs infested with *Ascaris suum.* However, sheep without previous exposure to pigs also have shown both an immediate skin and respiratory reaction to *Ascaris suum* antigen (Chen *et al.*, 1990). The reason for this reaction is not clear and the possibility of cross-reaction to nematodes such as *Toxocara canis* was eliminated in a study using enzyme linked immunoabsorbent assay (Chen *et al.*, 1990). There may not be a causal association between pre-exposure to *Ascaris suum* and the size of skin reaction and the difference could perhaps be attributable to some other environmental factor (Chen, 1990).

Sheep can be experimentally sensitised to *Ascaris suum* antigen by repeated intra-muscular injections of a mixture of *Ascaris* antigen and Freund's adjuvant (Wanner and Reinhart, 1978; Wanner *et al.*, 1979), intra-tracheal infusion of antigen (Bosse *et al.*, 1987) or by

inhalation of *Ascaris* antigen (Abraham, 1989; Kung *et al.*, 1980; Kleeberger *et al.*, 1985). Most of the sheep with a positive cutaneous reaction show an airway response to initial inhalation challenge with the *Ascaris suum* antigen (Wanner *et al.*, 1979; Chen *et al.*, 1990). However, the correlation between skin reaction and airway response is not significant (Wanner *et al.*, 1979; Kleeberger *et al.*, 1985; Chen *et al.*, 1990). Sheep develop an acute or dual airway response to inhaled *Ascaris* antigen, and the response is similar in several aspects to that is seen in human asthmatics (Wanner *et al.*, 1979; Kleeberger *et al.*, 1985; Chen *et al.*, 1990; Abraham *et al.*, 1994; 1997, O'Riordan *et al.*, 1997). Animals that show a dual response develop a prolonged airway hyperresponsiveness that remains for up to two weeks after a single antigen challenge (Abraham *et al.*, 1994).

3.3.2. Acute airway response

Acute airway response to *Ascaris* antigen challenge in most of the sensitised sheep developed 3-6 minutes after the beginning of the aerosol challenge, reached a maximum at 30 minutes and resolved within two hours after the antigen challenge (Wanner *et al.*, 1979; Bosse *et al.*, 1987). The changes in respiratory function consisted of an increase in airway resistance, a decrease in specific pulmonary conductance (Wanner *et al.*, 1979; Chen *et al.*,1990; Bosse *et al.*, 1987; Abraham, 1996; Abraham *et al.*, 1997), an increase in functional residual capacity (Wanner *et al.*,1979; Bosse *et al.*, 1987; Abraham, 1996), a decrease in dynamic lung compliance (Wanner *et al.*, 1979, Bosse *et al.*, 1987; Chen *et al.*,1990; Abraham. 1996) and in some cases a decrease in arterial oxygen tension (Wanner *et al.*, 1979; Bosse *et al.*, 1987; Abraham. 1996). The airflow obstruction in allergic sheep is accompanied by an increased functional residual capacity which is also a typical finding in asthmatics (Wanner *et al.*, 1979). Unlike dogs and monkeys, pulmonary hyperinflation

is observed in allergic sheep (Wanner *et al.,* 1979; Abraham, 1996), a finding also observed in human asthmatics (Cameron and Bateman, 1983). The reason for these differences between species is not clear, but may be related to differences in the efficiency of collateral ventilation of the lungs (Wanner and Abraham, 1982). Unlike dogs, monkeys and guinea pigs, species in which the collateral ventilation is high, pulmonary hyperinflation readily occurs in sheep and humans as they have poor collateral ventilation (Wanner and Abraham, 1982).

The main mediator of acute airway response in sheep is likely to be histamine derived from mast cells (Wanner *et al.*, 1979; Abraham *et al.*, 1981; Dworski *et al.*, 1989; Abraham, 1990, 1996). Antigen-induced bronchoconstriction is accompanied by a transient increase in histamine levels in arterial blood and bronchoalveolar lavage fluid (Wanner *et al.*, 1979). Dworski *et al.* (1989) observed an increase in histamine level in the bronchoalveolar lavage fluid (BAL), after challenging sheep with *Ascaris* antigen. In agreement with the above observations, several pharmacological studies have shown that acute airway response in sheep can be prevented either by blocking the release of mast cell mediators through administration of mast cell stabilisers or by blocking H_1-receptors using H_1-receptor antagonists (Abraham *et al.,* 1981; Weissberger *et al.,* 1981; Wanner and Abraham, 1982; Ahmed *et al.,* 1992; Abraham, 1996). However, there is evidence that mediators other than histamine may also contribute to the development of acute airway response in sheep (Abraham, 1996). Dworski *et al.* (1989) reported an increase in arachidonate metabolites in BAL fluid immediately after allergen challenge of sheep. Wanner *et al.* (1979) reported that about 20% of sheep with an acute response showed no change in arterial plasma histamine. These results indicate that histamine is not solely responsible for the development of an acute airway response in the sheep model of asthma

and some products of arachidonic acid may also be involved. Dworski *et al.* (1989) also observed a release of biologically active products such as thromboxane A_2 into BAL of sheep after the local challenge of lungs with *Ascaris* antigen. However, the importance of some of these products in the development of acute airway response in sheep is not clear. Failure to prevent the acute response in sheep by pretreatment with the prostaglandin synthetase inhibitor, indomethacin and anti-cholinergic agent atropine makes the role of these products in the development of the acute response in sheep uncertain (Abraham *et al.*, 1981; Weissberger *et al.*, 1981; Fugner, 1985). Unlike human asthmatics, the role of leukotrienes (LT) in the development of acute bronchoconstriction in sheep is not clearly defined (Abraham, 1996). In a study to determine the role of arachidonic acid metabolites in the development of acute airway response in sheep, Dworski *et al* (1989) observed that LT B_4 and LT C_4 were not significantly increased in BAL fluid after the instillation of *Ascaris suum* antigen into the lungs. Sheep with an acute response do not excrete significant amounts of LTE_4 in their urine (Abraham, 1996). Inhalation of peptidoleukotriene antagonist FPL 55712 and leukotriene antagonist FPL 57231 did not affect the acute response in sheep (Abraham *et al.*, 1981; Lanes *et al.*,1986; Abraham, 1996) indicating that leukotriene derivatives are not involved in the acute response.

The role of other mediators in the development of acute airway response in sheep has also been investigated. Administration of platelet activating factor (PAF) to allergic sheep induced an acute airway response which can be prevented by pretreatment with the PAF antagonist WEB-2086 (Fernandez *et al.*, 1989). Subsequently, Soler *et al.* (1989) found that pretreatment with WEB-2086 can also reduce or abolish the severity of acute allergen-induced airway responses and infiltration of inflammatory cells into BAL fluid. These results indicate that PAF is involved in the development of the acute airway response in

sheep. Administration of PAF can cause a significant fall in the circulation of cervical tracheal vasculature in sheep (Corfield *et al.,* 1991). The reduction in vascular resistance produced by the PAF was not affected by indomethacin, the leukotriene receptor antagonist FPL 55712 or a combination of H_1 - and H_2 -receptor antagonists mepyramine with cimetedine. However, the PAF antagonist WEB-2086 significantly reduced the vasodilatation produced by PAF (Corfield *et al.,* 1991).

As in humans, there were a greater number of eosinophils in BAL fluid from sheep with an acute response (Lanes *et al.,* 1986). Like human asthmatics, allergic sheep also show an impairment in tracheal mucus velocity after exposure to antigen (Wanner and Abraham, 1982; Abraham, 1996; O'Riordan *et al.,*1997). A decrease in mucus velocity in the trachea during *Ascaris* antigen induced bronchoconstriction in conscious sheep has been observed by Weissberger *et al.* (1981). Mucus transport depends on several factors such as ciliary motility, quantity and rheologic properties of mucus, and the periciliary fluid (Weissberger *et al.,* 1981). Alteration in one or more of these factors, during allergic bronchoconstriction, may result in impaired mucus transport (Weissberger *et al.,* 1981). Changes in viscosity and elasticity of mucus or an increase in the amount of mucus may also play an important role in the pathogenesis of impaired mucociliary dysfunction in human asthmatics (Weissberger *et al.,* 1981).

3.3.3 Late airway response

As observed in human asthmatics, some allergic sheep also showed a late bronchoconstriction 6-8 hours after the initial challenge with inhaled *Ascaris* antigen (Wanner *et al.,* 1979; Wanner and Abraham, 1982; Okayama *et al.,* 1989; Abraham *et al.,* 1994; Abraham. 1996; Abraham *et al.,* 1997). The late response in sheep is thought to be antigen specific and immunologically mediated (Wanner and Abraham. 1982, Abraham *et*

al., 1983, Delehunt *et al.*, 1984, Lanes *et al.*, 1986, Abraham. 1990, Abraham. 1996). The late response was predominant in central airways (Abraham *et al.*, 1983). Late airway response in sheep is mediator dependent and leukotrienes appear to be the principal mediators (Delehunt *et al.*, 1984; Russi *et al.*, 1984; Okayama *et al.*, 1989; Soler *et al.*, 1991; Abraham, 1988, 1990, 1996). Pretreatment with leukotriene antagonists blocked the late response in dual-phase responders (Delehunt *et al.*, 1984; Lanes *et al.*,1986). The involvement of leukotrienes in the development of late response is further supported by the findings of Okayama *et al.* (1989). They found that LT B_4 and LT C_4 levels in BAL fluid from sheep with a late response were significantly increased. Since the increase in histamine level in BAL fluid of dual responders was detected only during acute response, these findings indicate that leukotrienes could be the main mediators that contribute to the development of late airway response in sheep (Abraham, 1988, 1990, 1996). In addition to leukotrienes, other mast cell derived mediators may also contribute to the development of late airway response in sheep. The late response can be blocked by inhalation of cromolyn sodium prior to the antigen challenge indicating the involvement of mast cell-derived mediators (Abraham *et al.*, 1983; Abraham, 1988, 1990). BAL fluid from sheep with a late airway response appeared to have increased levels of inflammatory cells. BAL fluid from sheep with a dual response contained a higher number of neutrophils (Lanes *et al.*, 1986; Okayama *et al.*, 1989), a similar finding to that observed in humans experiencing late-phase reaction after allergen challenge (Kay and Corrigan, 1992).

3.3.4. Chronic airway response

Although the chronic airway responses (airway hyperreactivity which persists for several days) are not naturally found in sheep (Wanner *et al.*, 1990), they may be induced experimentally by repeated exposure to intra-tracheal *Ascaris* antigen (Bosse *et al.*, 1987).

This group reported a airway hyperreactivity that persisted for at least seven days after initial antigen challenge in sheep chronically sensitised with *Ascaris* antigen. In contrast to the acute and late responses, the chronic airway response predominantly affected the peripheral airways. Histamine levels, neutrophils and eosinophils were elevated in BAL fluid from sheep with a chronic airway response (Bosse *et al.*, 1987). This chronic airway response in sheep is a disorder quite comparable to that of humans with moderately severe chronic asthma (Bosse *et al.*, 1987).

3.3.5. Non- specific airway hyperresponsiveness (NAHR)

Non-specific airway hyperresponsiveness is a characteristic feature in asthmatics (Wanner and Abraham, 1982; Ahmed *et al.*, 1983; Lanes *et al.*, 1986; Corfield *et al.*, 1991; Lemanske and Busse, 1997). An increase in non-specific airway hyperresponsiveness similar to human asthmatics has been detected in sheep that develop acute (Wanner and Reinhart, 1978) or dual (Lanes *et al.*, 1986) responses to challenge with *Ascaris* antigen. This NAHR occurred after provocation with histamine (Ahmed *et al.*, 1983; Long *et al.*, 1985), carbacol (Abraham *et al.*, 1981) or methacholine (Wanner and Reinhart, 1978; Marek and Ulrner, 1995). The mechanism which causes NAHR to develop is not clear (Lanes *et al.*, 1986; Abraham, 1996). The NAHR in sheep with dual response is probably independent of the development of late airway response (Lanes *et al.*, 1986). Pharmacological studies suggested that different mechanisms are involved in the development of late airway response and NAHR. The development of NAHR can be prevented by both the cyclooxygenase inhibitor (indomethacin) and the leukotriene antagonist FPL-57231 (Lanes *et al.*, 1986). Only FPL-57231 caused a blockade of both the late airway response and NAHR in sheep, while indomethacin had no effect on the late response (Lanes *et al.*, 1986). In contrast, inhaled glucocorticosteroids, which are capable

of preventing both acute and late airway responses, failed to block NAHR in sensitised sheep (Lanes *et al.*, 1986). These results indicate that metabolites of arachidonic acid may also contribute to the development of non-specific airway hyperresponsiveness (Lanes *et al.,* 1986).

