Role of bradykinin in virus-induced airway inflammation

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Summary

Asthma is a chronic inflammatory disease of the airways and viral infections account for the majority of exacerbations and may play a role in its pathogenesis. Bradykinin levels are increased in the lungs of asthmatics and inhaled bradykinin produces bronchoconstriction in asthmatic but not in normal patients. In this study, guinea-pigs were inoculated with parainfluenza and influenza virus to establish airways inflammation and hyperreactivity. The role of bradykinin in the parainfluenza model was examined by using the tissue kallikrein inhibitor, FE999024, and the bradykinin B_2 receptor antagonist, MEN16132. Firstly, the effects of bradykinin inhalation in conscious guinea-pigs were characterized by using inhibitors of its breakdown and selective antagonists. Inhaled bradykinin produced a bronchoconstriction only after treatment with the inhibitors of angiotensin converting enzyme and/or neutral endopeptidase, captopril and phosphoramidon respectively. Inhaled bradykinin also increased inflammatory cell influx to the lungs when its breakdown was inhibited with both drugs. Cell influx and bronchoconstriction were blocked by the B_2 receptor antagonists icatibant and MEN16132. These responses were therefore B_2 receptor-mediated. In ovalbumin sensitized guinea-pigs, inhaled ovalbumin produced early and late asthmatic responses, inflammatory cell influx and airway hyperreactivity to histamine. These were inhibited by dexamethasone. Bradykinin caused bronchoconstriction without using metabolism inhibitors, indicating airways hyperreactivity to bradykinin. Parainfluenza-3 and influenza caused inflammatory cell influx and airways hyperreactivity to histamine. These were inhibited by FE999024, MEN16132 and dexamethasone. Parainfluenza-3 virus inoculated into sensitized guinea-pigs exacerbated the response to inhaled

ovalbumin, with a prolonged bronchconstriction replacing early and late phases. This was resistant to dexamethasone. This study supports a role for bradykinin in virus-induced lung inflammation and the use of inhibitors of bradykinin for potential treatment of airway inflammation.

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Chapter 1

Introduction

1.1 Asthma

Asthma is a chronic inflammatory disease of the airways. It is characterized by bronchial hyperresponsiveness, an influx of inflammatory cells, epithelial cells damage and bronchiolar fibrosis (Cohn *et al* 2005).

1.2 Clinical Categories

Atopic asthma is triggered by allergens and is usually diagnosed early in life. Atopic asthma is caused by an inappropriate Immunoglobulin-E (Ig-E)-mediated immune response in certain individuals (Lopez and Salvaggio 1987). Non-atopic asthma is caused by respiratory infection, exercise and drugs including aspirin and develops later in life. In non-atopic asthma there is no antibody or hyper-sensitivity reaction and IgE levels are normal. There is a third type of asthma caused by exposure to specific proteins or small chemicals at work and is called occupational asthma.

1.3 Inflammation

Asthma is a complex inflammatory disease of the airway which involves the release of a wide range of inflammatory mediators from a wide range of inflammatory and structural cells. Subjects who suffer from atopic or allergic asthma become sensitive to a specific allergen. When they are subsequently exposed to that allergen, asthmatic symptoms will occur. Sensitization occurs when allergens, such as those associated with house dust mites or pollens, are recognized by antigen presenting cells (APCs), such as macrophages, B cells and dentritic cells. IgE on the surface of APCs binds the antigen and causes it to be internalized by endocytosis. The antigen is processed and then

transferred to the cell surface as membrane bound histocompatibility complex class II (Banchereau and Steinman, 1998). The antigen is recognised by naïve T cells which are activated and differentiate into T helper type 1 (Th1) or T helper type 2 (Th2) cells. This differentiation is triggered by interleukin (IL)-2 which is released from naïve T cells. IL-2 has an autocrine function, causing proliferation of the cells that release it, giving rise to a clone of activated T cells. Whether T cells become Th1 or Th2 is dependent upon the presence of cytokines IL-12 and IL-4, respectively (Romagnani 1997). Th1 cells are responsible for killing intracellular parasites and are involved in autoimmune responses (Berger 2000). They secrete macrophage-activating cytokines and other cytokines such as interferon (IFN)-γ activate cluster of differentiation (CD)8+ T cells to become cytotoxic cells (Tc) that kill virally infected host cells. IFN-γ derived from Th1 cells inhibits Th2 cell function. Th2 cells are responsible for the development of antibody-mediated immune responses. Th2 cells cooperate with and activate B cells to proliferate and give rise to memory B cells (MB) and plasma cells (P) that secrete antibody and IL-4 from Th2 cells inhibit Th1 cell function. Antigen is taken in and processed by APCs, such as dendritic cells, that migrate to the lymph nodes and present antigen to the naïve T helper cells which are activated to Th2 cells (Fig. 1.1). These then release cytokines IL-4 and Il-13 which stimulate the production of allergen-specific IgE from B cells. These then bind to the surface of mast cells. IL-4 also induces the expression of high affinity allergen-specific IgE receptors (FcεRI) on the surface of mast cells. Monovalent binding of IgE causes increased FcεRI expression, increases mast cell resistance to apoptosis and can induce cytokine release (Knol 2006). Binding of the allergen-specific IgE to these

receptors marks the establishment of sensitization – subsequent exposure to that allergen leads to the allergic response.

