THE EFFECTS OF ACUTE AND CHRONIC ANTIGEN INHALATION ON AIRWAY INFLAMMATION AND FUNCTION AND ANTI-INFLAMMATORY DRUG INTERVENTIONS

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RHYS EVANS

DEPARTMENT OF PHARMACOLOGY

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SUMMARY

Asthma is a chronic inflammatory disease characterised by airway inflammation, bronchoconstriction, airway hyperresponsiveness (AHR) and airway remodelling. Most models of asthma focus on acute allergen challenges, where airway remodelling is absent. The thesis aimed to compare acute and chronic allergen challenge models of asthma and analyse the effects of anti-inflammatory drugs on these models.

Acute and chronic challenges with ovalbumin in conscious guinea pigs and mice caused impared lung function, measured as specific airway conductance (sG_{aw}) and enhanced pause (P_{enh}), respectively. This was characterised by early (EAR) and late (LAR) asthmatic responses, AHR and cellular influx. Multiple challenges with ovalbumin caused airway remodelling distinguished by increased bronchiolar collagen and goblet cells compared to control. No airway remodelling was observed in acute ovalbumin models.

Treatment with the corticosteroid, fluticasone propionate (FP), attenuated the LAR, AHR and cellular influx in all models. In both chronic ovalbumin models, FP treatment partially reversed airway remodelling, though not to naïve levels. This was also the case with treatment with the phosphodiesterase IV inhibitor, roflumilast. However, roflumilast also attenuated the EAR. Treatment with the iNOS inhibitor, GW274150F, reduced the LAR and AHR and showed some inhibition of cellular influx in the acute ovalbumin challenged animals. However, GW274150F was ineffective in chronic ovalbumin models. Lung oedema, assessed by magnetic resonance imaging in acute and chronic ovalbumin challenged guinea pigs, was increased and correlated temporarily with the LAR. Dexamethasone treatment attenuated the level of oedema though not to control levels.

Acute ovalbumin challenged models showed airway functional changes which were partially resolved with drug treatment. Chronic ovalbumin challenges provoked lung functional and structural changes which were attenuated by FP and roflumilast but not GW274150F. As GW274150F proved ineffective in clinical trials, the data in this thesis suggests that chronic ovalbumin challenge animals are better pre-clinical models of asthma.

CONTENTS

ABBREVIATIONS

3-NT = 3-nitrotyrosine

5'AMP = 5'adenosine monophosphate

AB/PAS = Alcian blue/periodic acid Schiff

AHR = Airway hyperresponsiveness

$Al(OH)_3$ = Aluminium hydroxide

APC = Antigen presenting cell

ATP = Adenosine trisphosphate

AUC = Area under the curve

BAL = Bronchoalveolar lavage

cAMP = Cyclic adenosine monophosphate

CBP = CREB binding protein

cGMP = Cyclic guanosine monophosphate

COX = Cyclooxygenase

$cPLA_2$ = Cytoplasmic phospholipase A_2

CREB = Cyclic adenosine monophosphate response element-binding protein

CysLT = Cysteinyl Leukotriene

DMSO = Dimethyl sulfoxide

EAR = Early asthmatic response

ECM = Extracellular matrix

ECP = Eosinophil cationic protein

EDN = Eosinophil derived neurotoxin

EGF = Epidermal growth factor

ELISA = Enzyme-linked immunosorbant assay

eNOS = Endothelial nitric oxide synthase

EPO = Eosinophil peroxidase

ET-1 = Endothelin-1

EtOH = Ethanol

FACS = Fluorescent-activated cell sorting

FE_{NO} = Level of nitric oxide in exhaled air

FEV_1 = Forced expired volume over one second of expiration

FP = Fluticasone propionate

FWBP = Flow whole-body plethysmograph

G_{aw} = Airway conductance

GM-CSF = Granulocyte-macrophage colony stimulating factor

GR = Glucocorticoid receptor

GRE = Glucocorticoid response element

GTP = Guanosine trisphosphate

HAT = Histone acetyltransferase

HDAC = Histone deacetylase

HRP = Horse radish peroxidase

ICAM = Intercellular adhesion molecule

IFN = Interferon

Ig = Immunoglobulin

IκBα = Inhibitor of kappa B - α

IKK2 = Inhibitor of IκB kinase-2

IL = Interleukin

IMS = Industrial methylated spirit

iNOS = Inducible nitric oxide synthase

I.P. = Intraperitoneal

LAR = Late asthmatic response

LPS = Lipopolysaccharide

LT = Leukotriene

MBP = Major basic protein

MCP = Monocyte chemotactic protein

mDC = Myeloid dendritic cell

MHC = Major histocompatibility complex

MIP = Macrophage Inflammatory Protein

MMP = Matrix metalloproteinases

MRI = Magnetic resonance imaging

NADPH = Nicotinamide adenine dinucleotide phosphate (reduced)

NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF = Nerve growth factor

nNOS = Neuronal nitric oxide synthase

NO = Nitric oxide

NOS = Nitric oxide synthase

O_2^- = Superoxide oxygen molecule

$OONO^-$ = Peroxynitrite

OPD = O-phenylenediamine dihydrochloride

OVA = Ovalbumin

PBS = Phosphate buffered saline

PBST = Phosphate buffered saline with tween

pDC = Plasmacytoid dendritic cell

PDE = Phosphodiesterase

PDGF = Platelet-derived growth factor

P_{enh} = Enhanced pause

PICP = Procollagen type I C-terminal peptide

PWBP = Pressure whole-body plethysmograph

RANTES = Regulated upon activation, normal T-cell expressed and secreted

R_{aw} = Airway resistance

RF = Radio frequency

S.E.M. = Standard error of the mean

sG_{aw} = Specific airway conductance

Tc CELL = T cytotoxic cell

TGF = Transforming growth factor

TGV = Thoracic gas volume

Th CELL = T helper cell

TNF = Tumor necrosis factor

TSLP = Thymic stromal lumphopoietin

VEGF = Vascular endothelial growth factor

Chapter 1

General Introduction

CHAPTER 1

1.1 ASTHMA

1.1.1 DEFINITION

Asthma has proven to be frustratingly difficult to define in an accurate and precise manner. The first definition came from Hippocrates (460-377 BC) who used the Greek word 'asthmaino' meaning panting or gasping. However, a link between asthma and bronchospasm was not made until Galen (130-201 AD). A definition of asthma continued to evolve. The most commonly quoted definition of asthma comes from the National Asthma Education and Prevention Program Export Panel Report 2, which described asthma as:

A chronic inflammatory disorder of the airways in which many cells and cellular elements play a role, in particular, mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough, particularly at night and in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli. (1987)

However, this does not begin to describe the complexity of the disease and it is now known not to be completely accurate as several patients with asthma show extremely poor reversibility (Bousquet *et al.*, 2000). It is believed that around 5.4 million Britons are receiving treated for asthma and there is a person with asthma in one of every five households (Asthma UK). It is estimated that the economic costs of asthma will exceed those of tuberculosis and HIV-AIDS combined (van Schalkwyk *et al.*, 2005).

1.1.2 PATHOPHYSIOLOGY

Asthma can be classified into two different forms, intrinsic or extrinsic asthma (Wardlaw *et al.*, 2002). Intrinsic, or non-atopic, asthma is triggered by something inside the body, not by an allergy. Respiratory infections, exercise, cold weather and certain drugs, such

as non-steroidal anti-inflammatory drugs, can cause a reflex in the upper airways leading to asthmatic symptoms. This form of the disease is not caused by hypersensitivity to an antigen or allergen and levels of immunoglobulin E (IgE) remain normal in comparison to non-asthmatics.

In contrast, extrinsic, or atopic/allergic, asthma is IgE mediated. It is commonly diagnosed early in life and is a result of hypersensitivity to an inhaled antigen such as dust mites, pollen or ovalbumin. Individuals who suffer from extrinsic asthma become sensitive to the allergen; whenever they encounter the allergen again asthmatic symptoms occur. As a result extrinsic asthma is described as being caused by two phases, the sensitisation phase and the effector phase (figure1).

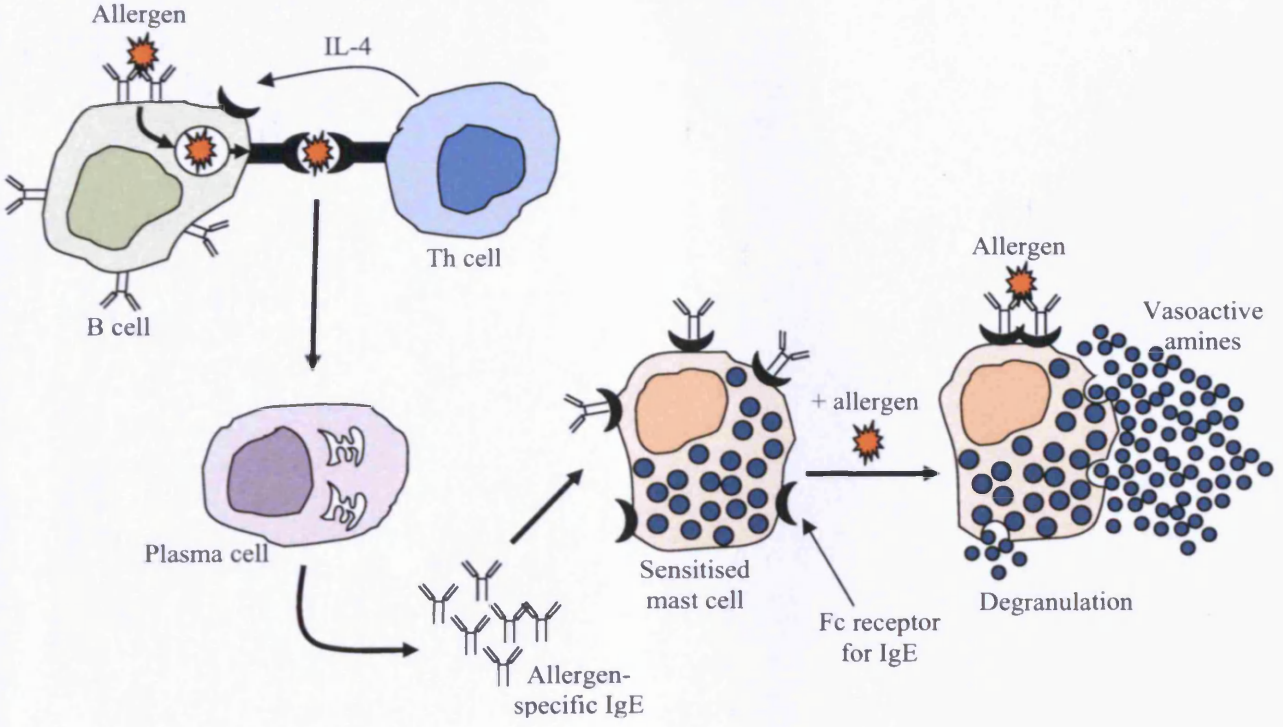


Figure 1 - Schematic diagram of how sensitisation to an allergen occurs and the effect that a repeated exposure to the allergen has. Picture adapted from Goldsby *et al.*, (2000). IgE = Immunoglobulin E; Th cell = T helper cell.

1.1.3 SENSITISATION PHASE

Circulating allergens can be recognised as foreign by antigen presenting cells (APC), such as macrophages, B cells and dendritic cells. IgE on the surface of the APC captures

the antigen and causes it to be internalised by phagocytosis or endocytosis. The antigen is then proteolysed into peptides and part of it is displayed on the membrane bound major histocompatibility complex (MHC) class II on the surface of the APC (Banchereau & Steinman, 1998). The antigen is then recognised by naïve T cells which then become activated. Once activated, the naïve T cells will differentiate into T helper 1 (Th1) or T helper 2 (Th2) cells. This differentiation is triggered by interleukin-2 (IL-2). Naïve T cells release IL-2 and at the same time develop surface receptors specific for it, once the receptor is active the differentiation occurs. Whether the cell becomes Th1 or Th2 is dependent on the presence of the autocrine cytokines IL-12 and IL-4 respectively (Romagnani, 1997). The Th1 response mainly activates certain T cells and macrophages through a cytokine profile that supports inflammation. The Th2 response activates B cells and immune responses dependent on antibodies (Goldsby *et al.*, 2000). TheTh1 response is responsible for killing intracellular parasites and is more involved in autoimmune conditions (Berger, 2000). In asthmatic patients a trend is seen toward the differentiation of Th2 cells.

Active Th2 cells cause sensitisation to the allergen by releasing the cytokines IL-4 and IL-13. IL-4 and IL-13 stimulate the production of allergen-specific IgE from plasma cells. IL-4 also induces the expression of FcεRI, or high affinity allergen-specific IgE, receptors on the surface of mast cells and basophils. Monovalent binding of IgE causes increased FcεRI expression, increases mast cells resistance to apoptosis and can induce cytokine release (Knol, 2006). Once the allergen-specific IgE binds to these receptors sensitisation has occurred, therefore exposure to the same allergen would lead to an asthmatic response.

1.1.4 EFFECTOR PHASE

The asthmatic response following re-exposure of a specific allergen is two-fold. The first response is an immediate bronchoconstriction and is known as the early asthmatic response (EAR). The second bronchoconstriction does not occur until around seven hours after allergen exposure and is known as the late asthmatic response (LAR). The responses are a result of the re-exposed allergen binding to the membrane-bound IgE on the sensitised mast cells or basophils. The bound IgE interacts and cross-links with the IgE

that bound to the mast cell or basophil during the sensitisation phase. Cross-linking of bound IgE leads to surface receptor clustering which induces a cascade of tyrosine phosphorylation events resulting in the activation of phospholipase Cγ and ultimately causing degranulation of the mast cells or basophils (Knol, 2006). Mast cells and basophil degranulation occurs rapidly, within 60-300 seconds (Holmes 1999 referenced by John 2007). Mast cells contain many inflammatory mediators, including histamine, tryptase, prostaglandins, leukotrienes and cytokines, which are released when mast cells are degranulated. Some of these mediators, such as histamine, act on the local airway receptors causing the immediate bronchoconstriction or EAR. The LAR is caused by some of the other mediators chemoattracting inflammatory cells, such as macrophages, eosinophils, lymphocytes and neutrophils which in turn attract more inflammatory mediators and cause bronchoconstriction (Durham & Kay, 1985).

1.2 AIRWAY INFLAMMATION

Airway inflammation is one of the defining characteristics of asthma, it can be present even in mild asthma (Laitinen *et al.*, 1996). Along with airway hyperresponsiveness, airway inflammation contributes to the common symptoms of asthma - chest tightness, wheezing, cough and shortness of breath. However, there are other mechanisms such as the activation of airway sensory nerves which can contribute to these symptoms, especially cough (Nasra & Belvisi, 2009). It has been established that the extent of inflammation correlates with the severity of the disease (Walter & Holtzman, 2005). Elevation of the number of inflammatory cells in the airways of patients who die of an asthma attack (*status asthmaticus*) was observed by Barnes (1996). Although some inflammatory cells predominate in causing inflammation of the airways no single cell can account for the complex pathophysiology of asthma. Inflammation is caused by a complex cascade of events involving various inflammatory cells and mediators.

1.2.1 MAST CELLS

As previously mentioned mast cells play a key role in the sensitisation and effector phase of asthma. The number of mast cells found in the broncho-alveolar lavage (BAL) fluid of

asthmatics is two- to six-fold higher than found in non-asthmatics (Hamid *et al.*, 2003). The percentage of mast cells expressing the cytokines IL-4, IL-5 and tumour necrosis factor (TNF)-α is also increased in asthmatics (Bradding *et al.*, 1994). These cytokines cause increased Th2/reduced Th1 cell production, reduced apoptosis and bronchial hyperresponsiveness respectively (Barnes, 2002a).

Mast cells contain cytoplasmic granules in which mediators such as histamine, tryptase, prostaglandin D2 (PGD2) and leukotriene C4 (LTC4) (Barnes, 2002a). The glycosaminoglycan, heparin is also stored by mast cells (Rose & Page, 2004). These mediators are released when the mast cells become degranulated. Degranulation is caused by the binding of an allergen to the allergen-specific IgE bound to the mast cell or by other indirect stimuli, such as in intrinsic asthma (Barnes, 1996). The released mediators cause bronchoconstriction, increased vascular permeability and leukocyte recruitment and activation, (Galli, 2000) all of which contribute towards airway inflammation.

1.2.2 MACROPHAGES

Macrophages are derived from blood monocytes and are the most prominent cell found in the BAL fluid of both asthmatic and non-asthmatic patients (Hamid *et al.*, 2003). As previously mentioned macrophages can act as an APC for presenting an allergen to a naïve T cell. Macrophages have the ability to play a role in both increasing and decreasing airway inflammation. They are able to release cytokines, which induce epithelial cells and fibroblasts to release chemoattractants and growth factors. Some of these chemoattractants such as RANTES (regulated upon activation, normal T-cell expressed and secreted) and monocyte chemotactic protein (MCP)-1 attract further macrophages and eosinophils. This would result in more macrophages acting as APCs and therefore inflammation would occur. On the other hand alveolar macrophages can have a suppressive effect on lymphocyte activity; however, this effect may be impaired after allergen exposure (Barnes, 1996; Hamid *et al.*, 2003).

1.2.3 EOSINOPHILS

Eosinophils play a predominant role in the late asthmatic response (Goldsby *et al.*, 2000). The number of eosinophils in the airways has been correlated to the severity of asthma

(Hamid *et al.*, 2003). In the bronchoalveolar lavage (BAL) fluid of sensitised and allergen challenged guinea pigs the number of eosinophils in the airways are shown to progressively increase after an hour until a peak of 24 hours (Toward & Broadley, 2004). This correlates with the late asthmatic response. Eosinophil recruitment is believed to occur by two exclusive pathways. The first is through the release of TNF-α by degraded mast cells, this acts on epithelial and endothelial cells causing the release of eosinophil-directed cytokines, such as granulocyte macrophage colony-stimulating factor (GM-CSF), and chemokines, such as eotaxin (Barnes, 2002a). The second pathway involves the release of IL-5 by recently activated naïve T cells. IL-5 provides the signal for eosinophils to mobilise from the bone marrow and it also enhances the chemoattractant potential of tissue chemokines (Barnes, 2002a). Once in tissue, eosinophils can amplify the inflammatory cascade by producing their own cytokines (Rothenberg, 1998).

Eosinophils are not very phagocytic but contain granules that hold highly charged and basic proteins. IL-3, IL-5, GM-CSF, IL-1β and platelet activating factor can degranulate eosinophils (Chapoval *et al.*, 1999) causing the release of the granule proteins. There are four types of granule proteins released from eosinophils; these are major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and eosinophil peroxidase (EPO). MBP, ECP and EPO are all able to cause histamine release from mast cells. ECP is also able to damage target cells by causing voltage-insensitive, ion non-selective pores therefore allowing an influx of potentially damaging ions such as calcium (Rothenberg, 1998). MBP is believed to have a pathogenic role in asthma as it has been shown to cause damage to airway epithelial cells and levels of MBP in the airway decline in correlation with improved airway function (Barnes, 2002a).

In addition to basic proteins, eosinophils can also produce LTC4, Platelet Activating Factor (PAF) (Chapoval *et al.*, 1999), reactive oxygen species (ROS), transforming growth factor (TGF) α and β, TNF- α, IL-1, IL-3, IL-5, IL-6 and GM-CSF (Busse & Lemanske, 2001) all of which can contribute to airway inflammation via various mechanisms such as airway smooth muscle contraction, increased vascular permeability and mucus secretion. They are also able to chemoattract more inflammatory cells by releasing the chemokines, eotaxin, Macrophage Inflammatory Protein (MIP)-1a and RANTES (Nickel *et al.*, 1999). A role for eosinophils in airway remodelling may also

exist as they produce fibrogenic growth factors, elastase and matrix metalloproteinases (MMP) (Bousquet *et al.*, 2000).

1.2.4 T-LYMPHOCYTES

T-lymphocytes are essential in the asthmatic inflammatory response, they can be grouped depending whether they have a CD4 or CD8 surface cell marker. Generally CD4+ cells are T helper (Th) cells, whereas CD8+ are T cytotoxic (Tc) cells. It is the Th cells that are presented an allergen by an APC and as a result become active. As previously mentioned Th cells can be further divided into Th1 and Th2 cells depending on the presence of various cytokines. Th2 cells are more frequently found in asthmatics and it is these cells that release IL-4 and IL-13 which lead to IgE release from plasma cells. The cytokines that T-lymphocytes release can also serve to attract and activated other cell types. Bronchial hyperresponsiveness and eosinophil number and activation has been shown to correlate with activated CD4+ lymphocytes (Robinson *et al.*, 1993). Lymphocytes may play a role in airway remodelling as there is some evidence that they stimulate the synthesis of matrix proteins such as collagen I, III and fibronectin (Postlethwaite *et al.*, 1992; Postlethwaite & Seyer, 1991).

1.2.5 NEUTROPHILS

Neutrophils do not have a clear role in asthma. Some studies have shown a rapid influx of neutrophils into the airways following a stimulus challenge in asthma models (Fabbri *et al.*, 1984; Matsumoto *et al.*, 1999). This influx was associated with bronchial hyperresponsiveness suggesting there is a potential role for neutrophils. Evidence of a neutrophilic asthma, where neutrophils predominate over eosinophils, exists (Wardlaw *et al.*, 2002) though results are controversial. Neutrophils contain reactive oxygen species and proteases, that are both capable of causing damage to the airways if released. Therefore, neutrophils have the potential to cause airway inflammation. In sensitised and allergen challenged guinea pigs airway neutrophils were shown to increase after an hour, peak at the end of the early asthmatic response and had subsided after 12 hours (Toward & Broadley, 2004) suggesting neutrophils may play a role in the early phase bronchoconstriction associated with asthma. There is a strong association between

neutrophilic inflammation of the airways and severe asthma (Jatakanon *et al.*, 1999; Wenzel *et al.*, 1997), steroid resistant asthma (Pavord *et al.*, 1999; Wenzel *et al.*, 1999) and the sudden onset of fatal asthma (Sur *et al.*, 1993).

1.2.6 DENDRITIC CELLS

The role of dendritic cells is primarily as APCs. As dendritic cells express high levels of MHC class II molecules they are more potent APCs than macrophages and B cells (Goldsby *et al.*, 2000). There are several different subtypes of dentritic cells, however, the two that have the most prominent role in asthma are myeloid dentritic cells (mDC) and plasmacytoid dendritic cells (pDC) (Barnes, 2002a). These cells are crucial in determining the outcome of allergen encounter in the lung (Lambrecht, 2008). Under normal conditions the outcome of inhalation of a harmless allergen would lead to immunological tolerance. The allergen would be taken up by both mDCs and pDCs. Under normal conditions the mDCs would be partly mature and the T-cell response they induce is characterised by cell division and not differentiation to effector cells (Barnes, 2002a) and therefore result in immunological tolerance. This process is regulated by pDCs, cyclooxygenase-2 derived prostaglandins and complement activation (Barnes, 2002a).

Th2 sensitisation to an allergen can also be caused by mDCs (Lambrecht, 2008). For this to occur it seems that mDCs need to be activated, or matured (Barnes, 2002a). Several factors can cause this. Toll-like receptor agonists, such as endotoxin, adjuvants, viral infection, overexpression of GM-CSF and cigarette smoke have all been implicated in causing Th2 sensitisation through activation of mDCs (Barnes, 2002a). Activation of mDCs can be direct, by factors such as low dose toll-like receptor agonists or adjuvants, or indirect (Barnes, 2002a). Indirect activation can be caused by factors leading to epithelial activation, e.g. cigarette smoke, increasing the mDC/pDC balance, e.g. GM-CSF overexpression, or by the loss of pDC tolerogenic function, e.g. viral infection (Barnes, 2002a).

Dendritic cells may also have a role in the effector phase of asthma as it appears that 'inflammatory' dendritic cells are necessary and sufficient for secondary immune responses to the allergen (Barnes, 2002a). The fact that dendritic cells have an

inflammatory role in asthma has led to the development of novel anti-allergic compounds aiming to combat this inflammatory effect (Lambrecht, 2008).

1.2.7 CYTOKINES AND GROWTH FACTORS

Cytokines and growth factors play a huge role in asthma, one that goes way beyond the scope of this thesis. Some have a pro-inflammatory role in asthma whereas some have the opposite. Table 1 describes the roles of some of the more important pro-inflammatory cytokines and growth factors in asthma.

Cytokine / Growth Factor	Effect in Asthma	Mechanism
IL-1β	Enhances disease	Increases inflammation
IL-4	Enhances disease	Increases IgE production and number of Th2 cells
IL-5	Enhances disease	Increases eosinophil number
IL-6	Enhances disease	Increases inflammation
IL-9	Enhances disease	Increases mast cell number
IL-10	Decreases disease	Decreases inflammation
IL-12	Reduces disease	Increases Th1 number
IL-13	Enhances disease	Increases IgE production and induces airway remodelling
IL-17	Enhances disease	Increases neutrophil number
IL-18	Reduces disease	Increases IFN-γ release
IL-25	Enhances disease	Increases Th2 number
EGF	Enhances disease	Induces mucus secretion
GM-CSF	Enhances disease	Increases eosinophil and neutrophil number
IFN-γ	Reduces disease	Decreases Th2 number
NGF	Enhances disease	Increases AHR
TGF-β	Enhances severe disease	Increases fibrosis
TNF-α	Enhances severe disease	Increases inflammation

TSLP	Enhances disease	Increases Th2 number
VEGF	Enhances disease	Induces angiogenesis

Table 1- A list of cytokines and growth factors involved in asthma and the response they cause. Adapted from (Barnes, 2008). IL = Interleukin; EGF = Epidermal growth factor; GM-CSF = Granulocyte-macrophage colony stimulating factor; IFN = Interferon; NGF = Nerve growth factor; TGF = Transforming growth factor; TNF = Tumour necrosis factor; TSLP = Thymic stromal lumphopoietin; VEGF = Vascular endothelial growth factor.

1.2.8 HISTAMINE

Histamine plays a fundamental role in causing airway inflammation and hyperresponsiveness in asthma. It is stored in, and then released from, mast cells through an IgE mediated pathway. Upon release, histamine constricts airway smooth muscle in the large and small airways, causes plasma exudation and bronchial vasodilation. Histamine mediates its effect through three subtypes of receptor, H_1, H_2 and H_3. Although all have been implicated in the airway response to histamine (Hill, 1990), it appears the main effect is through the H_1 receptor as this is the receptor that causes contraction of airway smooth muscle when active. The activation of macrophages and enhancement of eotaxin-induced chemotaxis of eosinophils are suggested as roles for histamine (Das *et al.*, 1997). More recently a histamine H4 receptor was identified and was suggested to have a role in the immune system (Zampeli & Tiligada, 2009). A H4 receptor antagonist, JNJ-7777120, has proved to be effective in animal models and is expected to enter clinical trials soon (Engelhardt *et al.*, 2009).

1.3 AIRWAY HYPERRESPONSIVENESS (AHR)

Asthmatic airways are prone to increased responsiveness to a variety of stimuli (1987). These stimuli are known as direct and indirect stimuli. Direct stimuli are pharmacological agents administered exogenously and act directly on specific bronchiolar smooth muscle receptors to cause constriction (O'Byrne *et al.*, 2009). These agents include histamine and methacholine which act on histamine (H_1) and muscarinic receptors respectively. Eicosinoid mediators such as prostaglandins, thromboxanes and leukotrienes can also

induce AHR (O'Byrne *et al.*, 2009). Indirect stimuli include natural stimulants such as cold air or exercise. Adenosine monophosphate (AMP) is also classified as an indirect stimulus. These stimulants indirectly cause a bronchoconstriction by causing the endogenous release of bronchoconstriction mediators from airway inflammatory cells (O'Byrne *et al.*, 2009). Direct and indirect stimuli contribute to the exacerbations of asthma by causing airway obstruction and bronchospasm. AHR is used as a test when diagnosing asthma. Subjects inhale a direct stimulus, such as histamine, and levels of bronchoconstriction are measured. Although non-asthmatics can respond to direct stimuli the dose required to elicit a response is far greater than would be required by asthmatics.

The term AHR encompasses both airway hyperreactivity and airway hypersensitivity. Airway hyperreactivity is commonly used erroneously to mean increased responsiveness to a variety of stimuli whereas it actual means a greater degree of closure in the airways (O'Connor *et al.*, 1999). Airway sensitivity is a decrease in the threshold of the airways to react to stimuli, therefore sensitive airways respond to a dose of stimuli that would have no effect on normal airways. Figure 2 demonstrates the shift to the left hypersensitive airways cause on a dose response curve and the increase in gradient that hyperreactive airways show. The figure of PC_{20} is the provocative concentration of the bronchoconstrictive agent required to cause 20% reduction in the forced expired volume over one second of expiration (FEV_1) (O'Connor *et al.*, 1999).

How AHR develops is not well known. There is evidence that an increased amount of MBP (released from eosinophils) leads to an increase in the number of damaged epithelial cells and an increase in AHR (Wardlaw *et al.*, 2002). It is possible that damage to the epithelial cells could lead to a greater exposure of irritant receptors situated on the airway nerves meaning that the airways become hyperresponsive to stimuli as there are a greater number of receptors for the stimuli to activate. These receptors are those activated by direct stimuli such as histamine (H_1), muscarinic and leukotriene receptors whose activation results in bronchoconstriction of the airway smooth muscle.

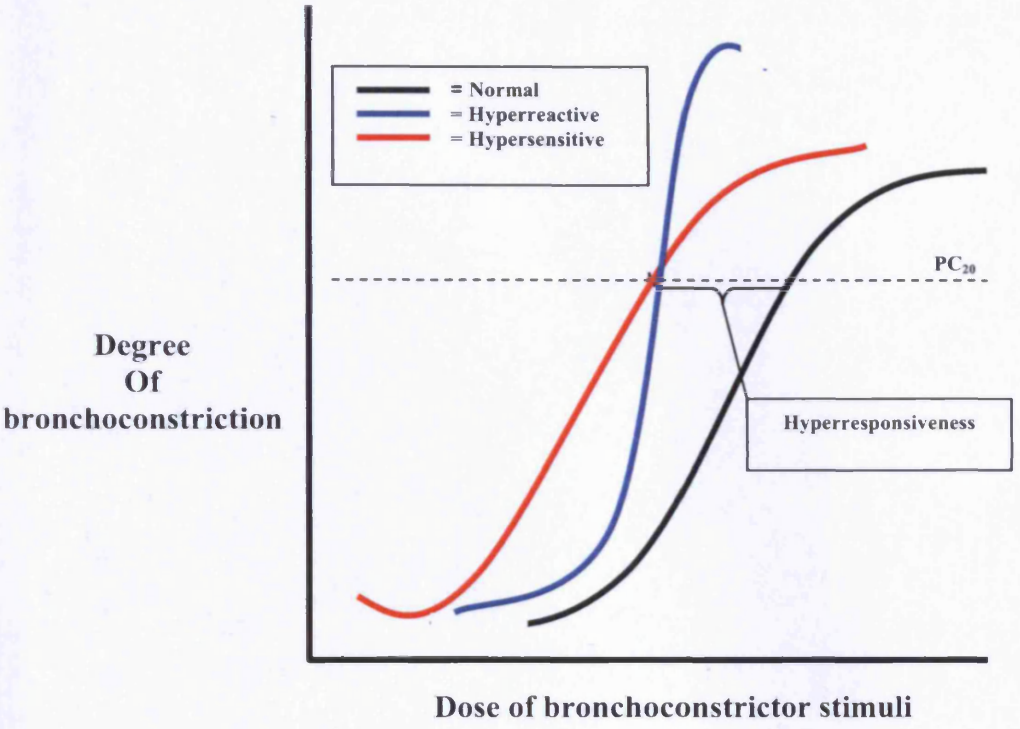


Figure 2 - Schematic diagram to demonstrate the differences between normal and asthmatic airways in response to an increasing dose of bronchoconstrictor agent. Figure adapted from (Lotvall *et al.*, 1998).

1.4 AIRWAY REMODELLING

The long-term inflammation caused by repeated asthmatic episodes and subsequent repair mechanisms can cause irreversible structural changes in the airways commonly seen as an increase in airway wall thickness (Bousquet *et al.*, 2000). Thickening has been observed in all layers of the asthmatic airway wall, a phenomenon known as 'airway remodelling' (Roberts, 1995). Hegele (2000) states that airway remodelling may exacerbate the chronicity and progression of asthma. This suggests that airway remodelling could be part of a vicious cycle that results in further thickening of the airway wall leading to more severe asthma.

The pathological changes of airway remodelling can be accounted for by four main changes in the airways (figure 3); subepithelial fibrosis, increased smooth muscle mass, goblet cell hyperplasia / metaplasia leading to excessive mucus secretion and

angiogenesis / increased vascularity. The combination of these features leads to permanent thickening of the airways which results in increased resistance to airflow which is exacerbated during bronchial contraction and bronchial hyperresponsiveness (Bousquet *et al.*, 2000). Goblet cell hyperplasia and subepithelial fibrosis has been observed in mild asthma, whereas increased smooth muscle mass appears to be mainly a feature of severe asthma (Woodruff & Fahy, 2002).

1.4.1 SUBEPITHELIAL FIBROSIS

Subepithelial fibrosis is defined as the thickening of the basement membrane caused by the deposition of extracellular matrix (ECM) proteins at the subepithelial space (Roche *et al.*, 1989). The role of the ECM is to provide structural support and act as a physical barrier in the airways. It is comprised of various molecules including collagen type I, III, IV and V, elastin, microfibrils, proteoglycans, laminin and fibronectins. Collagen is the most abundant protein in the ECM, with type I predominating. Type I gives structural support to the alveolar, bronchial and vascular walls. This structural support is through fine fibres called fibrils. Type III collagen is essential for type I collagen fibrillogenesis (Bienkowski *et al.*, 1990; Bradley *et al.*, 1974; Hay, 1995; Leblond & Inoue, 1989). The role of type IV collagen is to act as a structural scaffold for binding laminin and proteoglycans, leading to the development of the basement membrane (Leblond and Inoue 1989). Finally, the role of type V collagen in the lung is unclear.

Elastin causes the lungs to retain its shape after inspiration and expiration. It has been localised to the alveoli, pleural conducting airways and vascular tissues. Elastin can be degraded by the matrix metalloproteinases (MMP) 2 (gelatinase A), 9 (gelatinase B) and 12 (macrophage elastase). This is essential for tissue remodelling and physiological development. However, upregulation of the MMPs is believed to occur during asthma (Locke *et al.*, 2007), leading to decrease in lung structure but potentially preventing airway remodelling. Tissue inhibitor of metalloproteinases (TIMPs) breakdown metalloproteinases resulting in an increase in matrix proteins such as elastin. Whilst this is beneficial in a condition such as emphysema, in asthma an increase in TIMPs would cause an excess matrix accumulation in the asthmatic airways (Barnes, 2002a).

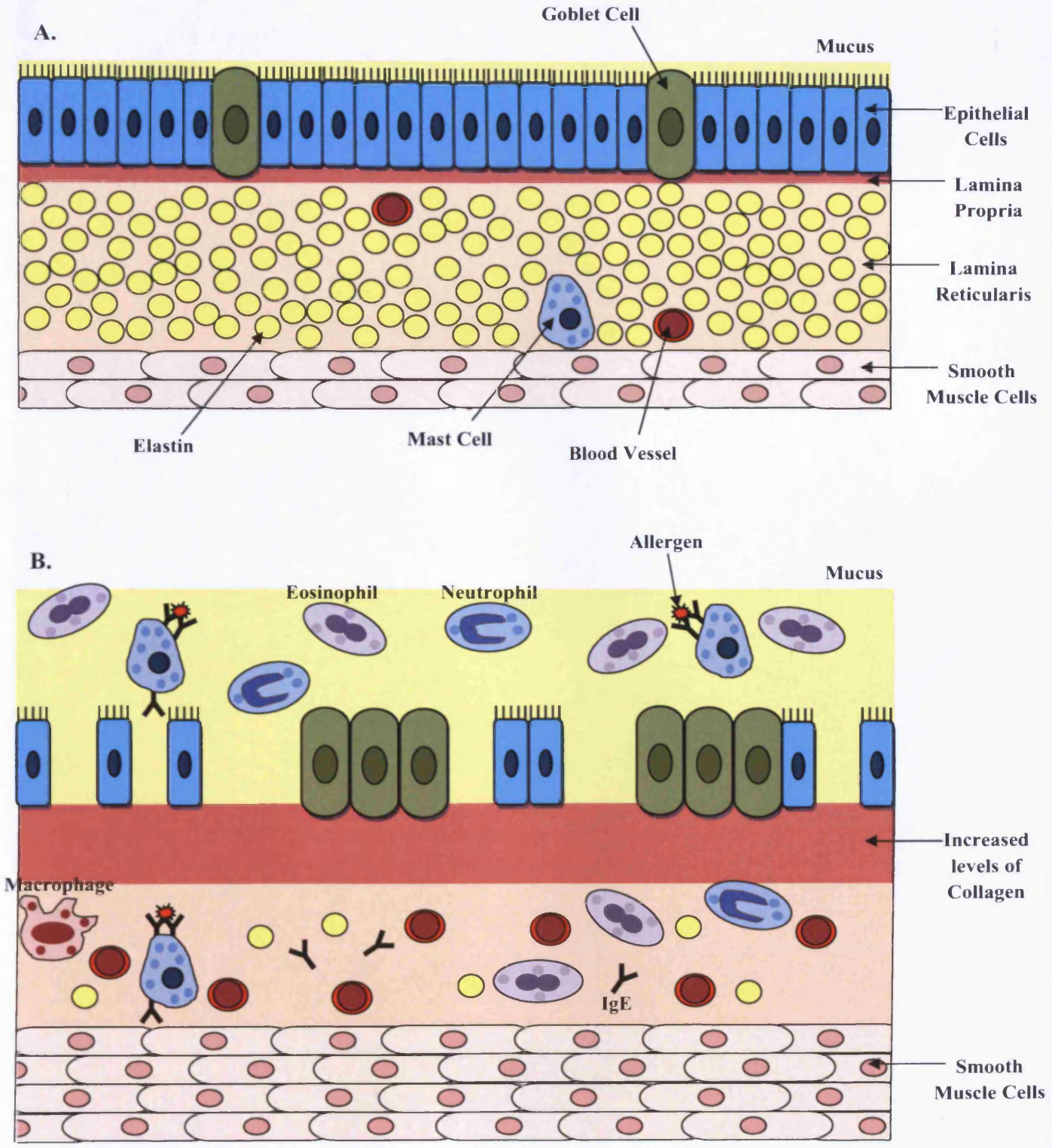


Figure 3 - A schematic diagram to show the difference between normal lung structure (A.) and an asthmatic lung after repeated allergen exposures (B.). Mucus levels are increased, as are goblet cells. Epithelial shredding has occurred allowing the inflammatory cells to penetrate the subepithelial layer. Levels of smooth muscle cells and blood vessels have increased whereas, elastin levels are dramatically reduced. A thickening of the lamina reticularis is also observed. Diagram adapted from (Hu *et al.*, 2007).

It is hypothesised that an imbalance between synthesis and degradation is what causes ECM protein deposition (Yamauchi, 2006) and as a result a 2-3 fold increase in the thickness of the lamina propria and reticularis (Brewster *et al.*, 1990; Jeffery *et al.*, 1989; Kuwano *et al.*, 1993; Roche *et al.*, 1989). Increased deposition of collagen types I, III and V, fibronectin, laminin, and elastin (Elias *et al.*, 1999; Freyer *et al.*, 2004; Freyer *et al.*, 2001; Laitinen *et al.*, 1997) have been identified as some of the causative molecules of this thickening.

Upregulation of fibroblasts seems partly responsible for subepithelial fibrosis. Fibroblasts maintain the ECM by synthesising the ECM proteins such as collagen. Gizycki *et al.*, (1997) suggest myofibroblasts, the intermediate between fibroblasts and smooth muscle cells, are responsible for matrix deposition in asthma as myofibroblast hyperplasia has been observed in the subepithelial layer of the airways. The increased level of fibroblasts in the airways appears to be a result of overexpression of TGF-β and platelet-derived growth factor (PDGF) (Krymskaya *et al.*, 2005). TGF-β can also cause the differentiation of fibroblasts into myofibroblasts and block matrix degradation by inhibiting proteolytic enzymes (Zhang *et al.*, 1996)

Chetta (1997) demonstrated that the thickness of the subepithelial layer is highly related to the severity of asthma but not related to the atopy or length of asthmatic history. However, Chu *et al.*, (1998) reject the claim that there is a link between subepithelial thickness and asthma severity.

1.4.2 INCREASED SMOOTH MUSCLE MASS

Smooth muscle mass is increased in both the large and the peripheral asthmatic airways. This increased mass is a result of hyperplasia (increase in the number of cells) and hypertrophy (increase in the size of cells) (Munakata, 2006). Whether increased smooth muscle mass is caused mainly by hyperplasia or hypertrophy is controversial. Heard and Hossain (1973) suggested that the increase is predominately a result of hyperplasia. In contrast Ebina *et al.*, (1993) describe two different observations. The first is increased muscle mass caused exclusively by hyperplasia restricted to the large airways, whereas the second is a result of smooth muscle thickening throughout the bronchial tree caused predominately by hypertrophy, with a small degree of hyperplasia in the large airways.

The exact mechanism that leads to the increase of smooth muscle mass is unknown. Barnes (1996) suggests stimulation of airway smooth muscle cells could be a result of growth factors such as PDGF or endothelin-1. TGF-β has the ability to promote or inhibit smooth muscle growth (Cohen *et al.*, 1997) suggesting that it could be involved. The factors that cause the increase of airway smooth muscle cause grand scale changes, with increases of 50-230% in fatal cases and 25-150% in nonfatal cases of asthma (James, 1997).

1.4.3 GOBLET CELL HYPERPLASIA AND METAPLASIA

Goblet cell hyperplasia and metaplasia is commonly observed in mild, moderate and severe asthma (Yamauchi, 2006). Ordonez *et al.*, (2001) reported that a statistical increase in goblet cells and stored mucin was observed in the airways of asthmatics compared to non-asthmatics. When an increased number of goblet cells release mucus then bronchial obstruction occurs as a result of the mucous plugging the airways. This is observed in the central and peripheral airways of sufferers of both chronic and severe asthma (Andoh *et al.*, 1992).

Rogers (1994) proposed that metaplasia from epithelial cells into goblet cells is a result of mucus gene expression. This increased expression can be triggered by both environmental pollutants and host factors (Levine, 1995). A component in cigarette smoke, acrolein, has been shown to cause expression of the epithelial MUC5AC gene and therefore cause metaplasia in rat models (Borchers *et al.*, 1999). Similarly, MUC5AC and MUC2 genes have been shown to be upregulated by IL-4, IL-9 and IL-13 using *in vivo* models (Temann *et al.*, 1997). The upregulation of these genes and resultant goblet cell hyperplasia leads to hypersecretion of mucus, which is a serious issue in asthma as it can plug the airways contributing to airflow limitation, airway hyperresponsiveness and in severe cases, mortality (Morcillo & Cortijo, 2006).

1.4.4 ANGIOGENESIS AND INCREASED VASCULARITY

More vessels and a greater percentage area of vasculature are observed in the lamina propria of asthmatics than there are in non-asthmatics (Orsida *et al.*, 1999). Whether this is primarily a result of angiogenesis (the formation of new blood vessels) or

microvasculature enlargement is unclear. The microenvironment in asthma has been shown to have the potential for angiogenesis and in fact Li and Wilson (1997) found changes suggesting new vessel formation in mild asthma. Several factors that are involved in angiogenesis have been identified, such as fibroblast growth factor (Montesano *et al.*, 1986), hepatocyte growth factor (Nakamura *et al.*, 1984) and PDGF (Ishikawa *et al.*, 1989).

On the contrary, a study of the membrane bronchioles by Kuwano *et al.*, (1993) suggests airway remodelling is a result of enlargement of the microvasculature. Whether angiogenesis or microvasculature enlargement predominate a correlation between number of airway wall blood vessels and asthma severity exists (Lazaar & Panettieri, 2003).

1.5 TREATMENT

Asthma therapeutics target inflammation, bronchoconstrictions and narrowing of the airways. Both symptom relievers and preventers are clinically available. The drugs used can be broadly classified as either bronchodilators or anti-inflammatories. Treatment is dependent on the severity of the disease, with mild asthma being treated with a short-acting bronchodilator whereas severe asthma is treated with oral steroids. Guidelines for the treatment of asthma were drawn up by the British Thoracic Society (Wu *et al.*, 2008) (table 2).

Short acting β_2-adrenoreceptor agonists are administered to control mild asthma as they are bronchodilators. Beta-agonists are the most effective bronchodilators clinically available as they are effective at protecting against the bronchoconstriction caused by exercise, cold air and allergen (Barnes, 1995). The binding of the agonists to the β_2-adrenoreceptor causes activation of the receptor; this leads to the stimulation of the enzyme adenylyl cyclase, which converts adenosine trisphosphate (ATP) into cyclic adenosine monophosphate (cAMP). The formation of cAMP causes relaxation of the bronchial smooth muscle via downstream signals. Two types of β_2-adrenoreceptor agonists are available, short acting and long acting. Short acting agonists, such as salbutamol, are used when required for symptom control, whereas long acting agonists, such as salmeterol, cause a prolonged bronchodilation for more than twelve hours

(Boulet, 2004). However, there have been studies that cast doubt on the safety of long acting β_2 agonists. It has been suggested that long acting β_2 agonists such as salmeterol may increase the risk of asthma deaths (Salpeter *et al.*, 2006). Recently concerns have been reduced as a result of reassuring data (Moore, 2009; Nelson *et al.*, 2009).

STEP 1 Mild Asthma	Inhaled short acting β_2 agonist e.g. salbutamol
STEP 2	+ Low dose inhaled corticosteroid e.g. fluticasone propionate
STEP 3	+ Increasing doses of inhaled corticosteroid + inhaled long acting β_2 agonist e.g. salmeterol
STEP 4 Severe Asthma	+ Oral steroids e.g. dexamethasone

Table 2 - Recommended guidelines for managing increasingly severe asthma in adults. Adapted from British Thoracic Society (Wu *et al.*, 2008).

Another method to control the immediate bronchoconstriction in asthma is to target the muscarinic receptor. In the airways acetylcholine is released from vagus nerves, when this binds with the muscarinic receptor contraction of the smooth muscle and increased secretion of mucus occurs (Katzung, 2004). Muscarinic receptor antagonists, such as ipratropium bromide, competitively inhibit this acetylcholine effect. Ipratropium bromide is a non-selective muscarinic receptor and therefore blocks the M_1-, M_2- and M_3-receptors (Barnes, 2001). However, this is not efficient as the M_3-receptor mediates the bronchoconstriction and is therefore the target, whereas the M_2-receptor inhibit acetylcholine release and is therefore beneficial (Barnes, 2002a). Thus far, M_3-selective anatagonists have proved difficult to develop (Maesen *et al.*, 1993). The fact that M_3-

receptors can cause mucus secretion suggests that the inhibition of these receptors is beneficial when it comes to controlling airway remodelling.

Phosphodiesterase (PDE) is another target for asthma therapeutics. PDE enzymes decrease intracellular levels of cyclic adenosine monophosphate (cAMP) by catalysing its degradation to inactive AMP. Inhibiting PDE causes increased intracellular levels of cAMP which has several effects such as relaxing smooth muscle and inhibition of inflammatory cell activity. Methylxanthines, such as theophylline, are non-selective phosphodiesterase inhibitors. Through this inhibition of PDE, levels of cAMP increase and smooth muscle relaxation and anti-inflammatory actions can occur therefore attenuating the early phase bronchoconstriction associated with asthma. It is possible that theophylline works through another mechanism as it can inhibit cell surface adenosine receptors (Katzung, 2004). Adenosine has been shown to cause contraction of airway smooth muscle and to provoke histamine release from mast cells (Katzung, 2004). Therefore, inhibiting adenosine would also attenuate the early bronchoconstriction.

There is increasing evidence that theophylline has anti-inflammatory effects in asthma such as activation of histone deactylase (HDAC), which increases anti-inflammatory gene transcription, (Barnes, 2002a), increased eosinophil apoptosis (Barnes, 2002a), inhibition of mediator release (Orange *et al.*, 1971) and inhibition of superoxide anion release from neutrophils (Nielson *et al.*, 1988). Whether this action is mediated through cAMP activation, inhibition of adenosine cell surface receptors or through another mechanism is unclear. The side-effects of theophylline has led to limited usage as the combination of β_2-agonists and corticosteroids has less detrimental effects (Barnes, 2002a). Theophylline is a non-selective PDE inhibitor; more recent developments have been the introduction of selective inhibitors of the PDE subtypes such as the PDE4 inhibitor, roflumilast (see chapter 6 for more information).

Some asthma therapeutics are effective at treating the late phase bronchoconstriction by having anti-inflammatory properties and are therefore also effective against AHR and cellular influx. The most powerful of this type of drug are the corticosteroids. Corticosteroids are the most commonly used drug in the treatment of asthma, their mechanism of action and effects in asthma are described in chapter 5. Another group of anti-inflammatory drugs focus on the inhibition of leukotrienes. Leukotrienes cause

airflow obstruction and bronchoconstriction. The leukotriene antagonist, montelukast, blocks leukotriene D_4 having a bronchoconstrictor response by binding to the Cysteinyl Leukotriene 1 (CysLT1) receptor on mast cells, smooth muscle and eosinophils, therefore attenuating the early asthmatic response (Finnerty *et al.*, 1992; Taylor *et al.*, 1991) and is in fact as effective as short acting β_2-agonists (Hui & Barnes, 1991; Reiss *et al.*, 1997) when administered orally. As well as a bronchoconstriction, CysLT1 receptor activation causes eosinophil recruitment, mucus secretion and plasma exudation (Barnes, 2002a). Therefore montelukast is also effective at inhibiting the late asthmatic response (Barnes, 2002a).

The expanding knowledge of asthma mechanisms and pathology correlates with an increase in anti-asthma therapeutics. Many of the recently developed compounds are highly specific to a certain mediator shown to have a role in asthma, examples of such include tyrosine kinase inhibitors, adhesion molecule inhibitors and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitors (Barnes, 2002a). Another class of potential anti-asthmatic drugs are inhibitors of inducible nitric oxide synthase (iNOS) which are described in chapter 7. Despite an increasing number of anti-asthmatic therapeutics, a combination of β_2 agonists and corticosteroids is still the main treatment for asthma (Barnes, 2002b) and it is believed that the current pharmacological treatment of asthma will not change for the next 10 years (Caramori *et al.*, 2009).

1.6 MODELS OF ASTHMA

The use of animal models of asthma is no new phenomenon. Although no animals exhibit asthmatic symptoms spontaneously, certain horses, cats and Basenji-greyhound cross dogs show some features (Barnes, 2002a). Consequently the most common method of developing a model of asthma is using allergen sensitisation and subsequent challenge. Several different allergens can be used to elicit an asthmatic condition, including house dust mite (Cates *et al.*, 2007), short ragweed extracts (Chapoval *et al.*, 1999) and ovalbumin (Smith & Broadley, 2007).

Allergen sensitisation and exposure has led to the development of several animal models of asthma in guinea pigs, mice, rats, monkeys and dogs. The similarity between the

pulmonary response and histological findings to antigen exposure in the airways of guinea pigs and humans led to the guinea pig being the model of choice (Nabe *et al.*, 1997). However, studies that use mice as models of asthma are becoming more common (Kumar & Foster, 2002; McMillan & Lloyd, 2004; Wegmann *et al.*, 2005). This is because mice are low cost, have a well-characterised immune system, knock-out mice are more achievable than other small species and antibodies for molecular studies in inflammatory mediators are readily available.

Several models of asthma concentrate on acute inflammation following antigen exposure (McMillan & Lloyd, 2004). These short-term models develop an inflammatory response which is limited to the proximal airways and as a result is not associated with the chronic pathological changes associated with airway remodelling (Wegmann *et al.*, 2005). Many studies also use anaesthetised animals (Johnson & Broadley, 1999; Underwood *et al.*, 1995), however, the possibility exists that the anaesthetic may interfere with the vagal tone or sensory reflex (Toward & Broadley, 2004).

Clearly there are several advantages and disadvantages between species when it comes to using them as a model of asthma. The advantages and disadvantages of guinea pig and mouse models of asthma are discussed in greater detail in chapters 3 and 4, respectively.

Ultimately no animal model can be completely perfect when it comes to representing human asthma; however, several models display many of the characteristics of asthma. Therefore, they have an important role as the first means of testing asthma therapeutics.

1.7 AIMS

The overall aims of this thesis are as follows:

- Develop acute and chronic models of asthma in guinea pigs and mice, which demonstrate the features of human asthma – early and late asthmatic responses to allergen challenge, airway hyperresponsiveness, airway inflammatory cell influx and changes in lung histology.
- Evaluate the effects of anti-inflammatory compounds belonging to the steroid, phosphodiesterase type IV inhibitor and inducible nitric oxide synthase inhibitor classes on these acute and chronic guinea pig and mouse models of asthma.
- Assess what effect acute and chronic ovalbumin challenges have on lung oedema in sensitised guinea pigs and whether corticosteroid treatment attenuates this by using magnetic resonance imaging.

Chapter 2

Methods

CHAPTER 2

2.1 MATERIALS AND EQUIPMENT

A full list of materials and equipment used can be found in Appendix 1. Ovalbumin (OVA) was the allergen of choice to cause the sensitisation and effector phase of asthma.

2.2 ANIMAL EXPERIMENTS

2.2.1 ANIMAL WELFARE

All animals were obtained through the Joint Animal Service department of Cardiff University from Harlan UK Ltd. Upon arrival the animals were given one week to habituate to their surroundings before any experiments began. They were housed under conventional conditions with a twelve hour light/dark cycle, maintained room temperature of 20°C±2°C and a humidity of 50%. All experiments were carried out in accordance with the Animal Scientific Procedures Act 1986, under valid Home Office project and personal licences. Experienced technicians were in charge of the welfare of the animals. Before beginning any experiments the animals were exposed to the system that measures their airway function to ensure it was not a novel environment and the animals were habituated when readings were taken. The guinea pigs used in chapter 8 were kept at GlaxoSmithKline, further details can be found in section 8.3.

2.2.1.1 GUINEA PIGS

Male Dunkin-Hartley guinea pigs (200-250g) were used for all research involving guinea pigs. They received commercial guinea pig pellets, supplemented with ascorbic acid, and drinking water *ad libitum*. The guinea pigs were housed in grid-floor cages in groups of six and in order to enrich the environment, a cardboard tube and hay was supplied. An n of 6 was used for all experiments.

2.2.1.2 MICE

Male BALB/c mice (20-25g) were the breed used for all mouse studies. They were housed in groups of six in plastic cages, with a sawdust base, and a removable grid roof.

Food and drinking water was supplied *ad libtum*. For environmental enrichment cardboard tubes were supplied. An n of 6 was used for all experiments.

2.2.2 SENSITISATION PROCESS

2.2.2.1 GUINEA PIGS

Guinea pigs in all studies were sensitised by an intraperitoneal injection of a mixture of OVA (100 μg) and aluminium hydroxide ($Al(OH)_3$) (100 mg) in phosphate-buffered saline (PBS). The mixture was stirred for two hours prior to injection to ensure the OVA and $Al(OH)_3$ were completely dissolved. $Al(OH)_3$ was used as an adjuvant to boost the immune response to OVA and promote the development of sensitization. 1 ml of the suspension was administered bilaterally to the guinea pigs on day 1 and day 5, all procedures then commenced on day 14. This method of sensitization was developed by Smith and Broadley (2007).

2.2.2.2 MICE

The sensitisation process for mice used the same OVA mixture as in guinea pigs. Intraperitoneal injections of 0.25 ml bilaterally were administered on days 1 and 5, with procedures commencing on day 14 (Fernandez-Rodriguez *et al.*, 2008).

2.2.3 MEASURING LUNG FUNCTION IN GUINEA PIGS

Throughout all studies non-invasive methods were employed using plethysmography, an airtight chamber, to measure airway function. For respiration to occur there must be a difference in pressure between the mouth or nose and the alveoli, this is known as the pressure difference. If the difference in pressure is divided by the airflow then the outcome is a measure of airway resistance (R_{aw}). However, airway conductance (G_{aw}) is regarded as a better measurement of airway function as this incorporates a change in transpulmonary pressure and lung tissue tension (Griffiths-Johnson *et al.*, 1988). In asthma differences in the alveoli pressure occur and therefore changes in the volume of air in the lung, or thoracic gas volume (TGV), occur. Taking this into account a value of G_{aw} – TGV is used for measuring airway function. This value is known as specific airway conductance (sG_{aw}).

The plethysmograph allows sG_{aw} to be calculated. The pressure in the sealed chamber, box pressure, will decrease and increase in line with the pressure changes between the mouth or nose and alveoli of the subject if the chamber is kept at a constant temperature (Boyle's Law) (West, 2005). Airflow is measured through a mask placed over the subject's mouth and nose. The full description of how sG_{aw} is derived, taking the TGV into account is found in Appendix 2.

Guinea pigs are very docile and as a result they can be restrained and have a mask placed over their snout without much complaint. As previously mentioned to measure sG_{aw} in guinea pigs a plethysmograph is used (figure 2.1). This is a perspex box with a removable endplate at one end. The guinea pig was placed in a neck restrainer, this slides into another restrainer that has high sides and therefore stops the guinea pig from moving its body. A perspex mask was attached to the full body restrainer; it was sealed to the snout of the guinea pig by means of a cut balloon. The restrainer was then placed into the plethysmograph and the endplate clamped shut making an airtight environment. The plethysmograph measures two variables, airflow and box pressure. Airflow was measured by using a mesh pneumotachograph that is inside the perspex mask. The mask is connected to a UP1 pressure transducer by means of a plastic tube. Box pressure measures the change in air pressure in the plethysmograph. It is connected to a UP2 pressure transducer by means of a plastic tube.

The pressure transducers are connected to a Biopac data system that converts the airflow and box pressure data into waveforms; this is displayed on the computer by using Acqknowledge software. Acqknowledge allows the analysis of the waveforms by comparing the gradients of airflow and box volume where the flow of the wave tends towards 0. The result is a value of specific airway conductance (sG_{aw}). One reading takes 5 seconds to record and an average of 8 breaths are captured in this time. An example of the waveform data obtained from the plethysmograph is shown in figure 2.1.

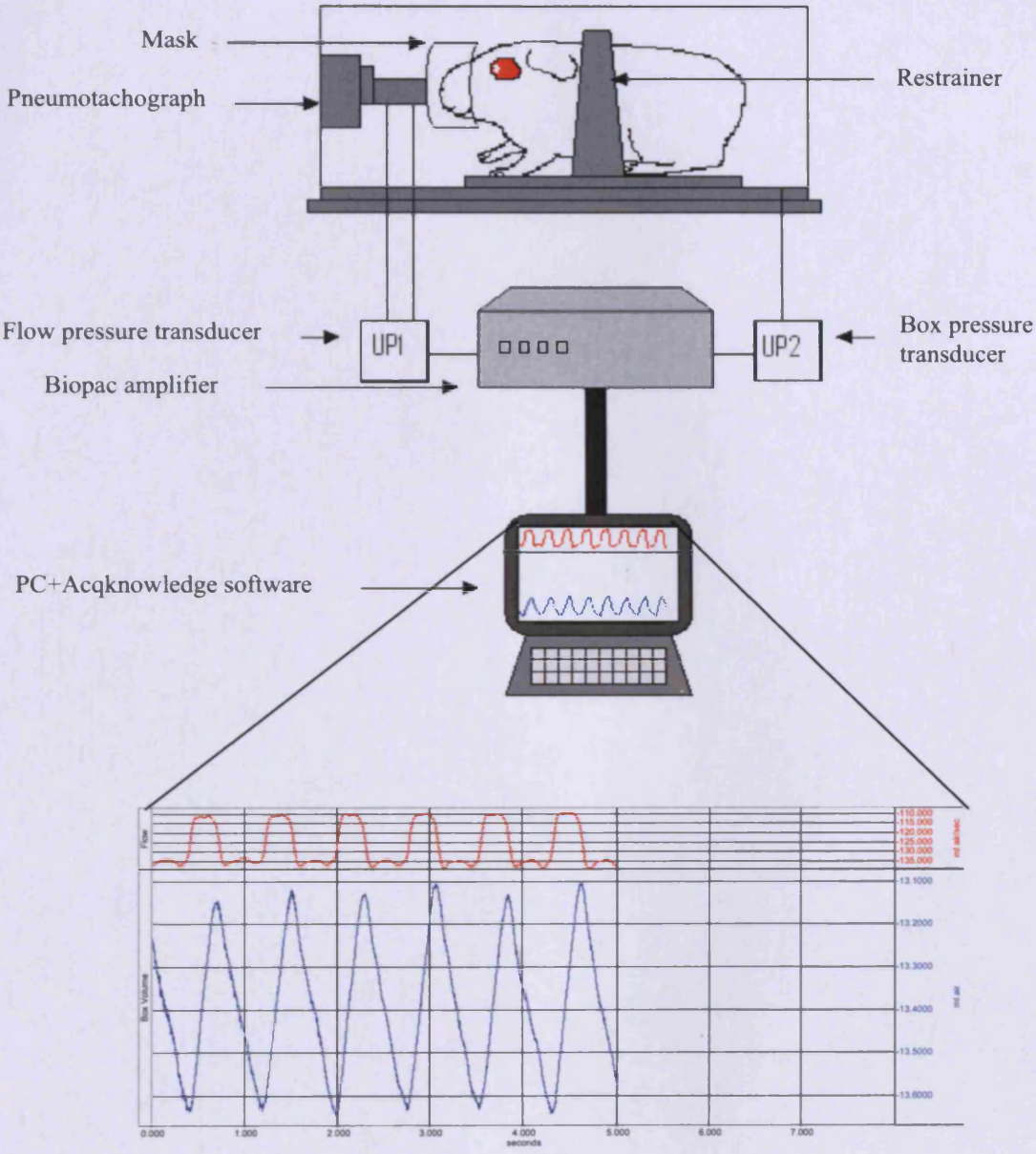


Figure 2.1 - A simplified schematic of the whole body plethysmograph and acquisition packs used to measure specific airways conductance sG_{aw}, in conscious, restrained guinea pigs. Flow rate is measured through a flow pressure transducer which is connected to the pneumotachograph found in the mask that is placed over the snout of the guinea pig. Changes in box pressure are also measured through a transducer. The two wave forms are captured using a Biopac amplifier, a value of sG_{aw} is then established using Acqknowledge software by comparing the gradients of flow rate (red) and box volume (blue) where the flow of the wave tends towards 0.

2.2.3.1 ACUTE CHALLENGE PROTOCOL

On days 14 and 16 guinea pigs were exposed to histamine to determine whether airway hyperresponsiveness was occurring, this is explained in greater detail in section 2.7.1. The effector phase of asthma was triggered by an allergen challenge on day 15. The guinea pigs were exposed to either OVA (0.01%) or a control solution (saline). Exposure was carried out in a stainless steel exposure chamber (40 cm diameter, 15 cm height) with a Wright nebuliser attached. The nebuliser delivered the OVA or saline at an air pressure of 20 lb p.s.i. and at a rate of 0.3 ml/min. The guinea pigs remained in the chamber for 1 hour, however, if they appeared to be distressed they were immediately removed from the chamber and exposure was considered complete. On day 16 following the second histamine challenge the guinea pigs were killed and a lavage was carried out. This allowed for total and differential cell counts to be obtained, following this the lungs were stored for histological analysis.

An additional group following the same protocol as the antigen-challenged group was run, however, this group was left for 72 hours after the test day before the second histamine challenge and lavage was performed. The purpose of this group was to observe whether any recovery occured in terms of airway hyperresponsiveness and cellular influx and histology when compared to the antigen-challenged group that had the lavage immediately after the final histamine exposure. Figure 2.2 highlights the various stages of the acute protocol for guinea pigs.

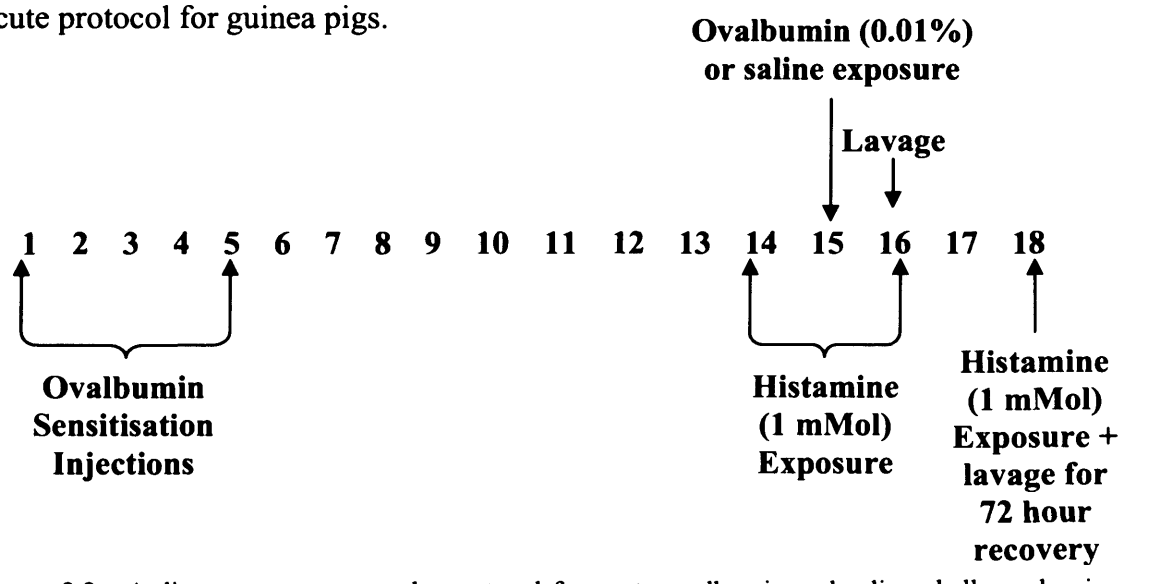


Figure 2.2 - A diagram to represent the protocol for acute ovalbumin and saline challenged guinea pigs. Lung function measurements are recorded on day 15 immediately following the OVA or saline exposure.

2.2.3.2 CHRONIC CHALLENGE PROTOCOL

Exposure for the chronic model was carried out using the same equipment. Once again, all exposures lasted 1 hour unless the guinea pig appeared distressed. Day 15 was the first exposure day for each protocol, the control group was exposed to nebulised saline, whereas the antigen-challenged group was exposed to OVA (0.01%). Further exposures occurred every 48 hours from day 17 to day 29, once again this was saline for the control group. However, the antigen-challenged group was exposed to a higher dose of OVA (0.1%), 30 minutes prior to this exposure the group were given an intraperitoneal injection of the histamine H_1 receptor antagonist, mepyramine (30 mg/ml). This protected against the fatal anaphylaxis that would be caused by massive histamine release as a result of the high level of OVA inhaled. The reason the high dose of OVA was used is because the guinea pigs would rapidly become tolerant to the lower dose and therefore the structural changes in the airways would be less likely to occur (John 2007).

Day 31 was the final exposure day and the day in which readings assessing lung function were taken. The control group were exposed to saline. The antigen-challenged group were exposed to OVA (0.1%), however, they had developed a tolerance to the high dose and therefore did not need mepyramine. In a similar way to the acute protocol a group was introduced that was allowed 72 hours to recover following the OVA challenge before the final histamine exposure and lavage were carried out. Figure 2.3 shows the chronic protocol for guinea pigs.

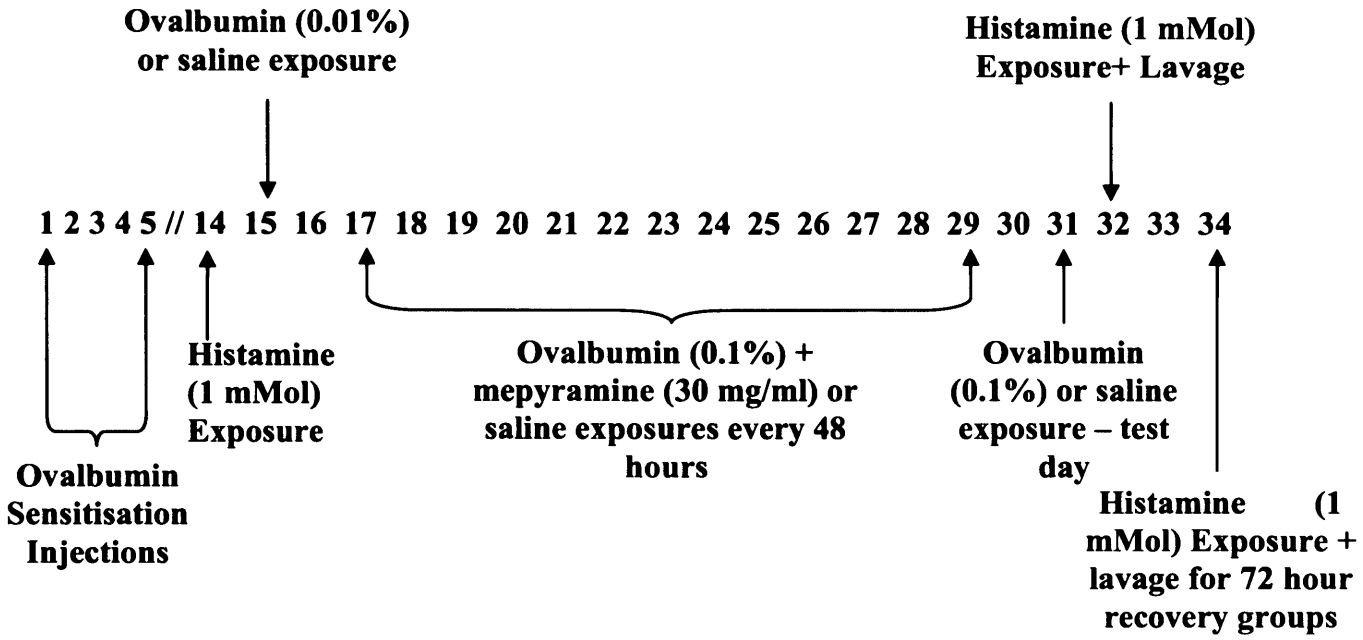


Figure 2.3 - A diagram to represent the protocol for chronic ovalbumin and saline challenged guinea pigs. Lung function is measured on day 30.

2.2.4 MEASURING LUNG FUNCTION IN MICE

As mice cannot be easily restrained an alternative method to sG_{aw} must be used to measure airway function. Generally, enhanced pause (P_{enh}) is the method of choice for measuring airway function in mice. As recording P_{enh} does not involve restraint the mouse is allowed to move freely around the airtight chamber. This is advantageous over sG_{aw} in the respect that as no restraint is involved stress is reduced and normal breathing patterns occur. Despite this, mice still need a great deal of habituation and as a result they were introduced to the chamber several weeks before any experiments began.

There is great controversy whether P_{enh} should be used as a measurement for airway function as it may not necessarily represent a change in the lower respiratory tract (Nakaya *et al.*, 2006). Hamelmann *et al.*, (1997) supports the use of P_{enh} by demonstrating that it correlates well with the pulmonary resistance measured with

conventional two-chamber plethysmography in anaesthetised animals under mechanical ventilation. However, using P_{enh} as a measurement of lung resistance is described as inappropriate by Adler *et al.,* (2004). The use of Penh is described in section 9.1.4. Although the use of P_{enh} is widely debated it prevents using a method of forced restraint on a mouse. The method by which P_{enh} is derived is explained fully in Appendix 3. The chamber (height - 8 cm; diameter - 8 cm) is part of a system designed by Buxco for measuring airway function in mice, giving a value of enhanced pause (P_{enh}). Figure 2.4 shows the Buxco chamber.

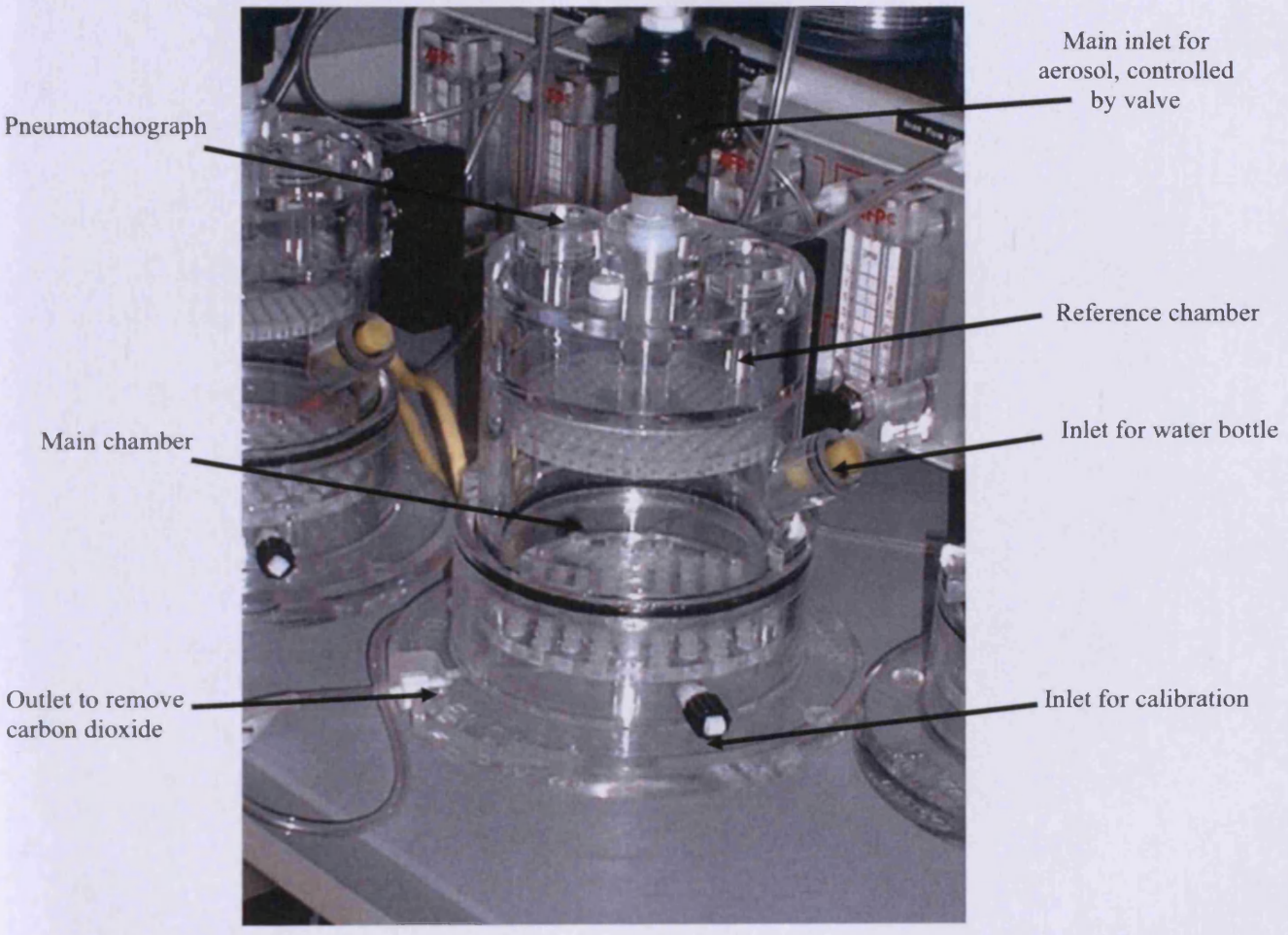


Figure 2.4 - A photograph of the Buxco chamber in which mice have free run whilst P_{enh} is being measured. Diagram taken from The Jackson Laboratory (http://pga.jax.org/images/buxco2.jpg).

2.2.4.1 ACUTE CHALLENGE PROTOCOL

In order to elicit the effector phase associated with asthma, mice were exposed to ovalbumin (0.5%) on day 15. On the previous day and day 16 the mice were challenged with methacholine (30 mg/ml) to determine the responsiveness of the airways. Like the guinea pig protocol, the OVA or saline challenge lasted for one hour. However, one OVA challenge is not sufficient to provoke an asthma-like inflammatory response in mice (Ohkawara *et al.*, 1997), consequently another hour-long challenge occurred four hours after the start of the first challenge. Exposures were carried out in a perspex box (38 cm length; 20 cm width; 20 cm height) using the same nebuliser as in the guinea pig protocols. The acute protocol for mice is summarised in figure 2.5.

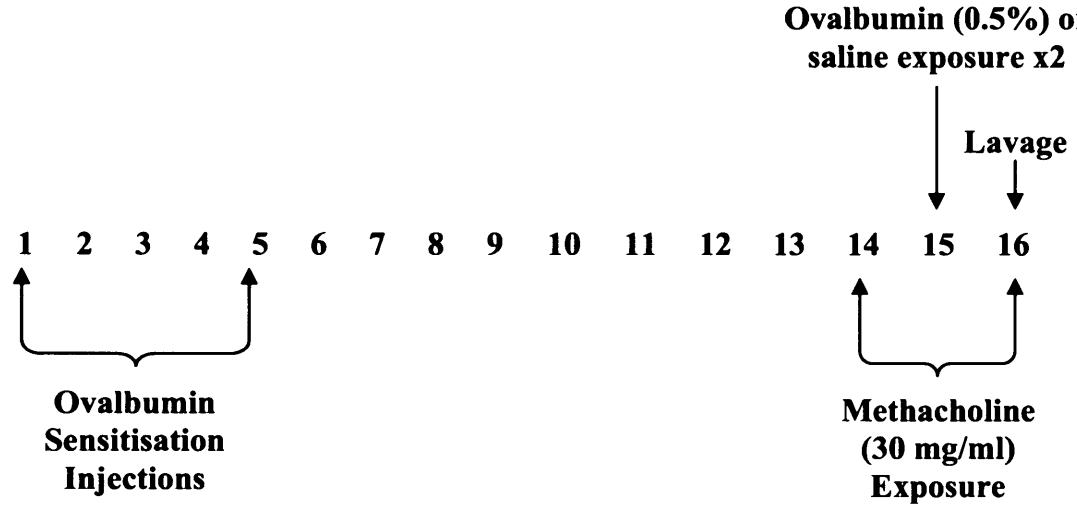


Figure 2.5 - A diagram to represent the protocol for acute ovalbumin and saline challenged mice.

2.2.4.2 CHRONIC CHALLENGE PROTOCOL

The chronic challenge protocol for mice was very different to the guinea pig protocol. After sensitisation (days 1 and 5) and the first methacholine exposure (10 mg/ml) (day 14), the mice were challenged three times a week for six weeks. They were challenged with OVA (2%) for 30 minutes, no protective drug, such as mepyramine, was needed as the mice could withstand the high dose of OVA immediately but did not become tolerant to it over time. The final challenge occurred on the first day of the seventh week (day 57)

and lasted for one hour, readings were taken on this day. Unlike the acute group only one challenge was needed on the test day. The same protocol was followed using saline for a control group. Figure 2.6 shows the chronic allergen challenge protocol for mice.

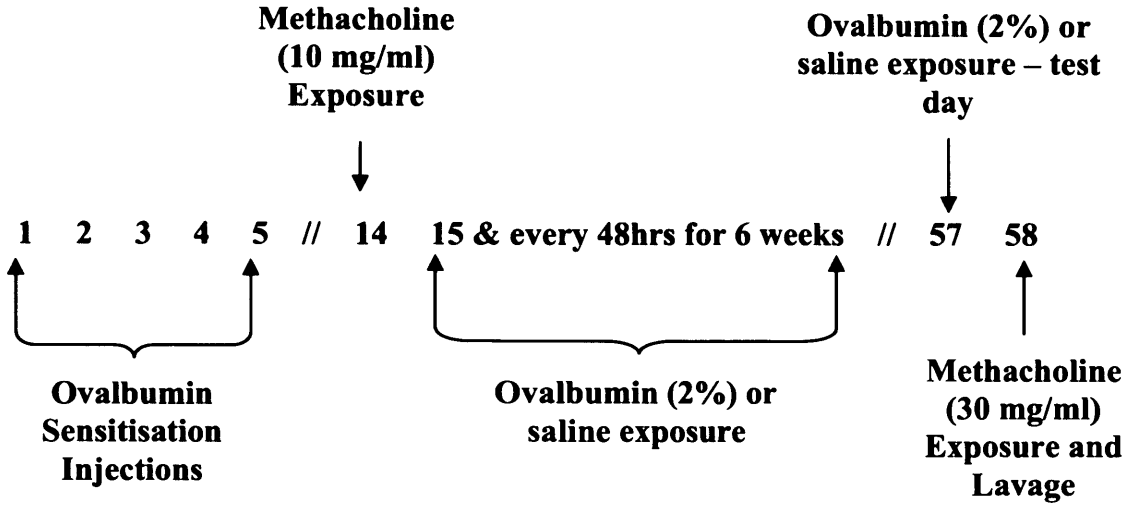


Figure 2.6 - A diagram to represent the protocol for chronic ovalbumin and saline challenged mice.

2.2.5 RECORDING EARLY AND LATE ASTHMATIC RESPONSES

2.2.5.1 GUINEA PIGS

Values for sG_{aw} were taken 0, 15, 30, 45 and 60 minutes after antigen exposure, then hourly until 12 hours with a final reading being taken at 24 hours. Before exposure a baseline value was recorded. The other sG_{aw} values at the different time-points were expressed as a percentage of baseline. If the percentage value was negative this signifies a trend towards bronchoconstriction whereas if it was positive it signifies bronchorelaxation.

The early asthmatic response (EAR) occurs within the first hour, however, it can often take up to 6 hours until the sG_{aw} values returns to baseline levels. To allow for variability in guinea pigs, a value of maximum bronchoconstriction between 0 and 6 hours was used. The same principles apply for the late asthmatic response (LAR), as a result maximum

bronchoconstriction values between 7 to 12 hours were used, to ensure the LAR is analysed correctly.

2.2.5.2 MICE

Prior to allergen challenge, baseline values of P_{enh} were recorded. In the opposite way to sG_{aw}, if a percentage change in P_{enh} values was positive this would signify bronchoconstriction, whereas negative values were bronchorelaxation. Values of P_{enh} following allergen challenge were recorded at 0, 20, 40, 60, 90 and 120 minutes, then hourly until 10 hours followed by a final reading at 19 hours for the acute protocol (24 hours after first challenge) or 24 hours for the chronic protocol. The EAR of mice does not peak until around 2 hours after exposure, whereas the LAR tends to occur at around 7 hours (Fernandez-Rodriguez *et al.*, 2008). In order to ensure maximum bronchoconstriction was being observed and to minimise variation between each mouse, a value of maximum percentage change in P_{enh} between 0-6 hours and 7-10 hours was used for analysis.

2.2.6 RECORDING AIRWAY RESPONSIVENESS

2.2.6.1 GUINEA PIGS

AHR was also measured by using plethysmography. Whether AHR occurs or not was determined by the airway responses to 1 mMol of histamine. 1 mMol of histamine was used as this is considered the subthreshold dose; the guinea pig should not show any reaction to it before OVA exposure but if there is AHR there should be a response (figure 2.7).

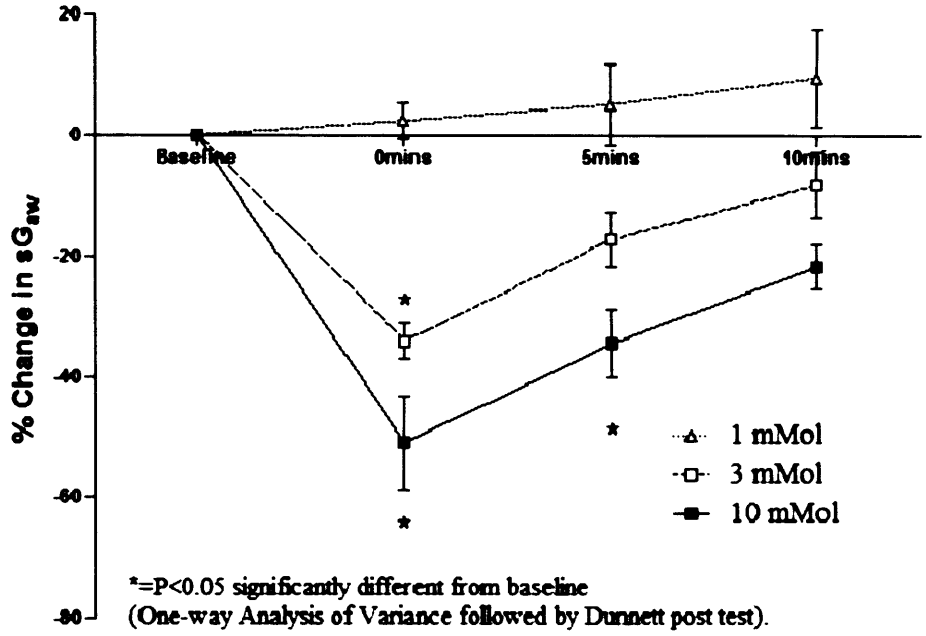


Figure 2.7 - A histamine dose of 1mmol is not significantly different from baseline values as seen with higher doses. Therefore 1 mMol is the best dose to use to induce airway hyperresponsiveness in antigen challenged guinea pigs.

Guinea pigs were given histamine by using a Wright nebuliser, instead of using a chamber the guinea pig was exposed using a plastic pipe (figure 2.8). The end of the nebuliser with the aerosol is attached to a J-bend at one end of the pipe; the other end leads out to an air filtration system, so as not to expose the surrounding environment to histamine. The pipe has a hole within three inches of the aerosolised end with a diaphragm over it by means of a balloon. The guinea pigs' snout was placed into this hole while the nebuliser was running and they are exposed to the histamine for 20 seconds. A baseline value, measured as sG_{aw}, was recorded before the histamine exposure and all other values were taken as a percentage of this. The time-points used were 0, 5 and 10 minutes after histamine exposure.

The histamine exposures occurred 24 hours pre-OVA or saline exposure (day 14) and 24 hours post-OVA or saline exposure (day 16) in acute models. In the chronic model histamine exposures occurred 24 hours pre-first exposure (day 14) and 24 hours post-final exposure (day 32). The purpose of performing a histamine exposure pre- and post-ovalbumin or saline exposure was two-fold. Firstly, if there was a significant bronchoconstriction pre-ovalbumin or saline exposure when comparing values to the

baseline reading then the guinea pig was already exhibiting some form of airway hyperresponsiveness and should not be used. Secondly, by comparing pre- and post-ovalbumin or saline exposure it was possible to determine whether ovalbumin or saline causes airway hyperresponsiveness.

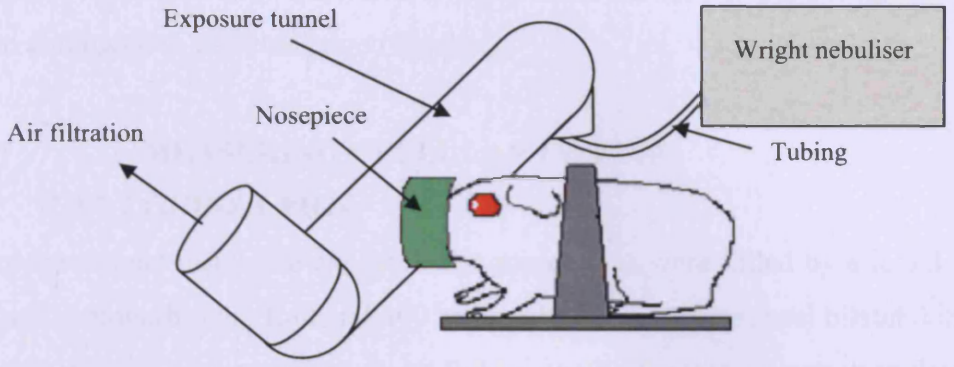


Figure 2.8 – A schematic diagram to show how guinea pigs are exposed to histamine. The Wright nebuliser causes 1 mMol histamine to be aerosolised through the exposure tunnel. The guinea pig is held with the nosepiece over its snout for 20 seconds. Any excess histamine exits through the air filtration system.

2.2.6.2 MICE

Hyperresponsiveness in mice was measured using methacholine (10 mg/ml or 30 mg/ml). 30 mg/ml was used for measuring hyperresponsiveness in the acute model and 10 mg/ml was used for the chronic model. These doses were used as they have previously been shown to cause a response in OVA challenged mice but not in saline challenged mice (Fernandez-Rodriguez *et al.*, 2008). The higher dose would cause fatal anaphylaxis in the chronic model mice hence 10 mg/ml was used. The exposures were carried out in the same Buxco chambers that were used to measure lung function.

The mice were challenged with methacholine both pre- and post-challenge. If a significant response to methacholine was observed pre-challenge then the animal exerting the response was removed from the experiment. The second methacholine challenge occurred after the final OVA challenge. The Buxco set up allowed the mice to be exposed to the methacholine whilst in the chamber. The protocol began with five minutes of baseline readings of which an average was taken. This was followed by one minute of

intermittent methacholine exposure (one second exposure followed by five seconds without exposure on a continuous cycle). Finally, another five minutes of readings were recorded and averaged. The mean for the post-methacholine value was then expressed as a percentage of baseline. When the results were graphed post-challenge, hyperresponsiveness was shown as a significant increase in percentage change in P_{enh} when compared to pre-challenged results.

2.2.7 MEASURING CELLULAR INFLUX

2.2.7.2 GUINEA PIGS

After the second histamine exposure the guinea pigs were killed by a lethal overdose of sodium pentobarbitone (Euthatal 400 mg/kg) by an intra-peritoneal bilateral injection. An incision into the neck was then made. Subsequently, the trachea was cannulated using a 5 cm length of intravenous polypropylene cannula. The lungs were then completely removed from the guinea pig. Saline solution (1 ml/100 g guinea pig weight) was instilled into the lungs through the cannula and then recovered three minutes later. This process was repeated and the two amounts of recovered lavage fluid were combined. The lungs were inflated with 10% buffered formaldehyde solution (2 ml/100 g guinea pig weight) via the cannula and then submerged in 10% buffered formaldehyde. They were stored ready for further histological studies.

In order to measure the level of cellular influx two different counts were carried out on the lavage fluid. Firstly, the total number of cells (per ml of lavage fluid) were counted using a Neubauer haemocytometer under a light microscope at 100 x magnification. 10 µl of bronchoalveolar lavage (BAL) fluid was pipetted beneath the coverslip on the haemocytometer, capillary action enabled the fluid to be evenly distributed. When viewed under a microscope two grids of 25 squares can be observed on the haemocytometer. The number of cells in 5 of the squares was counted, always using the same squares (top left, top right, middle, bottom left and bottom right), and the total figure was multiplied by 5 to give an estimated amount of cells in 25 averaged. This was repeated for the other grid on the haemocytometer and an average was taken. As each of the squares measure 0.04 mm^2 and 25 were counted, a figure of number of cells per 1 mm^2 was established. However, the depth of the chamber was 0.1 mm, therefore giving a

number of cells per 0.1 mm^3. As the number of cells per 1 ml (1 cm^3) was required, the cell count figure was multiplied by 10^4.

The second count was a differential count. 100 µl of the lavage solution was centrifuged at 1000 rpm for 7 minutes onto a glass slide using a cytospin. The slide was then stained using 0.15% Leishman's solution in 100% methanol. After 7 minutes it was removed from the solution and allowed to air-dry over night. Once dry, 200 cells were counted under a light microscope at 1000 x magnification to determine which leukocytes were present in the lavage fluid. The leukocytes counted were macrophages, eosinophils, lymphocytes and neutrophils (figure 2.9). Once all the cells had been counted the leukocyte populations were calcuated as a ratio of total cells in 1 ml.