2.3.6. Effects of pharmacological agents on allergic sheep

Many researchers have used pharmacological agents to characterise the airway hyperresponsiveness in sheep (Abraham *et al.*, 1981; Abraham, 1989; Ahmed *et al.*, 1983; Long *et al.*, 1985; Marek and Ulmer, 1995). The effect of various pharmacological agents on acute airway hypersensitivity in asthmatic and allergic sheep have been summarised in the Table 3.4.

Table 3.4. Effect of pharmacological agents on acute airway hypersensitivity in man and sheep (based on Fugner (1985) and Abraham (1996))

Drug or class of drugs	Inhibitory activity	
	Human	Sheep
β- agonist	Y	Y
Antihistamine	Y	Y
Antiserotonin	UK	V
Anticholinergic	V	N
FPL 55712	N	N
Steroids	Y	Y
Cromolyn Na	Y	Y
Nedocromil Na	Y	Y
LT / 5-LO inhibitors	V	V

Y = yes, N= no, V = variable, UK= unknown 5-LO = 5-Lypoxygenase, LT = Leukotriene

3.4 CONCLUSION

The principal reason for using animal models is to test hypotheses concerning the pathogenesis of the diseases and their treatment that cannot be tested in humans. Although there are advantages and disadvantages of using animal models, the advantages outweigh the disadvantages. An ideal model of human asthma would require:

a) non- specific airway hyperresponsiveness *in vivo,*

b) *in vitro* hyperresponsiveness of airway tissues,

c) a late response after allergen challenge,

d) effective therapeutic modalities identical to their effectiveness in humans,

e) increased lung volume during an allergen-induced hyperresponsiveness.

However, human asthma is a heterogeneous disease and strict criteria for animal models may be counter productive (Wanner *et al.*, 1990).

Among the animal models, the sheep is thought to be one of the closest to human asthma. Allergic airway responses are reproducible and consistent and the ovine model has many characteristics of human asthma (Wanner and Abraham, 1982). In many studies, sheep have been shown to have a natural skin sensitivity to *Ascaris suum* antigen (Wanner *et al.,* 1979; Okayama *et al.*, 1989; Chen *et al.,* 1990). Like human asthmatics (Cockcroft *et al.,* 1977) sheep also exhibit the late asthmatic response (Wanner and Abraham, 1982; Abraham, 1996; Abraham *et al.,* 1997) and non-specific airway hyperresponsiveness (Wanner and Abraham. 1982; Corfield *et al.*, 1991; Abraham. 1996) and responses to a variety of pharmacological agents (Fugner, 1985; Abraham, 1996) are largely common to both species. In addition, poor collateral ventilation in ovine lung is another similarity to the human situation and pulmonary hyperinflation occurs in both species (Wanner and Abraham, 1982; Abraham, 1996). The inflammatory mediators and cellular changes

associated with antigen-induced bronchoconstriction are similar in both humans and sheep (Wanner and Abraham, 1982; Abraham *et al.*, 1983; Okayama *et al.*, 1989; Abraham, 1990, 1996).

Studies of the sheep model indicate that airway responses to challenge with inhaled *Ascaris* antigen are mediated mainly by mast cell derived mediators (Wanner *et al.*, 1979; Abraham *et al.*, 1981; Dworski *et al.*, 1989; Abraham, 1990, 1996). While histamine is the major contributor, metabolites of arachidonic acid also contribute to the development of the acute response (Dworski *et al.*, 1989). The late response is mediated mainly by the leukotrienes (Delehunt *et al.*, 1984; Russi *et al.*, 1984; Okayama *et al.*, 1989; Soler *et al.*, 1991; Abraham, 1988, 1990, 1996).

Multiple physiological parameters can be measured in sheep and the same animal can be used repeatedly (Wanner *et al.*, 1979). The sheep model has the advantage that general anaesthesia is not required for the measurement of airway function and naso-tracheal intubation can be done with local anaesthesia (Wanner and Reinhart, 1978; Kleeberger *et al.*, 1985; Soler *et al.*, 1991; Abraham *et al.*, 1994; O'Riordan *et al.*, 1997). The use of sheep is, therefore, preferable to using many laboratory animal models of asthma in the study of allergic bronchoconstriction and especially for long-term observations (Wanner *et al.*, 1979).

Most studies using sheep have focused on the significance of inflammatory mediators in the development of allergic bronchoconstriction (Abraham, 1988, 1990, 1996; Chen, 1990). Factors responsible for any inherent difference in airway response between individual animals and the mechanisms which make one individual more responsive should be explored (Chen 1990).

CHAPTER 4

AIRWAY HYPERESPONSIVENESS AND MORPHOLOGICAL CHANGES INDUCED BY REPEATED EXPOSURE OF SHEEP TO *ASCARIS SUUM* ANTIGEN

4.1 INTRODUCTION

Several different animal species have been used as models of human asthma (Bai, 1998). Among the animal models developed so far, the '*Ascaris suum*-sensitive' sheep has been regarded by many authors as the closest model of human asthma (Wanner *et al.,* 1979, Wanner and Abraham, 1982; Okayama *et al.*, 1989; Chen, 1990; Abraham; 1996). However, like all animal models, the ovine model also has not exhibited all the pathological features seen in human asthmatics.

Repeated antigen exposures are required to reproduce the airway wall remodelling seen in human asthma (Bai, 1998). Although remodelling after chronic challenge has been reported in Brown Norway rats (Sapienza *et al.,* 1991), Hartley guinea pigs (Wang *et al.,* 1995) and cats (Padrid *et al.,* 1995), none of these models has been shown to reproduce all the pathological features of airway wall remodelling seen in human asthma, particularly the thickening of reticular lamina. There have, however, been recent reports of this thickening of reticular lamina in airways of rodents (Blyth *et al.*, 1996; Yoshikawa *et al.*, 1996; Palman *et al.,* 1997). In these studies, the thickening of the reticular lamina was induced either by repeatedly exposing sensitised mice to ovalbumin (Blyth *et al.*, 1996; Palman *et al.,* 1997) or infecting rat lungs with larvae of the canine round worm *Toxocara canis* (Yoshikawa *et al.*, 1996). *Toxocara* larvae produced the most marked reaction in the reticular layer but had the undesirable side effect of leaving the rat lung infected with

worms. Thus, any measurement of lung function would be affected not only by the thickening of the reticular lamina but also by the additional effects caused by the worms living in the lung.

In this study, repeated exposure of "A*scaris* sensitive sheep" to *Ascaris suum* antigen was used to attempt to develop an ovine model which had the structural changes that occur in human asthmatic airways. The thickening of sub-basement membrane connective tissue was regarded as important in this respect. Subsequently, it was envisaged that this model could be used to investigate the efficacy of asthma treatments in negating early the pathological and physiological changes associated with the disease.

4.2 MATERIALS AND METHODS

Animals

Twenty-two one-year old Romney-cross sheep of either sex weighing 21.5-39.2 kg (average weight 29.7kg) were obtained from the Massey University farm. For handling convenience these animals were used in groups of six.

Ethical consideration

All procedures had prior approval of the Animal Ethics Committee of the Massey University.

Antigen

Ascaris suum extract was purchased commercially (Greer Laboratories, Lenoir, NC) as a stock solution dilution of 1:10 concentration (206,000 to 218,000 Protein Nitrogen Units (PNU)/ml). A working solution for skin tests and antigen challenge was freshly prepared by diluting the stock with sterile phosphate-buffered saline (PBS).

Skin Test

All animals were skin tested for cutaneous reactivity using intradermal injection of *Ascaris* extract using the method recommended for human asthmatics by the Asthma and Allergic Disease Centre for the USA (Chai *et al.*, 1975). Five minutes prior to the skin test, each animal received an intravenous injection of 5 ml of 0.5% Evans blue (Merck Colour Index No 23860) dissolved in saline to improve the clarity of the reaction. 0.2 ml of three antigen concentrations (200, 2,000 & 20,000 PNU/ml) were injected intradermally using a 26 g 3/8" hypodermic needle to the shorn and shaved lateral chest wall 5-6 cm below the vertebral column. A control injection of 0.2 ml PBS was also injected 2-3 cm below the site of antigen injection. The immediate and the late cutaneous reactions were read using a 50 mm Vernier caliper (Mitsutoya, Japan) 30 minutes and 6-8 hours post-antigen injection

respectively. Since the resulting wheal was not always circular, the mean of the longest and the shortest diameters was considered as the diameter of the reaction. The degree of the reaction was determined by the difference between the diameter of the wheal produced at the site of antigen injection and the control site, and a difference of 5 mm or more was considered a positive reaction. In each group, the sheep with the lowest skin reaction was used as a non-sensitised control and the two 'positive skin reactors' that showed the most clear response were used as sensitised-control sheep and experimental sheep (allocated randomly). The remaining three sheep were returned to the farm. All animals were ear-tagged for identification and held in individual metabolic crates in either the animal holding room in the Veterinary Tower or Animal Physiology Unit, with food and water *ad libitum*.

Tracheal Fistulation

One week prior to the start of the experiment, a tracheostomy was performed under general anaesthesia. After the induction of general anaesthesia with 5% halothane (Fluothane, Zeneca Ltd, Cheshire, UK) using a face-mask the animals were intubated with a 9 mm cuffed endotracheal tube and positioned in dorsal recumbency. Anaesthesia was maintained with 1.5-2.5% halothane to effect. A long acting intra-venous analgesic ('Zenicarp' 0.7 mg/kg, C-Vet, Lanchashire, UK) was given to minimise the stressful effects of the surgery. The tracheostomy was performed according to the method described by Dueck *et al.* (1985). Briefly, a transverse skin incision was made at a level between the 3rd and 4th tracheal rings caudal to the cricoid cartilage. Three to four horizontal mattress retention sutures (5-0 nylon) were placed on both edges of the skin incision to join the tracheal wall to the skin. A transverse tracheal incision was then made between the two suture lines to generate an opening to the anterior one third of the trachea

and the retention sutures were then tied off. Cruciate-shaped retention sutures (3-0 silk) were placed on the lateral edges of the skin incision. A post-surgical antibiotic course (20 mg/kg Terramycin LA, New Zealand Vet, New Zealand) was given intra-muscularly for 5 days to minimise any post-surgical infections. The tracheal fistulae were closed with swabs and crepe bandages to allow normal nasal breathing between antigen exposures. Animals were closely monitored during the recovery period for any post-surgical complications and signs of infection were assessed by examining the surgical site daily and, also, by observing the general appearance and food intake of animals.

Animal restraint and handling during experiments

During each antigen/PBS exposure and measurement of physiological parameters animals were sedated with intra-muscular diazepam injection ('Pamlin' Parnnell Lab, New Zealand 0.3 mg/kg) to a level where they could barely support their body weight. They were supported in metabolic crates in sacking hammocks throughout the experimental procedure.

Enhancement of responsiveness of airways to the antigen

The respiratory allergic response of the two skin reactive animals in each group was enhanced by 3 antigen exposures at fortnightly intervals. Following infiltration of the fistula with 2% lignocaine HCl ('Lopaine', Ethical Agents, New Zealand), a 9 or 8.5 mm cuffed endotracheal tube was passed through the fistula allowing the animal to breathe without the gases passing through the nose. Once the breathing patterns of the animals were stabilised, baseline values of airflow (F), tidal volume (V_T) and intra-oesophageal pressure (P_{IO}) were recorded as described below. An aerosol of *Ascaris suum* antigen (82,000 PNU/ml) was delivered through the endotracheal tube using an ultrasonic nebuliser (De Vibliss, Pennsylvania, 0.5 ml/min) for 20 minutes and recordings of F, P_{IO}

and V_T (also the angle of the P_{IO} -F curve on the oscilloscope) were made before antigen exposure and at 5, 10, 15 & 20 minutes after the beginning of exposure. A minimum of five consecutive breaths were measured on each occasion. The nebuliser and the Fliesch pneumotachograph were connected to the endotracheal tube via a two-way valve, so that flow could be directed through either the nebuliser or pneumotachograph during aerosol delivery or airflow measurement respectively (Fig. 3.1). Once the antigen exposure was completed, the endotracheal tube was removed and the animal allowed to recover. The expired air of the sheep was passed through a vapour trap containing ice in order to precipitate any antigen aerosol remaining before voiding to the atmosphere (Fig. 4.1).