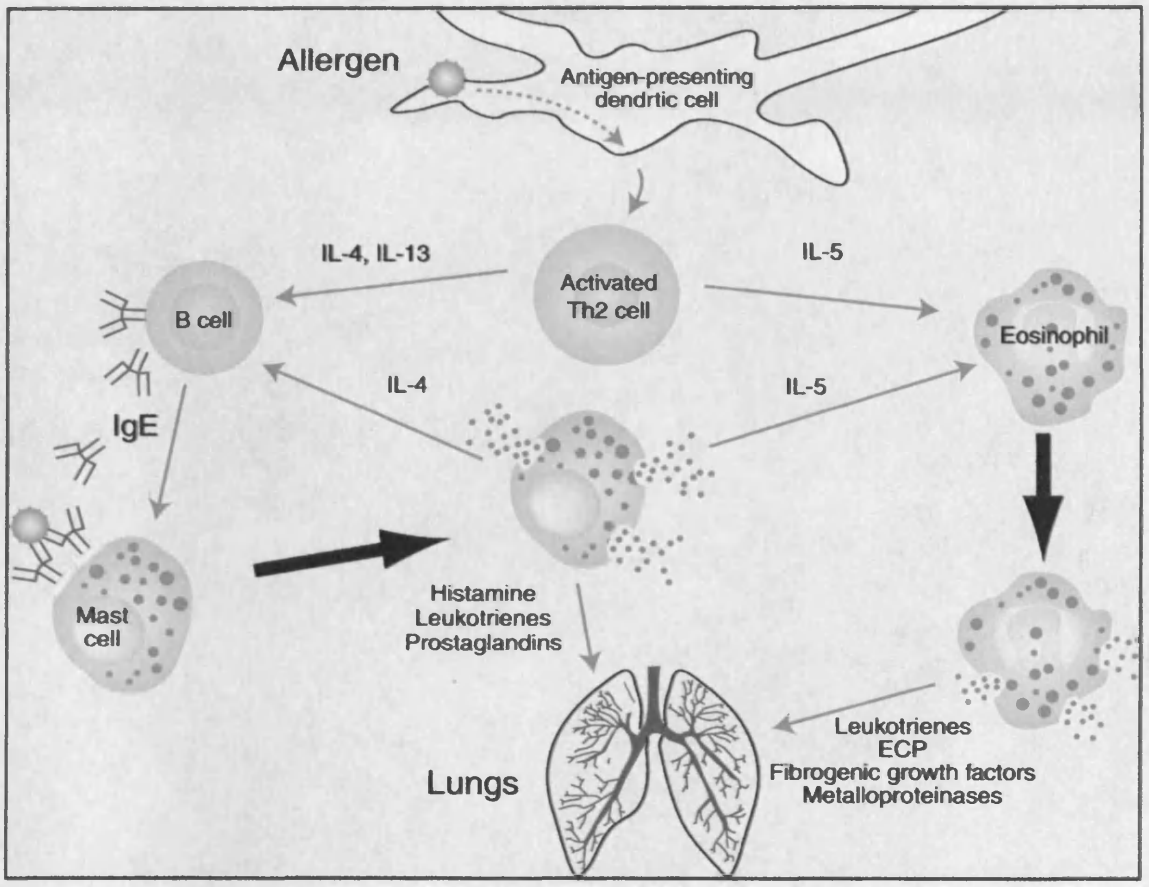


Figure 1.1

The allergic cascade in asthma: a Th2-immune response. Eosinophil cationic protein (ECP). Taken from Hendeles *et al* (2004).

There are two phases of the allergic response to allergen exposure – the immediate or early asthmatic response (EAR) and the late asthmatic response (LAR) which occurs about 7 hours later (Fig. 1.2). These responses arise as a result of re-exposure to the allergen which binds to the membrane-bound IgE on sensitized mast cells or basophils. The bound IgE interact and cross-link with the IgE that bound to the mast cell during the

sensitization phase. Cross-linking of bound IgE leads to clustering of surface receptors which promotes a cascade of tyrosine phosphorylation resulting in activation of phospholipase Cγ and mast cell degranulation (Knol, 2006). The mast cell releases a wide range of inflammatory mediators, including histamine, tryptase, prostaglandins, leukotrienes and cytokines. The histamine release mainly explains the EAR since it acts locally on airways smooth muscle to cause contraction and bronchoconstriction. IL-5 released from mast cells and from activated Th2 cells during the sensitization process recruits eosinophils and activates them to release mediators of inflammation and bronchospasm, including leukotrienes. They also release eosinophilic cationic protein and major basic protein (MBP) which may cause epithelial damage and thereby promote airways hyperreactivity (see later). Eosinophils also release fibrinolytic growth factors and matrix metalloproteinases (MMPs) which are involved in airways remodelling in asthma (Elias *et al* 1999). IL-4 also enhances the migration of eosinophils from the blood to the lungs via upregulation of endothelial vascular-cell adhesion molecule expression (VCAM-1). The LAR is caused by the attraction of inflammatory cells such as macrophages, eosinophils, lymphocytes and neutrophils into the lungs where they release mediators to cause bronchoconstriction (leukotrienes), bronchoconstriction and vasodilatation and vascular leakage of protein to cause oedema (Durham and Kay 1985).