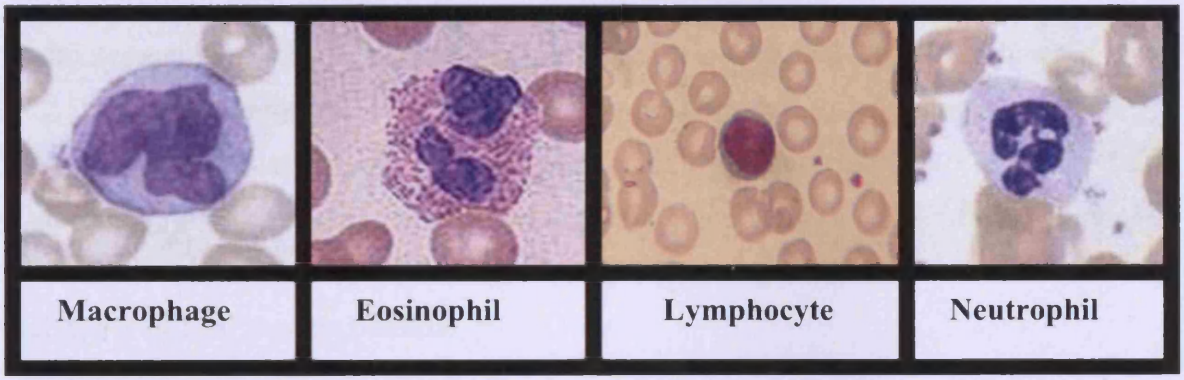


Figure 2.9 - Photographs of the four subtypes of leukocytes, stained with Leishman's solution, counted in a differential count. Diagram taken from (Gude *et al.*, 1982)

Macrophages are the most common inflammatory cells found in the BAL fluid (Hamid *et al.*, 2003). Macrophages are the largest cells found in the BAL fluid with a diameter of 15-20 µm (Gude *et al.*, 1982). Eosinophils are easily definable as they have a bi-lobed nucleus which stains blue. The cytoplasm is full of red/pink stained granules. They are not as big as macrophages, measuring 10-14 µm in diameter (Gude *et al.*, 1982). Lymphocytes, like macrophages, are mononuclear. They can vary in size from small (6-8 µm), medium (8-10 µm) and large (10 µm or more) (Gude *et al.*, 1982). However, the majority of them are small. As figure 2.10 shows they have a large spherical nucleus that stains a dark purple and a small blue cytoplasm. Neutrophils have a diameter measuring around 9-12 µm (Gude *et al.*, 1982). The nucleus stains dark purple and is multi-lobed,

made up of around two- to three-lobes. The cytoplasm contains granules that are smaller in size than those found in eosinophils, they stain a dark purple/pink colour.

2.2.7.2 MICE

As mice have a trachea with such a small diameter the tracheal internal wall was frequently damaged when cannulating causing the release of red blood cells and therefore contaminating the BAL fluid. Consequently, total cell counts were not carried out. Differential cell counts could still be undertaken and the method of retrieving the BAL fluid was the same as in guinea pigs with the exception that the mouse lungs were not removed until the BAL fluid was withdrawn. To prepare the slide for the differential count 200 µl of each sample of BAL fluid was centrifuged for 7 minutes at 1000 rpm at room temperature. After air drying, the slides were stained using Leishman's stain (0.15% in 100% methanol) for 20 minutes then rinsed with distilled water.

The same inflammatory cells were counted as in the guinea pig protocol (figure 2.9). Once again 200 cells were counted and expressed as a percentage of inflammatory cell. However, as no total cell count occurred then estimating total number of inflammatory cell types in 1 ml was not possible.

2.2.8 QUANTIFICATION OF SERUM IMMUNOGLOBULIN G LEVELS USING AN ENZYME-LINKED IMMUNOSORBENT ASSAY

This is described fully in section 3.3.6

2.2.9 HISTOLOGICAL ANALYSIS OF GUINEA PIG AND MOUSE LUNGS

The lungs were removed from the guinea pigs or mice following cellular analysis and they were stored in formaldehyde. Once enough samples were collected to carry out histological analysis a 3-5 mm section from the superior left lobe was cut 1 mm below the bronchus from each sample and stored in histology cassettes. To process the sections they were put through the following protocol:

50% industrial methylated spirit (IMS) – 1 hour

70% IMS – 1 hour
90% IMS – 1 hour
100% IMS – 1 hour 30 minutes
100% IMS – 1 hour 30 minutes
100% IMS – 1 hour 30 minutes
50% IMS:50% chloroform – overnight
Chloroform – 1 hour 30 minutes
Chloroform – 1 hour 30 minutes
Paraffin wax – 6 hours

To embed the lung samples in wax the processed samples were placed in metal histology moulds, molten paraffin wax was then poured into the moulds and then they were left to set on a cold plate. A microtome was used to cut 3-5 μm sections of the embedded lung samples and the sections were subsequently fixed to glass slides and allowed to dry overnight. Once dry the slides were stained with one of the following protocols:

Haematoxylin & Eosin for general morphology

Xylene – 5 minutes
100% IMS – 5 minutes
100% IMS – 5 minutes
90% IMS – 5 minutes
70% IMS – 5 minutes
Distilled water – 5 minutes
Mayer's Haematoxylin – 3 minutes
Rinse in distilled water
Running tap water – 5 minutes
Acid ethanol – dip 8-12 times to destain
Running tap water – 2 minutes
Distilled water – 2 minutes
Eosin – 30 seconds

90% IMS – 15 minutes

100% IMS – 15 minutes

Xylene – 45 minutes

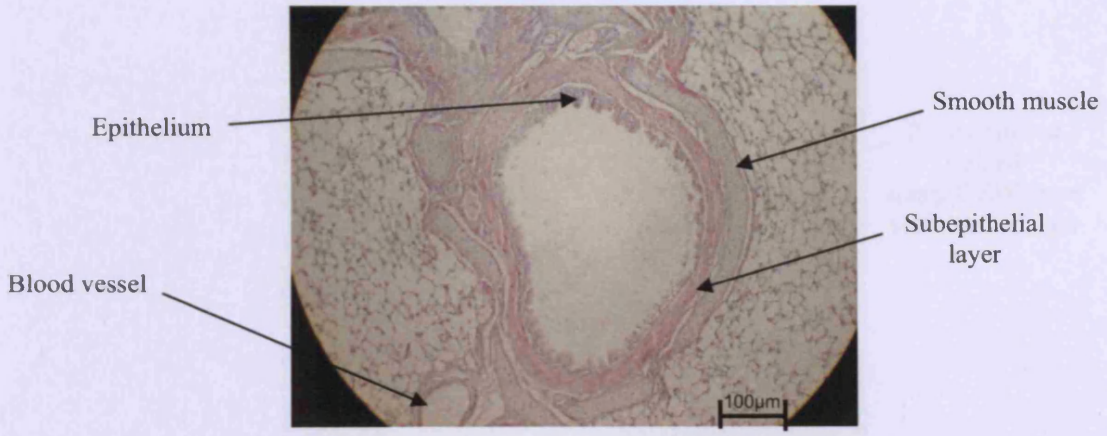


Figure 2.10 – A chronic ovalbumin challenged guinea pig bronchiole that has been stained with haematoxylin and eosin to show general morphology (1000x magnification).

Picrosirius red stain for collagen

Xylene – 5 minutes

100% IMS – 5 minutes

100% IMS – 5 minutes

90% IMS – 5 minutes

70% IMS – 5 minutes

Distilled water – 5 minutes

0.5 g sirius red in 500 ml picric acid – 60 minutes

0.01 M hydrochloric acid – 2 minutes

Rinse in distilled water

Mayer's haematoxylin – 30 seconds

Running tap water – 5 minutes

70% IMS – 5 minutes

90% IMS – 5 minutes

100% IMS – 5 minutes

100% IMS – 5 minutes

Xylene – 5 minutes

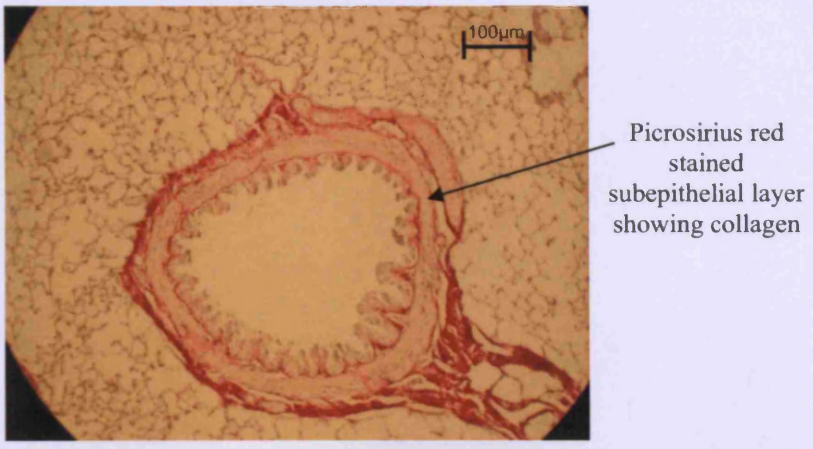


Figure 2.11 – A GW274150F treated chronic ovalbumin challenged mouse bronchiole that has been stained with picrosirius red to show collagen deposition (1000x magnification).

Alcian blue/periodic acid Schiff stain for goblet cells/presence of mucus

Xylene – 5 minutes

100% IMS – 5 minutes

100% IMS – 5 minutes

90% IMS – 5 minutes

70% IMS – 5 minutes

Distilled water – 5 minutes

1% Alcian blue dissolved in 3% aqueous acetic acid (pH 2.5) – 5 minutes

Running tap water – 5 minutes

Periodic acid (0.5%) – 5 minutes

Running tap water – 5 minutes

Distilled water – 5 minutes

Schiff's reagent – 10 minutes

Running tap water – 10 minutes

Mayer's haematoxylin – 20 seconds

Running tap water – 5 minutes

70% IMS – 5 minutes

90% IMS – 5 minutes

100% IMS – 5 minutes

100% IMS – 5 minutes

Xylene – 5 minutes

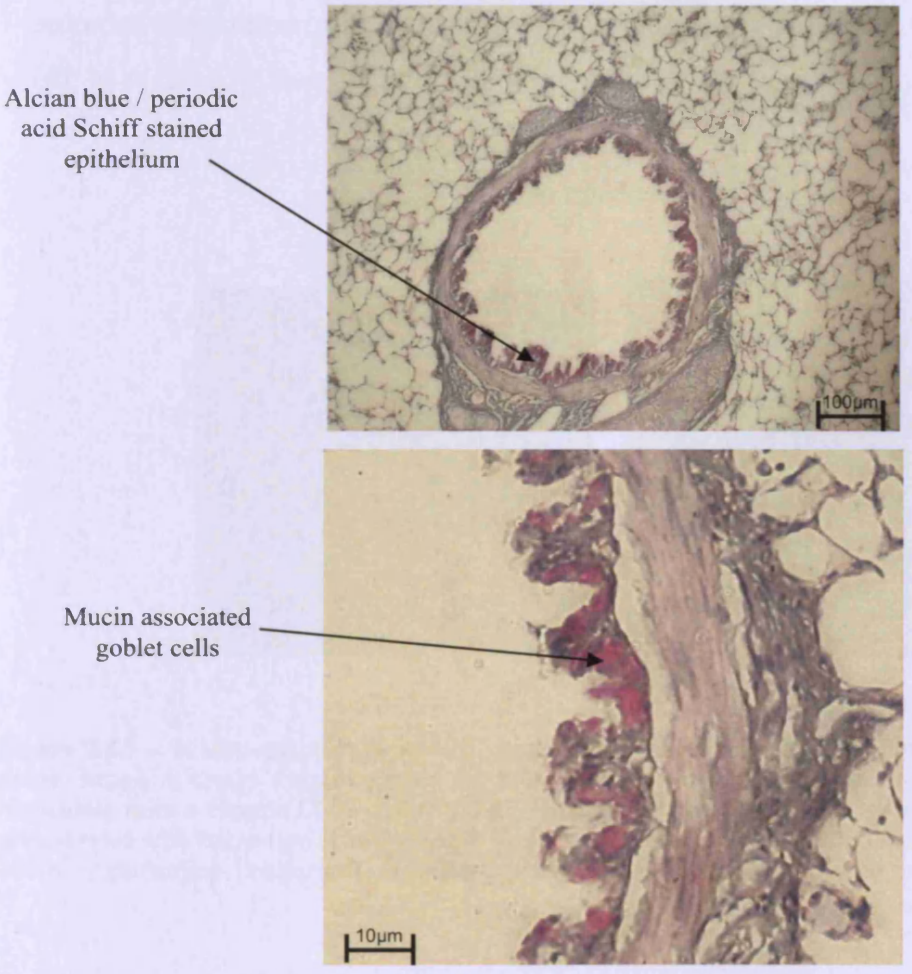


Figure 2.12 – A GW274150F treated chronic ovalbumin challenged mouse bronchiole that has been stained with alcian blue/periodic acid Schiff to show mucin associated goblet cells (1000x magnification). Goblet cells stain bright pink as shown in the zoomed in picture.

Following each of the staining protocols a drop of DPX mountant medium was added to the slide and a coverslip was placed on top ensuring no air bubbles were trapped underneath the slip. Once dry photographs of the slides were then taken using a

microscope at 1000x magnification and a digital camera with 3 times optical zoom. The purpose of including haematoxylin & eosin stain was for qualitative and semi-quantitative analysis. Firstly it showed the general morphology of the bronchiole and as a result enabled easy differentiation between different treatment groups. Secondly a semi-quantitative method was applied to score the number of inflammatory cells present in the peribronchiolar area (the periphery of the small airways). Each of the individual slides from each treatment protocol was assessed by eye at random using a scale which recorded the number of cells as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5) (Barends *et al.*, 2004).

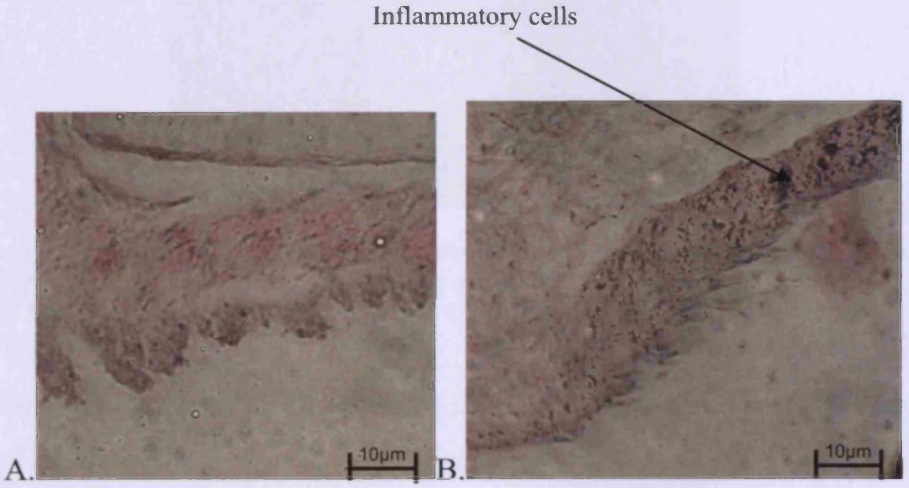


Figure 2.13 – A semi-quantitative scoring method is used for assessing inflammatory cell influx in the lung tissue. Image A shows a naïve guinea pig bronchiole that would be scored '1-minimal'. Image B shows a bronchiole from a chronic OVA challenged guinea pig that would be scored '4-marked'. The bronchioles have been stained with haematoxylin and eosin to show general morphology. Photographs of the images were taken at 1000x magnification. ImageJ software allows closer analysis of the bronchioles by increasing the magnification.

Picrosirius red stain is used to enable analysis of the amount of collagen found around the bronchiole. This method enabled further comparisons between acute and chronic OVA challenged animals and respective drug treatments and also helped determine whether airway remodelling had occurred. The sections of picrosirius red and AB/PAS stained bronchioles were both analysed using ImageJ software. To measure the percentage of

collagen in the bronchiole the lumen (including the epithelium) was drawn around as was the total bronchiole as shown in figure 2.13. The following formula was then applied:

Bronchiolar Collagen = 100 – [(Lumen area ÷ Total bronchiole area) x 100]

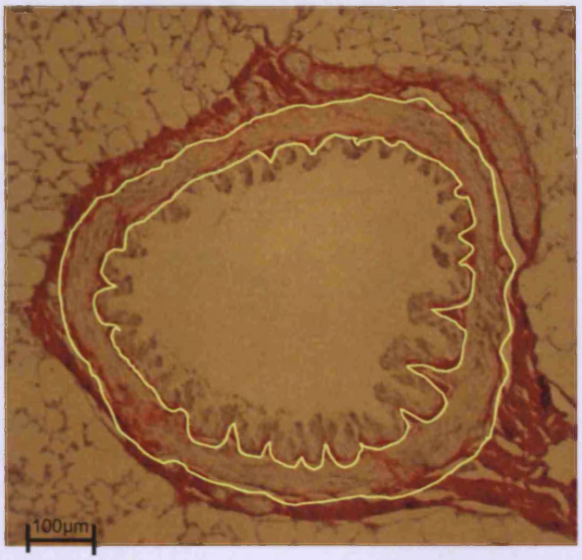


Figure 2.14 – To determine levels of bronchiolar collagen firstly the lumen area (including the epithelium) is drawn around, then the total bronchiole area. They are divided by each other and multiplied by 100. The value is then subtracted from 100. The bronchiole is that of a GW274150F treated chronic ovalbumin challenged mouse (1000x magnification).

Staining with AB/PAS identifies goblet cell associated mucin. As goblet cell hyperplasia is a feature of airway remodelling this staining protocol would also help determine the presence of the structural changes in the airways associated with chronic asthma. To measure the number of mucin-associated goblet cells in the epithelium a click/count method was applied using Image J. This involved counting each of the bright purple-stained mucin containing cells. Firstly the epithelium area was calculated by drawing around the lumen including and excluding the epithelium (figure 2.15) and using the following formula:

Epithelial area = Lumen including epithelium – lumen excluding epithelium

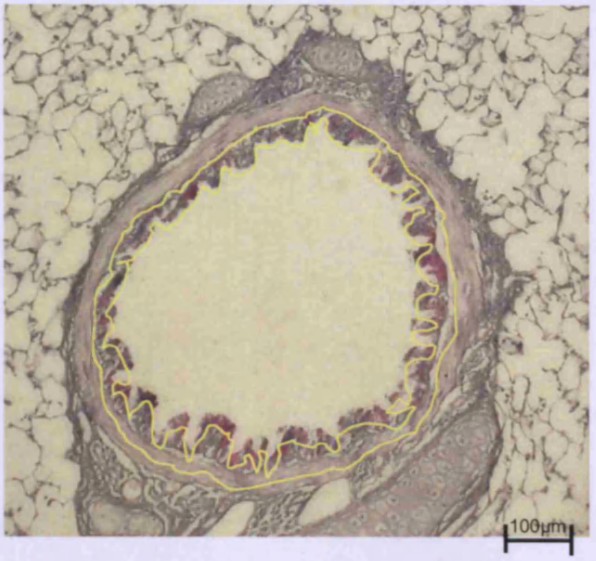


Figure 2.15 – The area of the epithelium is determined by drawing around the lumen (including the epithelium) then subtracting the lumen area (excluding the epithelium). The bronchiole is that of a GW274150F treated chronic OVA challenged mouse (1000x magnification).

Although this method will enable any differences between acute and chronic challenges to be observed it does not distinguish whether the differences were caused by goblet cell hyperplasia or hypertrophy or whether epithelial shedding is contributing. Therefore, the AB/PAS positive points were counted (figure 2.16) and the following formula was applied to determine the mucin-associated goblet cells per 10000 epithelial pixels:

$$\text{Mucin per 10000} = (\text{Number of AB/PAS+ points} \div \text{Epithelial area}) \times 10000$$

The outcome of this formula enabled any differences in goblet cell hyperplasia to be recorded.

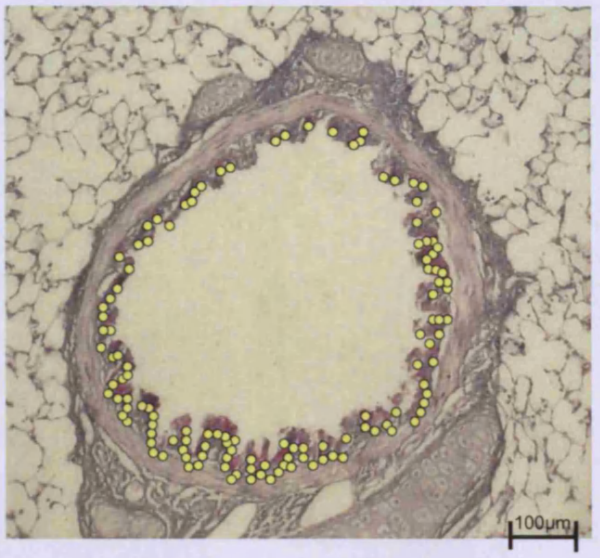


Figure 2.16 – Each goblet cell (shown as a yellow dot) is counted to ensure the differences in mucin associated goblet cells is a result of goblet cell hyperplasia not hypertrophy or epithelial shedding. The bronchiole is that of a GW274150F treated chronic OVA challenged mouse (1000x magnification).

2.2.10 MRI ANALYSIS

This is described fully in chapter 8

2.2.11 DRUG ADMINISTRATION

The effects that corticosteroids, a PDE4 inhibitor and an iNOS inhibitor have on acute and chronic models of asthma have been evaluated throughout this thesis. The drugs used were fluticasone propionate, dexamethasone, roflumilast and the iNOS inhibitor (GW274150F). Each of the drugs were administered 24 hours and 0.5 hours pre-ovalbumin challenge and 6 hours post-ovalbumin challenge. The methods of administration, dose and vehicle for each drug are detailed in table 2.1.

Drug	Method of Administration	Dose	Vehicle
Fluticasone Propionate	Nebulised Inhalation (15 minutes)	0.51 mg/ml	Saline – 40% DMSO – 30% Ethanol – 30%
Dexamethasone	Intraperitoneal	20 mg/kg	Saline – 50% DMSO – 50%
Roflumilast	Oral (Gavage Needle)	1 mg/kg	Methyl Cellulose (10mg in 10ml of sterile H_2O)
INOS inhibitor (GW274150F)	Oral (Gavage Needle)	5 mg/kg	Methyl Cellulose (10mg in 10ml of sterile H_2O)

Table 2.1 - List of the compounds used, administration route, dose and vehicle.

The fluticasone propionate vehicle of saline (40%): DMSO (30%): Ethanol (30%) was selected because there was difficulty in getting the drug to dissolve in saline and DMSO alone. Previous work in the laboratory had identified the saline:DMSO:ethanol mix as a vehicle for budesonide (Nevin & Broadley, 2004) and it was shown to have minimal side-effects whilst also ably dissolving the drug. Dexamethasone dissolved easily in saline (50%): DMSO (50%) (Toward & Broadley, 2004) as a result of which there was no need to introduce ethanol. Methyl cellulose was used as a vehicle as it is a viscous material that the drugs dissolved in easily. As a result of the viscosity the guinea pigs and mice took the drugs without any complaint or noticeable side-effects.

Fluticasone propionate was administered by inhalation to match the clinical route of administration (Barnes and Adcock, 2003). Clearly guinea pigs or mice cannot be given an inhaler so a 15 minute inhalation using a Wright nebuliser connected to a Perspex box measuring (38 cm length; 20 cm width; 20 cm height) was used. Dexamethasone was administered via the IP route because previous data had shown this route to inhibit successfully some of the features of asthma (Toward & Broadley, 2004). Roflumilast was given as an oral dose to match the clinical trial setting (Spina, 2008) and although limited

clinical work has been carried out on GW274150F it was agreed that the oral route would be the best option (Nials, personal communication).

2.3 STATISTICAL ANALYSIS

In all studies that were comparing two points a two-tailed t-test was used for statistical analysis. If three or more groups were being compared a one-way analysis of variance (ANOVA) followed by a Bonferroni post test was used. All results were plotted as mean±SEM and a P value of <0.05 was considered significant. Figures 3.2b and 4.1b show raw data. However these graphs do not take variations in baseline calibration values into account, therefore % change from baseline graphs were used elsewhere with raw values shown in the legends. As guinea pigs and mice have slight variations in when they have an EAR and LAR, three methods were used to assess these parameters. Firstly, values of sG_{aw} were plotted as a time course graph as % change from baseline, secondly, maximum bronchoconstriction values between 0-6 hours and 7-12 hours were recorded to account for the EAR and LAR, respectively. These were plotted as a histogram on the same graph as the time course plot. T Finally, area under the curve (AUC) analysis was carried out on the time course graph, assessing total AUC, EAR AUC (0-6 hours) and LAR AUC (6-24 hours). This allowed %.h to be measured and therefore offered another statistical approach. Graphs were drawn using GraphPad Prism 5 and results were analysed using GraphPad Instat 3.

Chapter 3

Analysis of guinea pig acute and chronic models of asthma

3.1 INTRODUCTION

3.1.1 GUINEA PIG MODELS OF ASTHMA

Guinea pigs have been used as models of pulmonary hypersensitivity for more than 100 years (Karol, 1994); however, the development of animal models of asthma is a more recent phenomenon (Cates *et al.*, 2007). In 1983 it was shown that guinea pigs that were exposed to the organic chemical toluene diisocyanate demonstrated both early and late phase bronchoconstrictions, hyperreactivity towards cholinergic agonists and production of hypersensitivity antibodies (Karol, 1983). This was an early model of occupational asthma.

Although guinea pigs have proved an important model of asthma like all other animal disease models their effectiveness in representing human conditions has to be carefully considered. There are several advantages of using guinea pigs for models of asthma compared to larger species such as sheep and dogs. Guinea pigs are reasonably inexpensive and are therefore a relatively cost efficient model. They are also small in size and therefore easy to use. Guinea pigs also have advantages over other small animal models such as rats and mice. As they are so docile they can be restrained and therefore accurate measurements of airway function can be recorded without a need for anaesthesia. They also readily respond to histamine in a similar manor to humans (Zosky & Sly, 2007).

There are also several disadvantages in using guinea pigs as models of asthma. A major criticism in using all small animal models is that they do not develop an asthma-like condition naturally and therefore usually require an adjuvant in order to have an immune response (Zosky and Sly 2007). It is argued that using an adjuvant neglects the fact that humans are generally exposed at the respiratory mucosa by aeroallergens and as a result this could have immunological consequences (Cates *et al.*, 2007). Although Nabe *et al.*, (1997) developed an aerosol sensitisation model an adjuvant was still required and sensitisation took 4 weeks.

Guinea pigs are also sometimes overlooked as models because of the poor availability of species-specific reagents. Therefore the role of genetics in asthma cannot be as well defined in this model as it can be in mice where there is a vast availability of species-specific reagents (Zosky and Sly 2007). One of the main fundamental differences between guinea pigs and

humans is that the main antibody present during a type 1 hypersensitivity reaction in guinea pigs is immunoglobulin (Ig) G1 whereas in humans it is IgE. Despite these disadvantages guinea pigs are still an important tool in understanding the mechanisms of asthma and in drug discovery and development.

3.1.2 GUINEA PIG OVALBUMIN MODELS

The majority of models of asthma use ovalbumin (OVA) as the allergen to provoke an immune response (Cates *et al.*, 2007). OVA models have been developed in both conscious (Smith & Broadley, 2007) and anaesthetised (Johnson & Broadley, 1999) guinea pigs, however, it is possible that anaesthetic may interfere with the vagal tone or sensory reflex (Toward & Broadley, 2004). The availability of whole-body plethysmographs means that lung function can easily be measured in conscious restrained guinea pigs using specific airway conductance (sG_{aw}) as a parameter (Griffiths-Johnson *et al.*, 1988).

Several studies have shown that sensitising and challenging a guinea pig with OVA results in reproducible airway constriction responses similar to those observed in human asthma (Smith and Broadley 2007; Nabe *et al.*, 1997, Karol 1994). Ideally a good model of asthma should display an early and late asthmatic response, airway hyperresponsiveness and cellular influx into the lungs (Smith and Broadley 2007). However, the chronic nature of asthma needs to be considered and the majority of guinea pig studies use only acute inflammation (Zosky and Sly 2007).

Studies that demonstrate chronic airway changes in the guinea pig airways after OVA challenge are rare, in fact Zosky and Sly (2007) state that there is no model in smaller laboratory animals that demonstrates both chronic remodelling and long-term airway hyperresponsiveness. It was not until recently that chronic OVA guinea pig models of asthma started appearing. Studies using multiple OVA challenges were shown to cause smooth muscle remodelling (Gosens *et al.*, 2005) and goblet cell hyperplasia (Bos *et al.*, 2007) but other features of asthma were not measured. More recently Bazan-Perkins, Sanchez-Guerrero (2009) showed airway remodelling, airway hyperresponsiveness and increased levels of cellular influx in the airways in a chronic guinea pig model, however, early and late asthmatic responses were not measured and airway hyperresponsiveness peaked around a third of the way through the study. Currently it seems there is a lack of guinea pig models which displays

early and late phase bronchoconstriction, airway hyperresponsiveness, cellular influx into the lungs and airway remodelling.

The reason for the lack of chronic guinea pig models of asthma could be because of the issue of tolerance. Sensitised animals that are chronically exposed to an allergen can develop tolerance and therefore have a reduced allergic response (Schramm *et al.,* 2003). Tolerance is somewhat of a double-edged sword. Studies have shown that exposure to cat dander during childhood can reduced the incidence of asthma in adulthood (de Meer *et al.*, 2004; Roost *et al.*, 1999). However, if an animal model develops tolerance to repeated allergen exposure, then it would make chronic changes in remodelling more difficult to establish. Increasing the dose of allergen used could go some way in preventing tolerance, though measures must be taken to prevent the anaphylaxis that a higher dose could cause in the animal models.

3.1.3 REQUIRED FEATURES OF ACUTE AND CHRONIC GUINEA PIG MODELS OF ASTHMA

As previously mentioned a good model of asthma should display early and late asthmatic responses, airway hyperresponsiveness, cellular influx into the airways and airway remodelling. These are all common features of human asthma and therefore if they can be replicated in a consistent manner the model could prove effective at investigating the mechanisms of asthma and potential therapeutics.

For more than 30 years it has been known that early and late phase bronchoconstrictions after a single dose of allergen have been observed in humans with atopic asthma (Pepys & Hutchcroft, 1975).The early phase bronchoconstriction, also known as early asthmatic response (EAR), occurs within 10 minutes, reaches a maximum after 30 minutes and recovers around 3 hours in human asthmatics (Gauvreau & Evans, 2007). A second bronchoconstriction known as the late phase bronchoconstriction or late asthmatic response (LAR) occurs between 4-8 hours in human asthmatics (Booij-Noord *et al.*, 1971). Therefore a model of asthma should show these responses during the same time points as seen in human asthma.

Airway hyperresponsiveness (AHR) is a key feature of asthma (Hargreave *et al.*, 1981) therefore it is extremely important that an animal model is able to display some form of AHR. A role for monitoring AHR in the long-term management of asthma was suggested by

Sont *et al.*, (1999), as a result it can be classified as one of the defining features of an asthmatic response. AHR is characterised by an increased sensitivity of the airways to a variety natural or pharmacological stimuli such as histamine and methacholine (Booij-Noord *et al.*, 1971). Therefore a sensitised and allergen exposed model of asthma should have an enhanced bronchoconstriction response when challenged with histamine or methacholine.

As asthma is a pulmonary inflammatory disorder an increase in inflammatory cells should be observed in the lungs. Macrophages, eosinophils, lymphocytes and neutrophils are among several different inflammatory cells that play a role in asthma and can therefore be found in the airways of asthmatics (Barnes, 1996). Lavaging the lungs is a method that has been used to analyse lung inflammatory cell levels in both humans (Calhoun *et al.*, 1991) and animals (Smith & Broadley, 2007), for that reason a good model of asthma should show increased inflammatory cell levels when it is lavaged. However, the number of inflammatory cells in the lavage fluid do not necessarily reflect the number of cells in lung tissue therefore some studies use histology as a method for assessing cell number (Barends *et al.*, 2004).

As mentioned, asthma is a chronic disorder but the majority of animal models are acute (Zosky and Sly 2007). These acute models do not show the same levels of airway remodelling that is observed in an asthmatic lung such as collagen deposition and goblet cell hyperplasia. Therefore if a model is going to give us a better understanding of the mechanisms of asthma and prove a better model for drug discovery and development it is clearly important that airway remodelling is present.

In order to ensure complete sensitisation has occurred and to confirm a hypersensitivity reaction it is worthwhile to quantifiably measure the levels of the hypersensitivity antibody in the serum of the animal model. In guinea pigs this is IgG1 but it is IgE in humans. Therefore running an enzyme-linked immunosorbant assay (ELISA) to evaluate guinea pig serum levels of IgG1 would be beneficial in improving the quality of the model.

3.2 AIMS AND OBJECTIVES

3.2.1 AIMS

The aim of this chapter was to assess early and late phase bronchoconstriction, airway hyperresponsiveness, cellular influx and lung histology in OVA sensitised acute and chronic guinea pig models of asthma. By measuring these characteristics, a comparison can be drawn between both models and in subsequent chapters the effects of anti-asthmatic compounds will be examined on these asthmatic features. The level of IgG in both acute and chronic models was assessed to ensure adequate sensitisation.

3.2.2 OBJECTIVES

- To investigate the effect that a single exposure of OVA or saline has on sensitised guinea pigs by measuring lung function, responsiveness to histamine and cellular influx.
- To investigate the effects of multiple exposures of OVA or saline on the same parameters mentioned in the previous point.
- To analyse the level of blood IgG found in naïve, saline challenged and OVA challenged guinea pigs by using an enzyme-linked immunosorbent assay.
- To assess bronchiole structure, collagen deposition and goblet cell hyperplasia by utilising histological techniques.

3.3 METHODS

The methods that were used are described in full detail in chapter 2; the following is a brief overview. For each study in this chapter six male Dunkin-Hartley guinea pigs (Harlan, UK) weighing 200-250 g were used. An n of six was used for each experimental group.

3.3.1 SENSITISATION

Guinea pigs were sensitised by an intra-peritoneal, bilateral injection of a suspension containing OVA (100 μg) and $Al(OH)_3$ (100 mg) in 1 ml of PBS on days 1 and 5.

3.3.2 OVALBUMIN CHALLENGES

Ovalbumin challenges for both acute and chronic groups were carried out in a stainless steel chamber with a Wright nebuliser attached. Exposures lasted 1 hour, however, if the guinea pig appeared distressed it was removed and exposure was considered complete.

3.3.2.1 ACUTE PROTOCOL

On day 15 (14 days subsequent to the first sensitisation injection), guinea pigs following the acute protocol were challenged with a single exposure of OVA (0.01%) or saline (see figure 2.2).

3.3.2.2 CHRONIC PROTOCOL

Guinea pigs that were following the chronic protocol were also challenged with an exposure of OVA (0.01%) or saline on day 15. Every 48 hours from days 17-29 guinea pigs were then challenged with an exposure of OVA (0.1%), this was preceded by 30 minutes with an intra-peritoneal, bilateral injection of mepyramine (30 mg/kg). On day 31 the guinea pigs were once again challenged with an exposure of OVA (0.1%), however, this time no mepyramine protection was required. Over the same period the control group of sensitised guinea pigs were challenged with 1 hour long saline exposures (see figure 2.3).

3.3.3 LUNG FUNCTION MEASUREMENTS

Lung function measurements were achieved by using conscious guinea pigs restrained in a whole-body plethysmograph. This allowed values of sG_{aw} to be obtained. Lung function measurements were acquired in the same way for both the acute and chronic challenged groups. On day 14 (prior to any OVA exposures) baseline values of sG_{aw} were recorded. Following the final OVA exposure, day 15 for acute groups and day 31 for chronic groups, further values were recorded. The time points were 0, 15 minutes, 30 minutes, 45 minutes and 60 minutes post-exposure, then hourly until 12 hours and a final reading at 24 hours. As the maximum bronchoconstriction of the LAR occurred at different times in each animal, the peak bronchoconstrictions between 7 and 12 hours were recorded. These were displayed along with the peak of the early phase (0-6 hours) as a histogram next to the time course plot.

3.3.4 AIRWAY HYPERRESPONSIVENESS MEASUREMENTS

Whole-body plethysmography in conscious guinea pigs was used to evaluate AHR. The response of the guinea pig to a nose-only nebulised solution of histamine (1 mMol for 20 seconds) determined the presence of AHR. Values of sG_{aw} were recorded prior to the histamine challenge and 0, 5 and 10 minutes post-challenge. The histamine response was assessed 24 hours before the first OVA challenge (day 14) and 24 hours after the final OVA challenge on day 16 for acute groups and day 32 for chronic groups.

To investigate the recovery rates of both the acute and chronic challenged groups two further groups were introduced. The first group followed the acute protocol but the final histamine challenge did not occur until 72 hours after the OVA challenge (day 18). The second group followed the chronic protocol and once again the final histamine challenge did not occur until 72 hours after the last OVA challenge (day 34).

3.3.5 TOTAL AND DIFFERENTIAL CELL COUNTS

Following the final histamine challenge the guinea pigs were administered a lethal dose of sodium pentobarbitone, the lungs were then removed and lavaged. Using the BAL fluid

recovered total and differential cell counts were then carried out using the methods described in chapter 2. A naive, non-sensitised, group of guinea pigs were introduced at this stage as a reference to establish the normal cell counts in a guinea pig.

3.3.6 HISTOLOGICAL ANALYSIS OF GUINEA PIG LUNGS

Histological techniques for the lungs are detailed in chapter 2. Briefly, 3-5 mm thick portions of the left superior lobe sliced 1 mm below the bronchus were processed into wax blocks. 6μm sections were then sliced using a microtome and fixed onto a glass slide. The slices were stained using either the haematoxylin and eosin, picrosirius red or AB/PAS protocols. The purpose of haematoxylin and eosin staining was two-fold. Firstly, the staining shows the general morphology of the bronchiole allowing for any structural changes to be easily observed. Secondly, the stain allowed the assessment of the number of inflammatory cells in the peribronchiolar space of the lung tissue by using a semi-quantifiable scoring method. The parameters for scoring were absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5) (Barends *et al.*, 2004). For the sections stained with picrosirius red or AB/PAS protocols the mean % of collagenous or AB/PAS positive area of the bronchiolar epithelium was determined respectively. ImageJ software was used to quantify the results.

3.3.7 QUANTIFICATION OF SERUM IMMUNOGLOBULIN G LEVELS USING AN ENZYME-LINKED IMMUNOSORBENT ASSAY

In order to ensure that sensitisation had occurred and to determine the differences in Immunoglobulin G (IgG) levels between the acute and chronic groups 1ml of blood was removed directly from the heart of the guinea pig following the administration of sodium pentobarbitone. The blood was left to coagulate overnight and then centrifuged at 18000g for 15 minutes at a temperature of 2°C. Serum was removed and stored at –80°C. Once enough samples were collected an anti-guinea pig OVA specific IgG1 enzyme-linked immunosorbent assay (ELISA) (figure 3.1) was run using the serum.

The ELISA plate was coated with 100 μg/well of OVA (10 μg/ml in phosphate buffered saline) and left overnight at room temperature. The following day the unbound antigen was washed off using phosphate buffered saline with tween (PBST) buffer three times. The plate

was then blocked using 1% non fat milk powder in PBST for 30 minutes, following this the plate was once again washed three times with PBST. 100 µl/well of serum (diluted 1:1000 in PBST) were added to the plate and left for 90 minutes. In order to monitor background colour change some wells had PBST containing no serum added.

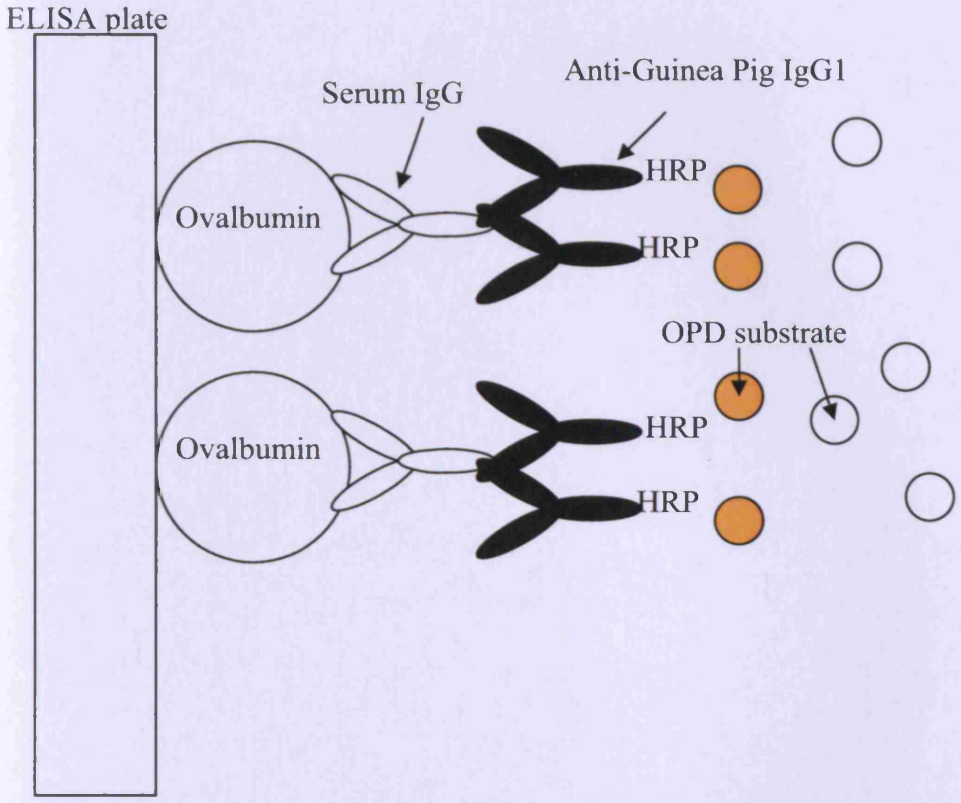


Figure 3.1 - A schematic of the indirect ELISA used to detect levels of IgG1 in the guinea pig serum. Ovalbumin binds to the ELISA plate and in turn is bound by the serum IgG. The enzyme-linked anti-guinea pig IgG1 antibody reacts with the serum IgG. An o-phenylenediamine dihydrochloride (OPD) substrate is added which causes a colour change when it binds the enzyme-linked antibody. IgG = Immunoglobulin G; HRP = Horse Radish Peroxidase.

Following the 90 minute period the plate was washed three times with PBST and then 50 µl/well of 1:500 dilution of the Horse Radish Peroxidase (HRP)-anti guinea pig IgG1 antibody was added and left for 60 minutes. After a final three washes with PBST, 50 µl/well of an o-phenylenediamine dihydrochloride (OPD) substrate was added. This comprised of 12.15 ml 0.1 M citric acid; 12.85 ml 0.2 M sodium phosphate; 25 ml distilled water; OPD (0.1 M citrate-phosphate buffer pH 5.0). OPD is a substrate for the peroxidise enzyme and as a result it yielded a yellow-brown colour when it reacted with the HRP conjugated to the anti-

guinea pig IgG1 antibody. As soon as the change had developed sufficiently 50 µl/well of sulphuric acid was added to stop the colour changing further (after around 15-20 minutes). Using a spectrophotometer the absorbance at 490 nm was determined for each of the samples. The more IgG detected by the ELISA the higher the colour changing unit.

3.4 RESULTS

3.4.1 EFFECT OF AN ACUTE OVALBUMIN CHALLENGE ON LUNG FUNCTION

Figure 3.2a represents the mean time course changes in sG_{aw} that sensitisation followed by a single OVA (0.01%) or saline challenge cause. In OVA sensitised and challenged guinea pigs a significant bronchoconstriction was observed immediately (-68.8±8.7%), this did not resolve to saline challenged levels until 5 hours following the OVA challenge. This bronchoconstriction was the EAR. 7 hours following the challenge a second significant bronchoconstriction was observed (-19.8±5.7%), the LAR, which recovered to saline challenged levels by 8 hours.

The mean of the peak early and late asthmatic responses are also represented in figure 3.2 as a histogram. An early phase bronchoconstriction was demonstrated in OVA challenged guinea pigs shown by a significant decrease in sG_{aw} compared to saline challenged guinea pigs (-71.2±6.5% compared to -8.9±1.5% respectively). This was also the case for levels of late phase bronchoconstriction (-25.2±6.7% compared to -1.3±0.8% respectively).

Figure 3.3 represents area under the curve analysis for both saline and OVA challenged sensitised guinea pigs. Throughout the whole time course OVA challenged showed a significantly greater area under the curve than saline challenged (977.3±111.8%.hr compared to 63.8±18.0%.hr). This was also true for the EAR time points (853.3±103.6%.hr compared to 55.2±18.9%.hr) and LAR time points (134.4±29.6%.hr compared to 4.2±1.5%.hr).

3.4.2 EFFECT OF AN ACUTE OVALBUMIN CHALLENGE ON AIRWAY RESPONSIVENESS TO HISTAMINE

Figure 3.4 demonstrates the effect of challenging sensitised guinea pigs with histamine (1 mMol for 20 seconds) before and after a saline or OVA exposure. Following a saline challenge guinea pigs show no significant bronchoconstriction when compared to the sG_{aw} values prior to the saline challenge. In the OVA exposed group there was a significant bronchoconstriction immediately after the histamine challenge when compared to the sG_{aw}

values taken prior to the OVA challenge (-18.8±2.4% compared to -3.4±5.3% respectively). This bronchoconstriction was still present 5 minutes after the histamine challenge (-6.8±0.6% compared to 1.7±3.0% respectively). By 10 minutes the guinea pigs had recovered to pre-OVA exposure levels.

In order to determine how long AHR lasts in acute challenged guinea pigs a group was added that followed the same protocol as the OVA (0.01%) challenged guinea pigs, but instead of the histamine challenge being carried out 24 hours after OVA exposure this group had the histamine challenge at 72 hours. Figure 3.5 shows that no AHR was present in guinea pigs that were given a longer recovery period.

3.4.3 EFFECT OF CHRONIC OVALBUMIN CHALLENGES ON LUNG FUNCTION

Figure 3.6 represents mean time course changes in sG_{aw} for OVA sensitised guinea pigs that had received nine challenges with OVA at 48 hour intervals. Saline controls received repeated saline exposures at the same intervals. Changes in sG_{aw} were assessed after the final OVA or saline exposure (day 31). The guinea pigs challenged with repeated OVA exposures showed an immediate bronchoconstriction of -71.6±2.6% which was significant when compared to saline challenged values. This bronchoconstriction did not resolve for 5 hours. A second significant bronchoconstriction occurred at 7 hours (-22.4±2.0%) this recovered to saline challenged levels at 9 hours.

When comparing mean of the peak bronchoconstriction values an early asthmatic response was observed in the OVA challenged group observed as a significantly greater bronchoconstriction than the saline challenged group (-71.6±2.6% compared to -5.7±0.8% respectively). This significantly greater decrease in sG_{aw} was again seen between 7-12 hours demonstrating that the OVA challenged group had a late asthmatic response (-22.4±2.0% compared to -1.3±0.5% respectively).

When area under the curve analysis was applied to both groups (figure 3.7) the OVA challenged group showed a significantly greater area than the saline challenged group overall (875.3±57.1%.hr compared to 32.0±8.9%.hr). This was also seen for the early asthmatic time period (691.4±29.4%.hr compared to 29.6±9.1%.hr) and the late asthmatic time period (187.3±31.5%.hr compared to 4.2±1.8%.hr).

3.4.4 EFFECT OF CHRONIC OVALBUMIN CHALLENGES ON AIRWAY RESPONSIVENESS TO HISTAMINE

Figure 3.8 highlights the changes in sG_{aw} from baseline following a histamine challenge (1 mMol for 20 seconds) preceding and following multiple saline or OVA exposures. No response to histamine was observed in saline challenged guinea pigs. An immediate significant bronchoconstriction was seen in guinea pigs that had received multiple OVA exposures compared to sG_{aw} values before they had been exposed to nebulised OVA (-40.5±0.8% compared to 1.5±0.8% respectively). This bronchoconstriction remained in place 5 (-33.0±1.5% compared to 0.7±0.8% respectively) and 10 (-10.0±3.1% compared to 1.4±1.6% respectively) minutes after the histamine challenge.

In figure 3.9 the level of recovery at 72 hours following the histamine challenge and the recovery at 24 hours is shown. There was still a significant bronchoconstriction immediately following the histamine at the 72 hour recovery point compared to before they received any OVA (-25.4±5.1% compared to -1.5±4.4% respectively). Despite the fact that AHR was still present at 72 hours it was not as severe as it was at 24 hours.

3.4.5 EFFECT OF ACUTE AND CHRONIC OVALBUMIN CHALLENGES ON TOTAL AND DIFFERENTIAL CELL COUNTS

Figure 3.10 represents the number of cells recovered in the BAL fluid of naïve, saline challenged and OVA challenged guinea pigs. A single and multiple OVA exposures caused a significant increase in the total number of BAL cells, as well as increase in macrophage, eosinophil, lymphocyte and neutrophil number compared to their respective saline challenged group. Multiple OVA exposures resulted in a significantly greater number of BAL cells than a single exposure ($11.1\pm0.3x10^6$ compared to $8.8\pm0.6x10^6$ respectively). This was also true for macrophage number ($4.4\pm0.2x10^6$ compared to $2.9\pm0.3x10^6$ respectively) and eosinophil number ($6.3\pm0.1x10^6$ compared to $5.2\pm0.3x10^6$ respectively). Despite the increase in inflammatory cells that multiple OVA exposures cause the BAL levels of neutrophils are slightly higher, though not significantly, in the acute model than the chronic model.

Figure 3.11 compares acute and chronic OVA challenged guinea pigs that were lavaged at either 24 hours or 72 hours. The total cell numbers between single challenged OVA guinea pigs was significantly greater than in the 24 hour recovery group compared to the 72 hour group ($8.8\pm0.6x10^6$ compared to $3.3\pm0.2x10^6$ respectively). This was also the case in macrophage number ($2.9\pm0.3x10^6$ compared to $1.8\pm0.1x10^6$ respectively), eosinophil number ($5.2\pm0.3x10^6$ compared to $1.4\pm0.1x10^6$ respectively) and neutrophil number ($0.5\pm0.1x10^6$ compared to $0.01\pm0.01x10^6$ respectively).

A significant increase in total cell numbers was also observed in the chronic OVA challenged group that had 24 hours recovery compared to the 72 hour recovery group ($11.1\pm0.3x10^6$ compared to $6.8\pm0.7x10^6$ respectively). Again this was true for the numbers of macrophages ($4.4\pm0.2x10^6$ compared to $2.8\pm0.2x10^6$ respectively), eosinophils ($6.3\pm0.1x10^6$ compared to $3.9\pm0.3x10^6$ respectively) and neutrophils ($0.3\pm0.05x10^6$ compared to $0.03\pm0.03x10^6$ respectively).

3.4.6 EFFECT OF ACUTE AND CHRONIC OVALBUMIN CHALLENGES ON LUNG HISTOLOGY

Figures 3.12 (A-E) shows a bronchiole stained with haematoxylin and eosin for a naïve, acute saline challenged, acute OVA challenged, chronic saline challenged and chronic OVA challenged guinea pig. These pictures highlight the general morphology of each of the bronchioles. The bronchioles of the naïve, acute saline challenged, acute OVA challenged and chronic saline challenged are all relatively similar with a small amount of smooth muscle, if any, a thin lamina propria and a small epithelial layer. The chronic OVA challenged guinea pig bronchiole shows a much greater level of airway smooth muscle, a thicker lamina propria and a larger epithelium compared to the other protocols.

Figure 3.13 shows the mean pathological scores for the number of inflammatory cells in the peribronchiolar space of naïve, acute saline challenged, acute OVA challenged, chronic saline challenged and chronic OVA challenged guinea pigs. Multiple exposures of OVA cause a significantly greater level of inflammatory cells in the lung tissue compared to repeated saline exposures (4.8 ± 0.3 compared to 1.0 ± 0.4 respectively). However, this increase in inflammatory cells is not a result of an OVA exposure. Repeated exposures are required for an increased score to be observed as shown by the significantly greater level of inflammatory

cells in the chronic OVA challenged guinea pigs compared to the acute OVA challenged guinea pigs (4.8±0.3 compared to 1.8±0.3 respectively).

Figure 3.14 (A-E) show picrosirius red stained bronchioles from the five protocols. The naïve, acute saline challenged, acute OVA challenged and chronic saline guinea pig bronchioles show limited red stained collagen. However, the chronic OVA challenged group show collagen throughout the lamina propria and smooth muscle. Figure 3.15 represents the percentage of bronchiolar collagen in naïve, saline and OVA challenged guinea pigs. No significant difference was observed between a single saline or OVA challenge. Multiple challenges of OVA caused a significantly greater percentage of bronchiolar collagen than multiple challenges of saline (58.1±5.0% compared to 19.7±3.5% respectively). When comparing both OVA challenges the chronic group had a significantly greater percentage of bronchiolar collagen than the acute group (58.1±5.0% compared to 16.9±1.7%).

Alcian blue and periodic acid Schiff stained guinea pig bronchioles are shown in figure 3.16 (A-E). The epithelial cells stain a dark purple whereas mucin associated goblet cells are a brighter pink colour. The naïve, acute saline challenged, acute OVA challenged and chronic saline guinea pig bronchioles have a very dark purple epithelium whereas the chronic OVA challenged group have a brighter pink epithelium suggesting the presence of goblet cells. Figure 3.17 shows the number of mucin associated goblet cells per 10,000 epithelial pixels for the same groups, again no significant differences were observed between acute challenged guinea pigs. Guinea pigs following a chronic OVA challenge protocol showed a higher number of mucin associated goblet cells per 10,000 epithelial pixels than those following a chronic saline challenge protocol (9.4±1.9 compared to 1.9±0.2 respectively). Chronic OVA challenged guinea pigs also show a higher number of mucin associated goblet cells per 10,000 epithelial pixels than acute OVA challenge guinea pigs (9.4±1.9 compared to 2.5±0.5%).

3.4.7 EFFECT OF ACUTE AND CHRONIC OVALBUMIN CHALLENGES ON BLOOD IgG1 LEVELS

Figure 3.18 represents the results of an anti-guinea pig OVA specific IgG1 ELISA in level of colour changing units measured at 490nm. All groups had an increase in intensity that was significantly greater than naïve guinea pigs. The acute OVA challenged group had a

significantly greater intensity than acute saline challenged (0.5±0.008 compared to 0.2±0.01 respectively). An even greater increase in colour changing units was observed between chronic OVA and chronic saline (2.2±0.06 compared to 0.7±0.04 respectively). The ELISA also demonstrated that repeated OVA challenges had a significantly greater effect on the colour intensity than a single OVA dose.

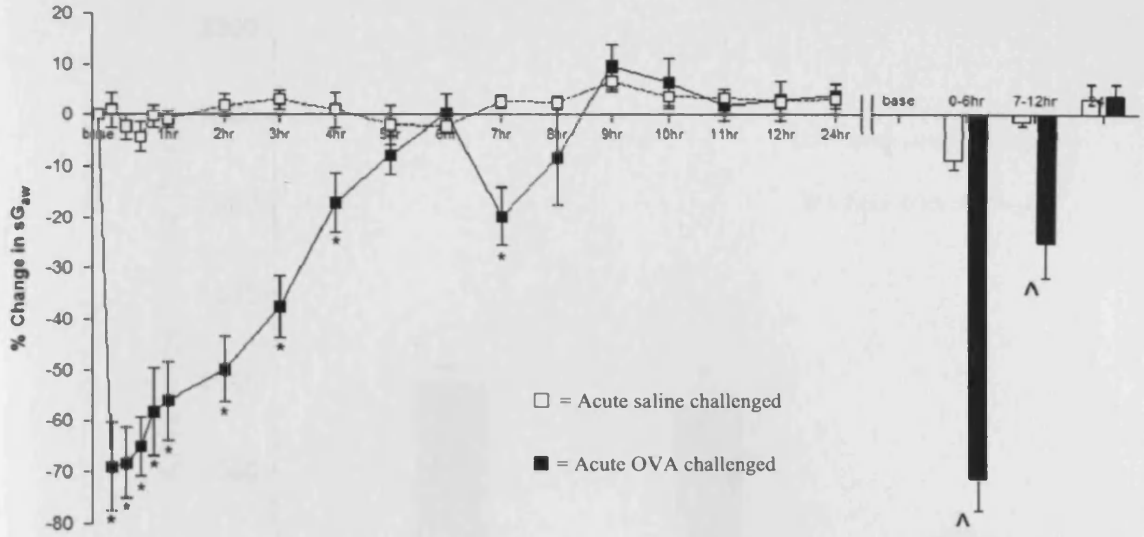


Figure 3.2a – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that were challenged with saline or 0.01 % OVA. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from saline challenged guinea pigs; ^ significant difference between saline and OVA challenged guinea pigs. Two-tailed T-test ($P<0.05$; n=6).

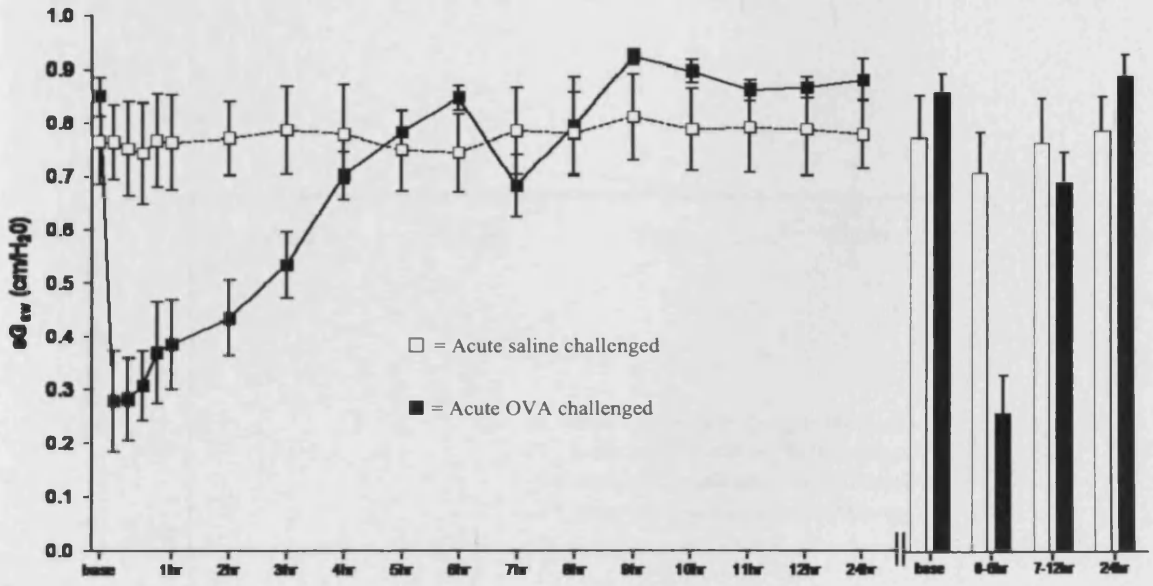


Figure 3.2b – Mean time-course raw values of sG_{aw} in OVA sensitised guinea pigs that were challenged with saline or 0.01 % OVA. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Raw OVA sG_{aw} values (cm/$H_2$0) – Base (0.85±0.04), 0-6 hours (0.25±0.07), 7-12 hours (0.68±0.06) and 24 hours (0.88±0.04). Raw control sG_{aw} values (cm/$H_2$0) – Base (0.77±0.08), 0-6 hours (0.70±0.08), 7-12 hours (0.76±0.08) and 24 hours (0.78±0.06).

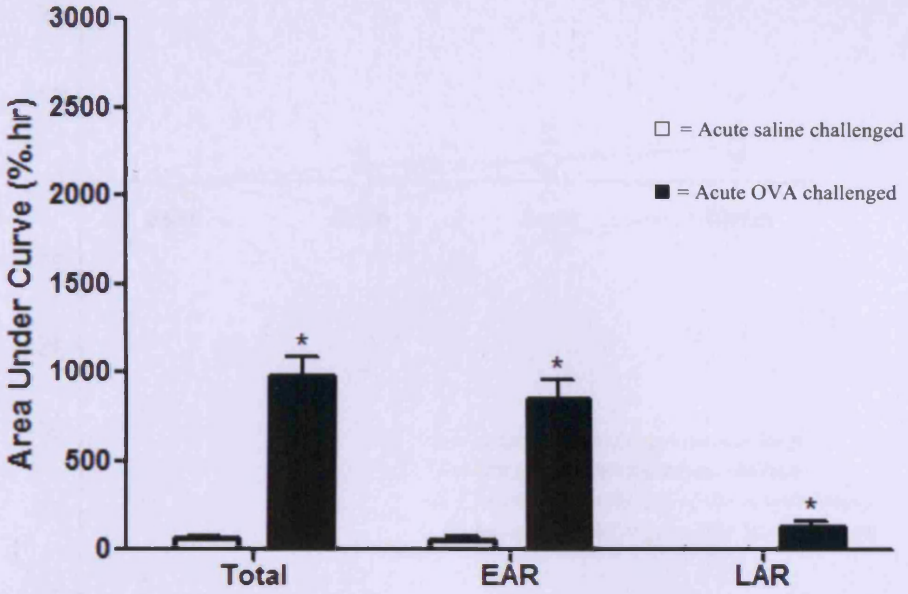


Figure 3.3 – Area under the curve analysis comparing OVA sensitised guinea pigs challenged with a single dose of saline or 0.01% OVA. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from saline challenged guinea pigs. Two-tailed T-test ($P<0.05$; n=6).

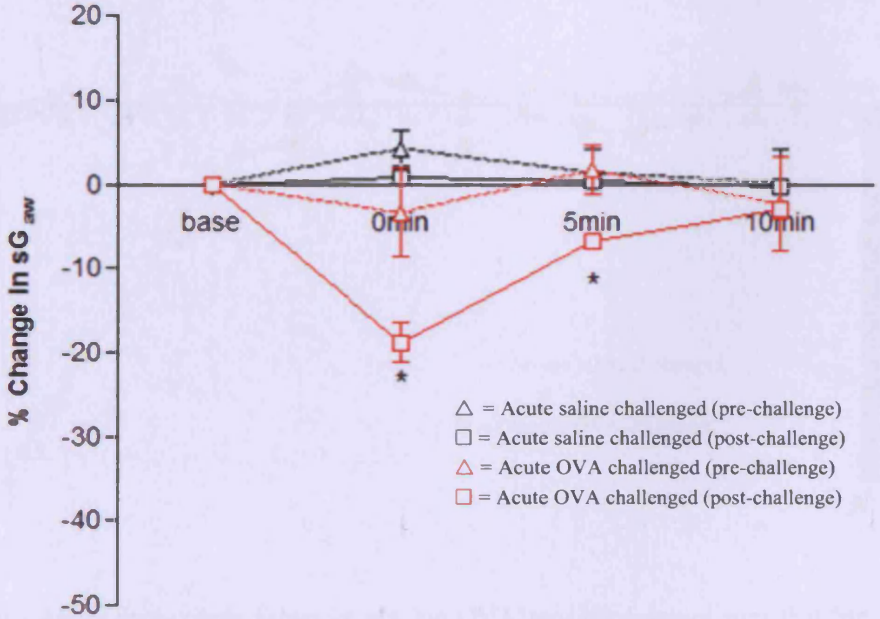


Figure 3.4. – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs challenged with saline or OVA (0.01%). Values were recorded 24 hours before saline or OVA challenge and again 24 hours post-saline or OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

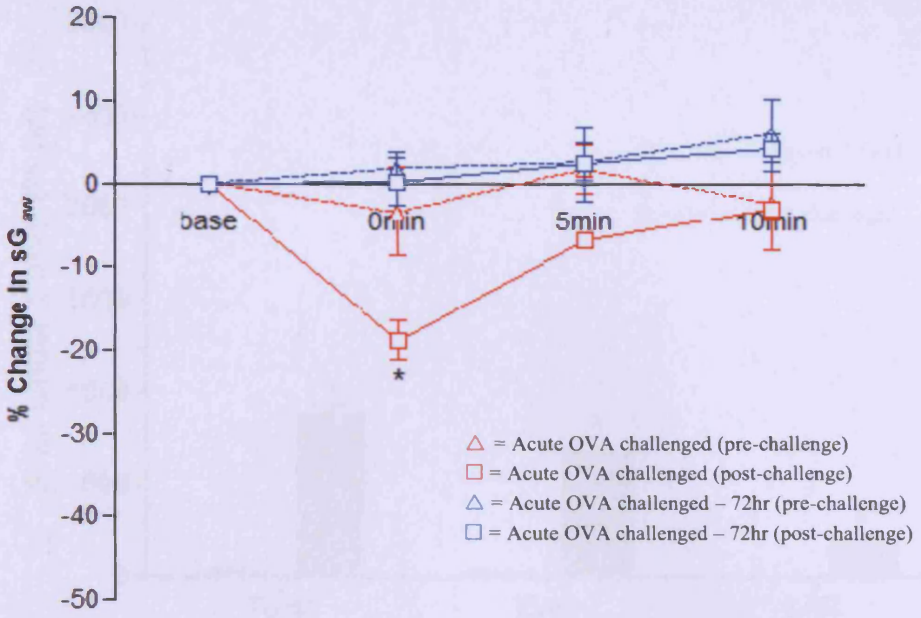


Figure 3.5 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs challenged OVA (0.01%). Values were recorded 24 hours before saline or OVA challenge and again 24 or 72 hours post- OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

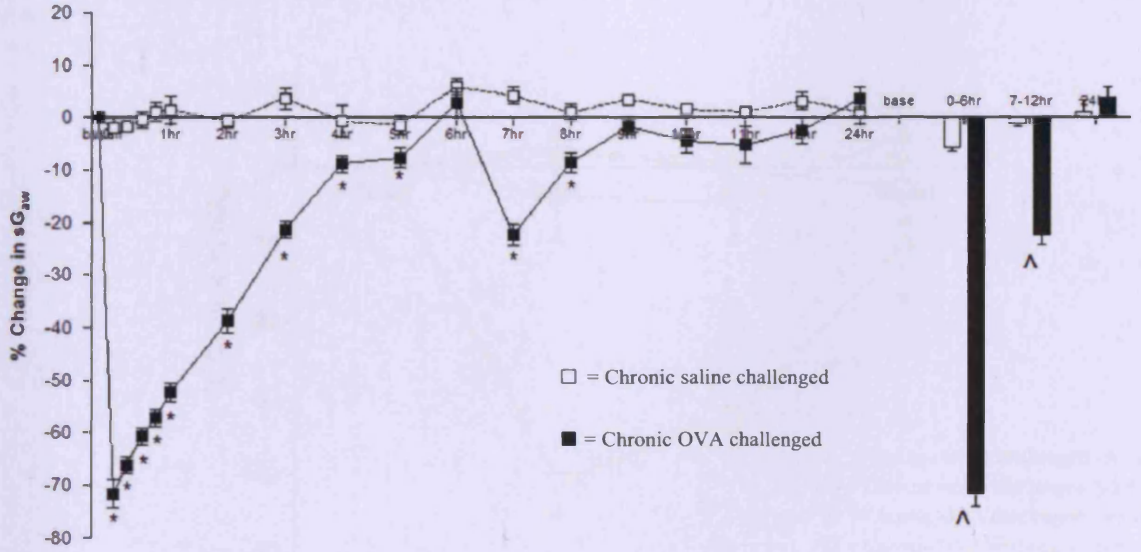


Figure 3.6 – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that had nine challenges at 48 hour intervals of saline or OVA . The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from saline challenged guinea pigs; ^ significant difference between saline and OVA challenged guinea pigs. Two-tailed T-test ($P<0.05$; n=6). Raw OVA sG_{aw} values (cm/H_20) – Base (0.72±0.01), 0-6 hours (0.21±0.02), 7-12 hours (0.56±0.01) and 24 hours (0.75±0.01). Raw control sG_{aw} values (cm/H_20) – Base (0.71±0.06), 0-6 hours (0.67±0.06), 7-12 hours (0.70±0.06) and 24 hours (0.72±0.06).

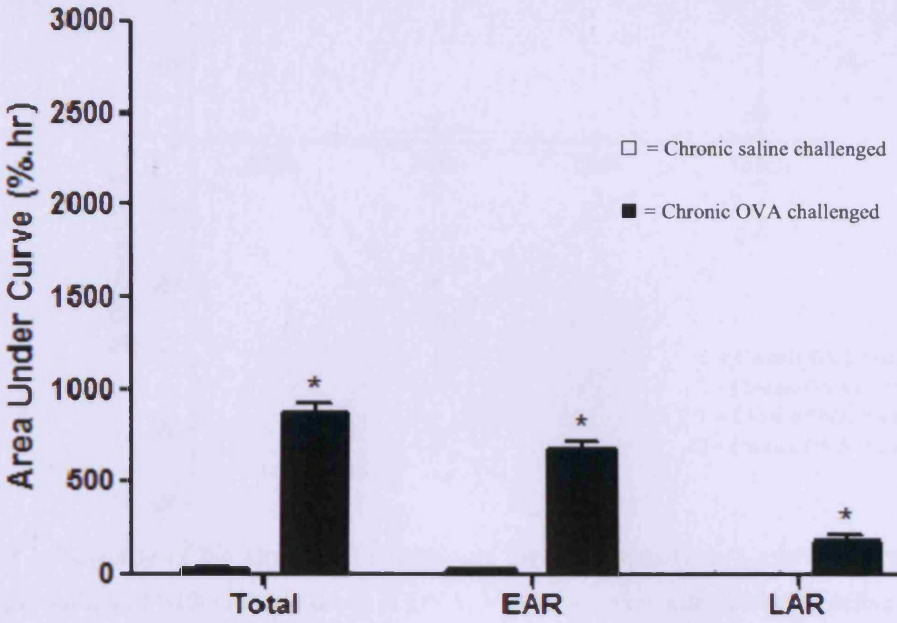


Figure 3.7 – Area under the curve analysis comparing OVA sensitised guinea pigs challenged with a chronic dosing of saline or OVA. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from saline challenged guinea pigs. Two-tailed T-test ($P<0.05$; $n=6$).

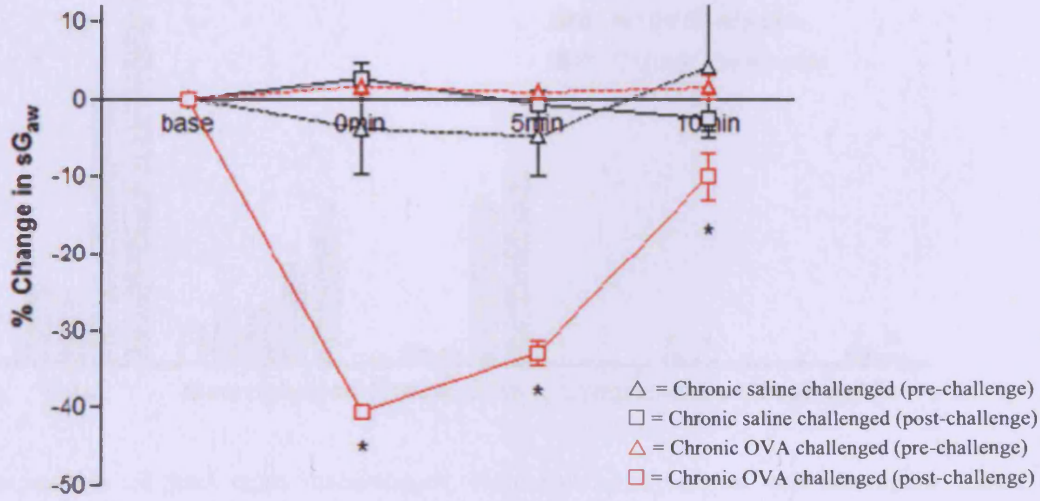


Figure 3.8 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs chronically challenged with saline or OVA. Values were recorded 24 hours before saline or OVA challenge and again 24 hours post-final saline or OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; $n=6$).

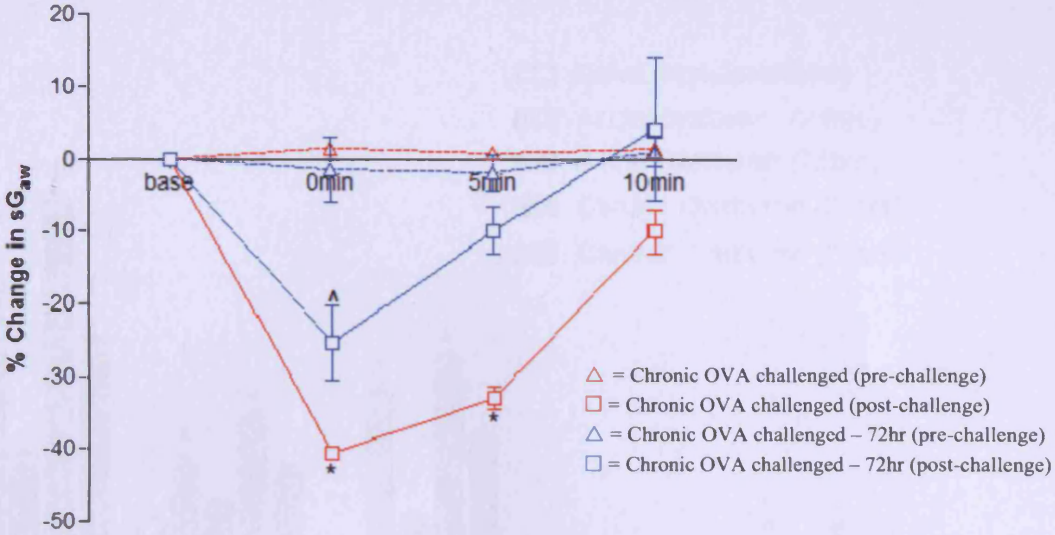


Figure 3.9 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs challenged with multiple doses of OVA. Values were recorded 24 hours before saline or OVA challenge and again 24 or 72 hours post-final OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; $n=6$).

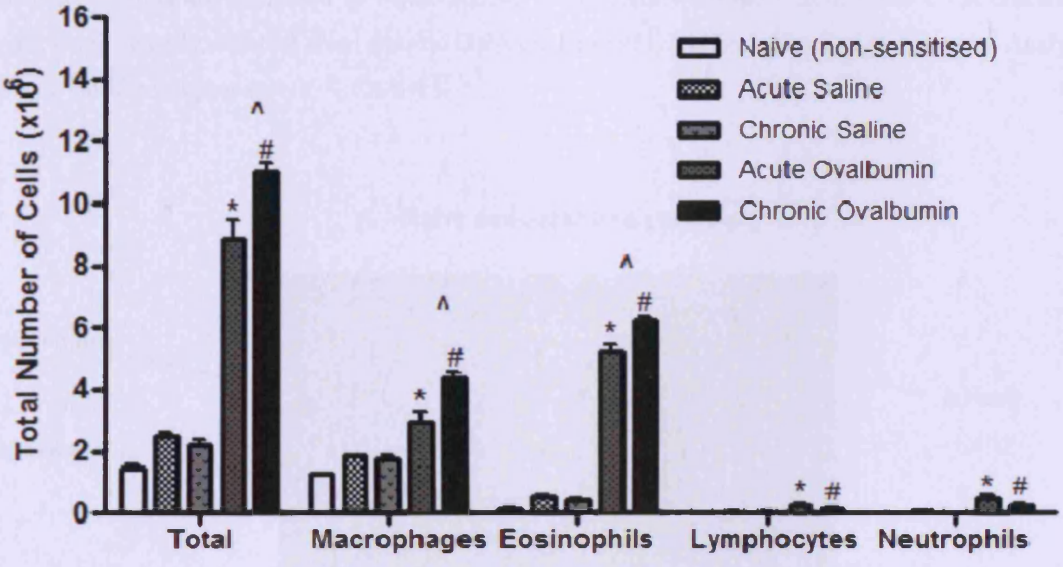


Figure 3.10 – The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised), saline challenged (acute and chronic) and OVA challenged (acute and chronic) guinea pigs. Results are expressed as mean±S.E.M. *significantly different from acute saline; #significantly different from chronic saline; ^significant difference between acute and chronic OVA challenged guinea pigs. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; $n=6$).

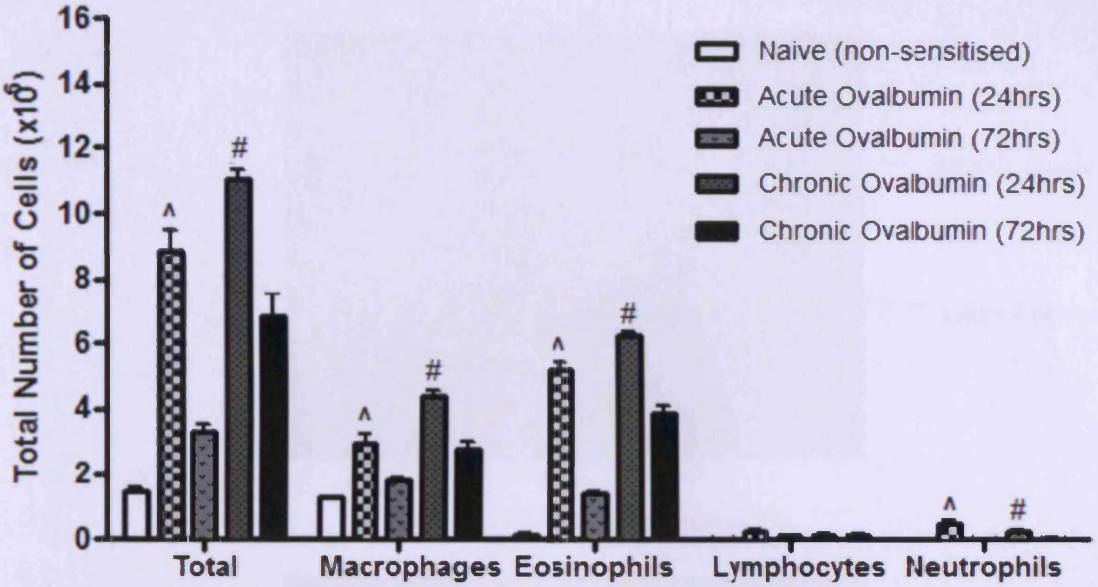
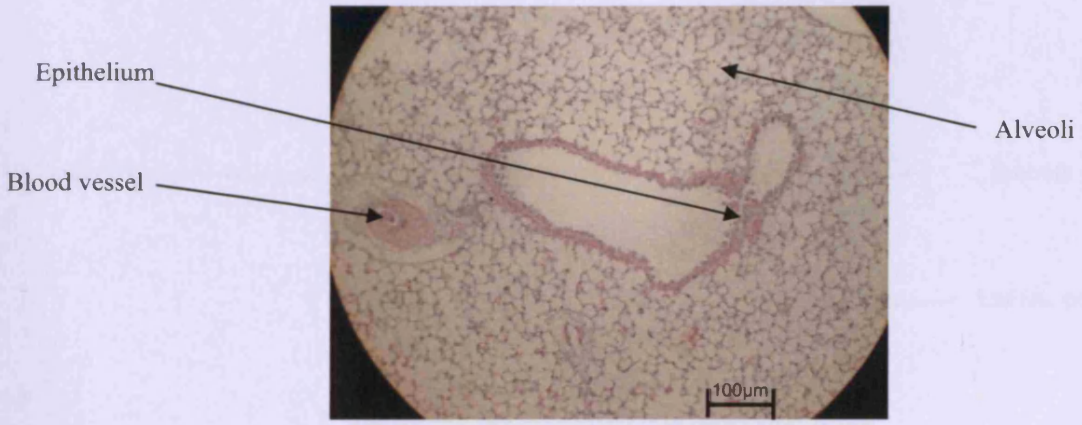
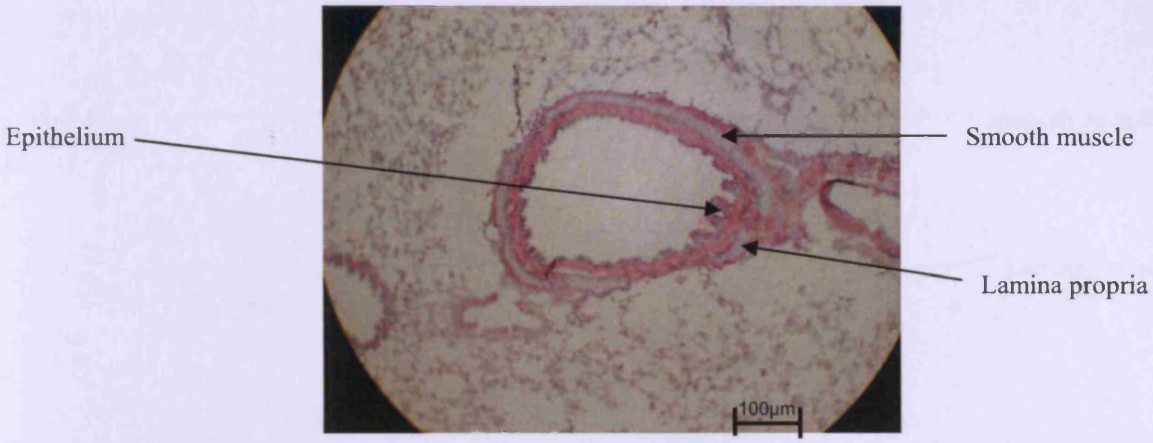


Figure 3.11 – The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised), and OVA challenged (acute and chronic) guinea pigs 24 or 72 hours post-final challenge. Results are expressed as mean±S.E.M. ^significantly different from acute OVA challenged (72 hours) guinea pigs; #significantly different from chronic OVA challenged (72 hours) guinea pigs. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; $n=6$).

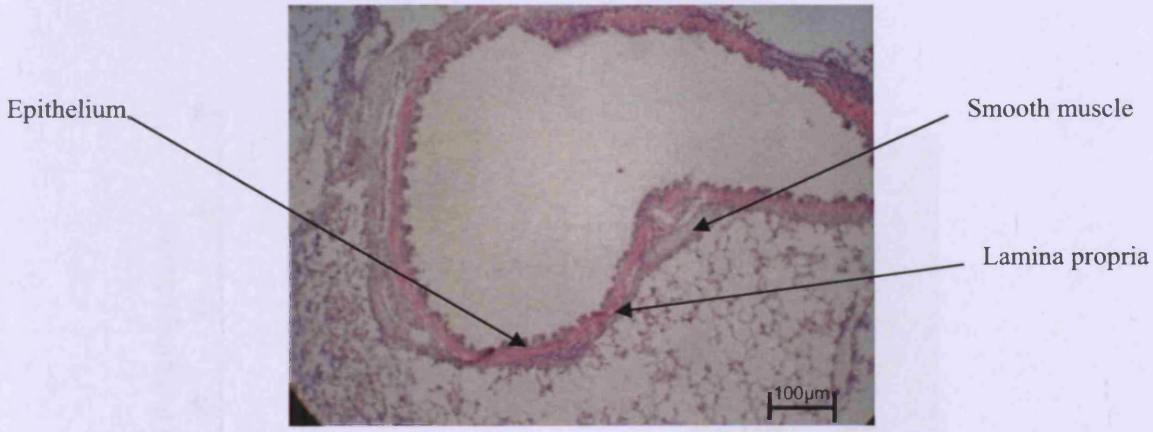
A – Naïve non-sensitised guinea pig



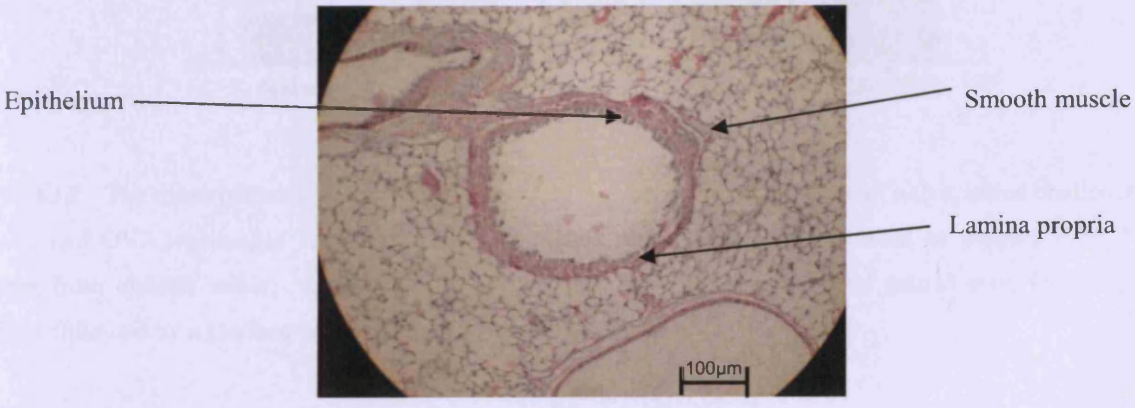
B – Acute saline challenged guinea pig



C – Acute OVA challenged guinea pig



D – Chronic saline challenged guinea pig



E – Chronic OVA challenged guinea pig

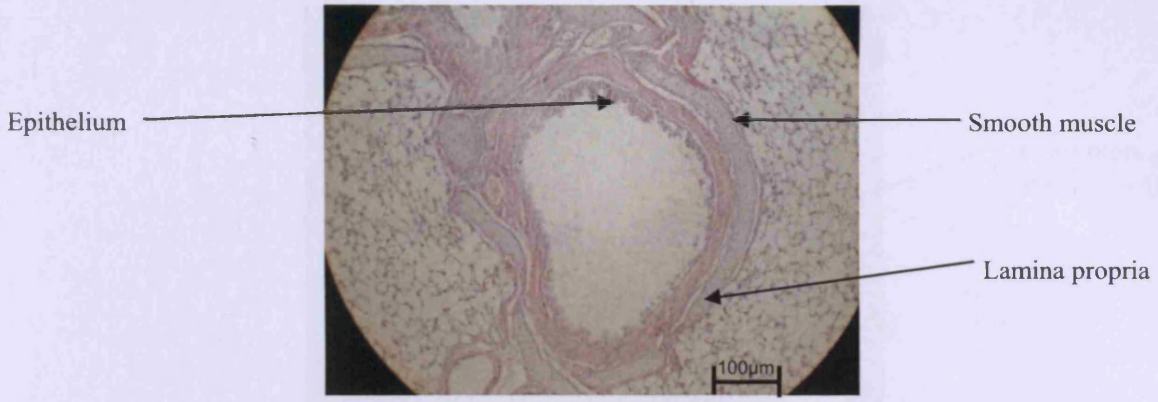


Figure 3.12 (A-E) – Bronchioles of naïve, saline challenged (acute and chronic) and OVA challenged (acute and chronic) guinea pigs stained with haematoxylin and eosin to display general morphology (1000x magnification)

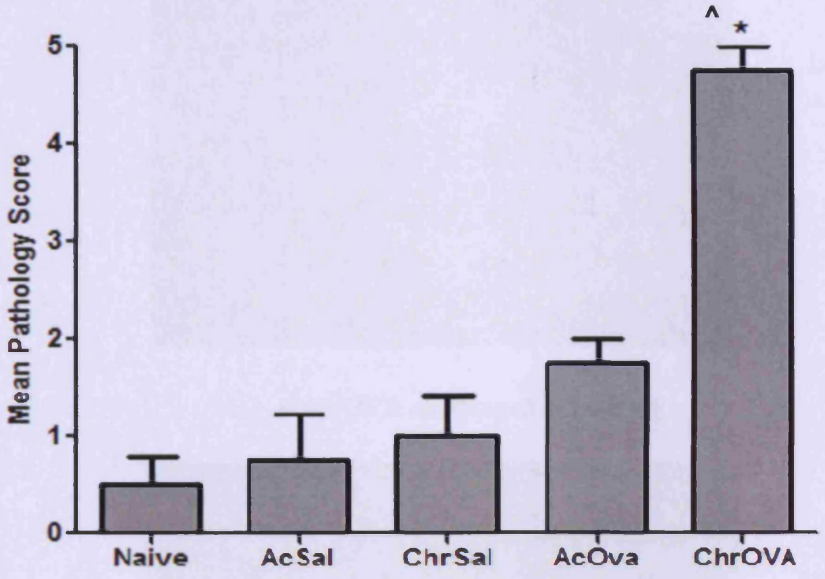
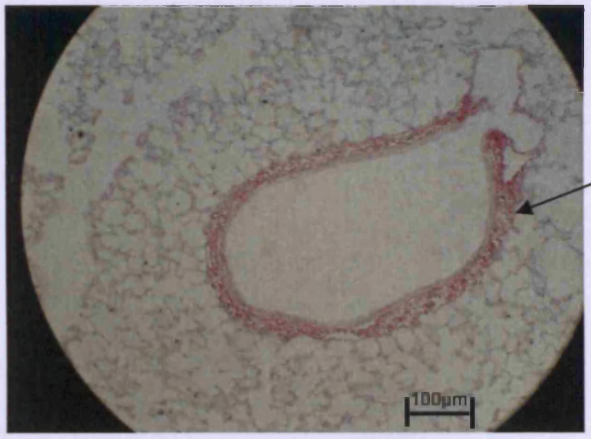


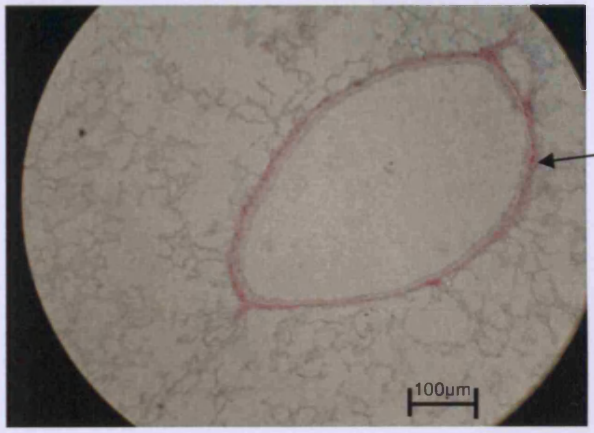
Figure 3.13 – The mean pathology score of cells found in the peri-bronchiolar space of naïve, saline challenged (acute and chronic) and OVA challenged (acute and chronic) guinea pigs Results are expressed as mean±S.E.M. *significantly different from chronic saline; ^significantly different from acute OVA challenged guinea pigs. One-way Analysis of Variance followed by a Dunnett post-test ($P<0.05$; $n=4$).