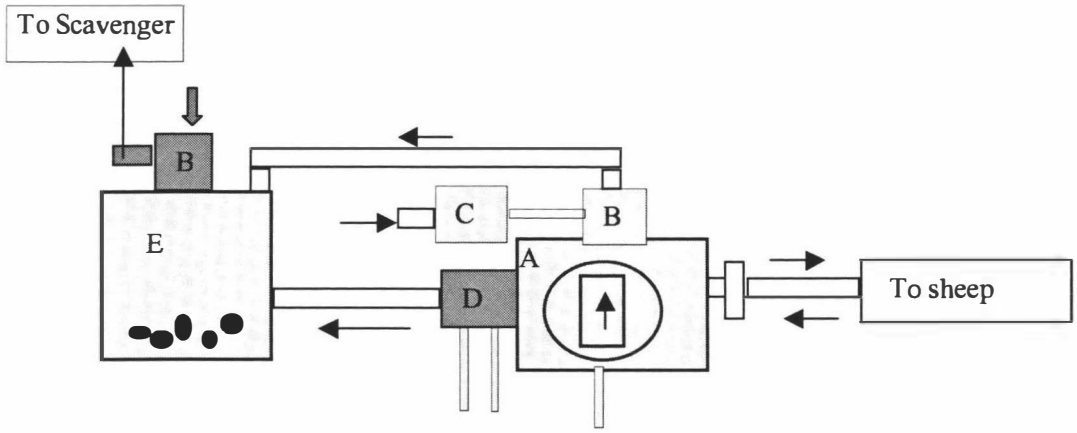


Figure 4.1. The arrangement of apparatus used for antigen/PBS exposures in conscious sheep. A= two-way valve, B= one-way respiratory valve, C= nebuliser, D= Pneumotachograph, E= Vapour trap, ⟶ = flow direction

Measurement of physiological parameters

To measure $P_{IO,}$ an oesophageal balloon catheter (made with a condom and a polyethylene tube with internal and external diameters 2 and 3 mm respectively) was advanced through one nostril until it reached the cardiac sphincter, withdrawn 5-10 cm and the balloon inflated with 2-3 ml of air.

Respiratory airflow (F) was measured using a No 2 Fliesch pneumotachograph and a differential pressure transducer (Model DP45-16, Validyne Engineering Corp., Ca, USA) that was connected to the proximal end of endotracheal tube using a two-way valve (Fig. 4.1).

Tidal volume (V_T) was obtained by integrating the flow signal using a respiratory integrator. The P_{IO} and F were recorded simultaneously on a Gould RS 3400 (Gould Inc. Ohio, USA) four-channel chart recorder (chart speed 5 mm/sec). The dynamic lung compliance (Cdyn) expressed in ml/ cmH_2O was calculated from the chart recorder by dividing the V_T by corresponding P_{IO} when the flow rate is zero (end expiration pause). The airway resistance (Raw) values were obtained by the method described by Mead and Whittenberger (1953) as modified by Nadel and Widdicombe (1962). Briefly, P_{IO} and F were displayed on an x/y oscilloscope to obtain the 'on line' changes in the airway resistance (Raw) by measuring the angle (θ) of the P_{IO}-flow curve using the following formula. The compliance effect of changing lung volume was negated by subtracting an electrical signal proportional to lung volume from the pressure signal in the respiratory integrator. The resistance of the endotracheal tube (0.041 and 0.0601 cmH_2O /l/sec for 8.5 and 9 mm respectively) was subtracted from the calculated Raw.

$$\text{Raw } (cmH_2O/l/sec) = \text{Tan theta} * \frac{\text{calibration of } P_{IO}/\text{ cm oscilloscope screen}}{\text{calibration of flow /cm oscilloscope screen}}$$

The chart recorder and the oscilloscope were calibrated prior to each exposure using 'point calibration'. Five and 10 cm water columns and atmospheric pressure (0 cm H_2O) were used as three points to calibrate pressure. The linearity of pressure calibration was established prior to the beginning of exposures by using different pressure levels at 1 cm

H_2O intervals and the response was linear between 0 and 10 cm H_2O. Air-flow (5 l/minute and no flow) through a variable area flow meter (Cole-Pamer Int. Illinois, USA) was used to calibrate the air-flow signal. The response of airflow was linear over a range of 0-8 l/min at 1 l/min intervals. Volume was calibrated by using either a 1 or 6 litre bell jar spirometer at volumes 0, 0.5, 1, 2, litres. The inlet of the spirometer was connected to a No 2 Fliesch pneumotachograph and a differential pressure transducer. The cylinder of the spirometer was moved vertically so that the pen recorded a volume of 1000 ml on the paper. The flow through the pneumotachograph generated a flow signal corresponding to a volume of 1000 ml that was recorded on the chart recorder as a volume of 1000 ml. The response was approximately linear over a range of 500-2000 ml.

Bronchial Provocation

In each group, one of the two sheep underwent two/three fortnightly exposures to antigen to enhance airway response was exposed to an aerosol of *Ascaris suum* antigen (82,000 PNU/ml) in sterile PBS daily for 14 days using the procedure described earlier. The other sensitised and non-sensitised sheep received the saline vehicle alone. All physiological parameters were recorded in the manner described previously.

On the last day of bronchial provocation, the antigen exposures were carried out under general anaesthesia. Following induction of anaesthesia (intra-venous Sodium Pentobarbitone 30 mg/kg, Virbac Laboratories, NZ), the animal was positioned in dorsal recumbency. The left or right femoral artery and the left and right femoral veins were catheterised using clear vinyl (1.5 and 2.0 mm internal and external diameters) and polyethylene (2.0 & 3.0 mm internal and external diameters) tubes (Critchly Electrical Products Pty Ltd, NSW, Australia), respectively. Anaesthesia was maintained by connecting one of the venous catheters to a saline drip containing 10 mg/ml Sodium

Pentobarbitone diluted from 'Pentobarb 500'(New Zealand Vet, NZ) administered at 4-6 drops/minute to effect. The other catheter was passed up the vein to the extent that it lay in the thorax at a level close to the heart. The intra-thoracic venous catheter and the femoral artery catheter were connected to blood pressure transducers (Model P23XL, Spectramed Inc., CA, USA) to measure central venous pressure (CVP) and systemic arterial blood pressure (SABP), respectively.

The skin overlying the right jugular vein was clipped and cleaned. A small skin incision was made over the jugular vein and a size 7f Swan-Ganz flow-directed thermodilution catheter (model 93-132-7f) was inserted into the vein using a percutaneous sheath introducing kit (model CC-350B-8.5F, Baxter Health Care Corp., Ca, USA). The balloon at the tip of the catheter was inflated with 1 ml of air and the catheter was gently advanced through the right side of the heart such that the tip of the catheter lay in the pulmonary artery. The position of the tip of the catheter was confirmed by the change in the distal tip pressure. The catheter was advanced further to a pulmonary artery wedge position to obtain pulmonary artery wedge pressure and the balloon was deflated. The catheter was kept patent by flushing periodically with heparinised 0.9% saline (25,000 IU Heparin per litre of saline (Leo Pharmaceutical Products, Denmark). The proximal end of the Swan-Ganz catheter was connected to a blood pressure transducer. Once the surgery was completed, the animal was transferred to the left-lateral recumbency and left until the heart rate and blood pressure became stable (approximately 20-30 minutes) before being exposed to antigen or PBS.

Once the pulmonary arterial pressure (Pap) was recorded, the balloon at the tip of the catheter was inflated with 0.5-1 ml of air and pulmonary arterial wedge pressure (Pwp) was obtained.

The cardiac output (CO) was measured using Fick's thermodilution method. The CO was obtained using a purpose built cardiac output computer (COM-1, American Edwards Laboratories, Ca, USA). A bolus of 5 ml ice cold 0.9% saline was injected into the Swan-Ganz catheter and CO was read from the cardiac output computer. Five consecutive measurements were obtained and the mean value obtained. Both left and right cardiac workloads were calculated using the following formula

Power (mW/kg) = (2.2216 (P_1 - P_2)* CO)/kg Body weight

Where 2.2216 is the unit conversion factor to express power in mW/kg and CO is the cardiac output in l/min, the corresponding measurements for P_1 and P_2 for left and right sides of the heart are given in table 4.1.

Table 4.1 Corresponding P_1 and P_2 for left and right sides of heart in the formula used for calculation of cardiac work load.

	Left side of the heart	**Right side of the heart**
P_1	Systemic arterial blood pressure in mmHg	Pulmonary arterial pressure in mmHg
P_2	Pulmonary wedge pressure in mmHg	Central venous pressure in mmHg

In addition to the airway measurements, SABP, CVP, Pap and Pwp and CO were also measured at five minutes intervals during the airway challenge on the last day of two-week exposure and recorded on the Gould 4 channel chart recorder.

Histological studies

At the end of the experiment animals were killed using excess intra-venous anaesthetic (10-15 ml of 'Pentobarb 500', NZ Vet, New Zealand) and necropsied. The carcass was dissected along the midline of the ventral neck. The ribs were removed from the vertebral column and sternum and the thoracic cavity was opened. The respiratory tract was dissected from the carcass by cutting through the trachea (just below the cricoid cartilage), muscles, connective tissues and pleural adhesions and transferred to a tray. Tissue blocks up to 2 x 1 x 0.5 cm in size were taken for histological studies from 10 predetermined airway levels (Fig. 4.2). Tissues were fixed by immersion in 10% neutral-buffered formalin for a minimum period of 24 hours, processed routinely for histology and embedded in paraffin wax. Sections of 3μm thickness were cut and stained with haematoxylin and eosin (H&E), periodic acid-shift/Alcian blue (PAS/AB) and Masson's trichrome (Culling *et al.*, 1985). All sections were coded and the person carrying out the measurements was 'blind' regarding which experimental group of sheep they belonged to. All morphometric studies were carried out using light microscopy. Peripheral airways with a mean internal diameter of 20-25 μm and with an aspect ratio (longest to shortest axis) of <3 were considered reasonable cross-sections of peripheral airways and used for measurements.

Epithelial thickness was determined by measuring the distance between the basement membrane and apical border of the epithelial cells at a magnification of *1000. Basement membrane thickness was obtained by measuring the distance from the base of the basal cell layer to the internal border of lamina reticularis. In addition, the number of eosinophils and goblet cells present were counted in the following way. The base of the basal cell

layer of the epithelium was placed in the middle of the microscopic field (10*40) and all the cells present in the entire field were counted. In all cases, five measurements were taken in each microscopic field and 20 such fields 200 μm apart were studied and the mean values were obtained. In the case of peripheral airways, several from each slide were used to obtain measurements from 20 fields.

The goblet cells were identified as the tall and columnar cells with abundant PAS-stained granules filling most of the cytoplasm between nucleus and the luminal surface. Eosinophils were identified as cells with granular eosinophilic cytoplasm with at least two nuclear lobules.