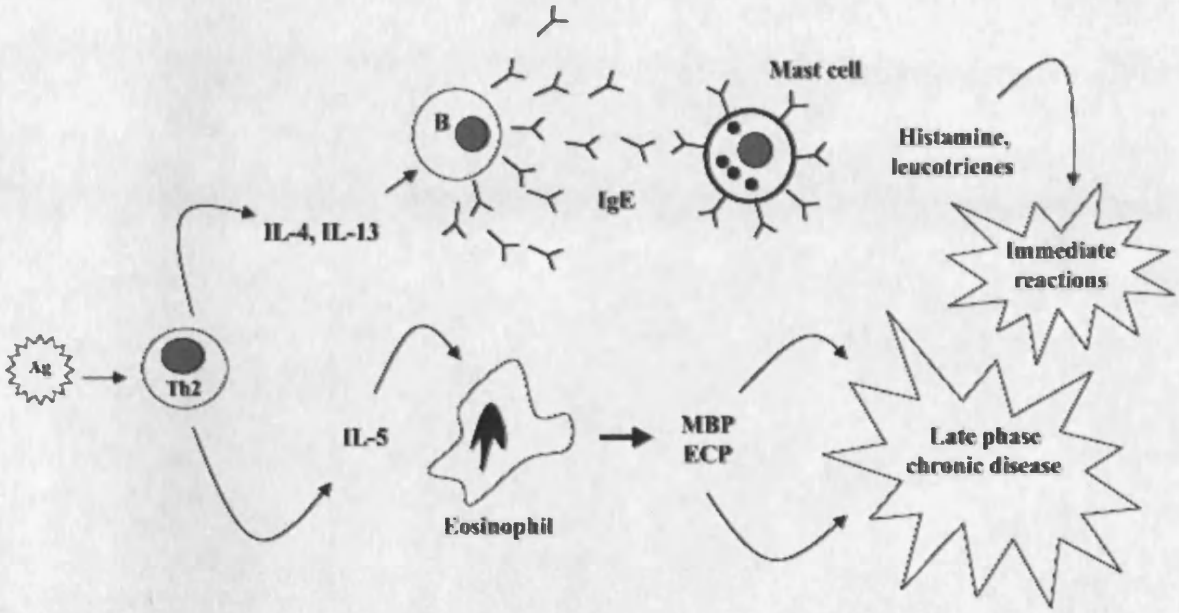


Figure 1.2

Induction of the immediate or early and late asthmatic responses after allergen challenge in atopic asthma. Major basic protein (MBP); eosinophil cationic protein (ECP); allergen (Ag). (From Biaze *et al* 2003).

1.4 Neurogenic inflammation

Inflammatory mediators such as bradykinin, histamine and prostaglandins released from mast cells act on sensory nerve endings to release the neuropeptides substance P (SP), neurokinin (NK) A and calcitonin gene-related peptide (CGRP) (Fig 1.3.) (Maggi 1991). These peptides exert inflammatory effects and the process is therefore referred to as neurogenic inflammation. They interact with tachykinin receptors which are classified as NK_1, NK_2 and NK_3 receptors (Brain and Cox 2006). The rank orders of potency for the naturally occurring neuropeptides are SP>NKA>NKB for NK_1 receptors, NKA>NKB>SP for NK_2 receptors and NKB>NKA>SP for NK_3 receptors (Alexander *et*

al 2009). SP and NKA cause raised vascular permeability (NK_1 receptors), vasodilatation (NK_1 receptors), bronchconstriction (NK_2 receptors), mast cell degranulation, inflammatory cell recruitment, and mucus secretion (NK1 receptors) (Maggi 1991). The epithelial shedding that occurs in asthma exposes the sensory neurones to the excitation by inflammatory mediators. Neurokinin antagonists have now been developed but are not currently used in the treatment of asthma, because they do not show sufficient efficacy.

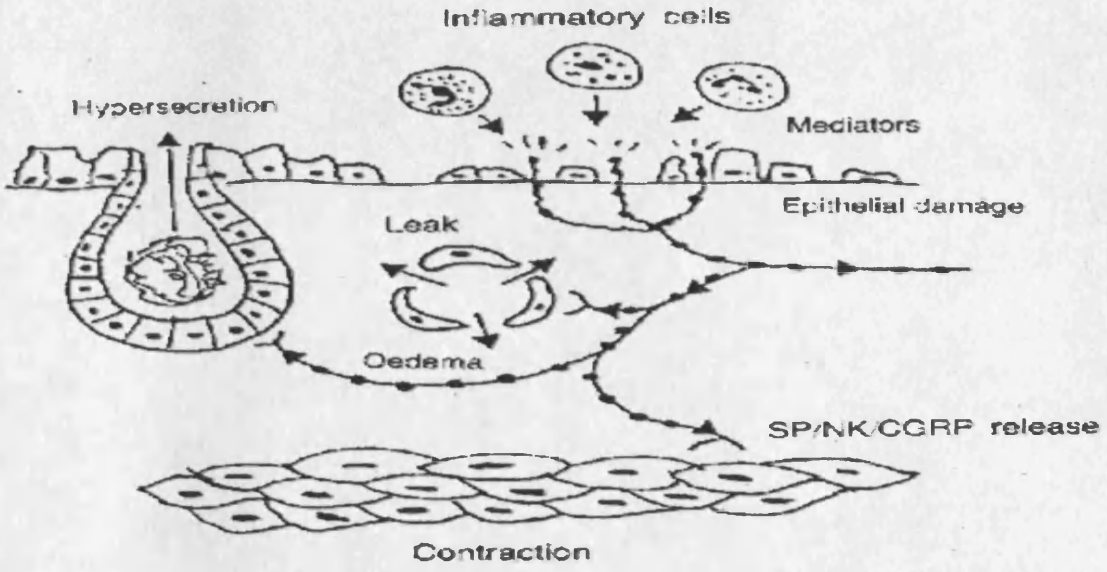


Figure 1.3

Neurogenic inflammation. Release of neuropeptides from sensory nerve endings.