A – Naïve non-sensitised guinea pig



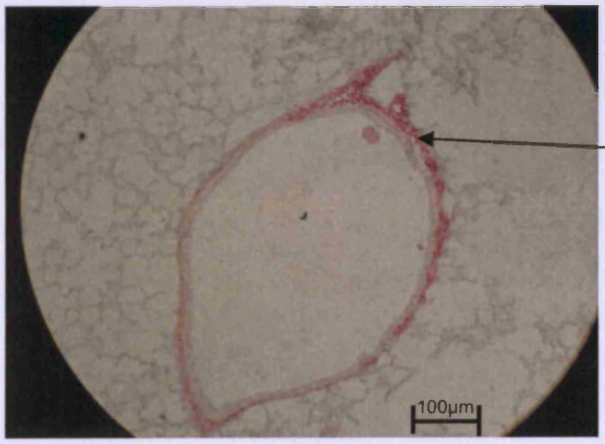
Lamina propria containing collagen

B – Acute saline challenged guinea pig



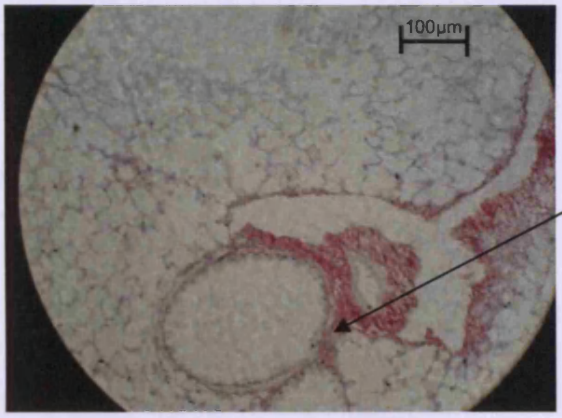
Lamina propria containing collagen

C – Acute OVA challenged guinea pig



Lamina propria containing collagen

D – Chronic saline challenged guinea pig



E – Chronic OVA challenged guinea pig

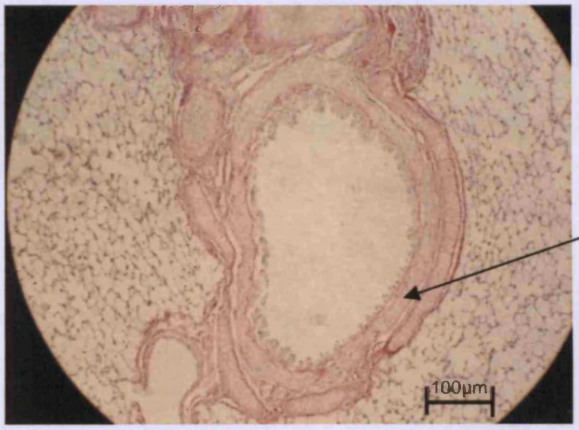


Figure 3.14 (A-E) – Bronchioles of naïve, saline challenged (acute and chronic) and OVA challenged (acute and chronic) guinea pigs stained with picrosirius red to display collagen (1000x magnification)

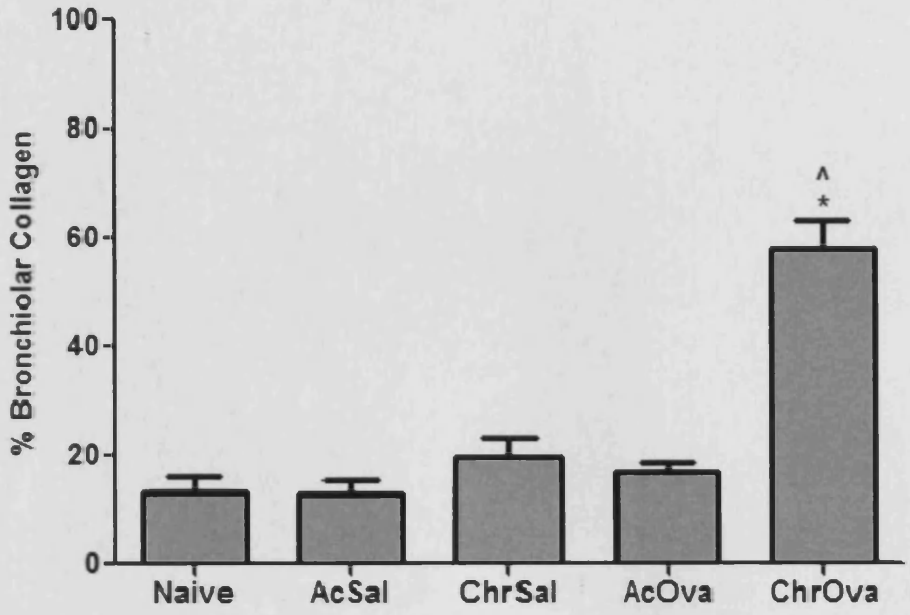
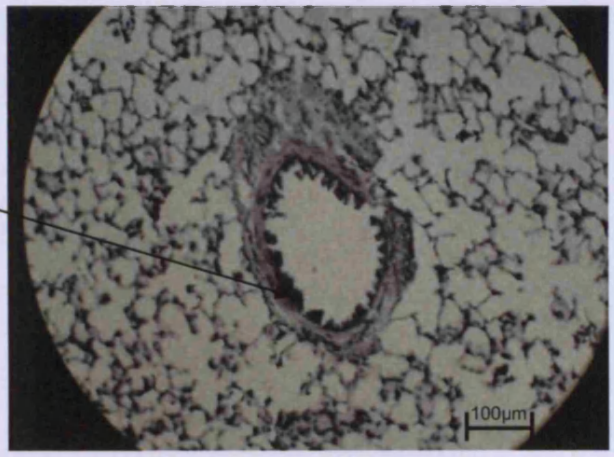
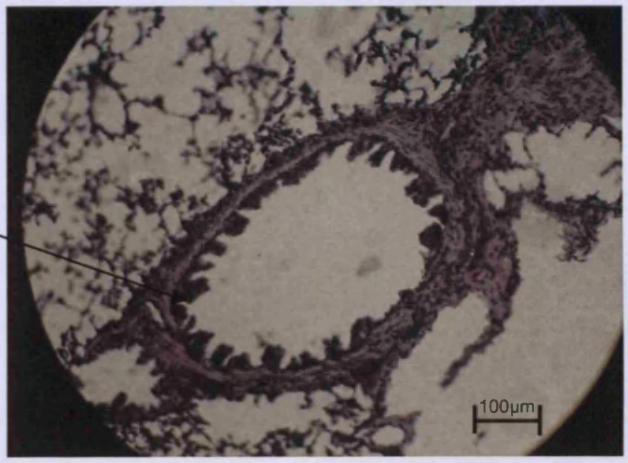


Figure 3.15 – Effect of saline (acute or chronic) or OVA (acute or chronic) challenge on percentage bronchiolar collagen in guinea pigs. Results are expressed as mean±S.E.M. *significantly different from chronic saline; ^significantly different from acute OVA challenged guinea pigs. One-way Analysis of Variance followed by a Dunnett post-test ($P<0.05$; $n=4$).

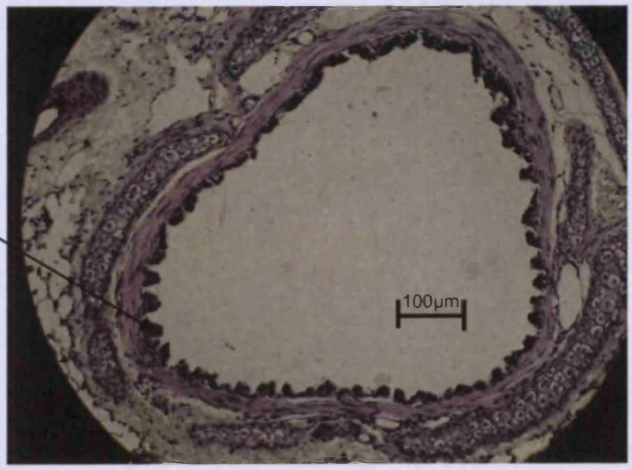
A – Naïve non-sensitised guinea pig



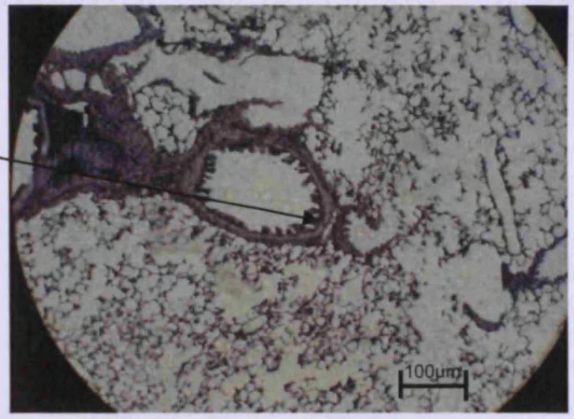
B – Acute saline challenged guinea pig



C – Acute OVA challenged guinea pig



D – Chronic saline challenged guinea pig



E – Chronic OVA challenged guinea pig

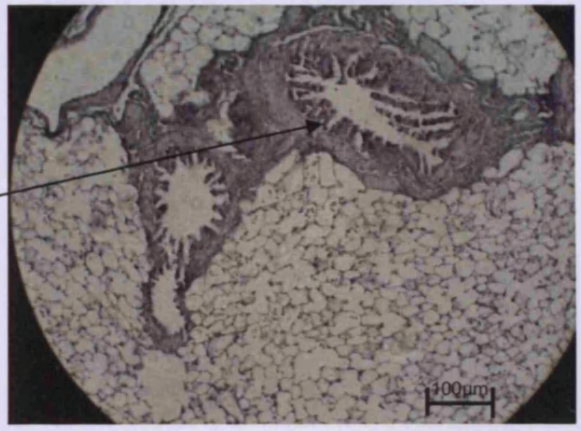


Figure 3.16 (A-E) – Bronchioles of naïve, saline challenged (acute and chronic) and OVA challenged (acute and chronic) guinea pigs stained with alcian blue and periodic acid Schiff to display goblet cells (1000x magnification).

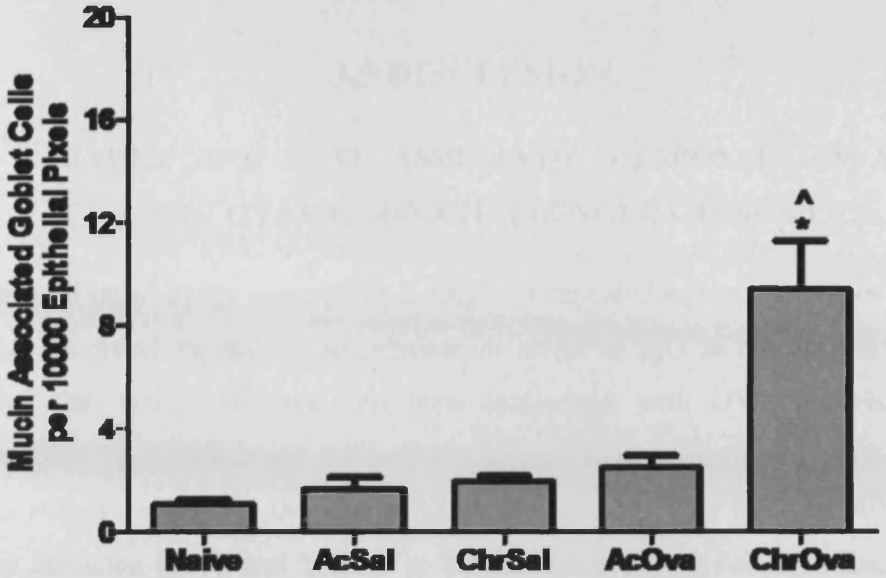


Figure 3.17 – Effect of saline (acute or chronic) or OVA (acute or chronic) challenge on number of mucin associate goblet cells per 10,000 epithelial pixels. Results are expressed as mean±S.E.M. *significantly different from chronic saline; ^significantly different from acute OVA challenged guinea pigs. One-way Analysis of Variance followed by a Dunnett post-test ($P<0.05$; n=4).

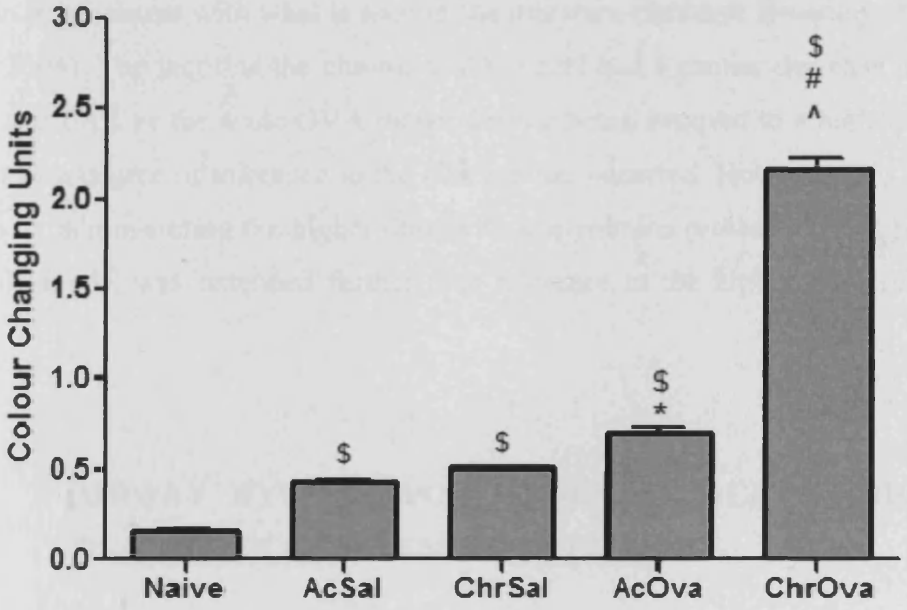


Figure 3.17 – Levels of IgG1 in the serums of naïve, saline (acute and chronic) and OVA (acute and chronic) challenged guinea pigs determined by ELISA. Results values of colour changing units measured at 490nm and are expressed as mean±S.E.M. *significantly different from acute saline; #significantly different from chronic saline; ^significantly different from acute OVA challenged guinea pigs; $significantly different from naïve. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

3.5 DISCUSSION

3.5.1 EARLY AND LATE ASTHMATIC RESPONSES IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS

When sensitised guinea pigs were given a single or repeated saline challenges no features of asthma were recorded except for an increase in levels of IgG in the chronic saline model. Guinea pigs that were sensitised and then challenged with OVA showed evidence of bronchoconstriction. A single and the last of a series of OVA exposures cause an immediate decrease in sG_{aw} representing the early asthmatic response. This recovers 4 hours after the exposure in the acute group and 5 hours in the chronic group. A second bronchoconstriction was observed after 7 hours in both groups which is consistent with the late asthmatic response time period. As with the early phase bronchoconstriction this response takes 1 hour longer to recover to control levels in the chronic group than the acute group.

Observing an EAR and LAR in acute challenged guinea pigs sensitised with 100 μg ovalbumin is consistent with what is seen in the literature (Smith & Broadley, 2007; Smith & Johnson, 2005). The fact that the chronic OVA model had a similar decrease in sG_{aw} during the EAR and LAR as the acute OVA model despite being exposed to a higher dose of OVA suggests that a degree of tolerance to the allergen has occurred. However, this tolerance was overcome by administering the higher dose with mepyramine protection. It is possible that if the chronic model was extended further then tolerance to the higher dose of OVA would occur.

3.5.2 AIRWAY HYPERRESPONSIVENESS IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS

AHR was observed in both acute and chronic OVA exposed guinea pigs that were challenged with a direct stimulus but not in the saline challenged groups suggesting that sensitisation alone does not cause AHR. A dose of 1 mMol histamine, which is sub-threshold for naïve guinea pigs (figure 2.7) and before OVA challenge, caused a response 24 hours post-OVA challenge in the acute group, however, when AHR was assessed after 72 hours it was not present. This proves that an acute challenge does not replicate the chronic nature of asthma.

In the chronic OVA challenged groups AHR was observed after 24 hours and AHR was still present after 72 hours. The chronic nature of AHR is something which is observed in asthma (Khor *et al.*, 2007). It has been suggested that persistent AHR is caused by airway inflammation and airway remodelling (O'Byrne *et al.*, 2009). However, Crimi *et al.*, (1998) state that the link between persistent AHR and inflammation is unclear and suggest it may be that airway remodelling or autonomic dysfunction are the main causes.

As the mechanisms that cause AHR are not well known it is possible that because the degree of AHR in the chronic OVA model was greater than the acute OVA model that this was the reason AHR was still present after 72 hours in the chronic model. However, although features that were present in both models, such as eosinophils, have been implicated in AHR (Wardlaw *et al.*, 2002) other factors that were only present in the chronic model, such as increased airway wall thickness, have been suggested to exacerbate AHR (Kasahara *et al.*, 2002). Therefore it is possible that these factors cause the delay in AHR recovery.

3.5.3 CELLULAR INFLUX IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS

Acute and chronic OVA challenge caused a mass influx of inflammatory cells into the airways as demonstrated in BAL fluid samples. This is understandable as inflammatory cell influx is a feature of human asthma (Barnes 1996). A great influx was observed in chronic challenged guinea pigs compared to acute which corroborates similar findings shown by Bazan-Perkins *et al.*, (2009). An increase was observed in macrophage number compared to control guinea pigs. Although macrophage number in control guinea pigs were at a greater level than eosinophils the opposite was true for the OVA challenged group. This is partly contradictory to findings in humans where macrophages were the most prominent cell in both asthmatics and non-asthmatics (Hamid *et al.*, 2003).

A mass increase in airway eosinophils was observed in both OVA challenged groups. This is consistent with human findings as patients with allergen-induced late-phase reactions had a significant increase in BAL eosinophil number (Diaz *et al.*, 1989). Eosinophils are believed to be involved in the development of the LAR (De Monchy *et al.*, 1985; Diaz *et al.*, 1989). This would explain why the numbers were so high 24 hours after challenge and could explain

the conflicting data with the Hamid *et al.,* (2003) study as it does not state at which point BAL samples were taken from asthmatics.

Lymphocytes are activated in response to an acute allergen challenge and produce high levels of cytokines which contribute to the asthmatic response (Barnes, 2008). This would explain why an increased level is seen in the BAL fluid of acute and chronic OVA challenged guinea pigs compared to vehicle. Neutrophil levels are also increased in OVA challenged guinea pigs compared to vehicle, however, they are not one of the prominent cell types. It seems unlikely that neutrophils have a prominent role in the LAR as a response can still be observed even after depletion of neutrophils (Hutson *et al.*, 1990). Toward and Broadley (2004) showed almost non-existent levels of neutrophils in the BAL fluid following an acute OVA exposure in sensitised guinea pigs after 24 hours but showed the peak neutrophil influx was during the EAR. This could explain why lower levels in neutrophils were observed in the chronic OVA model compared to the acute OVA model. The influx of neutrophils is likely to be maximal during the first EAR and diminish with each challenge.

When comparing the levels of cellular influx between 24 hour and 72 hour lavage time points there are significant reductions throughout all leukocyte subtypes except lymphocytes. In chronic OVA challenged guinea pigs the levels of eosinophils is still high, this goes someway in confirming the theory of Wardlaw *et al.*, (2002) that major basic protein released from eosinophils lead to an increase in damaged epithelial cells and AHR. The fact that the levels of cells have not completely returned to baseline after 72 hours suggests that complete recovery takes some time to occur and therefore these cells could be exacerbating asthma and making it the chronic condition it is.

3.5.4 HISTOLOGICAL CHANGES IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS

A vast difference was seen in chronic OVA challenged guinea pig bronchioles compared to the other protocols bronchioles. An increase in amount of smooth muscle, thickness of lamina propria and epithelium (possibly a result of goblet cell hyperplasia) was observed. These findings are similar to those by Bazan-Perkins *et al.,* (2009) who showed increased fibrosis in the guinea pig lung following nine OVA challenges. The number of inflammatory cells found in the lung tissue corroborated with the total cells counts observed in the BAL fluid. The

influx of these inflammatory cells, especially eosinophils, are likely to be the cause of the airway remodelling observed in the chronic OVA model.

When assessing the lamina propria for fibrosis a much greater percentage of bronchiolar collagen was observed in the chronic OVA challenged guinea pigs. This was also the case when assessing the number of mucin associated goblet cells. Goblet cell hyperplasia has occurred in the chronic OVA model. This model shows airway remodelling has occurred and is therefore a much more clinically relevant model of asthma than the acute OVA model.

3.5.5 IgG PRODUCTION IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS

An increase in IgG1 levels compared to naïve guinea pigs was observed in all models except the acute saline challenged guinea pigs suggesting that sensitisation to OVA occurred. The guinea pigs challenged with OVA had higher levels of IgG1 especially the chronic model. Although the OVA challenge would cause cross-linking of the antibodies and subsequent degranulation of the mast cells and cleavage of the antibody (Rauter *et al.*, 2008) at the same time the sensitisation process would also be reoccurring and thus new antibodies being created. The acute OVA challenged group had significantly greater levels of IgG1 than the acute saline challenged group suggesting that after an OVA exposure a greater level of antibodies may be produced than degraded. The chronic OVA exposed guinea pigs had significantly greater levels of IgG1 than all the other groups and this is likely to be a result of repeated stimulation of the immune system with each exposure of the allergen. If with each exposure more antibodies were produced than degraded then there would be an accumulation of antibodies. It was also possible that the higher dose of OVA that the chronic group receive caused the production of more IgG1 antibodies. A much greater level of IgG1 was observed in the chronic OVA group than the other groups suggesting that this model may be a better representation of human asthma than the acute OVA group although it is important to consider that IgE is the allergic antibody in humans.

3.5.6 GENERAL CONCLUSIONS

From these data it is clear that although the acute OVA guinea pig model displays several of the features of asthma it does not demonstrate airway remodelling. It seems that the chronic OVA guinea pig model is the first model to display EAR, LAR, long-term AHR, cellular influx and airway remodelling backed up by the statement by Zosky and Sly (2007) that stated there is no model in smaller laboratory animals that demonstrates both chronic remodelling and long-term airway hyperresponsiveness. It would seem that the chronic OVA model developed in this chapter has several advantages over other guinea pig models and indeed larger animal models as a pre-clinical model as it is cost effective and takes a relatively short time to develop. However, as previously mentioned all models of asthma must take into account the fact that it is not completely representative of human asthma. The chronic OVA model is sensitised then challenged nine times over the period of a month. Many asthmatics have suffered with asthma for years and the levels of airway remodelling are likely to be far more severe than observed in the chronic OVA model. Despite this, the chronic OVA model would enable more accurate research into asthma mechanisms and potential therapy than the acute OVA model.

Chapter 4

Analysis of mouse acute and chronic models of asthma

4.1 INTRODUCTION

4.1.1 MOUSE MODELS OF ASTHMA

In recent years mice models of asthma have become the most popular animal for modelling allergic airway responses (Zosky & Sly, 2007). This is because mice have several advantages over other models of asthma. The size of mice can be an advantage compared to larger models of asthma as they are easy to house together and therefore reduce cost. However, as mice are not as docile as guinea pigs they are not as easy to handle and as a result non-restrained measurements of lung function are employed. The main reasons why murine models have become so popular in asthma research is the availability of species-specific reagents (Karol, 1994) and transgenic mice (Hausding *et al.*, 2008). These allow further research into the mechanisms of asthma at both the immunological and genetic level respectively. The fact that IgE is the major anaphylactic antibody (Karol, 1994), as this is also the case in humans, serves as another advantage over guinea pigs where IgG is the prominent antibody.

Despite the advantages of mice models of asthma they have drawbacks. Mice are different to humans when it comes to mast cell degranulation. The primary mediator in human mast cell degranulation is histamine whereas in mice it is serotonin (Canning, 2003), as a result mice respond poorly to histamine (Zosky and Sly 2007). There is huge variation in the pattern of allergic inflammation between different strains of mice (Kumar *et al.*, 2008). For example, Shinagawa and Kojima (2003) found that A/J mice exhibited more marked changes after chronic intranasal challenges compared to BALB/c and C57BL/6 mice.

There are large anatomical differences between mice and humans. The fact that mice, and guinea pigs, are quadrupeds means the difference in posture compared to humans could influence the way the lung responds to flow limitation and therefore may have an impact on aerosol deposition (Zosky and Sly 2007). In addition to this, mice have poorly developed airway smooth muscle compared to humans (Karol 1994). Mice, like guinea pigs, generally require an adjuvant along with OVA to induce sensitisation. Although adjuvant-free protocols exist they require many injections to achieve sensitisation (Kumar *et al.*, 2008). This is because like most models of asthma they do not develop allergy naturally and therefore do

not completely represent human asthma. Despite these drawbacks mice have proved an excellent tool at investigating the mechanisms of asthma and will continue to do so.

4.1.2 MOUSE OVALBUMIN MODELS

Despite increasing studies that use house dust mite (Kim *et al.*, 2006), olive pollen (Batanero *et al.*, 2002) and Aspergillus fumigatus extract (Baelder *et al.*, 2005), ovalbumin is still the allergen of choice to provoke an immune response in the majority of studies modelling asthma (Cates *et al.*, 2007). Although using ovalbumin is sometimes criticised for not being as clinically relevant as other allergens, such as house dust mite, the fact that it can induce a consistent and robust inflammatory response leads to it being used extensively (Cates *et al.*, 2007).

The method of ovalbumin sensitisation and subsequent challenge differs greatly between different study groups and species (Kumar *et al.*, 2008). The dose of ovalbumin used to sensitise mice ranges from 1 μg to 8000 μg and varies between the route of administration used (Kumar *et al.*, 2008). With so much variation between sensitisation and exposure methods and, on top of this, strain variation it is no surprise that the key features observed vary dramatically. An example of this is how the differences in protocols alter the time of the early asthmatic response (EAR) can be shown by comparing the studies by Cieslewicz *et al.*, (1999) and Fernandez-Rodriguez *et al.*, (2008). An early and late phase bronchoconstriction was observed in ovalbumin sensitised and challenged mice in both studies, however, in the Cieslewicz *et al.*, (1999) study the EAR peaks at 15 minutes whereas in the Fernandez-Rodriguez *et al.*, (2008) study it did not peak until 2 hours. Variation needs to be carefully considered when developing and assessing a mouse model of asthma, as a result strain and protocol will be kept consistent throughout this thesis.

When developing a chronic model of a disease the issue of tolerance must be considered. As a result of tolerance the majority of inhaled challenges are short-term, which results in eosinophil accumulation and airway hyperresponsiveness (Kumar *et al.*, 2008). However, these models are associated with features that are not characteristics of human asthma such as perivascular and parenchymal inflammation of the lungs (Cohn, 2001). To ensure that tolerance to inhaled ovalbumin does not occur some models have been developed in which ovalbumin was delivered intermittently following sensitisation (Henderson *et al.*, 2002; Leigh

et al., 2002; Tanaka *et al.*, 2001; Temelkovski *et al.*, 1998). However, this approach does not completely inhibit tolerance as down-regulation of inflammation, airway hyperresponsiveness and structural airway changes were observed following repeated antigen exposure (Jungsuwadee *et al.*, 2004; Palmans *et al.*, 2000; Yiamouyiannis *et al.*, 1999).

Previous work in our laboratory developed a chronic mouse model that maintained a consistent dose of ovalbumin throughout and showed limited tolerance (Fernandez-Rodriguez *et al.*, 2008). Sensitisation followed by six weeks of ovalbumin challenges three times a week led to early and late asthmatic responses, airway hyperresponsiveness and cellular influx. However, this model required a higher dose of ovalbumin than the acute model to provoke asthma-like responses. Histological analysis will help determine the appropriateness of this model as a model of human asthma.

4.1.3 REQUIRED FEATURES OF ACUTE AND CHRONIC MOUSE MODELS OF ASTHMA

Models of asthma should be able to consistently replicate most, if not all, of the key features of asthma. These are early and late asthmatic responses (Pepys & Hutchcroft, 1975), airway hyperresponsiveness (Hargreave *et al.*, 1981), increased airway cellular influx (Barnes, 1996) and airway remodelling (McMillan & Lloyd, 2004). The model developed by Fernandez-Rodriguez *et al.,* (2008) demonstrated four of these features in both acute and chronic ovalbumin challenged mice. Airway remodelling was not assessed in their study. Airway remodelling has been observed in many mouse models of asthma (Blyth *et al.*, 2000; McMillan & Lloyd, 2004; Temelkovski *et al.*, 1998; Xisto *et al.*, 2005). However, these studies did not assess all of the other features of asthma. It appears that currently there is no chronic model of asthma that can demonstrate all the hallmarks of asthma.

The model by Fernandez-Rodriguez *et al.*, (2008) has also assessed the levels of IgE, the antibody involved in asthma, and ovalbumin-specific IgG in the serum of acute and chronic ovalbumin challenged mice. The study showed that sensitisation followed by ovalbumin challenge caused a significant increase in serum IgE compared to naïve mice and that sensitisation alone was enough to cause a significant increase in ovalbumin-specific IgG. Currently this model appears to demonstrate more hallmarks of asthma than any other mouse

model, therefore the protocol developed in the Fernandez-Rodriguez *et al.,* (2008) model will be used in this chapter.

4.2 AIMS AND OBJECTIVES

4.2.1 AIMS

The aim of this chapter was to sensitise mice to ovalbumin (OVA) and subsequently challenge them with acute or chronic exposures of OVA or saline. The response to the final OVA challenge was assessed by measuring early and late phase bronchoconstrictions, airway hyperresponsiveness, cellular influx and lung histology. The development and comparison of acute and chronic OVA mouse models allows for the assessment of potential asthmatic therapies in subsequent chapters.

4.2.2 OBJECTIVES

- To investigate the effect that an acute exposure of OVA or saline has on sensitised mice by measuring lung function, response to methacholine and percentage cellular influx.
- To investigate the effect that multiple exposures of OVA or saline has on the same parameters mentioned in the previous point.
- To assess bronchiole structure, collagen deposition and goblet cell hyperplasia in both acute and chronic challenged mice by utilising histological techniques.

4.3 METHODS

Chapter 2 describes the methods used in detail and the following is a brief overview. Male BALB/c mice (20-25g) were used for all mouse studies. Six mice were used per experimental group.

4.3.1 SENSITISATION

The sensitisation process for mice was OVA (100 μg) and $Al(OH)_3$ (100 mg) in phosphate buffered saline (PBS). Intraperitonael injections of 0.25 ml bilaterally were administered on days 1 and 5, with procedures commencing on day 14.

4.3.2 OVALBUMIN CHALLENGES

Mice were exposed to aerosolised OVA in a Perspex box (38 cm length; 20 cm width; 20 cm height). If a mouse appeared distressed during the exposure it was removed from the box and exposure was considered complete.

4.3.2.1 ACUTE PROTOCOL

The mice were exposed to two 1 hour OVA (0.5%) or saline inhalation challenges on day 15, there was a 4 hour gap between exposures.

4.3.2.2 CHRONIC PROTOCOL

Mice were challenged three times a week for six weeks with nebulised OVA (2%) or saline for 30 minutes. A final hour long challenge occurred on the first day of the seventh week (day 57).

4.3.3 LUNG FUNCTION MEASUREMENTS

The method for measuring lung function in mice is covered in chapter 2. Briefly, values of the lung function of unrestrained mice were measured as P_{enh} using a Buxco system. Values of P_{enh} following allergen challenge were recorded at 0, 20, 40, 60, 90 and 120 minutes, then

hourly until 10 hours followed by a final reading at 19 hours for the acute protocol (24 hours after first challenge) or 24 hours for the chronic protocol.

4.3.4 AIRWAY HYPERRESPONSIVENESS MEASUREMENTS

Airway hyperresponsiveness in mice was measured using methacholine. A 1 minute intermittent exposure of 30 mg/ml methacholine (one second exposure followed by five seconds without exposure) was used in the acute challenged mice and a 10 mg/ml dose over the same time period was used in the chronic model. Prior to the challenge 2 minutes of baseline readings were taken and subsequent to the challenge 5 minutes of readings were taken. The percentage change in P_{enh} from baseline was calculated. Methacholine challenges were carried out before the first ovalbumin exposure and 19 or 24 hours after the final ovalbumin exposure.

4.3.5 DIFFERENTIAL CELL COUNTS

As previously described in chapter 2 it was not possible to carry out a total cell count on mice BAL fluid, however, a differential cell count of macrophages, eosinophils, lymphocytes and neutrophils was obtained. Non-sensitised naïve mice were included as a reference only.

4.3.6 HISTOLOGICAL ANALYSIS OF MOUSE LUNGS

The techniques for histological analysis are described in chapter 2. Briefly, sections of the left superior lung measuring 3-5 mm were cut 1 mm below the bronchus and processed into wax blocks. Sections measuring 6 μm were then cut using a microtome and fixed onto a glass slide. The slices were stained with either the haematoxylin and eosin, picrosirius red or AB/PAS protocols. The haematoxylin and eosin staining shows the general morphology of the bronchiole allowing for any structural changes to be easily observed. Secondly, the stain allowed the semi-quantifiable analysis of the number of inflammatory cells in the peribronchiolar space of the lung tissue. The parameters for scoring were absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5) (Barends *et al.*, 2004). The sections stained with picrosirius red or AB/PAS protocols allowed the analysis of the mean % of

collagenous or AB/PAS positive areas respectively of the bronchiolar epithelium using ImageJ software.

4.4 RESULTS

4.4.1 EFFECT OF AN ACUTE OVALBUMIN CHALLENGE ON LUNG FUNCTION

Mice that were sensitised with OVA (100 μg) and challenged with saline showed no significant changes in lung function. However, if the sensitised mice are challenged with OVA (0.5%) changes were observed. A significant increase versus saline challenged mice was seen after 1 hour, this peaks after 2 hours (64.6±2.7%) and had recovered by 4 hours. A second increase in P_{enh} was observed at 7 hours (55.8±3.7%) which recovered by 8 hours. This is equivalent to an EAR and LAR and is shown in figure 4.1a. When comparing peak P_{enh} values, OVA challenged mice showed a significant increase in P_{enh} compared to saline challenged mice (64.6±2.7% compared to 20.4±5.0% respectively) suggesting the OVA challenged mice had an early phase bronchoconstriction. This was also true for the late phase bronchoconstriction time period (7-12 hours) (55.8±3.7% compared to 21.5±3.8% respectively).

Area under the curve analysis (figure 4.2) highlights the differences in %.hour between the sensitised mice challenged with an acute saline or OVA exposure. OVA mice showed a greater area than saline mice throughout the whole curve (1611.5±200.4%.hr compared to 677.9±199.2%.hr respectively). This was also the case when the area under the curve for the EAR time period (0-6 hours) (1024.5±151.8%.hr compared to 346.8±117.1%.hr respectively) and the LAR time period (7-12 hours) (550.2±62.9%.hr compared to 260.6±76.3%.hr respectively) was taken.

4.4.2 EFFECT OF AN ACUTE OVALBUMIN CHALLENGE ON AIRWAY RESPONSIVENESS TO METHACHOLINE

The response of mice after a single OVA challenge to 30 mg/ml methacholine (1 minute) is shown in figure 4.3. Saline challenged mice showed no significant difference post-saline challenges compared to pre-saline challenges. AHR was present in the OVA challenged mice post-OVA exposures as P_{enh} values were significantly greater when compared to pre-challenge levels (1203.9±115.6% compared to 2.2±3.3% respectively).

4.4.3 EFFECT OF CHRONIC OVALBUMIN CHALLENGES ON LUNG FUNCTION

Figure 4.4 represents the effects that multiple exposures of saline or OVA (2%) have on the lung function of mice sensitised with OVA (2%). Saline challenges have no significant effect on lung function, however, this is not the case for chronic OVA challenges. A significant bronchoconstriction was observed 1 hour after the final OVA challenge, which peaked at 2 hours (55.8±7.8%) and decreased almost to saline challenged levels at 4 hours. A second bronchoconstriction occurred after 6 hours, which peaked at 7 hours (47.5±10.1%) and recovered after ten hours. These two bronchoconstrictions represent the EAR and LAR respectively.

Peak P_{enh} values highlighted a significant increase in OVA challenged mice compared to saline challenged mice at the early phase (55.8±7.8% compared to 23.1±8.5% respectively) and late phase (47.5±10.1% compared to 1.7±4.3% respectively) bronchoconstriction time points. Area under the curve analysis is shown in figure 4.5. A significantly greater area was observed in chronic OVA challenged mice compared to chronic saline challenged mice throughout (1720.7±427.4%.hr compared to 236.6±150.5%.hr respectively) and also at the EAR (1132.4±282.3%.hr compared to 216.1±150.5%.hr respectively) and LAR (588.3±153.8%.hr compared to 23.4±17.5%.hr respectively) time points.

4.4.4 EFFECT OF CHRONIC OVALBUMIN CHALLENGES ON AIRWAY RESPONSIVENESS TO METHACHOLINE

Figure 4.6 shows that repeatedly challenging OVA sensitised mice with saline did not cause any hyperresponsiveness. However, following multiple OVA challenges, a response to 10 mg/ml methacholine (1 minute) was recorded in mice compared to the response pre-OVA challenge (362.6±16.1% compared to -10.3±2.2% respectively). Therefore, AHR was observed in sensitised mice following a chronic OVA exposure protocol.

4.4.5 EFFECT OF ACUTE AND CHRONIC OVALBUMIN CHALLENGES ON DIFFERENTIAL CELL COUNTS

Figure 4.7 represents the percentage of cells present in the BAL fluid of naïve, saline challenged and OVA challenged mice. More eosinophils were seen in acute OVA mice than in saline challenged mice (15.7±0.7% compared to 0.4±0.2% respectively). However, a significantly smaller percentage of macrophages were observed in acute OVA challenged mice compared to saline challenged (72.1±1.5% compared to 84.0±2.9% respectively), this is probably a result of the fact that there was a significantly greater percentage of eosinophils. A similar result was observed in chronically challenged mice with a greater percentage of eosinophils observed in the OVA challenged (12.5±1.0% compared to 0.2±0.1% respectively) and a lower percentage of macrophages seen in OVA challenged compared to saline challenged (72.8±1.9% compared to 89.3±1.8% respectively). No significant changes were observed in lymphocyte and neutrophil percentage influx.

4.4.6 EFFECT OF ACUTE AND CHRONIC OVALBUMIN CHALLENGES ON LUNG HISTOLOGY

Figure 4.8 (A-E) shows a bronchiole from a mouse in each of the five study groups in this chapter, naïve, acute saline, chronic saline, acute OVA and chronic OVA. The bronchioles have been stained with haematoxylin and eosin to show general morphology. Considerable structural changes were observed in the chronic OVA challenged mice compared to the other groups most noticeably an increase in epithelium thickness and the increased presence of smooth muscle. From the haematoxylin and eosin stained slides the number of inflammatory cells in the lung tissue can be assessed semi-quantifiably, shown in figure 4.9. A single OVA challenge caused a greater score of inflammatory tissue cells than a single saline challenge (2.0±0.0 compared to 0.8±0.3 respectively). However, when mice received nineteen OVA challenges the number of inflammatory cells in the lung tissue was significantly greater than a single OVA challenge (4.8±0.3 compared to 2.0±0.0 respectively). Chronic OVA challenged mice also had a greater number of lung tissue inflammatory cells than chronic saline challenged mice (4.8±0.3 compared to 1.0±0.4 respectively).

Bronchioles for the same five groups that were stained with picrosirius red to show collagen are shown in figure 4.10 (A-E). The chronic OVA challenged mice had a greater level of

collagen in the lamina propria than the other groups. The quantitative analysis of these bronchioles is shown in figure 4.11. This represents the percentage of bronchial collagen for each group. No significant change was observed between OVA and saline when a single challenge occurred. However, multiple OVA challenges caused an increase in percentage bronchiolar collagen compared to multiple saline challenges (51.5±5.2% compared to 14.2±4.4% respectively). When comparing the two OVA groups multiple challenges caused a significant increase compared to a single challenge (51.5±5.2% compared to 13.9±2.2% respectively).

Alcian blue / periodic acid Schiff stain bronchioles are shown in figure 4.12 (A-E). The epithelium of the naïve, acute saline, chronic saline and acute OVA mice was dark purple in colour suggesting the presence of mainly epithelial cells. However, the chronic OVA challenged mice had more bright purple stained cells in their bronchiolar epithelium suggesting the presence of mucin associated goblet cells. Figure 4.13 compares the number of mucin associated goblet cells per 10,000 epithelial pixels between naïve, acute saline, chronic saline, acute OVA and chronic OVA. Chronic OVA challenges caused a significant increase in the number of mucin associated goblet cells compared to acute OVA challenges (13.6±2.6 compared to 2.5±0.8 respectively) and chronic saline challenges (13.6±2.6 compared to 2.8±0.7 respectively). No significance was observed between both acute challenge groups.

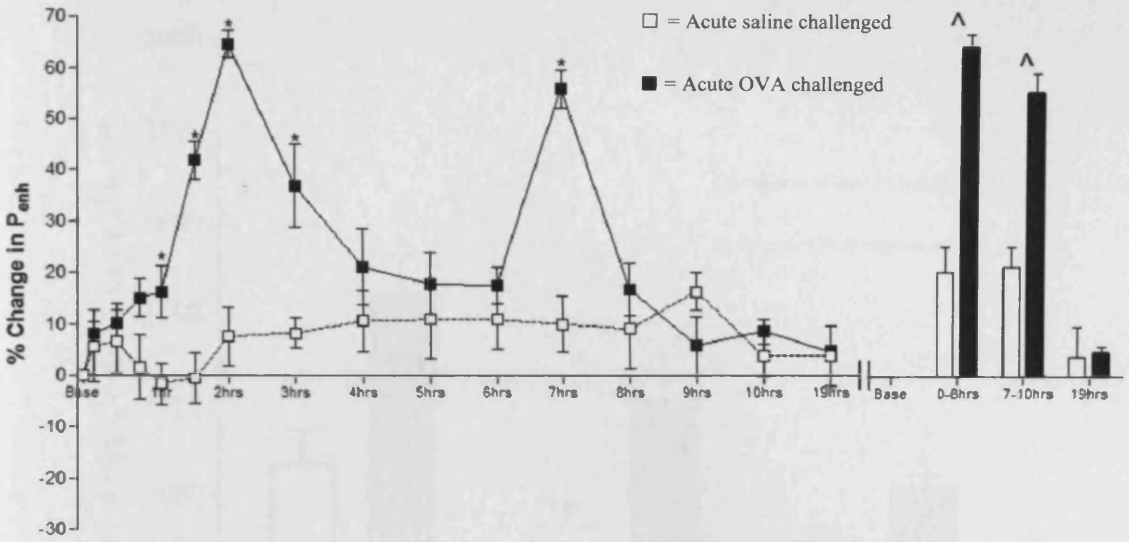


Figure 4.1a – Mean time-course values of P_{enh} in OVA sensitised mice that were challenged twice with saline or 0.5% OVA 4 hours apart. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 19 hours (24 hours after first exposure). Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from saline challenged mice; ^ significant difference between saline and OVA challenged mice. Two-tailed T-test ($P<0.05$; n=6).

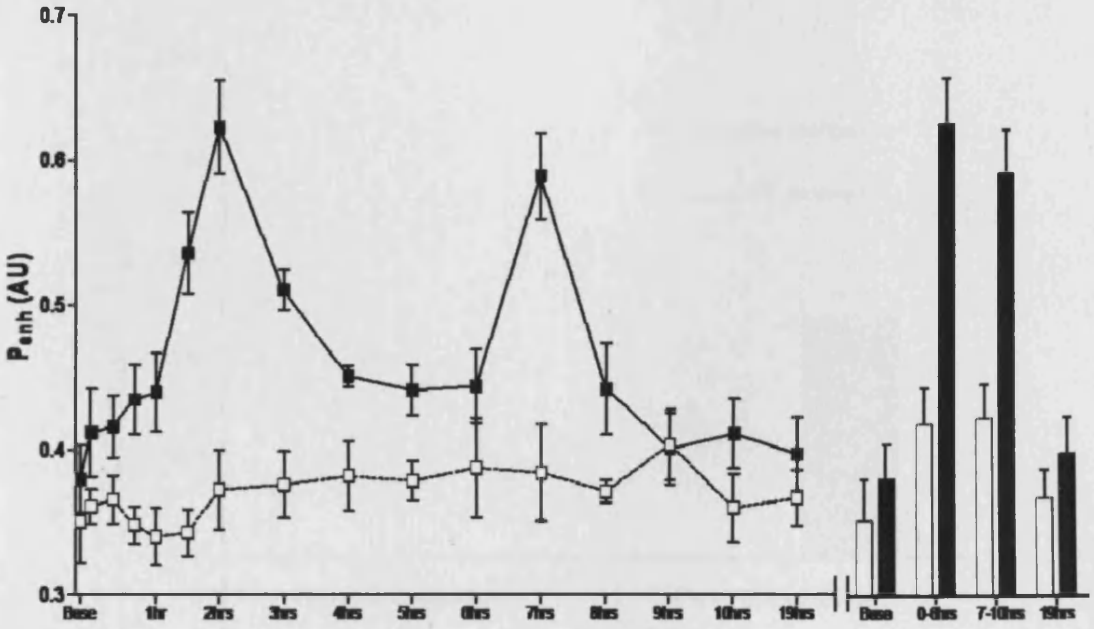


Figure 3.2b – Mean time-course raw values of P_{enh} in OVA sensitised mice that were challenged with saline or 0.01 % OVA. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Raw OVA P_{enh} values (AU) – Base (0.38±0.02), 0-6 hours (0.62±0.03), 7-12 hours (0.59±0.03) and 24 hours (0.40±0.03). Raw control P_{enh} values (AU) – Base (0.35±0.03), 0-6 hours (0.42±0.02), 7-12 hours (0.42±0.02) and 24 hours (0.37±0.02).

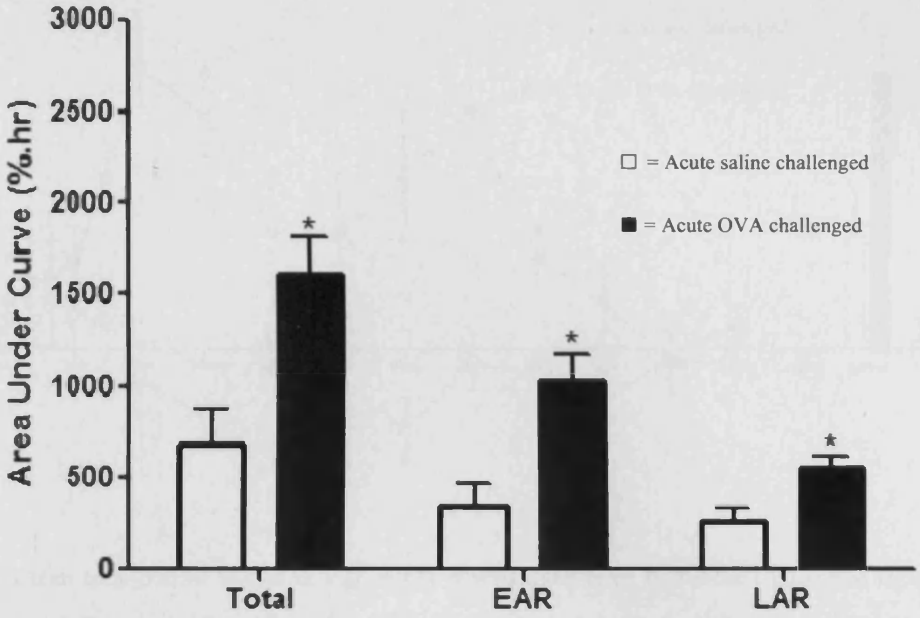


Figure 4.2 – Area under the curve analysis comparing OVA sensitised mice challenged with a two doses of saline or 0.5% OVA. Only positive peaks are considered, any peaks that have a negative value of P_{enh} are excluded. Total includes all negative peaks from 0-19 hours, EAR includes from 0-6 hours and LAR includes from 6-19 hours. Area under the curve is measured in %.hour. *significantly different from saline challenged mice. Two-tailed T-test ($P<0.05$; n=6).

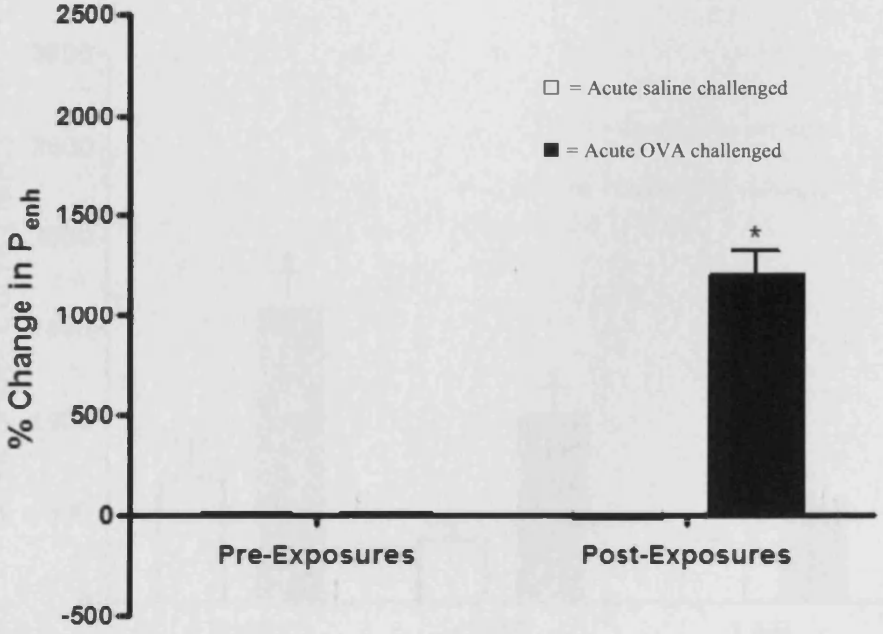


Figure 4.3 – Response of the airways to a 60 second intermittent dose of methacholine (30 mg/ml) in OVA sensitised mice challenged with saline or OVA (0.5%). Values were recorded 24 hours before saline or OVA challenge and again 24 hours post-saline or OVA challenge. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from pre-challenge values of P_{enh}. Two-tailed T-test ($P<0.05$; n=6).

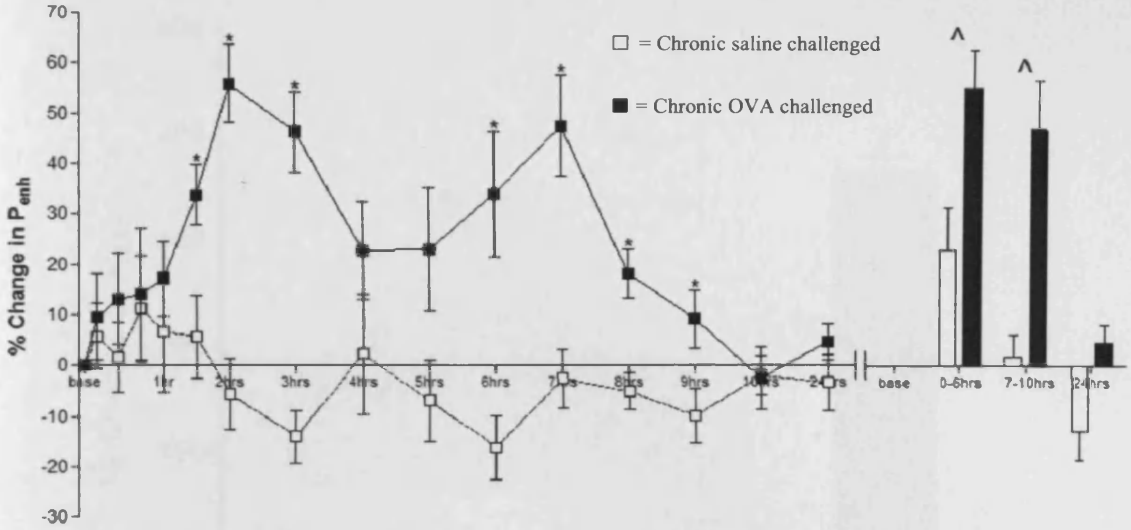


Figure 4.4 – Mean time-course values of P_{enh} in OVA sensitised mice that were challenged repeatedly with saline or 2% OVA three times a week for six weeks. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from saline challenged mice; ^ significant difference between saline and OVA challenged mice. Two-tailed T-test ($P<0.05$; n=6). Raw OVA P_{enh} values (AU) – Base (0.34±0.02), 0-6 hours (0.52±0.02), 7-12 hours (0.49±0.03) and 24 hours (0.31±0.01). Raw control P_{enh} values (AU) – Base (0.45±0.01), 0-6 hours (0.54±0.04), 7-12 hours (0.45±0.01) and 24 hours (0.39±0.02).

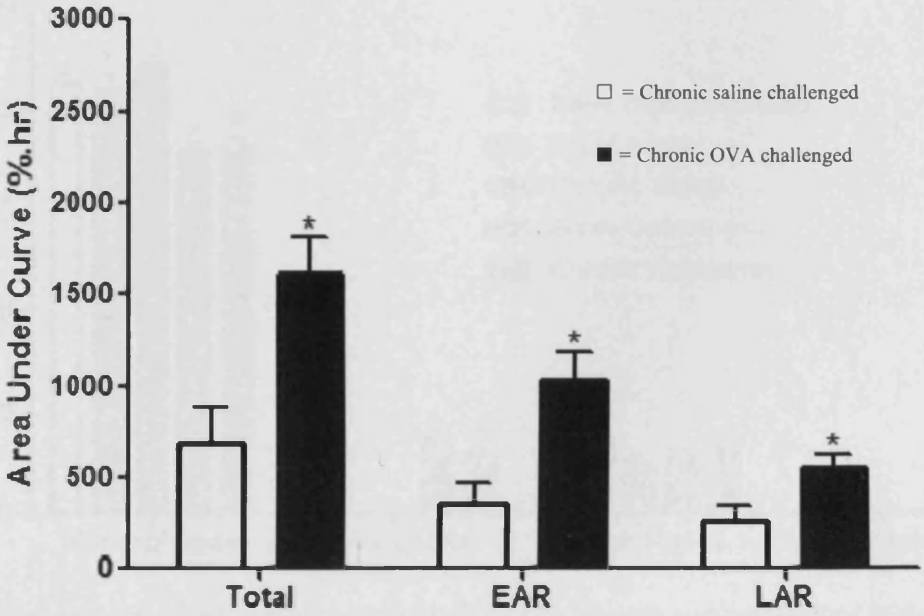


Figure 4.5 – Area under the curve analysis comparing OVA sensitised mice challenged with repeated doses of saline or 2% OVA. Only positive peaks are considered, any peaks that have a negative value of P_{enh} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from saline challenged mice. Two-tailed T-test ($P<0.05$; n=6).

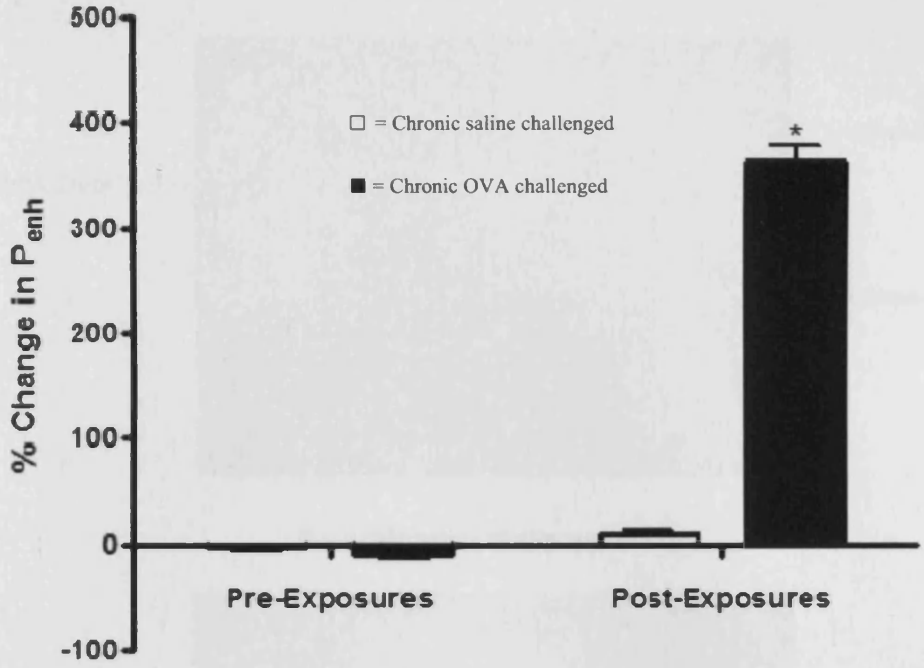


Figure 4.6. – Response of the airways to a 60 second intermittent dose of methacholine (10 mg/ml) in OVA sensitised mice challenged repeatedly over 7 weeks with saline or OVA (2%). Values were recorded 24 hours before the first saline or OVA challenge and again 24 hours post-final saline or OVA challenge. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from pre-challenge values of P_{enh}. Two-tailed T-test ($P<0.05$; $n=6$).

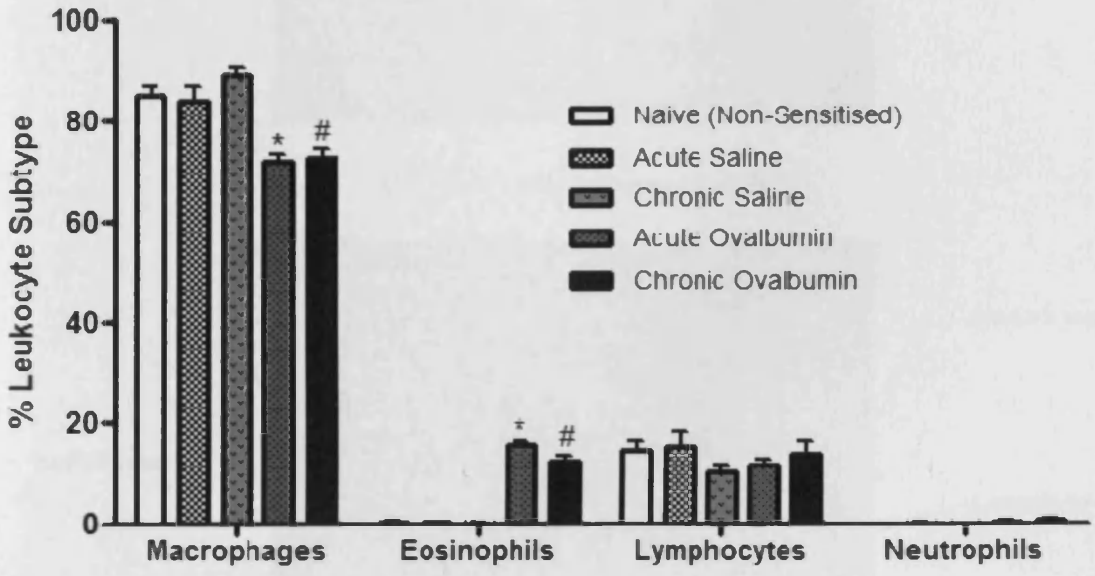
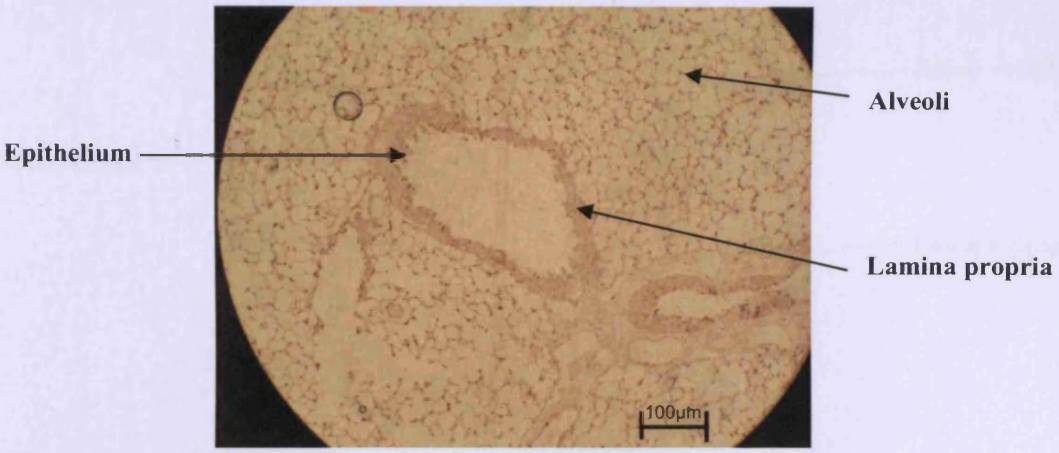
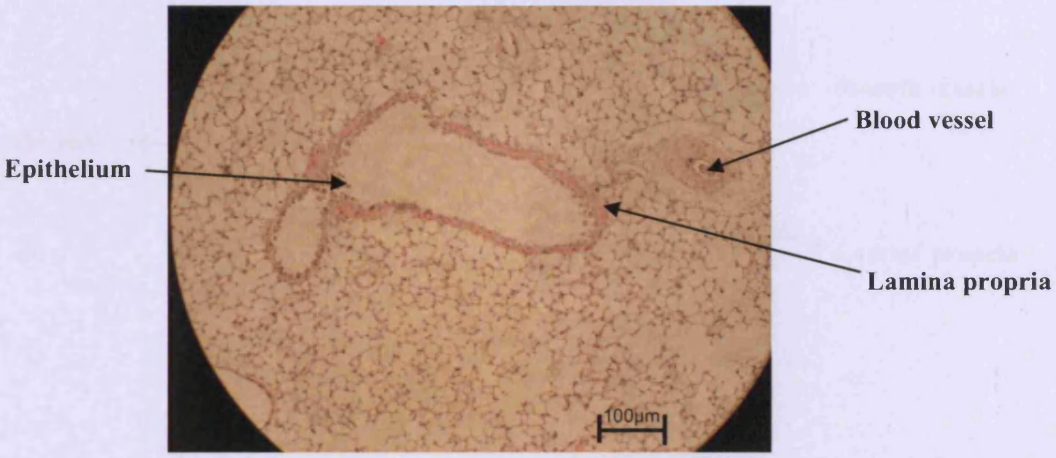


Figure 4.7 – The percentage of macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised), saline challenged (acute and chronic) and OVA challenged (acute and chronic) mice. Results are expressed as mean±S.E.M. *significantly different from acute saline; #significantly different from chronic saline; One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; $n=6$).

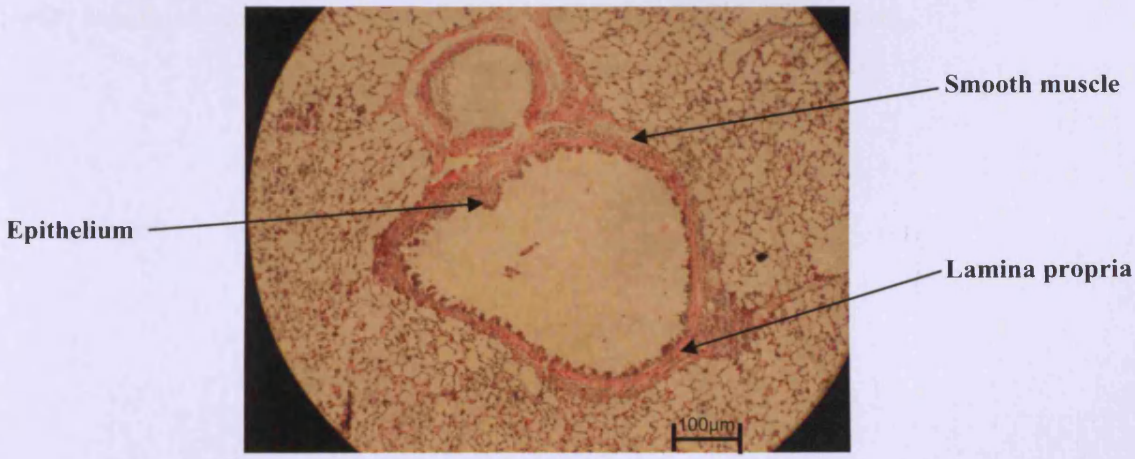
Naïve non-sensitised mouse



B – Acute saline challenged mouse



C – Acute OVA challenged mouse



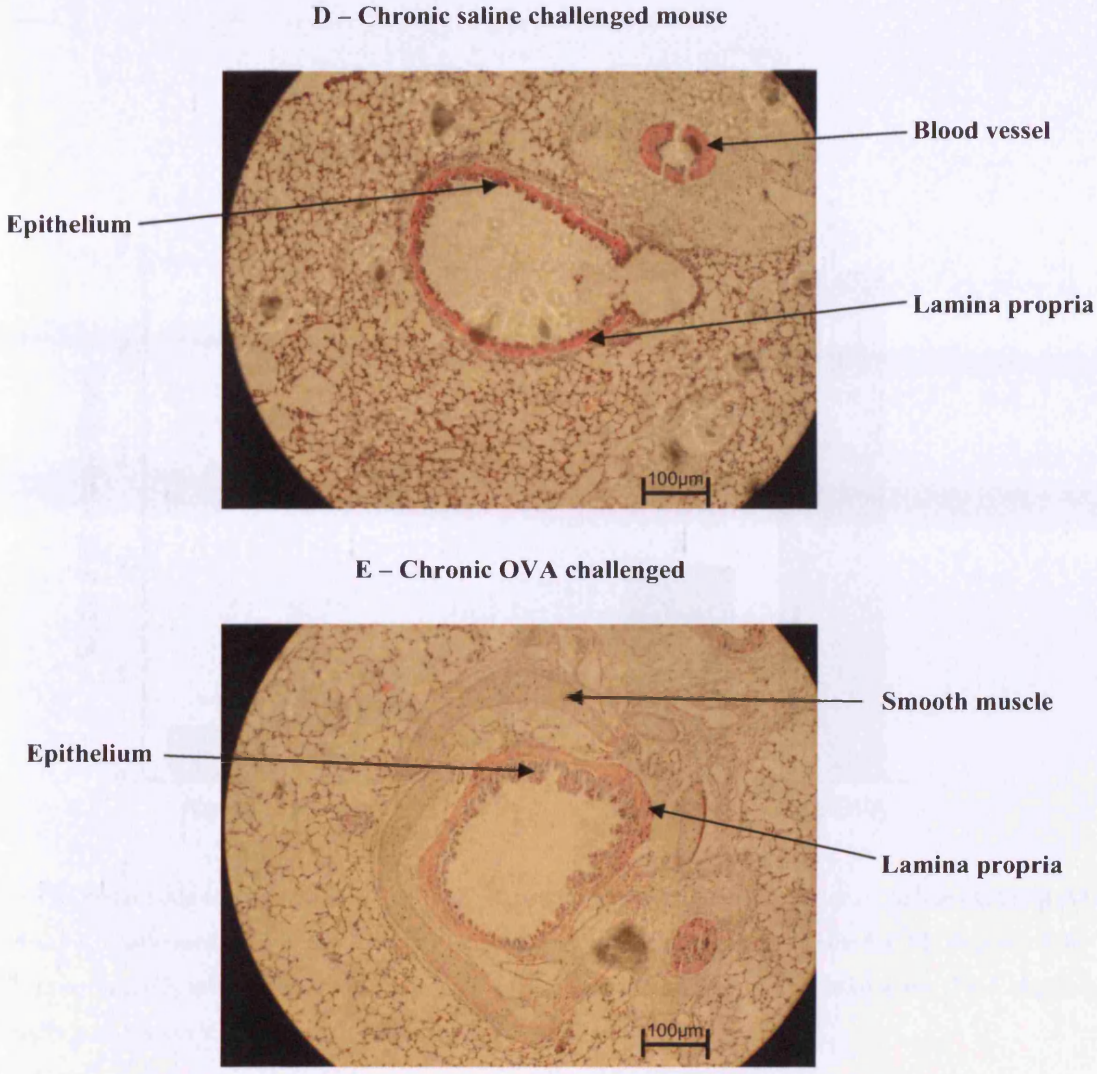


Figure 4.8 (A-E) – Bronchioles of naïve, saline challenged (acute and chronic) and OVA challenged (acute and chronic) mice stained with haematoxylin and eosin to display general morphology (1000x magnification).

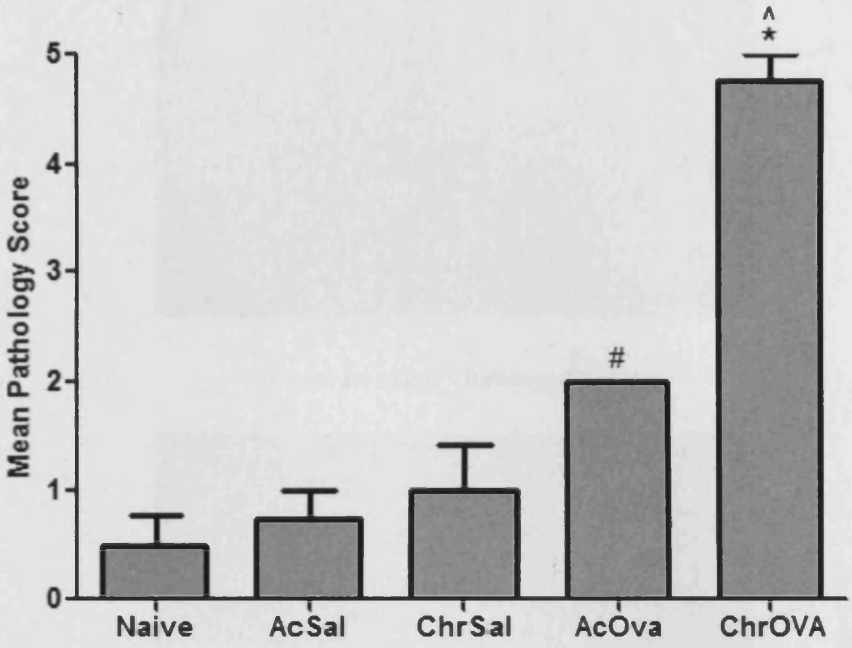
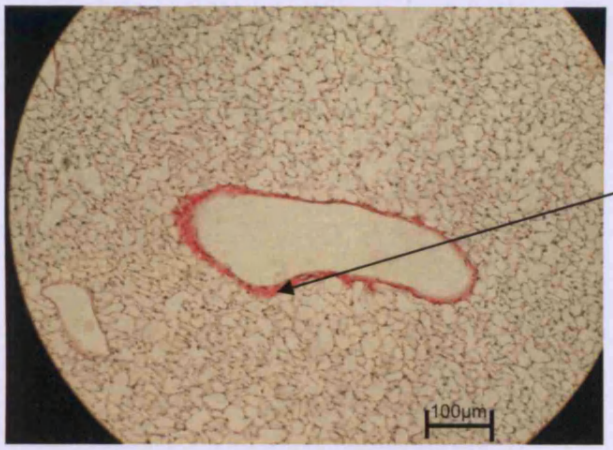
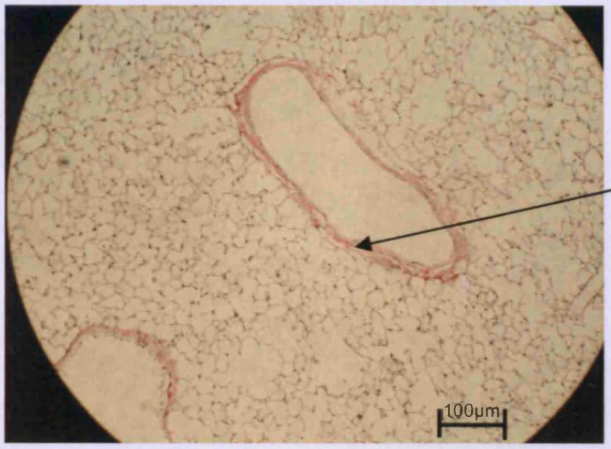


Figure 4.9 – The mean pathology score of cells found in the peri-bronchiolar space of naïve, saline challenged (acute and chronic) and OVA challenged (acute and chronic) mice. Results are expressed as mean±S.E.M. #significantly different from acute saline; *significantly different from chronic saline; ^significantly different from acute OVA challenged mice. One-way Analysis of Variance followed by a Dunnett post-test ($P<0.05$; n=4).

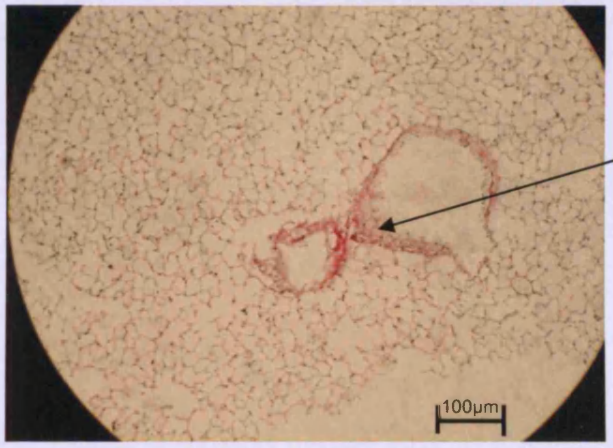
A – Naïve non-sensitised mouse



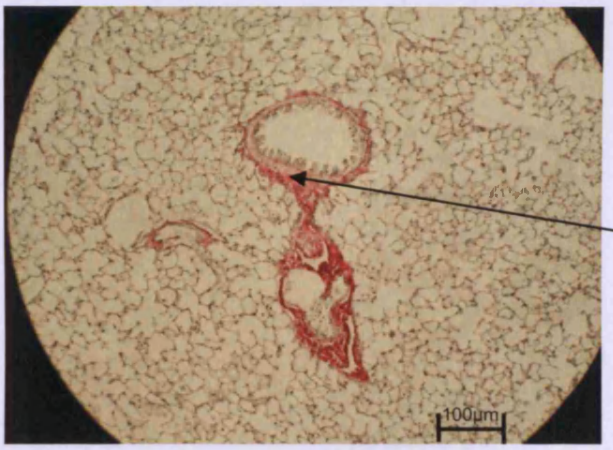
B – Acute saline challenged mouse



C – Acute OVA challenged mouse



D – Chronic saline challenged mouse



E – Chronic OVA challenged mouse

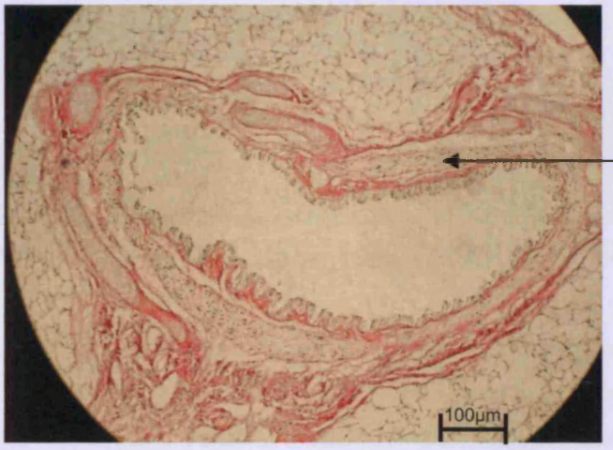


Figure 4.10 (A-E) – Bronchioles of naïve, saline challenged (acute and chronic) and OVA challenged (acute and chronic) mice stained with picrosirius red to display collagen (1000x magnification).

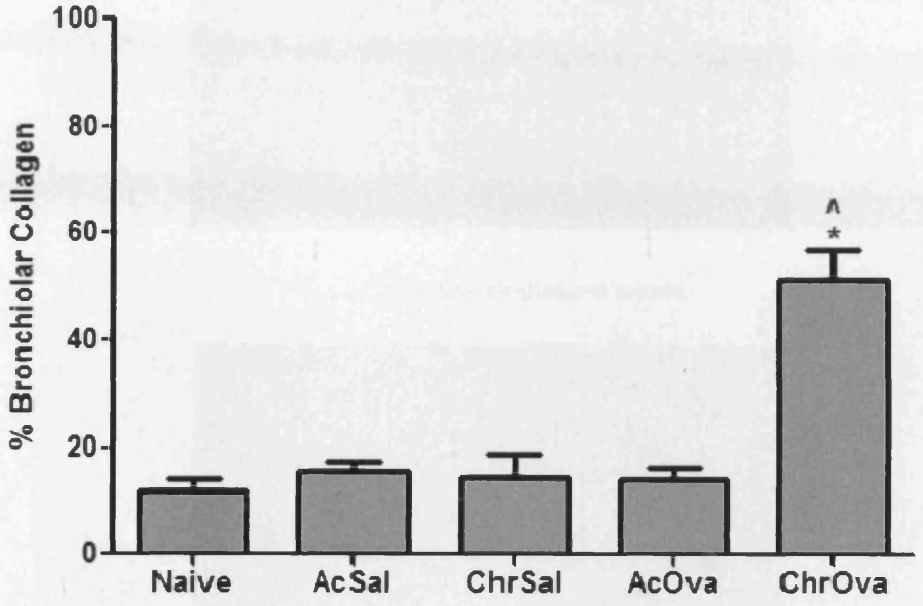
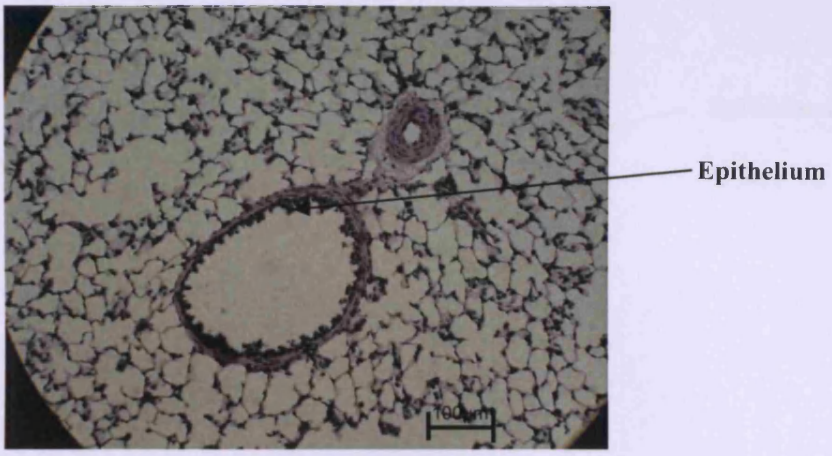
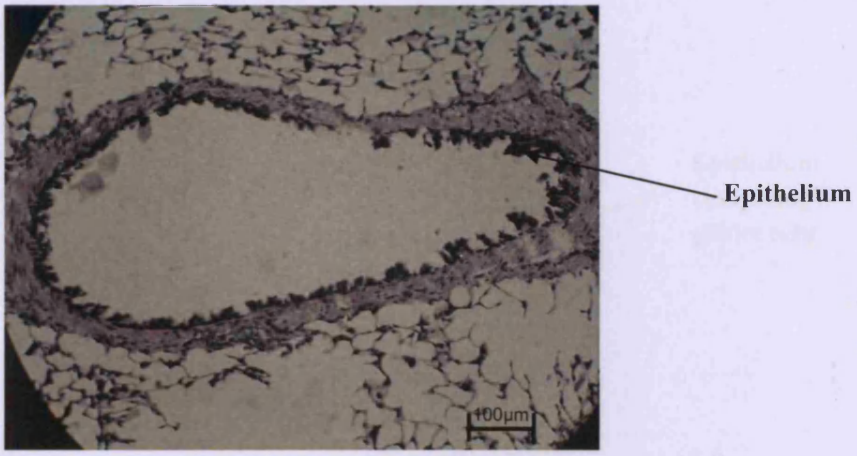


Figure 4.11 – Effect of saline (acute or chronic) or OVA (acute or chronic) challenge on percentage bronchiolar collagen in mice. Results are expressed as mean±S.E.M. *significantly different from chronic saline; ^significantly different from acute OVA challenged mice. One-way Analysis of Variance followed by a Dunnett post-test ($P<0.05$; $n=4$).

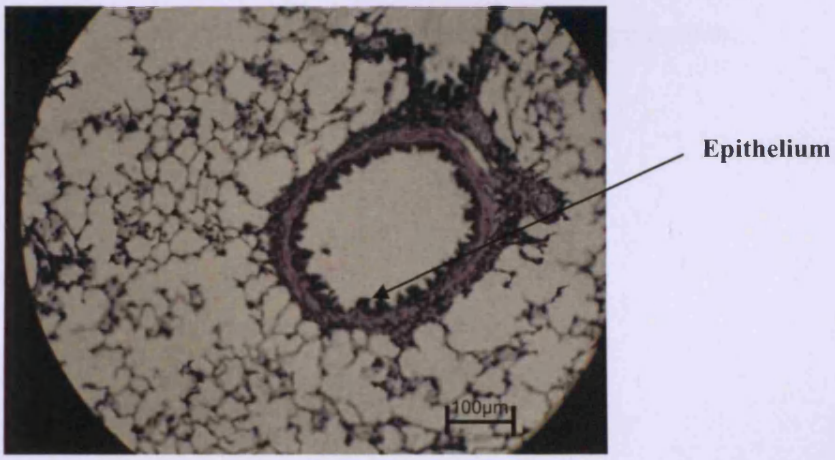
A – Naïve non-sensitised mouse



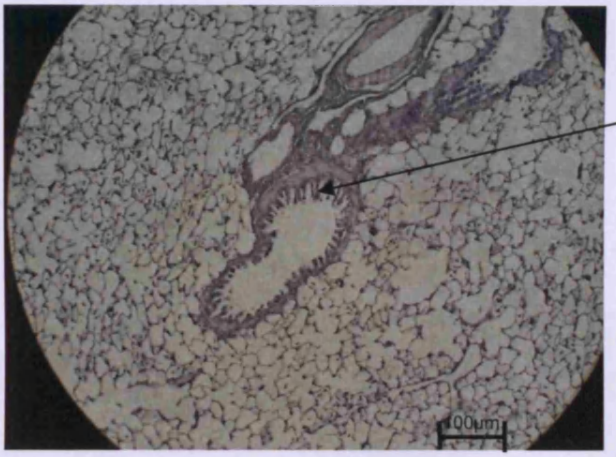
B – Acute saline challenged mouse



C – Acute OVA challenged mouse



D – Chronic saline challenged mouse



E – Chronic OVA challenged mouse

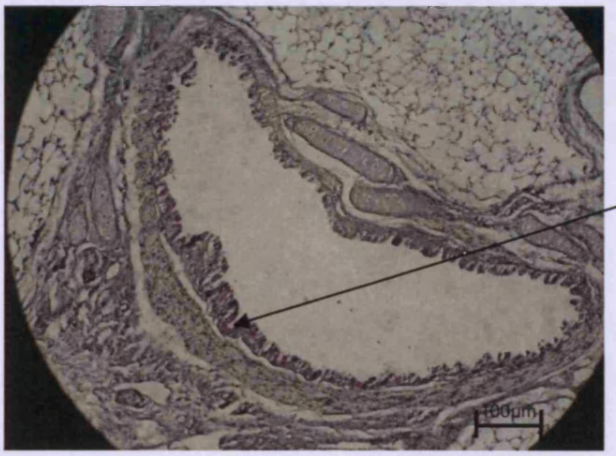


Figure 4.12 (A-E) – Bronchioles of naïve, saline challenged (acute and chronic) and OVA challenged (acute and chronic) mice stained with alcian blue and periodic acid Schiff to display goblet cells (1000x magnification).

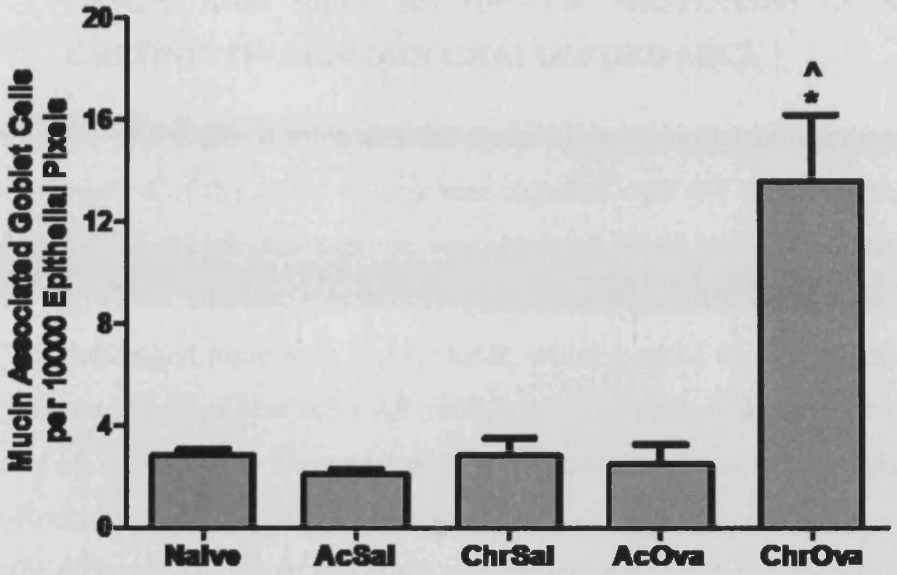


Figure 4.13 – Effect of saline (acute or chronic) or OVA (acute or chronic) challenge on number of mucin associate goblet cells per 10,000 epithelial pixels. Results are expressed as mean±S.E.M. *significantly different from chronic saline; ^significantly different from acute OVA challenged mice. One-way Analysis of Variance followed by a Dunnett post-test ($P<0.05$; n=4).

4.5 DISCUSSION

4.5.1 EARLY AND LATE ASTHMATIC RESPONSES IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED MICE

Saline challenges in sensitised mice did not cause bronchoconstriction indicating that there was no contamination of the saline or exposure chamber with OVA. However, in the groups challenged with OVA a biphasic response was observed. There was little difference observed between the two OVA challenge protocols when assessing EAR and LAR. The acute and chronic OVA challenged mice both had an EAR, which peaked at 2 hours, and LAR, which peaked at 7 hours. The fact that the EAR took 2 hours to peak is in contrast to the study by Cieslewicz *et al.,* (1999), who showed a peak at 15 minutes, but correlates with the study by Fernandez-Rodriguez *et al.*, (2008), upon which protocol the present study was based. The reason for the difference between this study and the Cieslewicz *et al.,* (1999) study could be a result of a number of factors. Firstly this study used male BALB/c mice obtained from Harlan, UK, the Cieslewicz *et al.,* (1999) study used female BALB/c mice obtained from The Jackson Laboratory, USA. It is possible that there could be differences between sex when it comes to the EAR in mice, also there could be differences between the strain of BALB/c mice as they were bred in different countries. There are also differences in the protocols and amounts of OVA used which could explain the differences.

It is unclear which study better reflects the true situation, however, a study by Choi *et al.,* (2005) also displayed a delayed EAR. There are explanations as to why the EAR could be delayed. As serotonin and not histamine is the primary mediator in mouse mast cell degranulation (Cannings 2003) and a relatively high dose of serotonin is require to cause a bronchoconstriction (Unpublished observations – Fernandez-Rodriguez *et al.*) it is possible that it takes longer for the levels of serotonin to reach a peak than histamine. Another explanation is that there could be a delay in the release of mediators that provoke mast cell degranulation (Fernandez-Rodriguez *et al.,* 2008). Mice have poorly developed airway smooth muscle compared to humans (Karol 1994) this may also be a factor in causing a delay. The fact that this model has a delay before the EAR and this is not seen in human asthma means that this model is a poor representation of the mechanisms of early phase bronchoconstriction in humans.

4.5.2 AIRWAY HYPERRESPONSIVENESS IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED MICE

Hyperresponsiveness of the airways was assessed by using the non-selective muscarinic receptor agonist methacholine. A dose of 30 mg/ml was used in acute OVA and saline challenged mice. Previous experiments have shown using this dose in chronic OVA challenged mice causes a severe bronchoconstriction response which was difficult to record (Fernandez-Rodriguez *et al.,* 2008); as a result a dose of 10 mg/ml was used. In both the acute and chronic saline challenged mice no difference was observed between pre- and post-saline challenge.

In the acute OVA challenged mice there was evidence of AHR following a methacholine challenge. Levels of P_{enh} increased by over 1,000 percent post-OVA challenge. AHR was also observed in the chronic OVA challenged mice. An increase in P_{enh} of almost 400 percent was observed after a methacholine challenge following the final OVA exposure. The fact that a lower dose of methacholine must be used in the chronic group suggests that the repeated challenges have increased the risk of bronchoconstriction occurring because of the increased hyperresponsiveness of the airways. The chronic OVA group may be more susceptible to the higher dose of methacholine because repeated OVA challenges will cause the loss of epithelial cells and therefore reduce the protection they give the airway wall.

4.5.3 CELLULAR INFLUX IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED MICE

After the final methacholine challenge the mice were euthanised and the trachea cannulated. As the mouse trachea is small it is difficult to insert the cannula without damage and subsequent contamination of the BAL fluid with red blood cells. When carrying out a total cell count it would be difficult to distinguish between red and white blood cells meaning the results would be inaccurate, because of this no total cell count was carried out. The drawback of not having a total cell count is that the BAL data must be presented as percentage of leukocytes. This is not an ideal measurement as an increased influx in one leukocyte subtype could mean that a smaller percentage of another leukocyte subtype is counted but this does not necessarily mean there is a decrease in the number of that subtype.

The issue of having no total cell count limits the effectiveness of the model as there could potentially be major changes in cell number which are not being observed. There are ways to overcome the issue of the trachea size. The mouse lung tissue was used to obtain a semi-quantifiable score of the total inflammatory cells in the lung tissue using histological analysis, unfortunately sections are not clear enough to distinguish the leukocyte subtypes.

A significant increase in the percentage of eosinophils present in the BAL fluid of acute and chronic OVA challenged mice was observed. This is to be expected as eosinophils drive the LAR which was observed in both models. Zosky and Sly (2007) reviewed models of asthma and stated that a drawback of mouse models is that they can have a massive influx of eosinophils, up to 60% of BAL fluid cells, and this is more reminiscent of allergic alveolitis. The fact that this model does not have the same magnitude of eosinophil influx strengthens the claim than it is more representative of human asthma that other mouse models. In both acute and chronic OVA treated mice a significantly decreased level of macrophages were observed compared to the respective saline challenged group. As mentioned this may not necessarily mean that there is a decrease in absolute number, but just a relative fall compared to the large increase in another subtype. In reality an increase in macrophage number would be expected.

4.5.4 HISTOLOGICAL CHANGES IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED MICE

Airway remodelling was observed in chronic OVA challenged mice but no other groups. It is clear to see from the haematoxylin and eosin stained sections that there is a vast increase in the thickness of the bronchiole wall of the chronic OVA challenged group compared to the other protocols. The scoring of the peribronchiolar inflammatory cells confirms that chronic OVA challenge caused increased influx of inflammatory cells into the lung. It is likely that these inflammatory cells contribute to the airway remodelling observed. Following staining with picrosirius red a large increase in bronchiolar collagen was observed both qualitatively and quantitatively in the repeated OVA challenged group. Evidence of airway remodelling was also observed in the alcian blue / periodic acid Schiff stained chronic OVA bronchioles where goblet cell hyperplasia was observed. These findings are also present in other studies following different OVA protocols (McMillan & Lloyd, 2004; Xisto *et al.*, 2005). The fact

that airway remodelling was observed in the chronic OVA model but not the acute OVA model suggests that the chronic model is a far better representation of human asthma.

4.5.5 GENERAL CONCLUSIONS

Models of asthma tend to have positive and negative features and this model is no exception. Firstly, there are limitations in using mice such as the fact that they respond poorly to histamine, there is huge variation between strains and anatomically they are very different to humans. However, species-specific reagents are available, transgenic mice are also readily available and IgE is the major-anaphylactic antibody making mice advantageous for models of asthma. As long as the limitations are considered when testing potential asthma therapeutics on mice then they can prove an excellent tool for asthma research.

This model was able to replicate the key features of the Fernandez-Rodriguez *et al.,* (2008) model. EAR, LAR, AHR and cellular influx, in the form of eosinophil recruitment, was observed. The fact that airway remodelling occurred in the chronic OVA model but not in the acute OVA model supports the method of chronic OVA challenging to develop a model of asthma as opposed to the commonly used single or double OVA challenge. Although the chronic model has a more time-consuming protocol it ultimately proves more efficient. Following a literature search it appears that this chronic OVA model is the only mouse model that displays changes in lung function, AHR, cellular influx and airway remodelling. The effect that current and potential asthma therapeutics has on the chronic OVA model and the acute OVA model will be assessed in the forthcoming chapters.

Chapter 5

Effect of corticosteroid treatment on acute and chronic models of asthma

CHAPTER 5

5.1 INTRODUCTION

5.1.1 CORTICOSTEROIDS

Corticosteroids, administered via inhalation, are recommended as the first-line therapy in treating persistent symptoms of asthma. Firstly a low dose is administered; if this does not relieve the symptoms then the dose is increased until control is achieved. Fluticasone propionate, budesonide and beclometasone are all examples of inhaled corticosteroids that have been used to relieve airway inflammation. However, in cases where asthma is severe and inhaled corticosteroids do not prove effective then orally administered steroids, such as dexamethasone, betamethasone and prednisolone, have been used (Wu *et al.*, 2008).

Corticosteroids can also reduce the number of the main inflammatory cells involved in the asthmatic reaction. Eosinophil apoptosis is increased when corticosteroids are administered (Meagher *et al.*, 1996) resulting in a decreased amount of circulating eosinophils. Viegas *et al.*, (2008) state that corticosteroids can also induce apoptosis in T-lymphocytes; this would result in the release of less cytokines such as IL-4, which play an important role in the exacerbation of the asthmatic response. The number of mast cells are reduced by corticosteroids during asthma (Jeffery *et al.*, 1992). Alveolar macrophages secrete chemokines and cytokines to chemoattract more macrophages to the airways resulting in increased inflammation. Corticosteroids can reduce this secretion (John *et al.*, 1998). Whether corticosteroids have an effect on neutrophils is debatable, it may be a case of reduced neutrophil number in severe asthma only (Belvisi, 2004). Kamei *et al*,. (1996) show that corticosteroids reduce the number of dendritic cells in the airways. The role of dendritic cells is to act as an antigen-presenting cell. Therefore reducing dendritic cell numbers leads to a reduction in the immune response.