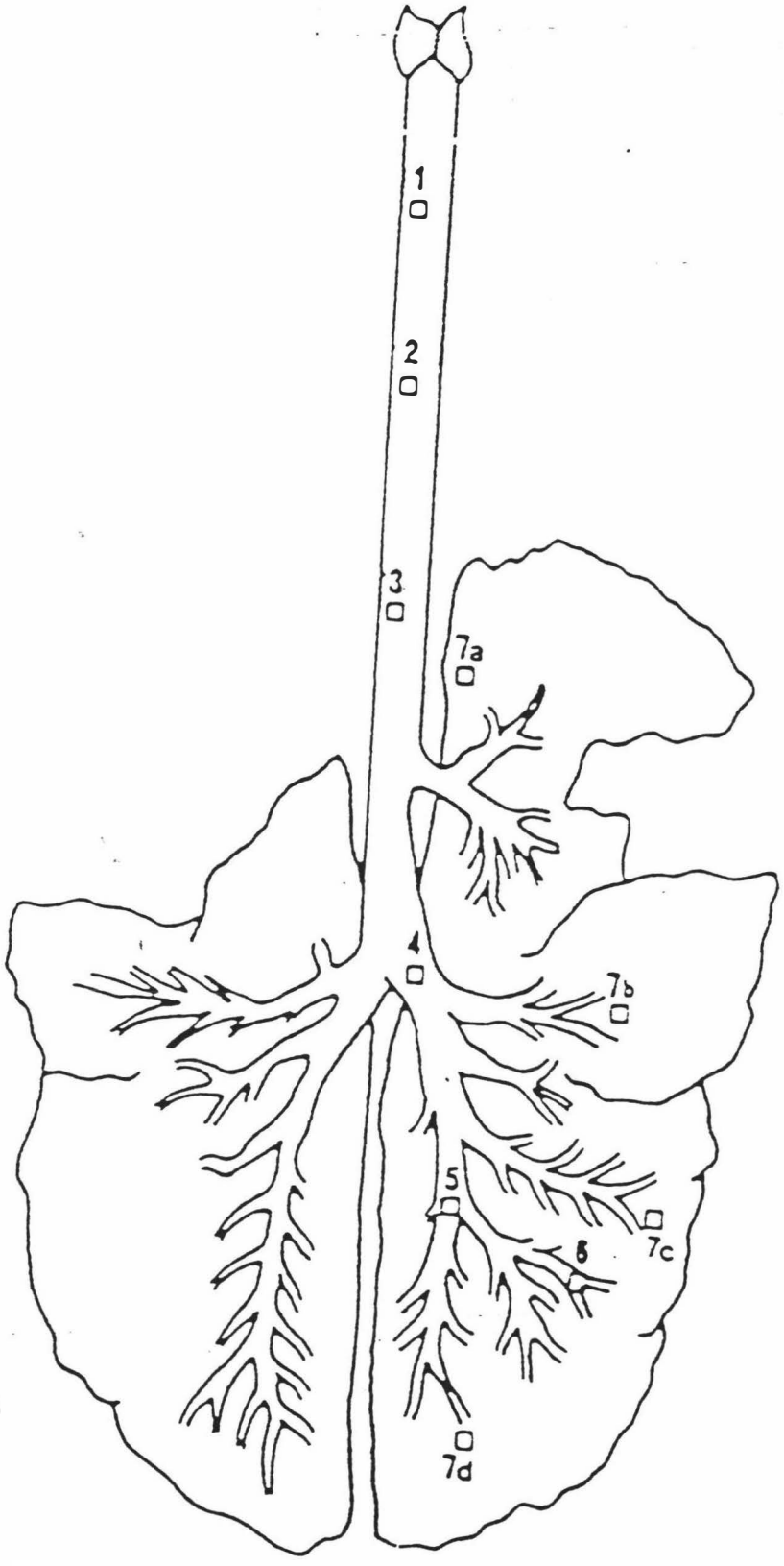
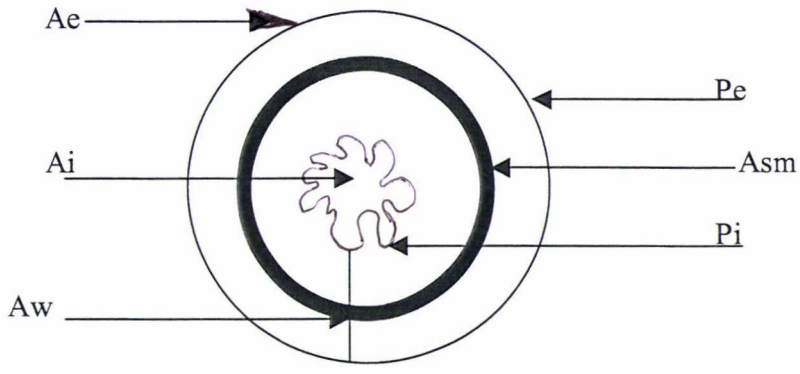


Figure 4.2 Diagram of the respiratory tract of sheep indicating the sites of tissue sampling. 1= upper trachea, 2= mid trachea, 3= lower trachea, 4= major bronchi, 5= lobar bronchi, 6= medium bronchi, 7a-d= bronchioles (Reproduced from Chen. 1990).

(a)



(b)

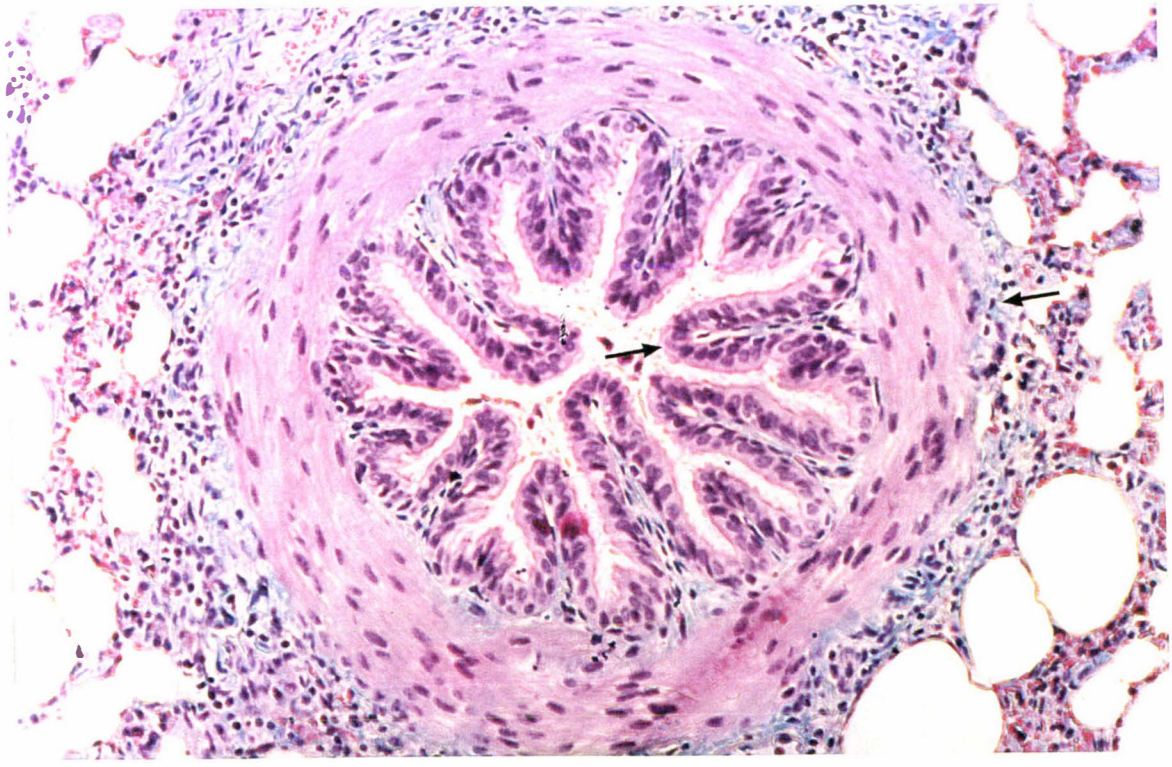


Figure 4.3. Schematic drawing (a) and a photomicrograph (b) of an airway illustrating the measurement that were made. Pi= internal perimeter, Ai= internal area, Pe= external perimeter, Ae= external area and Aw= airway wall area, Asm= area of smooth muscle. The external and internal perimeters are pointed at by arrows. Mason's trichrome. *50

Airway wall dimensions were measured using a light microscope with a side arm and a computer controlled digitising board (Jandel Scientific, CA, USA) and digitising software (Sigma Scan: Jandel Scientific, CA, USA). The internal perimeter (Pi) of the airway was used to determine the size of the airway used for measurements. The Pi is considered be relatively constant after smooth muscle contraction (James *et al.*, 1988). Cartilaginous and membranous airways with an internal perimeter between 3000 and 5000 μm, an aspect ratio of <3 (James *et al.*, 1988) were selected for the study. The following dimensions of the airway were measured (Fig. 4.3); The internal perimeter (Pi) and internal area (Ai) defined by the luminal surface of the epithelium, external perimeter (Pe) and external area (Ae) defined by the outer border of the adventitia. The area of smooth muscle (Asm) was measured by tracing around each smooth muscle bundle within the airway wall area (Aw). The perimeters and areas were measured in μm and μm^2 respectively. The airway wall area (Aw) was calculated by subtracting the internal area from the external area (Ae-Ai). The wall area was normalised to airway size by dividing the wall area by internal perimeter of the airway and Aw was expressed as area per unit length of internal perimeter. The percentage airway wall area occupied by smooth muscle (ASM%) was calculated as follows

$$ASM\% = Asm/Aw*100$$

The repeatability error of all the measured parameters, including cell counts, was obtained by repeatedly measuring a single airway 10 times and expressed as coefficient of variation (CV). The CV was less than 5% for all parameters measured except for eosinophil count and thickness of the basement membrane which were 6% and 7% respectively.

4.3 DATA ANALYSIS

All data were analysed using Microsoft Excel and Graphpad Prism software and presented as mean ± standard error of the mean. Statistical analysis was carried out using tests embedded in Graphpad Prism software. The effect of challenge over the two weeks was determined by one-way analysis of variance with Dunnett's multiple comparison test as a post test (if the P value was significant) to compare between groups. The changes in Raw and Cdyn from the baseline values during 20 minutes exposure and results of histological studies were compared using the Kruksal-Wallis test with Dunn's multiple comparison test as a post test (if the P value was significant) to compare between groups. A p value of $\leq$ 0.05 was considered significant in all tests.

4.4 RESULTS

4.4.1 Skin Test

Fourteen of 22 sheep tested (64%) had a positive reaction at 2000 PNU/ml antigen concentration. Two sheep with positive skin reaction (14%) also developed a late cutaneous reaction 5-6 hours later.

4.4.2 Changes in respiratory parameters

Airway resistance

Compared to PBS exposure, antigen exposure caused a significant increase ($p<0.001$) in the mean Raw from the baseline throughout two-week challenge in experimental sheep (Fig. 4.4). However, the magnitude of this increase varied between days. Occasionally PBS exposed sheep also appeared to show an increase in baseline values, but these did not reach a statistical significance (Fig. 4.4). The maximum increase in the mean Raw was 134% from the baseline in the antigen-exposed group.

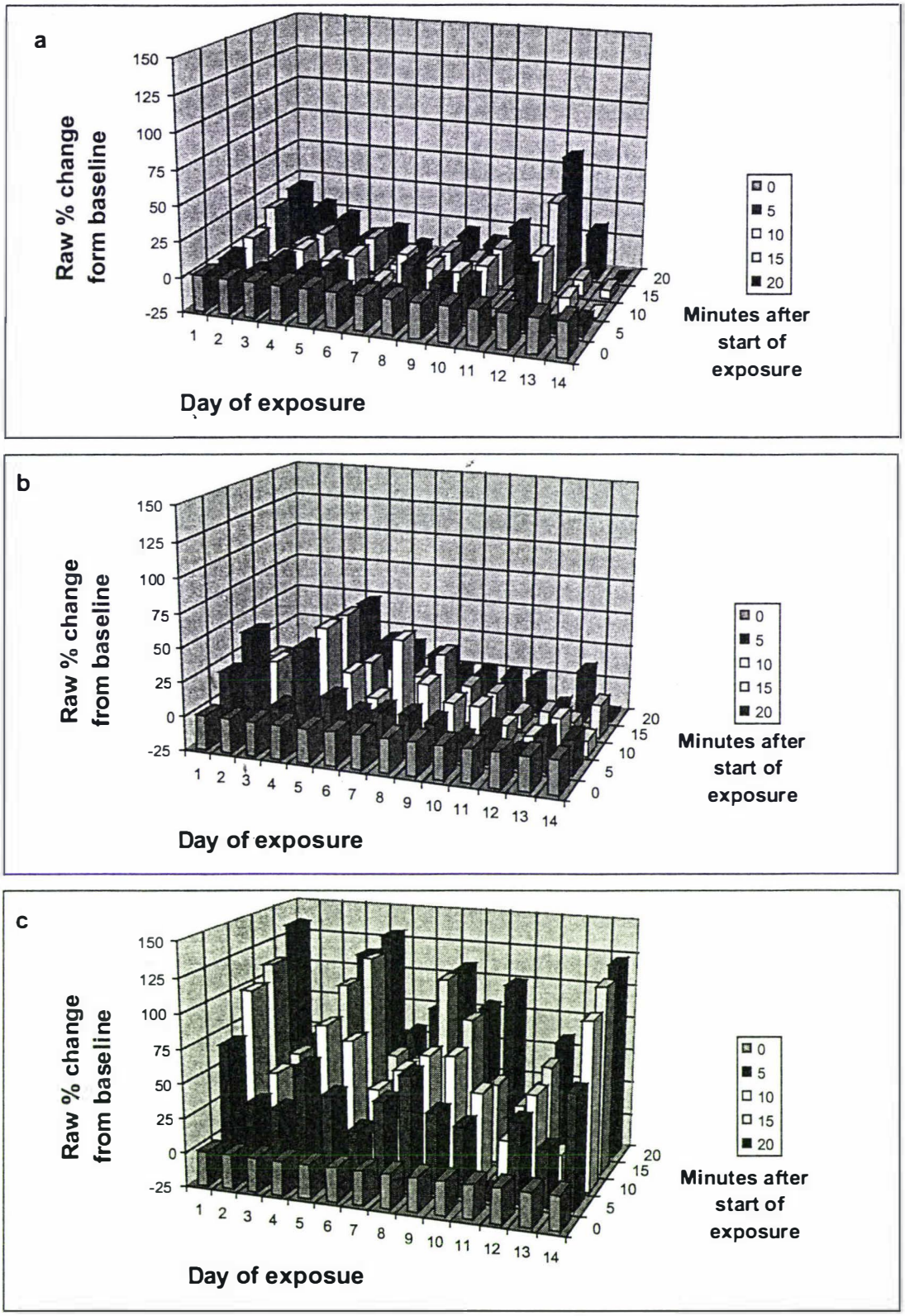


Figure 4.4. Percentage increase in airway resistance during 20 minutes exposure to *Ascaris* antigen/PBS daily for two weeks. a= Non-sensitised control, b= Sensitised control, c= Experimental. Time zero represents the baseline values. Antigen exposure caused an increase in baseline Raw during 20 minutes exposure throughout two week period.