1.5 Cells and mediators in asthma pathology

Mast cells

The numbers of mast cells in the bronchoalveolar lavage fluid of asthmatics is up to six-fold higher than in non-asthmatics (Hamid *et al* 2003).The fraction of mast cells that produce cytokines IL-4, IL-5 and tumour necrosis factor (TNF)-α is also increased in

asthmatics (Bradding *et al* 1994). The cytoplasmic granules of mast cells contain mediators of the asthmatic response, including histamine, tryptase, prostaglandin D_2 and leukotrienes C_4. Degranulation by the binding of allergen to the allergen-specific IgE bound to the mast cell releases these mediators to trigger the inflammatory cascade and bronchoconstriction, increased vascular permeability and leukocyte influx and activation. Also contained in mast cells is the glycosaminoglycan, heparin (Rose and Page 2004). There is clinical and experimental evidence that unfractioned and low molecular weight heparins may be benefical in airway inflammation (Diamant and Page 2000). The anionic nature of heparin allows rapid and spontaneous binding to cationic proteins (Motojima *et al* 1989), potentially neutralising the effects of damaging cationic species within the lung (Diamant and Page 2000). Heparin can bind to, and inhibit the effect of, a number of cytotoxic eosinophil-specific granule proteins including major basic protein, eosinophil cationic protein, and eosinophil peroxidase (Nilsson *et al* 1995). Heparin has been shown to affect various aspects of the immune response such as neutrophil chemotaxis and lymphocyte trafficking (Sy *et al* 1983). In clinical studies, heparin pre-exposure significantly reduced bronchoconstriction in asthmatic patients resulting from exposure to house dust mite (Bowler *et al* 1993) and exercise (Ahmed *et al* 1993).

Eosinophils

Eosinophils are involved in defence against parasites but the role they are most commonly associated with is in allergic disease and asthma. Eosinophilic infiltration of the lung is a characteristic of asthma and eosinophilic count is useful for regulating steroid dosage (Horn *et al* 1975). Eosinophilic protein can be detected in the sputum of

asthmatic patients (Fujimoto *et al* 1997). The eosinophil granule proteins MBP, ECP, eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase are involved in the shedding of the epithelium, the degranulation of mast cells and neural stimulation (Frigas *et al* 1991) (Bousquet *et al* 1990).

Macrophages

Macrophages are derived from blood monocytes and are the most abundant type of leukocyte found in the bronchoalveolar lavage fluid of both asthmatic and non-asthmatic subjects (Hamid *et al* 2003). Macrophages can serve as APCs to present the antigen to naïve T cells. They release a variety of cytokines which induce epithelial cells and fibroblasts to release chemoattractants and growth factors. These chemoattractants include regulated upon activation, normal T-cell expressed and secreted (RANTES) and monocyte chemotactic protein (MCP)-1 which attract further macrophages and eosinophils to the site of inflammation.

Neutrophils

Neutrophils contain reactive oxygen species and proteases (neutrophil elastase), both of which have the potential to cause damage to the lungs and to cause airways inflammation. After allergen challenge in sensitized guinea-pigs, neutrophils appear in the lavage fluid after 1 hour, peak at the end of the EAR and subside after 12 hours (Toward and Broadley 2004). There is an association between neutrophilic inflammation of the airways and severe asthma (Jatakanon *et al* 1999) and steroid-resistant asthma (Pavord *et al* 1999). Neutrophilia is otherwise more associated with chronic obstructive

pulmonary disease (COPD) (Cosio *et al* 2002) where the neutrophil elastase is responsible for the degradation of lung parenchyma and the consequent emphysema (Fujita *et al* 1990).

Dendritic cells

Dendritic cells are the primary APCs as they express high levels of major histocompatibility complex (MHC) class II molecules compared with macrophages and B cells. Those that have the most prominent role in asthma are the myeloid dentritic cells (mDC) and plasmacytoid dendritic cells (pDC) (Barnes 2002). Dendritic cells form a tightly meshed network throughout the epithelium and are therefore ideally suited to sample inhaled air for antigens (Kuipers and Lambrecht 2004). Dendritic cells take up antigens, process them to peptides and migrate to the lymph nodes where they present them to naïve T lymphocytes. In asthma, these develop into Th2 cells due to the release of the Th2-favouring cytokines, IL-4 and IL-13 (Barnes and Drazen 2002).

Cytokines

Cytokines are small protein mediators which play a crucial role in the co-ordination of inflammation in asthma. In asthma the airway wall is infiltrated by inflammatory cells including T lymphocytes of the Th2 phenotype, eosinophils, macrophages/monocytes, and mast cells. Th2 lymphocytes produce a wide range of cytokines, including IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-

CSF) (Barnes *et al* 1998). Some of the important cytokines in asthma are classified as follows:

- Lymphokines: IL-2, 3, 4, 5, 13, 15, 16 and 17
- Pro inflammatory cytokines: IL-1, 6 and 11, TNF-α, GM-CSF and stem cell factor (SCF)
- Inhibitory cytokines: Il-10, 1-ra, 12 and 18 and IFN-γ
- Growth factors: Platelet-derived growth factor (PDGF) and tumour growth factor (TGF)-β

Histamine

Histamine was the first mediator implicated in the pathogenesis of asthma. Histamine is synthesised and released from circulating basophils and mast cells in the airways. Most of the effects of histamine in the airways related to asthma are thought to be mediated by histamine H_1 receptors (Casale *et al* 1985). Histamine has many effects in the airways including contraction or airways smooth muscle, plasma exudation and mucus secretion (Barnes 2002). Inhaled histamine is used in asthmatics as a means of testing for airways hyperreactivity (AHR) since asthmatics are more sensitive to the bronchoconstrictor actions of histamine than non-asthmatics (Whicker *et al* 1988).

Leukotrienes

In asthmatics elevated levels of cysteinyl-leukotrienes (Cys-LT) have been detected in plasma, bronchoalveolar lavage fluid, and sputum samples obtained during spontaneous exacerbations of their asthma (Wenzel *et al* 1997). Cys-LTs are potent contractile agents

via the Cys-LT_1 receptor, having a 1000 times greater effect than histamine in human bronchi *in vitro* (Krell *et al* 1990). Cys-LTs can also increase mucus secretion (Hoffstein *et al* 1990) and in asthmatics causes eosinophilic infiltration (Laitinen *et al* 1993).