Corticosteroids do not have their role exclusively on inflammatory cells. They are also able to inhibit mucus secretion in the airways possibly by inhibiting the expression of the mucin genes MUC2 and MUC5AC (Liu *et al.*, 2004). This would reduce the occlusion of the airways caused by excess mucus release and thus make breathing easier. Epithelial cells release a vast amount of inflammatory mediators and hence are an excellent target for therapeutics. Figure 5.1 shows some of the mediators released by epithelial cells and how corticosteroids are able to reduce the secretion of these mediators. This all results in a marked

decrease in airway inflammation and as a result airway hyperresponsiveness is reduced (Barnes, 1990).

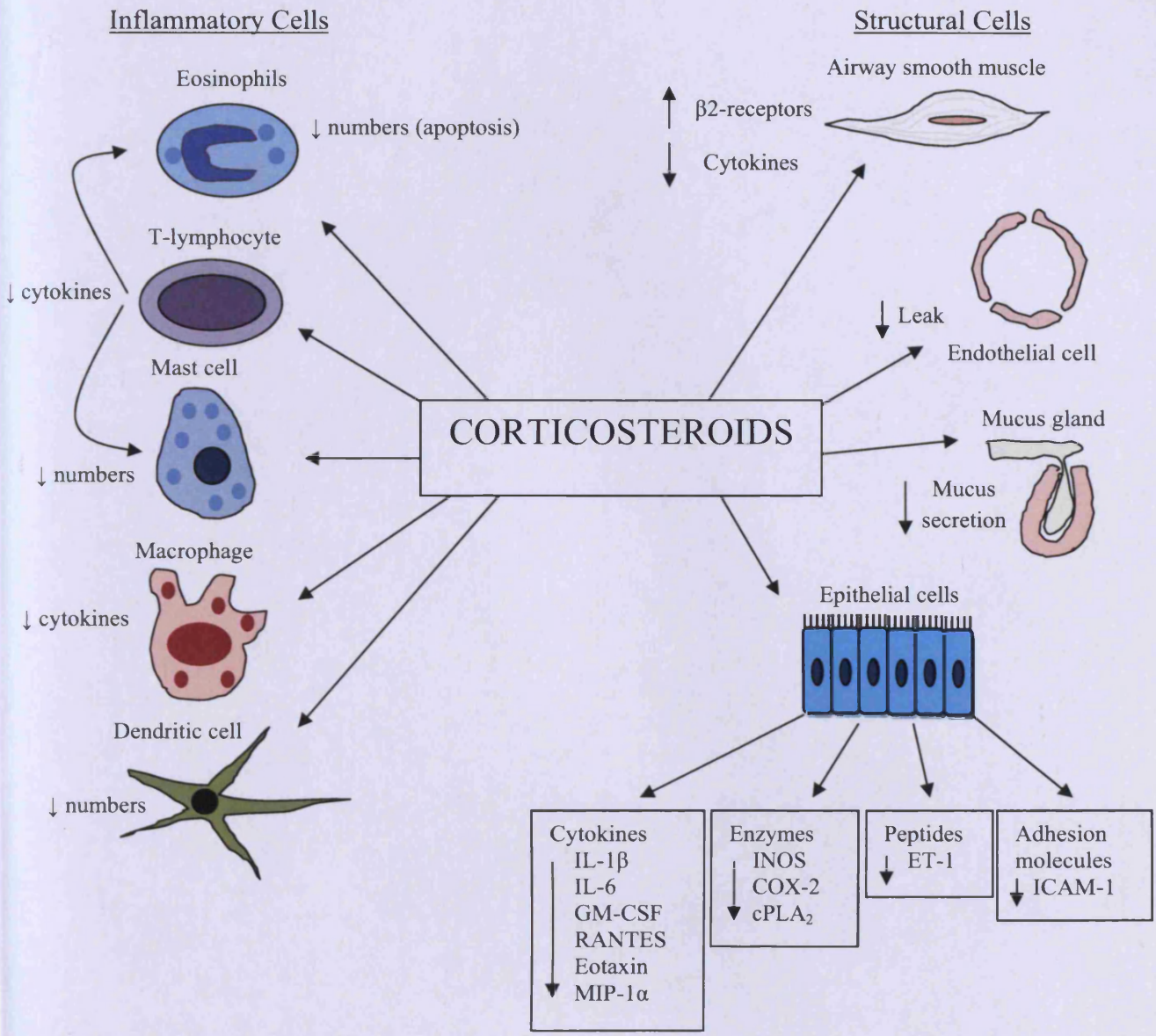


Figure 5.1: Schematic diagram to highlight the multiple roles that corticosteroids have on combating an asthmatic response. IL-1β = Interleukin 1β; IL-6 = Interleukin 6; GM-CSF = Granulocyte-Macrophage Colony Stimulating Factor; RANTES = Regulated upon Activation, Normal T Cell Expressed, and Secreted; MIP-1α = Macrophage Inflammatory Protein - 1α; INOS = Inducible Nitric Oxide Synthase; COX-2 = Cyclooxygenase-2; $cPLA_2$ = Cytoplasmic Phospholipase A_2; ET-1 = Endothelin-1; ICAM-1 = Intercellular adhesion molecule-1. Diagram adapted from Barnes (2002).

Corticosteroids do not have bronchodilator properties and as a result do not alleviate the EAR to allergen inhalation. To overcome this problem corticosteroids are often administered in

combination with inhaled β_2-agonists which are bronchodilators. The long-term administration of β_2-adrenergic receptor agonists causes downregulation and uncoupling of β_2-adrenoreceptors. This is overcome by administering the agonist in combination with corticosteroids (Mak *et al.*, 2002; Mak *et al.*, 1995). Corticosteroids also cause an increase in the expression of β_2-adrenergic receptors in the lung (Adcock *et al.*, 1996). The synergistic and complementary effect that these two drug classes have renders them arguably the most effective way to control asthma (Barnes, 2002).

Generally corticosteroids do not cause many harmful side effects. Dysphonia, hoarseness of the voice, and thrush are some of the more common complaints, which occur after taking inhaled corticosteroids. Some more severe systemic effects can occur such as osteoporosis and glaucoma but these are rare. One problem some asthmatics face is corticosteroid-resistant asthma; this means they do not respond to high doses of inhaled corticosteroids (McLeod *et al.*, 1985; Szefler & Leung, 1997). Reduced responsiveness to corticosteroids is a much more common problem; much higher doses must be used to treat airway inflammation (Barnes *et al.*, 2009).

5.1.2 MECHANISM OF ACTION

Corticosteroids are the only currently available therapy that can suppress the inflammation in asthmatic airways (Barnes & Adcock, 2003). This is done via two main mechanisms, transactivation and transrepression. Transactivation occurs when a high dose of corticosteroid is administered resulting in the formation of anti-inflammatory protein. The corticosteroid is able to cross the cell membrane of the target cell and bind to a cytosolic glucocorticoid receptor (GR). The binding causes structural changes in the receptor which causes transport of the receptor-corticosteroid complex to the nucleus. The complex then dimerises and binds to DNA at specific sequences known as the glucocorticoid response elements (GRE). The receptor-corticosteroid complex also interacts with coactivator molecules such as cyclic adenosine monophosphate response element-binding protein (CREB) – binding protein (CBP). CBP has intrinsic histone acetyltransferase (HAT) activity which is activated by the interaction of the complex. The result of the receptor-corticosteroid complex binding to both the GRE and CBP is an increase in gene transcription of anti-inflammatory genes. This leads to an increase in anti-inflammatory protein synthesis producing such proteins as interleukin-

10 and the α form of inhibitor of nuclear factor kappa B (NF-κB), inhibitor of kappa B-α (IκBα). A schematic diagram of transactivation is shown in figure 5.2.

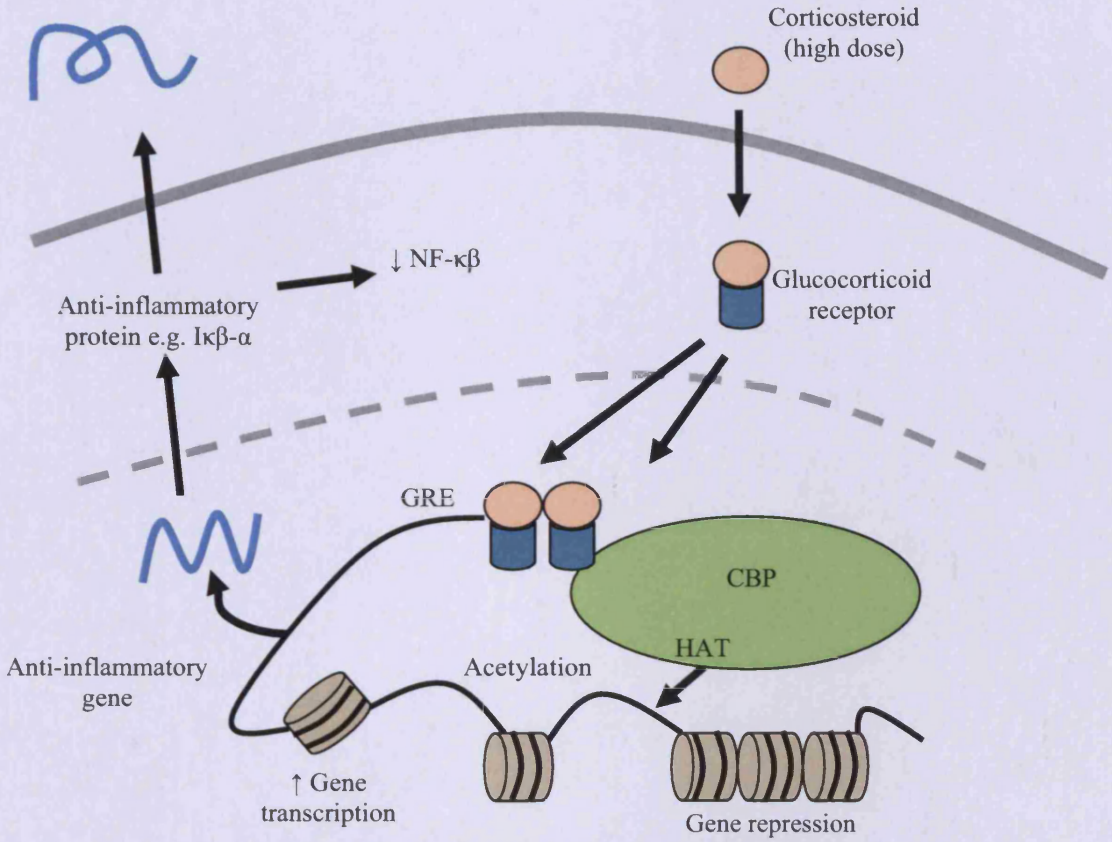


Figure 5.2: A high dose of corticosteroid passes through the cell membrane and binds with a glucocorticoid receptor causing the translocation of the complex to the nucleus. Here it dimerises and binds to GRE or CBP resulting in the increased transcription of anti-inflammatory genes. GRE = Glucocorticoid response elements; CBP = CREB (Cyclic adenosine monophosphate response element binding protein) – binding protein; HAT = Histone acetyltransferase; NF-κB = Nuclear factor kappa B; IκB-α = Inhibitor of kappa B - α Diagram adapted from Barnes and Adcock (2003).

If transactivation can be thought of as the switching on of anti-inflammatory genes then transrepression is the switching off of inflammatory genes. Transrepression occurs when a lower dose of corticosteroid is administered. Originally an inflammatory stimulus such as tumour necrosis factor-α (TNF-α) causes activation of inhibitor of IκB kinase-2 (IKK2) which in turn activates NF-κB. This causes the translocation of two NF-κB proteins, p50 and p65, to the nucleus where they bind to CBP. As CBP has intrinsic HAT activity the outcome

is increased gene transcription of inflammatory genes such as granulocyte-macrophage colony stimulating factor (GM-CSF) shown in figure 5.3.

Corticosteroids are able to inhibit this increase in gene transcription. Once administered the corticosteroids pass through the cell membrane and bind to the GR, like in transactivation, this causes the newly formed complex to transport to the nucleus and bind to the CBP. After binding the corticosteroid is able to prevent the formation of inflammatory genes by either directly inhibiting HAT activity or by recruiting histone deacetylase-2 (HDAC2) which causes gene repression by deacetylation. These two processes are shown in figure 5.3.

5.1.3 USE IN ASTHMA MODELS

Treatment with the corticosteroid fluticasone propionate (FP) was shown to have no effect on the EAR in both humans (Palmqvist *et al.*, 2005) and ovalbumin challenged guinea pigs (Lawrence *et al.*, 1998). This is expected as corticosteroids do not have bronchodilator properties. However, FP was able to inhibit the LAR in the same two studies. This inhibition is most likely a consequence of eosinophil apoptosis which is increased when corticosteroids are administered (Meagher *et al.*, 1996). In humans FP was also shown to decrease sputum levels of eosinophils and airways hyperresponsiveness to methacholine (Palmqvist *et al.*, 2005). This was also observed in ovalbumin sensitised and challenged guinea pigs that were treated with FP (Lawrence *et al.*, 1998). Treatment with the orally-administered corticosteroid dexamethasone has shown inhibition of eosinophil influx, LAR and AHR in sensitised guinea pigs that were exposed to ovalbumin (Toward & Broadley, 2002).

Corticosteroids potentially have a role in alleviating the structural airway remodelling observed in chronic asthma. A study by Feltis *et al.*, (2007) showed evidence of inhaled corticosteroids downregulating angiogenic remodelling in humans. It has also been shown that FP can increase the ratio of sputum metalloproteinase 9 (MMP-9) compared to tissue inhibitor of metalloproteinase-1 (TIMP-1) (Vignola *et al.*, 1998). MMP-9 is involved in the breakdown of extracellular matrix proteins, the build up of which leads to subepithelial fibrosis. Therefore an increase in MMP-9 over TIMP-1 results in the increased breakdown of extracellular matrix proteins and potential reduction in subepithelial fibrosis. However, a study in rats by Vanacker *et al.*, (2002) showed that FP had no measurable effect on airway remodelling at a clinically relevant dose and only when a higher dose, which caused systemic side-effects, was used then airway remodelling was inhibited.

By assessing the effect FP has on asthmatic parameters shown in both the acute and chronic guinea pig and mouse models developed in earlier chapters then it is possible to gain a better understanding of whether these models mimic the human response to a corticosteroid.

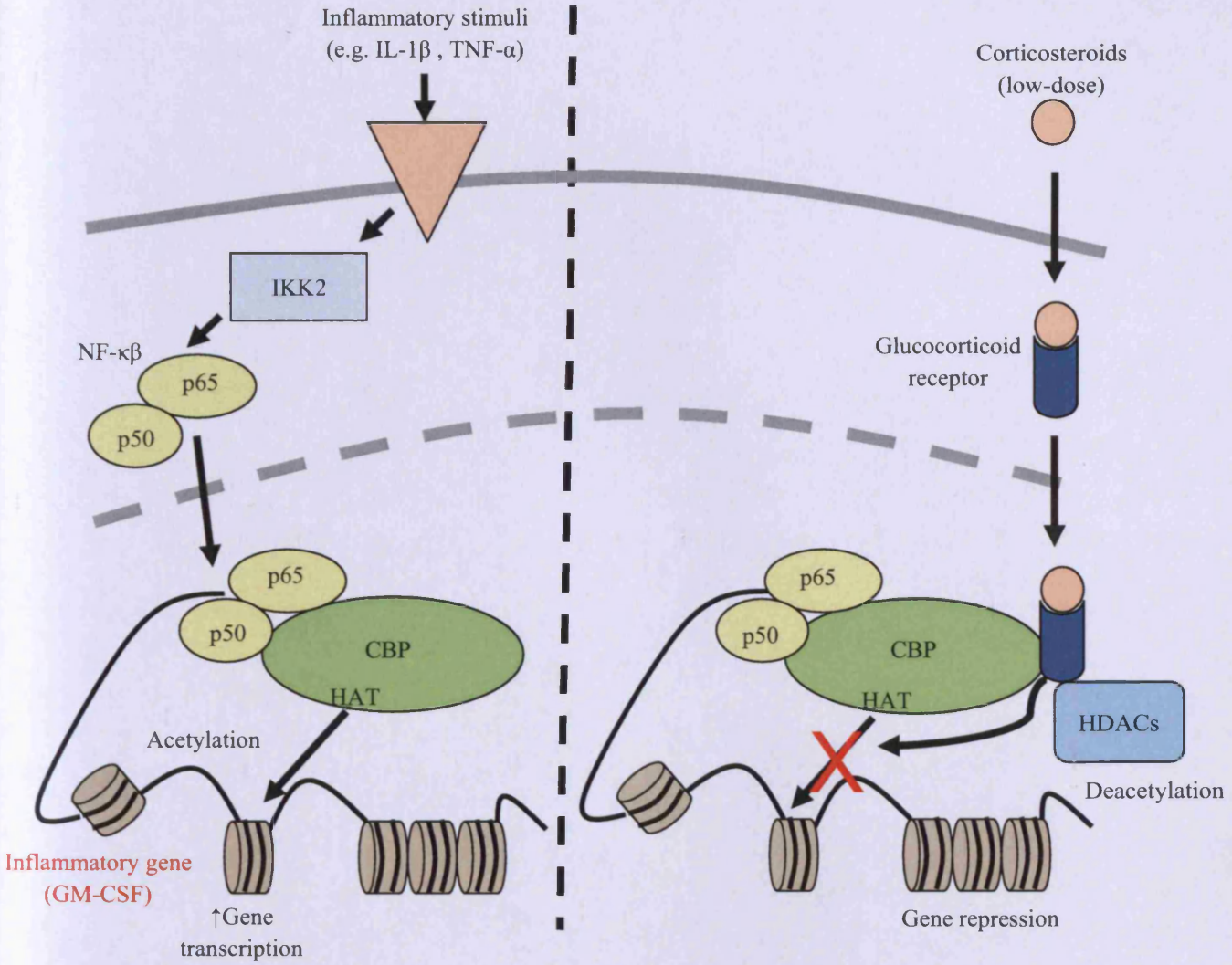


Figure 5.3: An inflammatory stimuli causes activation of IKK2 resulting in the translocation of p65 and p50 NF-κB dimers to the nucleus. This causes increased gene transcription of inflammatory genes. A low dose of corticosteroid passes through the cell membrane and binds with a glucocorticoid receptor causing the translocation of the complex to the nucleus. Here it binds to CBP causing inhibition of HAT activity. The complex can also bind to HDACs resulting in deacetylation and inflammatory gene repression. CRP = Cyclic adenosine monophosphate response element binding protein (CREB) – binding protein; HAT = Histone acetyltransferase; NF-κB = Nuclear factor kappa B; HDAC = Histone Deacetylase; IL-1β = Interleukin-1β; TNF-α = Tumour Necrosis Factor-α; IKK2 = Inhibitor of IκB Kinase 2; GM-CSF = Granulocyte-Macrophage Colony Stimulating Factor Diagram adapted from Barnes and Adcock (2003)

5.2 AIMS AND OBJECTIVES

5.2.1 AIMS

The aim of this chapter was to assess the effect of fluticasone propionate treatment compared to vehicle treatment on acute and chronic OVA challenged guinea pigs and mice. The parameters investigated were early and late phase bronchoconstriction, airway hyperresponsiveness, cellular influx and lung histology. This chapter will give an indication of how well the guinea pig and mouse models represent human asthma.

5.2.2 OBJECTIVES

- To investigate the effect that FP treatment has on acute OVA exposed guinea pigs and mice by measuring lung function, response to histamine and cellular influx compared to vehicle treatment.
- To investigate the effect that FP treatment has on chronic OVA exposed guinea pigs and mice by measuring lung function, response to histamine and cellular influx compared to vehicle treatment.
- To assess bronchiole structure, collagen deposition and levels of goblet cell hyperplasia in FP treated chronic OVA challenged guinea pigs and mice compared to vehicle.

5.3 METHODS

The methods for this chapter are covered in greater detail in chapter 2. Briefly, six male Dunkin-Hartley guinea pigs weighing 200-250 g and six male BALB/c mice weighing 20-25 g were used for each test group.

5.3.1 SENSITISATION

Both guinea pigs and mice were sensitised by an intra-peritoneal, bilateral injection of a suspension containing OVA (100 μg) and $Al(OH)_3$ (100 mg) in PBS on days 1 and 5. Guinea pigs were administered 1 mg/kg and mice 0.25 mg/kg.

5.3.2 OVALBUMIN CHALLENGES

Guinea pig OVA challenges for both acute and chronic groups were carried out in a stainless steel exposure chamber (40 cm diameter, 15 cm height) with a Wright nebuliser attached. The same nebuliser was used for challenging mice but they were exposed in a perspex box (38 cm length; 20 cm width; 20 cm height). The nebuliser delivered the OVA or saline at an air pressure of 20 lb p.s.i. and at a rate of 0.3 ml/min. Both animals were removed from the chamber and exposure was considered complete if they appeared to be distressed during the challenge.

5.3.2.1 ACUTE PROTOCOL – GUINEA PIGS

Guinea pigs were challenged with OVA (0.01%) on day 15 (14 days subsequent to the first sensitisation injection) for an hour.

5.3.2.2 CHRONIC PROTOCOL – GUINEA PIGS

To provoke a response to chronic OVA challenges the guinea pigs were intially challenged with an hour long OVA (0.01%) exposure on day 15. On days 17-29, every 48 hours, mepyramine (30 mg/kg) was administered then 30 minutes later an hour long challenge of

OVA (0.1%) occurred. Finally on day 31 OVA (0.1%) was administered for an hour without mepyramine. Lung function measurements were recorded immediately after this challenge.

5.3.2.3 ACUTE PROTOCOL – MICE

Two OVA (0.5%) challenges lasting an hour, separated by 4 hours, were used to provoke an acute response.

5.3.2.4 CHRONIC PROTOCOL – MICE

OVA (2%) was used throughout the chronic protocol. To begin with the mice were challenged with this dose for 30 minutes three times a week for six weeks. On the first day of the seventh week the exposure lasted an hour and lung function measurements were recorded after this.

5.3.3 LUNG FUNCTION MEASUREMENTS

Baseline values of sG_{aw} were recorded before the final OVA exposure and all subsequent results were taken as a percentage of these values. The results were obtained by using whole-body plethysmography. Following the final challenge values were taken after 0, 15, 30, 45 and 60 minutes, then hourly until 12 hours with a final reading taken at 24 hours.

To record lung function in mice an unrestrained system (Buxco), which calculates P_{enh} was used. Values were recorded 0, 20, 40, 60, 90 and 120 minutes post challenge, then hourly until 10 hours with a final recording at 19 hours (for acute) or 24 hours (for chronic). Prior to the challenge, baseline values of P_{enh} were taken and the subsequent results were taken as a percentage of this.

5.3.4 AIRWAY HYPERRESPONSIVENESS MEASURMENTS

5.3.4.1 GUINEA PIGS

Airway responses to histamine were assessed pre-OVA exposure and post-final OVA exposure. It was assessed by recording baseline sG_{aw} values, then exposing the guinea pigs to histamine (1 mMol for 20 seconds) and finally recording further sG_{aw} values 0, 5 and 10 minutes subsequent to the histamine exposure.

5.3.4.2 MICE

AHR was assessed in mice by evaluating their response to methacholine (30 mg/ml for acute, 10 mg/ml for chronic). 2 minutes of baseline values were recorded, followed by a minute of methacholine then finally 5 minutes of readings. The response to methacholine was recorded as a percentage of baseline. AHR was assessed on day 14 after the final OVA exposure.

5.3.5 TOTAL AND DIFFERENTIAL CELL COUNTS

5.3.5.1 GUINEA PIGS

After the final histamine challenge was complete the guinea pigs were euthanised and the trachea was cannulated. Following this the lungs were removed and saline was pumped into them and then removed to obtain BAL fluid. This was used to carry out a total and differential cell count.

5.3.5.2 MICE

BAL fluid was obtained from mice in the same way as guinea pigs. As the trachea was damaged when cannulated blood contaminated the BAL fluid meaning a total cell count could not be carried out. A differential cell count was still done using the BAL fluid.

5.3.6 DRUG ADMINISTRATION

FP was delivered at a dose of 0.51 mg/ml dissolved in saline (40%), DMSO (30%) and ethanol (30%). A 15 minute inhalation of the solution was administered 24 hours and 30 minutes before the final OVA exposure and 6 hours post-exposure.

5.3.7 HISTOLOGICAL ANALYSIS OF GUINEA PIG AND MOUSE LUNGS

3-5 mm thick portions of the left superior lobe sliced 1 mm below the bronchus were processed into wax blocks. 6μm sections were then sliced using a microtome and fixed onto a glass slide. The slices were stained with either the haematoxylin and eosin, picrosirius red or

AB/PAS protocols. This allowed staining for general morphology, collagen and mucin associated goblet cells, respectively. ImageJ software was used to quantify these results. The peribronchiole, periphery of the small airways, of sections that were stained with haematoxylin and eosin was scored for number inflammatory of cells present to evaluate whether the number of inflammatory cells differed in the lung tissue of various treatment groups. The parameters for scoring were on a semi-quantitative scale as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5) (Barends *et al.*, 2004).

5.4 RESULTS

5.4.1 LUNG FUNCTION OF ACUTE CHALLENGED GUINEA PIGS AND MICE

5.4.1.1 GUINEA PIGS

Figure 5.4 demonstrates that in guinea pigs that were administered the FP vehicle (saline-40%;DMSO-30%;ethanol-30%) an early phase bronchoconstriction was observed immediately following a single OVA challenge (-54.7±7.0%). FP treatment had no effect on this bronchoconstriction as an immediate decrease in sG_{aw} was observed in this group also (-65.9±2.2%). In fact no significant difference was observed between the two groups until the 7 hour time point. A late phase bronchoconstriction was present in the vehicle treated group (-25.5±5.1%) but FP treatment significantly reduced this (-1.2±3.2%).

When assessing the peak bronchoconstriction values between 0-6 hours no difference is seen between the two groups, however, between 7-12 hours FP treated guinea pigs had a significantly reduced value of sG_{aw} compared to vehicle treated (-4.5±2.0% compared to -26.0±4.9% respectively). Area under the curve analysis showed no difference in total or EAR area between the groups but as expected a significantly reduced area was seen after FP treatment compared to vehicle in LAR area (24.1 ±7.2%.hr compared to 163.8±45.4%.hr respectively) (figure 5.5).

5.4.1.2 MICE

Figure 5.6 represents the lung function of acute OVA challenged mice treated with vehicle or FP. An early phase bronchoconstriction seen as an increase in P_{enh} was observed for both vehicle treated (53.9±2.9%) and FP treated (53.6±4.0%) after 2 hours. This steadily recovered back to baseline. At 7 hours the vehicle treated group had a second bronchoconstriction (46.1±4.3%), however, FP treatment significantly reduced this (7.7±2.6%). FP continued to cause a significant reduction at 8 hours. When comparing peak bronchoconstrictions no difference was observed at the EAR time point. During the LAR time point FP treatment caused a significant reduction in the response to OVA compared to vehicle (11.8±1.9% compared to 46.6±4.2% respectively).

Area under the curve analysis is shown in figure 5.7. No significant variation was observed between the groups throughout the whole curve and during the EAR. However, FP treatment caused a significantly lower area under the curve compared to vehicle during the LAR (195.6±58.0%.hr compared to 555.9±101.1%.hr respectively).

5.4.2 AIRWAY HYPERRESPONSIVENESS OF ACUTE CHALLENGED GUINEA PIGS AND MICE

5.4.2.1 GUINEA PIGS

Assessment of AHR in acute OVA challenged guinea pigs treated with vehicle or FP is shown in figure 5.8. Vehicle treatment does not prevent AHR as a significant decrease in sG_{aw} after histamine inhalation was observed post-OVA challenge compared to pre-OVA challenge at both 0 (-40.4±9.4% compared to -0.7±0.7% respectively) and 5 (-16.4±7.3% compared to 0.1±1.1% respectively) minutes following histamine challenge. Treatment with FP caused complete inhibition of AHR as a response to histamine was observed pre- or post-OVA challenge.

5.4.2.2 MICE

The responses of acute OVA challenged mice treated with FP or vehicle to methacholine 30 mg/ml is shown in figure 5.9. The vehicle treated group show a significantly greater increase of percentage change in P_{enh} from baseline value post-OVA compared to pre-OVA challenge (1461.8±131.0% compared to 5.5±6.4% respectively). This was not the case for the FP treated mice as no significant difference was observed post-OVA challenge compared to pre-OVA challenge (5.9±4.4% compared to -9.2±4.4% respectively). FP treatment caused a significantly less value of P_{enh} post-OVA than vehicle treatment (5.9±4.4% compared to 1461.8±131.0% respectively).

5.4.3 LUNG FUNCTION OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

5.4.3.1 GUINEA PIGS

Following the final OVA challenge an immediate bronchoconstriction was observed in both FP (-47.8±7.4%) and vehicle (-54.3±10.8%) treated guinea pig as shown in figure 5.10. After these resolved a second bronchoconstriction was observed at 7 hours in the vehicle treated guinea pigs, however, FP treatment caused significant inhibition of this bronchoconstriction (-19.8±6.2% compared to -0.8±1.5% respectively). The maximum sG_{aw} values recorded between the EAR time point showed no significant difference between the two treatments, however, during the LAR time point FP treatment caused a significant decrease in sG_{aw} compared to vehicle (-3.1±1.0% compared to -28.1±2.6% respectively). Area under the curve analysis, displayed in figure 5.11, showed that the only difference seen between the two treatments was during the LAR, when the FP treated guinea pigs had a significantly smaller area than the vehicle treated (30.0±3.9%.hr compared to 252.3±20.4%.hr respectively).

5.4.3.2 MICE

Figure 5.12 represents the percentage change in P_{enh} values from baseline in chronic OVA challenged mice that were treated with FP or vehicle. A peak bronchoconstriction was observed after 2 hours in both the FP (64.8±4.5%) and vehicle (61.6±6.7%) treated groups. Although this steadily decreased a second bronchoconstriction was observed after 7 hours in the vehicle treated group, which was significantly inhibited by FP treatment (58.5±5.0% compared to 6.6±3.0% respectively). Maximum bronchoconstriction values show that FP was only significantly effective at reducing the level of the LAR compared to vehicle (13.1±1.8% compared to 58.5±5.0% respectively). Area under the curve analysis, shown in figure 5.13, highlights a significant difference in area between FP and vehicle throughout the whole curve (1261.2±65.3%.hr compared to 1934.0±220.0%.hr respectively) and at the LAR time point (210.2±38.6%.hr compared to 778.6±104.7%.hr respectively).

5.4.4 AIRWAY HYPERRESPONSIVENESS OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

5.4.4.1 GUINEA PIG

Figure 5.14 represents the effect of histamine challenge on chronic OVA exposed guinea pigs treated with FP or vehicle. In the vehicle treated guinea pigs a significant bronchoconstriction was observed post-exposure compared to pre-exposure at both 0 (-38.4±9.0% compared to 2.6±4.7% respectively) and 5 (-18.1±5.6% compared to 0.9%±3.0%) minutes after the histamine challenge. However, FP treatment effectively prevented this AHR as no significant difference was observed between sG_{aw} values taken pre- and post-OVA exposure.

5.4.4.2 MICE

The response of chronic OVA challenged mice that have been treated with vehicle or FP to methacholine (10 mg/ml) is shown in figure 5.15. The vehicle treated group show a significant increase in P_{enh} post-OVA challenge compared to pre-OVA challenge (374.4±31.2% compared to 7.9±4.1% respectively) suggesting AHR. FP treated mice had no significant difference post-challenge compared to pre-challenge (11.7±2.2% compared to -4.7±2.9% respectively). The effectiveness of FP at reducing P_{enh} after a histamine challenge was seen as a significant reduction compared to vehicle P_{enh} values post-OVA challenge (11.7±2.2% compared to 374.4±31.2% respectively).

5.4.5 CELL COUNTS IN ACUTE AND CHRONIC CHALLENGED GUINEA PIGS AND MICE

5.4.5.1 GUINEA PIG

Figure 5.16 represents the number of cells in BAL fluid of naïve and OVA challenged guinea pigs treated with vehicle or FP. The total number of cells in FP treated acute OVA challenged guinea pigs was significantly reduced compared to vehicle treated guinea pigs ($4.1\pm0.2x10^6$ compared to $6.2\pm0.6x10^6$ respectively). The same was true for FP treatment in chronic OVA challenged guinea pigs ($3.8\pm0.2x10^6$ compared to $15.1\pm0.6x10^6$ respectively). When comparing cellular subtypes a significant decrease was observed in eosinophil number when

the treatment was FP instead of vehicle in both acute ($1.7\pm0.1x10^6$ compared to $3.9\pm0.1x10^6$ respectively) and chronic ($1.5\pm0.1x10^6$ compared to $7.3\pm1.9x10^6$ respectively) OVA challenged guinea pigs. The only difference between the two FP treated groups was in neutrophil number, the chronic group showed a significantly lower level compared to the acute group ($0.1\pm0.03x10^6$ compared to $0.6\pm0.04x10^6$ respectively).

5.4.5.2 MICE

The percentage of leukocyte subtypes found in the BAL of naïve, vehicle treated and FP treated mice is shown in figure 5.17. A significant reduction in percentage of eosinophils was observed in FP treated mice compared to vehicle (3.3±1.2% compared to 14.8±1.8% respectively). A significant reduction in eosinophil percentage was also observed in chronic OVA – FP treated mice compared to chronic OVA – vehicle treated (2.0±0.4% compared to 13.0±0.7% respectively). However, a significantly greater percentage of macrophages were observed in the acute OVA – FP treated mice compared to the acute OVA – vehicle treated group (83.3±2.7% compared to 71.3±2.9% respectively).

5.4.6 LUNG HISTOLOGY OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

Figure 5.18 (A-C) show a bronchiole from naïve, chronic OVA – vehicle treated and chronic OVA – FP treated guinea pigs. General morphology is shown after haematoxylin and eosin staining. The vehicle treated guinea pigs have increased levels of smooth muscle, increased thickness of the lamina propria and a thicker epithelium compared to the naïve guinea pig bronchiole. Compared to vehicle the FP guinea pig shows less features of airway remodelling as there is less smooth muscle present and the epithelium and lamina propria is not as thick. When the bronchiole pictures are zoomed in leukocytes are visible in the lung tissue. Figure 5.19 shows the mean pathology scores for leukocyte infiltration of the same three groups. Scoring ranged from 0-5 with the higher the score the more inflammatory cells present in the peri-bronchiole. There were clearly more cells present in the both the OVA challenged groups compared to naïve guinea pigs. FP treatment significantly reduced the mean pathology score compared to vehicle (1.8±0.3 compared to 4.5±0.5). Figure 5.20 (A-C) shows haematoxylin and eosin stained bronchioles from naïve, chronic OVA – vehicle treated and chronic OVA – FP treated mice. The differences between the FP and vehicle

treated mice were more difficult to see than in the guinea pigs. However, there still appeared to be slight loss of smooth muscle and lamina propria thickness after FP treatment. The bronchioles for both treatment groups showed more airway remodelling than the naïve mice. The mean pathological score for these groups is found in figure 5.21 FP treatment caused significant reduction in peri-bronchiolar cell number compared to vehicle (1.8±0.3 compared to 4.5±0.5).

Figure 5.22 (A-C) shows the three guinea pig groups whose bronchioles have been stained with picrosirius red to assess percentage bronchiolar collagen. The vehicle treated OVA challenged group showed substantial red stained collagen in the lamina propria. The bronchiole of the FP treated guinea pig had less collagen than the vehicle treated guinea pig. The quantitative results are shown in figure 5.23 it is clear to see that FP causes a reduction in the percentage of bronchiolar collagen compared to vehicle treated guinea pigs (26.8±4.8% compared to 53.5±5.3% respectively). Figure 5.24 (A-C) shows naïve, chronic OVA with vehicle treatment and chronic OVA with FP treatment mouse bronchioles stained with picrosirius red. The area of red-stained collagen-containing lamina propria was greatest in the chronic OVA vehicle treated group. A more clear difference was observed between the two treatment groups, with FP treatment showed less collagen than vehicle treatment. Figure 5.25 represents the percentage of bronchiolar collagen found in the mouse groups. It shows that FP treatment significantly reduced the percentage of collagen compared to vehicle (27.9±6.4% compared to 55.3±8.1% respectively).

Figures 5.26 (A-C) show an AB/PAS stained bronchiole from each of the three guinea pig groups. Vehicle treated chronic OVA challenged guinea pigs showed strong bright pink staining of the epithelium, representing mucin associated goblet cells. FP treatment had less of the bright pink stained mucin associated goblet cells than vehicle treatment. The number of mucin associated goblet cells per 10,000 epithelial pixels is shown in figure 5.27. FP reduces the number compared to vehicle (3.3±0.3 compared to 8.2±0.8). AB/PAS stained bronchioles from the three mouse groups are shown in figure 5.28 (A-C). FP treatment reduced levels of mucin associated goblet cells compared to vehicle. This is confirmed quantifiably as FP significantly reduced the number compared to vehicle (4.2±0.7 compared to 9.6±1.6) (figure 5.29).

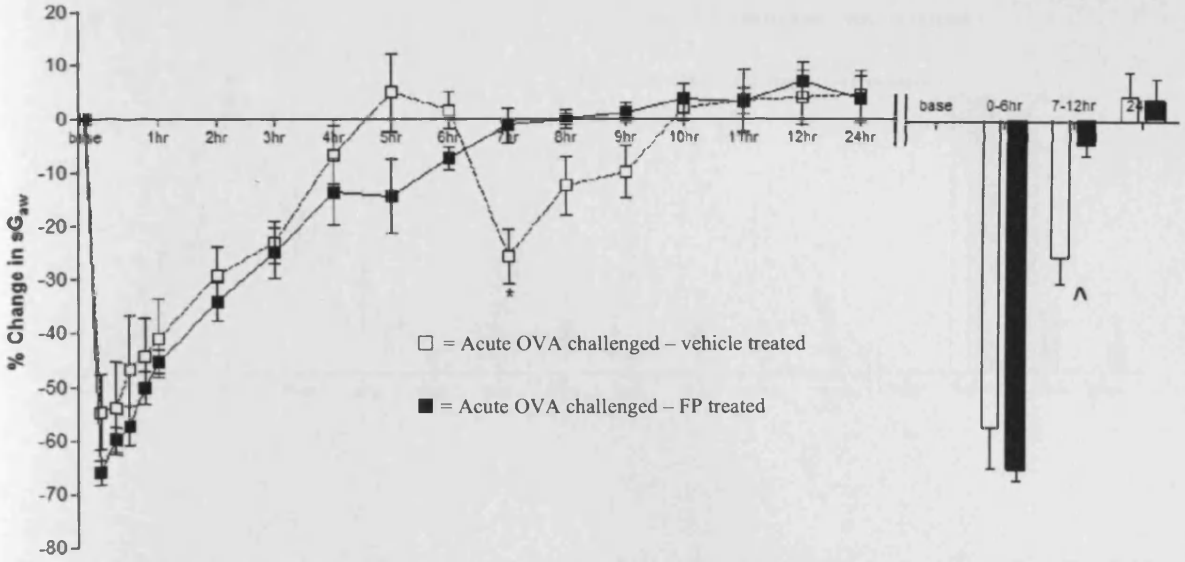


Figure 5.4 – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that were challenged with 0.01 % OVA and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from FP treated guinea pigs; ^ significant difference between vehicle and FP treatment. Two-tailed T-test ($P<0.05$; n=6). Raw FP treatment sG_{aw} values (cm/H_20) – Base (0.68±0.03), 0-6 hours (0.23±0.01), 7-12 hours (0.65±0.03) and 24 hours (0.71±0.06). Raw vehicle treatment sG_{aw} values (cm/H_20) – Base (0.67±0.08), 0-6 hours (0.29±0.07), 7-12 hours (0.48±0.04) and 24 hours (0.69±0.06).

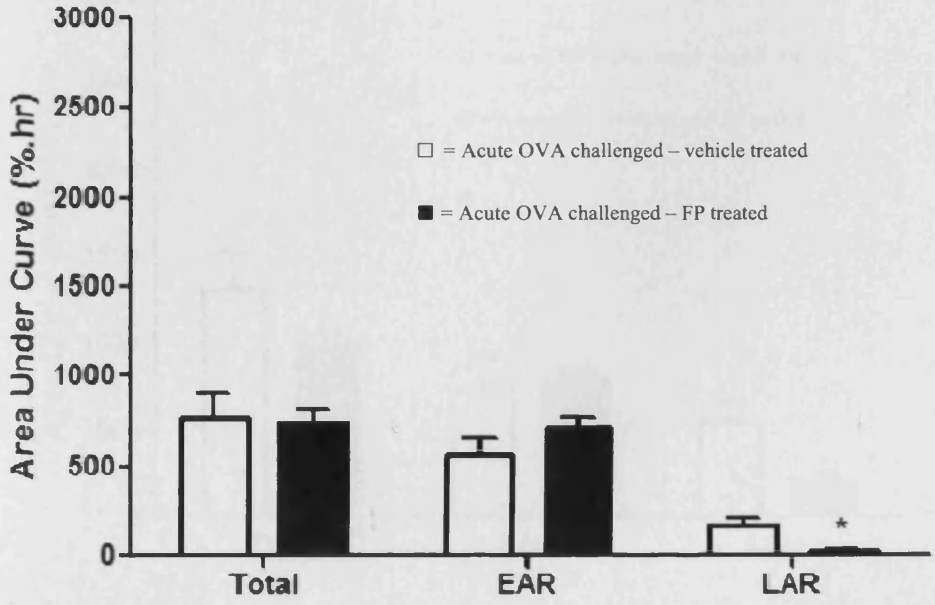


Figure 5.5 – Area under the curve analysis comparing OVA sensitised guinea pigs challenged with 0.01% OVA and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

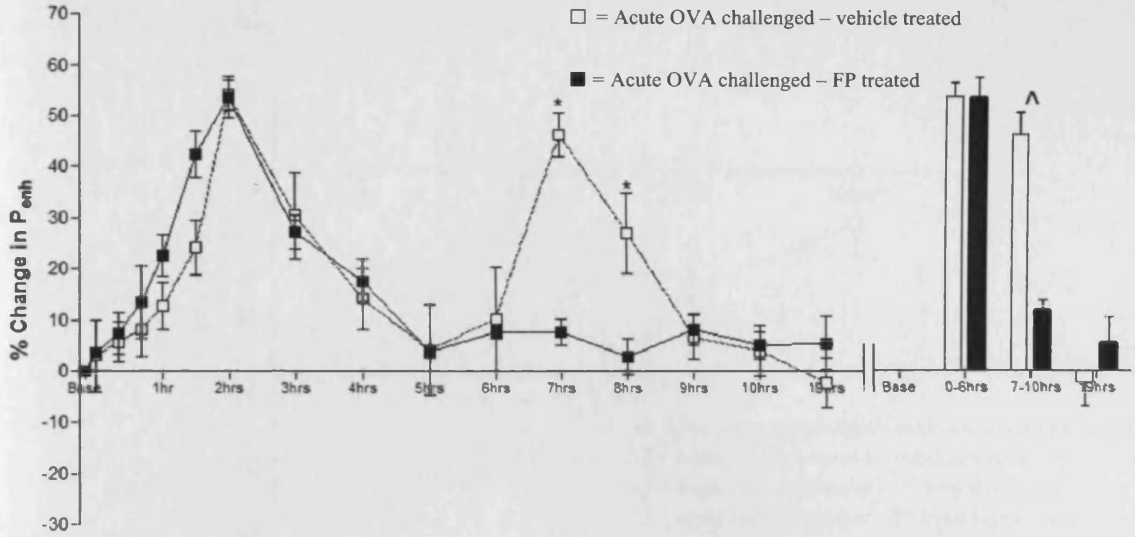


Figure 5.6 – Mean time-course values of P_{enh} in OVA sensitised mice that were challenged twice with 0.5% OVA 4 hours apart and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 19 hours (24 hours after first exposure). Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from FP treated mice; ^ significant difference between vehicle and FP treatment. Two-tailed T-test ($P<0.05$; n=6). Raw FP treatment P_{enh} values (AU) – Base (0.36±0.02), 0-6 hours (0.55±0.03), 7-12 hours (0.52±0.03) and 24 hours (0.35±0.03). Raw vehicle treatment P_{enh} values (AU) – Base (0.37±0.03), 0-6 hours (0.56±0.04), 7-12 hours (0.41±0.03) and 24 hours (0.38±0.03).

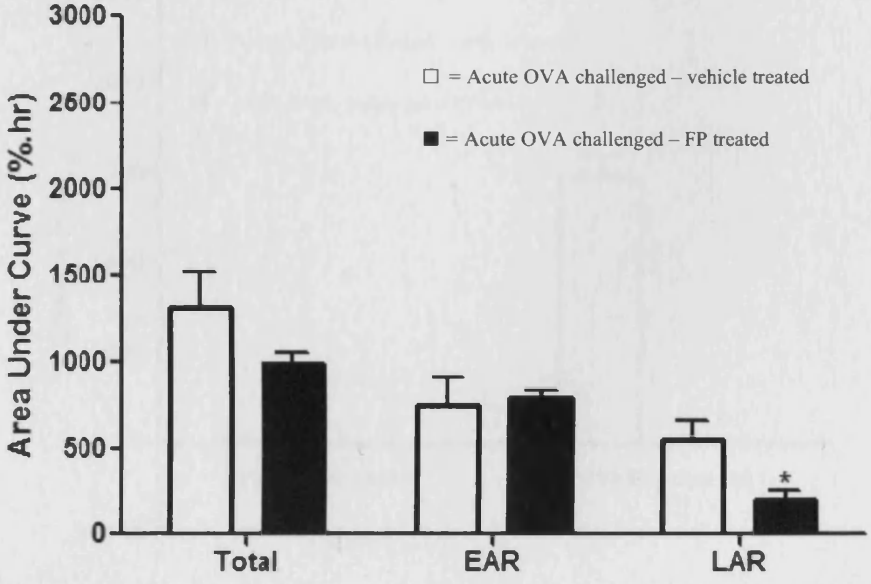


Figure 5.7 – Area under the curve analysis comparing OVA sensitised mice challenged with a two doses of 0.5% OVA and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only positive peaks are considered, any peaks that have a negative value of P_{enh} are excluded. Total includes all negative peaks from 0-19 hours, EAR includes from 0-6 hours and LAR includes from 6-19 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

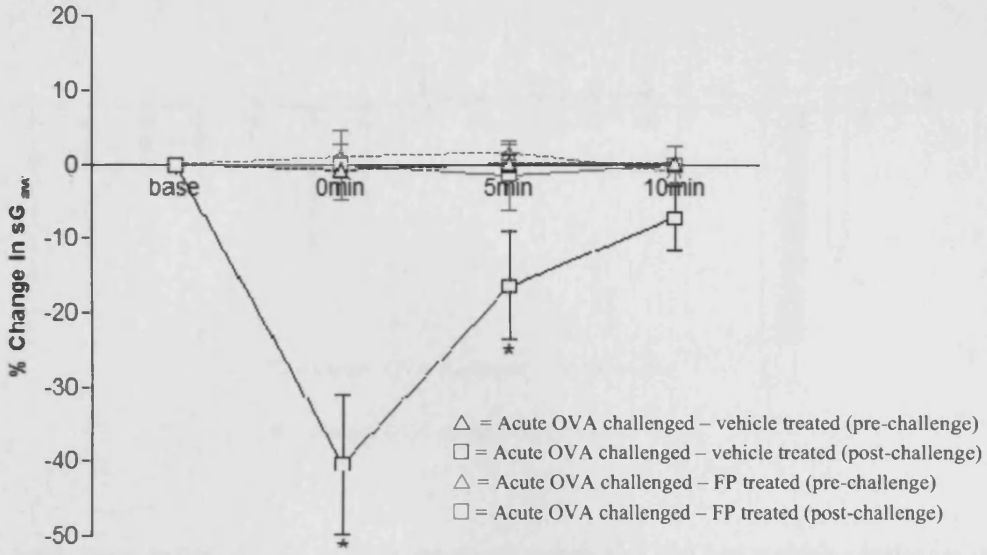


Figure 5.8 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs challenged with OVA (0.01%) and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post-OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

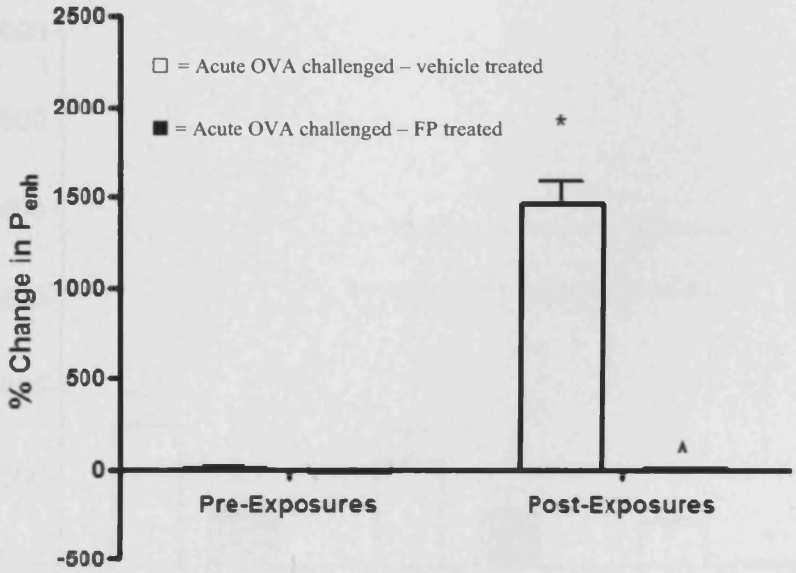


Figure 5.9 – Response of the airways to a 60 second intermittent dose of methacholine (30 mg/ml) in OVA sensitised mice challenged OVA (0.5%) and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post- OVA challenge. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from pre-challenge values of P_{enh};^significantly different from vehicle treated post-OVA. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

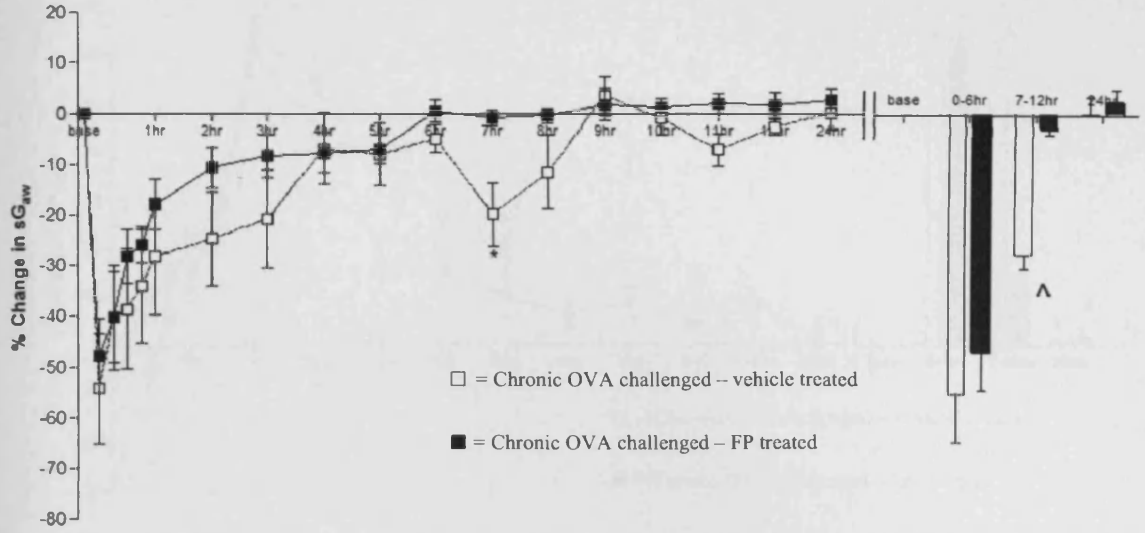


Figure 5.10 – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that had multiple challenges of OVA and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from FP treated guinea pigs; ^ significant difference between vehicle and FP treatment. Two-tailed T-test ($P<0.05$; n=6). Raw FP treatment sG_{aw} values (cm/H_20) – Base (0.66±0.03), 0-6 hours (0.35±0.06), 7-12 hours (0.64±0.02) and 24 hours (0.68±0.02). Raw vehicle treatment sG_{aw} values (cm/H_20) – Base (0.75±0.06), 0-6 hours (0.33±0.08), 7-12 hours (0.56±0.05) and 24 hours (0.75±0.05).

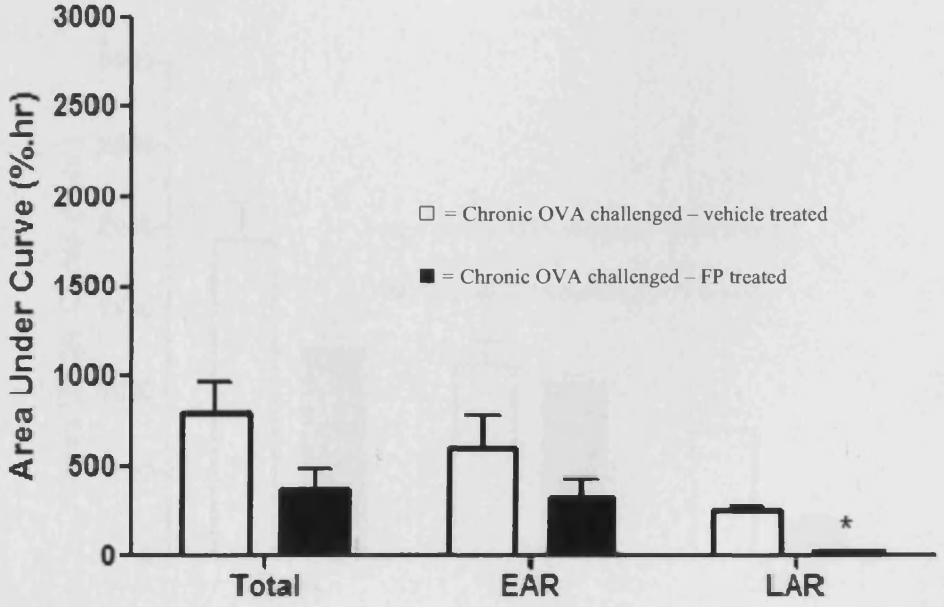


Figure 5.11 – Area under the curve analysis comparing OVA sensitised guinea pigs challenged with a chronic dosing of OVA and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treated guinea pigs. Two-tailed T-test ($P<0.05$; n=6).

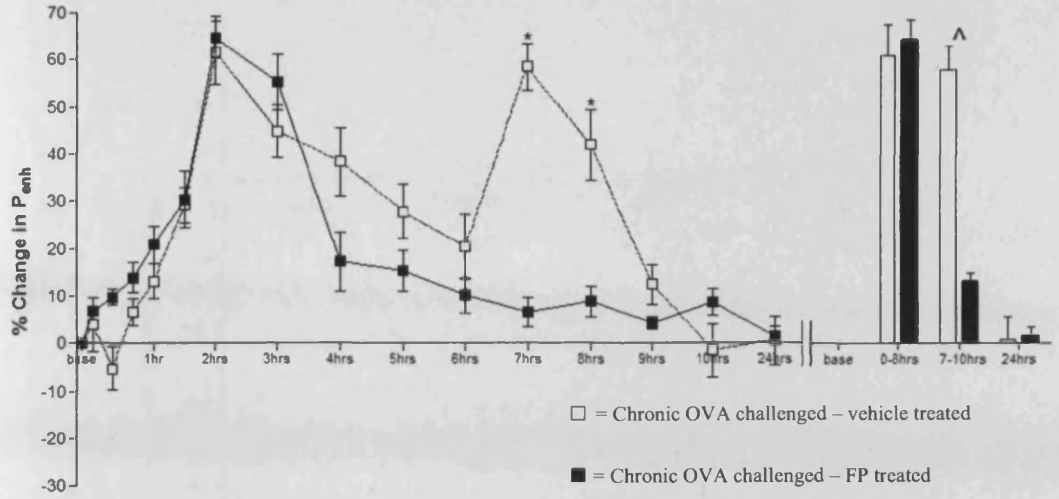


Figure 5.12 – Mean time-course values of P_{enh} in OVA sensitised mice that were challenged repeatedly with 2% OVA three times a week for six weeks and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different FP treatment; ^ significant difference between vehicle and FP treatment. Two-tailed T-test ($P<0.05$; n=6). Raw FP treatment P_{enh} values (AU) – Base (0.63±0.25), 0-6 hours (1.08±0.47), 7-12 hours (0.71±0.28) and 24 hours (0.64±0.25). Raw vehicle treatment P_{enh} values (AU) – Base (0.34±0.03), 0-6 hours (0.54±0.03), 7-12 hours (0.53±0.02) and 24 hours (0.34±0.02).

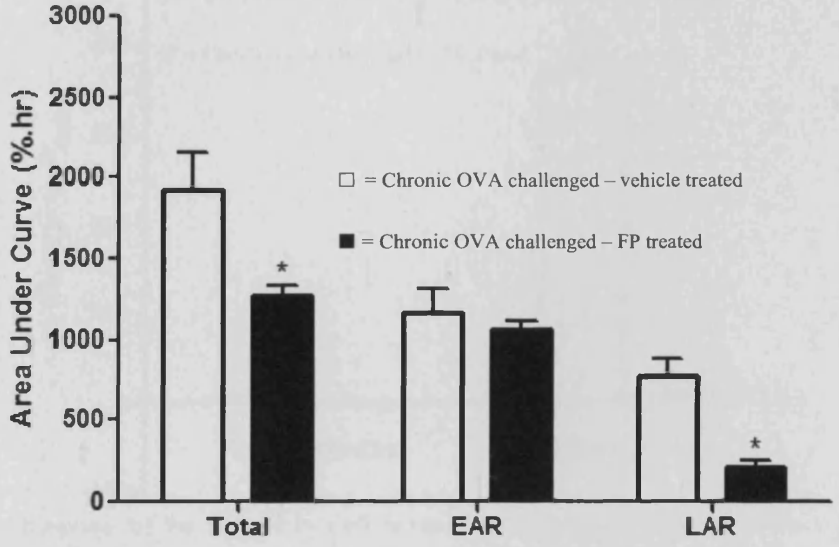


Figure 5.13 – Area under the curve analysis comparing OVA sensitised mice challenged with repeated doses of 2% OVA and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only positive peaks are considered, any peaks that have a negative value of P_{enh} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

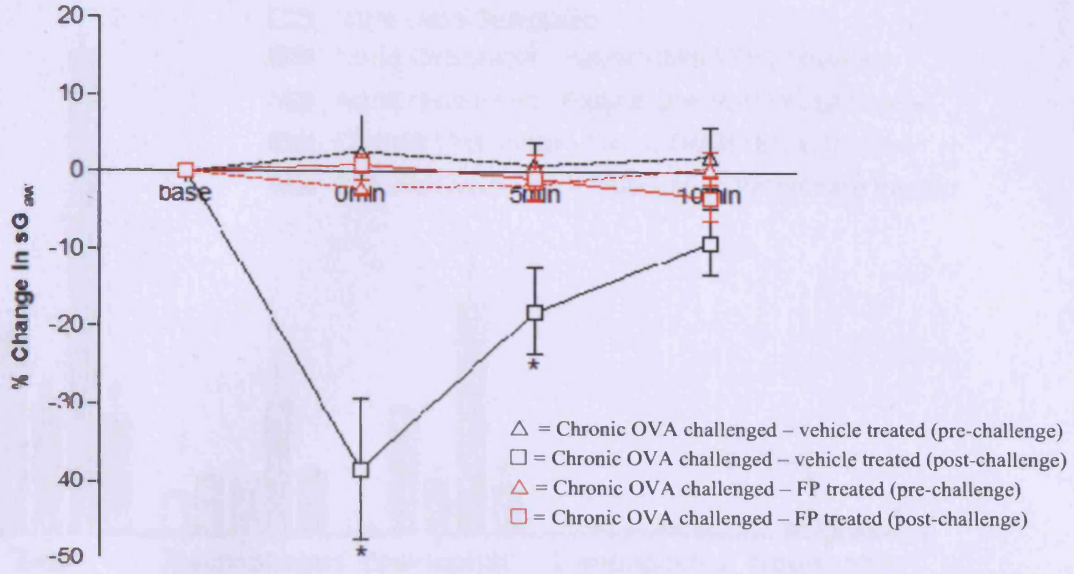


Figure 5.14 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs chronically challenged with OVA and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post-final OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

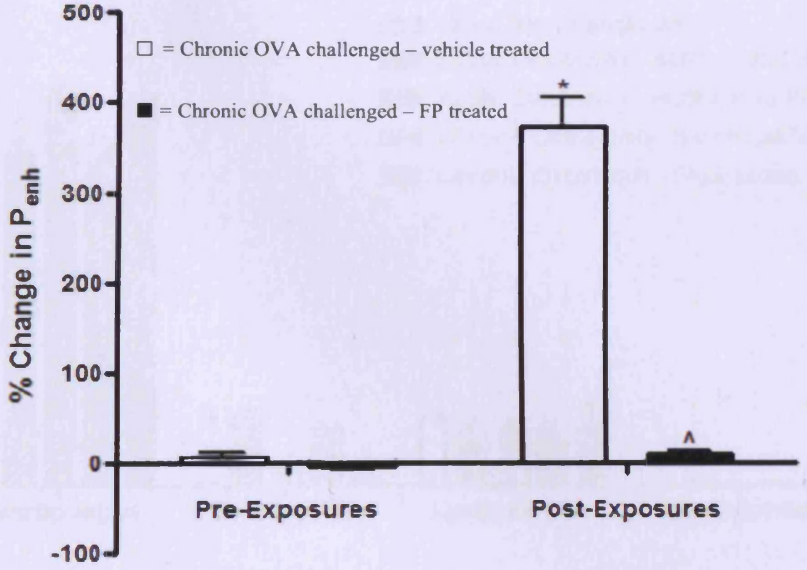


Figure 5.15. – Response of the airways to a 60 second intermittent dose of methacholine (10 mg/ml) in OVA sensitised mice challenged repeatedly over 7 weeks with OVA (2%) and treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before the first OVA challenge and again 24 hours post-final OVA challenge. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from pre-challenge values of P_{enh};^significantly different from vehicle treated post-OVA. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

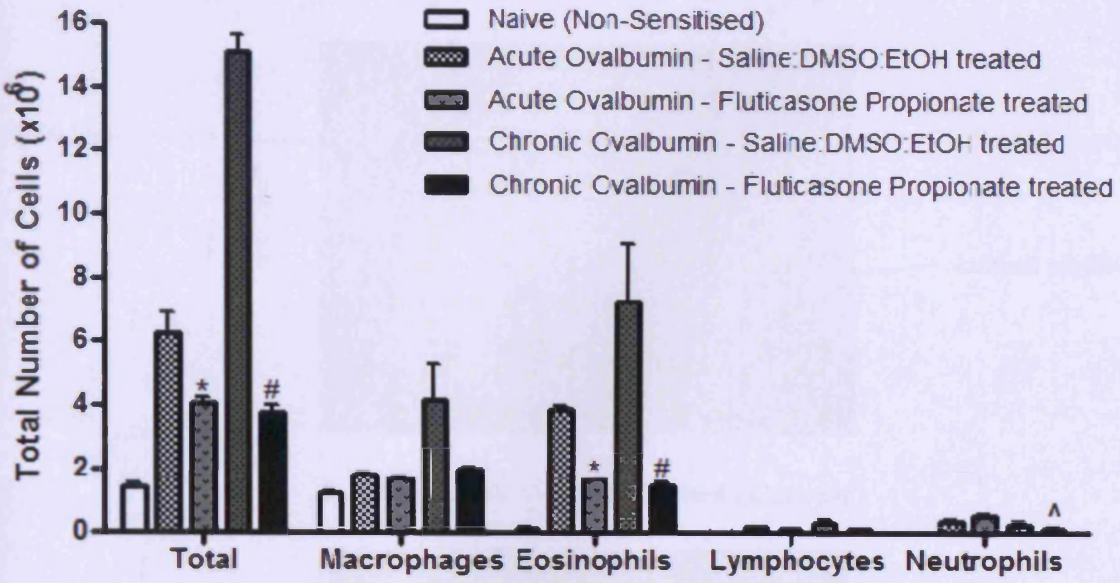


Figure 5.16 – The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised) and OVA challenged (acute and chronic) guinea pigs treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Results are expressed as mean±S.E.M. *significantly different from acute OVA – vehicle treated; #significantly different from chronic OVA – vehicle treated; ^significant different from acute OVA – FP treatment. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

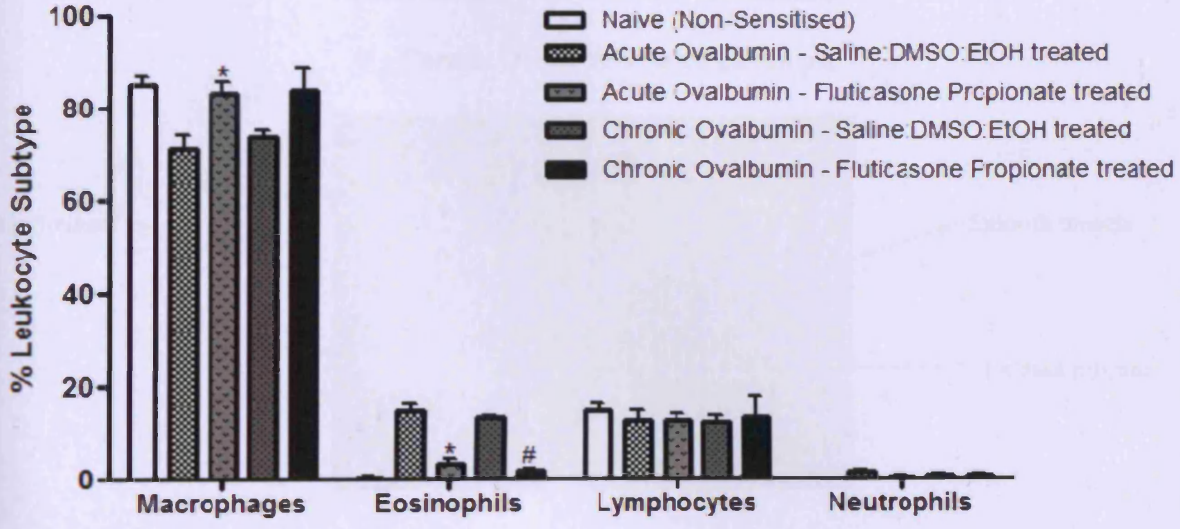
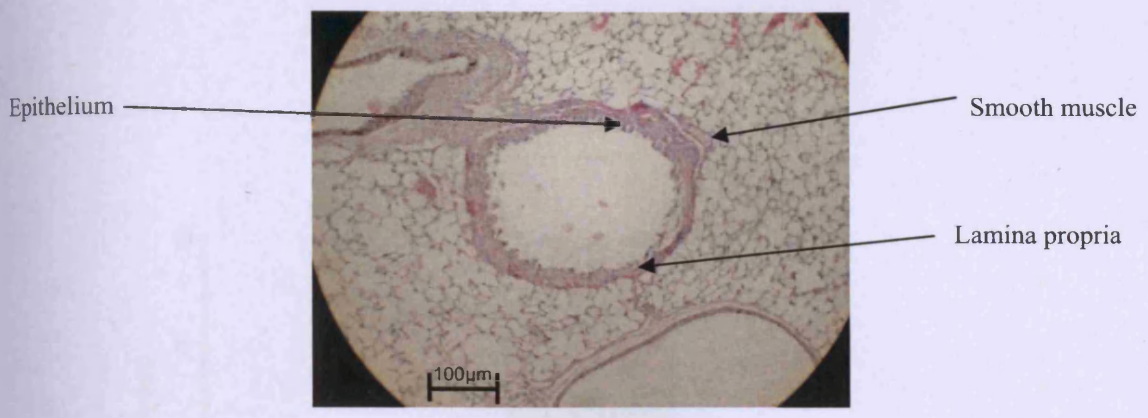
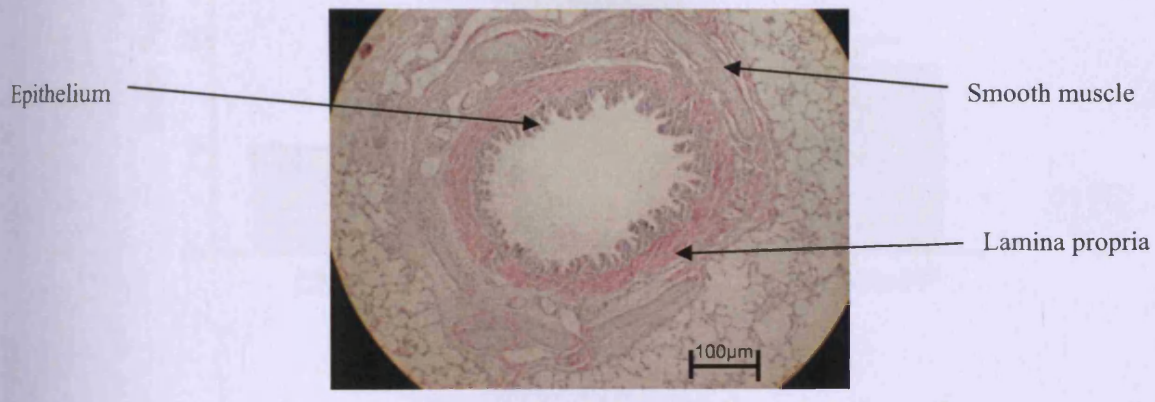


Figure 5.17 – The percentage of macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised) and OVA challenged (acute and chronic) mice treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge and OVA challenged (acute and chronic) mice. Results are expressed as mean±S.E.M. *significantly different from acute OVA – vehicle treated; #significantly different from chronic OVA – vehicle treated. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

A – Chronic saline challenged guinea pig



B – Chronic OVA – vehicle treated guinea pig



C – Chronic OVA – FP treated guinea pig

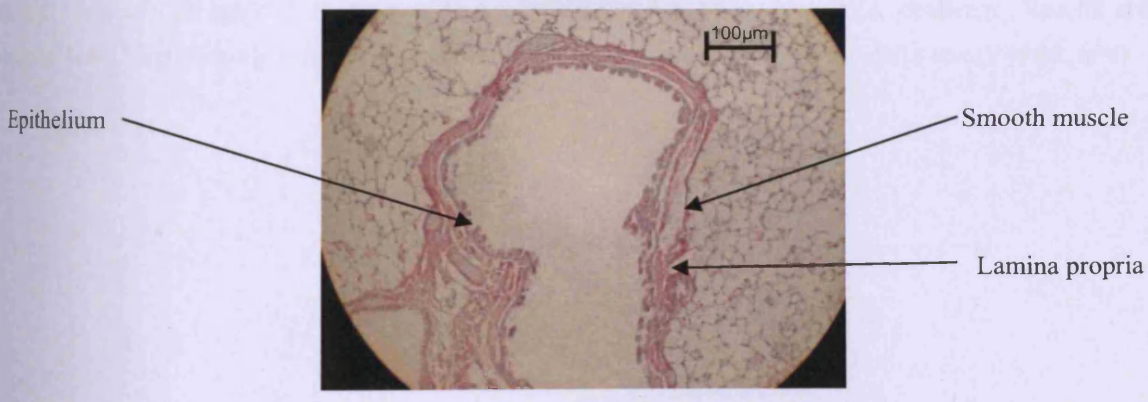


Figure 5.18 (A-C) – Bronchioles of chronic saline challenge, non-treated, guinea pigs and chronic OVA challenged guinea pigs treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with haematoxylin and eosin to display general morphology (1000x magnification).

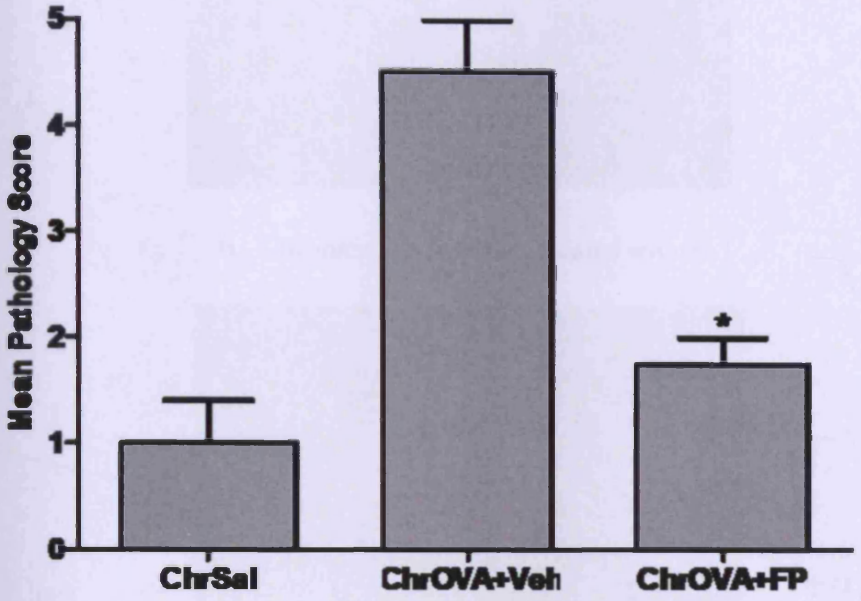
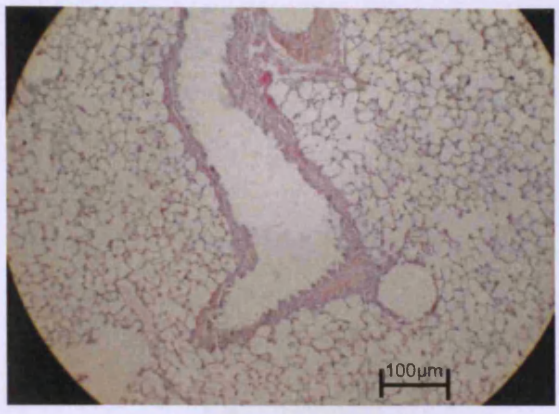
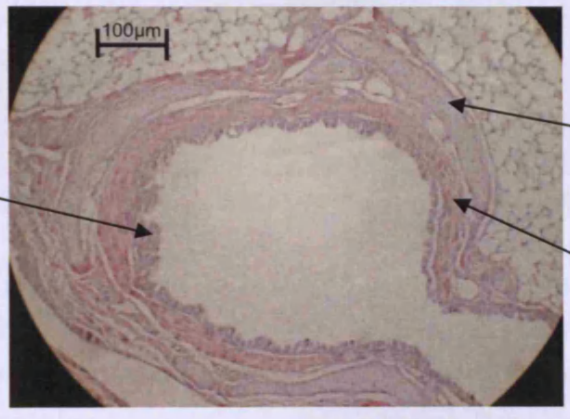


Figure 5.19 – The mean pathology score of cells found in the peri-bronchiolar space of naïve (non-sensitised) and OVA challenged (acute and chronic) guinea pigs treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Results are expressed as mean±S.E.M. *significantly different from chronic OVA – vehicle treated. Two-tailed t-test ($P<0.05$; $n=4$).

A - Chronic saline challenged mouse



B - Chronic OVA – vehicle treated mouse



C - Chronic OVA – FP treated mouse

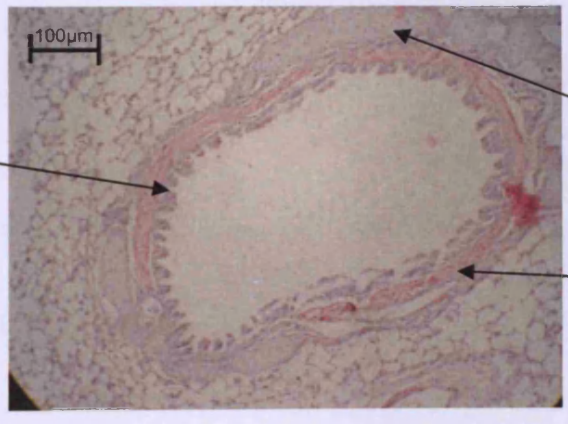


Figure 5.20 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with haematoxylin and eosin to display general morphology (1000x magnification).

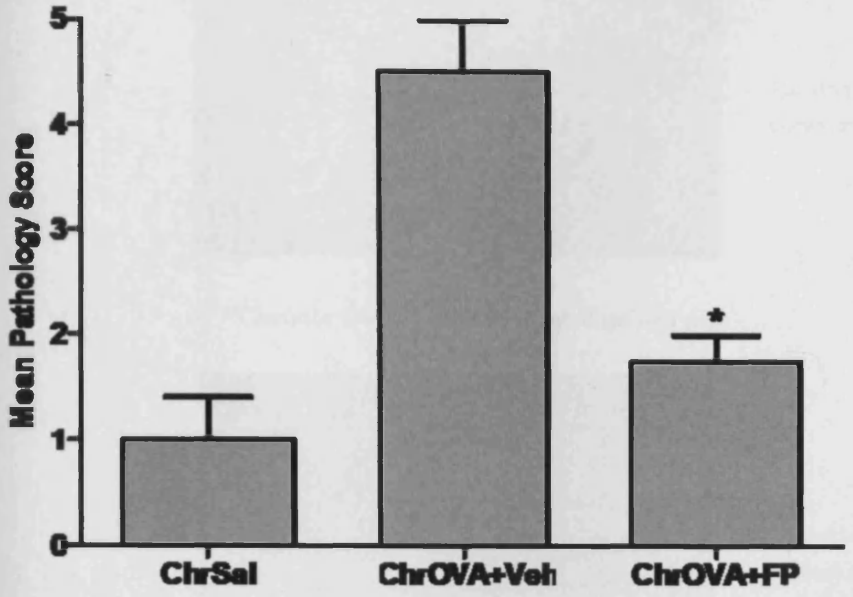
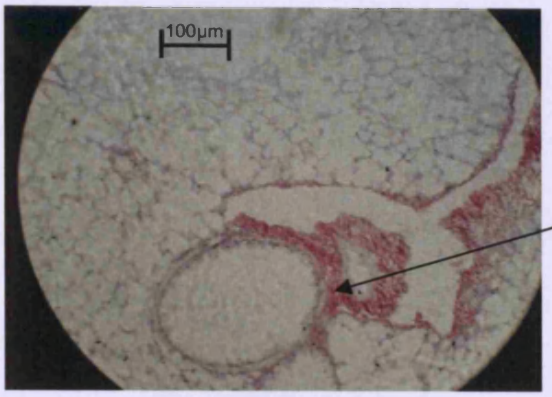
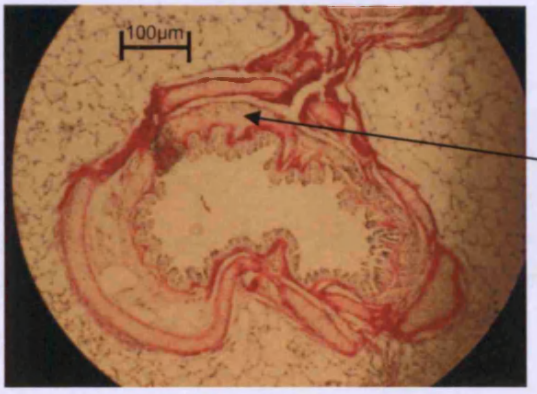


Figure 5.21 – The mean pathology score of cells found in the peri-bronchiolar space of naïve (non-sensitised) and OVA challenged (acute and chronic) mice treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.5 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Results are expressed as mean±S.E.M. *significantly different from chronic OVA – vehicle treated. Two-tailed t-test ($P<0.05$; $n=4$).

A - Chronic saline challenged guinea pig



B - Chronic OVA – vehicle treated guinea pig



C - Chronic OVA – FP treated guinea pig

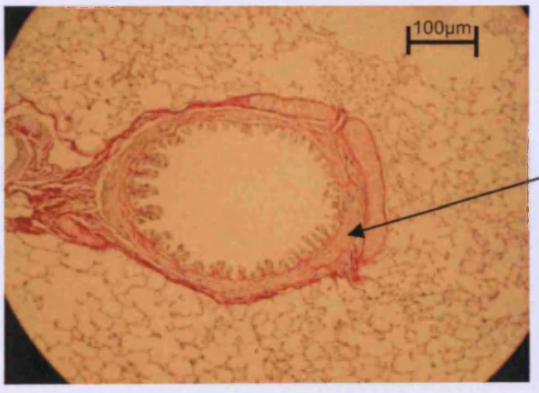


Figure 5.22 (A-C) – Bronchioles of chronic saline, non-treated, guinea pigs and chronic OVA challenged guinea pigs treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with picrosirius red to display collagen (1000x magnification).

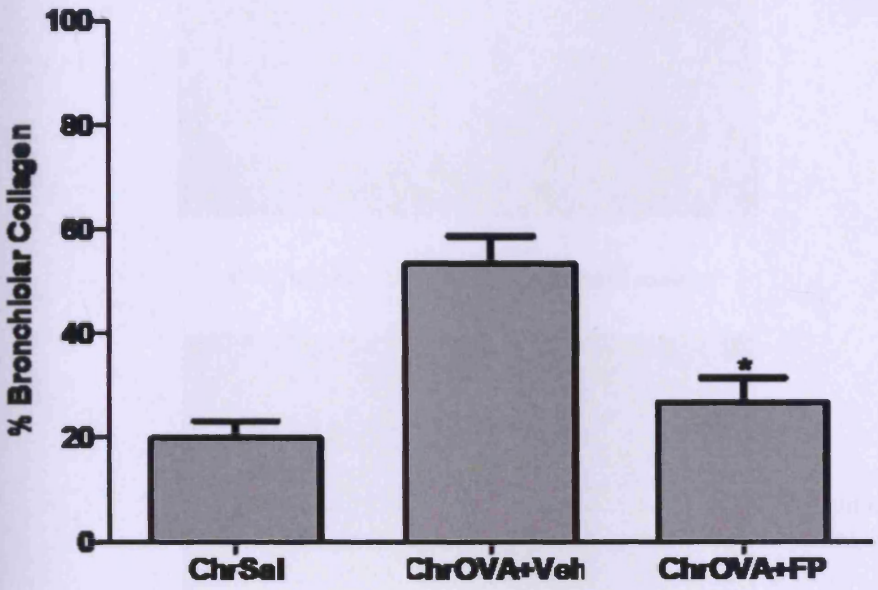
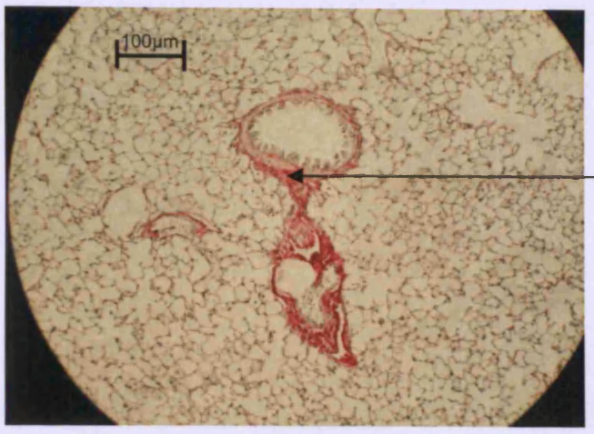
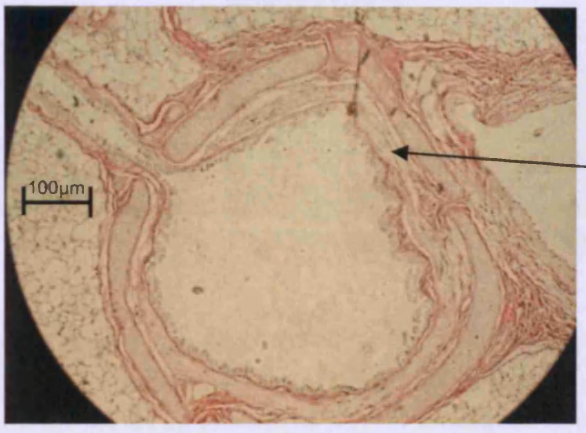


Figure 5.23 – Effect of a chronic OVA challenge and treatment with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on percentage bronchiolar collagen in guinea pigs. Results are expressed as mean±S.E.M. *significantly different from vehicle treatment. Two-tailed t-test ($P<0.05$; $n=4$).

A - Chronic saline challenged mouse



B - Chronic OVA – vehicle treated mouse



C - Chronic OVA – FP treated mouse

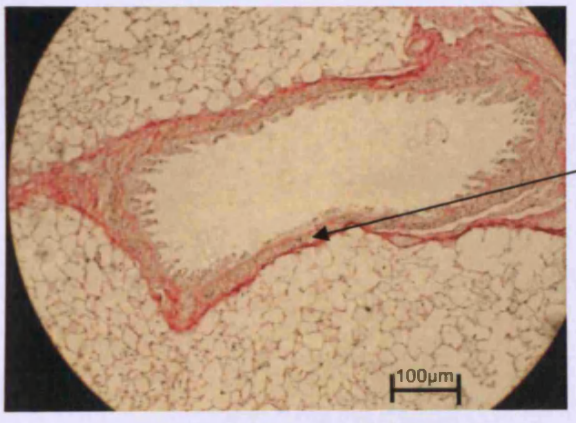


Figure 5.24 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with picrosirius red to display collagen (1000x magnification).

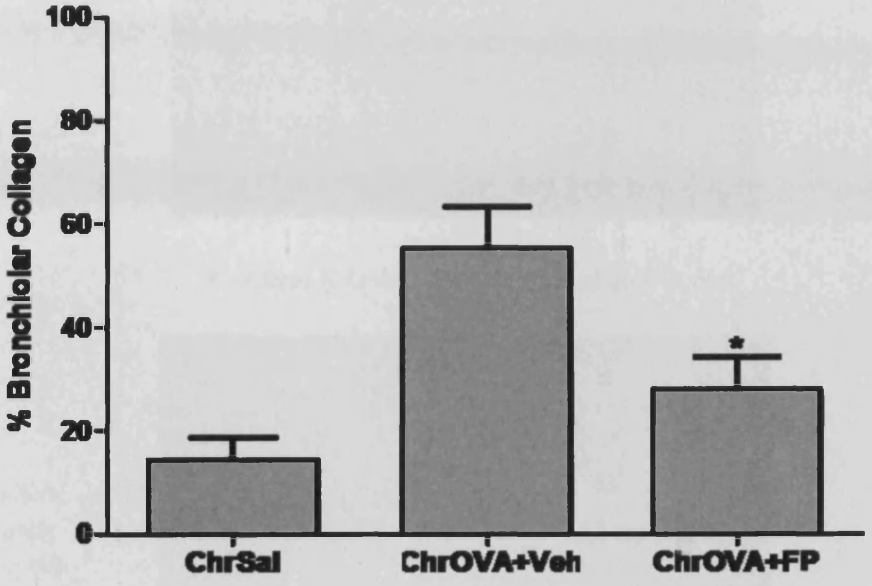
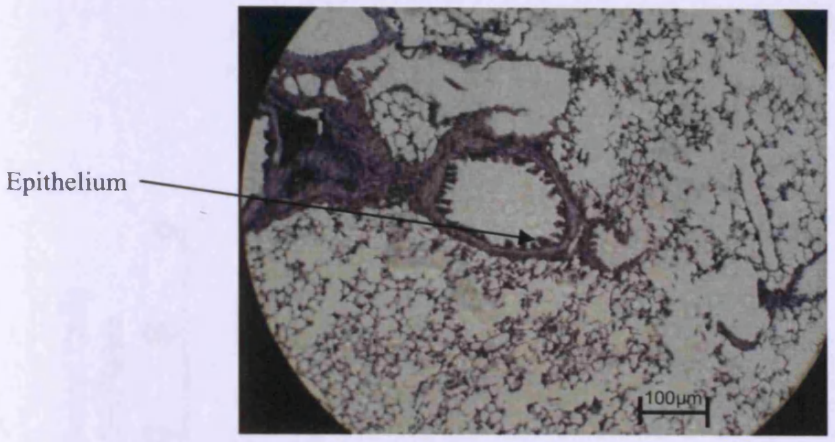
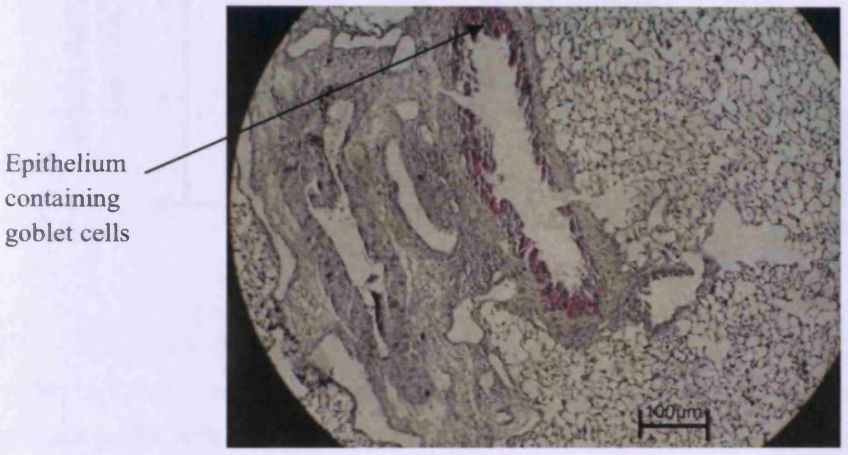


Figure 5.25 – Effect of a chronic OVA challenge and treatment with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on percentage bronchiolar collagen in mice. Results are expressed as mean±S.E.M. *significantly different from vehicle treatment. Two-tailed t-test ($P<0.05$; $n=4$).