The maximum increase in mean Raw was 62% and 48% from the baseline in non-sensitised and sensitised sheep respectively. At the end of the exposure period (day 14), antigen exposed sheep developed a significant increase in Raw ($P<0.001$) from the baseline throughout 20 minutes exposure (Fig. 4.5).

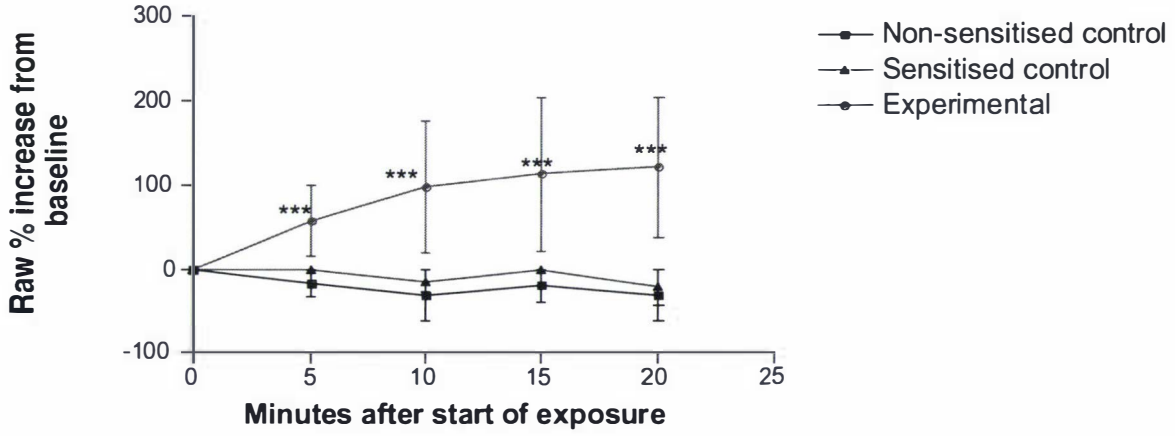


Figure 4.5. Changes in airway resistance at the end of the exposure period (day 14). Compared to sheep exposed PBS, there was a clear increase in Raw from the baseline in the antigen exposed sheep after being exposed to antigen daily for two weeks (*=p<0.001). Data presented as mean ± SEM.**

Dynamic lung compliance

The effect of two weeks antigen exposure on Cdyn was significantly different ($p<0.01$) from that of PBS exposure (Fig. 4.6). The mean Cdyn decreased from the baseline value consistently in antigen exposed sheep throughout the 20 minutes exposure period (Fig. 4.6). This decrease from the baseline was observed on most days of two weeks exposure period (Fig 4.6). However, on some days, the Cdyn also decreased from the baseline considerably in the non-sensitised control sheep (Fig 4.6).

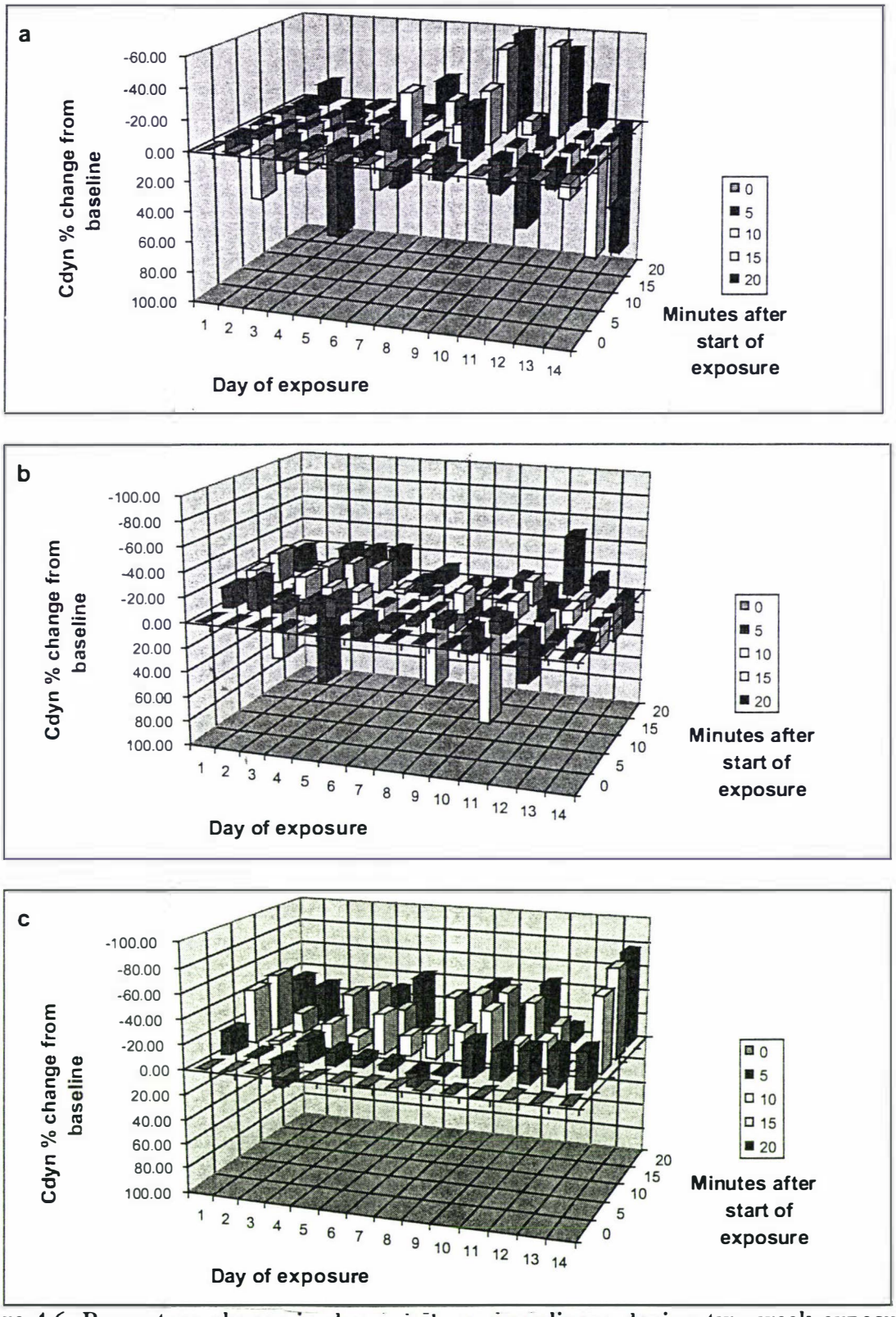


Figure 4.6. Percentage change in dynamic lung compliance during two week exposure to *Ascaris* antigen/PBS. a= Non-sensitised control, b= Sensitised control, c= Experimental. Minute zero represent the baseline values. Cdyn decreased from the baseline level during 20 minutes exposure throughout two week period in experimental sheep. To aid clarity a decrease in compliance is displayed as an upward displacement on the Y axis.

In sheep exposed to antigen, the maximum reduction in mean Cdyn was 70% of the baseline, while the non-sensitised and sensitised control groups showed a maximum reduction of 48.9 % and 59.91 % from the baseline respectively. On the last day of two-week antigen exposure (day 14), Cdyn in the experimental sheep significantly decreased ($p<0.01$) from the baseline level throughout 20 minutes exposure (Fig. 4.7).

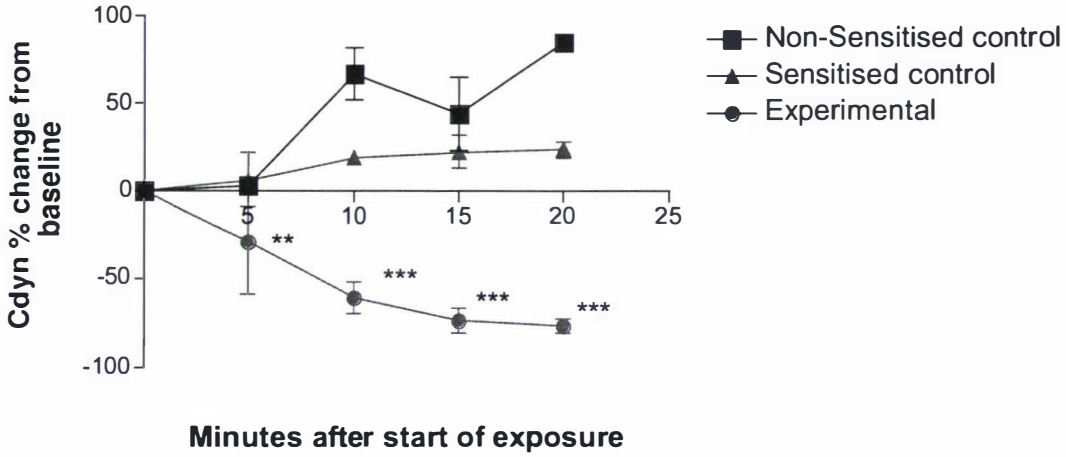


Figure 4.7. Changes in dynamic lung compliance at the end of the exposure period (day 14). Compared to sheep exposed to PBS, there was a clear decrease in Cdyn from the baseline in the antigen exposed sheep after being exposed to antigen daily for two weeks. Data presented as mean ± SEM. **=p<0.01, ***=p<0.001

4.4.3 Changes in cardiac power output.

The antigen exposure caused an increase in pulmonary arterial pressure which resulted in an increase in the mean work load of right side of the heart (Fig. 4.8a) in antigen-exposed sheep compared to PBS exposure ($p<0.05$). Cardiac power output of both sides of the heart was decreased from the baseline in the sensitised control group while there appeared to be a slight increase in the left side of the heart in non-sensitised control sheep. However, this apparent increase did not reach a statistical significance.

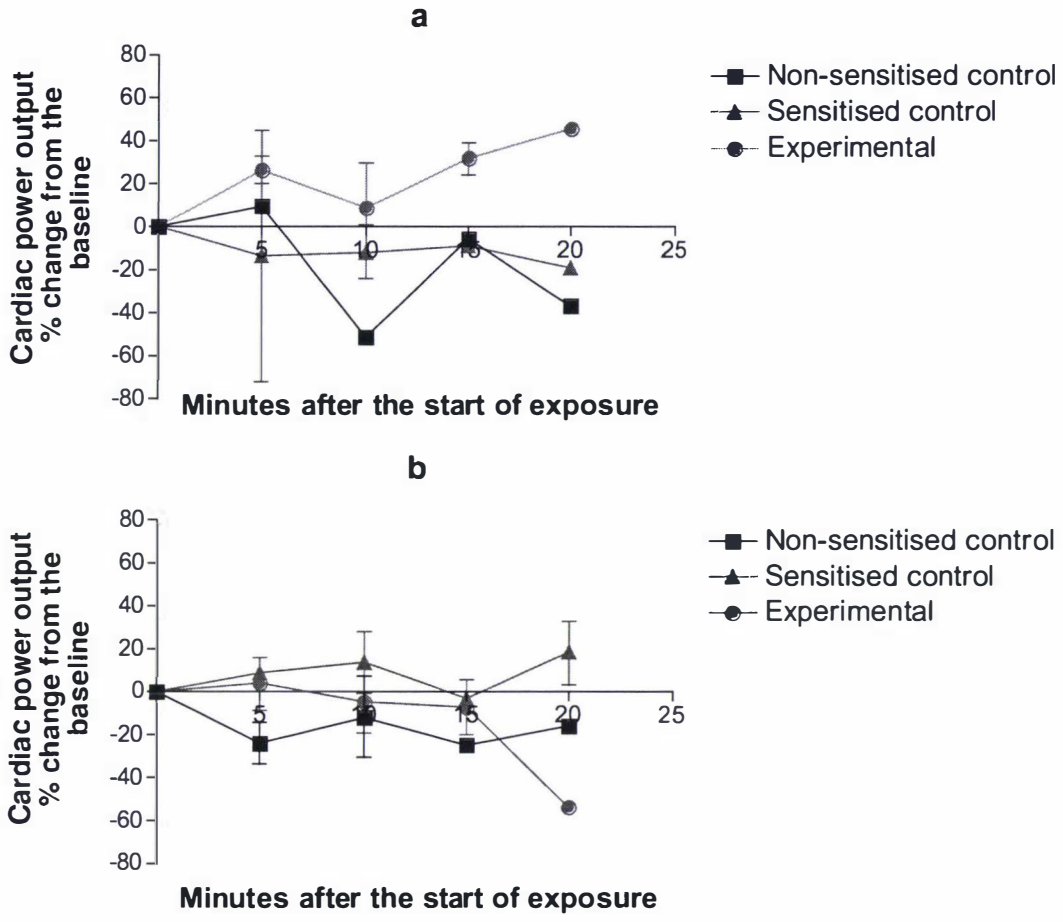


Figure 4.8. Percentage changes in cardiac power output (at the end of antigen/PBS exposure period) of right side (a) and left side (b) of the heart. Right side cardiac power output was increased in the antigen exposed sheep. Data presented as mean ± SEM.