Adenosine

Adenosine is a purine nucleoside formed from 5'- adenosine-monophosphate (AMP) when the oxygen demands of a tissue exceed the supply. Adenosine can therefore be released under conditions of hypoxia as occurs during an asthma attack and increased levels have been detected in the lungs during allergen bronchoconstriction. Raised levels have been found in bronchoalveolar lavage fluid (BALF) of asthmatic subjects (Driver *at al* 1983). Inhaled adenosine has no effect in normal subjects but in asthmatics it causes bronchoconstriction (Cushley *et al* 1983). Furthermore, in isolated airways tissue from asthmatic subjects, adenosine causes contraction of the bronchi whereas there is no response in tissues from non-asthmatics (Bjorck *et al* 1992). In guinea-pigs, inhaled adenosine has no effect but in sensitized animals, there is a bronchoconstriction (Thorne and Broadley 1994). Also, isolated tracheal and lung preparations from sensitized guinea-pigs show a contractile response whereas those from unsensitized animals show a bronchodilator effect (Thorne *et al* 1996). Inhalation of adenosine or 5'-AMP in sensitized guinea-pigs also causes an influx of inflammatory cells into the lungs (Spruntulis and Broadley 2001) and a late asthmatic response (Smith and Broadley 2008). It is likely that mast cell degranulation to release histamine is a major source of the bronchoconstriction by adenosine as AMP challenge of asthmatics provokes an increase in levels of mast cell mediators: histamine, prostaglandin (PG) D_2 and tryptase (Polosa *et*

al 1995). Adenosine A_{2A} receptors are on the surface of human mast cells and T lymphocytes (Fozard and Hannon 1999), and their activation suppresses the release of tryptase from human mast cells. Stimulation of adenosine A_2 receptors may therefore inhibit mast cell-mediated responses. In contrast, A_{2B} receptors are located on eosinophils and mast cells and their activation is associated with inflammatory cell influx (Smith and Broadley 2008).

Nitric Oxide

Nitric oxide (NO) is generated by the actions of nitric oxide synthase (NOS) which converts L-arginine to citrulline. NOS occurs in three forms; two constitutive (cNOS) forms (endothelial NOS, eNOS and neuronal NOS, nNOS) and an inducible form (iNOS) (Fig 1.4). iNOS is induced or activated by inflammatory stimuli including the cytokines IL-1β, TNF-α and IFN-γ and bacterial endotoxin (lipopolysaccharide). iNOS is found in eosinophils, macrophages, neutrophils and epithelial cells. Unlike cNOS which produces only picomolar concentrations of NO, iNOS produces nanomolar concentrations of NO, which is long lived and Ca^{2+}/calmodulin independent. NO has a wide spectrum of activities that are both pro- and anti-inflammatory. It is a bronchodilator through activation in bronchial smooth muscle of soluble guanylyl cyclase to generate cyclic guanosine monophosphate (cGMP). NO adversely affects inflammatory cell influx by increasing leukocyte chemotaxis and increasing microvascular permeability. It also interacts with reactive oxygen species to generate highly toxic peroxynitrite. These actions are probably involved in the induction of airways hyperreactivity (Nevin and Broadley 2002). Exhaled NO is a marker of asthma and there is a good correlation

between sputum eosinophils and airway hyperreactivity to inhaled methacholine (Jatakanon *et al* 1998).

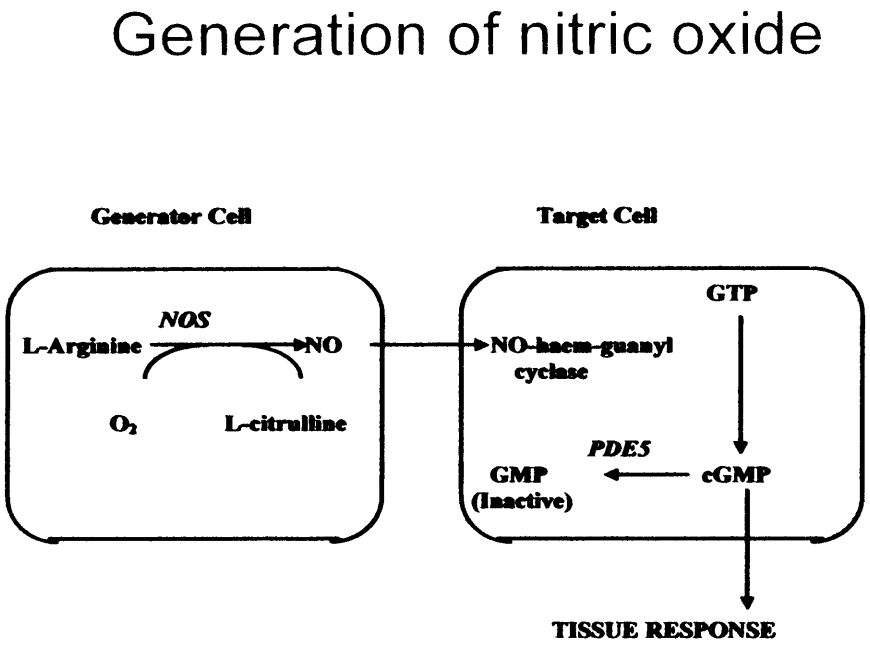


Figure 1.4

Generation of NO. NOS, nitric oxide; cGMP, cyclic guanosine monophosphate, phosphodiesterase (PDE) type 5.