A - Chronic saline challenged guinea pig



B - Chronic OVA – vehicle treated guinea pig



C - Chronic OVA – FP treated guinea pig

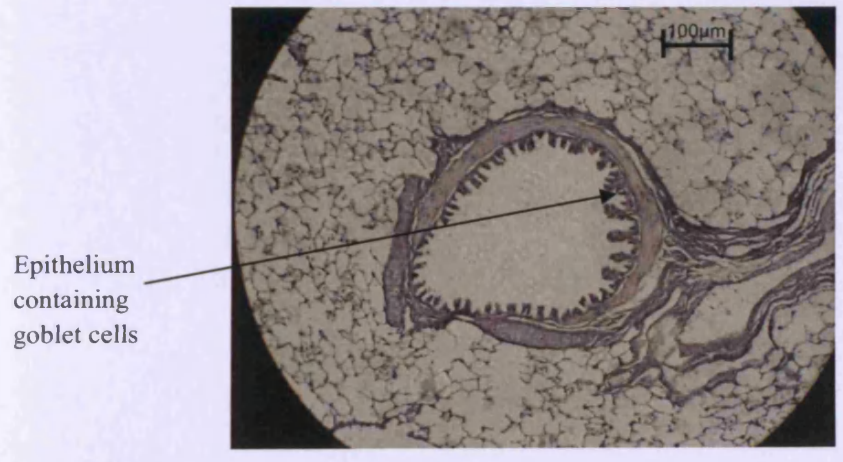


Figure 5.26 (A-C) – Bronchioles of chronic saline, non-treated, guinea pigs and chronic OVA challenged guinea pigs treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with alcian blue/periodic acid Schiff to display goblet cells

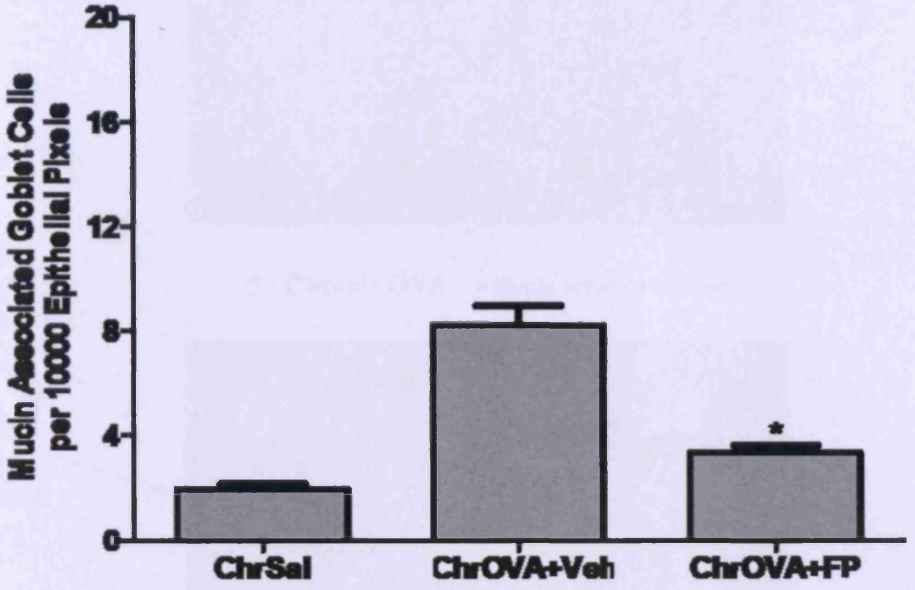
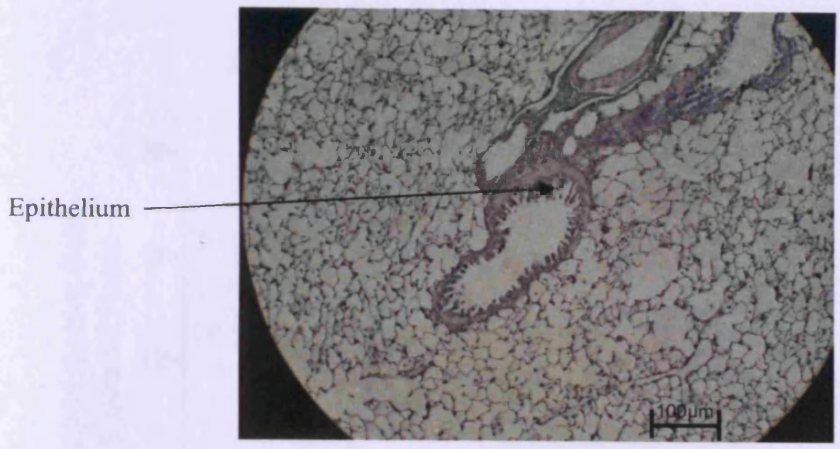
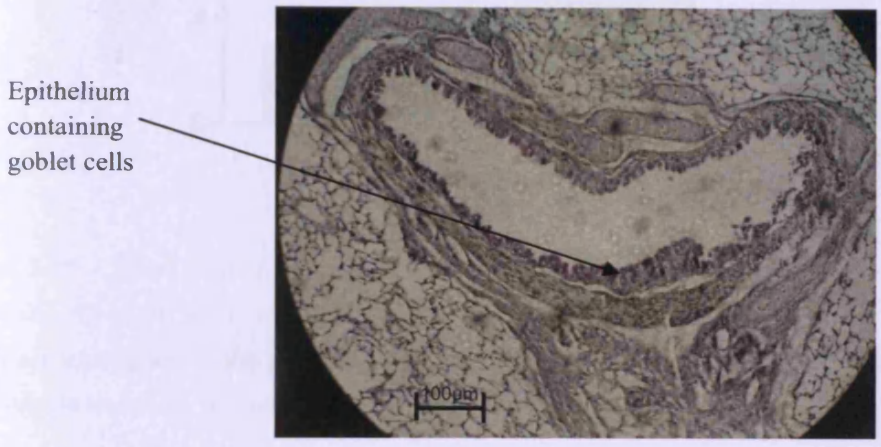


Figure 5.27 – Effect of a chronic OVA challenge and treatment with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on number of mucin associate goblet cells per 10,000 epithelial pixels. Results are expressed as mean±S.E.M. *significantly different from vehicle treatment. Two-tailed t-test ($P<0.05$; $n=4$).

A - Chronic saline challenged mouse



B - Chronic OVA – vehicle treated mouse



C - Chronic OVA – FP treated mouse

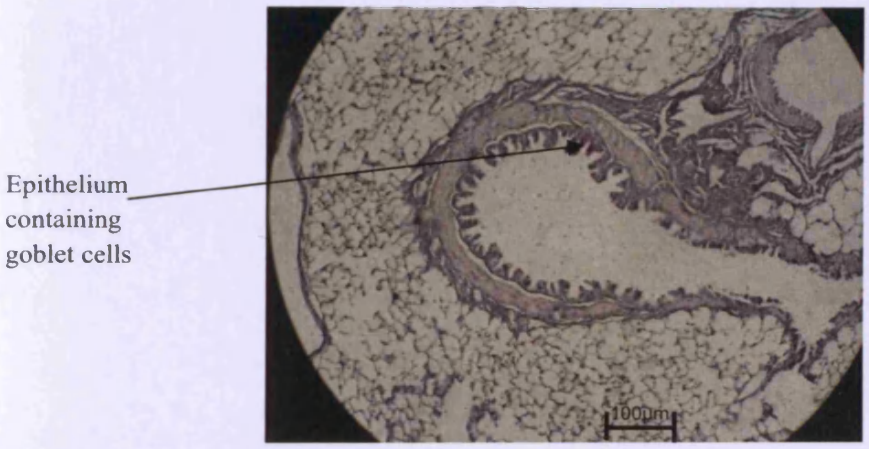


Figure 5.28 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice treated with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with alcian blue/periodic acid Schiff to display goblet cells (1000x magnification).

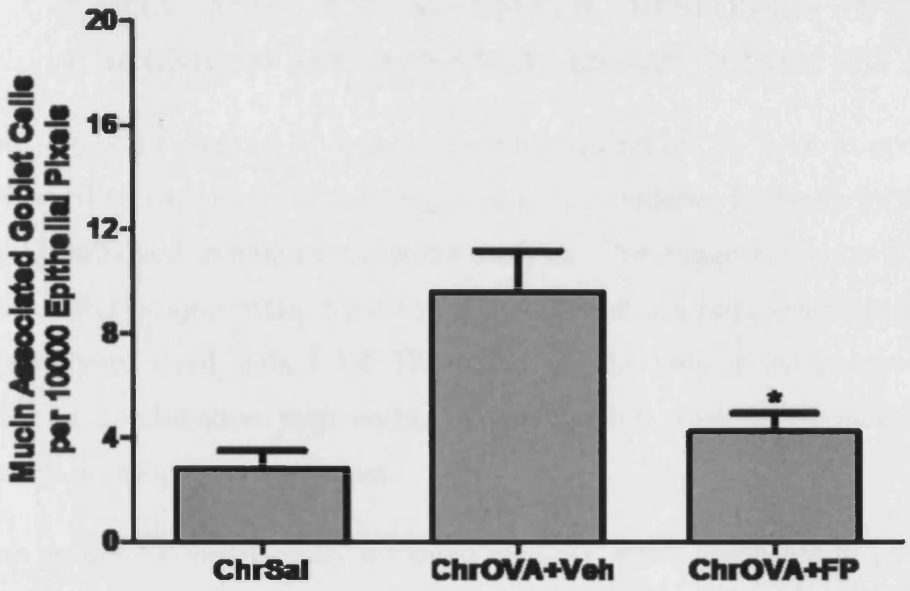


Figure 5.29 – Effect of a chronic OVA challenge and treatment with a 15 minute inhalation of saline/DMSO/Ethanol (40%/30%/30) or FP (0.51 mg/ml) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on number of mucin associate goblet cells per 10,000 epithelial pixels. Results are expressed as mean±S.E.M. *significantly different from vehicle treatment. Two-tailed t-test ($P<0.05$; $n=4$).

5.5 DISCUSSION

5.5.1 EARLY AND LATE ASTHMATIC RESPONSES IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIG AND MICE

As expected the corticosteroid FP had no inhibitory effect on the EAR in acute and chronic OVA challenged and sensitised guinea pigs and mice. Evidence is shown for this in both the time-course graphs and area under the curve analysis. This suggests that corticosteroids have little, if any, effect on preventing mast cell degranulation and reducing neutrophil number as these are heavily involved in the EAR. These findings also support the fact that administering a β_2 agonist in combination with corticosteroids would prove to be more effective than corticosteroids alone at treating asthma.

In the same groups FP significantly inhibited the LAR when compared to vehicle treatment. This follows what is seen in the literature in both animal models (Lawrence *et al.*, 1998) and humans (Palmqvist *et al.*, 2005). This finding is expected when considering the mechanism of action of corticosteroids. The fact that they are able to inhibit one of the main causes of the LAR, eosinophilia, and also inhibit gene transcription of inflammatory mediators such as leukotrienes and certain interleukins explains why they are so effective at preventing the second bronchoconstriction commonly observed in asthmatics. The acute and chronic guinea pig and mouse models showed the same response to FP treatment as humans suggesting they are possibly representative of asthma. However, the EAR and LAR are two of many features of asthma so the other parameters must be assessed before more conclusions can be drawn.

5.5.2 AIRWAY HYPERRESPONSIVENESS IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIG AND MICE

In acute and chronic OVA challenged guinea pigs that were given a histamine challenge after the final OVA challenge AHR was observed when vehicle was administered. However, in the groups administered with FP AHR was inhibited.

The finding that FP can inhibit AHR is no new phenomenon, studies by Toward and Broadley (2004) in guinea pigs have previously shown inhibition by dexamethasone. How

corticosteroids inhibit AHR is not so well known. The reason for the lack of knowledge of how AHR is inhibited is a result of the lack of understanding in the how AHR occurs in the first place. A strong correlation between exhaled nitric oxide, which is increased in asthmatics compared to non-asthmatics, and AHR has been found (Salome *et al.*, 1999). Eosinophils have also been implicated in AHR (Bradley *et al.*, 1991). An increase in eosinophil number would result in the increased release of major basic protein which would lead to epithelial damage, the removal of a diffusion barrier and ultimately AHR (Wardlaw *et al.*, 2002). The fact that corticosteroids have such a broad spectrum of anti-inflammatory effects means that they are effective drugs in treating several aspects of asthma, although sometimes the mechanism of how this is achieved is unclear.

5.5.3 CELLULAR INFLUX IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIG AND MICE

The total number of leukocytes found in the BAL fluid of acute and chronic OVA challenged guinea pigs that were treated with FP were reduced in number when compared to vehicle. This total decrease was mainly a result of the significant decrease in BAL eosinophil number that FP caused. To observe a decrease in eosinophil number is expected as corticosteroids cause increased eosinophil apoptosis and inhibit the recruitment of eosinophils to the airways by suppressing the production of chemotactic mediators and adhesion molecules (Barnes and Adcock 2003).

FP treatment in mice led to a decreased percentage of BAL eosinophils compared to vehicle. In acute OVA challenged mice treated with FP an increased percentage of macrophages was observed. However, this does not necessarily mean that the absolute number of macrophages has increased compared to vehicle. It is more likely to be a relative increase in proportion to eosinophils which were reduced. It is no surprise to find FP having such a prominent effect on eosinophils in both the guinea pig and mouse models as the LAR and AHR was inhibited in these models and eosinophils are believed to have an important role in both of these features.

5.5.4 LUNG HISTOLOGY IN CHRONIC OVALBUMIN CHALLENGED GUINEA PIG AND MICE

In chronic OVA challenged guinea pigs that were administered vehicle a clear build up of extracellular matrix proteins and smooth muscle can be observed compared to naïve guinea pigs. Qualitatively, it appears FP treatment reduces the level of extracellular matrix proteins and smooth muscle compared to vehicle treatment. This was also true in the mouse model. FP treatment was also able to significantly reduce the mean pathology score in both guinea pigs and mice. This is consistent with what was observed in the total BAL cell count for the guinea pigs and also suggests that although a greater percentage of macrophages were counted in mice there may be a lesser number present after FP treatment.

FP inhibited the levels of collagen found in the bronchiole of chronic OVA challenged guinea pigs and mice shown both quantifiably and quantitatively. A study in human asthmatics has previously shown that FP treatment significantly reduces sputum levels of procollagen type I C-terminal peptide (PICP), a marker of ongoing collagen type I deposition (Kai *et al.*, 2007). However, this study showed that levels were not reduced to control levels which is also true of the findings in the guinea pig and mouse models. There is some evidence that collagen is able to contribute to glucocorticoid resistance (Bonacci *et al.*, 2006) suggesting that corticosteroids may not always be effective at reducing collagen. As previously mentioned, corticosteroids are able to reduce mucous levels, therefore it is not surprising that a decrease in mucin associated goblet cells was observed after FP treatment in guinea pigs and mice compared to vehicle. OVA sensitised rats treated with FP have been shown to have reduced levels of goblet cells compared vehicle treated rats (Leung *et al.*, 2005).

5.5.5 GENERAL CONCLUSIONS

Inhaled corticosteroids are the most effective treatment for chronic asthma (Barnes, 2002). Corticosteroids are effective at inhibiting many inflammatory targets which is important for the control of asthma (Barnes and Adcock 2003). Attempts to find alternative treatments that are more specific have been unsuccessful (Barnes, 1999). The main drawback of using corticosteroids is that they are ineffective at preventing the EAR; this is overcome by administering β_2 agonists.

FP was effective at preventing the LAR and AHR in acute and chronic OVA challenged guinea pigs and mice. It was also able to reduce the number of eosinophils in the BAL fluid and partially reverse the airway remodelling observed in the chronic challenged models. All of these changes were observed after only three doses of FP. This suggests that it could be possible that long-term FP treatment could completely reverse airway remodelling. The fact that the results seen in the chronic OVA guinea pig and mouse model after FP are similar to those seen in humans strengthen the claim that they are effective models and would prove useful in a pre-clinical setting for the evaluation of new anti-asthma drugs.

Chapter 6

Effect of phosphodiesterase IV inhibitor treatment on acute and chronic models of asthma

6.1 INTRODUCTION

6.1.1 PHOSPHODIESTERASE IV INHIBITORS

Although corticosteroids are the most effective anti-inflammatory drugs used to treat asthma there are still cases of steroid insensitivity and systemic side-effects after long-term use. As a result there is a need for alternative therapies. Phosphodiesterase (PDE) 4 inhibitors have been in development as novel anti-inflammatory agents to treat asthma and chronic obstructive pulmonary disease (COPD) since the 1980s, however, to date none have been marketed (Spina, 2008). Despite the promising results in the treatment of asthma with PDE4 inhibitors in clinical trials and pre-clinical testing (Spina, 2008) they have major drawbacks when it comes to their side effects. Vomiting and nausea are a common symptom following PDE4 inhibitor treatment. If the dose is lowered to eradicate the side effects then it is less effective as an anti-inflammatory drug. Research in overcoming this problem is ongoing. As there are four known genes which code for PDE4 then a more selective approach could be the answer. Currently it seems that PDE4D plays an important role in inflammatory cell activation (Seybold *et al.*, 1998). Despite all of the problems with PDE4 inhibitors there is still plenty of optimism in the literature that using them to suppress inflammation is viable (Giembycz, 2008; Spina, 2008).

6.1.2 MECHANISM OF ACTION

PDE4 is an enzyme that acts specifically on cyclic adenosine monophosphate (cAMP) by hydrolysing cAMP into 5' adenosine monophosphate (5'AMP) (figure 6.1). cAMP is an intermediate, or second messenger, in the physiological responses to many hormones, neurotransmitters or drugs. It has been shown to play a key role in all types of cells involved in the pathophysiology of asthma (Nejman-Gryz *et al.*, 2006). cAMP is formed when a ligand binds to a transmembrane receptor causing the receptor to activate adenylyl cyclase usually via a G-protein mechanism. Adenylyl cyclase is an enzyme that breaks a bond in adenosine trisphosphate (ATP) leading to the formation of the cyclic phosphate cAMP. As cAMP is usually closely controlled by PDE4 the amount of cellular response is limited. However, if PDE4 is inhibited the intracellular level of cAMP increases causing a prolonged cellular response.

The mechanism of upregulation of PDE4 gene expression is unknown (Tang *et al.*, 2005), however it has been established that cAMP induces the expression of various PDE4 subtype genes (Conti & Jin, 1999). PDE4 can be activated by phosphorylation (Houslay, 2001), IL-2 (Crocker *et al.*, 2000), IL-3 (Ahmad *et al.*, 1999), IL-4 or interferon (IFN) γ (Li *et al.*, 1992).

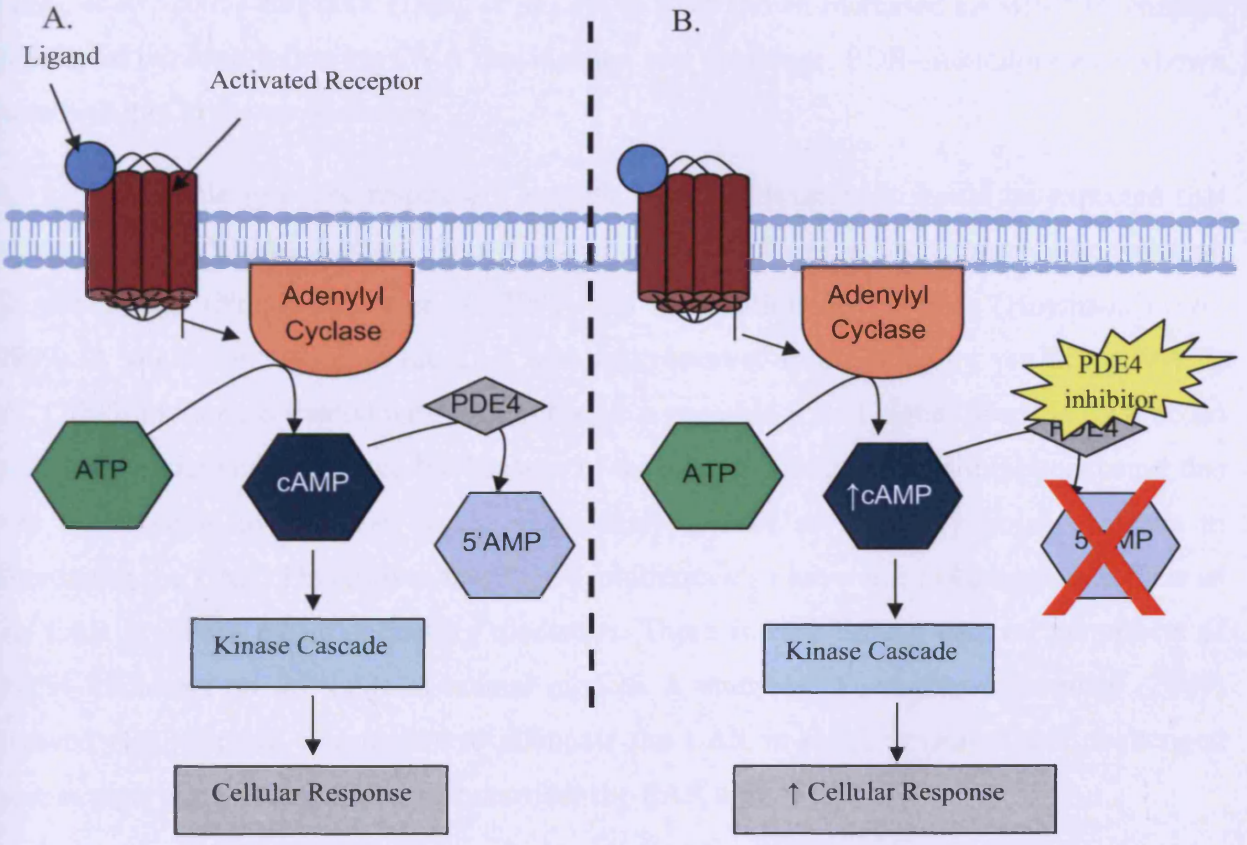


Figure 6.1 - Schematic diagram to demonstrate the effect that PDE4 inhibitors have. A ligand binds to a transmembrane receptor causing activation of adenylyl cyclase. This converts ATP into cAMP leading to a kinase cascade and ultimately the cellular response. PDE4 regulates this response and converts cAMP into 5'AMP (A.). PDE4 inhibitors are able to prevent the conversion of cAMP to 5'AMP. This leads to a greater cellular concentration of cAMP which in turn causes increased cellular response (B.). ATP = adenosine trisphosphate; cAMP = cyclic adenosine monophosphate; 5'AMP = 5'adenosine monophosphate; PDE4 = phosphodiesterase type IV.

The increased level of cAMP leads to several anti-inflammatory effects such as inhibition of the creation of reactive oxygen species in neutrophils and eosinophils amongst other roles (Nielson & Vestal, 1989). Smooth muscle relaxation in the respiratory system is also promoted by cAMP (Schramm & Grunstein, 1992). Clearly these effects would be beneficial in asthma making PDE4 an excellent target for therapeutics.

6.1.3 USE IN ASTHMA MODELS

PDE4 inhibitors have shown very promising effects in asthma. However, the effect of assessing PDE4 inhibitors in asthma has been confined to animals and clinical trial data as a result there is limited knowledge on long-term and large population effects. Studies in rats (Tang *et al.*, 2005) and mice (Deng *et al.*, 2006) have shown increased cAMP-PDE enzyme activity in the lung following OVA sensitisation and challenge. PDE4 inhibitors were shown to reduce this in the same studies.

As cAMP is able to cause respiratory smooth muscle relaxation it would be expected that treatment with PDE4 inhibitors should reduce the EAR. This has been shown with rolipram in guinea pigs (Nejman-Gryz *et al.*, 2006) and with roflumilast in mice (Hoymann *et al.*, 2009). A slight attenuation in the EAR was also observed in the study by van Schalkwyk *et al.*, (2005) in humans treated with roflumilast. It is possible with a higher dose the EAR could have been completely inhibited but because of the side-effects that PDE4 inhibitors cause this was not feasible to examine. In the same study roflumilast had very positive results in decreasing the LAR. This proves that PDE4 inhibitors also have anti-inflammatory effects as the LAR is driven by inflammatory mediators. There is very limited data on the effects of PDE4 inhibitors on the LAR in animal models. A study by Toward and Broadley (2004) showed that rolipram was unable to attenuate the LAR in OVA sensitised and challenged guinea pigs, many other studies just consider the EAR and AHR.

AHR is a common feature of asthma and as a result is one of the primary targets for asthma therapeutics. Several studies have shown PDE4 inhibitors are able to inhibit AHR in mice (Hoymann *et al.*, 2009), rats (Raeburn *et al.*, 1994), guinea pigs (Danahay & Broadley, 1998; Holbrook *et al.*, 1996; Howell *et al.*, 1993; Raeburn *et al.*, 1994; Santing *et al.*, 1995) and monkeys (Turner *et al.*, 1994). Roflumilast was shown to be effective at reducing AHR in humans following a histamine challenge (Louw *et al.*, 2007) further supporting the potential PDE4 inhibitors have as anti-inflammatory drugs to treat asthma. As eosinophils are believed to have a role in causing AHR (Wardlaw *et al.*, 2002) it is no surprise to find evidence that PDE4 inhibitors can reduce eosinophil BAL fluid number (Deng *et al.*, 2006). Kita *et al.*, (2008) have shown that in OVA sensitised and exposed mice a PDE4 and PDE1 inhibitor, KF19514, is able to reduce the number of eosinophils found in the lamina propria. Guinea pigs treated with rolipram also show a reduced influx of eosinophils (Nejman-Gryz *et al.*,

2006). This is also the case in mice treated with roflumilast (Hoymann *et al.*, 2009). Reductions in neutrophil numbers have also been associated with PDE4 inhibitors (Deng *et al.*, 2006).

Although the ability to reduce the inflammation associated with an asthmatic episode is extremely important when analysing a potential therapeutic, the ability of the drug to prevent or reverse airway remodelling caused by long-term inflammation is equally as important. Roflumilast can inhibit matrix protein deposition in human smooth muscle cells (Burgess *et al.*, 2006) and it can inhibit the increase of epidermal growth factor induced MUC5AC expression in human epithelial cells suggesting that PDE4 inhibitors may be able to combat airway remodelling. On top of this cyclic AMP may have an additional role in modulating airway smooth muscle hypertrophy and hyperplasia, which are common morphologic features of chronic asthma (Munakata, 2006).

6.2 AIMS AND OBJECTIVES

6.2.1 AIMS

The aim of this chapter was to assess the effect of the PDE4 inhibitor, roflumilast, compared to vehicle treatment on acute and chronic OVA challenged guinea pigs and mice. The parameters investigated were early and late phase bronchoconstriction, airway hyperresponsiveness, cellular influx and lung histology. This chapter will give an indication of how effective this relatively novel compound is against both acute and chronic guinea pig and mouse models that represent human asthma.

6.2.2 OBJECTIVES

- To investigate the effect of roflumilast treatment on acute OVA exposed guinea pigs and mice by measuring lung function, response to histamine and cellular influx compared to vehicle treatment.
- To investigate the effect that roflumilast treatment has on chronic OVA exposed guinea pigs and mice by measuring lung function, response to histamine and cellular influx compared to vehicle treatment.
- To assess bronchiole structure, collagen deposition and levels of goblet cell hyperplasia in roflumilast-treated chronic OVA challenged guinea pigs and mice compared to vehicle.

6.3 METHODS

Chapter 2 details the complete methods used for this chapter; the following is a brief overview. For each guinea pig study group in this chapter six male Dunkin-Hartley guinea pigs weighing 200-250 g were used. For the mouse studies, male BALB/c mice weighing 20-25g were used.

6.3.1 SENSITISATION

Guinea pigs and mice were sensitised by an intra-peritoneal, bilateral injection of a suspension containing OVA (100 μg) and $Al(OH)_3$ (100 mg) in PBS on days 1 and 5. The guinea pigs were administered 1 ml and the mice 0.25 ml.

6.3.2 OVALBUMIN CHALLENGES

Guinea pig OVA challenges for both acute and chronic groups were carried out in a stainless steel exposure chamber (40 cm diameter, 15 cm height) with a Wright nebuliser attached. The nebuliser delivered the OVA or saline at an air pressure of 20 lb p.s.i. and at a rate of 0.3 ml/min. The same nebuliser was used for mice OVA exposures but the chamber used was a Perspex box (38 cm length; 20 cm width; 20 cm height). If the animals appeared to be distressed at any stage during the exposure they were immediately removed and exposure was considered complete

6.3.2.1 ACUTE PROTOCOL – GUINEA PIGS

An hour exposure to OVA (0.01%) was used as the acute challenge in guinea pigs, this occurred on day 15 (14 days subsequent to the first sensitisation injection).

6.3.2.2 CHRONIC PROTOCOL – GUINEA PIGS

On day 15 the guinea pigs following the chronic protocol were challenged with OVA (0.01%) for an hour. Following this on days 17-29, every 48 hours, they were challenged

with OVA (0.1%). This challenge was preceded with an injection of mepyramine (30 mg/kg). Finally on day 31 an hour long exposure to OVA (0.1%), minus mepyramine, occurred.

6.3.2.3 ACUTE PROTOCOL – MICE

In order to provoke an acute response to OVA the mice were challenged with two OVA (0.5%) exposures lasting an hour each separated by a 4 hour gap on day 15.

6.3.2.4 CHRONIC PROTOCOL – MICE

For the chronic group the mice were challenged with OVA (2%) three times a week for six weeks for 30 minutes (18 challenges). On the first day of the seventh week (day 57), an hour long challenge occurred.

6.3.3 LUNG FUNCTION MEASUREMENTS

Lung function in guinea pigs was measured using whole-body plethysmography to obtain a value of sG_{aw}. Values were recorded at baseline, then 0, 15, 30, 45 and 60 minutes following the final exposure, then hourly until 12 hours with a final recording at 24 hours. All values were compared to baseline. Lung function in mice was recorded using an unrestrained chamber (Buxco) in order to obtain values of P_{enh}. Again a baseline value was recorded and all other readings were compared to this. Following the final exposure P_{enh} was recorded at 0, 20, 40, 60, 90 and 120 minutes and then hourly until 10 hours with a final reading at 19 hours for the acute protocol or 24 hours for the chronic protocol.

6.3.4 AIRWAY HYPERRESPONSIVENESS MEASUREMENTS

6.3.4.1 GUINEA PIGS

In order to determine whether AHR is present values of sG_{aw} were again used. Prior to OVA or saline exposures (day 14), the guinea pigs were challenged with histamine (1 mMol, 20 seconds) and recordings were taken 0, 5 and 10 minutes following the challenge. These values were compared to baseline values taken before histamine was administered. The same process was repeated immediately following the 24 hour reading taken after the final OVA or saline exposure.

6.3.4.2 MICE

To investigate airway responsiveness in mice 10 mg/ml methacholine was used in the acute challenged group and 30 mg/ml in the chronic challenged group. As with the guinea pigs this process of challenging occurred pre- and post-OVA or saline challenges. To obtain values in mice P_{enh} was used, firstly 2 minutes of baseline values were recorded and averaged. Following this a 1 minute intermittent methacholine challenge occurred and then P_{enh} values were recorded for 5 minutes and averaged. Percentage change from baseline was then recorded.

6.3.5 TOTAL AND DIFFERENTIAL CELL COUNTS

6.3.5.1 GUINEA PIGS

Guinea pigs were administered a lethal dose of sodium pentobarbitone following the final AHR test. The trachea was then cannulated and the lungs removed. Saline was instilled into the lungs then removed three minutes later. The fluid removed was used to carry out a total cell count and then was subsequently centrifuged and stained so a differential count could be carried out.

6.3.5.2 MICE

The methods for removing the BAL fluid and differential cell count were the same as in the guinea pigs. However, a total cell count was not carried out on mice as the BAL fluid was heavily tainted with blood as a result of damage to the trachea when removing the fluid.

6.3.6 DRUG ADMINISTRATION

Guinea pigs and mice were administered with 1 mg/kg roflumilast dissolved in methyl cellulose and delivered orally via a gavage needle. Administration occurred 24 hours and 0.5 hours before the final OVA or saline challenge and 6 hours post challenge.

6.3.7 HISTOLOGICAL ANALYSIS OF GUINEA PIG AND MOUSE LUNGS

3-5 mm thick portions of the left superior lobe sliced 1 mm below the bronchus were processed into wax blocks. Sections (6μm) were then sliced using a microtome and fixed onto a glass slide. The slices were stained using either the haematoxylin and eosin, picrosirius red or AB/PAS protocols to show general morphology, collagen deposition and mucin associated goblet cells respectively. The peribronchiole (surrounding the small airways) region of sections stained with haematoxylin and eosin were scored for numbers of inflammatory cells. The parameters for scoring were on a semi-quantitative scale as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5) (Barends *et al.*, 2004). The area of bronchiolar collagen and number of mucin associated goblet cells were calculated as a percentage using ImageJ software.

6.4 RESULTS

6.4.1 LUNG FUNCTION OF ACUTE CHALLENGED GUINEA PIGS AND MICE

6.4.1.1 GUINEA PIGS

Figure 6.2 represents sensitised guinea pigs challenged with a single OVA exposure that were treated with roflumilast or vehicle. The vehicle treated group had an immediate bronchoconstriction (-66.1±3.0%) which resolved around 4 hours. The roflumilast treated group also had an immediate bronchoconstriction (-36.3±3.4%) however, this was significantly smaller for the first 30 minutes of recording. At 7 hours the vehicle treated group had a second bronchoconstriction (-35.9±6.5%) which was not the case in the roflumilast treated group. When comparing the maximum bronchoconstriction values roflumilast significantly inhibits at the EAR time point compared to the vehicle group (-36.6±3.4% compared to -66.7±2.6% respectively) and at the LAR time point (-4.2±1.0% compared to -35.9±6.5% respectively).

Figure 6.3 shows that when comparing area under the curve analysis the roflumilast treated group have a significantly smaller area than the vehicle treated group throughout the entire test period (186.1±37.0%.hr compared to 792.6±193.2%.hr respectively), at the early phase (172.0±33.3%.hr compared to 403.9±88.3%.hr respectively) and at the late phase (22.4±7.3%.hr compared to 325.2±124.2%.hr respectively).

6.4.1.2 MICE

In figure 6.4 the effect of vehicle and roflumilast treatment on the lung function of acute OVA challenged mice is shown. After vehicle treatment, OVA showed an early phase peak increase in P_{enh} at 2 hours (63.9±2.5%). Roflumilast significantly reduced the OVA response at this time point (32.4±2.7%) and also at 90 minutes and 3 hours. At the late phase time points, vehicle treatment was unable to prevent a bronchoconstriction at 7 hours (59.9±3.1%). However, roflumilast significantly reduced this response (5.7±4.5%) and the significant reduction was still present after 8 hours. When comparing the effect that roflumilast and vehicle had on maximum bronchoconstriction values in acute OVA challenged mice a significant reduction at the EAR time point was seen in roflumilast treated mice compared to

vehicle treated mice (36.3±1.8% compared to 63.9±2.5% respectively). This was also true at the LAR time point (14.9±2.2% compared to 60.7±2.8% respectively).

Area under the curve analysis is shown in figure 6.5. Although no significant difference is seen throughout the total curve between the drug and vehicle treatments, roflumilast caused a significantly reduced area under the curve compared to vehicle at both the EAR time point (652.0±61.9%.hr compared to 972.0±77.1%.hr respectively) and LAR time point (224.3±44.0%.hr compared to 659.2±115.9%.hr respectively).

6.4.2 AIRWAY HYPERRESPONSIVENESS OF ACUTE CHALLENGED GUINEA PIGS AND MICE

6.4.2.1 GUINEA PIGS

Figure 6.6 represents the effect of challenging an acute OVA guinea pig with histamine. In the vehicle treated group a significant bronchoconstriction was observed immediately following the histamine in the post-challenge compared to pre-challenge (-21.1±3.6% compared to 0.9±1.9% respectively). This bronchoconstriction persisted for 5 minutes after the histamine (-15.2±3.1% compared to -0.1±0.9% respectively). Roflumilast treated guinea pigs showed no post-challenge response to histamine.

6.4.2.2 MICE

The effect of roflumilast and vehicle treatment on AHR in mice is shown in figure 6.7. Vehicle treated mice had a massive increase to 30 mg/ml methacholine post-OVA challenges compared to pre-OVA challenges (1335.6±97.6% compared to -6.9±2.6% respectively). Roflumilast treatment significantly inhibited the AHR observed in the vehicle treated group (13.8±3.1% compared to 1335.6±97.6% respectively).

6.4.3 LUNG FUNCTION OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

6.4.3.1 GUINEA PIGS

The effect of roflumilast and vehicle treatment on the lung function response to the final OVA challenge of chronic challenged OVA guinea pigs is shown in figure 6.8. In the vehicle treated group an immediate bronchoconstriction was observed (-70.0±2.0%) which recovered at around 6 hours. Roflumilast treatment was unable to prevent an immediate bronchoconstriction (-44.8±10.2%) however, it was significantly less than in the vehicle group up to 3 hours. Roflumilast treatment was also able to reduce the bronchoconstriction usually seen after 7 hours, however, the vehicle group still had this reduction in sG_{aw} (-31.2±4.9%). When comparing the effect of roflumilast treatment to vehicle treatment on maximum bronchoconstriction values, a significant reduction was observed at the early phase time point (-44.8±10.2% compared to -70.4±1.1% respectively) and late phase time point (-4.3±0.7% compared to -31.2±4.9%).

6.4.3.2 MICE

If the lung function curve is analysed using area under the curve, as shown in figure 6.9, then roflumilast treatment significantly reduced the total area compared to vehicle treatment (305.5±41.4%.hr compared to 1094.6±136.5%.hr respectively). This was also true during the EAR (268.8±55.1%.hr compared to 831.0±28.1%.hr respectively) and LAR (53.0±17.2%.hr compared to 305.5±101.6%.hr respectively).

Figure 6.10 shows that chronic OVA challenged mice treated with vehicle had an early phase bronchoconstriction at 2 hours (65.7±3.3%). Roflumilast treatment was able to significantly reduce this though a response was still observed (32.5±3.5%). A late phase bronchoconstriction was seen in the vehicle treated group at 7 hours (61.6±2.0%). Roflumilast was also able to reduce this response (12.0±1.8%) and still had a significantly smaller response after 8 hours. When assessing maximum bronchoconstriction values, roflumilast treatment significantly reduced values of P_{enh} compared with vehicle treatment (37.0±2.5% compared to 65.7±3.3% respectively) and this is also the case for the LAR (13.1±1.9% compared to 61.6±2.0% respectively).

Area under the curve analysis, as shown in figure 6.11, highlights the area reduction that roflumilast treatment has compared to vehicle treatment throughout the curve (1048.9±76.7%.hr compared to 1546.8±135.8%.hr respectively), during the EAR (672.4±36.9%.hr compared to 942.9±79.3%.hr respectively) and the LAR (277.1±49.9%.hr compared to 703.9±62.9%.hr respectively).

6.4.4 AIRWAY HYPERRESPONSIVENESS OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

6.4.4.1 GUINEA PIGS

Figure 6.12 represents the effect of a histamine challenge on roflumilast or vehicle treated, chronic OVA exposed guinea pigs. Roflumilast inhibited AHR as no significant difference in sG_{aw} was observed between pre- and post-OVA exposure after histamine. However, in the vehicle treated group a significant bronchoconstriction was observed post-OVA exposure compared to pre-OVA exposure immediately following a histamine challenge (-33.8±5.9% compared to -1.3±2.0% respectively) and this bronchoconstriction was still present 5 minutes after the histamine challenge (-22.1±1.9% compared to 0.5±1.2% respectively).

6.4.4.2 MICE

The response of chronic OVA challenged, roflumilast or vehicle treated mice to 10 mg/ml methacholine is shown in figure 6.13. Vehicle treated mice clearly demonstrated AHR post-OVA challenge as a significant bronchoconstriction was observed compared to pre-OVA challenge (387.5±12.6% compared to -2.2±5.5% respectively). Treatment with roflumilast inhibited the AHR observed post-OVA challenge in the vehicle treated mice (28.4±5.0% compared to 387.5±12.6% respectively).

6.4.5 CELL COUNTS IN ACUTE AND CHRONIC CHALLENGED GUINEA PIGS AND MICE

6.4.5.1 GUINEA PIGS

Figure 6.14 highlights the effects of roflumilast and vehicle treatment on cellular BAL fluid levels in acute and chronic challenged guinea pigs. In the acute OVA challenged group

roflumilast significantly decreases the total number of cells compared to vehicle treatment ($3.4\pm0.3x10^6$ compared to $6.7\pm0.5x10^6$ respectively). This is also true for levels of eosinophils ($1.3\pm0.3x10^6$ compared to $4.9\pm0.2x10^6$ respectively). In the chronic OVA challenged group roflumilast also decreases the total number of cells compared to vehicle ($3.8\pm0.1x10^6$ compared to $10.9\pm0.3x10^6$ respectively) and eosinophil number ($1.4\pm0.1x10^6$ compared to $5.7\pm0.3x10^6$ respectively). However, in the chronic group roflumilast also decreases the number of macrophages ($1.6\pm0.1x10^6$ compared to $4.7\pm0.3x10^6$ respectively) and neutrophils ($0.1\pm0.03x10^6$ compared to $0.3\pm0.4x10^6$ respectively) compared to vehicle. The levels were restored to the same levels as observed in acute OVA roflumilast treated guinea pigs.

6.4.5.2 MICE

Figure 6.15 represents the BAL fluid differential cell counts for acute and chronic OVA challenged mice treated with roflumilast or vehicle. In the acute challenged mice roflumilast significantly reduced the percentage of eosinophils compared to vehicle (1.2±0.2% compared to 16.7±1.0% respectively). As a consequence there was a corresponding significant increase in the percentage of macrophages compared to vehicle (87.0±1.0% compared to 69.8±2.0% respectively). This was also the case in the chronic challenged group. Roflumilast again showed a significant reduction in the percentage of eosinophils compared to vehicle (2.5±0.4% compared to 13.9±1.1% respectively). Therefore, a greater percentage of macrophages observed following roflumilast treatment compared to vehicle (86.0±2.6% compared to 72.3±1.1% respectively). No differences were observed in lymphocyte or neutrophil percentage for both groups.

6.4.6 LUNG HISTOLOGY OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

Figure 6.16 (A-C) shows a haematoxylin and eosin stained guinea pig bronchiole that was naïve, chronic OVA challenged – vehicle treated or chronic OVA challenged – roflumilast treated. Roflumilast treatment reduced the amount of smooth muscle present around the bronchiole compared to vehicle treatment. The epithelium of the roflumilast treated guinea pig also appeared thinner compared to the vehicle treated guinea pig, however, there did not appear to be much difference in the thickness of the lamina propria between both groups. The

number of inflammatory cells found in the peribronchiolar space of each of these groups have been scored and analysed and the outcome is shown in figure 6.17. Roflumilast treatment significantly reduces the number of lung inflammatory cells compared to vehicle (2.3±0.3 compared to 4.8±0.3). However, roflumilast is unable to reduce the cells to naïve levels.

Figure 6.18 (A-C) represents the bronchioles of mice that are either naïve or chronic OVA challenged treated with vehicle or roflumilast stained with haematoxylin and eosin to show general morphology. Roflumilast treatment appeared to reduce the levels of smooth muscle and decrease the thickness of the epithelium and lamina propria compared to vehicle treatment. The scoring of inflammatory cells found in the peribronchiolar space in these groups is shown in figure 6.19. The number of cells found in the vehicle treated group is significantly reduced with roflumilast treatment (4.8±0.3 compared to 1.8±0.3) though not to naïve levels.

Picrosirius red stained guinea pig bronchioles that are naïve, chronic OVA – vehicle treated or chronic OVA – roflumilast treated are shown in figure 6.20 (A-C). Decreased levels of collagen were observed compared to vehicle following roflumilast treatment. The percentage of bronchiolar collagen in these guinea pigs is represented in figure 6.21. Treatment with roflumilast significantly reduces bronchiolar collagen compared to vehicle treatment (23.2±6.0% compared to 46.7±2.9% respectively) in chronic challenged guinea pigs.

Picrosirius red stained bronchioles for the same three treatment groups in mice are shown in figure 6.22 (A-C). These show that roflumilast treatment reduces the level of collagen compared to vehicle. The percentage of bronchiolar collagen for each of these three groups is represented in figure 6.23. It shows that roflumilast treatment significantly reduced the amount of bronchiolar collagen compared to vehicle treatment (25.9±4.7% compared to 59.4±2.8% respectively).

Naïve, chronic OVA – vehicle treated and chronic OVA – roflumilast treated guinea pig bronchioles that were stained with alcian blue/periodic acid Schiff are shown in figure 6.24 (A-C). Less goblet cells were observed in the bronchioles of roflumilast treated guinea pigs compared to vehicle. The number of mucin associated goblet cells per 10,000 epithelial pixels in naïve, chronic OVA – vehicle treated or chronic OVA – roflumilast treated guinea pigs is shown in figure 6.25. Roflumilast treatment significantly reduces the number of goblet cells compared to vehicle treatment (3.6±0.3 compared to 9.1±1.7).

The mouse stained bronchioles are shown in figure 6.26 (A-C). Mice treated with roflumilast appear to have less goblet cells (stained bright pink) than vehicle treated mice. In figure 6.27 the effect that roflumilast and vehicle treatment has on the mucin associated goblet cells of chronic OVA challenged mice is shown. The graph shows that roflumilast significantly reduced the number of mucin associated goblet cells per 10,000 epithelial pixels compared to vehicle (3.4±0.8 compared to 10.3±2.3).

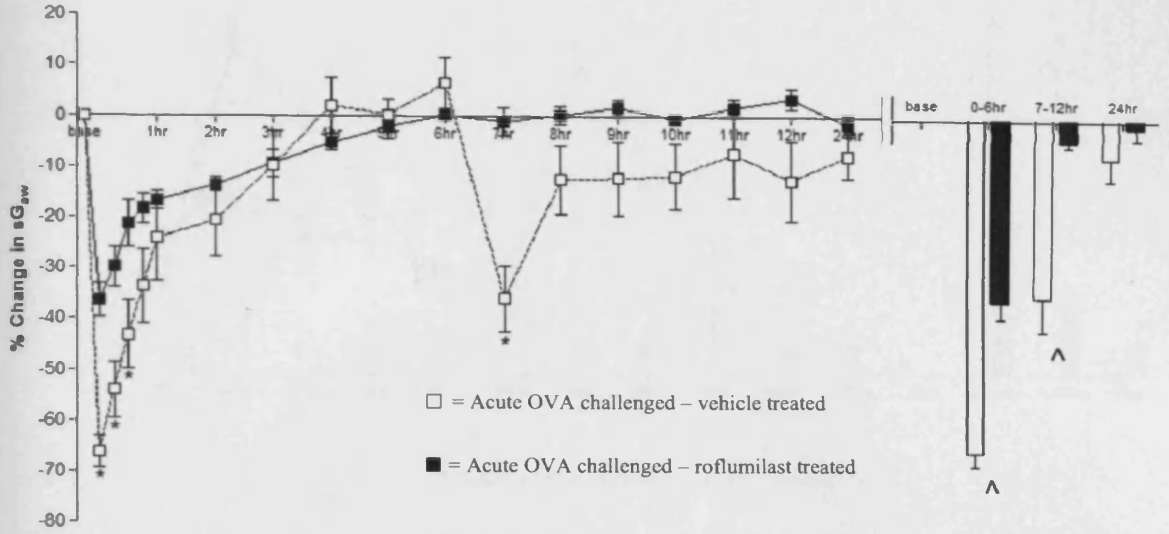


Figure 6.2 – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that were challenged with 0.01 % OVA and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from roflumilast treated guinea pigs; ^ significant difference between vehicle and roflumilast treatment. Two-tailed T-test ($P<0.05$; n=6). Raw roflumilast treatment sG_{aw} values (cm/H_20) – Base (0.67±0.04), 0-6 hours (0.42±0.02), 7-12 hours (0.64±0.03) and 24 hours (0.66±0.05). Raw vehicle treatment sG_{aw} values (cm/H_20) – Base (0.62±0.03), 0-6 hours (0.21±0.02), 7-12 hours (0.40±0.05) and 24 hours (0.57±0.06).

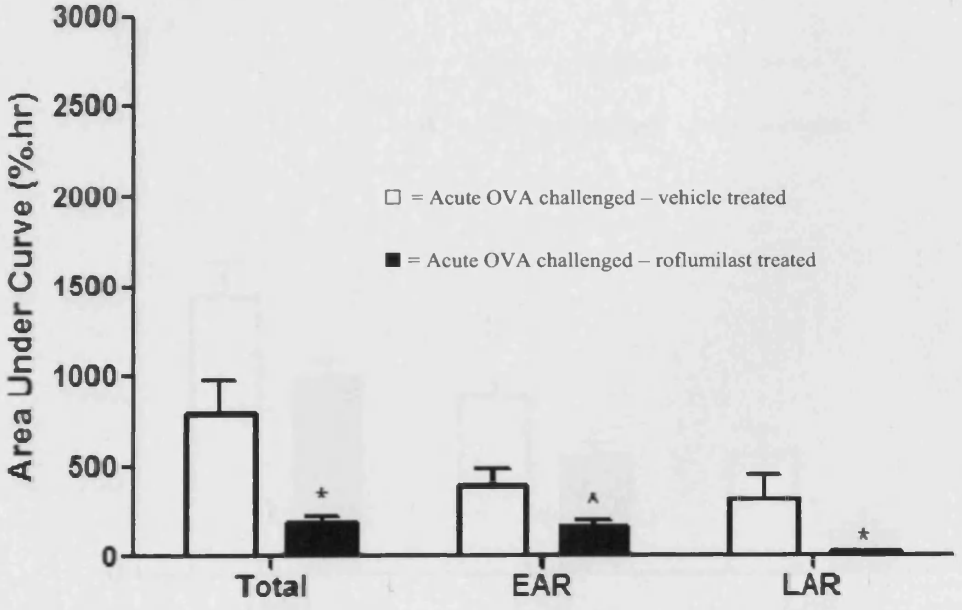


Figure 6.3 – Area under the curve analysis comparing OVA sensitised guinea pigs challenged 0.01% OVA and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

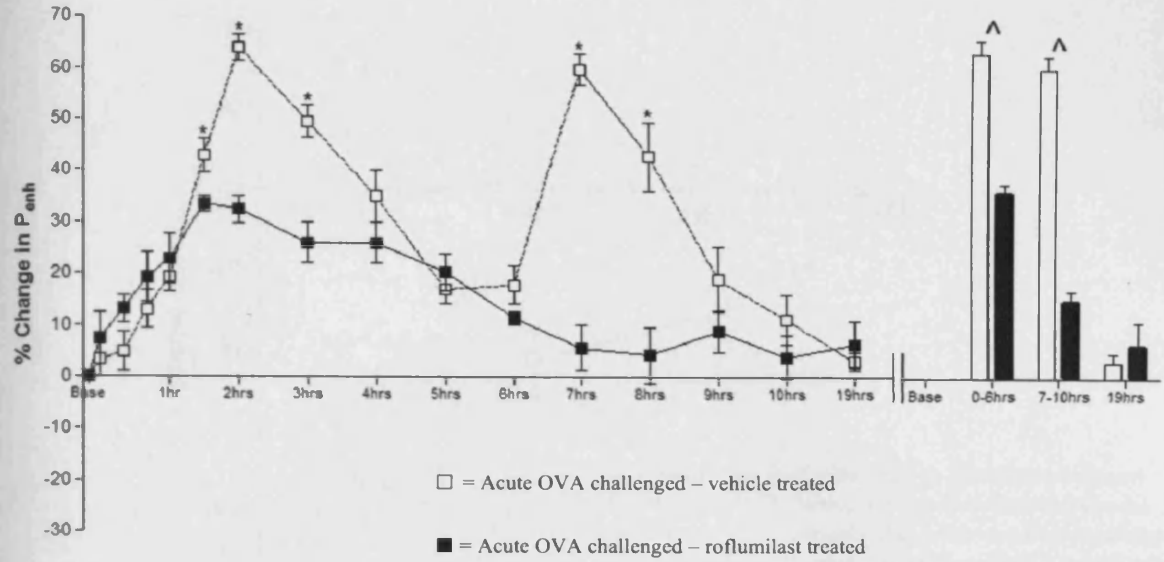


Figure 6.4 – Mean time-course values of P_{enh} in OVA sensitised mice that were challenged twice with 0.5% OVA 4 hours apart and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 19 hours (24 hours after first exposure). Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from roflumilast treated mice; ^ significant difference between vehicle and roflumilast treatment. Two-tailed T-test ($P<0.05$; n=6). Raw roflumilast treatment P_{enh} values (AU) – Base (0.32±0.03), 0-6 hours (0.44±0.04), 7-12 hours (0.37±0.03) and 24 hours (0.34±0.03). Raw vehicle treatment P_{enh} values (AU) – Base (0.30±0.01), 0-6 hours (0.49±0.02), 7-12 hours (0.48±0.02) and 24 hours (0.31±0.01).

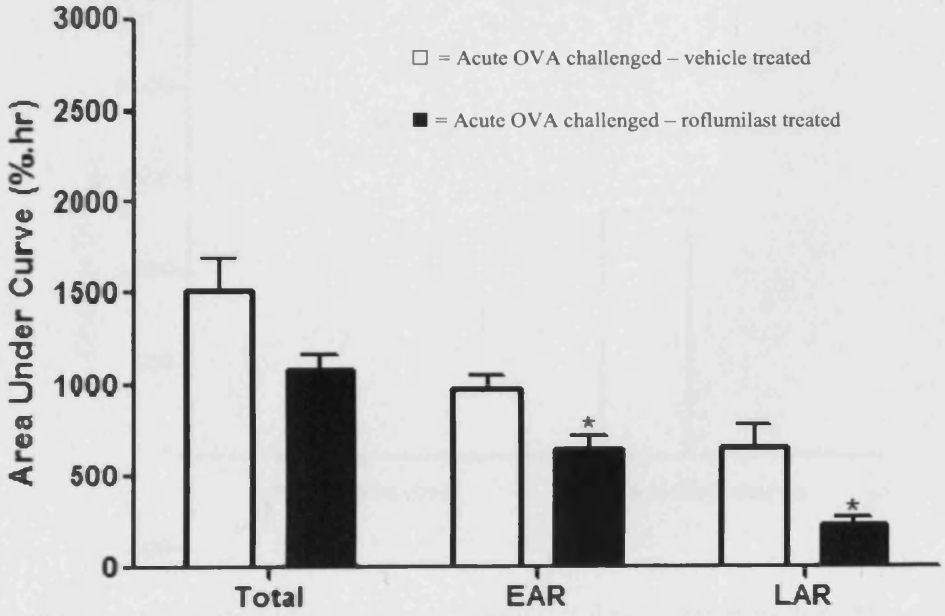


Figure 6.5 – Area under the curve analysis comparing OVA sensitised mice challenged with a two doses of 0.5% OVA and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only positive peaks are considered, any peaks that have a negative value of P_{enh} are excluded. Total includes all negative peaks from 0-19 hours, EAR includes from 0-6 hours and LAR includes from 6-19 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

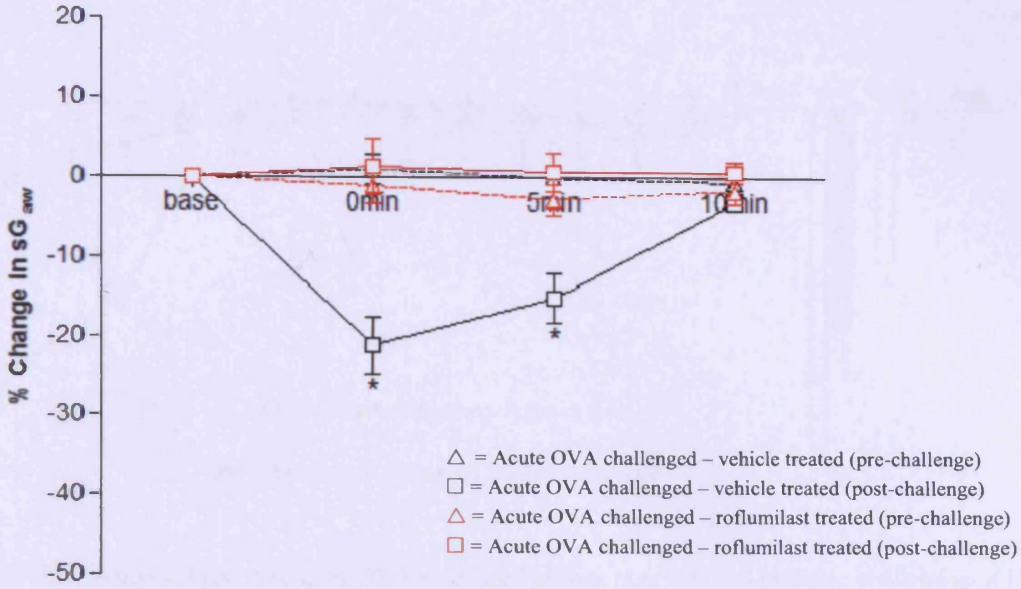


Figure 6.6 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs challenged with OVA (0.01%) and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post-OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

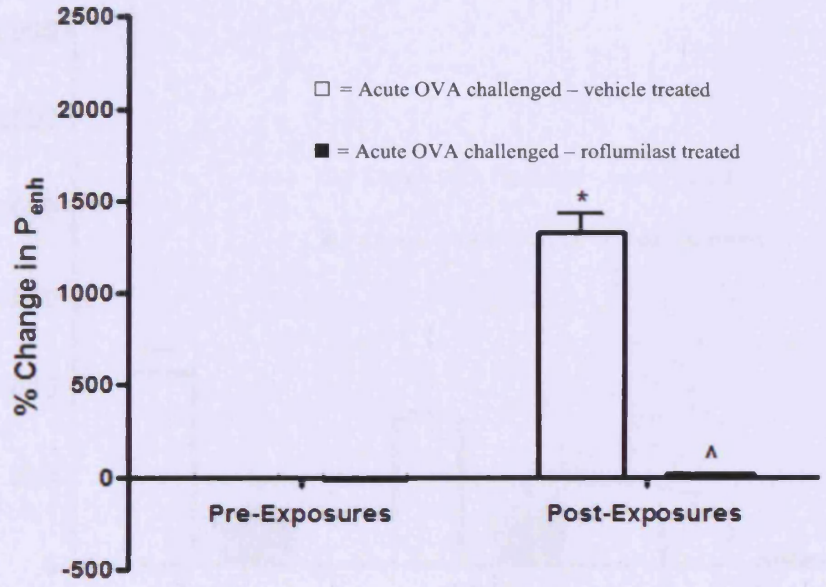


Figure 6.7 – Response of the airways to a 60 second intermittent dose of methacholine (30 mg/ml) in OVA sensitised mice challenged OVA (0.5%) and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post- OVA challenge. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from pre-challenge values of P_{enh}; ^significantly different from vehicle treatment post-OVA challenge. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

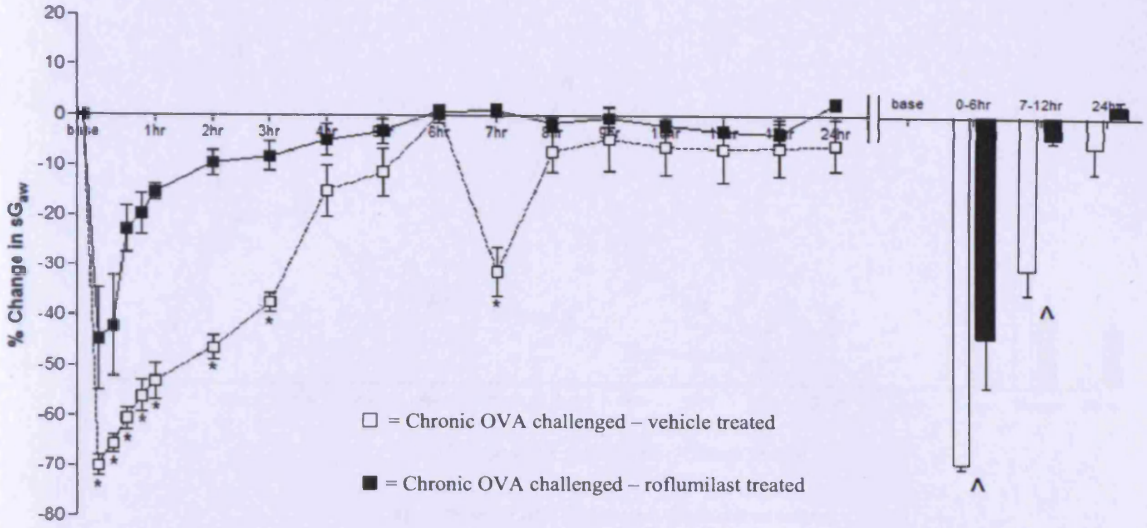


Figure 6.8 – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that had multiple challenges of OVA and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from roflumilast treated guinea pigs; ^ significant difference between vehicle and roflumilast treatment. Two-tailed T-test ($P<0.05$; n=6). Raw roflumilast treatment sG_{aw} values (cm/H_20) – Base (0.69±0.02), 0-6 hours (0.38±0.07), 7-12 hours (0.66±0.02) and 24 hours (0.71±0.02). Raw vehicle treatment sG_{aw} values (cm/H_20) – Base (0.42±0.03), 0-6 hours (0.12±0.01), 7-12 hours (0.30±0.01) and 24 hours (0.39±0.01).

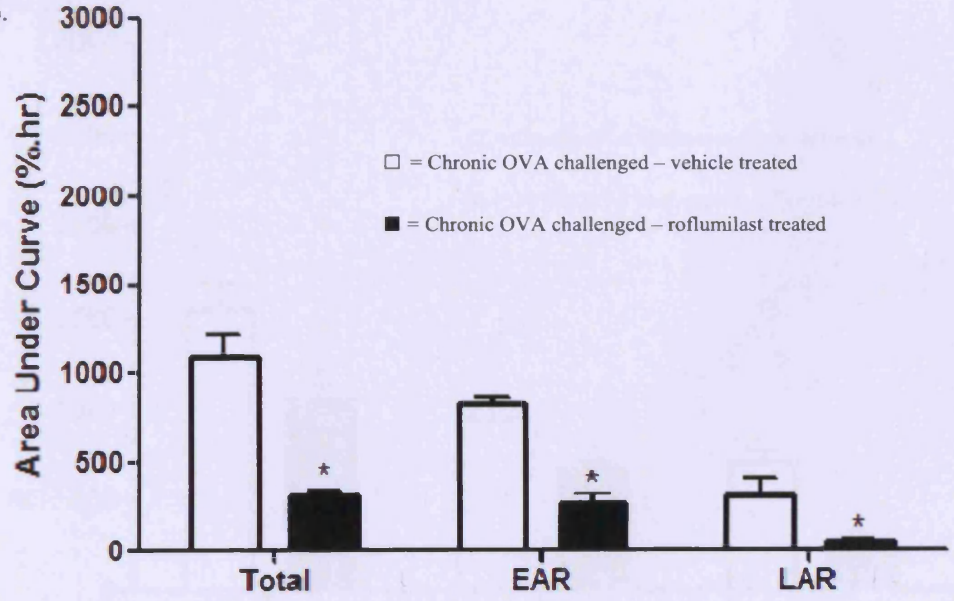


Figure 6.9 – Area under the curve analysis comparing OVA sensitised guinea pigs OVA sensitised guinea pigs challenged with a chronic dosing of OVA and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

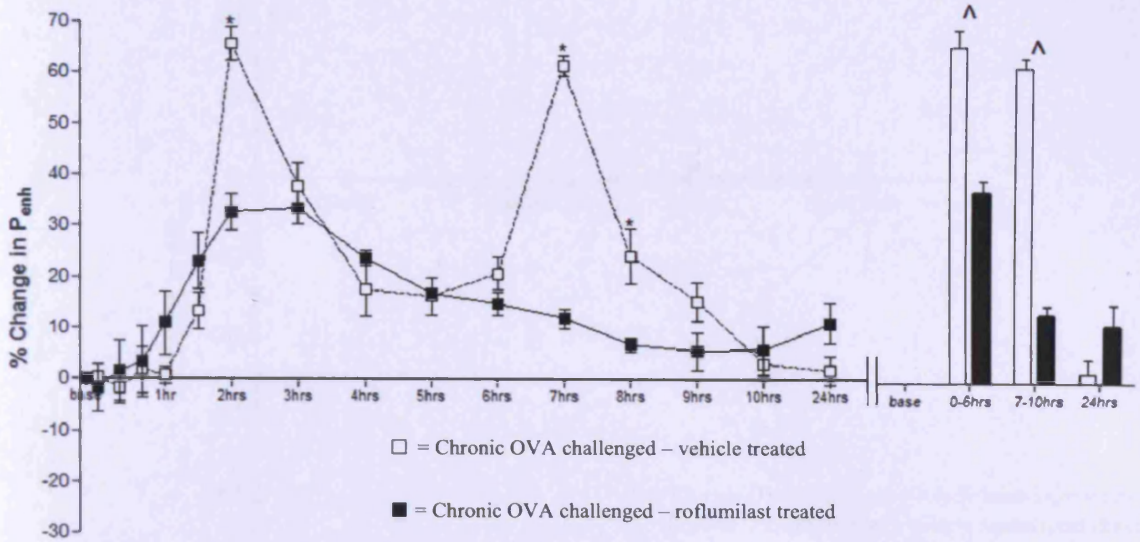


Figure 6.10 – Mean time-course values of P_{enh} in OVA sensitised mice that were challenged repeatedly with 2% OVA three times a week for six weeks and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 19 hours (24 hours after first exposure). Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from roflumilast treated mice; ^ significant difference between vehicle and roflumilast treatment. Two-tailed T-test ($P<0.05$; n=6). Raw roflumilast treatment P_{enh} values (AU) – Base (0.30±0.01), 0-6 hours (0.42±0.01), 7-12 hours (0.34±0.01) and 24 hours (0.34±0.02). Raw vehicle treatment P_{enh} values (AU) – Base (0.35±0.02), 0-6 hours (0.58±0.03), 7-12 hours (0.56±0.03) and 24 hours (0.36±0.02).

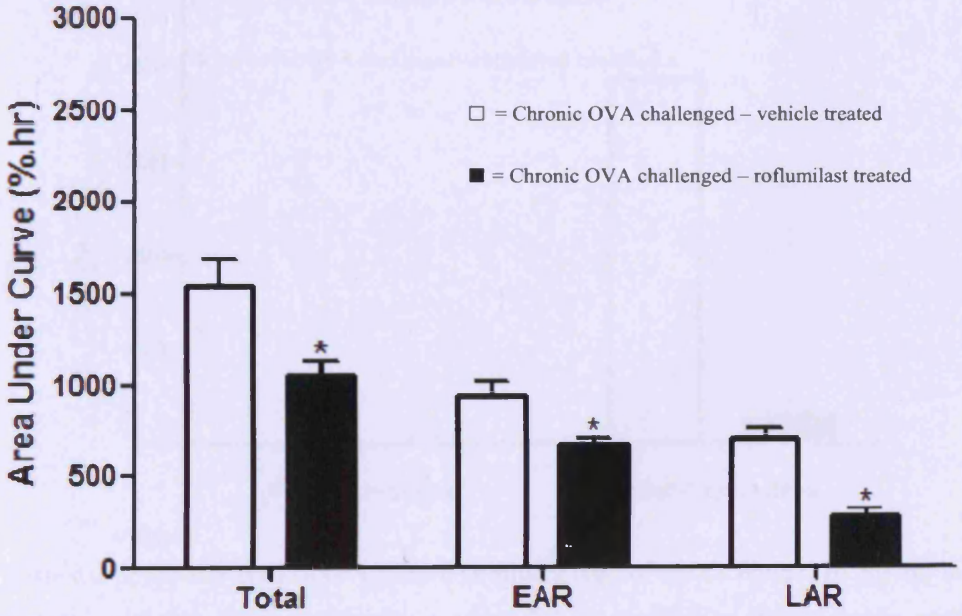


Figure 6.11 – Area under the curve analysis comparing OVA sensitised mice challenged with repeated doses of 2% OVA and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only positive peaks are considered, any peaks that have a negative value of P_{enh} are excluded. Total includes all negative peaks from 0-19 hours, EAR includes from 0-6 hours and LAR includes from 6-19 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

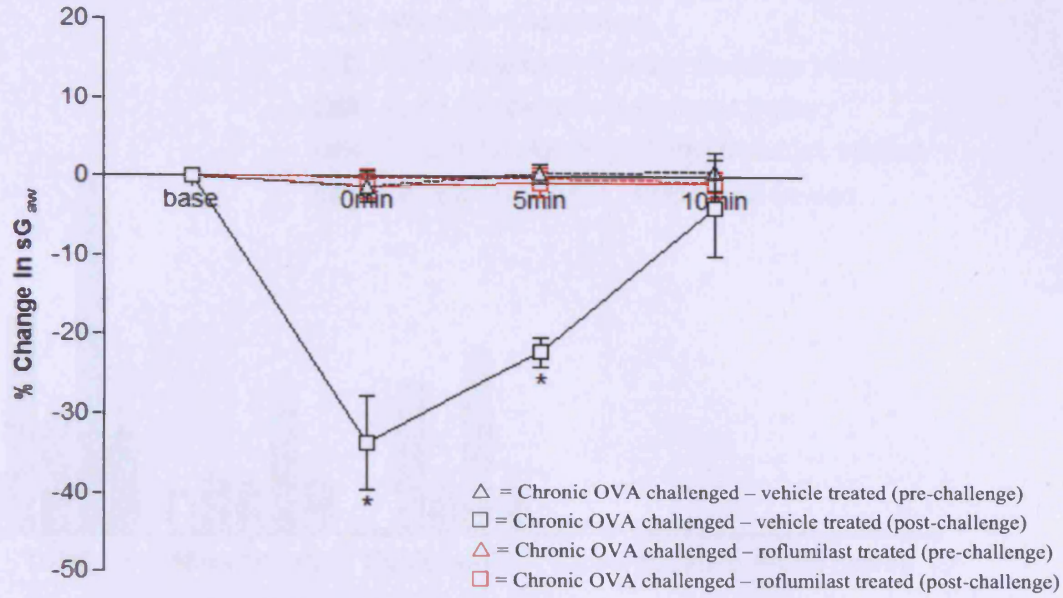


Figure 6.12 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs chronically challenged with OVA and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post-OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

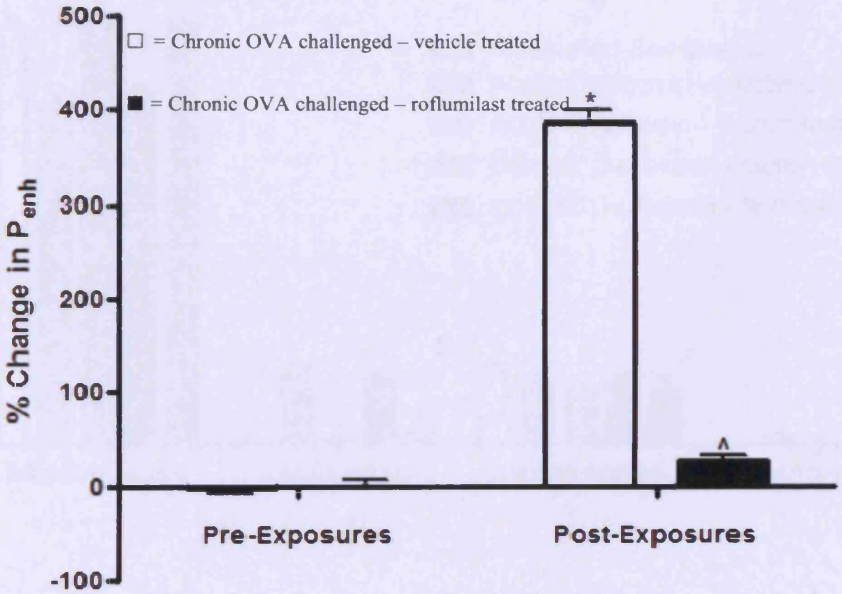


Figure 6.13 – Response of the airways to a 60 second intermittent dose of methacholine (10 mg/ml) in OVA sensitised mice challenged repeatedly with 2% OVA three times a week for six weeks and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post- OVA challenge. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from pre-challenge values of P_{enh}; ^significantly different from vehicle treatment post-OVA challenge. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

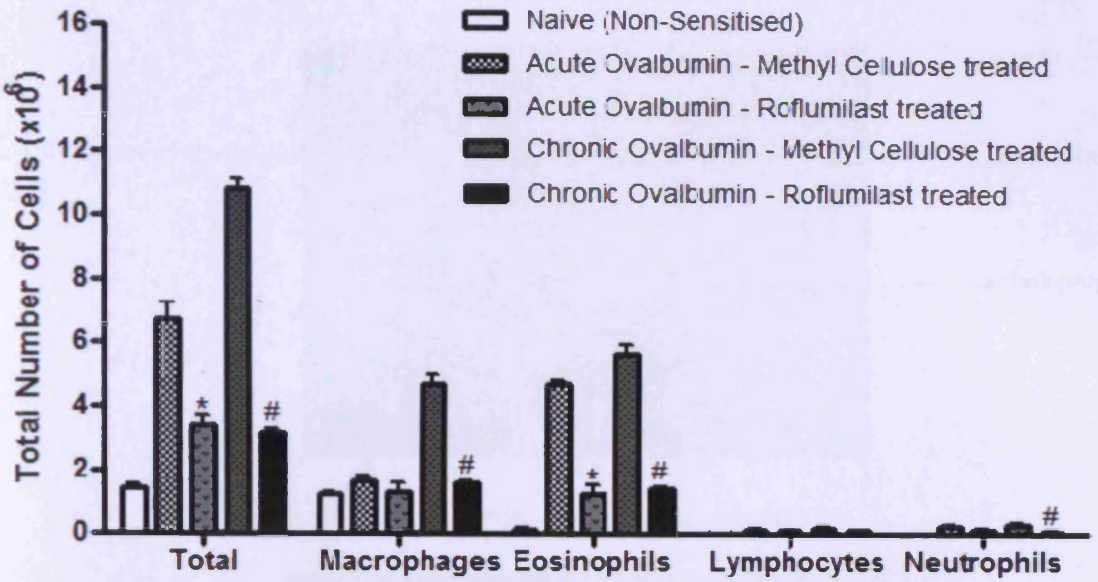


Figure 6.14 – The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised) and OVA challenged (acute and chronic) guinea pigs orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Results are expressed as mean±S.E.M. *significantly different from acute OVA – vehicle treated; #significantly different from chronic OVA – vehicle treated. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

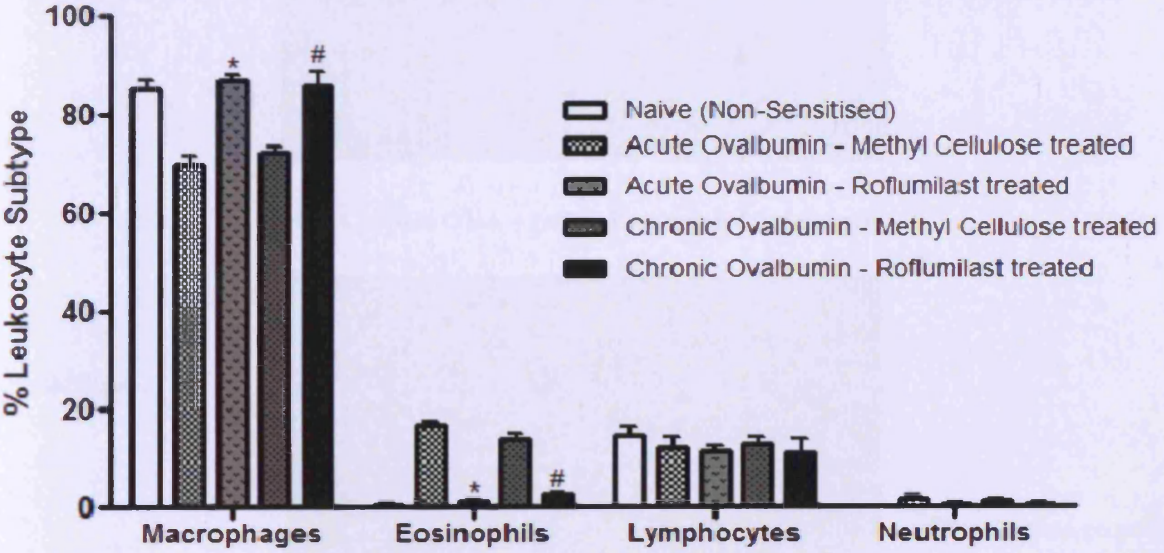
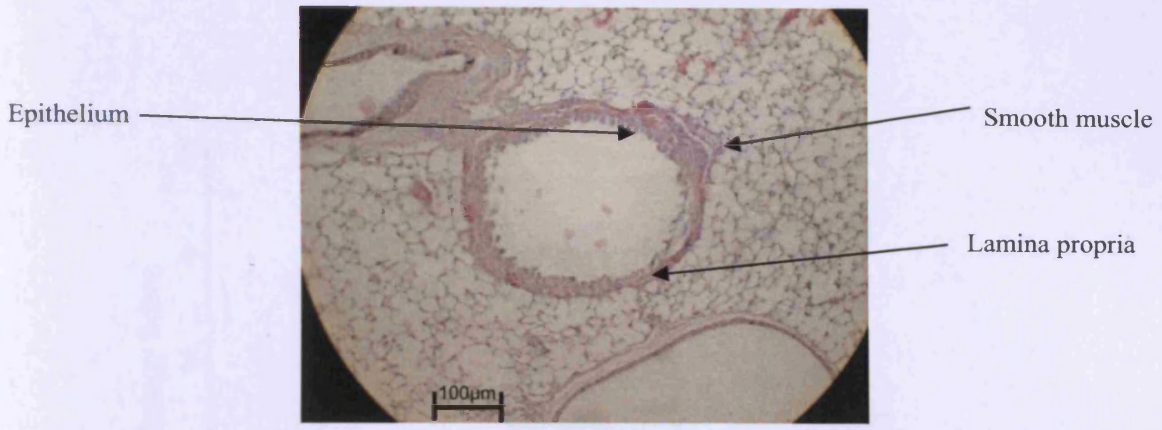
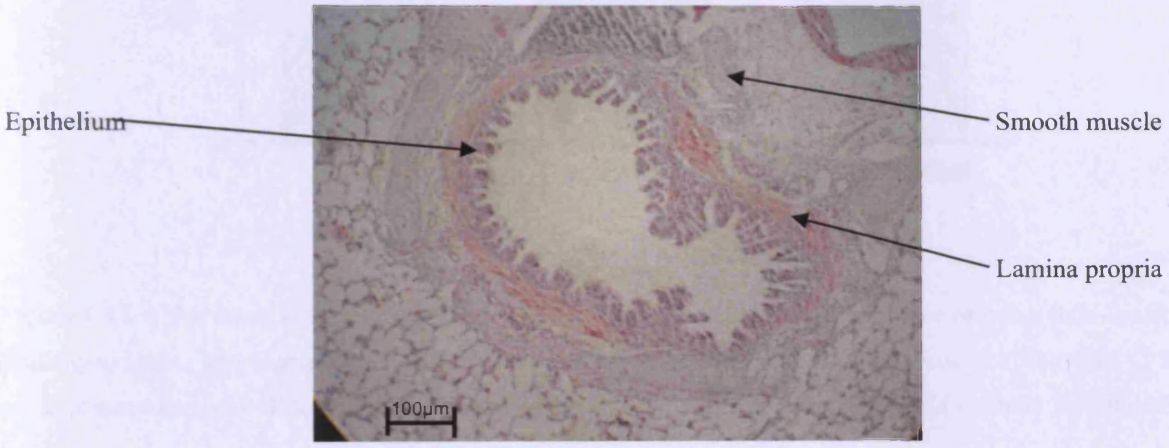


Figure 6.15 – The percentage of macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised) and OVA challenged (acute and chronic) mice orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Results are expressed as mean±S.E.M. *significantly different from acute OVA – vehicle treated; #significantly different from chronic OVA – vehicle treated. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

A – Chronic saline challenged guinea pig



B – Chronic OVA – vehicle treated guinea pig



C – Chronic OVA – roflumilast treated guinea pig

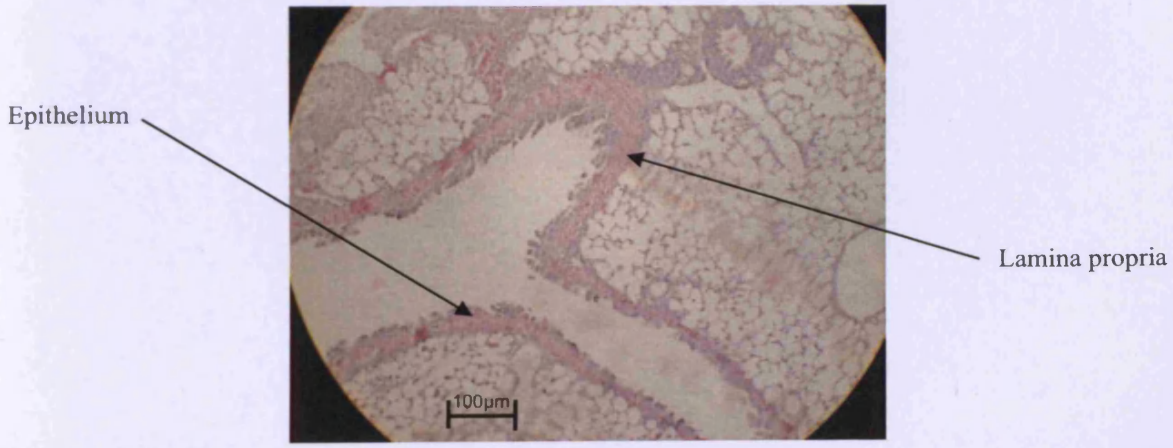


Figure 6.16 (A-C) – Bronchioles of chronic saline challenge, non-treated, guinea pigs and chronic OVA challenged guinea pigs orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with haematoxylin and eosin to display general morphology (1000x magnification).

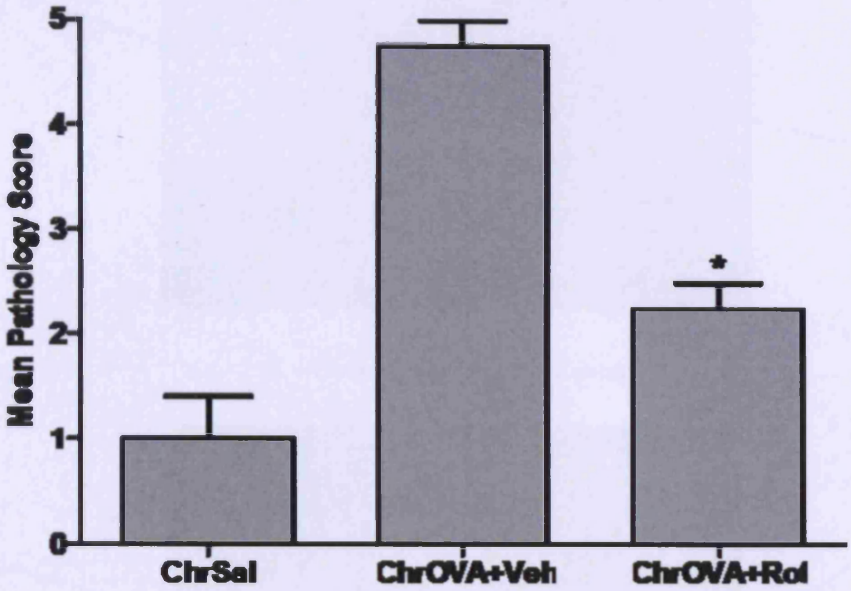
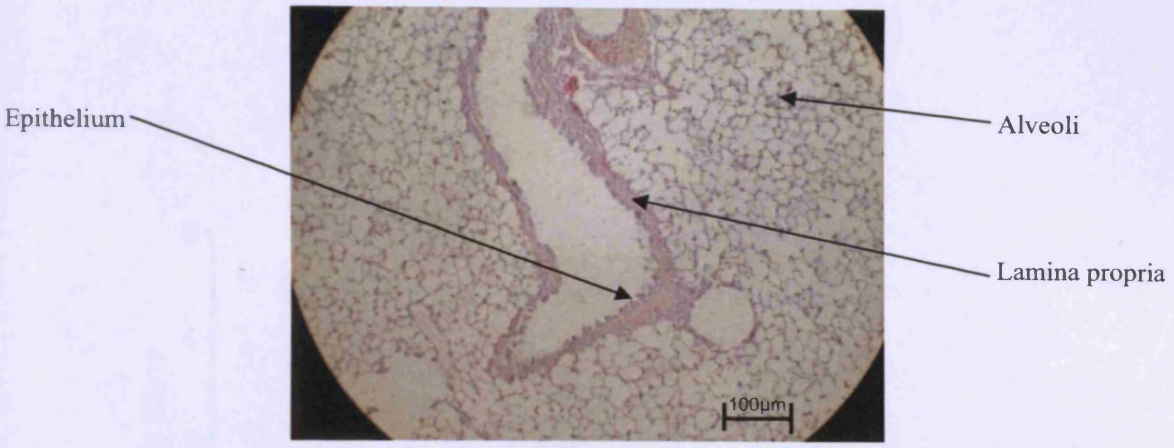
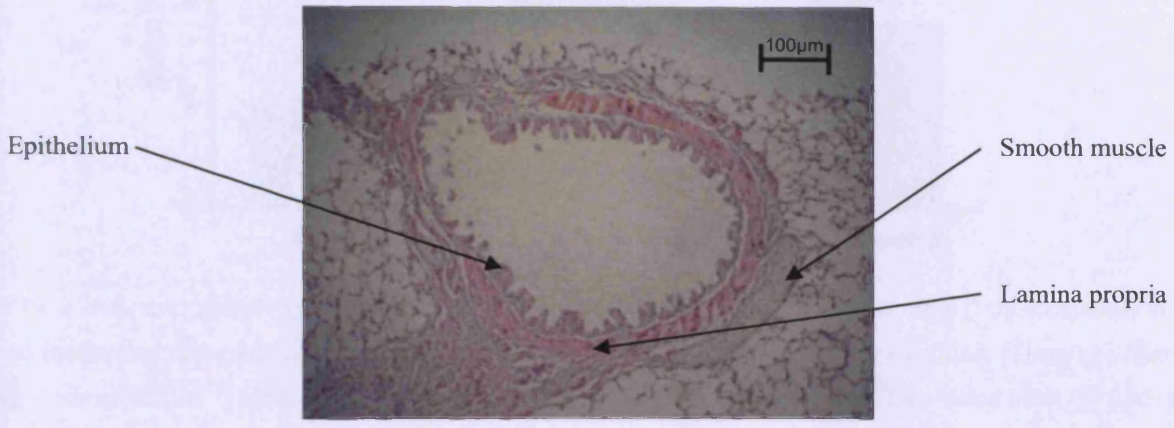


Figure 6.17 – The mean pathology score of cells found in the peri-bronchiolar space of naïve (non-sensitised) and OVA challenged (acute and chronic) guinea pigs treated orally treated with methyl cellulose or roflumilast (1 mg/kg). Results are expressed as mean±S.E.M. *significantly different from chronic OVA – vehicle treated. Two-tailed t-test ($P<0.05$; n=4).

A – Chronic saline challenged mouse



B – OVA – vehicle treated mouse



C – OVA – roflumilast treated mouse

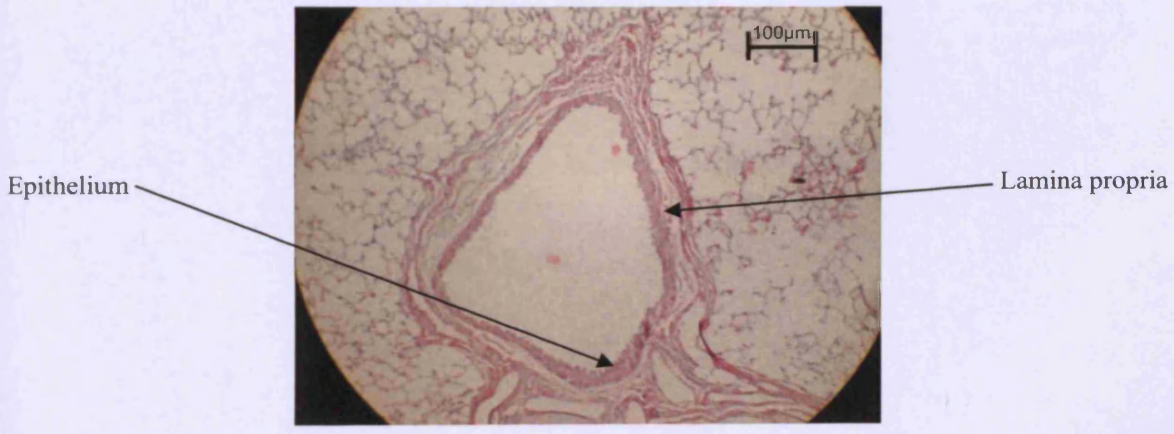


Figure 6.18 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with haematoxylin and eosin to display general morphology (1000x magnification).

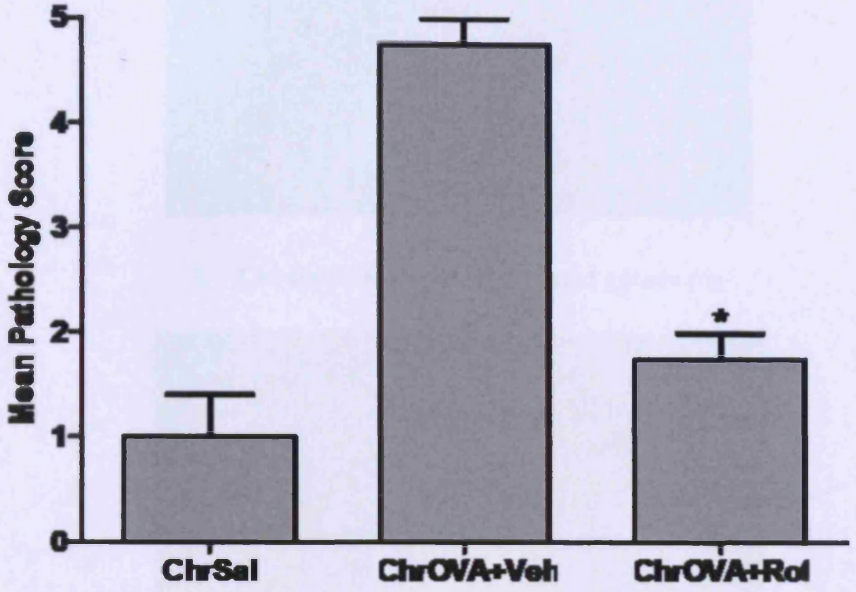
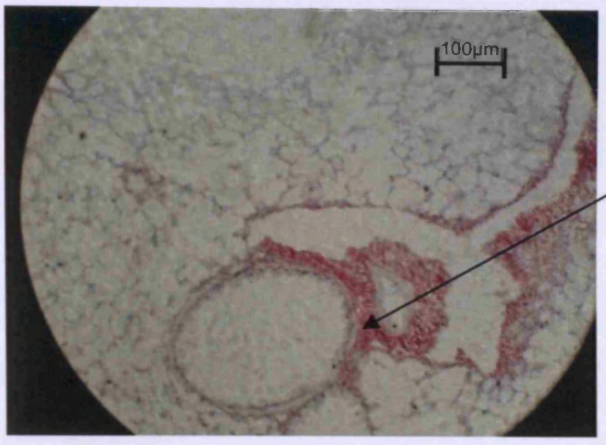
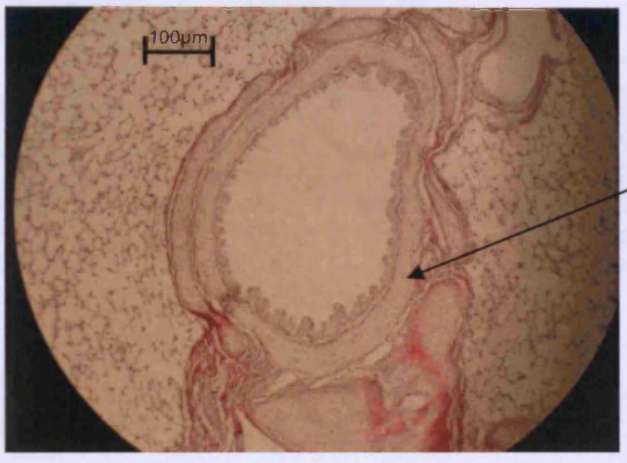


Figure 6.19 – The mean pathology score of cells found in the peri-bronchiolar space of naïve (non-sensitised) and OVA challenged (acute and chronic) mice treated orally treated with methyl cellulose or roflumilast (1 mg/kg). Results are expressed as mean±S.E.M. *significantly different from chronic OVA – vehicle treated. Two-tailed t-test ($P<0.05$; $n=4$).

A – Chronic saline challenged guinea pig



B – Chronic OVA – vehicle treated guinea pig



C – Chronic OVA – roflumilast treated guinea pig

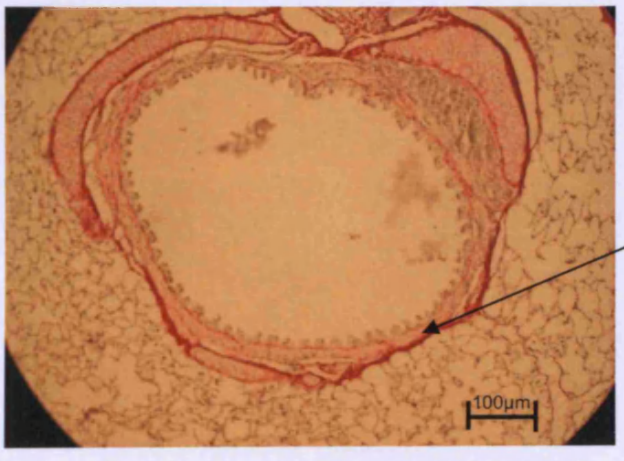


Figure 6.20 (A-C) – Bronchioles of chronic saline, non-treated, guinea pigs and chronic OVA challenged guinea pigs orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with picrosirius red to display collagen (1000x magnification).