4.4.4 Post-mortem findings

The tracheostomy site showed no gross signs of either infection or inflammation in any of the sheep. There were pleural adhesions in two of the non-sensitised control sheep; one of which had a congested right lung with consolidation on right cranial lobe. One of the sensitised control sheep also had congested right lung. Two sheep (sensitised and non-sensitised controls) had frothy mucus in trachea and major bronchi while one of the experimental sheep had blood-stained thick mucus in the right nasal cavity. The same experimental sheep had a congested right lung with diffuse peribronchial collapse.

4.4.6 Morphometric studies

Epithelial thickness.

There was no damage or sloughing of the airway epithelium. The quantitative comparison of the mean epithelial thickness at various levels of the respiratory tract is illustrated in the figure 4.9. Experimental sheep had a significantly thinner epithelium ($p<0.05$) compared to non-sensitised control sheep in mid trachea, lobar bronchi and bronchioles (Fig. 4.9). The sensitised control group also had significantly thinner epithelium in lobar bronchi compared to non-sensitised control sheep. The mean epithelial thickness was higher in experimental sheep in lower trachea while in major and medium bronchi the mean epithelial thickness was not remarkably different between three groups compared to PBS exposed sheep (Fig. 4.9).

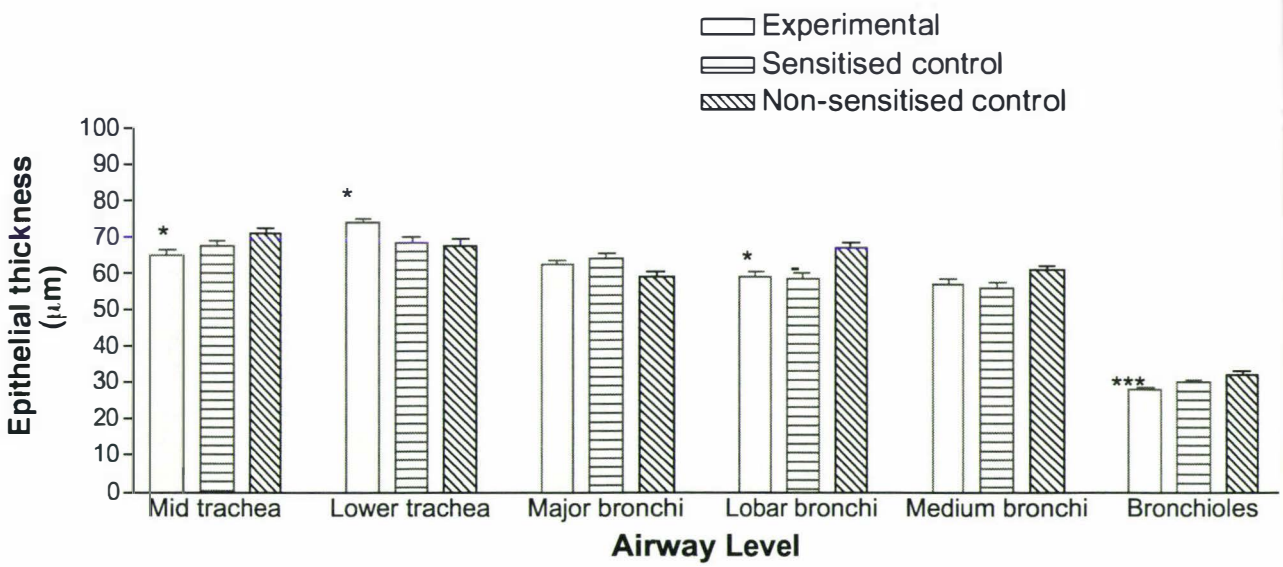


Figure 4.9. Comparison of the epithelial thickness at different levels of respiratory tract of sheep sensitised and challenged with *Ascaris* antigen (experimental), Sensitised with *Ascaris* antigen and challenged with PBS (Sensitised control) and with no sensitisation and challenged with PBS (non-sensitised control) for 14 days * = experimental vs non-sensitised control, + = experimental vs sensitised control, - = sensitised vs non-sensitised controls. */ +++/ --- = $p<0.001$, **/ ++/ -- = $p<0.01$, */ +/- = $p<0.05$. Data presented as mean ± SEM.**

Thickness of the 'Pseudo-Basement Membrane' (PBM).

The mean 'pseudo-basement membrane' (PBM) thickness at various levels of the respiratory tract is presented in the figure 4.10. In the antigen-exposed group, the PBM thickness was higher in mid-trachea ($p<0.001$) and medium and lobar bronchi ($P<0.01$) compared to the PBS exposed sheep. However, at the lower level of the trachea PBM in antigen exposed group was significantly different ($P<0.01$) only from the sensitised control sheep (Fig. 4.10). The PBM thickness was not significantly different between three groups in major bronchi and bronchioles. It was, however apparently increased in all airway levels.

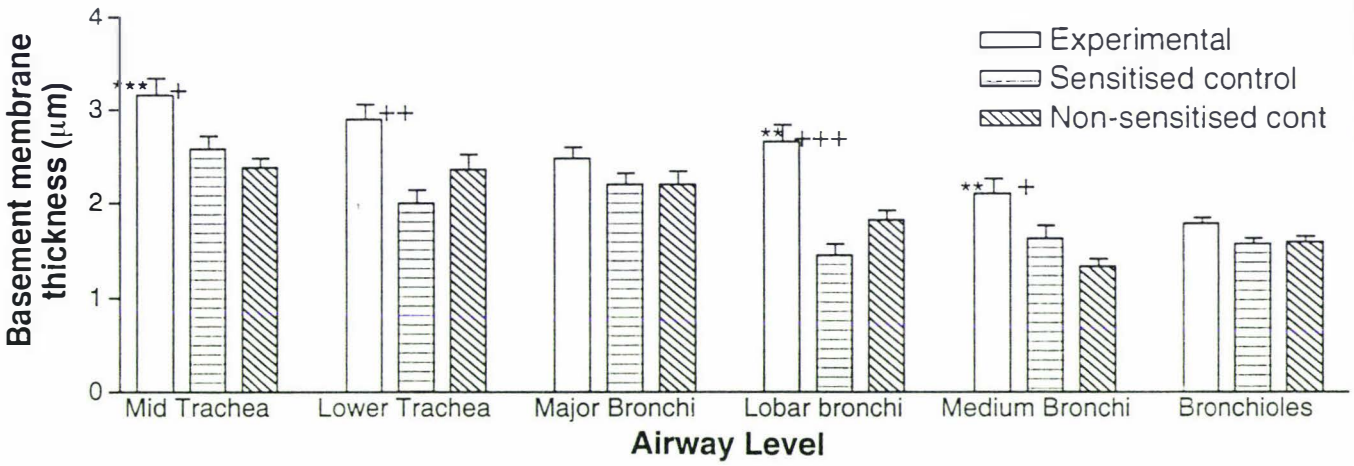


Figure 4.10. Comparison of the basement membrane thickness at different levels of respiratory tract of sheep sensitised and challenged with *Ascaris* antigen (experimental), Sensitised with *Ascaris* antigen and challenged with PBS (Sensitised control) and with no sensitisation and challenged with PBS (non-sensitised control) for 14 days. * = experimental vs non-sensitised control, + = experimental vs sensitised control, - = sensitised vs non-sensitised controls. */ +++/ --- = $p<0.0001$, **/ ++/ -- = $p<0.001$, */ +/- = $p<0.05$. Data presented as mean ± SEM.**

Eosinophil count.

The most abundant inflammatory cell type in all three groups was the eosinophil. In experimental sheep, the eosinophil count per microscopic field (10*40) was significantly higher ($p<0.001$) in most levels of the respiratory tract compared to control sheep (Fig. 4.11). Eosinophil infiltration was relatively pronounced in the trachea compared with the lower level of the respiratory tract (Fig. 4.11).

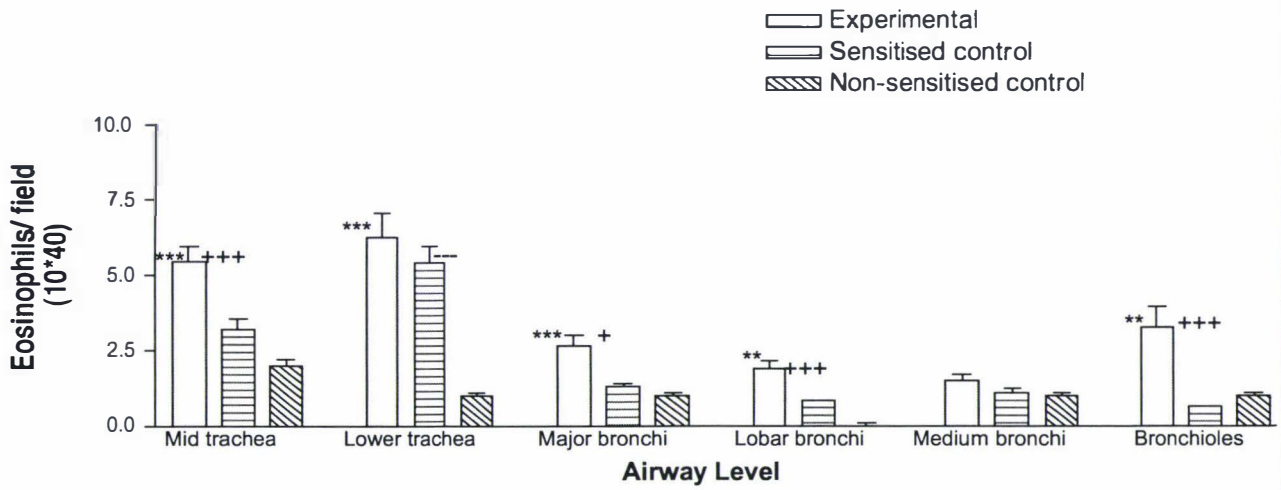


Figure 4.11. Comparison of the eosinophil counts/ field at different levels of respiratory tract of sheep sensitised and challenged with *Ascaris* antigen (experimental), sensitised with *Ascaris* antigen and challenged with PBS (sensitised control) and with no sensitisation and challenged with PBS (non-sensitised control) for 14 days. * = experimental vs non-sensitised control, + = experimental vs sensitised control, - = sensitised vs non-sensitised controls. */ +++/ --- = $p<0.001$, **/ ++/ -- = $p<0.01$, */ +/- = $p<0.05$. Data presented as mean ± SEM.**

Goblet cell count

There was a significant increase ($P<0.01$) in goblet cell numbers in the epithelium of sheep exposed to antigen at all levels of the respiratory tract examined (Fig. 4.12 and 4.13). This increase was highest in the upper levels of the respiratory tract. One of the sensitised control sheep (airway response enhanced with antigen exposure and exposed PBS daily for 14 days) also showed an increase in goblet cells particularly in the lower trachea and bronchioles.