Bradykinin

Bradykinin is a nine amino acid peptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) formed from the plasma precursor kininogen during inflammation and tissue injury. Large precursor kininogens are cleaved by serine proteinases, known as kininogenases, to release the active kinins, bradykinin and kallidin (Lys-BK) (Hall 1992). Bradykinin has

both direct and indirect actions in the airways. It produces bronchoconstriction, increased mucus secretion, stimulates cholinergic and sensory nerve endings, causes oedema due to increased microvascular leakage and promotes cough. The stimulation of sensory nerve endings causes the release of neuropeptides (SP, NKA and CGRP) resulting in neurogenic inflammation (see previously) and cough (Ellis and Fozard 2002). In humans, bradykinin-induced bronchoconstriction is inhibited by indomethacin, a non-selective cyclo-oxygenase (COX-1 and COX-2) inhibitor although aspirin has no effect (Fuller *et al* 1987). This indicates that it is mediated in part by the prostanoid products of COX, such as thromboxane (Tx) A_2 (Polosa *et al* 1990). The bronchoconstriction in humans is also due to activation of cholinergic nerves which release acetylcholine onto bronchiolar muscarinic M_3 receptors, since it is blocked by the muscarinic antagonist, atropine (Fuller *et al* 1987). Bradykinin is also able to release histamine from mast cells. The responses to bradykinin of guinea-pigs appear to be closer to those of humans than other species (Ellis and Fozard 2002). The actions of bradykinin in the airways is the subject of this thesis and its formation, metabolism, responses on the airways and receptors will be discussed in more detail later in this introduction and in Chapter 5.

1.6 Treatment of Asthma

β_2-Adrenoceptor agonists

Inhaled β_2-adrenoceptor agonists are the best available bronchodilators for asthma. They act directly on β_2 adrenoceptors on airway smooth muscle to dilate the bronchi. β_2 agonists bind to and activate the β_2 adrenoceptors, causing the activation of adenylyl cyclase and the formation of cyclic adenosine monophosphate (cAMP). cAMP is an

intracellular mediator, release of which causes smooth muscle relaxation and bronchodilatation. There are two types of β_2 agonists used in the treatment of asthma, short acting and long acting. Salbutamol and terbutaline are short acting drugs which are used on an as needed basis to control immediate symptoms of bronchoconstriction. Salmeterol and formeterol are long acting (at least 12 hours) and are used for maintenance therapy and to control nocturnal symptoms (Ullman and Svedmyr 1988). There has been a suggestion that long acting β-adrenoceptor agonists such as salmeterol might increase the risk of asthma deaths (Salpeter *et al* 2006). However, these concerns have subsided in the light of newer trials and analysis (Moore 2009; Nelson *et al* 2009).

Corticosteroids

Corticosteroids are the most effective treatment for controlling chronic asthma. They are recommended as the first-line therapy in treating persistent symptoms that derive from the inflammatory response of asthma. Fluticasone proprionate, budesonide and beclometasone are examples of inhaled corticosteroids. Where asthma is severe and resistant to the effects of inhaled corticosteroids, orally administered steroids, such as dexamethasone, prednisolone and betametasone are used. Steroids are not regarded as bronchodilator agents and therefore do not reduce the EAR following allergen challenge. They target the inflammatory process and reduce the numbers of inflammatory cells involved in the asthmatic response. Mast cells are reduced (Jeffery *et al* 1992). Corticosteroids reduce the secretion of cytokines and chemokines from lung macrophages (John *et al* 1998). Corticosteroids only reduce neutrophil numbers in asthma rather than COPD (Belvisi 2004). Corticosteroids also reduce the number of dentritic cells that serve

as APCs (Kamei *et al* 1996). Corticosteroids also inhibit the transcription of a wide range of inflammatory mediators, the enzymes that produce them and their receptors. They inhibit cytokines, enzymes involved in generating mediators, such as iNOS and COX-2. Corticosteroids inhibit mucus production that is elevated in asthma probably by inhibition of the mucin genes, MUC2 and MUC5A (Liu *et al* 2004).

Steroids act on the glucocorticoid receptor (GR), which is highly expressed in airway epithelial and vascular endothelial cells. The majority of the actions of steroids are mediated through changes in gene transcription. Corticosteroids bind to the GR and this homodimer is transported to the nucleus where it undergoes a conformational change and binds to a glucocorticoid responsive element (GRE) on the target gene leading to increased or decreased gene transcription. The homodimer exerts anti-inflammatory effects via two proposed mechanisms, direct and indirect.

Indirect: This mechanism involves an effect on histone acetylation in the DNA/histone complex of the DNA. The generation of inflammatory mediators (cytokines, inflammatory enzymes, receptors) through increased gene transcription is initiated via stimulation of nuclear factor κB (NF-κB) and activator protein-1 (AP-1) by TNFα, IL-1β. Binding of these transcription factors to the DNA/histone complex results in histone acetylation of N-terminal lysine residues by intrinsic histone acetyl transferase (HAT). This opens out the DNA/histone complex and allows activation of transcription and mRNA formation. This generates the inflammatory products. Closing and inactivation of this process is via deacetylation by histone deacetylase-2 (HDAC-2). Corticosteroids exert their anti-inflammatory effects by inhibition of HAT and stimulation of HDAC-2.

Direct: The steroid/receptor complex interacts with a number of elements (GREs, cyclic adenosine monophosphate response element binding protein (CBP), leading to activation of genes encoding ANTI-inflammatory proteins such as MAP kinase phosphatase (MKP-1) and IκB-α (an inhibitor of the pro-inflammatory transcription factor, NF-κB)). This pathway is now regarded as less important than the indirect mechanism (Barnes *et al* 2005).

Steroids therefore inhibit the transcription of many cytokines that are involved in inflammation including IL-1, TNFα, GM-CSF, IL-3, IL-4, IL-5, IL-6 and IL-8 (Guyre *et al* 1988). Steroids can also act indirectly by blocking the effects of cytokines. Steroids can inhibit the synthesis of cytokine receptors including the IL-2 receptor (Grabstein *et al* 1986). Several cytokines produce their effect by activating transcription factors such as AP-1 and $NF_{\kappa}B$. TNFα activates both AP-1 and $NF_{\kappa}B$ and this activation is counteracted by steroids through decreased transcription of TNFα (Barnes and Adcock 1997).