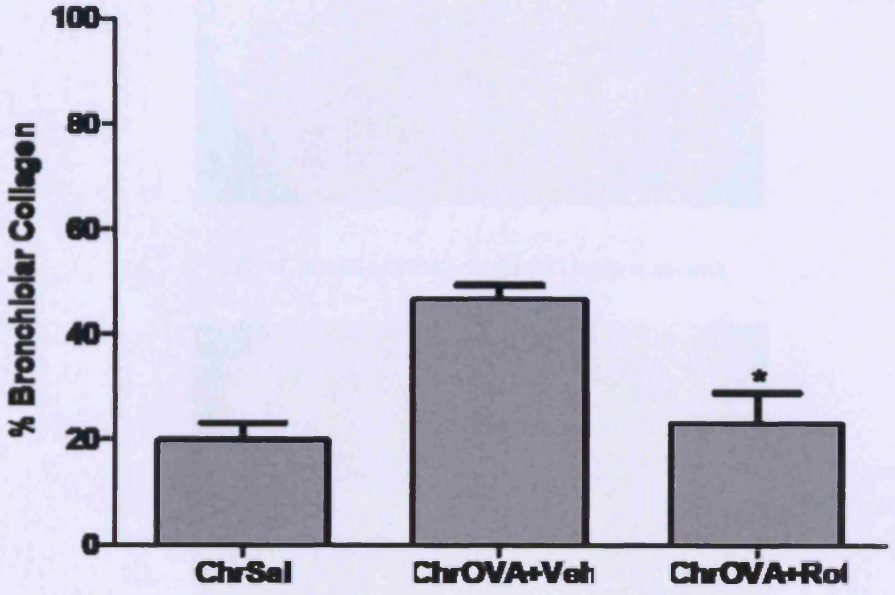
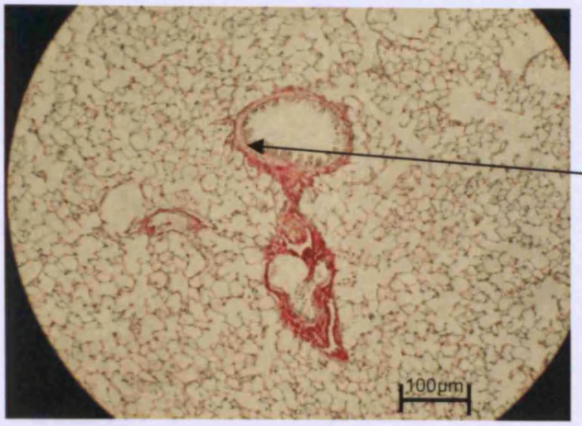
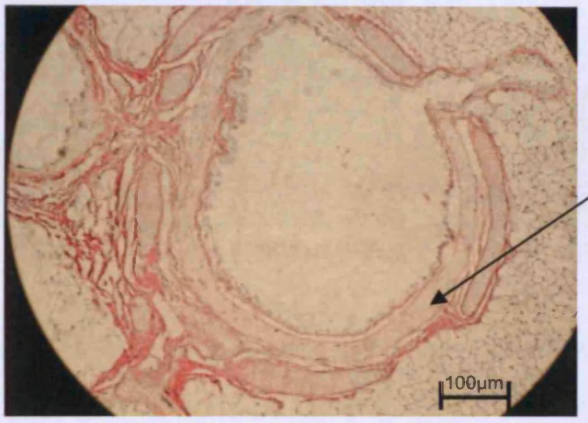


Figure 6.21 – Effect of a chronic OVA challenge and treatment of methyl cellulose or roflumilast (1 mg/kg) administered orally 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on percentage bronchiolar collagen in guinea pigs. Results are expressed as mean±S.E.M. *significantly different from vehicle treatment. Two-tailed t-test ($P<0.05$; n=4).

A - Chronic saline challenged mouse



B – Chronic OVA – vehicle treated mouse



C – Chronic OVA – roflumilast treated mouse

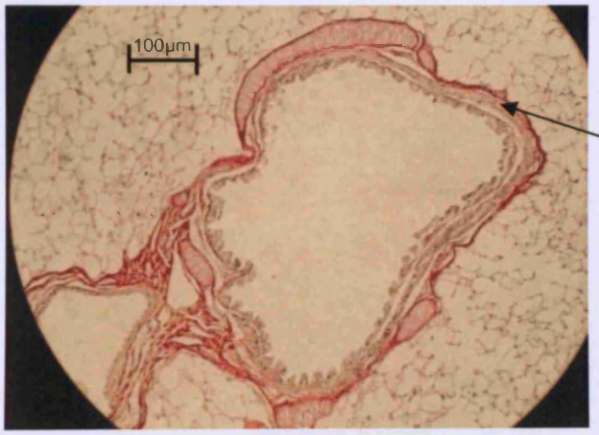


Figure 6.22 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with picrosirius red to display collagen (1000x magnification).

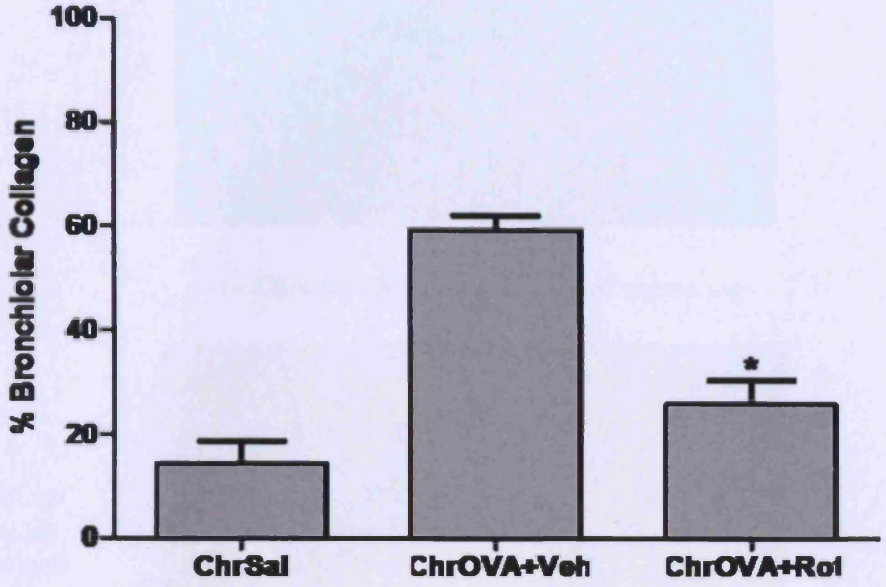
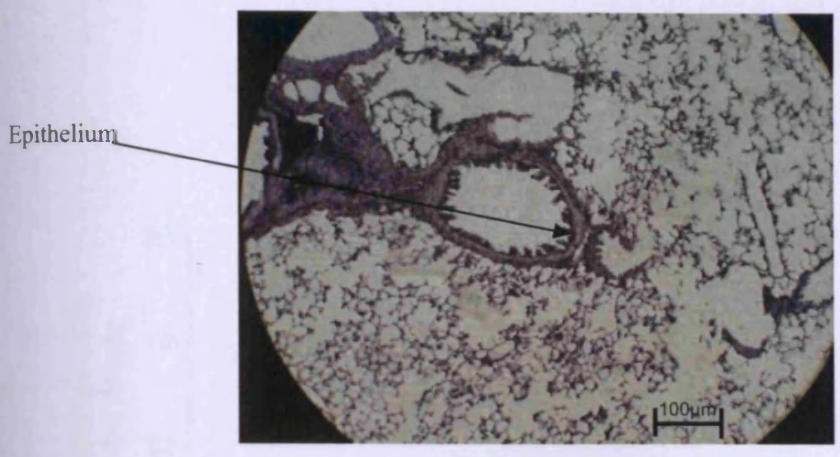
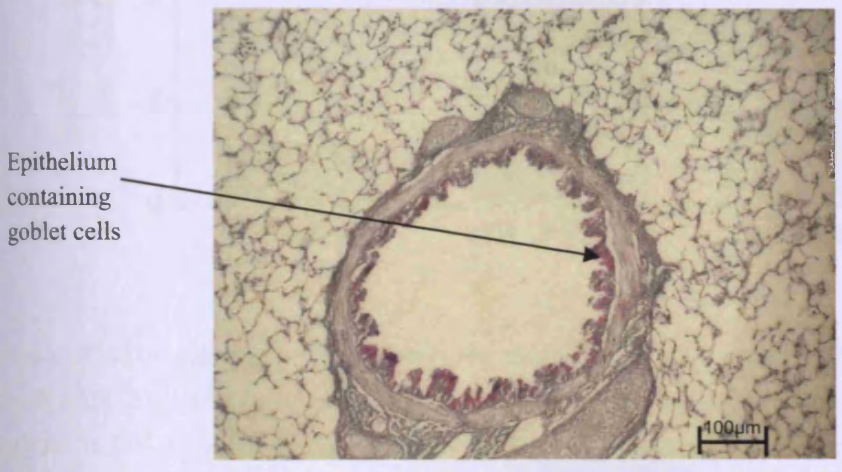


Figure 6.23 – Effect of a chronic OVA challenge and treatment of methyl cellulose or roflumilast (1 mg/kg) administered orally 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on percentage bronchiolar collagen in mice. Results are expressed as mean±S.E.M. *significantly different from vehicle treatment. Two-tailed t-test ($P<0.05$; $n=4$).

A - Chronic saline challenged guinea pig



B – Chronic OVA – vehicle treated guinea pig



C – Chronic OVA – roflumilast treated guinea pig

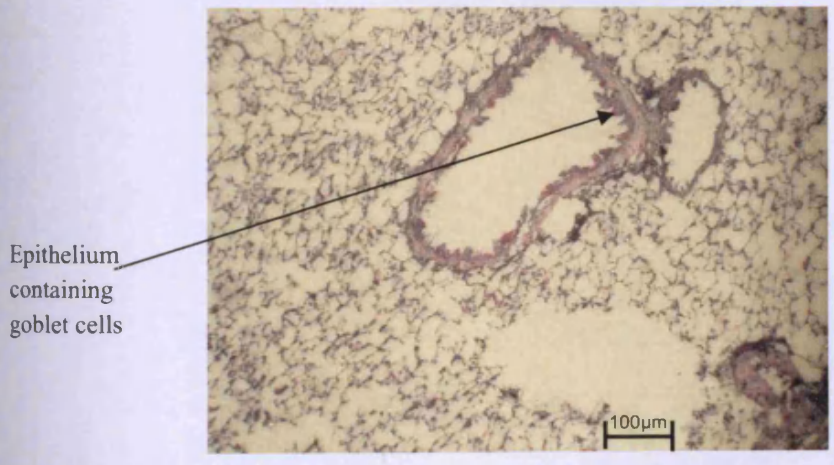


Figure 6.24 (A-C) – Bronchioles of chronic saline, non-treated, guinea pigs and chronic OVA challenged guinea pigs orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with alcian blue/periodic acid Schiff to display goblet cells (1000x magnification).

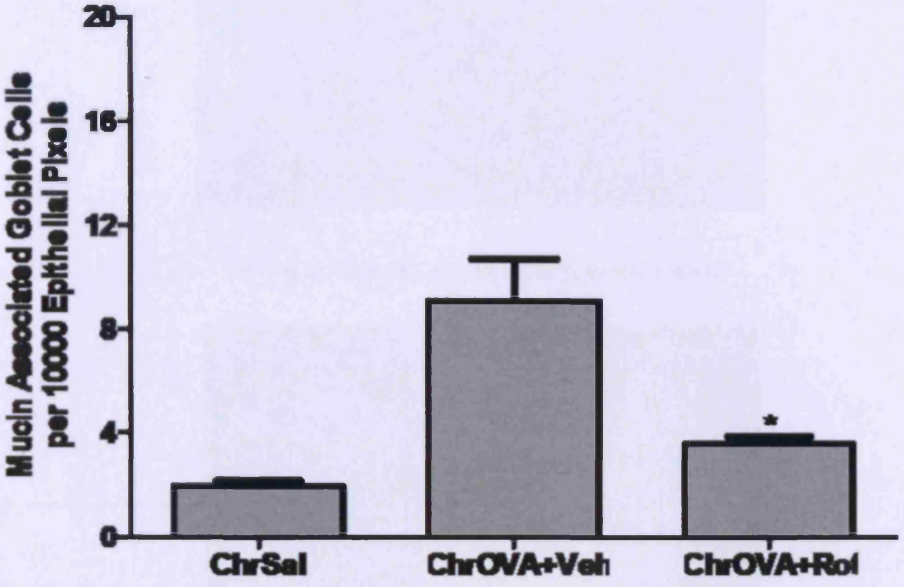
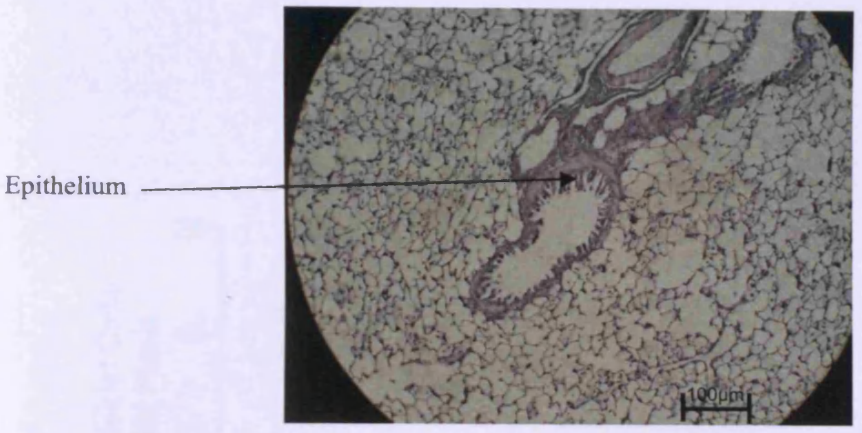
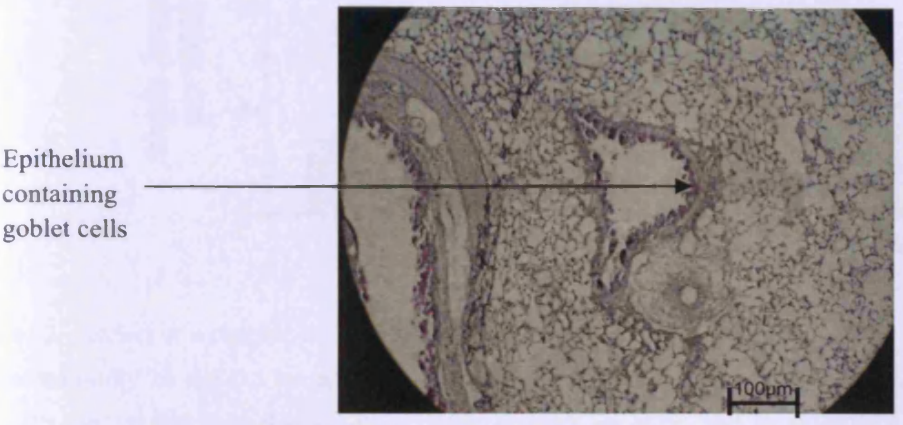


Figure 6.25 – Effect of a chronic OVA challenge and treatment and treatment of methyl cellulose or roflumilast (1 mg/kg) administered orally 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on number of mucin associate goblet cells per 10,000 epithelial pixels in guinea pigs. Results are expressed as mean±S.E.M. *significantly different from vehicle treatment. Two-tailed t-test ($P<0.05$; $n=4$).

A - Chronic saline challenged mouse



B – Chronic OVA – vehicle treated mouse



C – Chronic OVA – roflumilast treated mouse

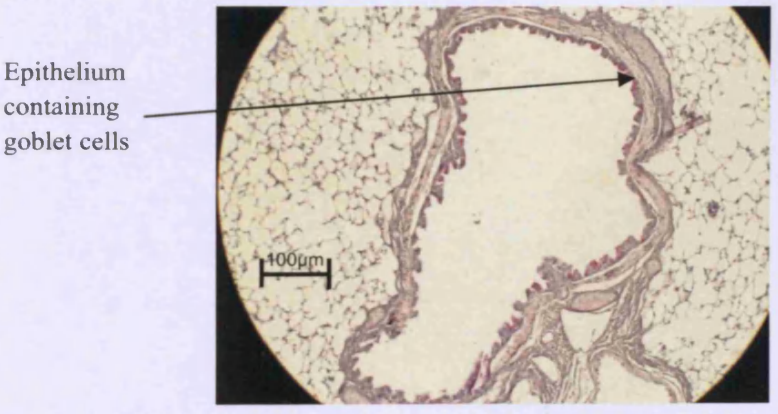


Figure 6.26 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with alcian blue/periodic acid Schiff to display goblet cells (1000x magnification).

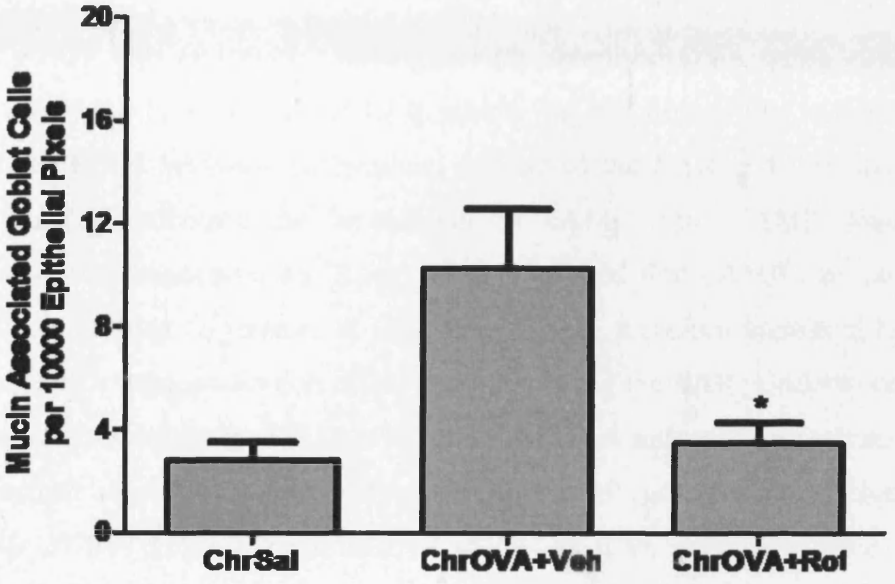


Figure 6.27 – Effect of a chronic OVA challenge and treatment and treatment of methyl cellulose or roflumilast (1 mg/kg) administered orally 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on number of mucin associate goblet cells per 10,000 epithelial pixels in mice. Results are expressed as mean±S.E.M. *significantly different from vehicle treatment. Two-tailed t-test ($P<0.05$; $n=4$).

6.5 DISCUSSION

6.5.1 EARLY AND LATE ASTHMATIC RESPONSES IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS AND MICE

Sensitised guinea pigs and mice challenged with nebulised OVA using either an acute or chronic protocol led to an EAR and LAR which was not affected by oral methyl cellulose treatment. The PDE4 inhibitor, roflumilast, attenuated the EAR and virtually abolished the LAR. Roflumilast prevents the breakdown of cAMP into 5'AMP causing increased intracellular cAMP concentration. It has been suggested that cAMP can cause respiratory smooth muscle to relax (Schramm & Grunstein, 1992), therefore increased levels of cAMP could be causing a bronchodilation effect and attenuating the EAR. Underwood *et al.*, (1998) showed that a PDE4 inhibitor, SB 207499, inhibited OVA induced contractions on guinea pig isolated tracheal strips but had no effect on exogenous agonist-induced contractions. This suggests SB 207499 inhibits the release of mediators from mast cells but does not exert a direct bronchodilation. The inhibition of mast cell mediator release would contribute to a reduction in the EAR. Therefore there may be two separate mechanisms in how roflumilast attenuated the EAR.

In asthmatic patients treated with the PDE4 inhibitor CDP840 twice daily for 9.5 days the LAR was reduced by 30% (Harbinson *et al.*, 1997). It is possible that the decrease in BAL fluid eosinophil levels that PDE4 inhibitors cause (Deng *et al.*, 2006) results in no LAR. Eosinophils are one of the main causes of the LAR as their degranulation or activation causes the release of several inflammatory mediators (Chapoval *et al.*, 1999) and creation of reactive oxygen species (Nielson & Vestal, 1989). The guinea pigs and mice in this chapter that were treated with roflumilast showed inhibition of the LAR compared to vehicle treatment despite the fact that they were only administered drug three times. This suggests that either the chronic changes in the animal models are not as severe as in humans or roflumilast is a more potent PDE4 inhibitor than CDP840.

The findings in this chapter are similar to those found in human asthmatics treated with roflumilast (van Schalkwyk *et al.*, 2005). However, the findings are contradictory to those found by Toward and Broadley (2004) who showed that rolipram was unable to attenuate the LAR in acute OVA challenged guinea pigs. The fact that roflumilast reduced the LAR in

these models suggests it has anti-inflammatory properties, this is corroborated by the fact that ciclamilast has been shown to inhibit tumour necrosis factor (TNF)-α (Deng *et al.*, 2006). TNF-α can activate NF-κB which was shown in chapter 5 to be able to cause inflammatory gene transcription.

6.5.2 AIRWAY HYPERRESPONSIVENESS IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS AND MICE

AHR was observed in both acute and chronic OVA challenged guinea pigs and mice treated with vehicle. Roflumilast treatment was able to inhibit the AHR provoked by a histamine (guinea pigs) or methacholine (mice) challenge. Previous studies have shown that PDE4 inhibitors can prevent AHR in animal models of asthma (Danahay & Broadley, 1998; Holbrook *et al.*, 1996; Howell *et al.*, 1993; Raeburn *et al.*, 1994; Santing *et al.*, 1995) and human asthmatics (Louw *et al.*, 2007). As previously mentioned the exact mechanisms of how hyperresponsiveness occurs is unknown, however, the epithelial shedding that occurs during asthma would lead to the airways being more sensitive to allergens and could possibly be a factor in AHR. Epithelial shedding can be caused by major basic protein (Barnes, 2002) and reactive oxygen species (Hirata *et al.*, 1996) released from eosinophils. As increased levels of cAMP leads to the inhibition of the creation of reactive oxygen species in neutrophils and eosinophils (Nielson & Vestal, 1989) it is possible that PDE4 inhibitors prevent AHR via this route at least in the acute OVA challenged models. It is possible that roflumilast prevents AHR by blocking histamine and methacholine, however, this scenario is unlikely as one would expect to see little, if any, EAR if this was the case.

6.5.3 CELLULAR INFLUX IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS AND MICE

Total leukocyte BAL fluid number was significantly reduced in roflumilast treated guinea pigs and mice compared to vehicle. Previous data has shown that PDE4 inhibitors can reduce eosinophil (Hoymann *et al.*, 2009; Nejman-Gryz *et al.*, 2006) and neutrophil (Deng *et al.*, 2006) number so this is no surprise. A significant reduction of eosinophil number and percentage was observed in guinea pigs and mice respectively. Eosinophils can be recruited to the airways by the cytokine eotaxin (Nejman-Gryz *et al.*, 2006) which has been shown to

be inhibited by PDE4 inhibitors (Deng *et al.*, 2006). This inhibition could explain why no LAR or AHR was observed in roflumilast treated animals.

Although an increase in macrophage BAL percentage was observed in mice this is likely to be misleading and a result of eosinophil percentage decrease. Chronic OVA challenged guinea pigs treated with roflumilast showed a reduction in macrophage number whereas the acute OVA challenged group did not. This suggests that chronic inflammation causes an increase in macrophages which is attenuated by PDE4 inhibitors. There is little evidence to link PDE4 inhibitors and macrophage reduction, however, Mehats *et al.*, (2008) showed that rolipram was able to reduce the increase in monocyte chemotactic protein (MCP)-1. As MCP-1 chemoattracts macrophages this could explain the reduction observed in the chronic OVA guinea pigs.

There is a large amount of evidence that PDE4 inhibitors can reduce neutrophil numbers in the airways (Deng *et al.*, 2006), this is likely a result of the PDE4 inhibiting TNF-α, which can cause influx of neutrophils (Nejman-Gryz *et al.*, 2006). However, no reduction in neutrophils was observed in any of the groups in this chapter. This lack of impact on neutrophil number is probably a result of the low number of neutrophils counted in the first place. Neutrophils are predominately involved in the EAR so it is likely by the time a lavage was carried out most of the neutrophils have been removed or undergone apoptosis.

6.5.4 LUNG HISTOLOGY IN CHRONIC OVALBUMIN CHALLENGED GUINEA PIGS AND MICE

Chronic OVA challenges cause massive structural changes to the bronchioles of OVA sensitised guinea pigs and mice such as an increase in epithelial and lamina propria thickness and increased levels of smooth muscle. It is clear to see in the haematoxylin and eosin stained bronchioles that although roflumilast treatment attenuates these changes they are still considerably different from naïve bronchioles. When the number of inflammatory cells in the peribronchiolar space were scored chronic OVA vehicle treated guinea pigs and mice show the greatest amount of cells. Roflumilast is able to significantly decrease the number of inflammatory cells found in the lung tissue. Previous data by Kita *et al.*, (2008) showed that a PDE4 inhibitor, KF19514, was able to reduce the number of inflammatory cells in the lamina propria of OVA exposed mice and data above shows that roflumilast can reduce the

inflammatory cells found in the BAL fluid so the fact it can have a role in decreasing inflammatory cells in the peribronchiolar space was expected.

Roflumilast alleviated the airway remodelling caused by repeated asthmatic episodes in several ways. As shown in this chapter roflumilast can significantly inhibit the production of collagen caused by chronic OVA challenges. Although an increased amount of collagen was observed in the picrosirius red stained bronchioles that were treated with roflumilast compared to naïve, the fact that roflumilast is able to somewhat decrease collagen production will lead to a decreased thickening of the bronchiole wall and thus make breathing easier for asthmatics. It is likely that these findings also apply for human asthmatics as Burgess *et al.*, (2006) showed that roflumilast inhibits collagen I.

Increased mucus in the airways is a serious consequence of asthma as it can cause airway occlusion. Mucus is released from goblet cells that increase in number during chronic asthma. Mucin associated goblet cells appear bright pink when stained with alcian blue/periodic acid Schiff. It is clear to see that chronic OVA exposures causes increased numbers mucin associated goblet cells in vehicle treated guinea pigs and mice. Roflumilast treatment significantly reduces the number of goblet cells therefore decreasing the amount of airway mucus. Goblet cells can increase in number by undergoing metaplasia from epithelial cells. It has been shown that the interleukins 4, 9 and 13, which increase in number during an asthmatic episode, can upregulate MUC5AC genes in epithelial cells (Temann *et al.*, 1997). MUC5AC upregulation can cause the metaplasia of epithelial cells to goblet cells (Rogers, 1994). However, roflumilast has been shown to inhibit this upregulation in human epithelial cells (Mata *et al.*, 2005).

6.5.5 GENERAL CONCLUSIONS

The fact that roflumilast can inhibit all of the features of asthma assessed in these models suggest they could have both bronchodilator or mast cell inhibiting effects, as they reduce the EAR, and anti-inflammatory effects, as they reduce the LAR, AHR, cellular influx and remodelling. Although roflumilast was unable to completely reverse airway remodelling the administration points must be considered. Roflumilast was only administered before the final OVA exposure in the chronic groups, therefore airway remodelling had probably developed by then suggesting that roflumilast is reversing and not preventing. It is possible that if

roflumilast was given before, or immediately after, every OVA exposure then a greater reduction in airway remodelling and even EAR may be observed. Considering roflumilast was only administered three times throughout the whole study, therefore the effect that it has was very impressive. This explains why there is so much interest in PDE4 inhibitors as a potential alternative to corticosteroids in asthma therapy.

Currently no PDE4 inhibitor has passed phase III clinical trials and therefore there are none commercially available. The reason for this is the side-effects that PDE4 inhibitors cause such as vomiting and nausea. Several approaches have been tried to overcome these side-effects. Administrating lower doses of PDE4 inhibitors have been tried but lack of efficacy was observed (Giembycz, 2008). Another alternative is a different route of administration. Giving a drug orally means that it only reaches the required destination after passing through the stomach, it is possible that it is this fact that causes the side-effects. As a result administration as an inhalation has been trialled though thus far the results have been disappointing (Spina, 2008). Further research has focused on the subtypes of PDE4 suggesting that it is possible that the inhibition of a PDE4 subtype may cause the side-effect to dimish (Spina, 2008).

Chapter 7

Effect of inducible nitric oxide inhibitor treatment on acute and chronic models of asthma

7.1 INTRODUCTION

7.1.1 NITRIC OXIDE

Nitric oxide (NO) is a free radical gas that acts as a multifunctional cell messenger. It is involved in many physiological and pathological processes in the body and has roles ranging from neurotransmission to vasodilation of blood vessels. NO is formed from L-arginine, oxygen and nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) in the presence of NO synthase (NOS) (figure 7.1).

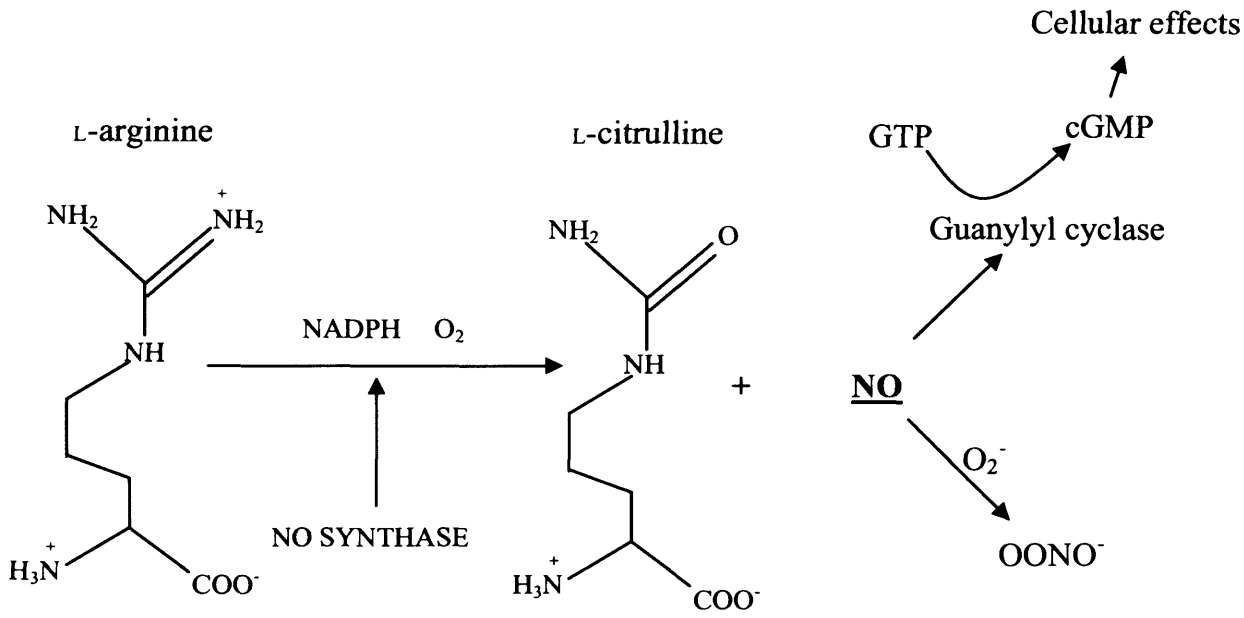


Figure 7.1 – A diagram to show the formation of nitric oxide from L-arginine, NADPH and oxygen catalysed by the enzyme NO synthase and some of the roles nitric oxide can have. NO = Nitric oxide; GTP = Guanosine trisphosphate; cGMP = Cyclic guanosine monophosphate; O_2^- = Superoxide oxygen molecule; $OONO^-$ = Peroxynitrite. Diagram adapted from Hancock (2005).

There are three forms of NOS, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). The enzymes eNOS and nNOS are calcium-dependent and are produced constitutively (Jagnandan *et al.*, 2005), whereas iNOS is calmodulin-independent. The production of new iNOS can be stimulated by the presence of interferon (IFN) – γ, interleukin (IL) – 1 and lipopolysaccharide (LPS) amongst other things (Hancock, 2005).

NO gets released by nerve stimulation of the parasympathetic nervous system (Belvisi *et al.*, 1991). The enzyme guanylyl cyclase is one of the main cellular targets of NO. NO binds to the enzyme to activate it which leads to the production, and subsequent intracellular increase in concentration, of cyclic guanosine monophosphate (cGMP). Normally, cGMP is converted

into guanosine monophosphate GMP by phosphodiesterase V to regulate the intracellular concentration of cGMP (Hancock, 2005). There are a number of cellular effects that an increased concentration of cGMP can cause, such as muscle relaxation.

7.1.2 THE ROLE OF NITRIC OXIDE IN ASTHMA

Increased levels of NO in exhaled air (FE_{NO}) are found in asthmatics compared to healthy individuals (Alving *et al.*, 1993), with the source of NO appearing to be epithelial cells and macrophages (Arock *et al.*, 1994; Warner *et al.*, 1995). However, whether NO has a beneficial or detrimental effect is hotly debated (Mathrani *et al.*, 2007). There is evidence that NO is somewhat of a double-edged sword depending on concentration (Toward & Broadley, 2004). The pro-inflammatory and anti-inflammatory effects that NO can have are shown in table 7.1.

Pro-inflammatory	• ↑ eosinophil infiltration • ↑ vascular permeability • ↑ airway narrowing • ↓ eosinophil apoptosis
Anti-inflammatory	• ↓ leukocyte chemotaxis and function • ↑ smooth muscle relaxation • ↓ mast cell activation • ↑ eosinophil apoptosis
Airway Hyperreactivity	• ↑↓ depending on degree of iNOS activation
Mucus Secretion	• ↑↓ secretion

Table 7.1 - The roles that nitric oxide play in asthma can be both beneficial and detrimental. Table adapted from (Nevin & Broadley, 2002)

Another pro-inflammatory issue that is caused by NO is the formation of peroxynitrite. The reactive oxygen species that are produced from the increased number of leukocytes in the airways react with excess NO to form the tissue damaging agent, peroxynitrite (Saleh *et al.*,

1998). Peroxynitrite has been shown to induce airway hyperresponsiveness in guinea pigs (Sadeghi-Hashjin *et al.*, 1996) and damage pulmonary epithelial cells (Zhu *et al.*, 2000). Peroxynitrite is highly reactive and as a result readily adds a nitro group to tyrosine to form the more stable product 3-nitrotyrosine (3-NT) (Saleh *et al.*, 1998). Lungs from mild asthmatics (Saleh *et al.*, 1998) and those who died of *status asthmaticus* (Kaminsky *et al.*, 1999) showed increased levels of 3-NT compared to non-asthmatics. Peroxynitrite or tyrosine nitration causes several pro-inflammatory factors in asthma such as increased number of prostaglandins, increased levels of NF-κB and steroid resistance (Barnes, 2002). However, the conversion also appears to have anti-inflammatory effects, causes collagen degradation and makes the glucocorticoid receptor steroid sensitive (Barnes, 2002), supplying more evidence for the fact that NO has both beneficial and detrimental roles.

Nitric oxide could play a role in airway remodelling as some of the relevant cellular and molecular events needed for remodelling are induced by NO. Gabazza *et al.*, (2000) described a correlation between nitrite/nitrate concentration and airway wall thickness. As a result of the detrimental effects and the fact that levels of NO have been shown to increase within thirty minutes of an allergen challenge (Mehta *et al.*, 1997) there has been increased research into NO inhibitors as asthma therapeutics.

7.1.3 INDUCIBLE NITRIC OXIDE INHIBITORS AND THEIR ROLE AS POTENTIAL ASTHMA THERAPEUTICS

In order to reduce levels of NO, targeting the production of the molecule is a sound approach. Several drugs have been created that either selectively target each subtype of NOS or target all subtypes in a non-selective manner. As levels of NO increase during asthma iNOS appears to be the main cause of this increase (Mathrani *et al.*, 2007), therefore a great deal of focus has gone into finding more out about the role of iNOS and developing iNOS inhibitors. In order to evaluate the role of iNOS in asthma, knock-out (KO) mice deficient of iNOS have been developed. However, the findings in these mice are just as contradictory as NO itself. A study by Duguet *et al.*, (2001) in KO C57BL/6 mice showed iNOS was pro-inflammatory and pro-eosinophillic. The following year Koarai *et al.*, (2002) showed iNOS had no effect on inflammation or AHR and the year after that Kenyon *et al.*, (2003) showed that iNOS was anti-inflammatory and it in fact reduced AHR. Both studies were also in KO C57BL/6 mice.

The use of iNOS inhibitors has done little to establish whether NO should be a realistic target for anti-asthma drugs. Several studies on mice have been carried out using 1400W, a slow, tight binding, highly selective inhibitor of iNOS (Garvey *et al.*, 1997). Iijima *et al.*, (2001) tested 1400W on male A/J mice and demonstrated iNOS to be pro-eosinophillic but anti-neutrophillic. Koarai *et al.*, (2000) also showed iNOS to be pro-eosinophillic and to increase AHR in BALB/c mice since 1400W inhibited these endpoints. The finding that iNOS increased during airways inflammation induced by allergen was corroborated by Muijsers *et al.*, (2001) using 1400W in male BALB/c mice. However, a study by Richard *et al.*, referenced by Mathrani *et al.*, (2007) showed that iNOS decreased AHR, since 1400W treatment exacerbated the AHR.

GW274150F is a highly selective iNOS inhibitor (Alderton *et al.*, 2005). Recently it was assessed as a potential asthma therapeutic providing further conflicting data on the effectiveness of iNOS inhibitors in asthma. Knowles *et al.*, (2007) showed GW274150F was able to inhibit AHR and total cell infiltration in mice and the LAR in guinea pigs but had no effect on the LAR and eosinophillia in rats. Despite these findings GW274150F went into clinical trials in mild asthmatics and although iNOS was effectively inhibited by GW274150F, there was no effect on AHR or inflammatory cell number (Singh *et al.*, 2007). As GW274150F was able to inhibit AHR and total cell influx in acute asthma models of mice but had no effect on AHR in humans this suggests that either airway remodelling or the vast differences between the pulmonary structure of mice and humans accounts for these findings. If chronic allergen exposure and airway structural changes cause iNOS inhibitors to be ineffective this suggests that the use of acute animal models of asthma for drug research has severe limitations and should be reviewed.

7.2 AIMS AND OBJECTIVES

7.2.1 AIMS

The aim of this chapter was to assess the effect of treatment with an iNOS inhibitor (GW274150F) compared to vehicle treatment on acute and chronic OVA challenged guinea pigs and mice. The parameters investigated were early and late phase bronchoconstriction, airway hyperresponsiveness, cellular influx and lung histology. This chapter will give an indication of how effective this novel compound is against both acute and chronic guinea pig and mouse models.

7.2.2 OBJECTIVES

- To investigate the effect that GW274150F treatment has on acute OVA exposed guinea pigs and mice by measuring lung function, response to histamine and cellular influx compared to vehicle treatment.
- To investigate the effect that GW274150F treatment has on chronic OVA exposed guinea pigs and mice by measuring lung function, response to histamine and cellular influx compared to vehicle treatment.
- To assess bronchiole structure, collagen deposition and levels of goblet cell hyperplasia in GW274150F treated chronic OVA challenged guinea pigs and mice compared to vehicle.

7.3 METHODS

The methods used in the chapter can be found in greater detail in chapter 2. In this chapter six male Dunkin-Hartley guinea pigs weighing 200-250 g and six male BALB/c mice weighing 20-25 g were used. The animals were purchased from Harlan (UK) and housed at Cardiff University.

7.3.1 SENSITISATION

Guinea pigs and mice were sensitised by an intra-peritoneal, bilateral injection of a suspension containing OVA (100 μg) and $Al(OH)_3$ (100 mg) in 1 ml in guinea pigs and 0.25 ml in mice of PBS on days 1 and 5.

7.3.2 OVALBUMIN CHALLENGES

A stainless steel exposure chamber (40 cm diameter, 15 cm height) was used for OVA challenges in both acute and chronic guinea pigs. A Perspex box measuring (38 cm length; 20 cm width; 20 cm height) was used for mice. A Wright nebuliser delivered the OVA or saline at an air pressure of 20 lb p.s.i. and at a rate of 0.3 ml/min. The animals were removed from the chamber if they appeared to be distressed during exposure and the challenge was considered complete.

7.3.2.1 ACUTE PROTOCOL – GUINEA PIGS

On day 15 (14 days subsequent to the first sensitisation injection) the guinea pigs were challenged with OVA (0.01%) for 1 hour.

7.3.2.2 CHRONIC PROTOCOL – GUINEA PIGS

The process begins the same way for the chronic protocol as the acute protocol with a OVA (0.01%) hour long challenge on day 15. Following this on days 17-29 the guinea pigs were challenged with OVA (0.1%) preceded with mepyramine (30 mg/kg) protection. On day 31 an hour long challenge of OVA (0.1%) without mepyramine occurred, this was the day lung function was measured.

7.3.2.3 ACUTE PROTOCOL – MICE

Two OVA (0.5%) exposures lasting an hour each separated by 4 hours were used to challenge the mice in an acute manner. These occurred on day 15.

7.3.2.4 CHRONIC PROTOCOL – MICE

OVA (2%) was again used to challenge the mice. However, to mimic the chronic features of asthma exposures occurred for 30 minutes three times a week for six weeks (18 challenges). On the first day of the seventh week (day 57) the mice were challenged for an hour and lung function measurements were taken immediately following this.

7.3.3 LUNG FUNCTION MEASUREMENTS

Values of sG_{aw} were used as guinea pig lung function measurements. They were obtained by using whole-body plethysmography. Before the final OVA challenge a baseline value of sG_{aw} was recorded, all subsequent values were taken as a percentage of this. After the exposure values were recorded at 0, 15, 30, 45 and 60 minutes, then every hour until 12 hours with a final reading at 24 hours.

To measure lung function in mice values of P_{enh} were used, obtained by using an unrestrained chamber (Buxco). Like with the guinea pigs a baseline value was recorded and all subsequent results were taken as a percentage of this. Values were recorded 0, 20, 40, 60, 90 and 120 minutes post-challenge, then hourly until 10 hours with a final reading at 19 hours for the acute protocol or 24 hours for the chronic protocol.

7.3.4 AIRWAY RESPONSIVENESS MEASUREMENTS

7.3.4.1 GUINEA PIGS

1 mMol of histamine (20 seconds) was used to detect responsiveness in guinea pig airways. Histamine challenges occurred before the first OVA exposure and after the final OVA exposure. Baseline readings of sG_{aw} were recorded, the histamine then was administered via a nose-only inhalation. Readings of sG_{aw} were then recorded 0, 5 and 10 minutes after the histamine challenge.

7.3.4.2 MICE

The effect of methacholine (10 mg/ml for acute challenged, 30 mg/ml for chronic challenged) in mice assessed airway responsiveness. Measurements were taken 24 hours before the first OVA exposure and 24 hours after the final OVA exposure. To detect the presence of AHR 2 minutes of baseline values were recorded, followed by 1 minute of methacholine and then 5 minutes of readings. The post-methacholine values were averaged and taken as a percentage of mean baseline values.

7.3.5 TOTAL AND DIFFERENTIAL CELL COUNTS

7.3.5.1 GUINEA PIGS

Once the final histamine challenge has finished the guinea pigs were euthanised, the trachea cannulated and the lungs removed. Saline was then pumped into the lungs and withdrawn obtaining BAL fluid. A total and differential cell count was then carried out using the BAL fluid.

7.3.5.2 MICE

As the BAL fluid gets contaminated with blood when cannulating the trachea no total cell count can be carried out. The BAL fluid is still used to carry out a differential cell count by centrifuging it onto slides and staining.

7.3.6 DRUG ADMINISTRATION

5 mg/kg of GW274150F was dissolved in methyl cellulose and administered orally via a gavage needle 24 and 0.5 hours before the final OVA exposure and 6 hours post exposure.

7.3.7 HISTOLOGICAL ANALYSIS OF GUINEA PIG AND MOUSE LUNGS

Slices measuring between 3 to 5 mm were cut 1 mm below the bronchus of the left superior lobe and then processed into wax blocks. A microtome was used to cut 6μm sections of the processed lung samples and they were then fixed onto a glass slide. The slides were stained with using either the haematoxylin and eosin, picrosirius red or AB/PAS protocols to stain for general morphology, collagen and mucin associated goblet cells respectively. Using imageJ software the percentage of bronchiolar collagen and number of mucin associated goblet cells were calculated. The peribronchiole, periphery of the small airways, of sections that were stained with haematoxylin and eosin was scored for number of cells present to evaluate whether the number of inflammatory cells differed in the lung tissue of various treatment groups. The parameters for scoring were on a semi-quantitative scale as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5) (Barends *et al.*, 2004).

7.4 RESULTS

7.4.1 THE LUNG FUNCTION OF ACUTE CHALLENGED GUINEA PIGS AND MICE

7.4.1.1 GUINEA PIGS

Figure 7.2 represents the lung function of acute OVA challenged guinea pigs treated with vehicle or GW274150F. The vehicle treated group showed an immediate bronchoconstriction (-66.1±3.0%) which recovered around 4 hours. A bronchoconstriction was also observed immediately after the OVA challenge in the GW274150F treated group (-69.8±2.6%), this resolved around 5 hours. A second bronchoconstriction was observed at 7 hours in the vehicle treated group, however, GW274150F significantly inhibited this (-35.9±6.5% compared to -1.8±3.7% respectively). When comparing maximum bronchoconstriction no significant difference between drug treatment and vehicle occurred during the EAR. GW274150F significantly reduced the LAR compared to vehicle (-5.6±2.8% compared to -35.9±6.5% respectively). Area under the curve analysis is shown in figure 7.3. No significant difference was seen between the drug treatment and vehicle for all time points.

7.4.1.2 MICE

In vehicle and GW274150F treated mice a peak bronchoconstriction was observed 2 hours following a single OVA challenge as shown in figure 7.4 (63.9±2.5% and 65.7±1.9% respectively). Although the values of P_{enh} for both groups do not decline to baseline value they steadily decrease until 7 hours when they both had a second bronchoconstriction. However, this time the GW274150F treatment led to significant inhibition of the bronchoconstriction compared to vehicle treatment (28.1±3.3% compared to 59.9±3.1% respectively). The maximum EAR bronchoconstriction values showed no significant difference between both treatment groups. For the LAR GW274150F significantly reduced the maximum bronchoconstriction values compared to vehicle (34.3±3.6% compared to 60.7±2.8% respectively). Figure 7.5 shows area under the curve analysis for acute OVA challenged, GW274150F or vehicle treated mice. No significant difference was observed between GW274150F and vehicle treatment throughout each point.

7.4.2 AIRWAY HYPERRESPONSIVENESS OF ACUTE CHALLENGED GUINEA PIGS AND MICE

7.4.2.1 GUINEA PIGS

Figure 7.6 shows the response of acute OVA challenged, vehicle and GW274150F treated guinea pigs to histamine (1 mMol, 20 seconds). Vehicle treatment did not prevent a significant bronchoconstriction that was observed immediately following the histamine in the post-challenge compared to pre-challenge (-21.1±3.6% compared to 0.9±1.9% respectively). This bronchoconstriction persisted for 5 minutes after the histamine (-15.2±3.1% compared to -0.1±0.9% respectively). In the GW274150F treated guinea pigs, there was no bronchoconstriction post-OVA challenge.

7.4.2.2 MICE

The effect of 30 mg/ml methacholine on vehicle or GW274150F treated mice pre- and post-OVA challenge is displayed in figure 7.7. AHR was observed in the vehicle treated mice represented by a significant increase in P_{enh} post-OVA challenge compared to pre-OVA challenge (1335.6±97.6% compared to -6.9±2.6% respectively). GW274150F treatment significantly attenuated AHR compared to vehicle treatment (279.6±78.6% compared to 1335.6±97.6%). However, the response to mechacholine in GW274150F treated mice was still significantly greater than the pre-OVA level (279.6±78.6% compared to 2.2±4.2% respectively).

7.4.3 LUNG FUNCTION OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

7.4.3.1 GUINEA PIGS

Figure 7.8 represents the lung function of chronic OVA challenged guinea pigs treated with vehicle or GW274150F. Following the final OVA challenge an immediate bronchoconstriction occurred in both the vehicle (-70.0±2.0%) and GW274150F (-60.6±3.2%) treated groups. These resolved at 6 hours and 5 hours respectively. On the 7 hour mark a second bronchoconstriction was again observed in both the vehicle (-31.2±4.9%) and GW274150F (-32.2±7.5%) treated groups. When comparing maximum bronchoconstriction values no significant difference was seen between vehicle treatment and GW274150F treatment. Area under the curve analysis, represented in figure 7.9, again showed no significant difference between both vehicle and GW274150F treated groups throughout all time points.

7.4.3.2 MICE

In chronic OVA challenged mice an early phase bronchoconstriction was observed after 2 hours following either vehicle treatment (65.7±3.3%) or GW274150F treatment (56.0±4.7%) as shown in figure 7.10. Although this increase in P_{enh} steadily resolved it never reached baseline levels and a second bronchoconstriction was observed after 7 hours in both vehicle treated (61.6±2.0%) and GW274150F treated mice (51.1±5.8%). If the maximum bronchoconstriction values for the EAR and LAR are both analysed then GW274150F treatment caused no significant difference from vehicle treatment. This was also the case when area under the analysis was considered (figure 7.11).

7.4.4 AIRWAY HYPERRESPONSIVENESS OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

7.4.4.1 GUINEA PIGS

Figure 7.12 shows AHR was observed in the vehicle treated group as a significant decrease in sG_{aw} was observed when comparing post-OVA challenge results with pre-OVA challenge results at both 0 (-33.8±5.9% compared to -1.3±2.0% respectively) and 5 (-22.1±1.9% compared to 0.5±1.2% respectively) minutes. GW274150F treatment was unable to prevent this bronchoconstrictor response following histamine (1 mMol, 20 sec) in post-OVA challenged guinea pigs compared to pre-OVA challenged (-27.5±5.6% compared to 0.6±0.5% respectively). This significant bronchoconstriction was still present 5 minutes after the histamine challenge (-10.8±3.6% compared to 0.2±0.8% respectively).

7.4.4.2 MICE

Figure 7.13 represents the effect of challenging vehicle and GW274150F treated mice with 10 mg/ml methacholine before and after repeated OVA challenges. AHR was observed in the vehicle treated group as the mice show a significantly greater level of percentage change from baseline post-OVA challenge compared to pre-OVA challenge (387.5±12.6% compared to -2.2±5.5% respectively).GW274150F was unable to reduces the level of AHR and a significant P_{enh} increase was observed post-OVA challenge compared to pre-OVA challenge (397.7±40.0% compared to -10.5±2.8% respectively).

7.4.5 CELL COUNTS IN ACUTE AND CHRONIC CHALLENGED GUINEA PIGS AND MICE

7.4.5.1 GUINEA PIGS

The effect of GW274150F or vehicle treatment on the BAL cellular level in acute and chronic OVA challenged guinea pigs is shown in figure 7.14. GW274150F treatment significantly reduced the total number of cells compared to vehicle treatment in both acute ($4.4\pm0.5x10^6$ compared to $6.7\pm0.5x10^6$ respectively) and chronic ($8.9\pm0.5x10^6$ compared to $10.9\pm0.3x10^6$ respectively) OVA challenged guinea pigs. In GW274150F treated guinea pigs the total number of cells counted was also significantly greater after multiple OVA challenges compared to a single OVA challenge ($8.9\pm0.5x10^6$ compared to $4.4\pm0.5x10^6$ respectively). In acute OVA challenged guinea pigs significantly more macrophages were counted after GW274150F treatment compared to vehicle treatment ($2.8\pm0.3x10^6$ compared to $1.7\pm0.2x10^6$ respectively), however, the opposite was true for eosinophil number ($1.3\pm0.3x10^6$ compared to $4.9\pm0.2x10^6$ respectively). In the chronic groups GW274150F had no significant effect on eosinophil number compared to vehicle but the level was significantly greater than acute OVA guinea pigs treated with GW274150F ($4.9\pm0.3x10^6$ compared to $1.3\pm0.3x10^6$ respectively). In terms of neutrophil number, GW274150F treatment significantly reduced the number of cells compared to vehicle treatment in both acute ($0.09\pm0.02x10^6$ compared to $0.3\pm0.04x10^6$ respectively) and chronic ($0.1\pm0.03x10^6$ compared to $0.3\pm0.04x10^6$ respectively) OVA challenged guinea pigs.

7.4.5.2 MICE

Figure 7.15 represents the percentage of macrophages, eosinophils, lymphocytes and neutrophils found in the BAL fluid of acute and chronic OVA challenged mice that have been treated with vehicle or GW274150F. No significant difference was observed between either challenge protocol or either treatment throughout all the cellular subtypes.

7.4.6 LUNG HISTOLOGY OF CHRONIC CHALLENGED GUINEA PIGS AND MICE

Figures 7.16 (A-C) show naïve, chronic OVA – vehicle treated and chronic OVA – GW274150F treated guinea pig bronchioles stained with haematoxylin and eosin. This staining shows the general morphology of the bronchioles. The guinea pigs treated with either GW274150F or vehicle both had a thicker lamina propria and epithelium and more smooth muscle than the naïve guinea pigs. GW274150F treatment did not seem to make a great deal

of difference to the bronchiole structure compared to vehicle treatment. Figure 7.17 demonstrates the scored value of inflammatory cells found in the peribronchiolar space, the greater the number the more cells. Treatment with GW274150F has no significant effect on the number of cells compared to vehicle treatment (4.0±0.4 compared to 4.6±0.3). Figure 7.18 (A-C) shows bronchioles from mice which are either naïve or chronic OVA challenged treated with vehicle or GW274150F. The figures show the general morphology of the bronchiole as it has been stained with haematoxylin and eosin. As with the guinea pigs, the mice that were treated with drug or vehicle showed the characteristics of airway remodelling. The stained bronchioles also enabled the number of inflammatory cells in the peribronchiolar space to be scored (figure 7.19). Treatment with GW274150F had no significant effect on cell scored compared to vehicle treatment in chronic OVA challenged mice (4.8±0.3 compared to 4.8±0.3).

Figures 7.20 (A-C) show guinea pig bronchioles stained with picrosirius red to assess collagen deposition. Figure 7.21 represents the percentage of bronchiolar collagen in the same three groups. GW274150F is unable to significantly alter the amount of collagen compared to vehicle (53.3±2.6% compared to 46.7±2.9%). The bronchioles of mice that have undergone the same treatments and staining protocol are shown in figure 7.22 (A-C). The level of bronchiolar collagen in these mice are shown quantitatively in figure 7.23 GW274150F treatment had no effect compared to vehicle treatment on reducing bronchiolar collagen percentage (49.7±7.9% compared to 59.4±2.8%).

The bronchioles of naïve, chronic OVA – vehicle treated and chronic OVA – GW274150F treated guinea pigs are shown in figure 7.24 (A-C). The bronchioles have been stained with alcian blue/periodic acid Schiff to show the amount of mucin associated goblet cells per 10,000 epithelial pixels. The data in quantitative form is shown in figure 7.25. GW274150F treatment had no significant effect compared to vehicle treatment (8.8±1.0 compared to 9.1±1.7). The stained bronchioles of mice that underwent the same protocols are shown in figure 7.26 (A-C). When the mucin associated goblet cells are counted and analysed (figure 7.27) the data show that GW274150F treatment was not significantly different from vehicle treatment (9.7±2.9 compared to 10.3±2.3).

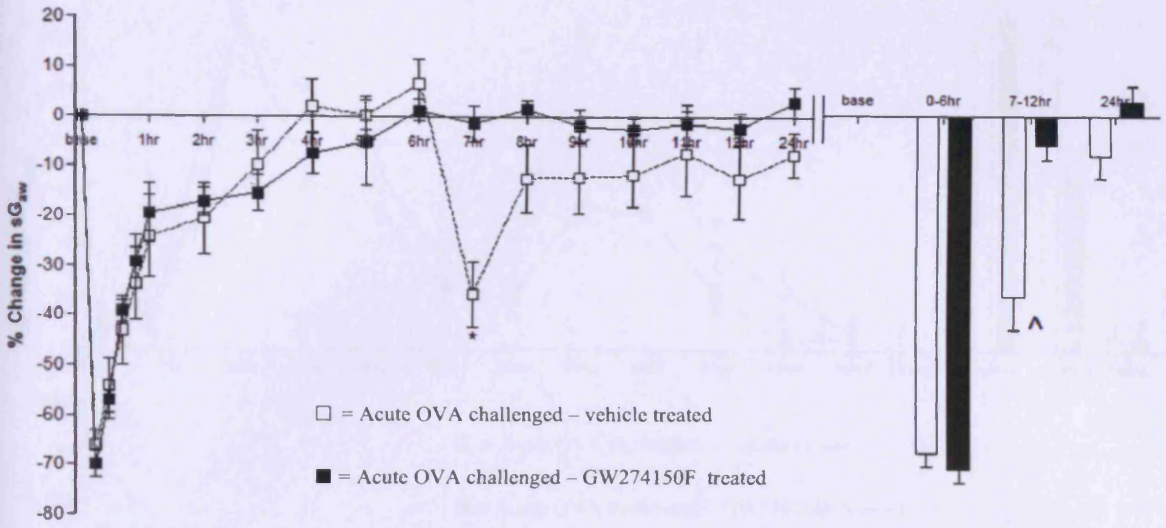


Figure 7.2 – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that were challenged with 0.01 % OVA and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from GW274150F treated guinea pigs; ^ significant difference between vehicle and GW274150F treatment. Two-tailed T-test ($P<0.05$; n=6). Raw GW274150F treatment sG_{aw} values (cm/H_20) – Base (0.80±0.06), 0-6 hours (0.24±0.03), 7-12 hours (0.75±0.04) and 24 hours (0.81±0.04). Raw vehicle treatment sG_{aw} values (cm/H_20) – Base (0.62±0.03), 0-6 hours (0.21±0.02), 7-12 hours (0.40±0.05) and 24 hours (0.57±0.06).

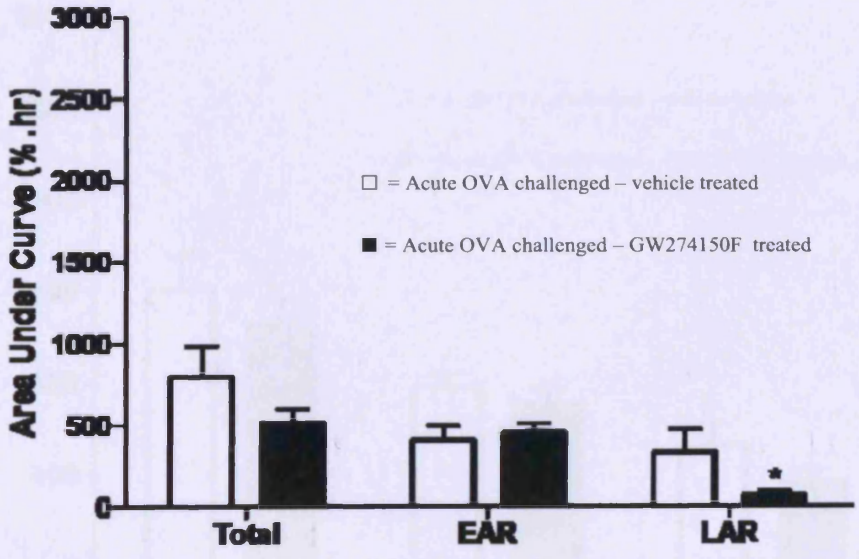


Figure 7.3 – Area under the curve analysis comparing OVA sensitised guinea pigs challenged 0.01% OVA and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

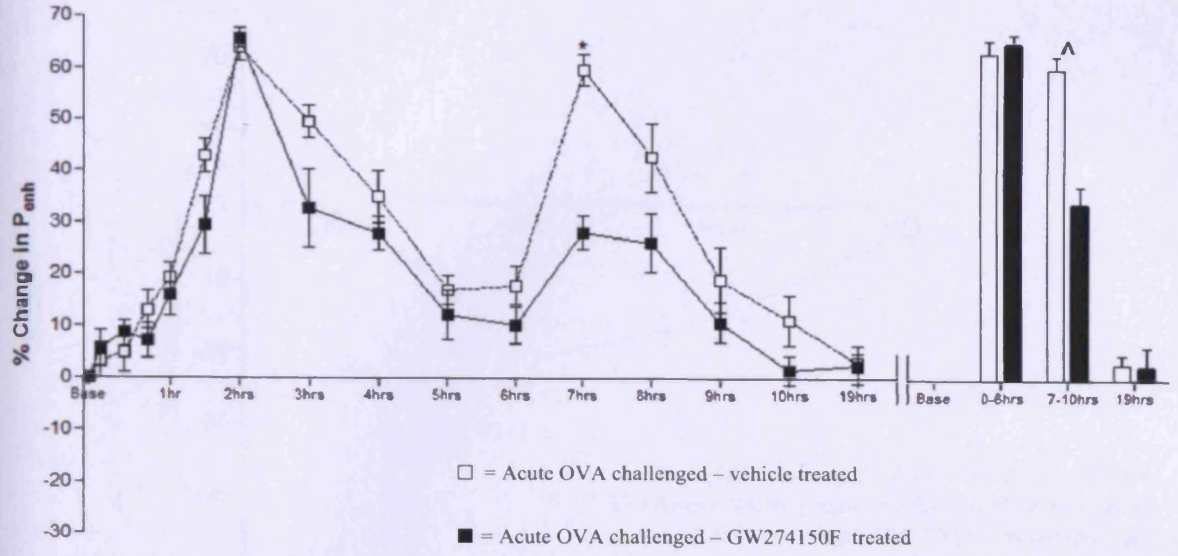


Figure 7.4 – Mean time-course values of P_{enh} in OVA sensitised mice that were challenged twice with 0.5% OVA 4 hours apart and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 19 hours (24 hours after first exposure). Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from GW274150F treated mice; ^ significant difference between vehicle and GW274150F treatment. Two-tailed T-test ($P<0.05$; n=6). Raw GW274150F treatment P_{enh} values (AU) – Base (0.36±0.02), 0-6 hours (0.59±0.02), 7-12 hours (0.48±0.03) and 24 hours (0.37±0.02). Raw vehicle treatment P_{enh} values (AU) – Base (0.30±0.01), 0-6 hours (0.49±0.02), 7-12 hours (0.48±0.02) and 24 hours (0.31±0.01).

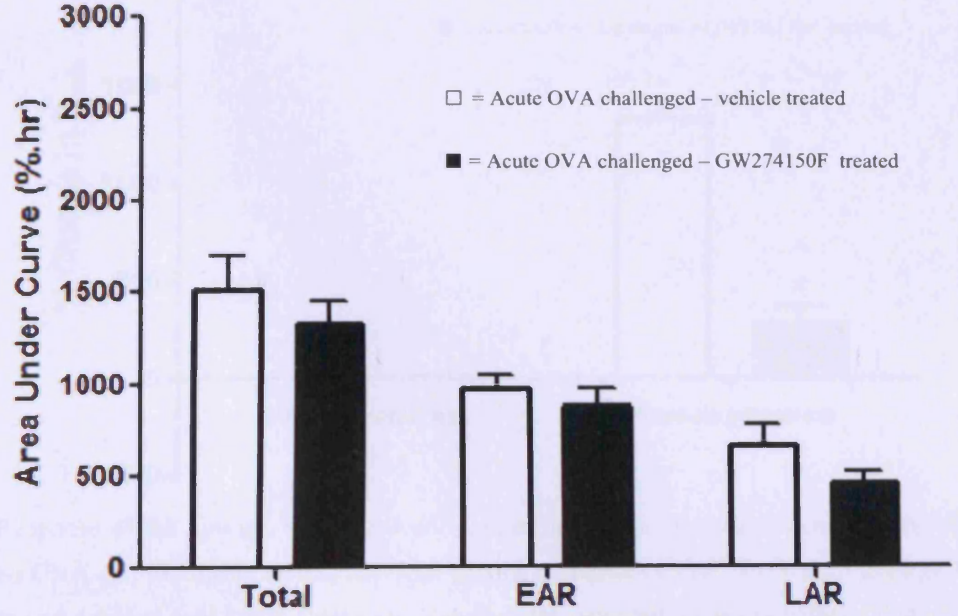


Figure 7.5 – Area under the curve analysis comparing OVA sensitised mice challenged with two doses of 0.5% OVA and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only positive peaks are considered, any peaks that have a negative value of P_{enh} are excluded. Total includes all negative peaks from 0-19 hours, EAR includes from 0-6 hours and LAR includes from 6-19 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

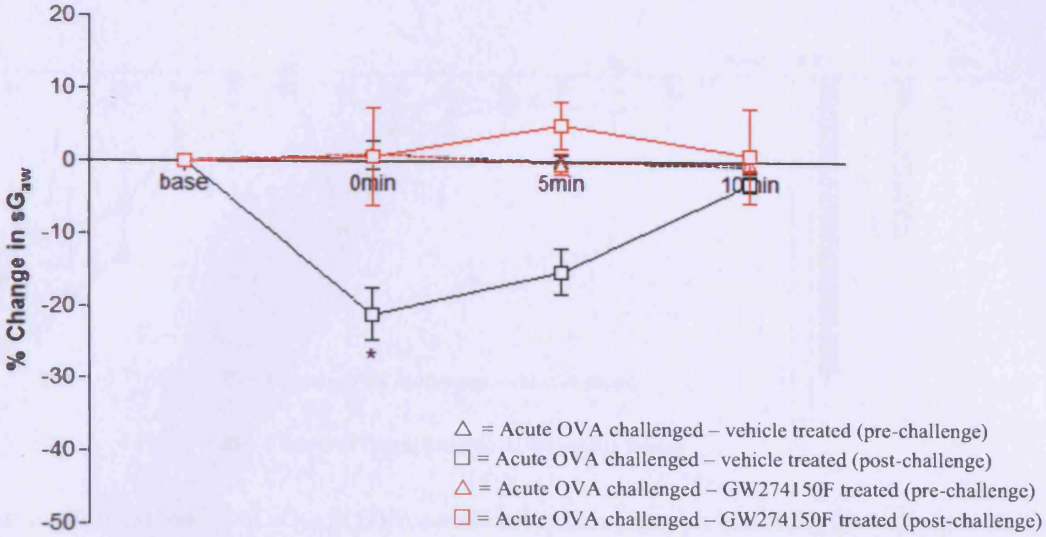


Figure 7.6 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs challenged with OVA (0.01%) and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post-OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

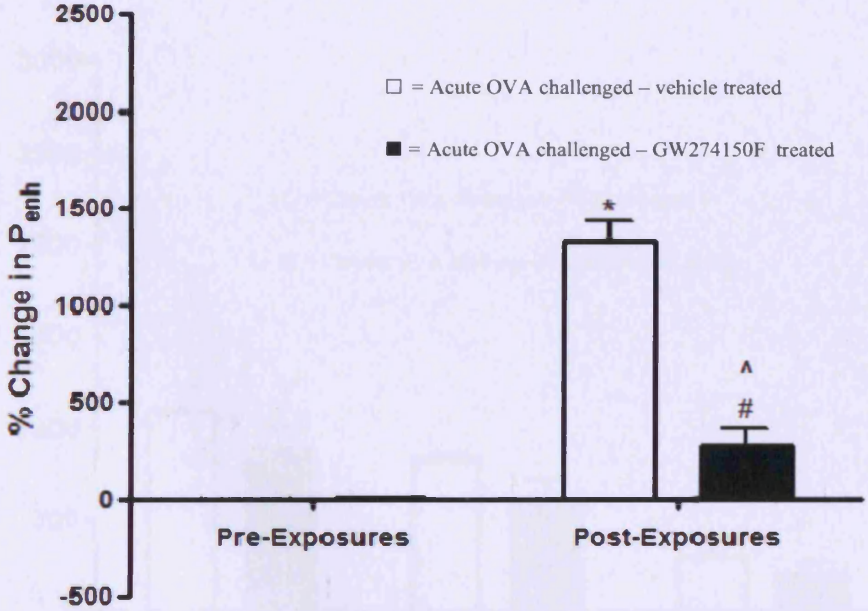


Figure 7.7 – Response of the airways to a 60 second intermittent dose of methacholine (30 mg/ml) in OVA sensitised mice challenged OVA (0.5%) and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post- OVA challenge. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from pre-challenge – vehicle treated; # significantly different from pre-challenge – GW274150F treated ^significantly different from vehicle treatment (post-challenge). One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

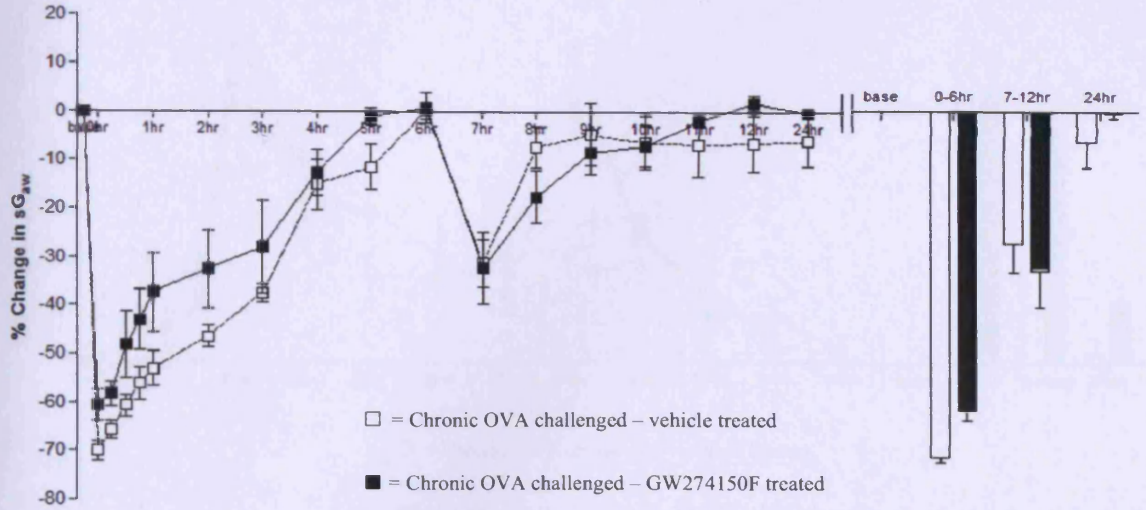


Figure 7.8 – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that had multiple challenges of OVA and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. (n=6). Raw GW274150F treatment sG_{aw} values (cm/H_20) – Base (0.71±0.03), 0-6 hours (0.28±0.01), 7-12 hours (0.48±0.04) and 24 hours (0.71±0.03). Raw vehicle treatment sG_{aw} values (cm/H_20) – Base (0.42±0.03), 0-6 hours (0.12±0.01), 7-12 hours (0.30±0.01) and 24 hours (0.39±0.01).

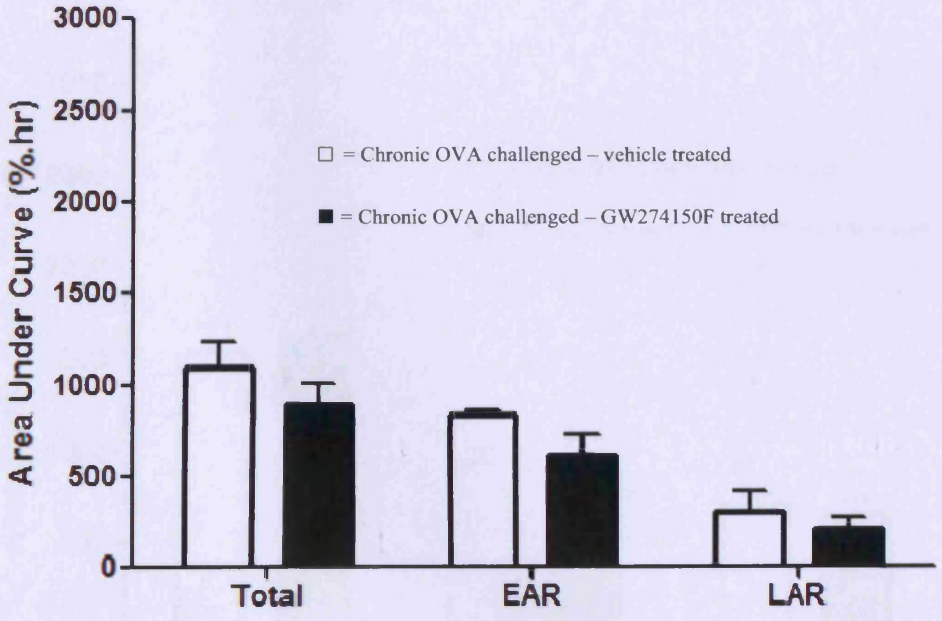


Figure 7.9 – Area under the curve analysis comparing OVA sensitised guinea pigs OVA sensitised guinea pigs challenged with a chronic dosing of OVA and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. (n=6).

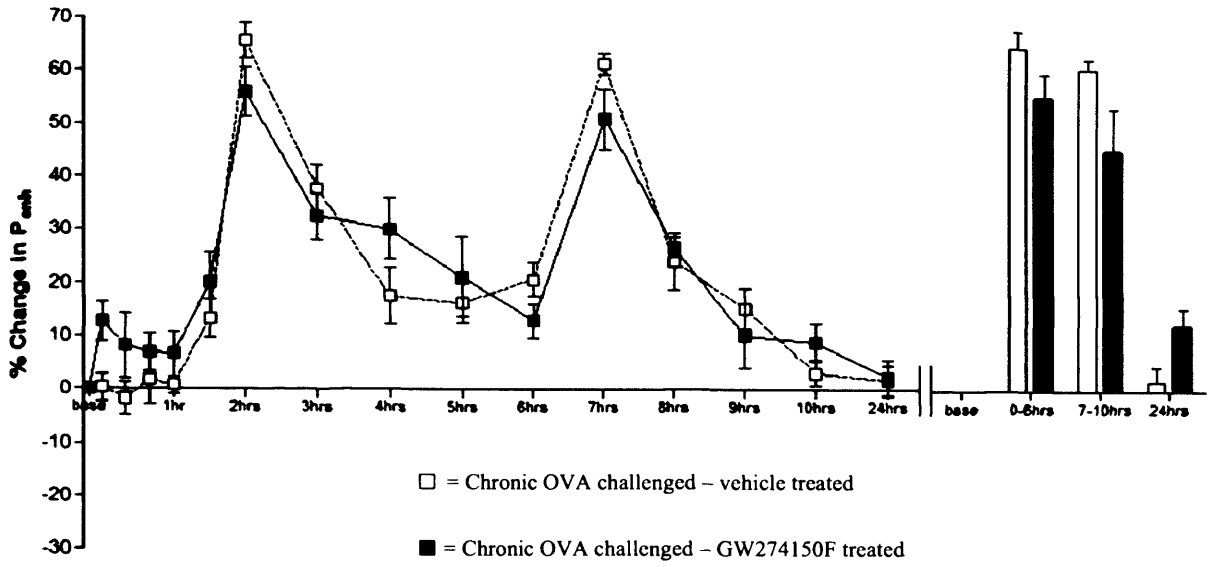


Figure 7.10 – Mean time-course values of P_{enh} in OVA sensitised mice that were challenged repeatedly with 2% OVA three times a week for six weeks and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 19 hours (24 hours after first exposure). Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. (n=6). Raw GW274150F treatment P_{enh} values (AU) – Base (0.34±0.01), 0-6 hours (0.53±0.02), 7-12 hours (0.49±0.03) and 24 hours (0.38±0.01). Raw vehicle treatment P_{enh} values (AU) – Base (0.35±0.02), 0-6 hours (0.58±0.03), 7-12 hours (0.56±0.03) and 24 hours (0.36±0.02).

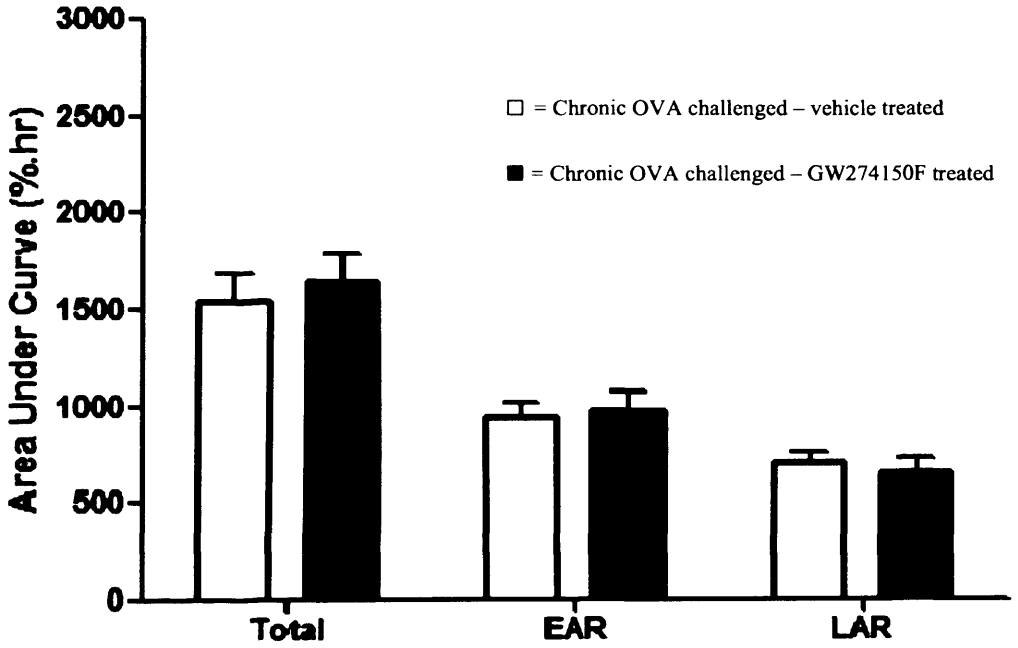


Figure 7.11 – Area under the curve analysis comparing OVA sensitised mice challenged with repeated doses of 2% OVA and treated orally with methyl cellulose or GW274150 (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only positive peaks are considered, any peaks that have a negative value of P_{enh} are excluded. Total includes all negative peaks from 0-19 hours, EAR includes from 0-6 hours and LAR includes from 6-19 hours. Area under the curve is measured in %.hour.(n=6).

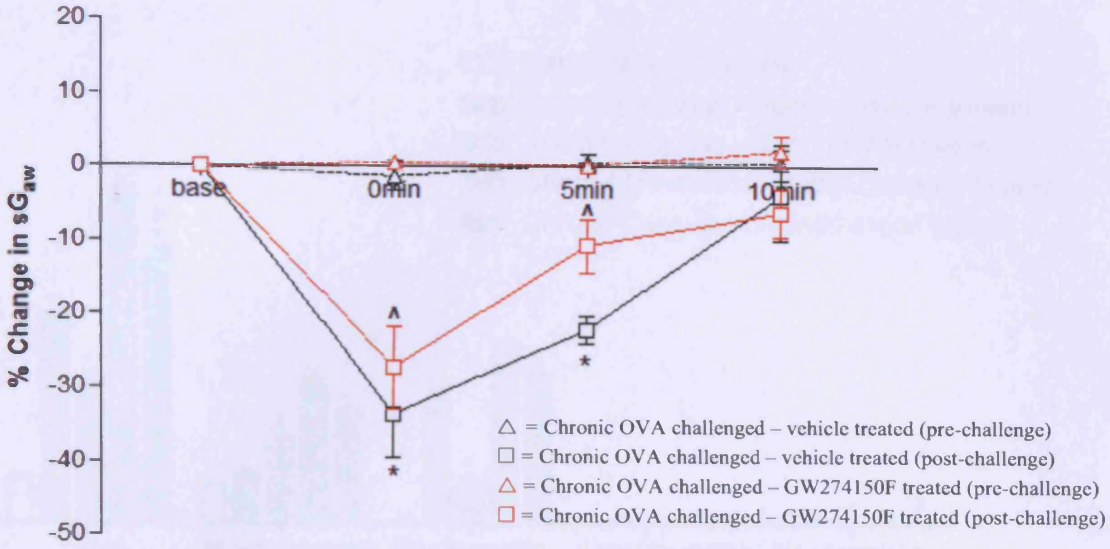


Figure 7.12 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs chronically challenged with OVA and treated orally with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post-OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from vehicle pre-challenge values of sG_{aw}.; ^ significantly different from GW274150F pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

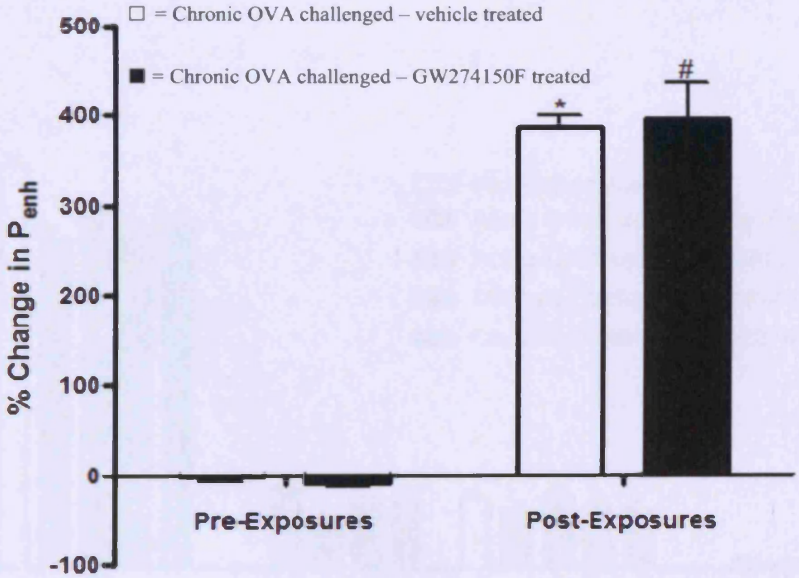


Figure 7.13 – Response of the airways to a 60 second intermittent dose of methacholine (10 mg/ml) in OVA sensitised mice challenged repeatedly with 2% OVA three times a week for six weeks and treated orally with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post- OVA challenge. Mean changes in P_{enh} are expressed as mean±S.E.M. percentage change from baseline where a positive value represents a bronchoconstriction. *significantly different from vehicle pre-challenge; #significantly different from GW274150F pre-challenge P_{enh} value. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

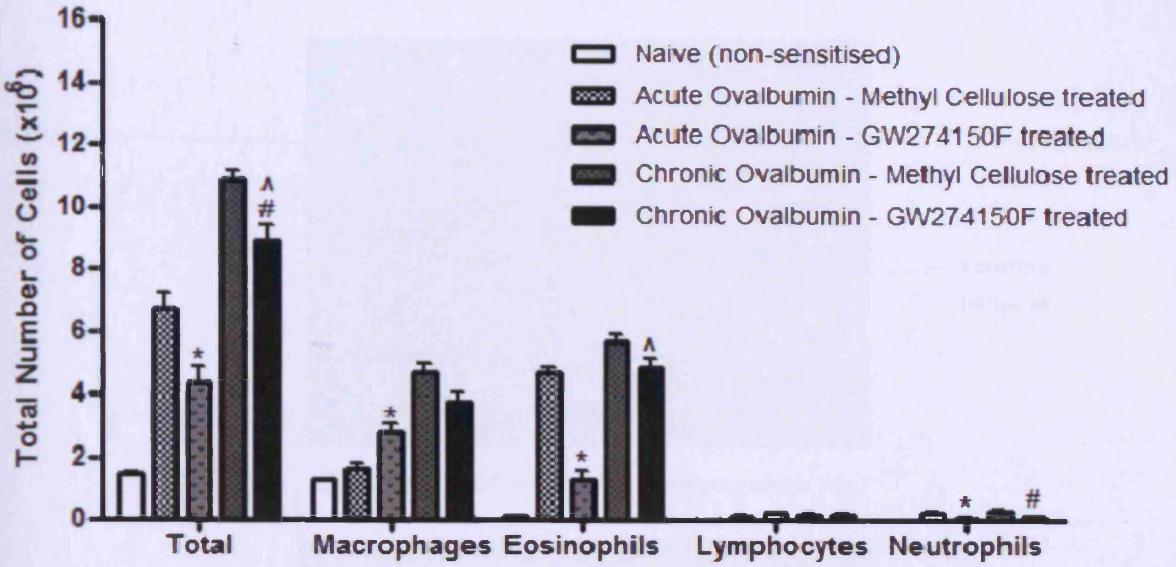


Figure 7.14 – The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised) and OVA challenged (acute and chronic) guinea pigs orally treated with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Results are expressed as mean±S.E.M. *significantly different from acute OVA – vehicle treated; #significantly different from chronic OVA – vehicle treated; ^ significantly different from acute OVA – GW274150F treated. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

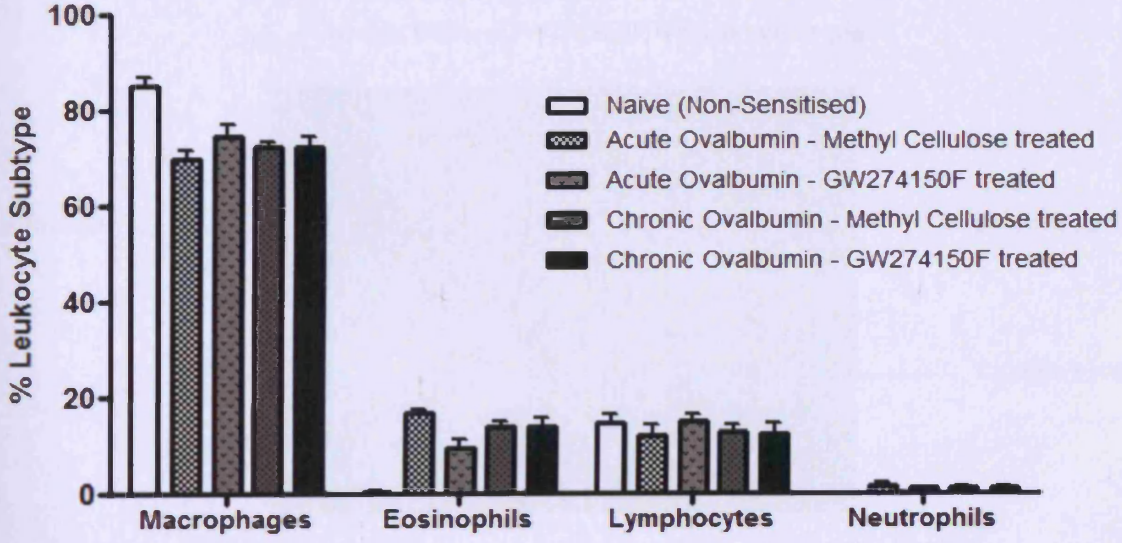
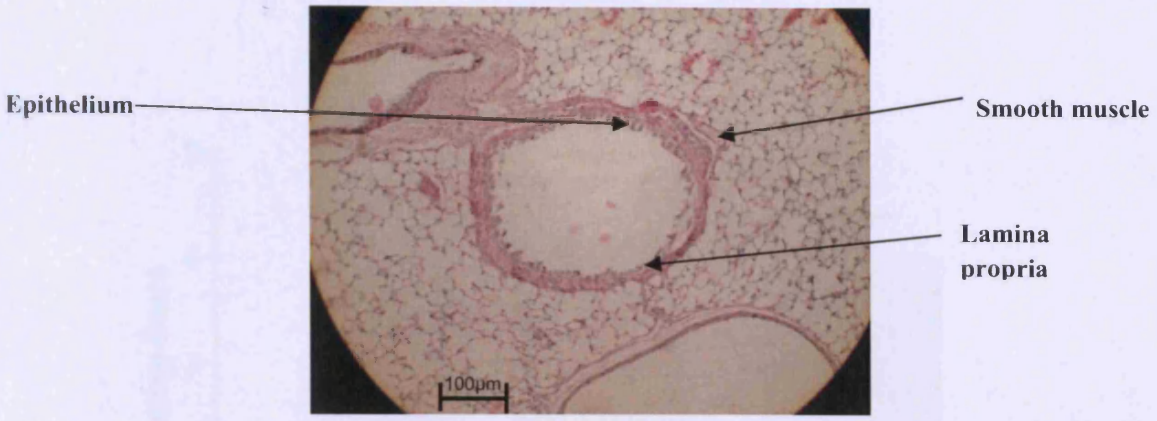
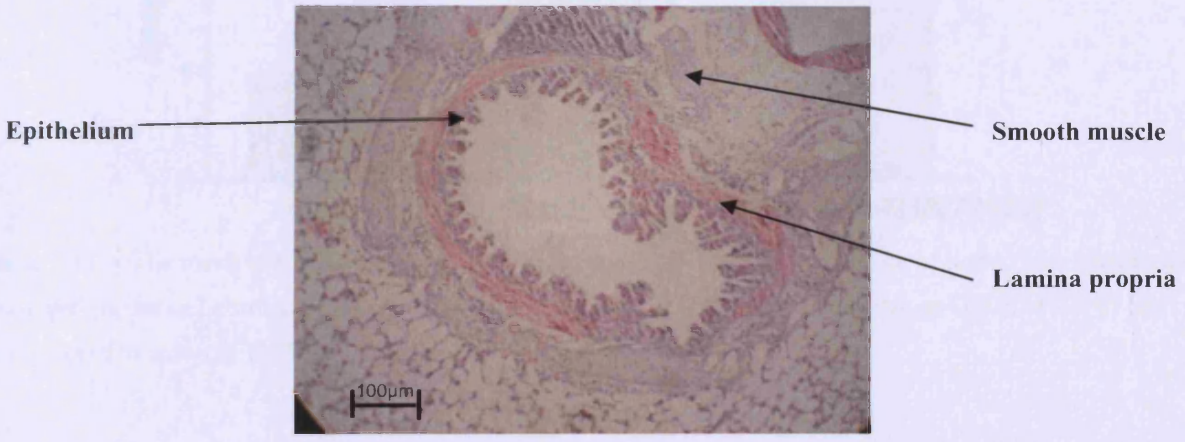


Figure 7.15 – The percentage of macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised) and OVA challenged (acute and chronic) mice orally treated with methyl cellulose or roflumilast (1 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge and OVA challenged (acute and chronic) mice. Results are expressed as mean±S.E.M. (n=6).

A – Chronic saline challenged guinea pig



B – Chronic OVA – vehicle treated guinea pig



C – Chronic OVA – GW274150F treated guinea pig

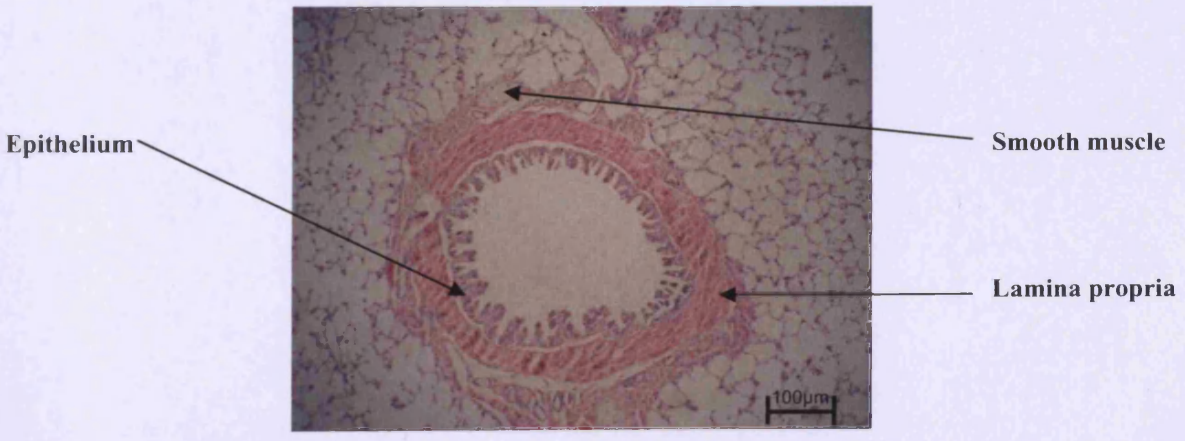


Figure 7.16 (A-C) – Bronchioles of chronic saline challenge, non-treated, guinea pigs and chronic OVA challenged guinea pigs orally treated with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with haematoxylin and eosin to display general morphology (1000x magnification).