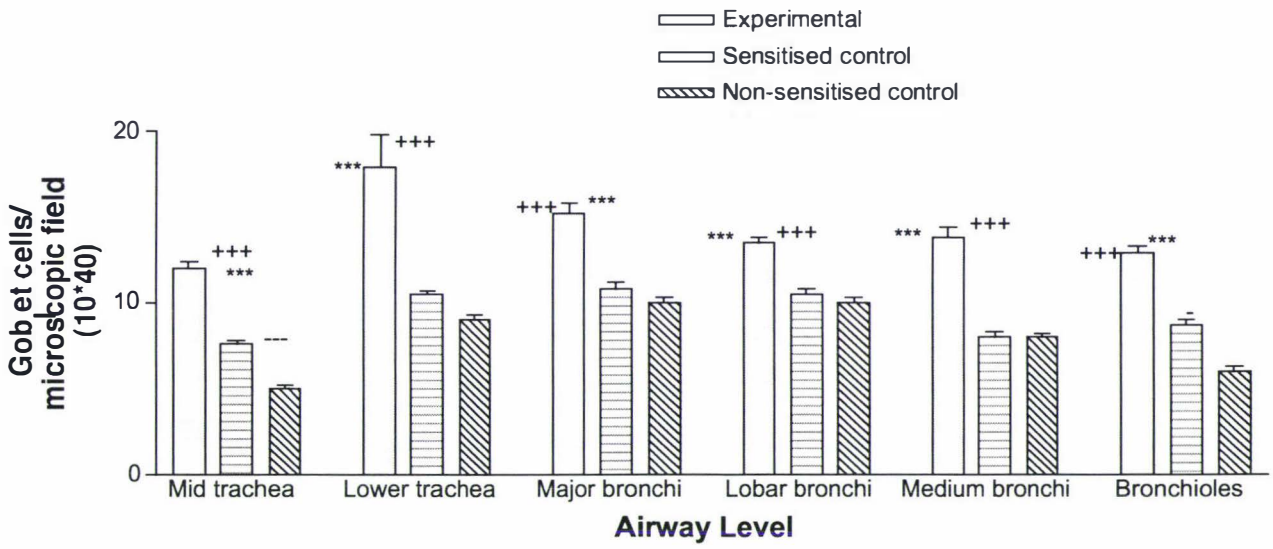
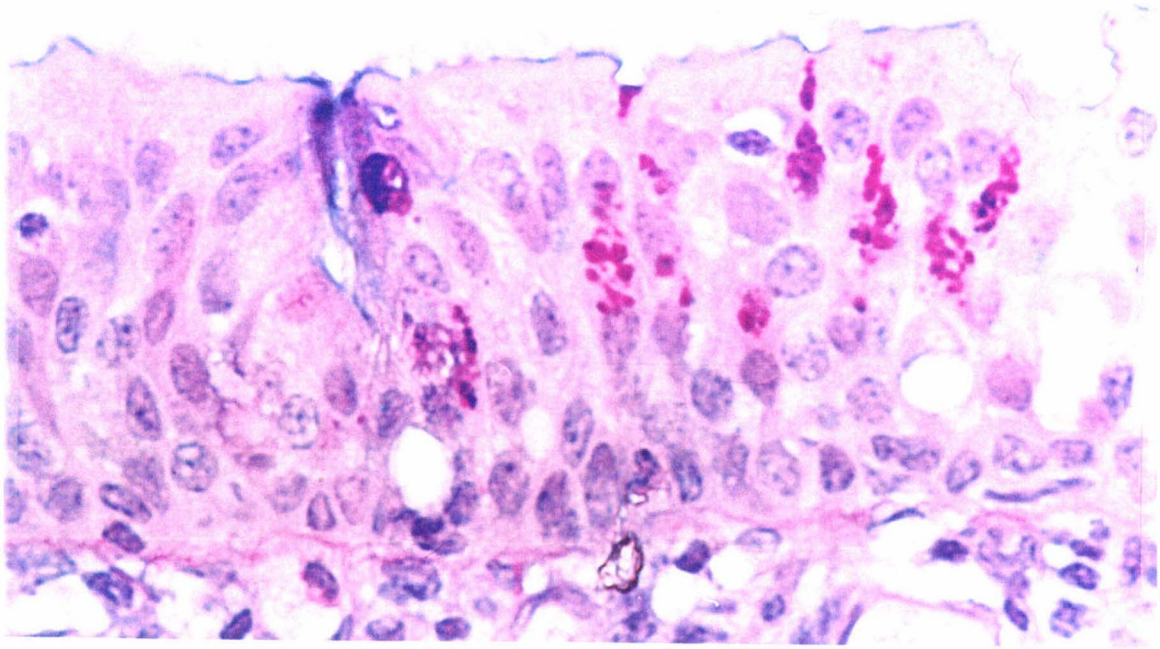


Figure 4.12. Goblet cell/microscopic field at various levels of respiratory tract of sheep sensitised and challenged with *Ascaris* antigen (experimental), sensitised with *Ascaris* antigen and challenged with PBS (sensitised control) and with no sensitisation and challenged with PBS (non-sensitised control) for 14 days. Experimental sheep showed goblet cell hyperplasia at all levels. * = experimental vs non-sensitised control, + = experimental vs sensitised control, - = sensitised vs non-sensitised controls. */ +++/ --- = p<0.001, **/ ++/ -- = p<0.01, */ +/- =p<0.05. Data presented as mean ± SEM.**

a



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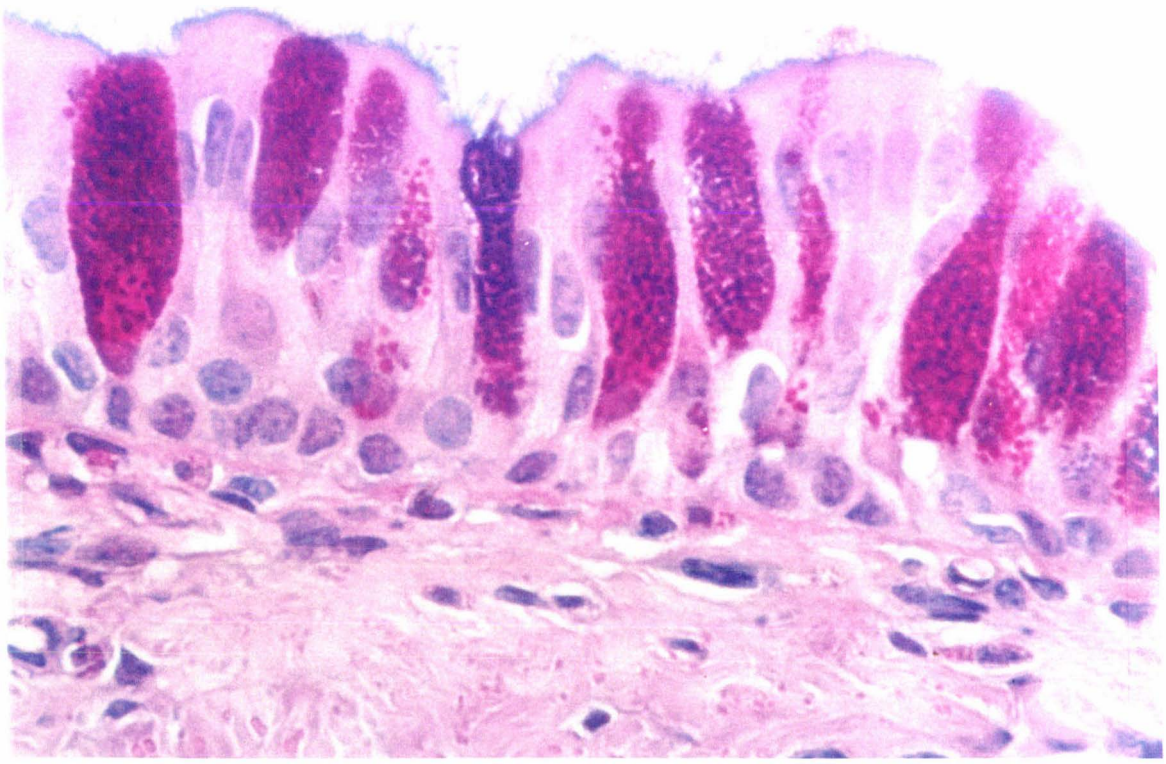

Figure 4.13 Epithelial goblet cells in the tracheal mucosa of PBS exposed (a) and antigen exposed sheep (b). The antigen exposed sheep regularly had more goblet cells compared to the control sheep. Alcian Blue/Periodic Acid Shift . *200.

Airway smooth muscle

The percentage smooth muscle (ASM%) in the airway wall cross sectional area of three groups of sheep is given in the figure 4.14. The ASM% was increased significantly ($P<0.01$) in the membranous bronchioles in the antigen exposed group. The ASM% was not significantly different in the bronchi.

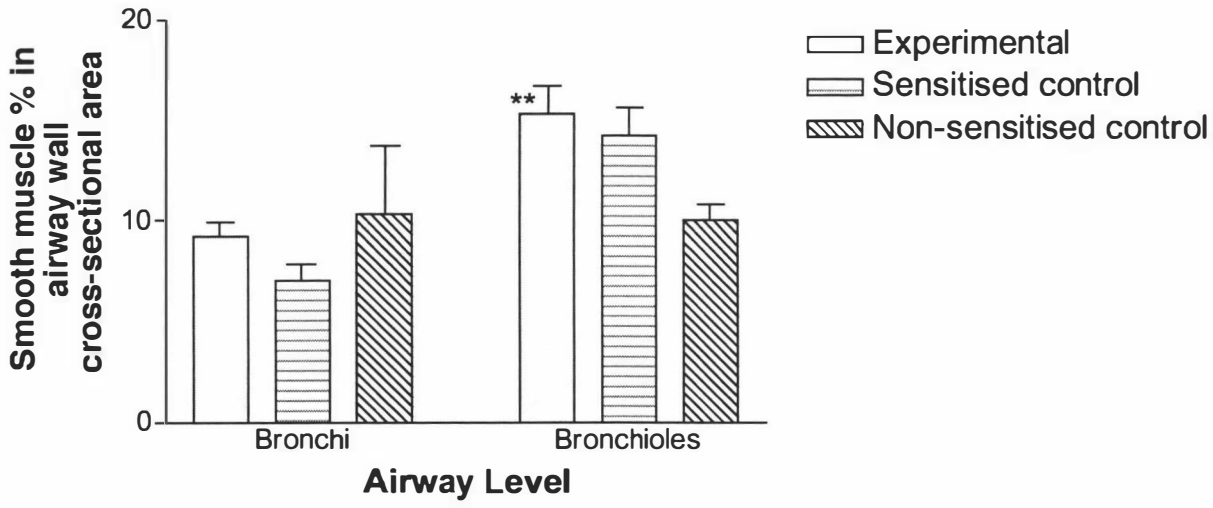

Figure 4.14. Percentage smooth muscle in the airway cross-sectional area in bronchi and bronchioles of sheep sensitised and challenged with *Ascaris* antigen (experimental), Sensitised with *Ascaris* antigen and challenged with PBS (Sensitised control) and with no sensitisation and challenged with PBS (non-sensitised control) for 14 days. * = experimental vs non-sensitised control. Data presented as mean ± SEM.

4.5 DISCUSSION

Present study indicates that the immediate cutaneous response to *Ascaris suum* antigen is common in sheep, a finding observed in several other studies (Wanner *et al.,* 1979; Okayama *et al.,* 1989; Chen,1990). The study also demonstrated that conscious sheep can be used to measure airway mechanics under sedation and that they will tolerate an endotracheal tube inserted through a tracheal stoma for a considerable length of time. The intermittent tracheotomy appeared to be a good alternative where the use of nasotracheal tube in conscious animals is not feasible.

Although allergic airway sensitivity to inhaled *Ascaris* antigen has been established in several species including sheep (Booth *et al*., 1970, Pare *et al*., 1976; Hirshman *et al*., 1980; Wanner and Abraham, 1982; Hamel *et al*., 1986; Okayama *et al*., 1989; Abraham, 1996), there are no reports of previous attempts to expose the sensitised sheep regularly on a daily basis to *Ascaris* antigen. However, there have been a limited number of reports where sensitised sheep was subjected to multiple exposures on a less frequent exposure protocols with mixed results (Kleeberger *et al*., 1985; Wagner *et al*., 1985; Bosse *et al*., 1987).

The magnitude of the increase in Raw in experimental sheep in this study was not consistent over the two weeks period. The reason for this observation is not clear. Raw changes in this study were less than those reported in previous studies (Wanner *et al*., 1979; Abraham *et al*., 1983; Deluhunt *et al*., 1984; Okayama *et al*., 1989; Chen *et al*., 1990). These discrepancies could be due to the differences in experimental protocols, methods used for the measurement and analysis of airway function or the inherent

pulmonary immunological status and morphology of the sheep studied (Wanner *et al.*, 1979; Yanta *et al.*, 1981). Although repeated antigen exposures have been shown to enhance the airway response to antigen in several species (Abraham *et al.*, 1981; Wagner *et al.*, 1985; Bosse *et al.*, 1987; Gundel *et al.*, 1990; Misawa and Chiba, 1993; Blyth *et al.*, 1996; Nabe *et al.*, 1997), there have also been reports where a diminished response was observed in immunised sheep (Kleeberger *et al.*, 1985) and guinea pigs (Andrew *et al.*, 1984). One explanation given for this diminished response is that repeated antigen challenge may deplete local specific immunoglobulin E and/or mast cell mediators thereby reducing the response to allergen (Cockcroft, 1983). Similarly, Kleeberger *et al.* (1985) found that 21% reduction in mast cell density in sheep one week after the final (5^{th}) fortnightly antigen exposure and suggested this as the possible reason for diminished response that was observed. The reduction in mast cell density in their study may have been sufficient to reduce the quantity of mast-cell-derived mediators released by subsequent antigen exposures and thereby reducing the bronchial response. The Cdyn change in the present study is higher than the values previously reported in sheep (Abraham *et al.*, 1981, 1983; Kleeberger *et al.*, 1985) and comparable to values observed in the study by Chen *et al.* (1990). The same system was used to measure respiratory parameters in the current study to those in the study by Chen *et al.* (1990) and this could probably be the reason for the similarities observed in respiratory parameters. The change in Cdyn is thought to be mainly brought about changes in the peripheral airways (Wanner *et al.*, 1979). It is well known that, like human asthmatics, allergic sheep also develop a late airway response which begins 4-12 hours after the immediate response and usually maximal after 5-6 hours (Abraham *et al.*, 1983; Abraham, 1996). The late response was not examined in this study during biweekly sensitisation or continuous daily challenge.