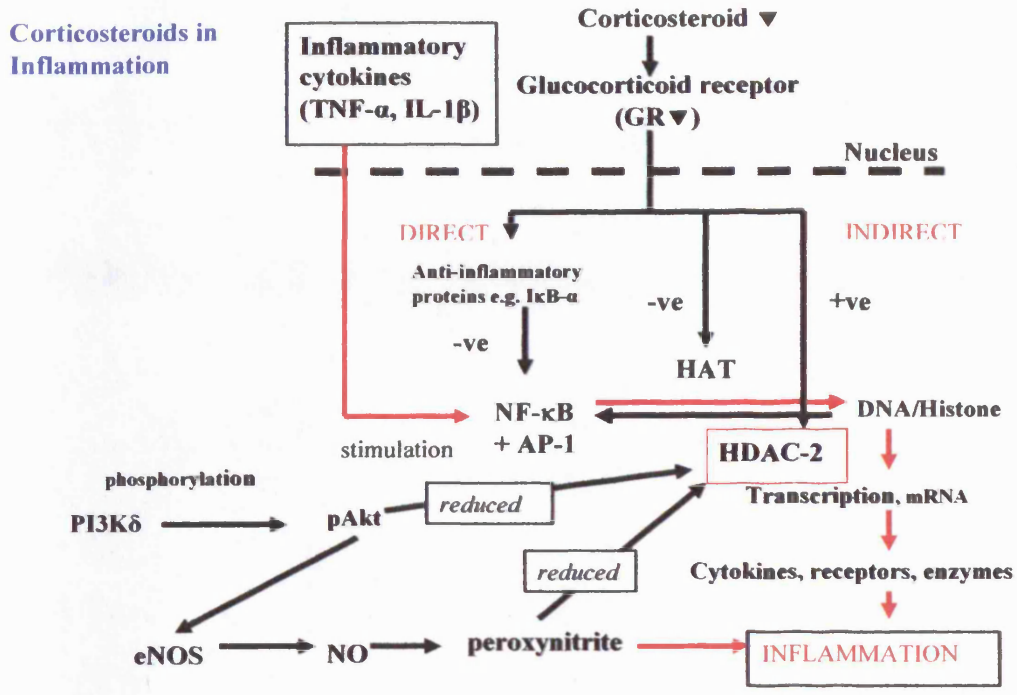


Figure 1.5

Pathways for the action of corticosteroids in inflammation.

Steroid resistance

Resistance to the anti-inflammatory effects of corticosteroids occurs in about 5% of asthmatics and in most COPD patients. Smoking asthmatics show a high degree of resistance. Steroid-resistance has been shown to be associated with a reduced level of HDAC-2 activity (Marwick *et al* 2007), which normally suppresses the inflammatory process. It is thought that oxidative stress, nitration by peroxynitrite and ubiquination reduce HDAC-2 activity. Recently, it was reported that phosphorylation of Akt by phosphatidylinositide 3-kinase (PI3K) also reduces the activity of HDAC-2 (Fig. 1.5). Phosphorylation of Akt is increased in COPD and targeting it may be a useful means of limiting exacerbations of COPD (Bozinovski *et al* 2006). Inhibition of PI3K (specifically

PI3Kδ) by IC87114 results in reversal of steroid resistance and attenuates allergic inflammation and hyperresponsiveness in mice (Lee *et al* 2006). Theophylline also appears to activate HDAC-2 and restore steroid sensitivity probably by inhibition of PI3Kdelta.

Methylxanthines

Theophylline is the prototypical methylxanthine which has been used for many decades and is one of the most widely used drugs for mild to moderate asthma. Despite its long history, the molecular mechanism of action remains unclear but several mechanisms have been proposed. It is widely held that the bronchodilator effect of theophylline is due to inhibition of PDE, which breaks down cAMP. This would result in increased cAMP levels, which would cause bronchodilatation and inhibit inflammatory cell migration and activation. Other mechanisms may be involved as the degree of inhibition of PDE is small at concentrations of theophylline which are clinically relevant (Bergstrand 1980). Other mechanisms for the bronchodilator effect include antagonism of adenosine receptors. Although inhaled adenosine has little effect on normal human airways, it causes bronchoconstriction in asthmatic subjects (Cushley *et al* 1984). Recent evidence suggests that theophylline is an antagonist of A_{2B} adenosine receptors. Like enprofylline, a selective A_{2B} antagonist, theophylline inhibits the release of IL-8 from human mast cells (Feoktistov and Biaggioni 1995). This would result in an inhibition of inflammatory cell activation and influx into the airways. Theophylline is also receiving a resurgence in interest as it may reverse steroid resistance, through activation of histone deacetylases (HDAC) (see above and Fig. 1.5) (Barnes 2004).

Anti-leukotrienes

Cysteinyl leukotrienes are potent bronchoconstrictors and may promote eosinophil mediated inflammation. Anti-leukotrienes are drugs that block their synthesis by inhibiting the enzyme 5'lipoxygenase (zileuton) or antagonists which block the cysteinyl leukotriene receptor (montelukast, pranlukast and zafirlukast). These drugs have been shown to reduce allergen, exercise and irritant induced asthma (Drazen *et al* 1999).