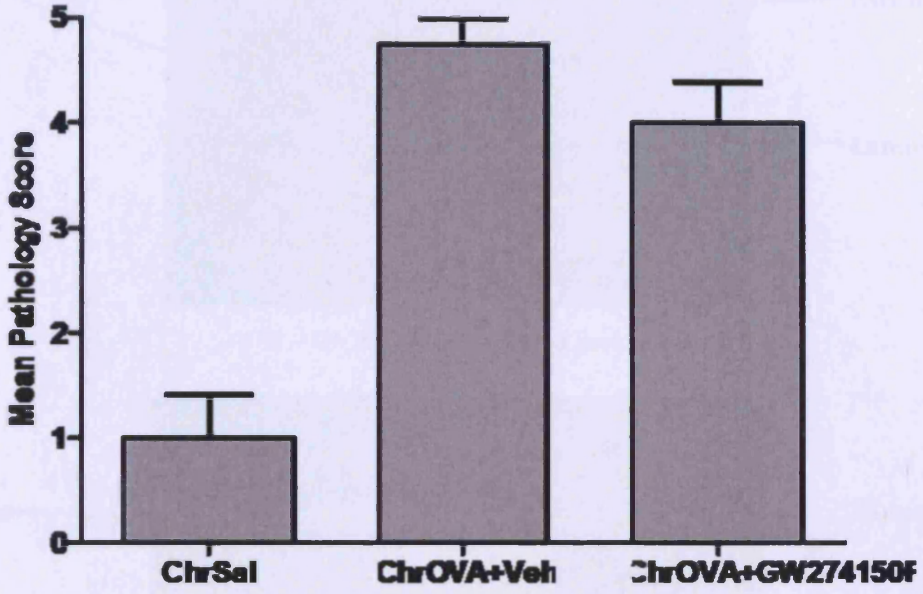
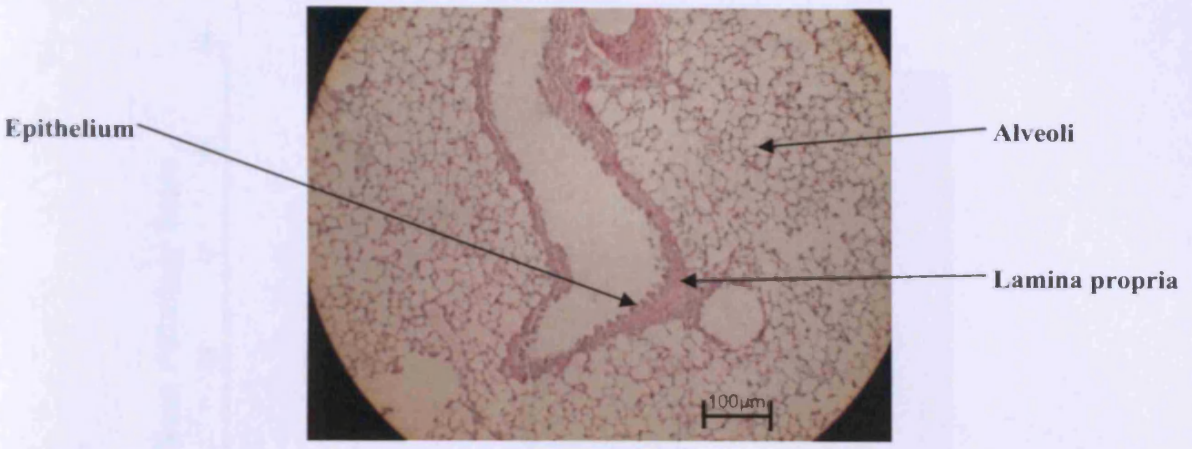
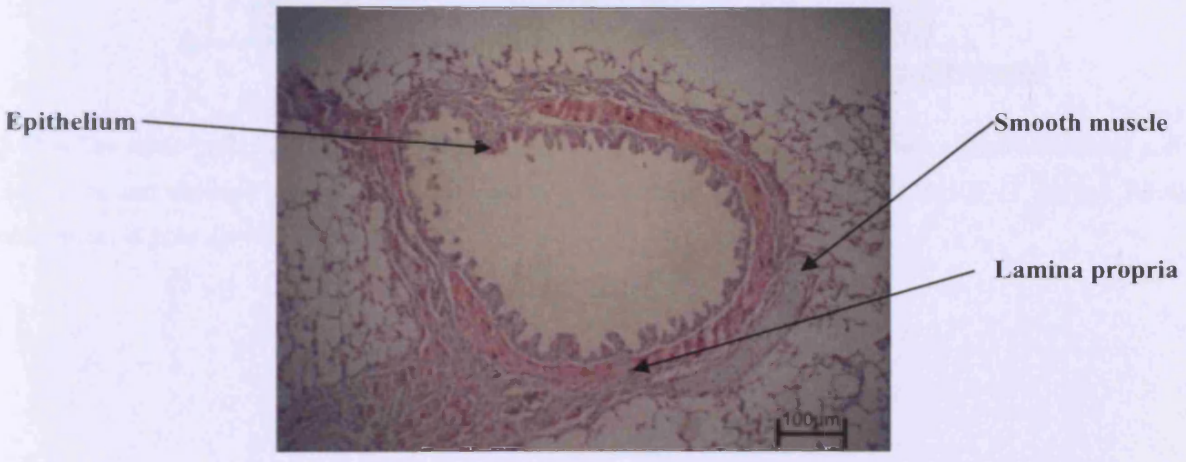


Figure 7.17 – The mean pathology score of cells found in the peri-bronchiolar space of naïve (non-sensitised) and OVA challenged (acute and chronic) guinea pigs treated orally treated with methyl cellulose or GW274150F (5 mg/kg). Results are expressed as mean±S.E.M. (n=4).

A – Chronic saline challenged mouse



B – OVA – vehicle treated mouse



C – OVA – GW274150F treated mouse

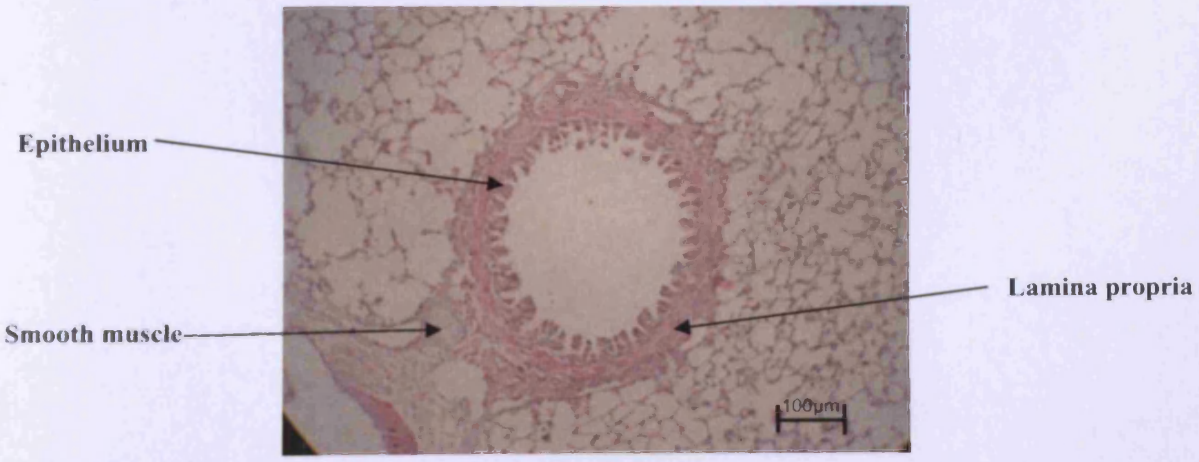


Figure 7.18 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice orally treated with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with haematoxylin and eosin to display general morphology (1000x magnification).

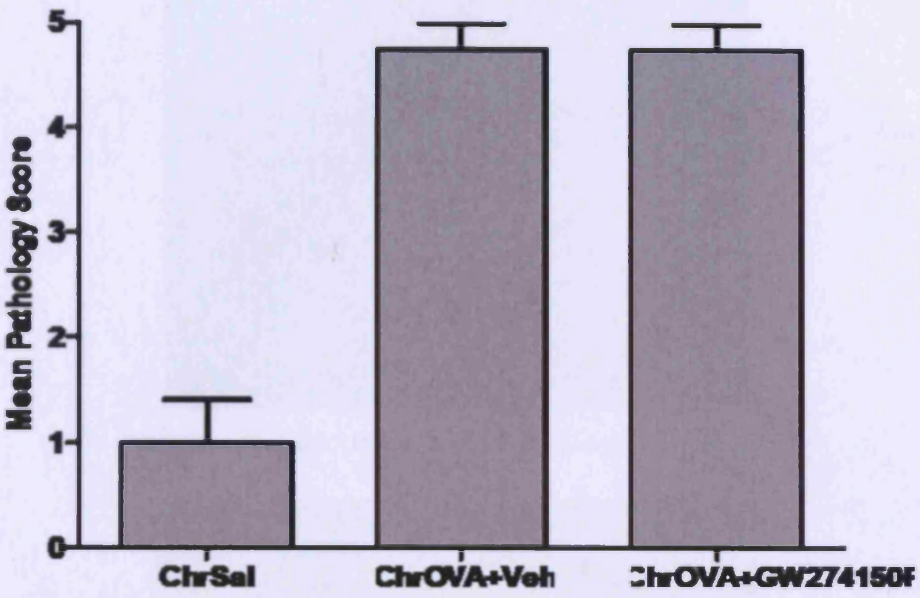
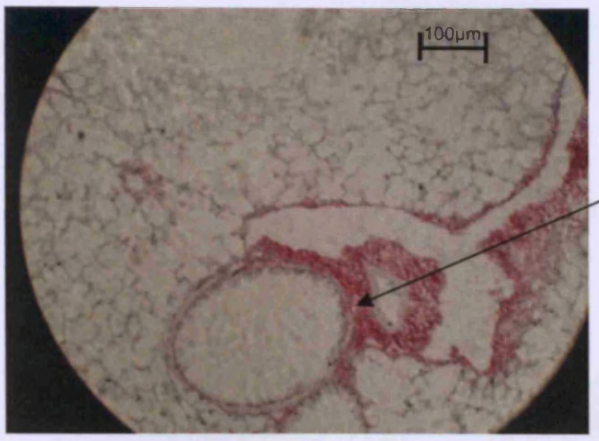
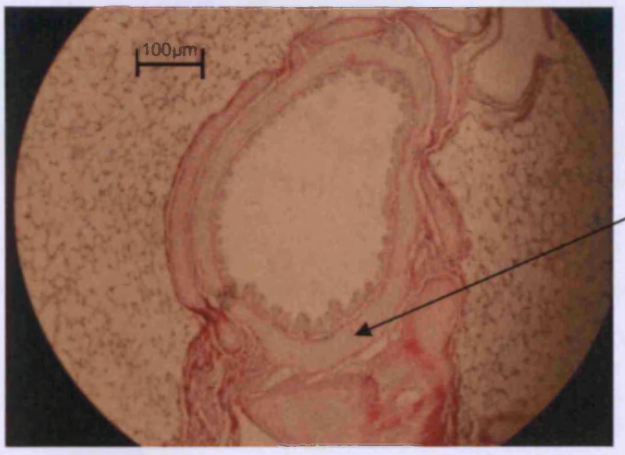


Figure 7.19 – The mean pathology score of cells found in the peri-bronchiolar space of naïve (non-sensitised) and OVA challenged (acute and chronic) mice treated orally treated with methyl cellulose or GW274150F (5 mg/kg). Results are expressed as mean±S.E.M. (n=4).

A – Chronic saline challenged guinea pig



B – Chronic OVA – vehicle treated guinea pig



C – Chronic OVA – GW274150F treated guinea pig

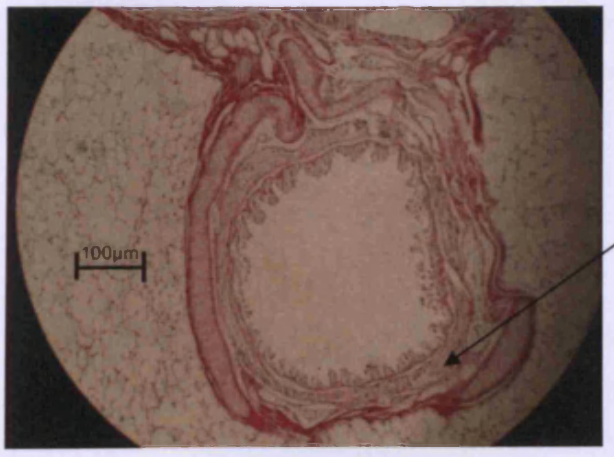


Figure 7.20 (A-C) – Bronchioles of chronic saline, non-treated, guinea pigs and chronic OVA challenged guinea pigs orally treated with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with picrosirius red to display collagen (1000x magnification).

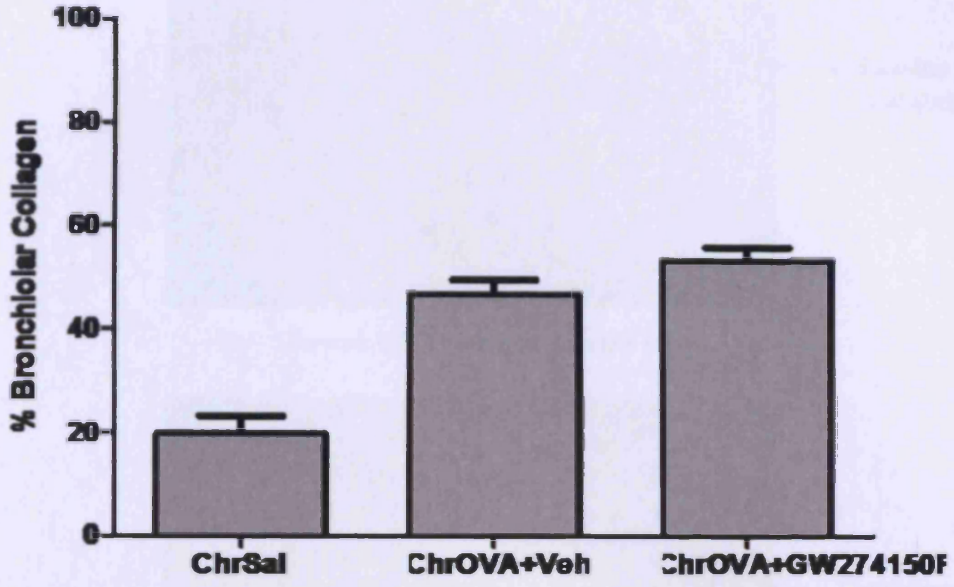
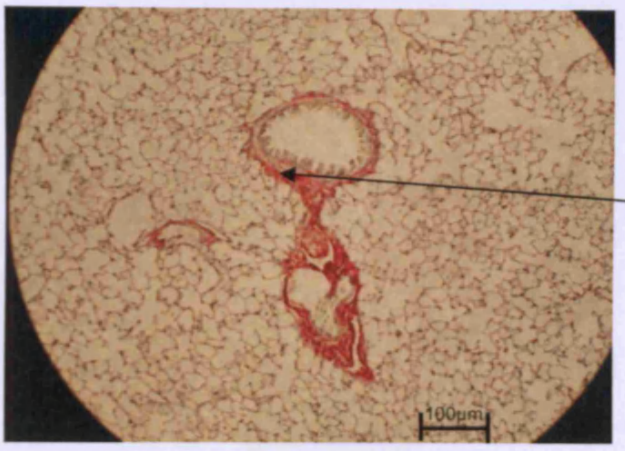


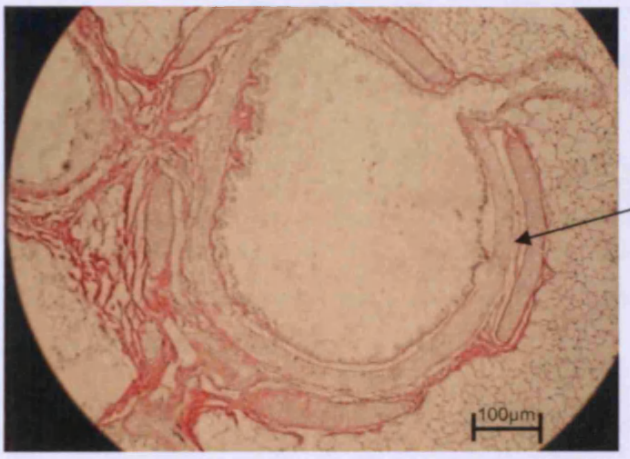
Figure 7.21 – Effect of a chronic OVA challenge and treatment of methyl cellulose or GW274150F (5 mg/kg) administered orally 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on percentage bronchiolar collagen in guinea pigs. Results are expressed as mean±S.E.M. (n=4).

A - Chronic saline challenged mouse



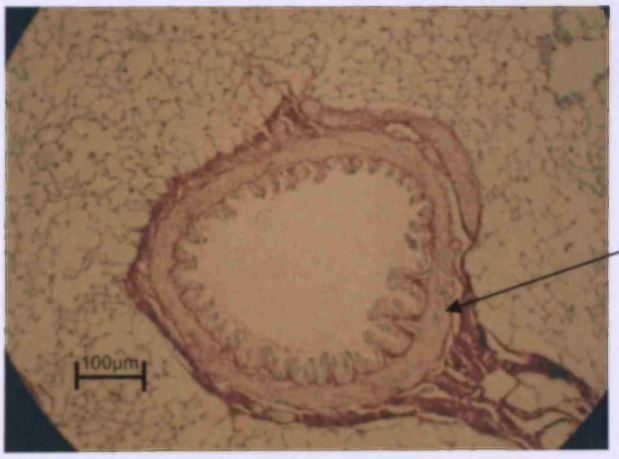
Lamina propria containing collagen

B – Chronic OVA – vehicle treated mouse



Lamina propria containing collagen

C – Chronic OVA – GW274150F treated mouse



Lamina propria containing collagen

Figure 7.22 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice orally treated with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with picrosirius red to display collagen (1000x magnification).

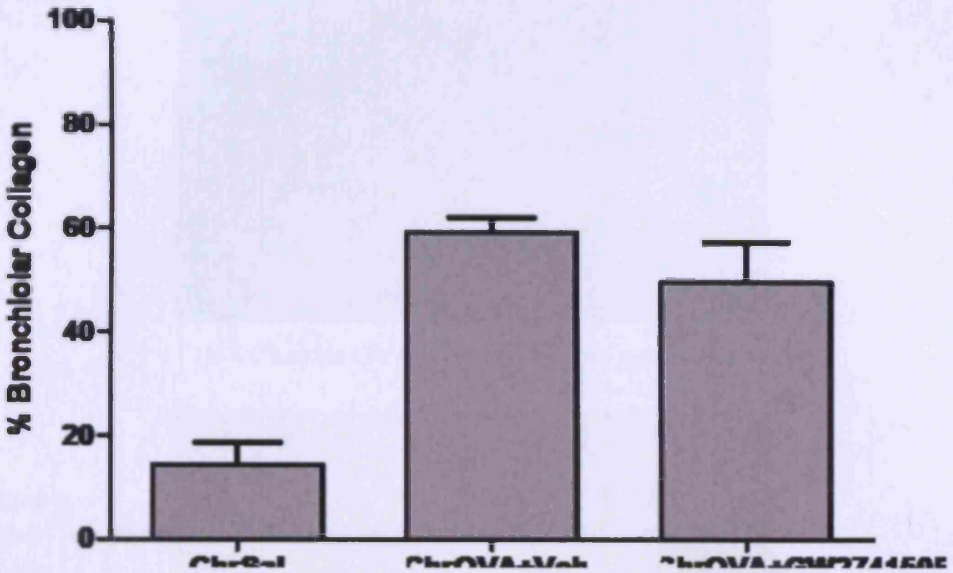
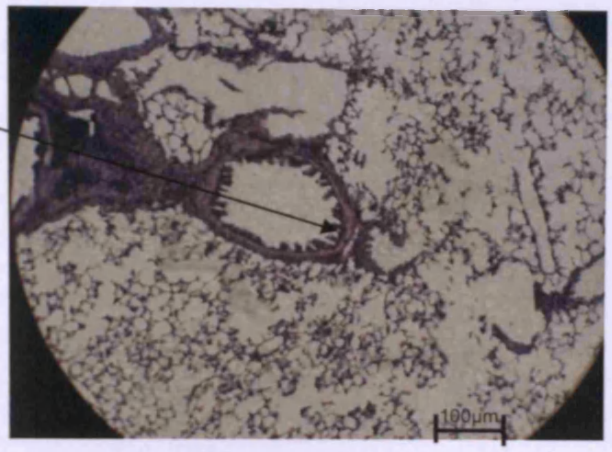
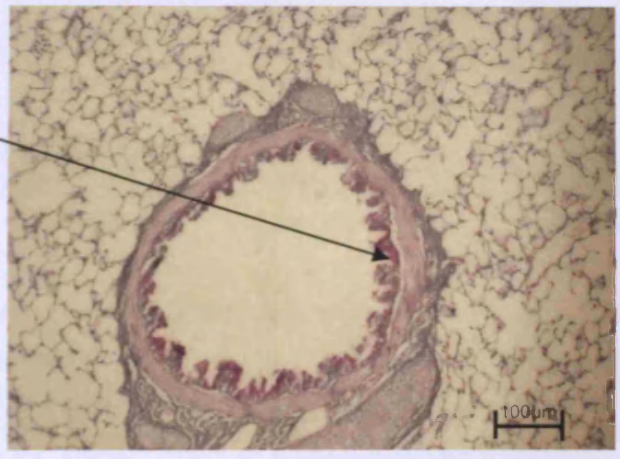


Figure 7.23 – Effect of a chronic OVA challenge and treatment of methyl cellulose or GW274150F (1 mg/kg) administered orally 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on percentage bronchiolar collagen in mice. Results are expressed as mean±S.E.M. (n=4).

A - Chronic saline challenged guinea pig



B – Chronic OVA – vehicle treated guinea pig



C – Chronic OVA – GW274150F treated guinea pig

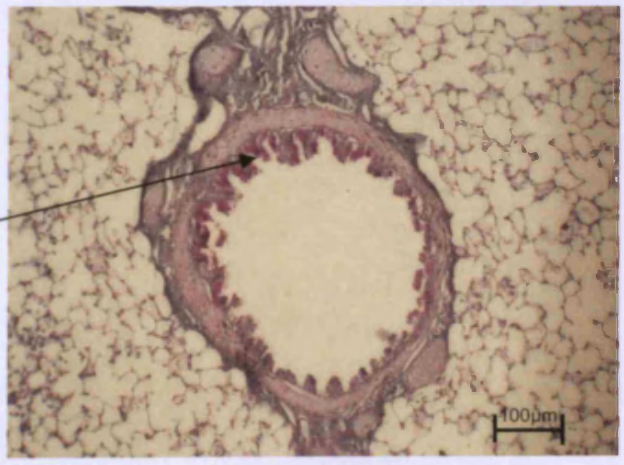


Figure 7.24 (A-C) – Bronchioles of chronic saline, non-treated, guinea pigs and chronic OVA challenged guinea pigs orally treated with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with alcian blue/periodic acid Schiff to display goblet cells (1000x magnification).

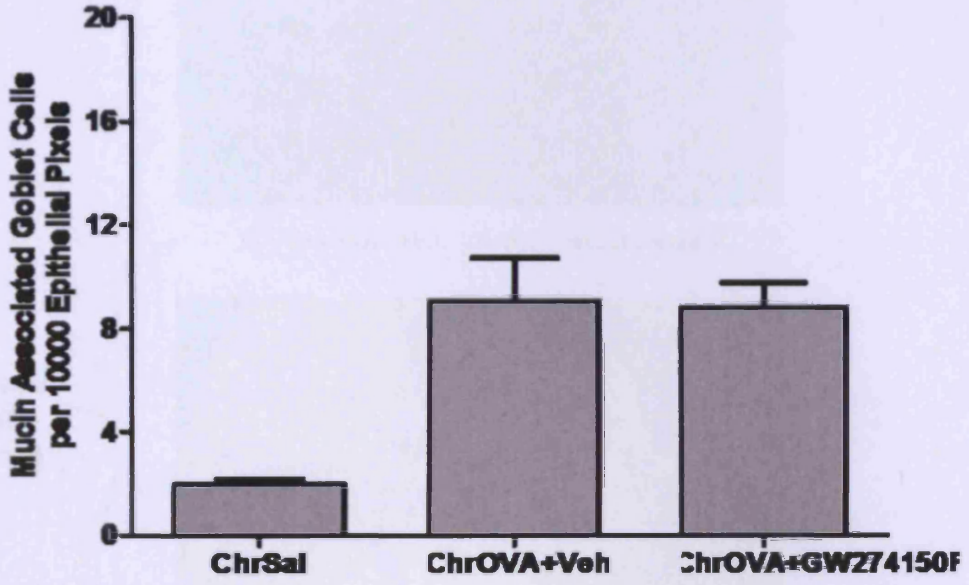
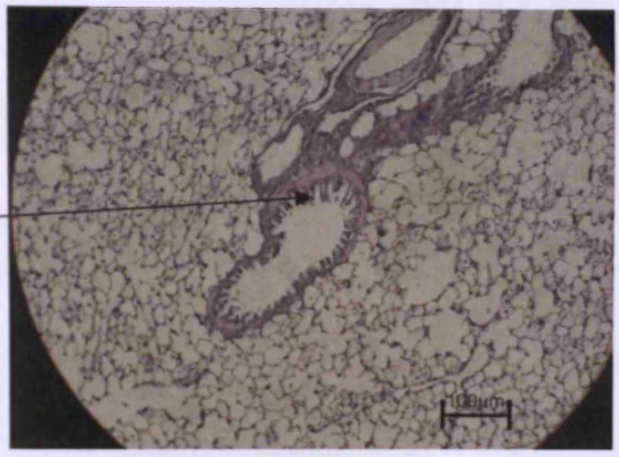
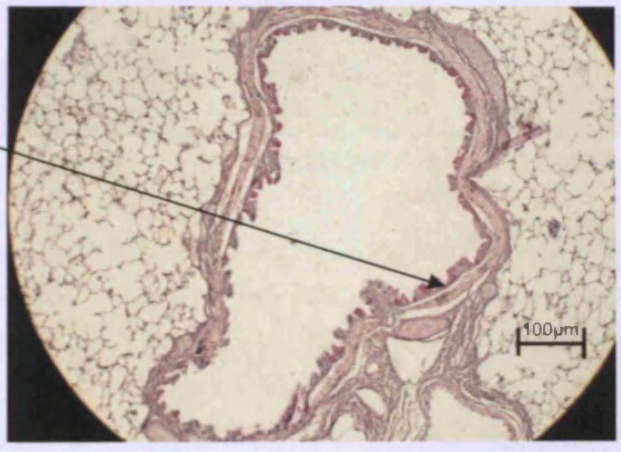


Figure 7.25 – Effect of a chronic OVA challenge and treatment and treatment of methyl cellulose or GW274150F (5 mg/kg) administered orally 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on number of mucin associate goblet cells per 10,000 epithelial pixels in guinea pigs. Results are expressed as mean±S.E.M. (n=4).

A - Chronic saline challenged mouse



B – Chronic OVA – vehicle treated mouse



C – Chronic OVA – GW274150F treated mouse

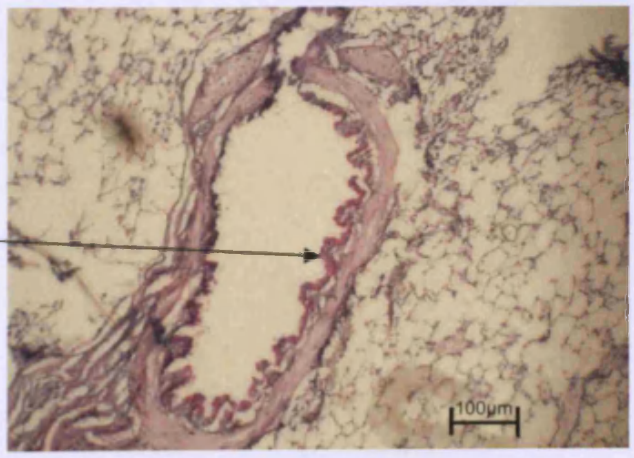


Figure 7.24 (A-C) – Bronchioles of chronic saline, non-treated, mice and chronic OVA challenged mice orally treated with methyl cellulose or GW274150F (5 mg/kg) 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge stained with alcian blue/periodic acid Schiff to display goblet cells (1000x magnification).

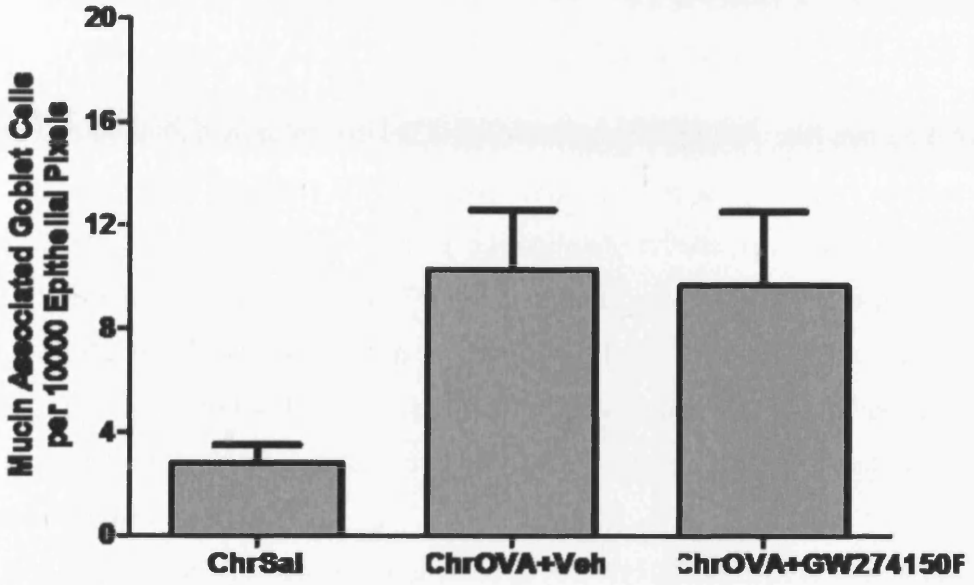


Figure 7.27 – Effect of a chronic OVA challenge and treatment and treatment of methyl cellulose or GW274150F (5 mg/kg) administered orally 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge on number of mucin associate goblet cells per 10,000 epithelial pixels in mice. Results are expressed as mean±S.E.M. (n=4).

7.5 DISCUSSION

7.5.1 EARLY AND LATE ASTHMATIC RESPONSES IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIG AND MICE

Guinea pigs and mice that were sensitised and challenged with OVA still had an EAR despite treatment with the iNOS inhibitor GW274150F. NO can activate guanylyl cyclase which in turn forms cGMP and ultimately results in cellular effects including smooth muscle relaxation. Therefore, the fact that GW274150F had no effect on the EAR was not surprising. Treatment with iNOS inhibitors could in fact be detrimental in terms of the EAR as it could be inhibiting the bronchodilation effect that NO has. However, no evidence for iNOS inhibitors potentiating the EAR was observed in these models suggesting that NO may not be having a bronchodilator effect.

In acute OVA challenged guinea pig and mice GW274150F had some effect on the LAR. When comparing values of sG_{aw} against time for vehicle versus GW274150F it is evident, and statistically shown, that the LAR is attenuated. This was also the case when maximum values of sG_{aw} for the LAR and area under the curve was assessed.. A similar result was observed in acute OVA mice, although it is clear to see that GW274150F is having an effect on the LAR it is not reducing it to basal levels and the area under the curve is not significantly different from vehicle. Previous work with the same iNOS inhibitor showed it could inhibit the LAR in guinea pigs but not rats (Knowles *et al.*, 2007). However, in order for the LAR to be reduced in guinea pigs a dose of 30 mg/kg was used which is a great deal more than the dose used in clinical trials.

GW274150F had no effect on the LAR in the chronic OVA challenged guinea pigs and mice. This suggests that if GW274150F was having an anti-inflammatory effect in the acute model it is lost after airway remodelling has occurred. It appears that although increased levels of nitric oxide are observed in asthmatics it is not nitric oxide that is causing the LAR. Whilst it is possible that nitric oxide is just one of a number of mechanisms involved in the LAR the evidence shown here suggest this role may only be in mild asthma where chronic airway inflammation and remodelling has not occurred. Evidence for this theory is corroborated by the fact that treatment with GW274150F had no effect on the LAR in humans with mild asthma (Singh *et al.*, 2007).

7.5.2 AIRWAY HYPERRESPONSIVENESS IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIG AND MICE

The inhibition of iNOS by GW274150F causes a significant reduction in the AHR observed when acute OVA challenged guinea pigs and mice are treated with vehicle, although in mice the level of P_{enh} is still significantly greater than pre-challenge. These findings suggest that GW274150F may be having an anti-inflammatory effect, possibly by having a protective role on the pulmonary epithelial cells. Studies have shown the iNOS generated NO increases AHR (Komlosi *et al.*, 2006; Muijsers *et al.*, 2001) and treatment with an iNOS inhibitor can inhibit AHR (Knowles *et al.*, 2007). These findings support the findings in the acute OVA challenge groups in this chapter. However, some studies have shown iNOS derived NO decreases AHR (Kenyon *et al.*, 2003) which is in direct contrast to the findings here.

If GW274150F is having a protective effect in acute asthma, that effect is abolished in chronic asthma as in both the guinea pig and mouse chronic OVA model AHR was observed after GW274150F treatment. It appears that the epithelial shedding and influx of inflammatory mediators associated with chronic asthma cannot be reversed or inhibited by GW274150F treatment. AHR was still present in human asthmatics treated with GW274150F, however, the drug was able to reduce levels of NO (Singh *et al.*, 2007). This suggests that either NO is involved in causing AHR in mild asthma but not chronic asthma or GW274150F has an effect on something unrelated to NO in mild asthma which attenuates AHR. Although there is a strong correlation between exhaled nitric oxide and AHR (Downie *et al.*, 2007; Salome *et al.*, 1999) it would appear that NO itself is not a direct cause of AHR.

7.5.3 CELLULAR INFLUX IN ACUTE AND CHRONIC OVALBUMIN CHALLENGED GUINEA PIG AND MICE

Treatment with GW274150F significantly reduced total cell number and eosinophil number in the BAL fluid of acute OVA challenged guinea pigs. However, these reductions were still greater than naïve levels. The reduction in eosinophils by GW274150F is likely to explain why a reduction in LAR and AHR was observed in this model. GW274150F treatment appeared to cause an increase in macrophages this could be a result of the fact that NO can reduce leukocyte chemoattraction and function (Nevin & Broadley, 2002). In the chronic OVA challenged guinea pigs there was a slight reduction in total cell count after GW274150F

treatment. It is likely that this was caused by the significant reduction in neutrophils that was also observed. A reduction in neutrophils after iNOS inhibitor treatment was also observed in the acute guinea pig model and has been shown previously in the literature in Candida-induced acute lung injury (Ohsugi *et al.*, 2007).

No significant difference was observed in eosinophil numbers between the vehicle and GW274150F treated chronic OVA challenged guinea pigs. This could explain why the iNOS inhibitor was unable to have an effect on the LAR or AHR in the chronic guinea pig model. The fact that GW274150F was unable to inhibit eosinophillia in the chronic model but was in the acute model resulted in significantly greater levels of total inflammatory cells and eosinophils in the BAL fluid of the chronic group.

In both acute and chronic OVA mice GW274150F was unable to significantly alter the percentage of inflammatory cells in the BAL fluid compared to vehicle treatment. This could be misleading as total cell counts were not carried out. Judging by the fact that GW274150F inhibited the LAR and AHR in the acute guinea pigs and significantly reduced eosinophil numbers in guinea pigs suggests that it may in fact be reducing eosinophil influx in acute OVA challenged guinea pigs. Other studies in mice have shown that iNOS is pro-eosinophillic (Iijima *et al.*, 2001; Koarai *et al.*, 2002) further corroborating this theory. As GW274150F was unable to attenuate the LAR and AHR in chronic OVA challenged mice suggests that it is having no effect on eosinophil influx. If GW274150F had a pronounced effect on total cell count in chronic challenged models it would show in the lung histology (discussed in the next section). In human studies GW274150F had no significant effect on inflammatory cell number and differential cells numbers (Singh *et al.*, 2007). There was no significant reduction in neutrophil number as observed in the guinea pig model. This could either be a result of the difference between humans and guinea pigs or it could be a dosing effect as in the human studies dosing lasted 14 days.

7.5.4 LUNG HISTOLOGY IN CHRONIC OVALBUMIN CHALLENGED GUINEA PIG AND MICE

Quantifiably it is quite clear to see that GW274150F has little if no effect on the structural changes in the airways that are caused by chronic OVA challenge. Therefore it is no surprise to see that GW274150F has proved a poor asthma therapeutic in the chronic OVA guinea pig

and mouse models and also in human asthmatics. Several components of airway remodelling such as increased airway vascularity (Orsida *et al.*, 1999) and airway wall thickness (Kasahara *et al.*, 2002) have been suggested to contribute AHR. This could explain why GW274150F was able to inhibit AHR in the acute models but not in the chronic models.

When the number of inflammatory numbers in the peribronchiolar space were scored GW274150F had no significant effect on score compared to vehicle in both the guinea pigs and mice. In guinea pigs this does not correlate with what was observed in the BAL fluid as a slight decrease in total cell number was seen, however, it may account for why the LAR and AHR was not inhibited by GW274150F. No significant differences were observed in cell number in the chronic challenged mice compared to vehicle in both the BAL fluid and peribronchiolar space. This suggests that either iNOS is not a pro-eosinophillic as some studies suggest (Iijima *et al.*, 2001; Koarai *et al.*, 2002) or that GW274150F is unable to inhibit the cellular effects of iNOS.

No significant change was observed in collagen deposition or number of mucin associated goblet cells in the GW274150F stained bronchioles compared to vehicle. If the iNOS inhibitor is having no effects on the key components of airway remodelling then it is understandable why the drug did not work in clinical trials. There is evidence that the iNOS inhibitor 1400W is able to attenuate airway remodelling (Starling *et al.*, 2009) suggesting this drug may have some potential as a future asthma therapeutic. However, this study was carried out in lung strips from guinea pigs and may not be reproducible *in vivo*. Despite that it is also possible that iNOS may be a feasible target and GW274150F is unable to prevent the effects of iNOS activation. This theory regarding GW274150F is supported by the findings that there was no reduction in 3-NT in GW274150F treated asthmatics compared to placebo (Singh *et al.*, 2007).

7.5.5 GENERAL CONCLUSIONS

The role of NO in the airways is somewhat of an enigma, there is evidence that it can be both pro-inflammatory and anti-inflammatory (Nevin and Broadley 2002). It is possible that the effect of NO is dependent on whether it is coming from iNOS or eNOS. Another explanation is that the effect of NO could be dependent on the dose present, i.e. the higher the dose the more damage it causes to the airways. Despite the confusing nature of NO it is known that

asthmatics exhale more NO than non-asthmatics and therefore there has been suggestions that reducing levels of NO could help alleviate some of the symptoms of asthma (Singh *et al.*, 2007; Mathrani *et al.*, 2007). Inhibitors of NO synthase, the enzyme that catalyses the formation NO, have been evaluated in animal models of asthma with mixed results (Mathrani *et al.*, 2007). GW274150F, an inhibitor of iNOS that had relatively promising results in animal asthma models was entered into clinical trials but was not effective in human asthmatics.

GW274150F was able to inhibit the LAR, AHR and some cellular influx in acute OVA models of asthma, however, it had little effect in the chronic OVA models. It is likely the main causes of the failure of GW274150F in the chronic model was that it was unable to inhibit eosinophil influx and airway remodelling. In the chronic model airway remodelling is allowed to develop before any drugs are administered. Therefore it is possible that although GW274150F is unable to reverse airway remodelling it could prevent it if administration occurred before each OVA challenge. However, fluticasone propionate and roflumilast were both administered at the same point in the protocol as GW274150F and these drugs were able to reverse airway remodelling and other features associated with asthma. Some subject will already have some degree of airway remodelling before treatment is administered, as a result a drug which can reverse airway remodelling would be favoured over a drug that may be able to prevent exacerbation.

This study proves that the chronic OVA model of asthma is a much closer representation of human asthma than the acute OVA model. GW274150F treatment had the same outcome in the parameters that were assessed in the chronic model and humans with mild asthma with the exception of total cell influx in the guinea pigs. GW274150F appeared to be quite an effective anti-inflammatory in the acute OVA model and as such a potential asthma therapy. These results were therefore misleading. Using the chronic OVA model, although time consuming and originally more expensive, could prove cost effective in the long-term as it would be unlikely that a drug that fails in chronic OVA animal models would work in humans or be put into clinical trials.

Chapter 8

MRI analysis of acute and chronic models of asthma

8.1 INTRODUCTION

8.1.1 MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging (MRI) is a non-invasive technique which is used extensively for imaging areas of the human body such as the brain. MRI uses magnetic fields and electromagnetic radio frequency waves to produce anatomical images with excellent contrast between tissues, water and fat. MR imaging has also been used in research involving animal models because it is a non-invasive technique (Kraft *et al.*, 2004; Tigani *et al.*, 2007) and is a useful tool to investigate inflammation.

8.1.1.1 THEORY OF MRI

Conventional MRI detects protons between tissue, water and fat. Protons are positively charged particles found in the nucleus of an atom. When a subject is placed in a MRI scanner the magnetic force causes the protons to align themselves with a static magnetic field (B_0), with the magnet either in a parallel or anti-parallel nature. As parallel alignment requires less energy than anti-parallel there are more parallel aligned protons than anti-parallel. Protons are always oscillating, when an external magnetic force is applied the protons move in a cone shape, a process called precessing (figure 8.1). The faster a proton precesses the higher the precession frequency. A stronger magnetic current causes a higher precession frequency and therefore the protons precess quicker. The precession frequency can be calculated using the Larmor equation:

$$\omega_0 = \gamma B_0$$

The precession frequency (ω_0) is equal to the gyromagnetic ratio (γ) which in protons is 42.5 MHz/T multiplied by the strength of the external magnetic field (B_0).

As protons precess very quickly there are magnetic forces in opposing directions that cancel each other out. However, as there are more parallel aligned protons these are left over. Protons have components along x, y and z axis and as a result parallel aligned protons that are precessing the left of the cone can cancel out protons precessing to the right of the cone. The only direction which protons cannot be cancelled out is in the direction of the external magnetic force (x-axis). Here all the magnetic forces add up to make a magnetic vector in the external magnetic field direction. This is known as longitudinal magnetisation (figure 8.1).

However, in cases where the nuclei have even number of protons it would be possible for all protons to cancel each other out and longitudinal magnetisation would not occur. Therefore in MRI the best nuclei to use are those with an odd amount of protons. As hydrogen only has one proton and is abundant throughout the body it is commonly used for MRI.

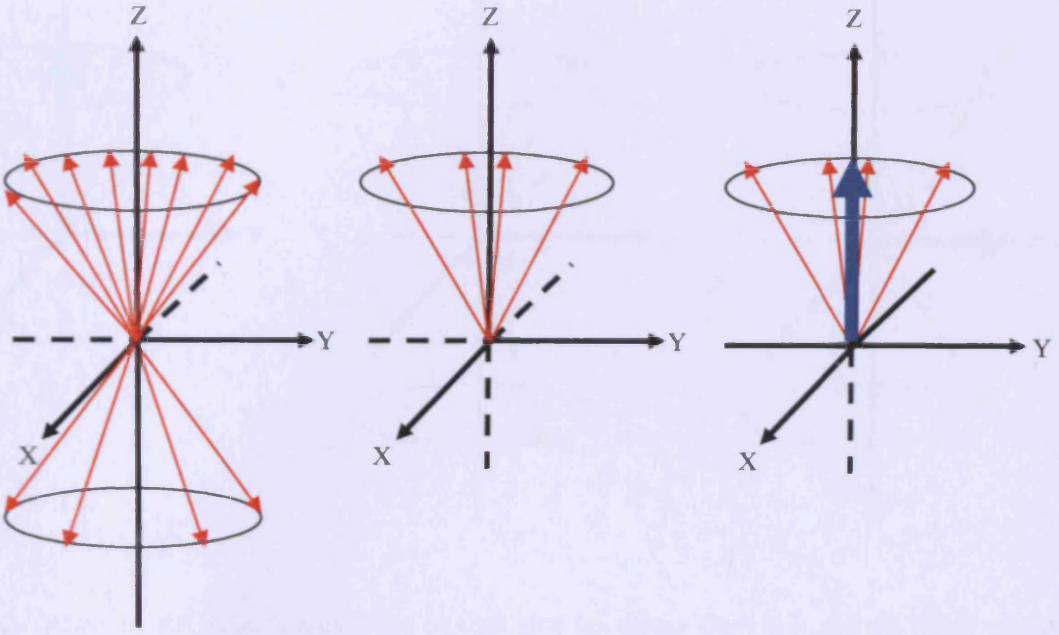


Figure 8.1: Protons are spinning in a cone along the z-axis, a process called precessing. More protons precess in a parallel direction as this requires less energy. The protons that precess in an anti-parallel direction get cancelled out by those in a parallel direction. The parallel protons still have forces along the y and x axis, these become cancelled out by corresponding parallel protons leaving magnetic force in the z-axis direction. This is known as longitudinal magnetisation. Diagram adapted from Schering (1990).

The longitudinal magnetic forces cannot be measured as it is parallel to the external magnetic field, therefore a radio wave is sent into the MRI machine. The radio wave is a short burst of an electromagnetic wave known as the RF (radio frequency) pulse. The purpose of the RF pulse is to disturb the precessing protons. In order for the RF pulse to do this it needs to have the same frequency as the protons so it can exchange energy with them. As the precession frequency can be calculated by using the Larmor equation this also calculates the required frequency of the RF pulse. Once the RF pulse and protons have the same frequency they can exchange energy, a process called resonance. As some protons gain extra energy from the RF pulse they switch from parallel alignment to anti-parallel and therefore cancel out their corresponding proton. This results in decreased longitudinal magnetisation. The RF pulse also causes the protons to align in the same directions so they are 'in phase'. This causes the

magnetic vectors to add up in this direction, a process known as transversal magnetisation (figure 8.2).

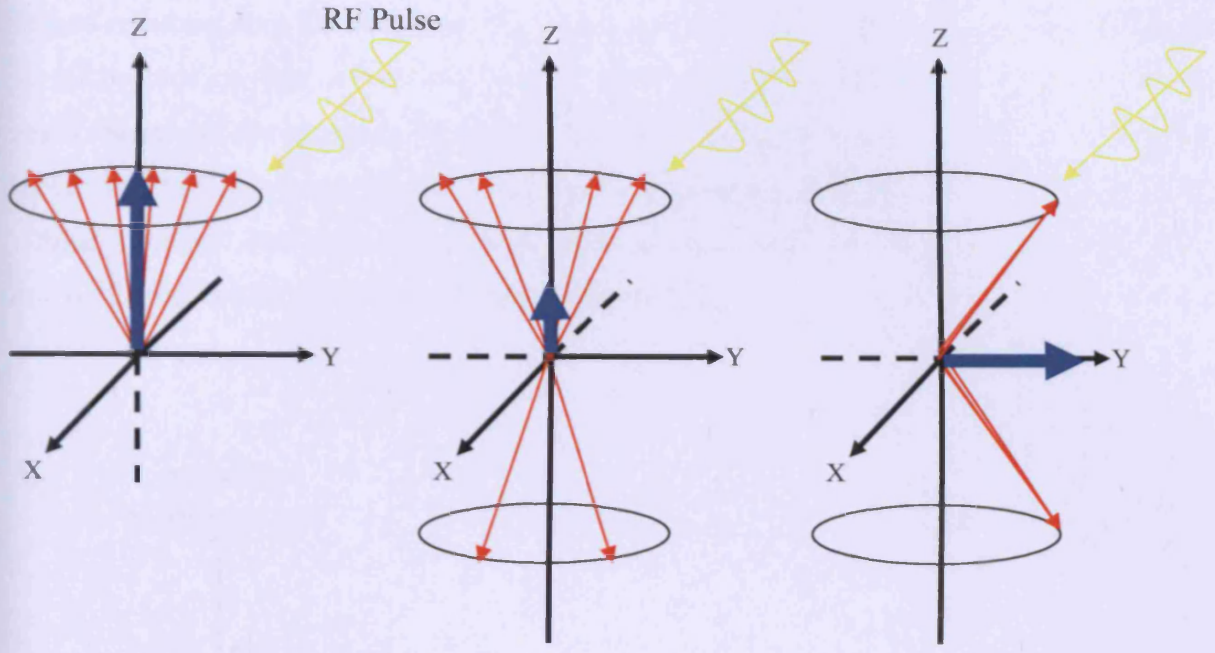


Figure 8.2: When an RF pulse is introduced protons pick up energy from it, a process called resonance. This causes some protons to become anti-parallel and results in them cancelling the z-axis magnetic field of the parallel protons, reducing longitudinal magnetisation. The RF pulse also causes the protons to precess together or 'in phase'. This establishes a new magnetic force along the x-y plane called transversal magnetisation. Diagram adapted from Schering (1990).

The newly established magnetic vector moves with the precessing protons at the precession frequency. This constant moving and changing causes an electric current. There is also a moving magnetic vector within the MRI machine which induces an electric current in an antenna, this is the MRI high intensity oedemic signal. As the transversal magnetic vector precesses it comes towards then away from the antenna at the precession frequency. Therefore the MRI signal has the precession frequency. However, before an image can be established from the signal it is necessary to know where in the body the signal comes from. In order to discover where the signal comes from the magnetic field which the subject is placed in has different strengths at each point of the cross section of the subject. As precession frequency depends on magnetic field if the magnet strength varies then the MR signal will also have different frequencies.

When capturing an image the RF pulse is switched off. As a result the transverse magnetisation starts to decrease, a process called transversal relaxation, and the longitudinal magnetisation starts to increase, known as longitudinal relaxation. This is caused by the protons returning to a lower energy state and resorting back to parallel alignment. However, not all protons do this at the same time. As the protons steadily become parallel they no longer cancel out the magnetic vectors of the other parallel protons. This eventually results in transversal magnetisation disappearing and longitudinal magnetisation resorting to the original strength. The time this process takes to occur is known as longitudinal relaxation time or T_1 and can be plotted as a T_1 curve (figure 8.3).

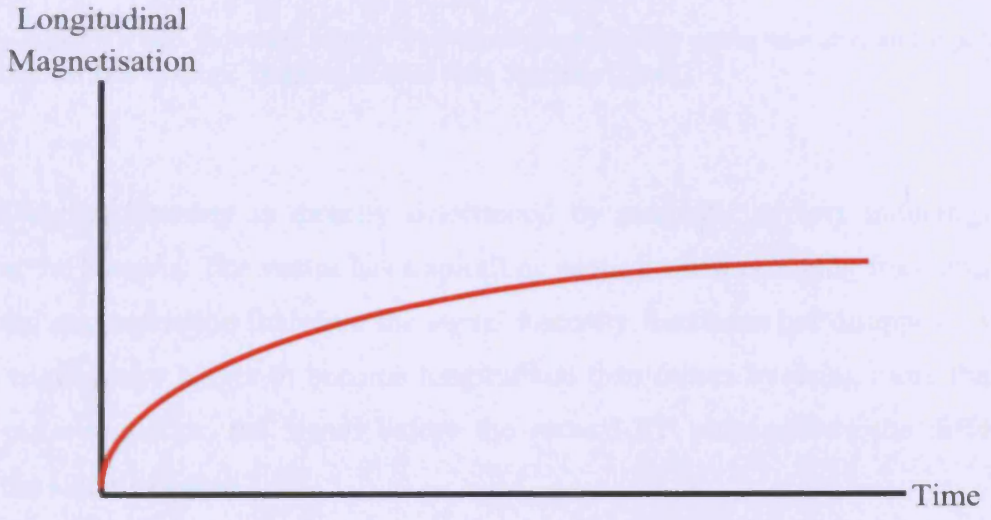


Figure 8.3: A graph which shows the level of longitudinal magnetisation versus time after an RF pulse is turned off. This is known as a T_1-curve. Diagram adapted from Schering (1990).

A T_1 curve only shows increase in longitudinal magnetisation. However, transversal magnetisation is also decreasing. This can be plotted as a T_2 curve (figure 8.4). The time that T_1 and T_2 takes establishes the characteristics of tissue. Liquids have a long T_1 and T_2 process whereas fat has a shorter T_1 and T_2. The T_1 process is longer in water as it takes protons longer to get rid of the RF pulse-supplied energy to the surrounding lattice as the molecules move more rapidly than larger fat molecules. The T_2 process is quicker in fat as larger molecules do not move around so much so not so many magnetic fields are cancelled out and protons can get out of phase easier than in water.

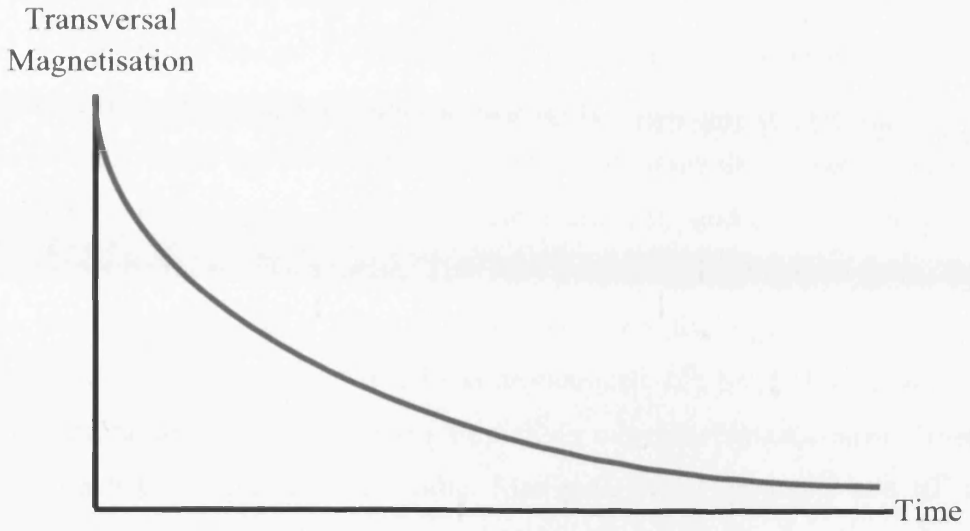


Figure 8.4: A graph which shows the level of transversal magnetisation versus time after an RF pulse is turned off. This is known as a T_2-curve. Diagram adapted from Schering (1990).

The MRI signal intensity is directly determined by magnetic vectors inducing electrical currents in the antenna. The vector has a spiralling motion when changing from transversal to longitudinal magnetisation therefore the signal intensity fluctuates but disappears with time. As some tissues take longer to become longitudinal than others by using more than one RF pulse, a pulse-sequence, the signal before the second RF pulse allows the differentiation between the signals (figure 8.5).

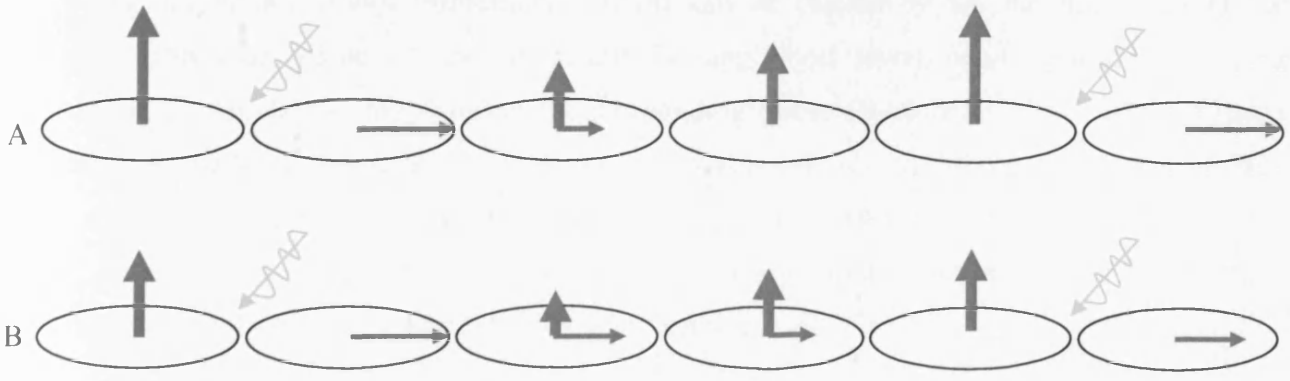


Figure 8.5: If more than one RF pulses are used a differentiation between magnetic vectors can be observed when the second RF pulse is added. This allows for differentiation between tissues. Here tissue B has a longer T_1-curve and as a result longitudinal magnetisation has not recovered to the point of tissue A. Diagram adapted from Schering (1990).

This time between RF pulses is known as time to repeat or TR. If a shorter TR is used the differences in signal intensity between the tissues is determined by their difference in T_1 and thus gives a T_1 weighed image. To obtain a T_2 weighed image a different process is used. If the RF pulse usually applied is 90° then a certain time after this is turned off (TE/2) a 180° pulse is sent. This makes the protons turn around, at this stage the slower protons are in front of the faster protons, however, the faster protons catch up and a strong signal is observed from the protons being in phase again. The 180° pulse is also called an echo, therefore TE stands for time to echo. If a short TE is used then the signal will be stronger but differentiation between the tissue will be less pronounced. If a long TE is used the difference will be more pronounced but the signal will pick up more background noise. Multiple slices will result in a better signal to noise ratio. Manipulation of gradients and RF pulses will allows any slide or orthogonal direction within the subject to be imaged. After these processes have occurred a mathematical process called Fourier transformation allows the analysis of the signals. The signals can be assigned to a certain location in the slice allowing the image to be reconstructed. MRI theory adapted from Schering (1990).

8.1.2 OEDEMA

The purpose of using MRI analysis in this chapter is to assess the levels of pulmonary oedema in the acute and chronic guinea pig models of asthma. Pulmonary oedema is defined as an abnormal accumulation of fluid in the extravascular compartments of the lung (Gluecker *et al.*, 1999). Pulmonary oedema can be caused by an increase in either the permeability or hydrostatic pressure within the lung blood vessels resulting in fluid escaping the microvasculature and entering the surrounding tissue (Beckmann *et al.*, 2001). Plasma leakage, oedema formation and mucus hypersecretion all contribute to luminal airway narrowing and therefore impair lung function (Ble *et al.*, 2008). Therefore using MR imaging could prove a useful tool in evaluating oedema formation in asthmatics and also to determine the effect that potential therapeutics have on oedema.

Several studies have used MR imaging on animal models of asthma. Ovalbumin (OVA) sensitised and challenged Brown-Norway rats showed increased oedema 6 hours after challenge. This oedema increased to a maximum at 48 hours and then decreased at 96 hours (Tigani *et al.*, 2007). This study also showed that oedema peaked 6 hours after repeated challenges and then steadily decreased. However, the peak level of oedema decreased after

each challenge suggesting that tolerance could have occurred. Further studies in Brown-Norway rats showed a correlation between MRI high intensity oedemic signals and BAL eosinophil number following OVA sensitisation and challenge (Tigani *et al.*, 2002). In OVA sensitised mice an increase in oedema was observed 24 hours after the second OVA challenge (Ble *et al.*, 2008). However, like in the rats the MRI high intensity oedemic signal became less intense after each challenge.

8.1.3 DEXAMETHASONE

Dexamethasone is a corticosteroid which is recommended for moderate to severe asthma (Shefrin & Goldman, 2009). However, although it is an extremely potent corticosteroid, the side-effects associated with dexamethasone limit its use (Ducharme *et al.*, 2003). Despite this dexamethasone has been commonly used in animal models of asthma and has been effective at reducing the LAR, AHR and cellular influx associated with asthma (Kumar *et al.*, 2003; Toward & Broadley, 2004). The mechanism of action of corticosteroids is described in greater detail in chapter 5; however, there is evidence that corticosteroids can prevent pulmonary oedema in animal models of asthma (Beckmann *et al.*, 2001; Tigani *et al.*, 2002). By using a potent corticosteroid it is possible to assess whether levels of oedema can be reduced and also whether the reduction of the late asthmatic response correlates with oedema reduction.

8.2 AIMS AND OBJECTIVES

8.2.1 AIMS

The aim of this chapter was to assess the level of lung oedema in acute and chronic OVA challenged guinea pigs at various time points to determine whether repeated challenges causes greater oedema and whether time after challenge makes a difference. The effect of dexamethasone treatment compared to vehicle treatment on acute OVA challenged guinea pigs on lung oedema was determined using MRI. The effect that dexamethasone has on the early and late phase bronchoconstriction, airway hyperresponsiveness, cellular influx was also assessed.

8.2.2 OBJECTIVES

- To investigate what effect, if any, that acute and chronic OVA exposures in sensitised guinea pigs has on levels of lung oedema using MRI
- To investigate the effect that dexamethasone treatment has on acute OVA exposed guinea pigs by measuring lung function, response to histamine and cellular influx compared to vehicle treatment.
- To investigate what effect, if any, that dexamethasone treatment has on the levels lung oedema in acute OVA exposed guinea pigs MRI.

3.3 METHODS

In this study the sensitisation, exposures and subsequent MR imaging was carried out at GlaxoSmithKline in Harlow. For each study group six male Dunkin-Hartley guinea pigs (Harlan, UK) weighing 150-175 g were used. The guinea pigs were ordered in at a lower weight to ensure the chronic group could fit comfortably into the MRI machine. The conditions in which the guinea pigs were kept were the same as in the animal service laboratories in Cardiff (detailed in chapter 2). All animal work carried out in GlaxoSmithKline was carried out by technicians. During this period I was controlling the imaging and running the MRI machine.

The studies involving lung function measurements and cellular influx after dexamethasone treatment were carried out in Cardiff. Six male Dunkin-Hartley guinea pigs (Harlan, UK) weighing 200-250 g were used for each group.

8.3.1 SENSITISATION

Guinea pigs were sensitised by an intra-peritoneal, bilateral injection of a suspension containing OVA (100 μg) and $Al(OH)_3$ (100 mg) in 1 ml of PBS on days 1 and 5.

8.3.2 OVALBUMIN CHALLENGES

OVA challenges for both acute and chronic groups were carried out in a stainless steel exposure chamber (40 cm diameter, 15 cm height) with a Wright nebuliser attached. The nebuliser delivered the OVA or saline at an air pressure of 20 lb p.s.i. and at a rate of 0.3 ml/min. The guinea pigs remained in the chamber for 1 hour, however, if they appeared to be distressed they were immediately removed from the chamber and exposure was considered complete. These methods were the same at both Harlow and Cardiff.

8.3.2.1 ACUTE PROTOCOL – GUINEA PIGS

On day 15 the guinea pigs were challenged with a 1 hour exposure to OVA (0.01%).

8.3.2.2 CHRONIC PROTOCOL – GUINEA PIGS

The guinea pigs were exposed to a 1 hour exposure to OVA (0.01%) on day 15. On days 17-29 (every 48 hours) the guinea pigs were exposed to OVA (0.1%) for 1 hour, this was preceded with an injection of mepyramine (30 mg/kg). On the final day (day 31) a 1 hour exposure of OVA (0.1%) was carried out.

8.3.3 MR IMAGING OF GUINEA PIG LUNGS

In order to capture MR images of lungs a Bruker 4.7 Tesla magnet was used. The magnet had a 11.6 cm gradient insert and a 72 mm coil and cradle. Images with a 7.2 cm field of view, slice thickness of 1.8 mm and interslice distance of 2.4 mm were taken.

Before the guinea pigs were placed in the MRI machine they were anaesthetised with isofluorane (1-4%) and oxygen/air as the carrier for the isoflourane, this was maintained throughout the whole time they were in the machine via an actively scavenged open face mask unit. Respiration was monitored using a transducer. The guinea pigs were placed in the supine position for imaging. As reproducing the position of the guinea pig for each scan was important for comparison of the images two scout images were recorded to check the positioning of the axial, coronal and sagittal views. Baseline images were captured before any challenges took place, subsequent to the final exposure images were taken 15 minutes, 4, 7 and 25 hours. The following images show the slices captured in one scan.

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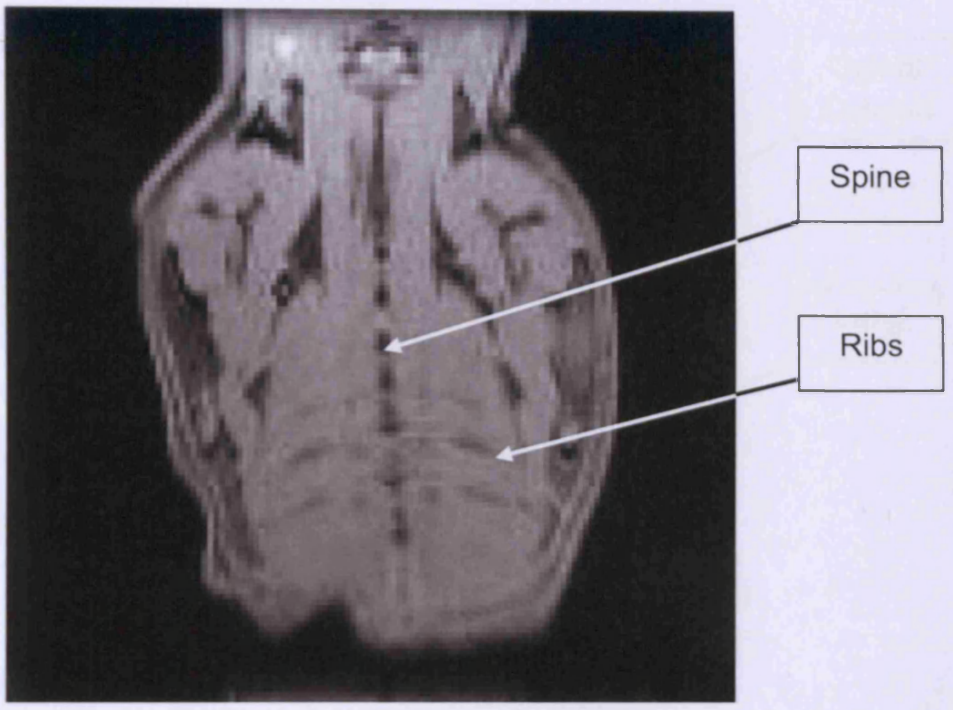

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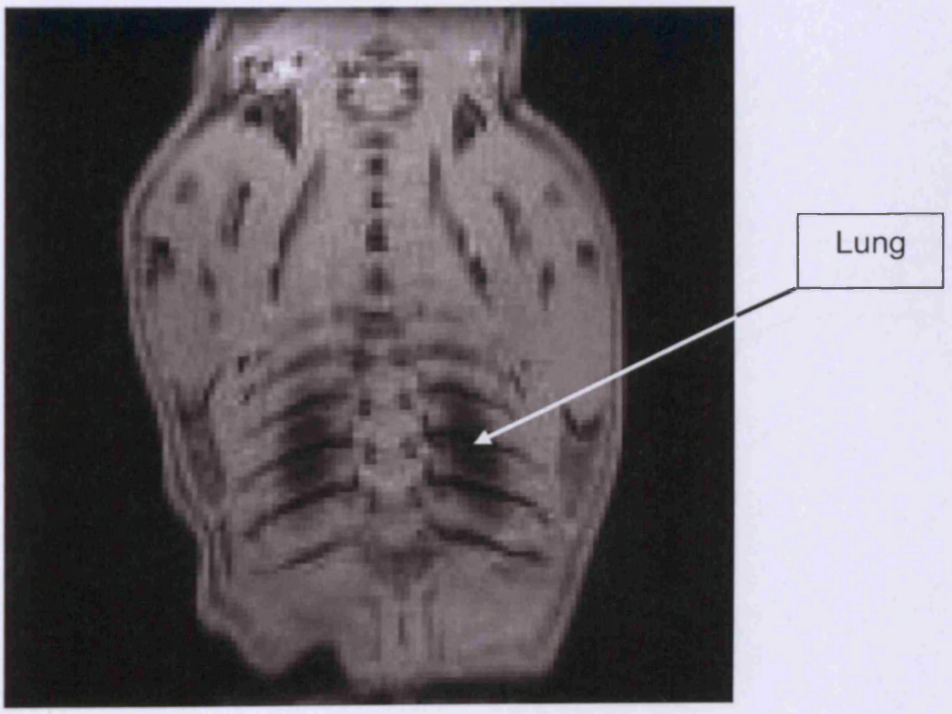

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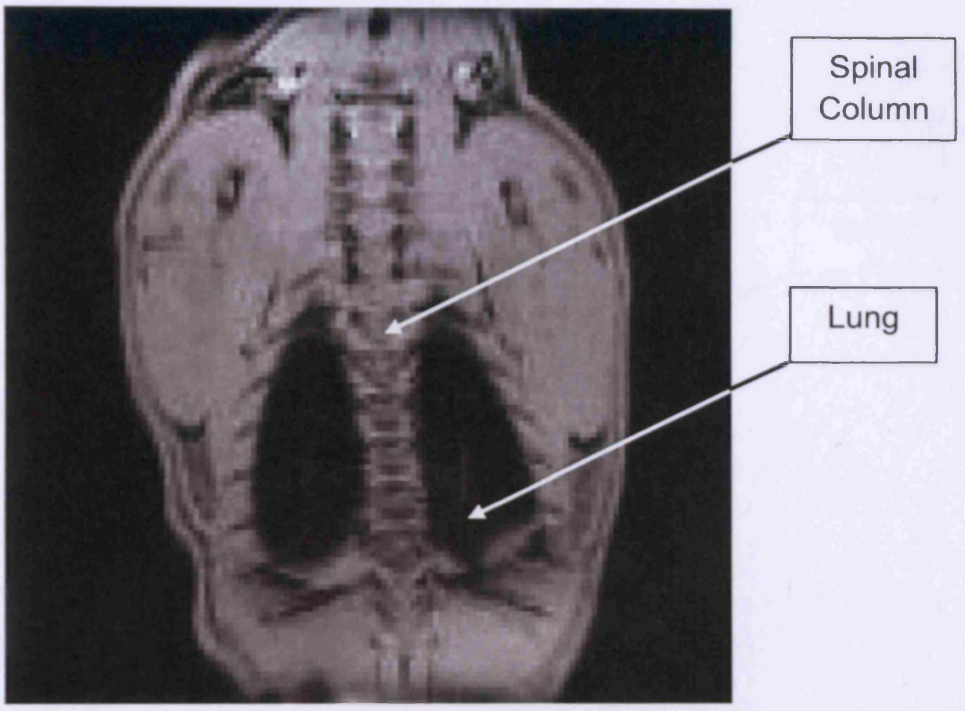

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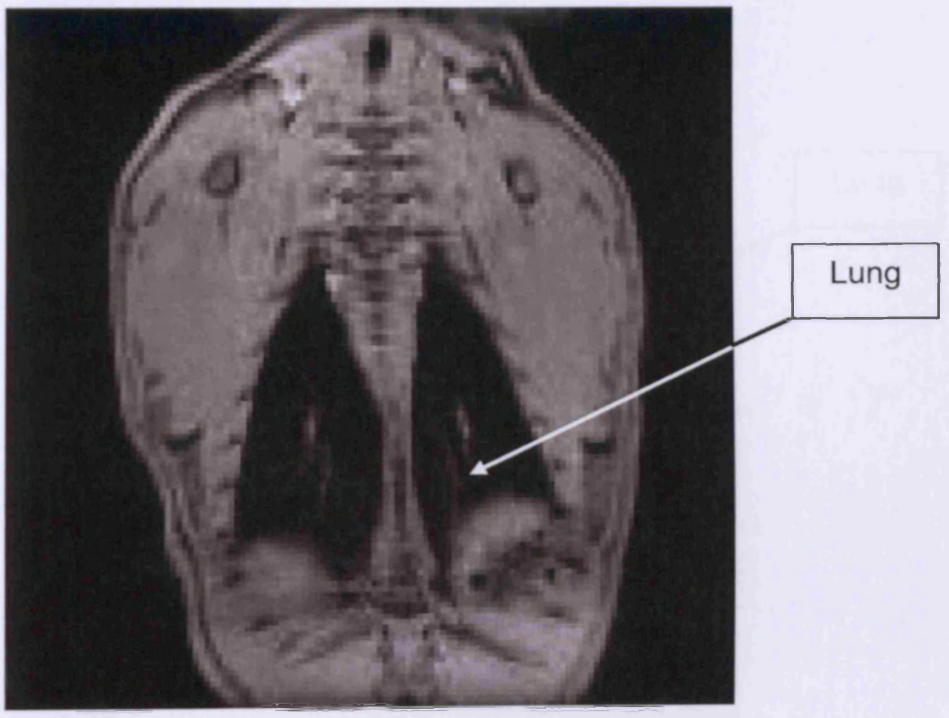

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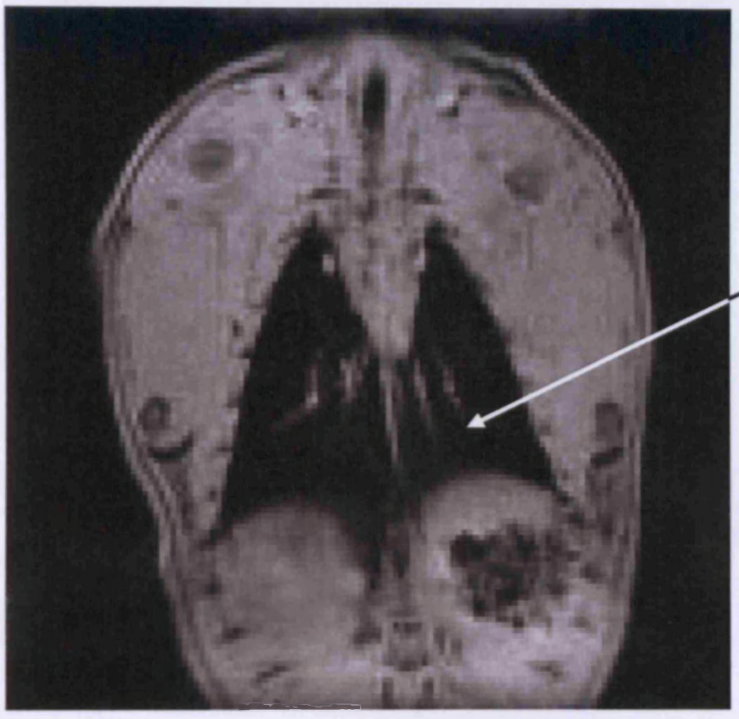

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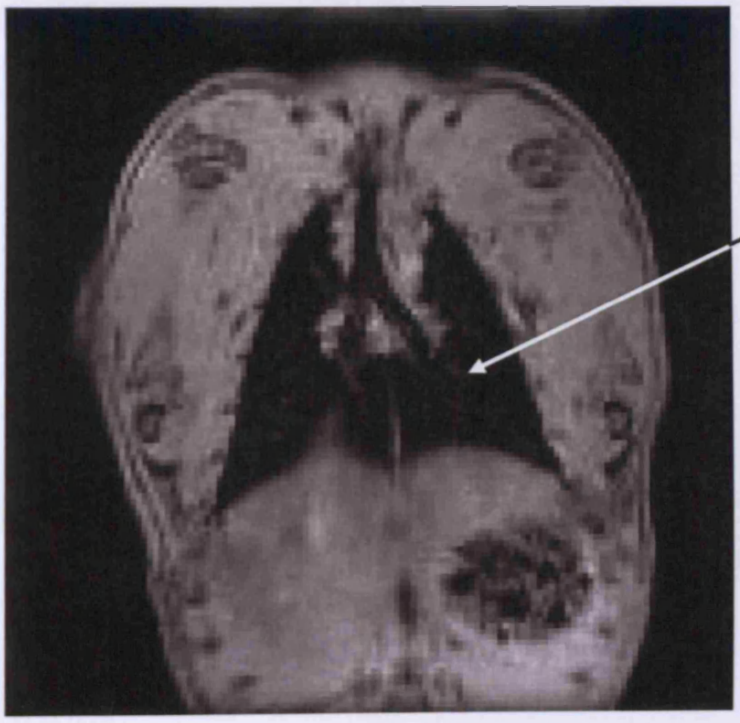

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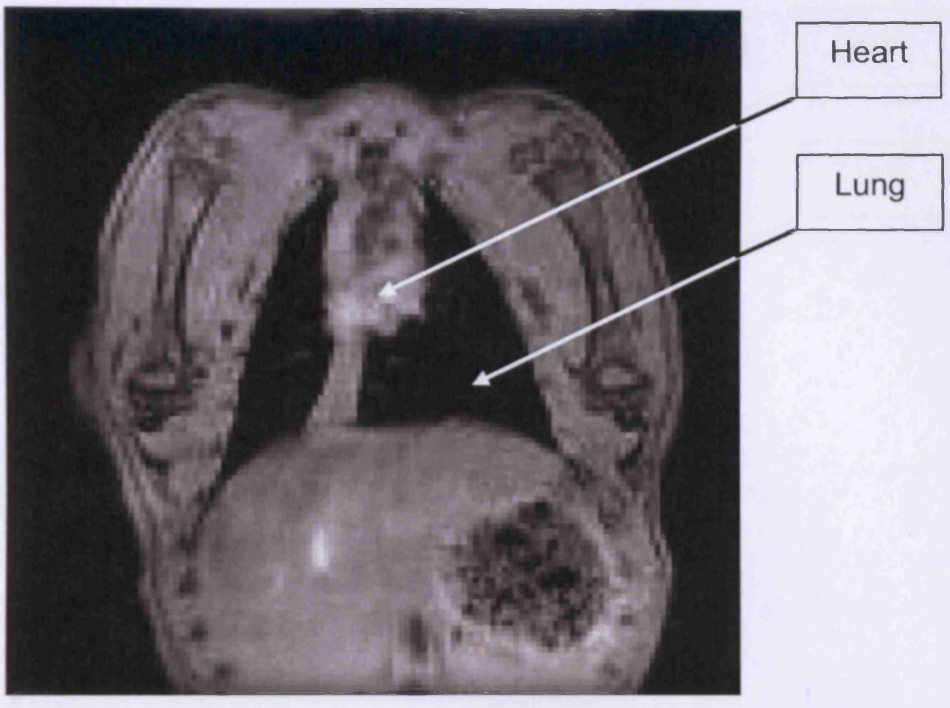

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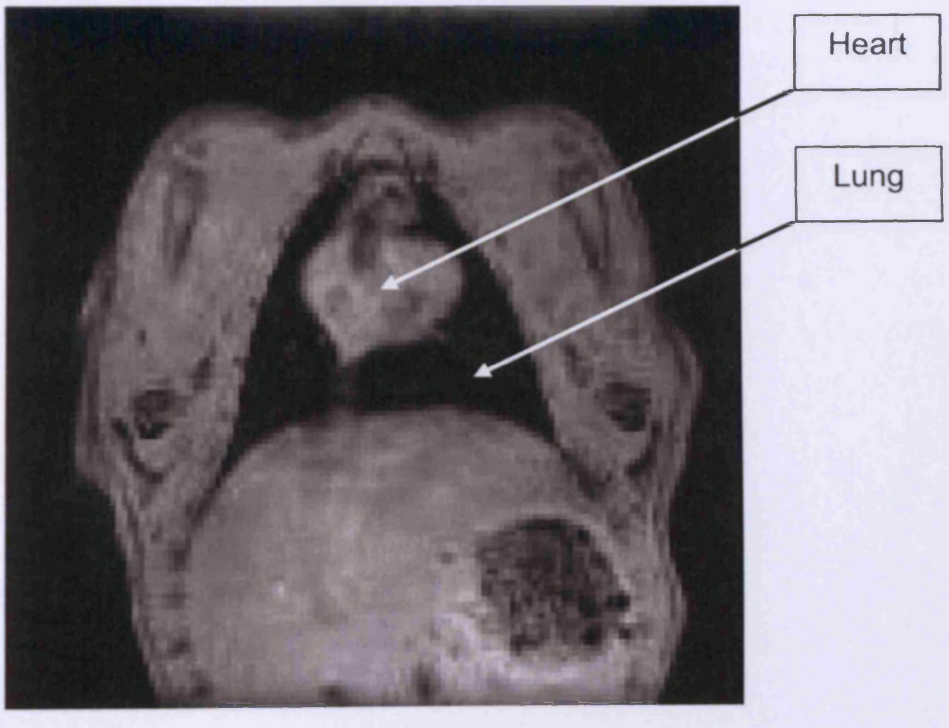

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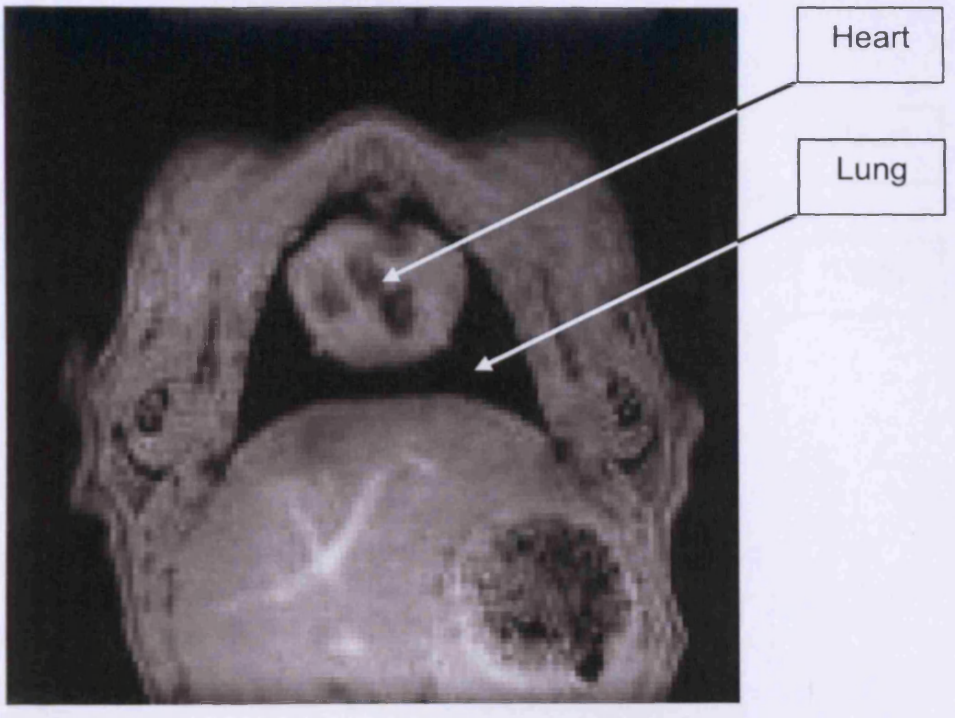

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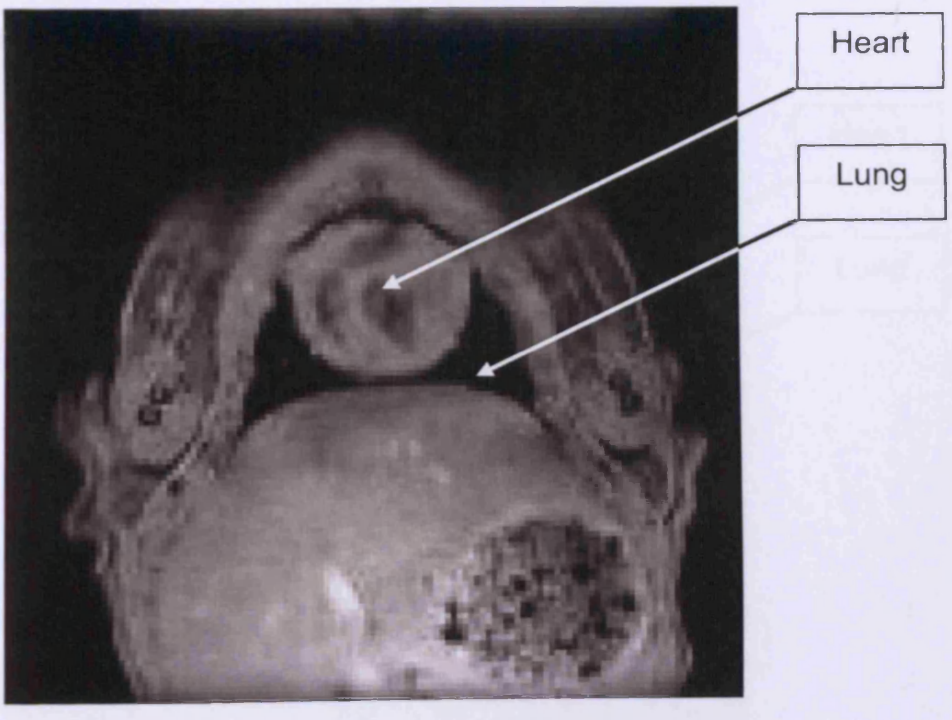

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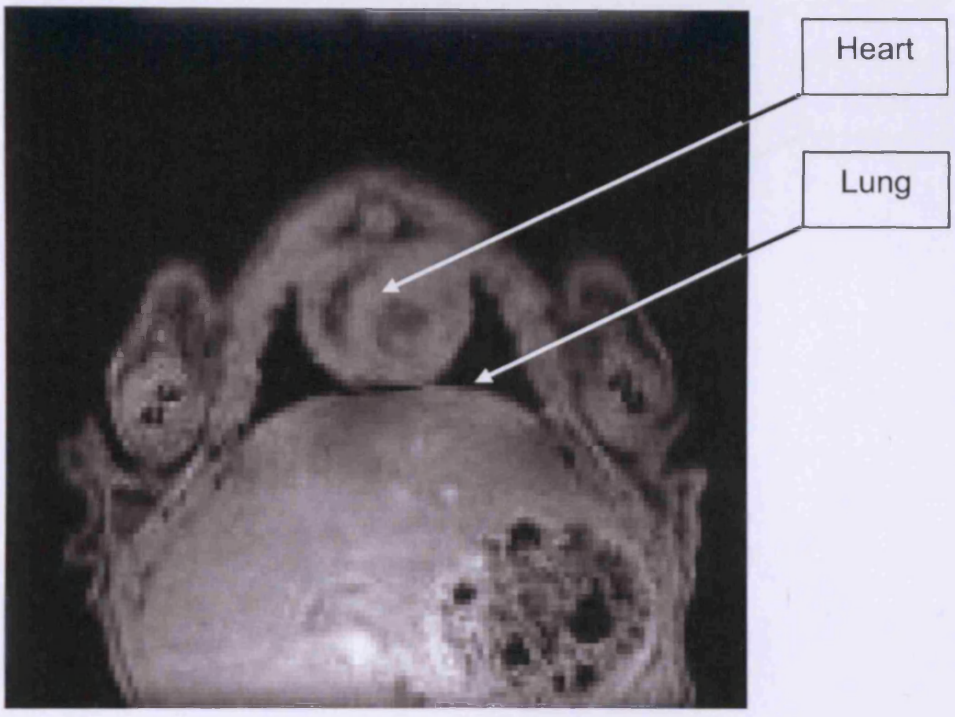

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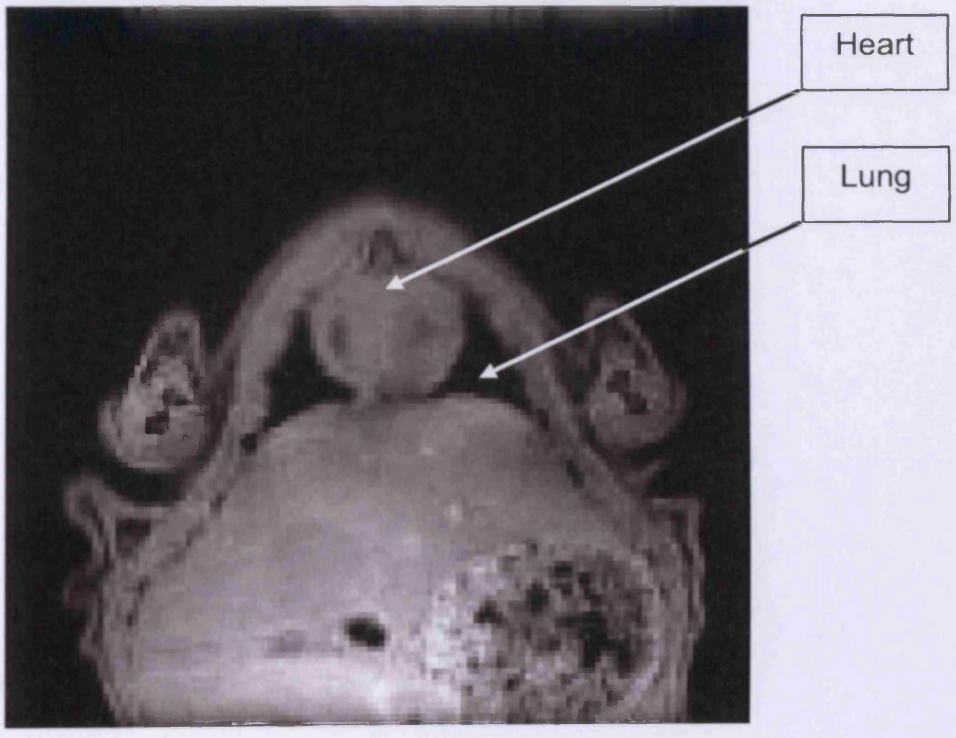

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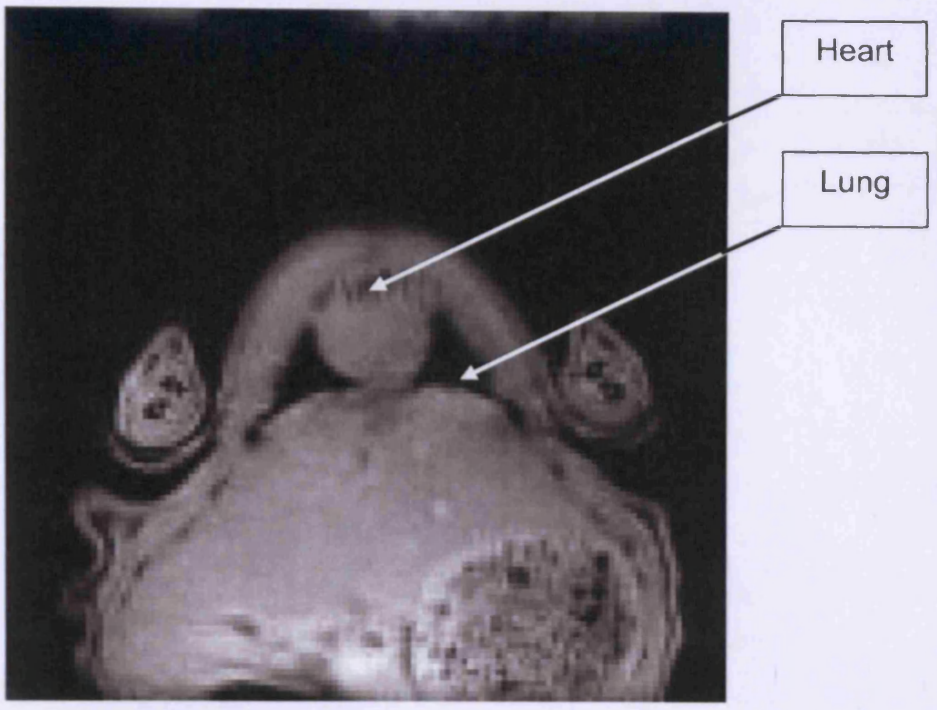

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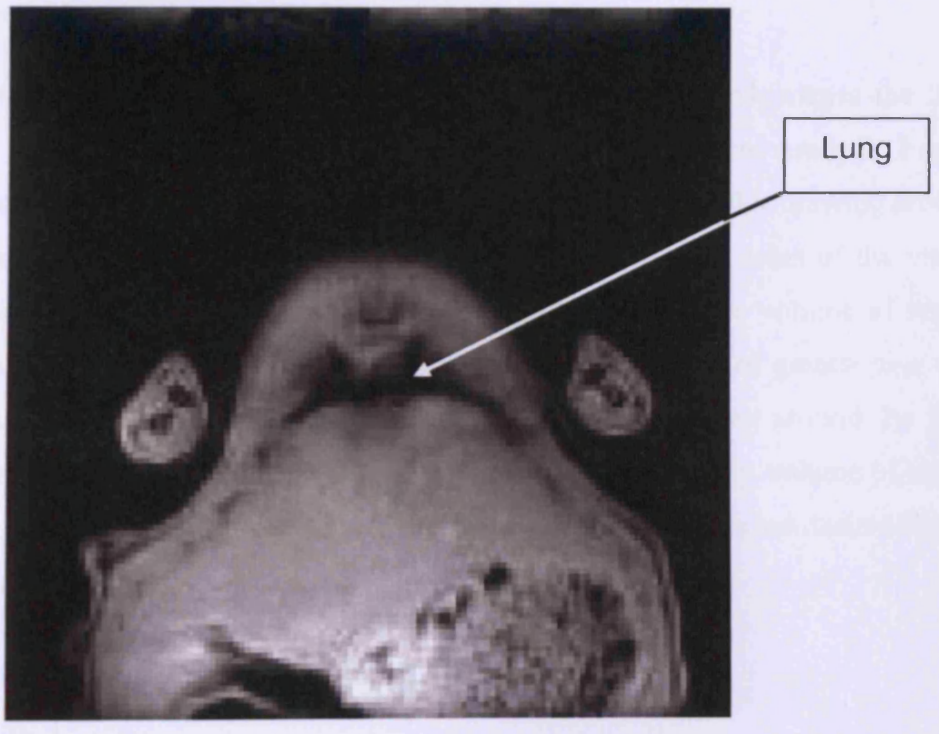

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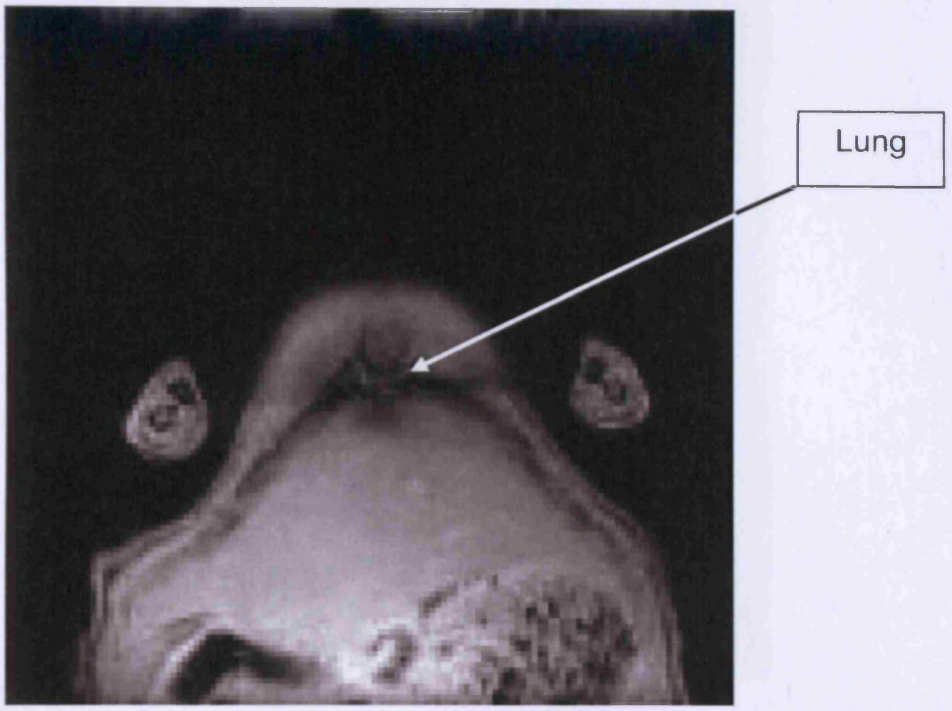
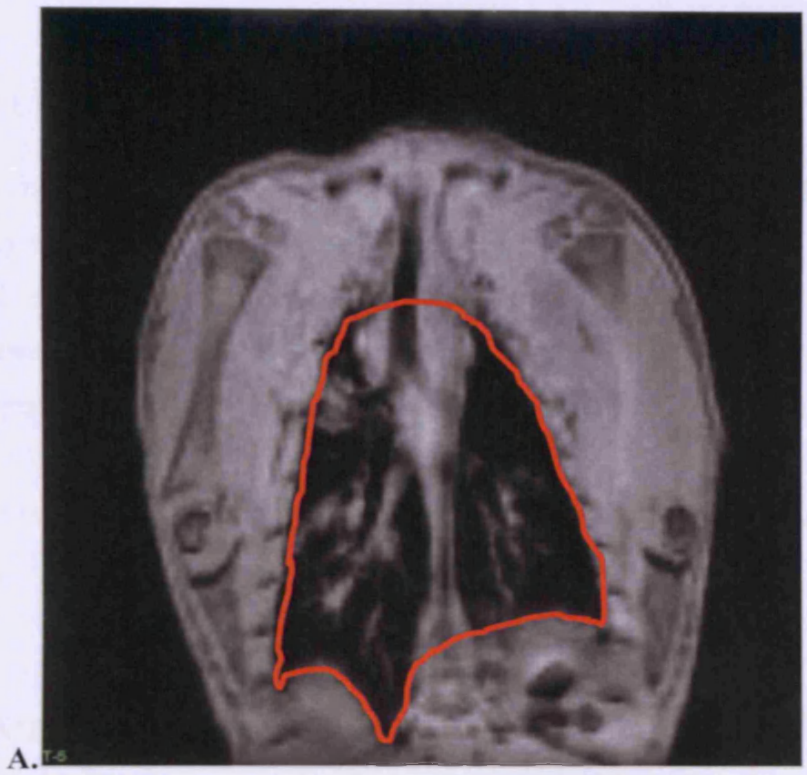
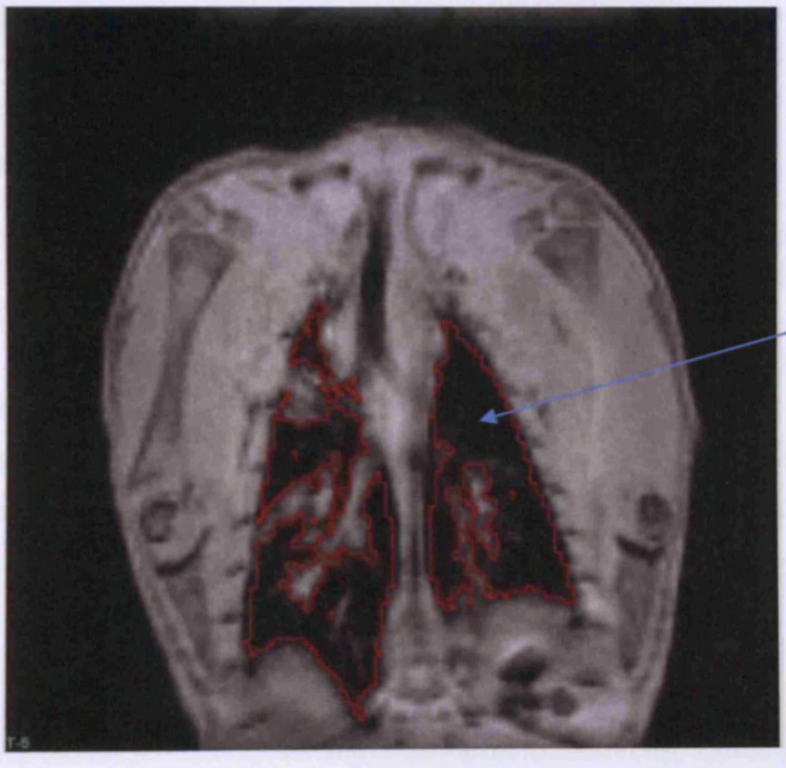


8.3.4 MRI ANALYSIS OF GUINEA PIG LUNGS

In order to analyse the level of oedema seen in the lungs for each of the images the lung cavity was drawn around for every slice taken using the computer program Analyze. Figure 8.6a shows an example of an image with the lung cavity drawn around. After drawing around the lungs a threshold is applied to the image by eye, this then allows all areas of the image that is not a certain intensity to be excluded (shown in figure 8.6b). The volume of bright intensity in the lung image can then be recorded in mm^3. A naïve group of guinea pigs was introduced to establish the baseline volume of bright intensity as drawing around the lung cavity also includes the heart which appears bright. Therefore, theoretically volume of bright intensity observed which is greater than the naïve volume (greater than the red dashed line in the graphs) is a result of oedema.