Although blood gas analyses were not performed in this study, the increase in the pulmonary blood pressure during the last day of antigen exposure indicated that the animals may have been hypoxic during antigen exposure. An increase in pulmonary blood pressure has also been observed in sheep breathing 13% O_2 during airway challenge with *Ascaris suum* antigen (Kung *et al.,* 1980). Similarly, Sylvester *et al.* (1979) found that in the constantly perfused pig lung pulmonary arterial oxygen tension progressively increased with decreasing alveolar oxygen tension. Systemic hypoxia has been observed in severe asthmatic episodes in humans (McFadden and Lyones, 1968; Hori, 1985).

The congestion of the right lung in some of the sheep could possibly be due to the fact that sheep were left on lateral recumbency through out the last day of the experiment. The presence of bloody thick mucus in the nasal cavity of one of the sheep could possible be a result of tissue damage caused by the insertion of oesophageal balloon catheter.

Damage and loss of airway epithelial cells are common pathological features in human asthma (Dunnill, *et al.,* 1969; Jeffery *et al.,* 1989). Although repeated exposure to antigen can be expected to cause damage to the epithelium, there was no obvious damage or epithelial sloughing of the airways in any of the sheep used in this study. The epithelium was significantly thinner in antigen-exposed sheep in the mid trachea, lobar bronchi and bronchioles. Although thinning of airway epithelium is not considered a common feature of airways in patients with mild asthma (Lozewics *et al.,* 1990), it has previously been reported in some atopic asthmatics (Jeffery *et al.,* 1989). A thinner epithelium has been reported in sheep challenged with *Ascaris suum* antigen (Chen, 1990) and in lobar bronchi of dogs with airway hyperresponsiveness (Yanta *et al.*, 1981). The epithelium forms a

continuous barrier separating the deeper tissues of the airways from irritants, allergens and mediators in the airway lumen. Because of the evidence of epithelial damage in asthma, the protective effect of this barrier could be expected to be impaired, increasing the accessibility of irritants to subepithelial tissues (Jacoby, 1997). The variations in airway responsiveness could reflect the variations in the thickness of this barrier (Yanta *et al.*, 1981). However, this hypothesis is based only on the physical thickness of the barrier. Other factors such as permeability may also be important in determining the response to inhaled mediators (Yanta *et al.,* 1981). Opening of the tight junctions is an important factor in the epithelial permeability (Boucher *et al.*, 1980). It has been shown that injuries to the epithelium caused by agents such as cigarette smoke exposure can increase mucosal permeability in humans (Jones *et al.*, 1980) and animals (Simani *et al.*, 1974, Boucher *et al.*, 1980). This may be related to the changes in tight junctions (Simani *et al.*, 1974, Boucher *et al.*, 1980).

One of the commonly reported abnormalities of human asthma is the thickening of the reticular layer beneath the epithelial basement membrane. However, there are only a few reports on this thickening of the "pseudo-basement membrane" in animal models. There have been no reports to date on thickening of basement membrane in allergic sheep. It has been claimed that thickening of airway basement membrane is rarely seen in sheep (Maurer *et al.,* 1981). However, in the current study, the antigen-exposed sheep showed a thicker PBM in the trachea, medium and lobar bronchi (Fig. 4.10). These observations indicate that repeated airway challenge of sheep with *Ascari suum* antigen can be used as a model to study features airway wall remodelling observed in human asthma.

The inflammatory cell infiltration in human asthma consists predominantly of eosinophils (Cutz *et al.*, 1978; Frigas *et al.*, 1986; Beasley *et al.*, 1989; Smith. 1992; Caroll *et al.*, 1996; Oddera *et al.*, 1998; Vignola *et al.*, 1998). However, neutrophils were seen as the major cell type in acute asthma in the study by Sur *et al*, (1993). Eosinophils have been identified as the major type of inflammatory cells in experimental asthma in animal models such as the sheep (Abraham *et al.*, 1985; 1988), primates (Gundel *et al.*, 1990) and rodents (Blyth *et al*, 1996, Yoshikawa *et al.*, 1996; Kung, 1998). In the present study, antigen-exposed sheep had a significantly higher eosinophil cell count in all levels of the respiratory tract examined. However, eosinophils are not the only inflammatory cell type involved in experimental asthma in sheep. Lanes *et al.* (1986) observed that a higher number of eosinophils in broncho-alveolar lavage (BAL) fluid of sheep with an acute response and a higher number of neutrophils in sheep with a dual response 24 hours after the antigen challenge. In another study, Okayama *et al.* (1989) reported that allergic sheep showed an increase in neutrophils in BAL fluid. In the present study, neutrophils were only observed occasionally.

Goblet cell hyperplasia has been described in the airways of asthmatics with varying degree of severity of the disease (Salvato, 1968; Dunnill *et al.*, 1969; Cutz *et al.*, 1978, Hogg, 1984; Aikawa *et al.*, 1992; Laitinen *et al.*, 1993; Laitinen and Laitinen, 1995). Although this has not been quantitatively confirmed in biopsies from patients with mild asthma (Lozewics *et al.*, 1990), it is described as the most discernible finding in the airway epithelium of newly diagnosed asthmatics (Laitinen *et al.*, 1993). Normal human airway epithelium has approximately 3-5 ciliated cells to each goblet cell (Rhodin, 1966; McDowell *et al.*, 1978). However, in asthma number of goblet cells greatly exceeds the

number of ciliated cells (Laitinen and Laitinen, 1992). Antigen exposure in the present study caused a significant increase in the number of goblet cells in the airway epithelium (Fig. 4.12). Goblet cell hyperplasia has also been observed in airways of hyperresponsive dogs (Yanta *et al.,* 1981), ovalbumin-exposed mice (Blyth *et al.*, 1996) and in cats exposed to *Ascaris suum* antigen (Padrid *et al.,* 1995). Goblet cell hyperplasia may play an important role in mucus hypersecretion and thereby airway obstruction with mucous plugs in severe asthma (Hogg, 1984; Jacoby, 1997; Tamaoki *et al.*, 1997; Lou *et al.,* 1998).

The percentage smooth of muscle in cross sectional airway wall area was significantly higher in membranous bronchioles of antigen-exposed sheep in the present study. An increase in smooth muscle thickness of the airways of patients who died of asthma has been reported by several authors (Huber and Koessler, 1922; Dunnill *et al.*, 1969; Cutz *et al.,* 1978; Carroll. *et al.,* 1993; Redington and Howarth, 1997). Similarly, an increase in the amount of smooth muscle has been reported in animal models of asthma including sheep (Chen, 1990; Sapienza *et al.*, 1991; Padrid *et al.*, 1995; Bai, 1998). In many of these studies the amount of smooth muscle may have been over-estimated since the airways of asthmatics are usually contracted and narrowed at post-mortem examinations (Seow *et al.*, 1998). During airway narrowing, the external diameter of the airway will decrease, albeit slightly less than internal diameter (Seow *et al.*, 1998). In contrast to the accepted view, some studies on subjects who had asthma but died of other causes suggest that there is no significant increase in the amount of smooth muscle in airways (Sobonya, 1984; Carroll *et al.*, 1993; Thomson *et al.* 1996; Thomson and Schellenberg, 1998). However, on closer examination of data from the study by Sobonya (1984), only very small airways were studied and when the muscle in the bronchi walls was expressed as percentage of wall area, there was an increase in asthmatic subjects (Black and Johnson, 1996).

It has been suggested that this increase in airway smooth muscle can account for the excessive airway narrowing seen in asthma (Moreno *et al.,* 1986; James *et al.*, 1989). An increase in the amount of smooth muscle results in a thicker airway wall. It has been proposed that, for a similar degree of muscle shortening, considerably greater luminal narrowing can result in an airway with a thicker wall than in a normal airway (Moreno *et al.,* 1986; James *et al.,* 1989; Hogg, 1993). Lambert *et al.* (1993) using a mathematical model have attempted to determine the importance of increased smooth muscle mass as a contributor to exaggerated airway narrowing. They developed a computer model in which they were able to examine the impact of theoretical changes in components of airway wall on airway narrowing and concluded that smooth muscle mass may be the single most important contributory factor to exaggerated airway narrowing. However, the assumptions made in this study (such as that the maximal airway smooth muscle tension remained constant) remain to be tested.

Although all the functional and morphological changes studied are not significantly different between anigen-exposed sheep and control sheep, most of them are similar to those seen in human asthmatics.

CHAPTER 5

CONCLUSIONS

An ideal model of human asthma should have most, if not all, the characteristics of bronchial asthma. The purpose of developing such an animal model is to reproduce the human disease in order to study its pathophysiology and pathogenesis.

The animal models discussed in the chapter 3 do not exhibit all the features of human asthma. Particularly the 'pseudo-basement membrane' thickening that occurs in chronic asthma and alters the mechanical properties of the airways during bronchospasms has only been reported in rodents (Blyth *et al.,* 1996; Palmans *et al.,* 1997). Although eosinophilic airway inflammation and an increase in sensitivity and responsiveness to antigen challenge have been established in several animal models of asthma, repeated challenge with antigen used in the current study was required to reproduce airway wall remodelling seen in human asthma (Bai. 1998).

Although the *Ascaris suum* sensitised sheep is regarded by many authors as the closest animal model of human asthma (see:- Abraham, 1996, and references therein), its use to study the chronic effects of asthma was restricted in that it did not have the structural changes associated with asthma. The results of the morphometric studies of the present study showed that the sheep subjected to repeated antigen antigen exposure had a thinner epithelium with increased number of goblet cells and eosinophils, higher percentage of smooth muscle in the airway wall cross-section and, more importantly, the thickened PBM in some levels of the respiratory tract. The structural changes produced in this study extend the use of the sheep as an animal model of human asthma. However, this increase

in the thickness of 'pseudo-basement membrane', which is possibly the most interesting new feature of the model, was not consistent throughout the respiratory tract and more selective procedures such as immunocytochemistry using specific antibodies to its constituents should be used to establish this observation.

The results of this study also demonstrated that the immediate skin and airway responses to *Ascaris suum* antigen are similar to that which occur in human asthma. Sensitised sheep respond to an antigen challenge to the respiratory system with an increase in Raw and decrease in Cdyn. Concurrent with these airway changes, the sheep also showed an increase in right ventricular work-load which was taken as an indication that sheep were hypoxic during the antigen challenge.

Although not examined in this study, the late airway response is an important feature in human asthma. It has been observed in several studies overseas that most of the sheep with an acute airway response also develop a late response 6-8 hours post exposure (Abraham *et al.*, 1983; Lanes *et al.*, 1986; Okayama *et al.*, 1989). Further studies to establish this feature using local sheep could provide additional information on this phenomena.

Because of its advantages as an experimental model, sheep have been used as a model of asthma in several countries. The ability to use sheep in the conscious state avoids the unwanted side effects caused by the use of general anaesthesia. Since more than fifty percent of the sheep tested for the study had a positive skin reaction to the antigen both control and test animals are readily available in New Zealand. Sheep are reasonably cheap

and easily accessible in New Zealand and therefore provide an excellent choice of a model for human asthma and other respiratory diseases.

Animal models have played an important role in understanding the pathogenesis and physiological changes that occur in asthma. Studies using animal models will lead to new ways of intervening in the pathogenesis of the disease. All the airway physiological functions and morphological changes observed in sheep in this study have been well documented in human asthmatics and are likely to contribute individually and/or in combination to the development of asthma.

Since many of the morphological changes observed in human asthmatic airways can be reproduced in the sheep model that has been repeatedly challenged with *Ascaris suum* antigen, this model has the potential use to test theories about the airway wall remodelling seen in human asthma and/or the effect of medical intervention on this chronic response.

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