A.



B.

Figure 8.6: To analyse the level of lung oedema the lung cavity was firstly drawn around (A.). Following this a threshold was applied by eye, this allows all areas of dark intensity to be excluded (B.), this is air space in the lungs. The volume of bright intensity, which is solid tissue or fluid, is then measured in mm^3.

8.3.5 LUNG FUNCTION MEASUREMENTS

Lung function methods are described in detail in section 2.2.3. Briefly, whole-body plethysmography was used to determine values of sG_{aw} from which changes in lung function can be observed. Baseline values were recorded before the guinea pig was exposed to OVA, following the exposure values were recorded at 0, 15 minutes, 30 minutes, 45 minutes and 60 minutes post-exposure, then hourly until 12 hours with a final reading at 24 hours. In order to capture both the early and late asthmatic responses maximum bronchoconstriction values between 0-6 hours and 7-12 hours were recorded and displayed as a histogram next to the time course plot.

8.3.6 AIRWAY RESPONSIVENESS MEASUREMENTS

Airway responsiveness was measured by investigating the response of guinea pigs to 1 mMol histamine (20 seconds). Exposures to histamine occurred 24 hours pre-OVA exposure and 24 hours post-OVA exposure. Readings of sG_{aw} were taken at baseline and 0, 5 and 10 minutes following the histamine exposure.

8.3.7 TOTAL AND DIFFERENTIAL CELL COUNTS

After the final histamine exposure the guinea pigs were administered a lethal dose of sodium pentobarbitone and their lungs were lavaged. The recovered BAL fluid was used to carry out a total and differential cell count. These methods are fully described in section 2.2.7.

8.3.8 DRUG ADMINISTRATION

Dexamethasone was administered by an intraperitoneal bilateral injection at a dose of 20 mg/kg. The vehicle used was saline and DMSO in a 50:50 mix. Administration occurred at three separate times, 24 hours and 30 minutes before the OVA exposure and 6 hours after the exposure.

8.4 RESULTS

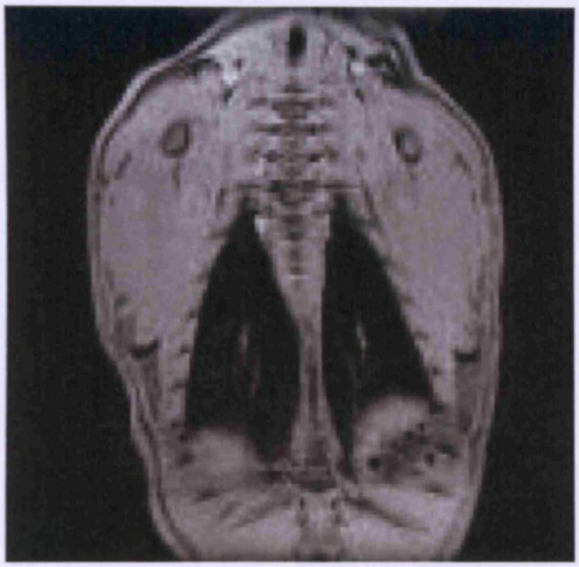
8.4.1 MRI ANALYSIS OF ACUTE AND CHRONIC CHALLENGED GUINEA PIGS

Figures 8.7 (A-E) show a single slice taken from naïve, saline challenged and OVA challenged guinea pigs 7 hours post-exposure. Figure 8.8 represents the effect that sensitising a guinea pig with OVA and challenging it with a single exposure of saline or OVA had on the volume of bright intensity in its lungs. A significant difference was observed between the OVA challenged and saline challenged guinea pigs at 4 hours ($6.4\pm0.2x10^3$ mm^3 compared to $4.8\pm0.2x10^3$ mm^3 respectively), 7 hours ($7.6\pm0.2x10^3$ mm^3 compared to $5.1\pm0.2x10^3$ mm^3 respectively) and 25 hours ($6.5\pm0.2x10^3$ mm^3 compared to $4.5\pm0.2x10^3$ mm^3 respectively) following exposure.

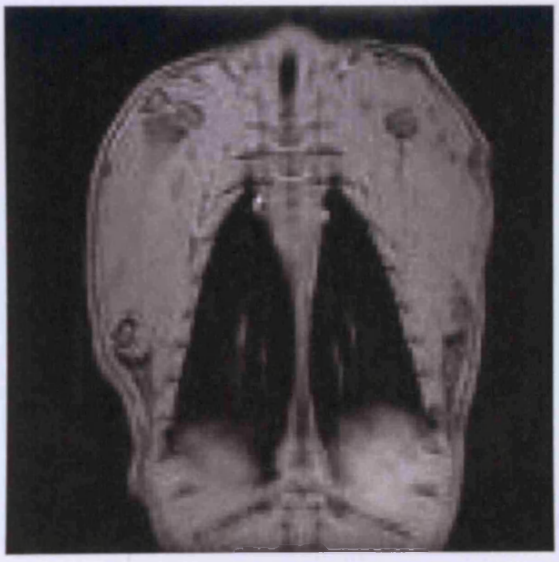
In figure 8.9 the effect of chronic exposures of saline or OVA on the volume of bright intensity in the lungs of sensitised guinea pigs is shown. Multiple challenges of OVA caused a significantly greater intensity than multiple challenges of saline at baseline ($7.3\pm0.4x10^3$ mm^3 compared to $5.5\pm0.1x10^3$ mm^3 respectively), 15 minutes ($7.7\pm0.6x10^3$ mm^3 compared to $5.4\pm0.2x10^3$ mm^3 respectively), 4 hours ($8.9\pm0.6x10^3$ mm^3 compared to $5.9\pm0.2x10^3$ mm^3 respectively), 7 hours ($9.0\pm0.7x10^3$ mm^3 compared to $6.0\pm0.2x10^3$ mm^3 respectively) and 25 hours ($7.4\pm0.5x10^3$ mm^3 compared to $5.7\pm0.2x10^3$ mm^3 respectively) following the final exposure.

Figure 8.10 shows the comparison between acute and chronic challenged guinea pigs. At the baseline time point multiple exposures of OVA caused a significantly greater volume of bright intensity than a single OVA challenge ($7.3\pm0.4x10^3$ mm^3 compared to $4.0\pm0.08x10^3$ mm^3 respectively). This was also true 15 minutes ($7.7\pm0.6x10^3$ mm^3 compared to $5.1\pm0.2x10^3$ mm^3 respectively) and 4 hours ($8.9\pm0.6x10^3$ mm^3 compared to $6.4\pm0.2x10^3$ mm^3 respectively) after the final challenge. However, no significant difference was seen at the 7 hour and 25 hour time points.

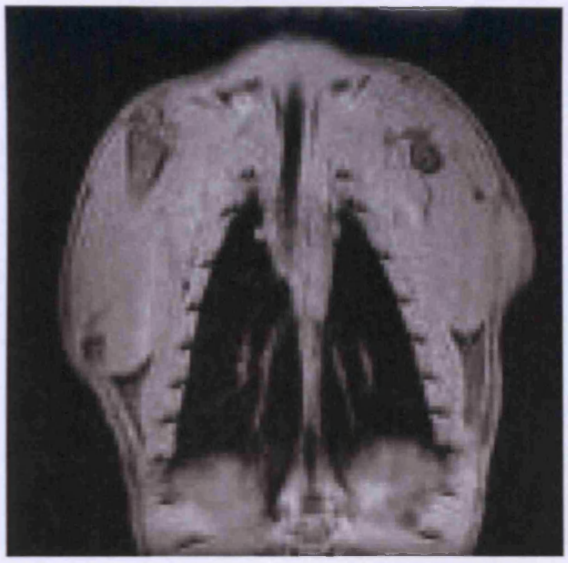
A. Naïve (non-sensitised)



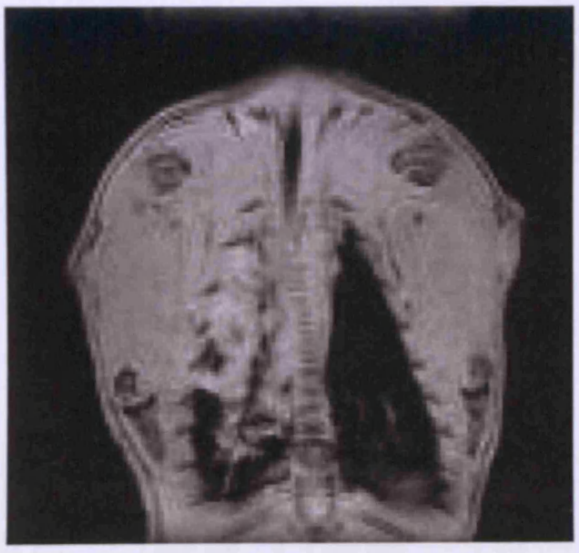
B. OVA sensitised – Acute saline challenged



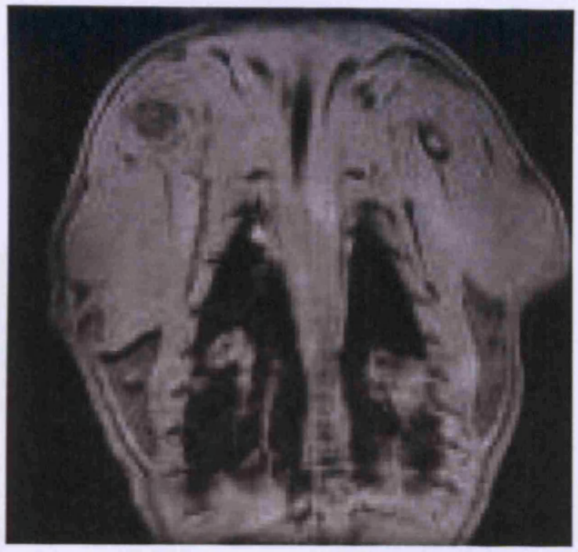
C. OVA sensitised – Chronic saline challenged



D. OVA sensitised – Acute OVA challenged



E. OVA sensitised – Chronic OVA challenged



Figures 8.7 (A-E): A single slice showing the MR image of the lungs of naïve and OVA sensitised guinea pigs challenged with a single or multiple exposures of saline or OVA.

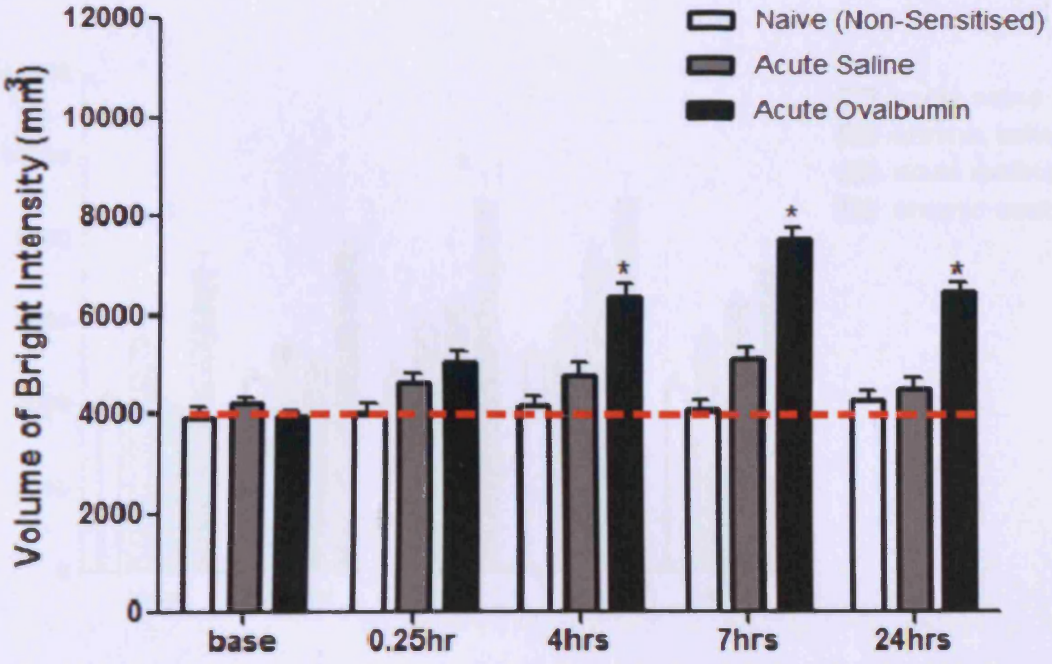


Figure 8.8: The volume of bright intensity in naïve or OVA sensitised acute saline or OVA challenged guinea pigs. The red line highlights the basal level of bright intensity, this is tissue such as the heart. *significantly different from acute saline challenged guinea pigs. Two-tailed T-test ($P<0.05$; $n=6$).

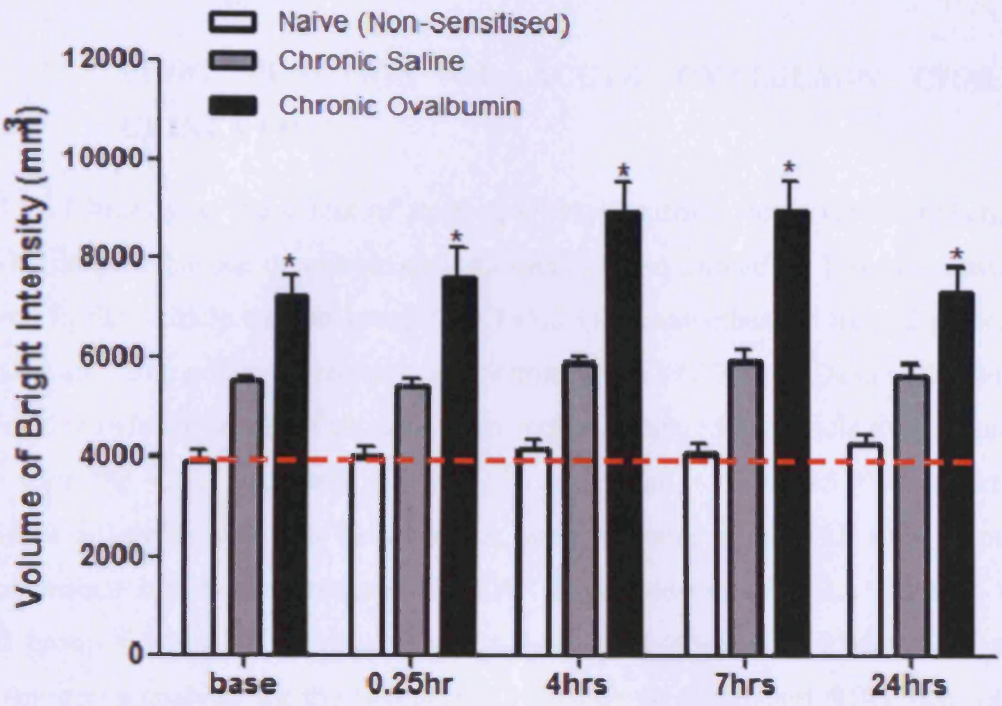


Figure 8.9: The volume of bright intensity in naïve or OVA sensitised chronic saline or OVA challenged guinea pigs. The red line highlights the basal level of bright intensity, this is like caused by tissue such as the heart. *significantly different from chronic saline challenged guinea pigs. Two-tailed T-test ($P<0.05$; $n=6$).

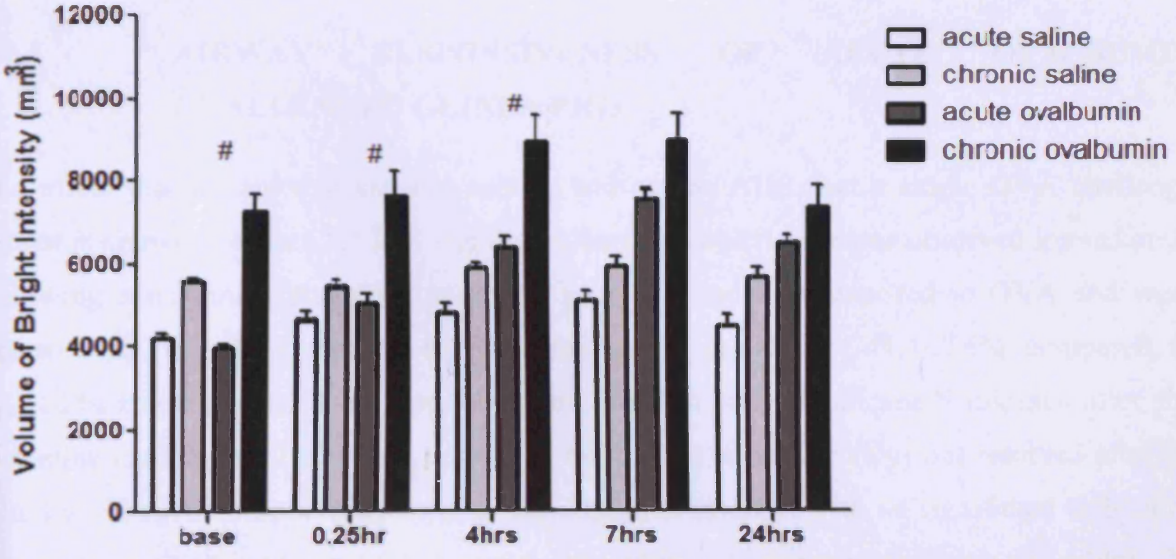


Figure 8.10: The volume of bright intensity in OVA sensitised guinea pigs challenged with a single or multiple saline or OVA exposure. #significant difference between acute and chronic OVA challenge. Two-tailed T-test ($P<0.05$; $n=6$).

8.4.2 LUNG FUNCTION OF ACUTE OVALBUMIN CHALLENGED GUINEA PIGS

Figure 8.11 highlights the effect of treating OVA sensitised, acute OVA challenged guinea pigs with dexamethasone or vehicle on lung function. An immediate bronchoconstriction was observed in the vehicle treated group (-59.1±5.3%), dexamethasone treated guinea pigs also showed an immediate bronchoconstriction (-56.8±7.7%). Dexamethasone treated significantly reduced levels of bronchoconstriction compared to vehicle treated guinea pigs 7 hours after the OVA exposure (11.7±3.9% compared to -20.2±5.9% respectively). No significant difference was seen between the two treatments at the EAR time point, however, dexamethasone significantly reduced the LAR compared to peak sG_{aw} values in the vehicle treated group (-5.9±1.2% compared to -26.3±4.8% respectively). Figure 8.12 shows area under the curve analysis for the two groups in which no significant difference was observed for total area and EAR area. However, dexamethasone treated guinea pigs had a significantly smaller area under the curve at the LAR time point than the vehicle treated group (37.0±18.4%.hr compared to 249.4±19.3%.hr respectively).

8.4.3 AIRWAY RESPONSIVENESS OF ACUTE OVALBUMIN CHALLENGED GUINEA PIGS

The effect that dexamethasone and vehicle had on the AHR that a single OVA challenge causes is shown in figure 8.13. A significant bronchoconstriction was observed immediately following a histamine challenge in guinea pigs that had been exposed to OVA and were treated with vehicle compared to the same group pre-OVA (-41.4±7.6% compared to 0.1±1.2% respectively). This bronchoconstriction was still significant 5 minutes after the histamine challenge (-24.5±4.6% compared to -1.0±2.1% respectively) but resolved after 10 minutes. Dexamethasone caused complete inhibition of AHR with no significant difference between pre-OVA and post-OVA exposures after a histamine challenge (1 mMol, 20 seconds).

8.4.4 TOTAL AND DIFFERENTIAL CELL COUNTS IN ACUTE OVALBUMIN CHALLENGED GUINEA PIGS

Figure 8.15 represents the number of cells found in the BAL fluid of acute OVA challenged guinea pigs treated with dexamethasone or vehicle. Dexamethasone treatment significantly reduced the total number of BAL cells compared to vehicle treatment ($2.9\pm0.2x10^6$ compared to $6.2\pm0.6x10^6$ respectively). This was also true for eosinophil number ($2.0\pm0.05x10^6$ compared to $1.8\pm0.08x10^6$ respectively) and lymphocyte number ($0.05\pm0.01x10^6$ compared to $0.2\pm0.03x10^6$ respectively).

8.4.5 MRI ANALYSIS OF ACUTE OVALBUMIN CHALLENGED GUINEA PIGS TREATED WITH DEXAMETHASONE

Figures 8.16 (A-B) show a single slice taken from acute OVA challenged guinea pigs treated with dexamethasone or vehicle at 7 hours post-exposure. Figure 8.17 represents the volume of bright intensity in the lung cavity of OVA sensitised, acute OVA challenged guinea pigs treated with either dexamethasone (20 mg/kg) or vehicle (saline–50%:DMSO–50%). Dexamethasone significantly reduces the volume of bright intensity compared to vehicle treated guinea pigs 7 hours after an OVA exposure ($6.4\pm0.3x10^3$ mm^3 compared to $9.1\pm1.0x10^3$ mm^3 respectively).

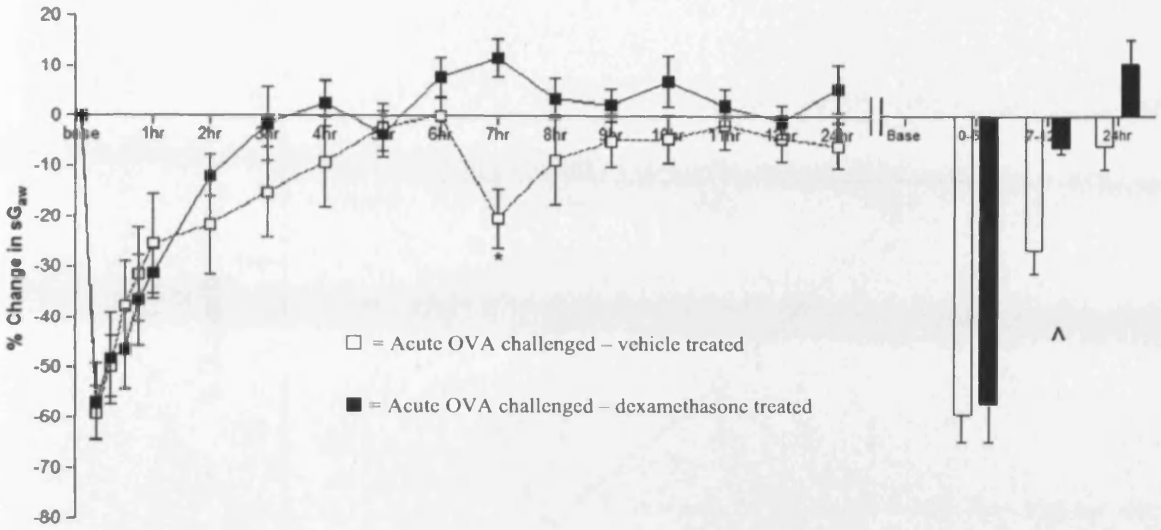


Figure 8.11 – Mean time-course values of sG_{aw} in OVA sensitised guinea pigs that were challenged with 0.01 % OVA and treated with saline/DMSO (50%/50%) or dexamethasone 20 mg/kg) administered i.p. 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The histogram represents maximum bronchoconstriction values during baseline, EAR, LAR and 24 hours. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from dexamethasone treated guinea pigs; ^ significant difference between vehicle and dexamethasone treatment. Two-tailed T-test ($P<0.05$; n=6). Raw dexamethasone treatment sG_{aw} values (cm/H_20) – Base (0.55±0.03), 0-6 hours (0.23±0.03), 7-12 hours (0.52±0.03) and 24 hours (0.61±0.04). Raw vehicle treatment sG_{aw} values (cm/H_20) – Base (0.56±0.02), 0-6 hours (0.22±0.02), 7-12 hours (0.40±0.03) and 24 hours (0.53±0.04).

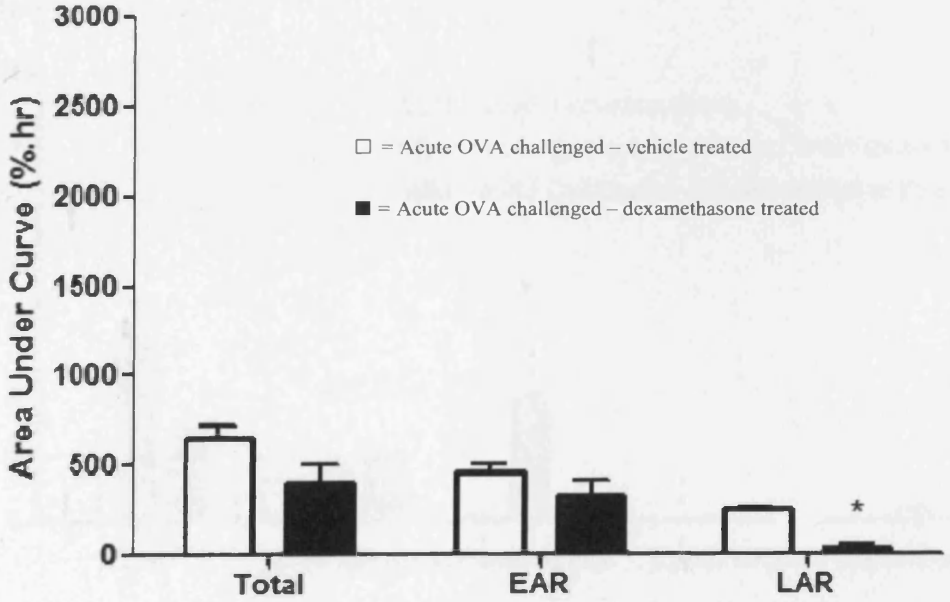


Figure 8.12 – Area under the curve analysis comparing OVA sensitised guinea pigs challenged with 0.01 % OVA and treated with saline/DMSO (50%/50%) or dexamethasone 20 mg/kg) administered i.p. 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Only negative peaks are considered, any peaks that have a positive value of sG_{aw} are excluded. Total includes all negative peaks from 0-24 hours, EAR includes from 0-6 hours and LAR includes from 6-24 hours. Area under the curve is measured in %.hour. *significantly different from vehicle treatment. Two-tailed T-test ($P<0.05$; n=6).

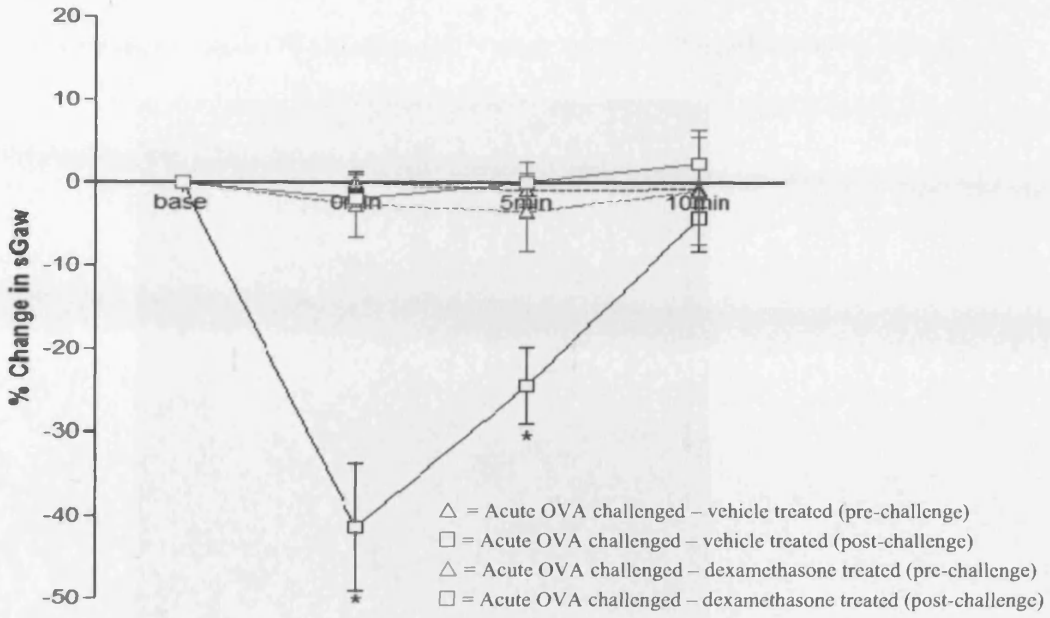


Figure 8.13 – Response of the airways to a nose-only histamine exposure (1 mMol for 20 seconds) in OVA sensitised guinea pigs challenged with 0.01 % OVA and treated with saline/DMSO (50%/50%) or dexamethasone 20 mg/kg) administered i.p. 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Values were recorded 24 hours before OVA challenge and again 24 hours post-OVA challenge. Mean changes in sG_{aw} are expressed as mean±S.E.M. percentage change from baseline where a negative value represents a bronchoconstriction. *significantly different from pre-challenge values of sG_{aw}. Two-tailed T-test ($P<0.05$; n=6).

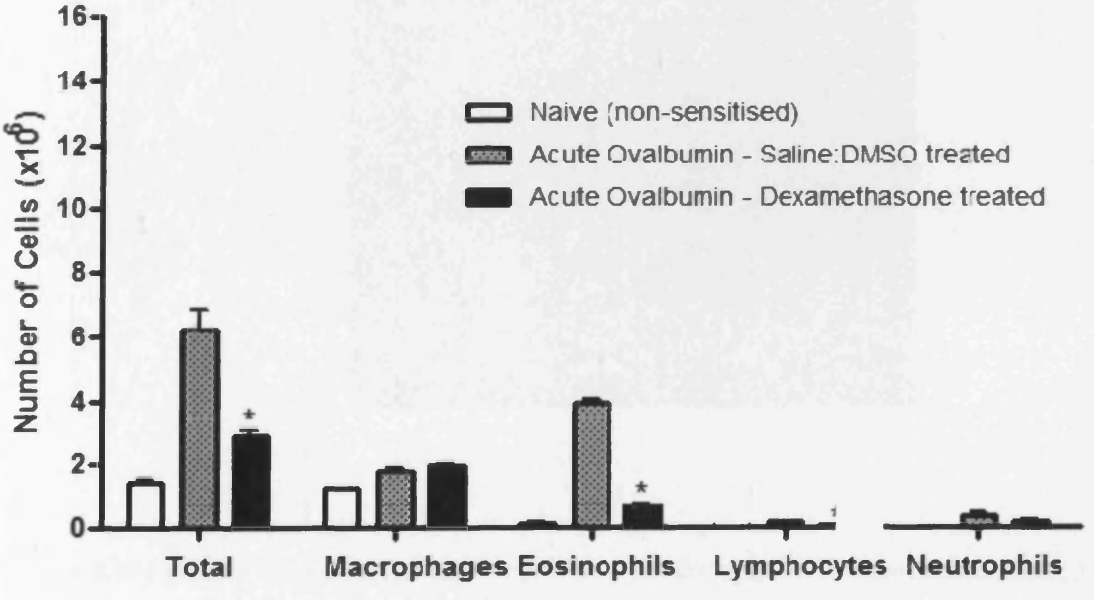
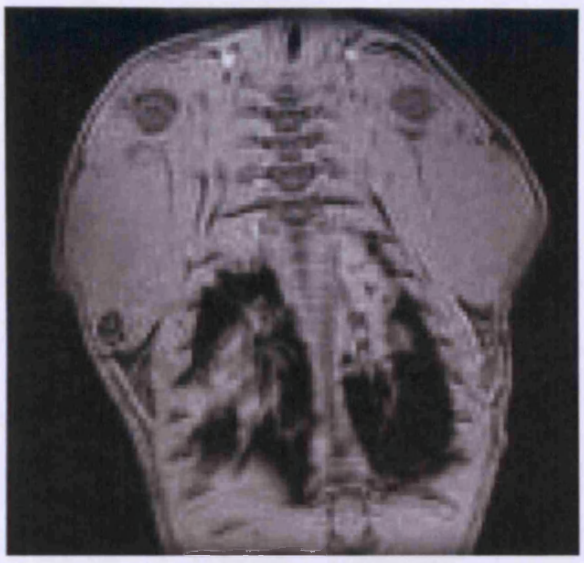
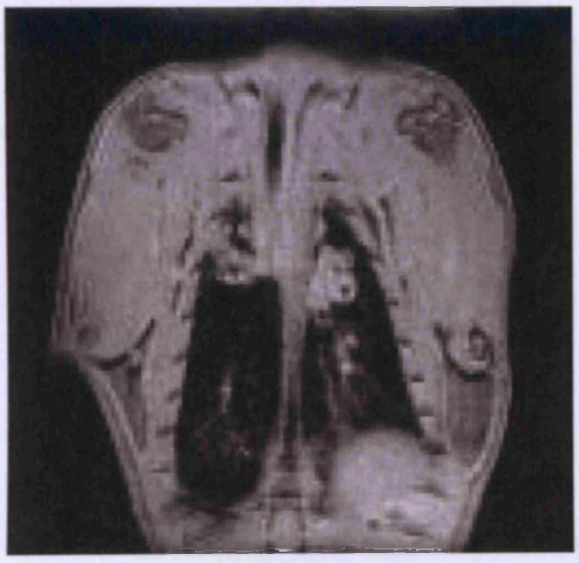


Figure 8.14 – The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar (BAL) fluid of naïve (non-sensitised) and acute OVA challenged guinea pigs treated with saline/DMSO (50%/50%) or dexamethasone 20 mg/kg) administered i.p. 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. Results are expressed as mean±S.E.M. *significantly different from acute OVA – vehicle treated. Two-tailed T-test ($P<0.05$; n=6).

A. OVA sensitised – Acute OVA challenged: Vehicle (saline–50%:DMSO–50%) treated



B. OVA sensitised – Acute OVA challenged: Dexamethasone (20 mg/kg) treated



Figures 8.15 (A-B): A single slice showing the MR image of the lungs of OVA sensitised guinea pigs challenged with a single OVA exposure and treated with saline/DMSO (50%/50%) or dexamethasone 20 mg/kg) administered i.p. 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge.

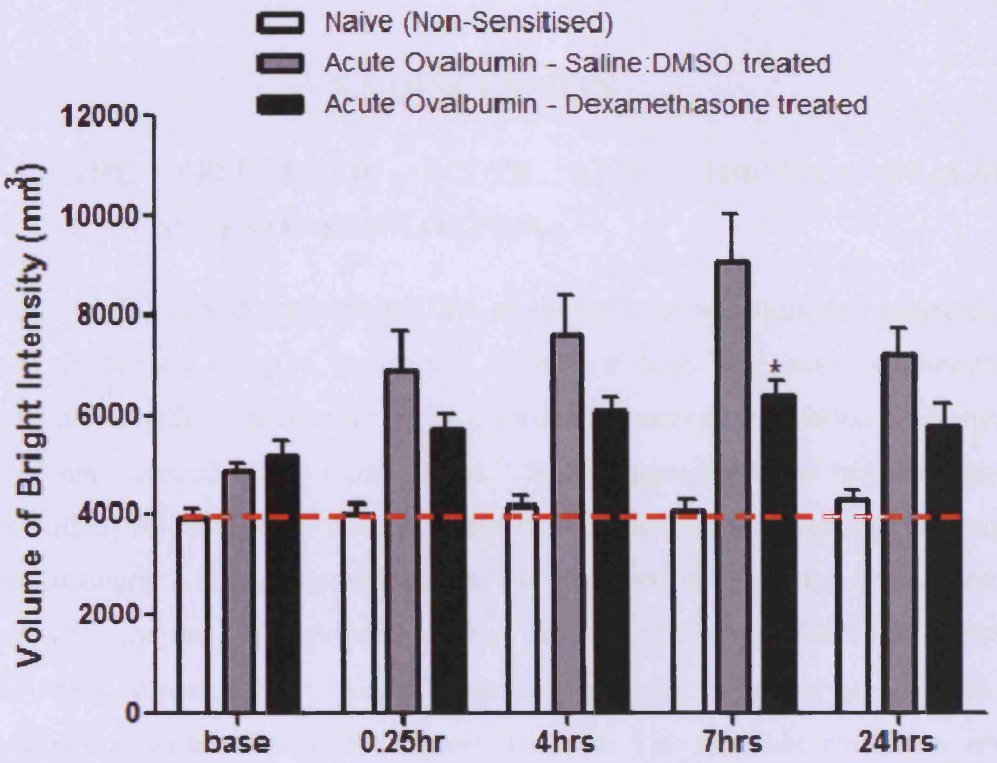


Figure 8.16: The volume of bright intensity in naïve and OVA sensitised acute OVA challenged guinea pigs treated with saline/DMSO (50%/50%) or dexamethasone 20 mg/kg) administered i.p. 24 and 0.5 hours pre-OVA challenge and 6 hours post-OVA challenge. The red line highlights the basal level of bright intensity, this is like caused by tissue such as the heart. *significantly different from vehicle treated guinea pigs. Two-tailed T-test ($P<0.05$; n=6).

8.5 DISCUSSION

8.5.1 THE EFFECT OF ACUTE AND CHRONIC OVALBUMIN EXPOSURES ON LUNG OEDEMA

Sensitisation with OVA and subsequent OVA challenge caused pulmonary oedema. In the acute OVA challenged group a significant difference from the saline challenged was observed at 4 hours with the maximum levels of oedema observed at 7 hours. At 24 hours the levels of oedema were reduced compared to at 7 hours suggesting some recovery, however, levels were still significant greater than the saline challenged group suggesting recovery takes longer than 24 hours. Although some oedema was observed in the saline challenged group this is somewhat expected as the guinea pigs were challenged in a high fluid environment and also saline does cause a very slight immune response as observed by maximum bronchoconstriction values shown in chapters 3 and 4. The fact that maximum levels of oedema were observed at 7 hours suggests oedema may contribute to the LAR.

The chronic OVA challenged group show significantly greater levels of oedema throughout all time points compared to saline challenge. The fact that there is an underlying level of oedema even at the baseline time-point is likely to be a consequence of repeated challenges not allowing the resolution of the oedema and inflammation to control levels. Despite the high levels of basal oedema in the chronic OVA challenged group the levels of oedema still increased to a maximum level after 7 hours. However, the increase from baseline levels to 7 hours is not as pronounced as was observed in the acute OVA group suggesting a maximal level of oedema could be reached possibly a result of the remodelling that occurs in the chronic airway (see chapter 3).

When levels of oedema were compared between acute and chronic challenges a significantly greater baseline level was observed in the chronic OVA challenged group compared to acute OVA challenge. However, whether this is exclusively a result of repeated OVA challenges causing more oedema or whether the fact that the chronic challenge guinea pigs were larger cannot be confirmed for certain. It is likely a result of the repeated challenges because although the chronic group have bigger lungs there would be less space for oedema because of the chronic remodelling of the airways.

8.5.2 THE EFFECT OF DEXAMETHASONE TREATMENT ON EARLY AND LATE ASTHMATIC RESPONSES IN ACUTE OVALBUMIN CHALLENGED GUINEA PIGS

Dexamethasone treatment had no effect on the early asthmatic response that was observed following an acute OVA challenge in the vehicle treated group. Evidence for this is displayed in both a time-course graph with maximum bronchoconstriction values and using area under the curve analysis. This was expected as dexamethasone is not a bronchodilator and as a result corticosteroids are often administered in combination with β_2 agonists to compensate for this.

As observed with fluticasone propionate in chapter 5, dexamethasone significantly inhibited the late asthmatic response in comparison to vehicle treatment confirming the anti-inflammatory nature of the drug. Dexamethasone is an oral steroid and is used to treat severe asthma (Bonnans *et al.*, 2003) so the fact that it is so effective against the late asthmatic inflammation was expected. Previous data has confirmed the ability of dexamethasone to attenuate the late asthmatic response in this acute OVA guinea pig model (Toward & Broadley, 2004).

8.5.3 THE EFFECT OF DEXAMETHASONE TREATMENT ON AIRWAY HYPERRESPONSIVENESS IN ACUTE OVALBUMIN CHALLENGED GUINEA PIGS

AHR was observed in vehicle treated guinea pigs that were sensitised and exposed once to OVA. However, when levels of airway responsiveness were assessed in dexamethasone treated guinea pigs a significant bronchoconstriction was not observed. Therefore, dexamethasone, like fluticasone propionate, is able to attenuate the hyperresponsiveness observed in the airways of acute OVA exposed guinea pigs following a histamine challenge. It is likely that dexamethasone has a protective effect in the same way as fluticasone propionate, however, as the exact mechanisms of AHR are unclear how dexamethasone prevents AHR is not completely understood. As discussed in chapter 5 nitric oxide has been implicated as a cause of AHR (Salome *et al.*, 1999) as have eosinophils (Bradley *et al.*, 1991), therefore it is likely that dexamethasone works by inhibiting one, if not both, of these

factors. It is unlikely that dexamethasone had an effect by inhibiting the histamine administered to assess the responsiveness of the airways as if dexamethasone has anti-histamine properties it would be more effective against the early asthmatic response. Attenuation of AHR by dexamethasone has been previously shown in animal models of asthma (Kumar *et al.*, 2003; Toward & Broadley, 2004).

8.5.4 THE EFFECT OF DEXAMETHASONE TREATMENT ON CELLULAR INFLUX IN ACUTE OVALBUMIN CHALLENGED GUINEA PIGS

Eosinophils play a major role in causing the lung inflammation observed in asthma with the number of eosinophils correlating with the severity of asthma (Hamid *et al.*, 2003). Corticosteroids increase eosinophil apoptosis and also inhibit the recruitment of eosinophils to the airways (Barnes & Adcock, 2003). A large influx of eosinophils was observed following an OVA challenge in the vehicle treated group compared to naïve guinea pigs. This influx was significantly reduced by dexamethasone. This would explain why a reduction in total number of inflammatory cells in the BAL fluid was observed after dexamethasone treatment compared to vehicle. Toward and Broadley (2004) previously showed that dexamethasone was able to significantly reduce eosinophil numbers in this model. It is possible that this reduction in eosinophils is responsible for the attenuation of the late asthmatic response and AHR usually observed.

8.5.5 THE EFFECT OF DEXAMETHASONE TREATMENT ON LEVELS OF LUNG OEDEMA IN ACUTE OVALBUMIN CHALLENGED GUINEA PIGS

Challenging OVA sensitised guinea pigs with a single OVA exposure caused pulmonary oedema. A maximum level of oedema was observed after 7 hours, at the same time as the LAR. Pre-treating a guinea pig with dexamethasone caused a significant reduction in the levels of oedema at 7 hours compared to vehicle treatment. Despite this levels of oedema were still higher than naïve guinea pigs suggesting that complete eradication of pulmonary oedema is not required for late phase attenuation. The volume of bright intensity was greater at baseline levels than naïve guinea pigs; it is highly unlikely that sensitisation has any effect

on oedema so the only possible explanation of this is the fact that the drug treated guinea pigs were older. Although at basal levels there will be no oedema present the entire lung cavity is considered when analysing volume of bright intensity, tissue such as the heart will show up with a bright intensity. The drug treated guinea pigs were a few weeks older than the naïve and as such would have had a larger heart explaining the greater basal level of bright intensity. Ideally only considering bright intensity in the lungs would be the ideal scenario, however, in an oedema filled lung it is very difficult to distinguish what is lung and what is heart and as a result the entire cavity was analysed. In hindsight scanning the naïve guinea pigs at the same age of the drug treated guinea pigs would have been a better option.

8.5.6 GENERAL CONCLUSIONS

Previous chapters have shown that OVA sensitisation and challenge cause early and late asthmatic responses, AHR, cellular influx and airway remodelling (in the chronic challenge protocol). This chapter confirms that the OVA sensitisation and challenge also induces pulmonary oedema which appears to correlate with the LAR. This LAR oedema can be significantly inhibited by corticosteroid treatment. This is not the first time LAR and oedema correlation has been observed as antigen-induced LAR was associated with pulmonary oedema previously in rats (Miyagawa *et al.*, 2009). However, it appears that this is the first study to show pulmonary oedema that can be attenuated with corticosteroid treatment in OVA challenged guinea pigs.

Eosinophils, which play a key role in asthma, have been suggested to contribute towards oedema (Tigani *et al.*, 2002). As eosinophils play a key role in the LAR this could explain the correlation between oedema and the LAR. Oedema has been associated with inducing hyperresponsiveness (Hargreave *et al.*, 1986) as have eosinophils (Bradley *et al.*, 1991) further corroborating the role of eosinophils in causing pulmonary oedema. Levels of eosinophils found in BAL fluid correlate with levels of MRI high intensity oedemic signal and in rats pre-treated with budesonide a reduced level of eosinophils and MRI signal was observed (Tigani *et al.*, 2002). It is extremely likely that dexamethasone works through the same mechanism as budesonide, where by inhibiting eosinophil influx decreased levels of oedema, LAR and AHR are observed. However, despite dexamethasone treatment a level of oedema which peaked at 7 hours was still observed suggesting that either oedema can also occur through an eosinophil-independent mechanism or that a low level of eosinophils are

needed to cause oedema as dexamethasone treatment did not reduce eosinophil level to that of naïve guinea pigs.

Although oedema has been shown to develop in the lungs of asthmatics (Beasley *et al.*, 1989; Laitinen *et al.*, 1985) in some animal models the levels of oedema appear to reduce after multiple challenges (Ble *et al.*, 2008; Tigani *et al.*, 2007). The Tigani study implies that the reduced leakage is a result of increased vessel wall thickness following repeated allergen challenge. However, the formation of new blood vessels, angiogenesis, is associated with chronic asthma (Orsida *et al.*, 1999) suggesting that there should be more plasma leakage from an increased number of blood vessels and therefore more oedema. It is likely that the decreased levels of oedema in these studies is a result of allergen tolerance. Oral tolerance in sensitised animals has been shown to decrease oedema formation (Ruiz Schutz *et al.*, 2009) further corroborating that tolerance is the reason less oedema was observed in the Tigani and Ble studies.

The fact that oedema was observed in the chronic OVA model, as it is in asthmatics, suggests it is an extremely useful pre-clinical model of asthma. The use of MRI for assessing the effect of anti-inflammatory drugs could prove to be a useful tool not only in animal models but also clinically. It provides a non-invasive way of accurately assessing the levels of oedema in the lung, a factor which appears to be highly correlated with levels of pulmonary inflammation. One issue of using MRI is that images can be distorted because of the guinea pigs spontaneous breathing, however, taking repetitive measurements allows for a better MRI high intensity oedemic signal and therefore a clearer image and overcomes this issue. Although the increased levels of oedema correlated with the LAR, nasal congestion cannot be ruled out as a potential cause of changes in sG_{aw} values using this method. In order to rule this out imaging the nasal passage over the same time periods as the lungs would be a worthwhile experiment. However, this was not possible during this thesis because of MRI machine availability.

Chapter 9

General Discussion

9.1 MAIN AIMS AND METHODS

Asthma is a chronic disorder that causes massive social and economic cost. However, very few models of asthma, used to evaluate the effects of potential asthma therapeutics pre-clinically, take into account the chronic nature of asthma. The main aim of this thesis was to develop a chronic guinea pig and mouse model that displayed many of the features of asthma and to compare this with the acute model that is more commonly used in research (Zosky & Sly, 2007). A second aim was to investigate the effects that current and novel asthma therapeutics have on these models. The parameters used for comparison were lung function, airway response to histamine, inflammatory cell influx, lung histology, response to a variety of anti-inflammatory drugs and assessment of lung oedema (guinea pig only). This thesis showed clear distinction between acute and chronic challenge protocols most noticeably the presence of airway remodelling in the chronic model but not in the acute model. These guinea pig and mouse chronic models appear to be the first to display early and late asthmatic responses, airway hyperresponsiveness, cellular influx and airway remodelling all in the same animal. Several other models have displayed some, but not all, of these features using various similar and different protocols (Bazan-Perkins *et al.*, 2009; Fernandez-Rodriguez *et al.*, 2008; Gosens *et al.*, 2005). There are many different protocols, with differing results, that are used on animal models to try and provoke an asthma-like inflammatory response.

9.1.1 SENSITISATION

All models in this thesis were sensitised with ovalbumin (OVA) and aluminium hydroxide dissolved in phosphate buffered saline and administered via an intraperitoneal injection. Although this route has been commonly used (Fernandez-Rodriguez *et al.*, 2008; Smith & Broadley, 2007; Toward *et al.*, 2004) it is argued that this route neglects the fact that humans are sensitised by being exposed to allergens via the respiratory mucosa (Cates *et al.*, 2007). Cates *et al.*, (2007) suggest this may have a number of significant immunological consequences. Studies have tried to overcome this issue by sensitising with an inhalation of OVA (Hamelmann *et al.*, 1997). However, this route is rarely successful (Cates *et al.*, 2007). It appears that the method of administration can influence the host response (Kumar *et al.*, 2008). Mice sensitised via the intraperitoneal route have been shown to have higher levels of

IgE than those sensitised via intranasal administration (Kurup *et al.*, 1992; Zhang *et al.*, 1997).

Another source of controversy in the method of sensitisation employed throughout this thesis is the use of an adjuvant. The adjuvant, aluminium hydroxide, was used to boost the immune response to OVA. Adjuvants usually induce a strong Th2-biased response with high levels of total and antigen-specific type 1 hypersensitivity reaction antibodies produced (Kumar *et al.*, 2008). A study by Nakae *et al.*, (2007) showed that AHR and airway inflammation are mast cell-independent when an adjuvant is used, however, without an adjuvant these parameters are mast cell-dependent. It is likely when an adjuvant is used that the EAR is still mast cell-dependent as the bronchoconstriction occurs immediately after allergen challenge. Adjuvant-free protocols exist but require many injections to achieve sensitisation (Blyth *et al.*, 1996; Hessel *et al.*, 1997).

Despite the argument that the method of sensitisation used in this thesis does not follow the same route as human sensitisation the vast majority of studies use this method (Cates *et al.*, 2007). The fact that an increased level of IgG1 was observed in the guinea pig models following sensitisation suggests that intraperitoneal administration of OVA does provoke an immune response. Further support of this method of sensitisation is that early and late bronchoconstrictions, AHR, cellular influx, airway oedema and airway remodelling, all features of human asthma, were all observed in the chronic OVA guinea pig model. Currently there are no studies comparing and contrasting the effects that various methods of allergen administration and the use, or absence, of adjuvants have on sensitisation and the features of asthma observed in the chronic OVA model following further allergen challenge.

9.1.2 CHALLENGE PROTOCOLS

As with the process of sensitisation there are many different protocols established using allergen challenges to provoke asthma-like responses. Although there are differences between routes of administration most studies use inhalation of aerosol as the route of choice (Kumar *et al.*, 2008). However, the main differences between studies are the number of challenges. It is the number of challenges which determine whether a protocol is acute or chronic. Most inhalation challenge models are short-term, or acute, and typically consist of one or several challenges over a period of time lasting not much more than a week (Kumar *et al.*, 2008). The

acute guinea pig protocol used in this thesis was optimised by Smith and Broadley (2007) and consisted of a single challenge following sensitisation. To provoke a bronchoconstriction in the acute OVA mouse model, two inhalations separated by four hours was used. This follows a protocol developed by Fernandez-Rodriguez *et al.*, (2008) adapted from Ohkawara *et al.*, (1997). Although there are other acute protocols which lead to some of the features of asthma the model by Fernandez-Rodriguez *et al.*, (2008) appeared to be the first to show a biphasic bronchoconstriction followed by AHR and correlating cellular influx.

The main reason why acute models of asthma are more commonly used than long-term chronic models is that long-term exposure to an allergen can cause tolerance (Schramm *et al.*, 2003). Although, the exact mechanisms behind tolerance are unclear an element of adaptation needs to be implemented before the chronic features of asthma can be observed in animal models. In the case of guinea pigs in this thesis, a ten-fold higher dose of OVA was used following the original OVA exposure and sensitisation. This protocol was developed in these laboratories by John (2007). The higher dose of OVA would cause an anaphylactic response as a result of increased histamine release. To combat this response the histamine H_1 receptor antagonist mepyramine was used. However, by the day of the final exposure (day 31) mepyramine protection was no longer needed highlighting the level of tolerance that the guinea pig model underwent. It is possible that if the chronic model was extended longer than 31 days then the dose of OVA used would have to be increased again to provoke an asthmatic response.

Chronic murine models of asthma take around 8 weeks to develop (Kumar *et al.*, 2008). However, long-term administration of a high concentration of allergen can lead to the development of parenchymal inflammation a condition which is markedly different from asthma (Kumar & Foster, 2002). Long-term allergen administration can also lead to down-regulation of the inflammation, AHR and structural airway changes as a result of tolerance (Jungsuwadee *et al.*, 2004; Palmans *et al.*, 2000; Yiamouyiannis *et al.*, 1999). To overcome these issues long-term exposures to a low dose of allergen are now being used (Fernandez-Rodriguez *et al.*, 2008; Kumar *et al.*, 2008). Although models using this approach have been relatively successful at preventing tolerance and displaying some of the features of asthma none have shown EAR, LAR, AHR, cellular influx and airway remodelling. The model developed by Fernandez-Rodriguez *et al.*, (2008) was able to show a biphasic inflammatory response, AHR and increased levels of eosinophils. However, airway remodelling was not assessed in this model. By using this protocol and assessing airway remodelling, along with

the other parameters previously measured, it was possible to develop a mouse model of asthma that could prove more effective for pre-clinical modelling.

9.1.3 OUTCOMES

9.1.3.1 GUINEA PIGS

Challenging a sensitised guinea pig with OVA on day 15 provoked an inflammatory response characterised by an early and late phase bronchoconstriction. Treatment with a corticosteroid, fluticasone propionate, PDE4 inhibitor, roflumilast or an iNOS inhibitor, GW274150F, attenuated the late phase bronchoconstriction. However, only roflumilast had an inhibitory effect on the early bronchoconstriction. The chronic OVA model also had an inflammatory response characterised by an early and late asthmatic response. Once again only roflumilast was able to reduce the level of bronchoconstriction during the EAR. Fluticasone propionate and roflumilast attenuated the LAR but GW274150F was ineffective against the bronchoconstriction.

24 hours following the final OVA exposure guinea pigs were challenged with histamine (1 mMol, 20 secs) to assess airway responsiveness. The acute OVA challenged guinea pigs had a bronchoconstriction following histamine challenge representing AHR. The level of responsiveness decreased to baseline levels after 72 hours. The AHR was inhibited by each of the three drugs. Chronic OVA challenges caused AHR characterised by a bronchoconstriction following a histamine challenge. In contrast to the acute model, AHR was inhibited with fluticasone propionate and roflumilast treatment but not with GW274150F. AHR was still present 72 hours after the final OVA challenge.

Following the final histamine challenge the guinea pigs were killed and lavaged enabling a total and differential cell count to be carried out. Acute OVA challenged guinea pigs had significantly greater levels of total cells and each of the subtypes assessed (macrophages, eosinophils, lymphocytes and neutrophils) than control guinea pigs. 72 hours after challenge a significant reduction was observed in total cell number and macrophage, eosinophil and neutrophil number. However, the levels of cells still appeared greater than naïve guinea pig levels. Fluticasone propionate, roflumilast and GW274150F only reduced the total number of cells and number of eosinophils. The chronic OVA challenged guinea pigs had significantly greater levels of total cells and all of the subtypes compared to control. The total number of

inflammatory cells, macrophages, eosinophils and neutrophils all significantly decreased when the guinea pigs were given 72 hours to recover following the final OVA exposure. When comparing drug treatments against the chronic OVA model roflumilast was the most efficient by decreasing levels of total cells, macrophages, eosinophils and neutrophils. Fluticasone propionate treatment reduced total cells and eosinophil number and GW274150F only affected total cells and neutrophils.

The chronic OVA challenged protocol was the only one to cause airway remodelling in the airways of sensitised guinea pigs. Airway remodelling was characterised by an increase in pulmonary inflammatory cells, increased smooth muscle, increased levels of collagen found in the lamina propria and goblet cell hyperplasia. An increase in pulmonary cells in the chronic OVA challenged group compared to control was observed following staining of the respective bronchioles with haematoxylin and eosin. Treatment with fluticasone propionate and roflumilast significantly decreased the mean pathological score, however, GW274150F had no significant effect. The bronchioles of the chronic OVA challenged guinea pig had a thicker lamina propria and epithelium and increased levels of smooth muscle than control. Treatment with fluticasone propionate appeared to reduce all of these features. Only the amount of smooth muscle and thickness of the epithelium was affected by roflumilast treatment and GW274150F only appeared to reduce the level of smooth muscle.

MRI analysis in guinea pigs showed the levels of oedema reached a peak that correlated with the LAR. Compared to control animals, the acute OVA challenged guinea pigs showed significantly greater levels of oedema 4, 7 and 25 hours following challenge. The chronic OVA challenged guinea pigs had significantly greater levels of oedema compared to control at each of the time points suggesting that there were underlying levels of oedema before the final OVA challenge. Despite the high levels of baseline oedema in this model, levels still increased following challenge and peaked at 7 hours. At baseline, 0.25 and 4 hours post-final OVA challenge the chronic OVA model had significantly greater levels of oedema than the acute OVA model. However, at 7 and 25 hours following challenge the differences between the two groups were not significant. This suggests that oedema does not disappear as quickly as it is formed and therefore this is likely to be why such a high baseline level of oedema was observed in the chronic OVA group. Treatment with the corticosteroid, dexamethasone, was able to attenuate the LAR, inhibit AHR and reduce the total number of cells and macrophages in the acute OVA model. When the effect of dexamethasone on the levels of oedema in the acute OVA challenged guinea pigs were compared to vehicle a significant reduction was

observed at the 7 hour mark. No other significant differences were observed throughout the other time points though it is clear to see that despite dexamethasone treatment the levels of oedema are still greater than naïve guinea pig levels. The effect of dexamethasone treatment on the chronic OVA guinea pig model was not assessed.

9.1.3.2 MICE

Mice sensitised with OVA and then challenged twice (4 hours apart) on day 15 have a biphasic bronchoconstrictor response. The first response is the EAR. This is slightly delayed compared to the guinea pigs possibly because serotonin (the primary mediator in mice mast cell degranulation) takes longer than histamine to reach a level that causes bronchoconstriction (unpublished observations – Fernandez-Rodriguez *et al.*). The second bronchoconstriction represents the LAR. The effects of fluticasone propionate, roflumilast and GW274150F were also assessed on the mouse models. Roflumilast attenuated the EAR and LAR in the acute OVA challenged mouse. However, fluticasone propionate and GW274150F were only effective against the LAR. The chronic OVA mouse model was challenged with three OVA challenges a week for six weeks (18 challenges) followed by a final challenge on the first day of the seventh week. This protocol provoked a biphasic airways function response characterised by an EAR and LAR. Once again it was only roflumilast that was able to have any effect on the EAR. Roflumilast was also able to attenuate the LAR as was fluticasone propionate, however, GW274150F was ineffective.

The responsiveness of the airways was assessed in the acute and chronic models using 30 mg/kg or 10 mg/kg of methacholine respectively. A significant level of AHR was observed following OVA challenge in the acute group. This AHR was significantly attenuated by each of the drugs administered, however, GW274150F was the least effective and a degree of AHR was still present. The chronic OVA group were challenged with a lower dose of methacholine so not to cause a fatal level of bronchoconstriction. AHR was observed post-final OVA challenge following methacholine challenge though because of the lower dose the level of AHR was not as great as in the acute OVA model. Fluticasone propionate and roflumilast treatment attenuated the levels of AHR, however, GW274150F was ineffective.

Following the final methacholine challenge the mice were killed and lavaged. However, as mice have small trachea, damage and contamination with blood frequently occurs and as a

result a total cell count could not be accurately carried out. Therefore the only means of assessing the bronchoalveolar lavage fluid was by using a differential cell count to determine the ratio of inflammatory cell subtypes present in the airways. The acute and chronic OVA challenged mice had a significantly greater percentage of eosinophils compared to control. However, the percentage of macrophages was apparently significantly decreased in the two OVA challenged groups compared to control. It is likely that this is not a real decrease in macrophages but a consequence of the increased percentage of eosinophils. Fluticasone propionate treatment significantly reduced the percentage of eosinophils in the acute OVA challenged mice; with a resulting apparent increase in macrophage percentage also observed. This was also the case for roflumilast treatment. GW274150F had no effect on cell percentages in the acute OVA challenged mice. In the chronic OVA challenged model a significant decrease in eosinophil percentage was observed following fluticasone propionate treatment. Roflumilast was also able to significantly decrease eosinophil percentage in the chronic OVA challenged model, however, a corresponding increase in macrophage percentage was observed. Once again, GW274150F had no effect on cell percentages.

No features of airway remodelling were observed in the acute OVA challenged mouse model, although a significant increase in mean pathological score was observed compared to control. The chronic OVA challenged mice also had a significantly greater mean pathological score compared to control and also had a significantly greater level of pulmonary tissue cells than the acute OVA challenged group. Chronic OVA challenges caused structural changes in the airways, or airway remodelling, characterised by increased levels of smooth muscle, increased thickness of the lamina propria by extracellular matrix proteins such as collagen and goblet cell hyperplasia. Compared to vehicle, fluticasone propionate treatment caused a reduction in the level of smooth muscle, collagen and goblet cells, though not to the same levels as naïve mice. Roflumilast treatment was also able to reverse the features of airway remodelling observed in the chronic OVA challenged mice compared to vehicle treatment. GW274150F had no effect on the structural changes observed in the chronic OVA mouse model and therefore was ineffective at reversing airway remodelling.

9.1.4 THE USE OF P_{enh}

Whole-body unrestrained plethysmography was used to measure airway responsiveness in conscious mice. Enhanced pause (P_{enh}), a non-dimensional parameter based on a

characteristic change in expiratory waveshape of the unrestrained plethysmograph box signal (Lomask, 2006), was used as an indicator of bronchoconstriction. Whole-body plethysmography measures two changes in the box pressure: a reduction caused by the inspiration of air by the animal and an increase because of the lungs expanding (Buxco Research Systems, 2006 referenced by Fernandez-Rodriguez *et al.*, 2008). Normally these flows cancel each other out. However, in conditions such as asthma the changes in box pressure during expiration are more pronounced than during inspiration (Dorsch *et al.*, 1981). P_{enh} has been used repeatedly as an indicator of airway responsiveness to bronchoconstrictors (Archer *et al.*, 2004; Fernandez-Rodriguez *et al.*, 2008; Lee *et al.*, 2005).

Despite this, the validity of P_{enh} as a marker of airway responsiveness has been questioned (Adler *et al.*, 2004; Lundblad *et al.*, 2002). Lundblad *et al.*, (2002) claim that the unrestrained plethysmograph waveform, and parameters derived from it (P_{enh}), are dominated by conditioning and are essentially unrelated to resistance. However, a paper by Lomask (2006) discusses the mathematics of unrestrained plethysmography as applied to two types of whole-body plethysmographs: a sealed chamber (pressure whole-body plethysmograph, PWBP) and a chamber with a pneumotachograph on its wall (flow whole-body plethysmograph, FWBP). Lomask (2006) describes how the PWBP waveform is largely dominated by conditioning, thus supporting the claim by Lundblad (2006) that unrestrained plethysmography and P_{enh} are unrelated to resistance. However, Lomask (2006) goes on to describe how the effects of resistance are evident in the FWBP waveform and P_{enh} is derived from this waveform. Lomask (2006) shows that the changes in the FWBP waveform which occur after methacholine challenge cannot be down to conditioning and are not just changes in respiratory timing but can be quantified by P_{enh}. Therefore it would seem that the use of P_{enh} is acceptable in chambers with a pneumotachograph, which is what was used in this thesis. How P_{enh} is derived can be found in Appendix 3.

9.2 EXPERIMENTAL LIMITATIONS

As with all models of asthma, guinea pigs and mice have limitations and therefore should not be thought of as having asthma. Guinea pigs or mice do not develop asthma, but this thesis has entailed provoking an inflammatory response that resembles the features of asthma. Therefore a result or response in one of these models may not be the same in human

asthmatics. Despite this, animal models have still proved very important in understanding the mechanisms and potential targets of asthma.

Aside from the obvious differences between humans and guinea pigs, such as the fact that humans are bipedal, arguably one of the major downfalls of using guinea pig models of asthma is the lack of species-specific reagents. Even though IgG1 is the major antibody in type 1 hypersensitivity allergies in guinea pigs IgE is still present. In this thesis OVA-specific IgG1 was assessed however, there is only one ELISA kit commercially available to measure OVA-specific IgE in guinea pigs. Establishing reproducible results using this kit proved to be difficult and as a result the data obtained was not included in this thesis. If one wanted to investigate the role of cytokines in the guinea pig model of asthma this would prove very difficult because of the limited amount of kits to do so. These issues are overcome by using the mouse model as the immune system in mice is well characterised and several species-specific reagents are commercially available.

The mouse model is also not without problems. Mice are inquisitive by nature and therefore require a great deal of conditioning and acclimatising to the Buxco chamber before readings can be taken. The measurement of P_{enh} by whole-body plethysmography also leads to intense criticism of the mouse model; this is discussed in the previous section.

Throughout this thesis total bronchoalveolar lavage (BAL) fluid cell counts were included for guinea pigs but not mice. This was because the size of the mouse trachea made it very difficult to cannulate without damage resulting in the contamination of the BAL fluid with red blood cells. As a consequence of this the differential cell count was presented as the percentage of inflammatory cell subtypes. This meant if one subtype was to increase in percentage then another subtype would show a corresponding decrease in percentage to compensate. This led to the situation like in the OVA challenged mice that eosinophil percentage was significantly greater than control but macrophage percentage was significantly decreased. This made it appear that there were less macrophages in the OVA challenge BAL fluid compared to the saline challenge BAL fluid. In reality this probably was not the case as OVA challenged guinea pigs have increased numbers of macrophages compared to control. However, without the total cell count one cannot say accurately whether OVA challenge in mice caused increased numbers of macrophages. The issue was partially overcome by the scoring of inflammatory cells in the lung tissue of the mice. This suggested that OVA challenged mice had a greater influx of inflammatory cells to the lung tissue

compared to their respective controls. However, it was not possible to distinguish leukocyte subtype using this method.

Another limitation of this thesis is the accuracy of the histological and MRI analysis. These methods both involved a degree of analysis by eye; therefore an element of human error is possible. Although every effort was made to avoid bias when analysing, such as randomising the histological and MRI images assessed, it is possible a degree of subconscious bias may still occur. Using an independent worker to carry out blind analysis would overcome this issue, unfortunately this was not possible at the time. The fact that all histological analysis was 2-dimensional means it is not as accurate as 3-dimensional analysis. However, this would have been too time consuming for this thesis and it is likely that most of the changes in the airways are represented using 2-dimensional analysis.

9.3 FURTHER WORK

If there were no time or financial constraints there are several studies that this thesis may have benefitted from. Firstly, all work was carried out in male animals. It would have been interesting to determine whether the same effects occur in female guinea pigs and mice as were observed in male guinea pigs and mice in this thesis. Clearly asthma affects both males and females, therefore if there is a fundamental difference in how one sex reacts to a drug it would be important to discover this, for example whether the steroid oestrogen has some effect on the inflammation in asthma.

The dosing schedule of the drugs was 24 and 0.5 hours before the final OVA challenge and 6 hours after in the chronic model. This measured the ability of the drug to reverse any changes observed. Although fluticasone propionate and roflumilast proved to be rather effective against airway remodelling it would have been interesting to administer them before each of the OVA challenges and therefore determine whether they have preventative properties. It is possible that if this method was employed there would be a lack of airway remodelling in the chronic model. The dosing schedule that was used during this thesis was not very clinically relevant and was only suitable for assessing what changes the drugs can have if they are administered before and during an asthmatic episode. In reality it would be almost impossible to predict an asthmatic episode was about to happen and therefore administer drugs 24 hours and 30 minutes before it occurred. Employing either the preventative method or a randomised

schedule of drug administration would improve clinical relevance. Usually drugs are administered immediately after an asthmatic episode. It would have been interesting to assess what effect roflumilast would have using this dosing schedule, as it was shown to have some effectiveness against the EAR. However, for the purpose of this thesis the dosing schedule used was satisfactory.

When it came to the MRI work at GlaxoSmithKline, assessing the effect of dexamethasone on the levels of oedema in the chronic OVA model was not possible. This was partially a result of time and secondly the guinea pigs were becoming too big to fit in the magnet of the MRI. Therefore, without financial or time limitations it would be interesting to carry out this experiment, this would determine whether levels of oedema can be reduced in the chronic OVA model. Currently the chronic OVA guinea pig model only lasts 31 days, extending this period may result in either further tolerance to the higher OVA dose or greater levels of airway remodelling. With more time it could have been worthwhile to investigate this and any potential changes in recovery times.

Another experiment this thesis could benefit from is analysing the pulmonary levels of 3-nitrotyrosine (3-NT) protein to determine the levels of nitrosylation of lung proteins as an index of nitric oxide production could be a worthwhile experiment. This would determine whether GW274150F was inhibiting iNOS. If *in vivo* elevated 3-NT proteins were still present in the lung tissue after GW274150F treatment this would indicate that it was unable to completely inhibit iNOS. However, if the proteins were not present this would suggest GW274150F is effective at reducing iNOS and that iNOS induced NO does not have a major role in causing the asthmatic response. This experiment was attempted though the results were inconclusive and therefore left out of this thesis.

9.4 CLINICAL RELEVANCE

Asthma is a chronic inflammatory disorder that affects of 300 million people worldwide (WHO). Many of the advances in the understanding of the pathobiology of asthma have come from provoked models of airway inflammation in animals (O'Byrne *et al.*, 2009). Although a few drugs have shown efficacy in animals but not humans, such as GW274150F in the acute OVA model, animal models are the only system available for modelling the *in vivo* processes of human asthma (Zosky & Sly, 2007). Currently short-term, or acute, models of asthma are

commonly used (Zosky & Sly, 2007); however, these models have significant limitations and do not take the chronic nature of asthma into account (Kumar & Foster, 2002). The fact that the acute OVA model only has one challenge, and the drugs were administered 24 hours and half hour before this challenge, is equivalent to trying to treat a sensitised individual before their first asthmatic episode.

The chronic OVA models of asthma developed in this thesis demonstrated lung inflammation, AHR, inflammatory cell influx and airway remodelling. It seems likely that this is the first time that all of these features have been observed in a guinea pig and a mouse model. Therefore, these models are extremely relevant for pre-clinical evaluation of potential mechanisms of asthma and subsequent therapy.

There are criticisms of the method of sensitisation and use of an adjuvant as detailed in section 9.1.1. However, as of yet these methods have not been proven to cause a vastly different immune response than the one observed in asthma. Research has been carried out to try and find a more clinically relevant allergen than ovalbumin such as house dust mite (Kim *et al.*, 2006), olive pollen (Batanero *et al.*, 2002) and Aspergillus fumigatus extract (Baelder *et al.*, 2005). However, ovalbumin is still the allergen of choice to provoke an immune response in the majority of studies modelling asthma (Cates *et al.*, 2007) as these other models of asthma are not able to produce the same features observed in ovalbumin models.

Perhaps the best evidence that the chronic OVA models are more clinically, or pre-clinically, relevant than the acute OVA models was the response to GW274150F treatment. In the acute OVA models GW274150F was quite effective at reducing the LAR, AHR and cellular influx. However, in the chronic OVA models, GW274150F was not effective though the dosing schedule was the same. This was also the case in clinical trials as GW274150F had no effect on AHR or inflammatory cells number (Singh *et al.*, 2007). This suggests the chronic OVA model is a better representation of human asthma than the acute OVA model, possibly because airway remodelling occurs in the chronic OVA model.

Chapter 10

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10.1 REFERENCES

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APPENDIX 1

EQUIPMENT

Amplifier (Biopac systems TCI 100) – Biopac Systems Inc., Santa Barbara, CA, USA.

Cannula – Fisher Scientific UK, Loughborough, UK.

Centrifuge (Mistral 3000) – Mistral, UK.

Cold Plate – Fisher Scientific UK, Loughborough, UK.

Coverslip – Surgipath Europe Ltd, Peterborough, UK.

Digital Camera (Camedia C4040ZOOM) – Olympus, London, UK.

ELISA Plate – Greiner Bio-One Ltd., Stonehouse, UK.

Exposure Chamber (Guinea Pigs) – Buxco Research Systems, Winchester, UK.

Exposure Chamber (Mice) – Buxco Research Systems, Winchester, UK.

Exposure Tunnel for Airway Responsiveness – In-house built

Glass Slides – Surgipath Europe Ltd, Peterborough, UK.

Histology Cassettes – Surgipath Europe Ltd, Peterborough, UK.

Microscope (Olympia – BH-2) – Olympus, London, UK.

Microtome – Mistral, UK.

MRI Machine – Bruker Corporation, Coventry, UK.

Neubauer Haemocytometer – Supe-rior, Marienfeld, Germany.

Plate Reader (ELISA) – Sunrise Systems, Cambridge, UK.

Pressure Transducers (UP1 and UP2) – Pioden Controls Ltd., Canterbury, UK.

Wax Dispenser – Fisher Scientific UK, Loughborough, UK.

Whole Body Plethysmograph (Gunea Pigs) – In-house built

Whole Body Plethysmograph (Mice) – Buxco Research Systems, Winchester, UK.

Wright Nebuliser (Pulmostar) – Sunrise Medical Ltd., Wollaston, UK.

MATERIALS

Acid alcohol – Surgipath Europe Ltd, Peterborough, UK.

Alcian blue – Surgipath Europe Ltd, Peterborough, UK.

Aluminium hydroxide – Sigma, Poole, UK.

Aqueous acetic acid – Sigma, Poole, UK.

Chloroform – Sigma, Poole, UK.

Citric acid – Sigma, Poole, UK.

Dexamethasone – Sigma, Poole, UK.

Dimethyl sulfoxide – Sigma, Poole, UK.

DPX mountant – Surgipath Europe Ltd, Peterborough, UK.

Eosin – Surgipath Europe Ltd, Peterborough, UK.

Ethanol – Sigma, Poole, UK.

Fluticasone propionate – GlaxoSmithKline, Stevenage, Herts, UK.

Formaldehyde – Fisher Scientific UK, Loughborough, UK.

Guinea Pigs – Harlan, UK.

GW274150F – GlaxoSmithKline, Stevenage, Herts, UK.

Histamine – Sigma, Poole, UK.

Hydrochloric acid – Sigma, Poole, UK.

IgG Antibody Horse radish peroxidise Conjugated – Biocompare, Harrogate, UK.

Industrial methylated spirit – Surgipath Europe Ltd, Peterborough, UK.

Isoflourane – Abbott Animal Health, Maidenhead, UK.

Leishman's solution – Sigma, Poole, UK.

Mayer's haematoxylin – Surgipath Europe Ltd, Peterborough, UK.

Mepyramine – Sigma, Poole, UK.

Methacholine – Sigma, Poole, UK.

Methanol – Fisher Scientific UK, Loughborough, UK.

Methyl cellulose – Sigma, Poole, UK.

Mice – Harlan, UK.

Milk (Powdered, non-fat) – Shop bought.

O-phenylenediamine dihydrochloride – Sigma, Poole, UK.

Ovalbumin – VWR International Ltd., Leicestershire, UK.

Paraffin wax – Surgipath Europe Ltd, Peterborough, UK.

Periodic acid – Surgipath Europe Ltd, Peterborough, UK.

Phosphate buffered saline – Fisher Scientific UK, Loughborough, UK.

Picric acid – Sigma, Poole, UK.

Roflumilast – GlaxoSmithKline, Stevenage, Herts, UK.

Schiff's reagent – Surgipath Europe Ltd, Peterborough, UK.

Sirius red – Sigma, Poole, UK.

Sodium pentobarbitone (Euthatal) – Merial, Harlow, UK.

Sodium phosphate – Sigma, Poole, UK.

Sterile water – Sigma, Poole, UK.

Sulphuric acid – Sigma, Poole, UK.

Tween – Sigma, Poole, UK.

Xylene – Surgipath Europe Ltd, Peterborough, UK.

COMPUTER PROGRAMS

Acqknowledge – Biopac Systems, Inc., Goleta, CA, USA.

Analyze – AnalyzeDirect, Inc., Overland Park, KS, USA.

GraphPad Instat 3 – GraphPad software, Inc., La Jolla, CA, USA.

GraphPad Prism 5 – GraphPad software, Inc., La Jolla, CA, USA.

ImageJ – National Institute of Health - http://rsbweb.nih.gov/ij/index.html

APPENDIX 2 – CALCULATION OF sG_{aw}

Airways resistance is based on laminar flow in straight, smooth-walled tubes. The physical law that describes such a flow is Poiseuille's Law, thus pressure drop due to friction (ΔP) is defined as:

$$1.\ \underline{\Delta P = 8\mu l v/\pi r^4}$$

(μ=viscosity; l = length of tube; r = radius; v=flow rate)

The airways however can consist of many branches and bends and are subject to dynamic changes in the radius of the tubes due to bronchoconstrictions and secretions. Therefore as airflow increases it becomes more turbulent and conversely as it approaches zero becomes more laminar.

Flow through the airways is induced by the alveolar pressure difference (P_d) i.e. the difference between mouth pressure (P_m) and alveolar pressure (P_{alv}).

$$P_d = P_m - P_{alv}$$

Airways resistance (R_{aw}) is defined as the relationship between instantaneous flow (v) and alveolar pressure difference.

$$R_{aw} = P_d/v$$

Conductance is the reciprocal of resistance, therefore airways conductance (G_{aw}) is defined as:

$$G_{aw} = R_{aw}^{-1} = v/P_d$$

To compare between different subjects the differences in thoracic gas volume (TGV) must be eliminated, hence specific airway conductance (sG_{aw}) is used.

$$2.\ \underline{sG_{aw} = v/P_d.TGV}$$

Plethysmograph – In a sealed box at a constant temperature, changes in pressure are related to changes in volume according to Boyle's Law, i.e. the volume of a gas changes inversely with the changes in pressure at a constant temperature.

$$P_1.V_1 = P_2.v_2$$

or

$$P.V = (P+\delta P).(V-\delta V)$$

by multiplying the two brackets

$$P.V = P.V - P.\delta V + V.\delta P - \delta P.\delta V$$

As $\delta P.\delta V$ is negligible

$$V.\delta P = P.\delta V$$

For a sealed plethysmograph changes in the alveolar pressure difference will be seen as changes in box pressure. Box pressure changes result from the difference in chest volume changes (V_c) and respired air volume changes (V_r) at atmospheric pressure (P_{atm}), corrected for saturated water vapour pressure (P_{svp}). So from Boyle's Law:

$$3.\ \underline{\delta P_d.TGV = \delta(V_c-V_r).(P_{atm}-P_{svp})}$$

For Poiseuille's Law to be true, in order to have laminar flow, v must tend towards zero, i.e. at flow manoeuvre reversal (end inspiration or end expiration). Conventionally end expiration is frequently used since TGV approximates to the functional residual capacity (FRC). Hence any changes in Pd must be small, i.e. δP_d which in turn will induce small changes in flow (δv).

Therefore substituting 2. into 3. gives:

$$sG_{aw} = \delta v/\delta(V_c-V_r).(P_{atm}-P_{svp})$$

Using computerised data acquisition it is possible to measure $\delta(V_c-V_r)$ as the slope of the change in box volume where flow tends towards zero. It is then possible to record the simultaneous change in flow (δv).

$$P_{atm} = 1010cmH_2O$$

$$P_{svp} = 63cmH_2O$$

Therefore by knowing δv and $\delta(V_c-V_r)$ from the slopes of the flow and box volume traces respectively sG_{aw} can be calculated:

$$sG_{aw} = \delta[\text{flow}]/\delta[\text{box volume}].947.cfs^{-1}cmH2O^{-1}$$

cf is a correction factor to account for the volume of air displaced by the guinea pig (V_{gp}). The density of a guinea pig (P_{gp}) is approximately $1.07kgl^{-1}$ and the volume of the plethysmograph chamber (V_{box}) is 5.5 litres. Thus:

$$V_{gp} = W_{gp}/ P_{gp} \text{ litres}$$

The net volume of the plethysmograph:

$$V_n = V_{box} - V_{gp} \text{ litres}$$

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$$cf = (V_{box}-V_{gp})/V_{box}$$

$$cf = 1 - (V_{gp}/V_{box})$$

$$cf = 1 - W_{gp}/ P_{gp}.V_{box}$$

$$cf = 1 - W_{gp}/5.885$$

Therefore:

$$\underline{\mathbf{sG_{aw} = \delta[flow]/\delta[box\ volume].947.1\text{-}W_{gp}/5.885\ cmH_2O^{-1}}}$$

Adapted from John (2007).

APPENDIX 3 – CALCULATION OF P_{enh}

The waveform from a flow whole-body plethysmograph can be separated into two regions: The first region contains the transition from inspiration to expiration. Region #2 follows region #1 but does not include the transition (Lomask, 2006). The ratio between the mean height of the two regions will respond to resistance. The comparison is made by the parameter Pause (Lomask, 2006).

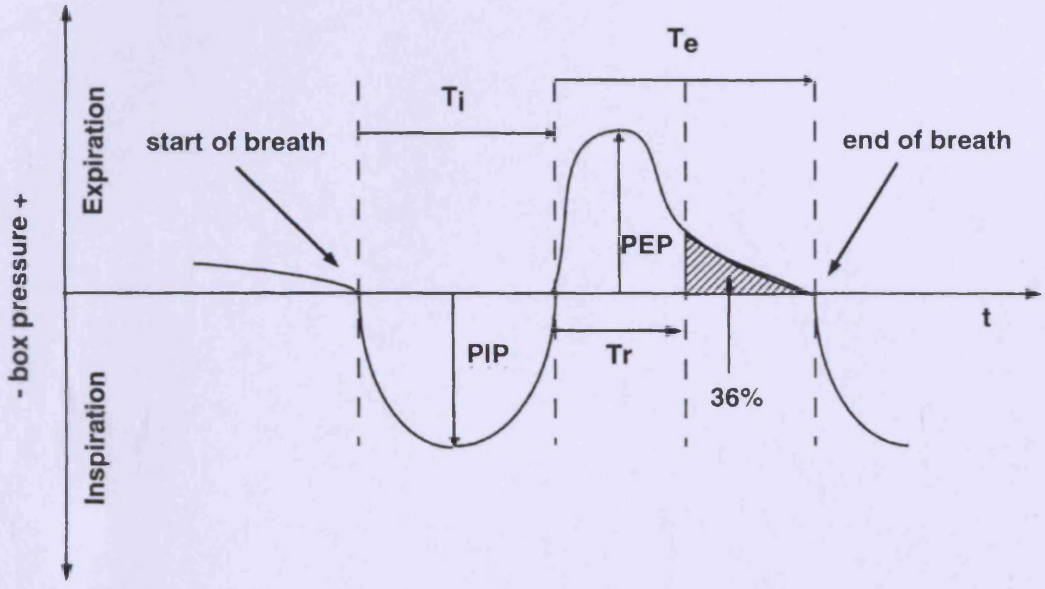

Figure A: A schematic diagram to show the box pressure wave in inspiration (down) and expiration (up). Using the parameters on this diagram P_{enh} can be derived. Diagram from Hamelmann *et al.,* (1997). Ti = Time of inspiration; Te = Time of expiration; Tr = Relaxation time; PIP = Peak inspiration pressure; PEP = Peak expiration pressure.

Figure A shows a box pressure wave in inspiration and expiration. The amount of time taken from the start to the end of inspiration is Ti. Time of expiration (Te) is the time from the end of inspiration to the start of the next inspiration. The time it takes for the pressure to decay to 36% of the total expiratory pressure signal is known as relaxation time (Tr). This may serve as a correlate to the time constant of the decay of volume signal to 36% of the peak volume in passive expiration (Hamelmann *et al.,* 1997). The transition of the flow of an animal from inspiration to expiration will take place during the Tr interval (Lomask, 2006). Therefore the following formula is used to calculated Pause:

$$\underline{\text{Pause} = (\text{Te-Tr})/\text{Tr}}$$

Levels of peak inspiration pressure (PIP) and peak expiration pressure (PEP) are used to calculation enhanced pause (P_{enh}). Conditioning during expiration is significantly less than during inspiration; therefore a change in resistance will be more prominent in the expiratory phase (Lomask, 2006). Using the ratio of PEP to PIP will enhance the sensitivity of Pause, in other words:

$$\underline{\mathbf{P_{enh}\ (Enhanced\ pause) = (PEP/PIP)\ x\ Pause}}$$