Muscarinic receptor antagonists

The parasympathetic nervous system is the main autonomic innervation of airways smooth muscle and reflex bronchoconstriction arises from sensory stimulation by a range of agents. There is evidence for enhanced parasympathetic activity in airways disease but most particularly in COPD (Roffel and Zaagsma 1995). The bronchoconstrictor actions of the parasympathetic innervation are mediated through the release of acetylcholine (ACh) onto muscarinic receptors of the M_3 subtype. These receptors also mediate an increase in mucus secretion from epithelial goblet cells. M_2 muscarinic receptors are located on the parasympathetic nerve endings where their stimulation causes inhibition of transmitter release; that is a negative-feedback effect. Anticholinergic drugs are not particularly effective against allergic challenge in asthma, but they do inhibit the augmented mucus secretion which occurs in asthma. They are more effective in COPD. The main anticholinergic compounds used in the treatment of asthma and COPD are ipratropium and tiotropium. The latter is longer acting and licensed for COPD but not suitable for relief of acute bronchospasm (British National Formulary 2009). They exert their effects by antagonising the M_3 muscarinic receptors involved in bronchoconstriction

resulting in bronchodilatation. Ipratropium does not discriminate between M_3 and M_2 receptors and therefore also blocks the negative-feedback which will enhance transmitter release and offset the antagonism of M_3 receptors on the smooth muscle.

1.7 Bradykinin

Synthesis and metabolism of bradykinin

Bradykinin is a nonapeptide formed by the cleavage of its precursor, high molecular weight kininogen (HMWK) by the kininogenase enzymes, which include tissue and plasma kallikrein (Fig 1.6). Another kinin, lysyl-BK is a decapeptide, formed by kininogenase enzymes, and then rapidly converted to bradykinin by aminopeptidase-N. Brasykinin is degraded by a number of kininase enzymes including ACE, NEP and carboxypeptidase-N, though not all these enzymes are present in the airways (Bhoola *et al* 1992). ACE has been localized to the endothelium, but it is thought to be present in the airways as inhibition of ACE by captopril enhances bradykinin induced bronchoconstriction. NEP is localized to the epithelium and is therefore likely to be the most important kininase in the airways. Inhibition of NEP by phosphoramidon potentiates the bronchoconstriction to bradykinin. Decreased NEP levels due to damage to the epithelium as seen in asthma and in viral infections is likely to be a contributory factor to the bronchial hyper-responsiveness seen in these conditions. The lack of removal of bradykinin by the epithelial enzymes allows its effects to be enhanced. Carboxypeptidase-N is not thought to play a role in the airways, as inhibition of this enzyme has no effect on bradykinin-induced bronchoconstriction (Ichinose and Barnes 1990).

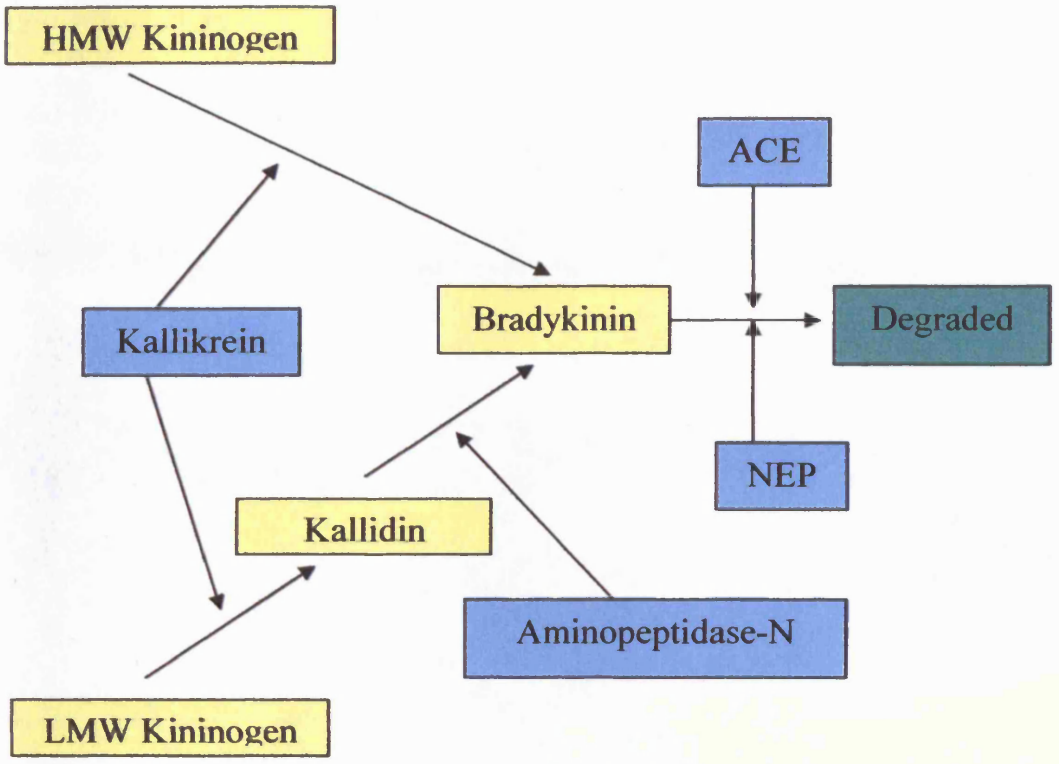


Figure 1.6

A simplified diagram of the synthesis and metabolism of bradykinin in the airways.

Bradykinin and asthma

Bradykinin is thought to be an important mediator of asthma. Inhalation of bradykinin causes bronchoconstriction in asthmatic patients, but has little or no effect in non-asthmatics (Fuller *et al* 1987). Kinin levels are increased in BALF of asthmatics after allergen challenge (Christiansen *et al* 1992).

Allergen challenge in asthmatics in the airways is associated with an immediate (<30mins) and a delayed (6-24hours) increase in BALF tissue kallikrein activity (Christiansen *et al* 1987). The source of the immediate increase in tissue kallikrein activity is likely to be submucosal cell gland secretions triggered by secretagogue mediators including elastase (Scuri *et al* 2001). Increases in macrophages and neutrophils coincide with the delayed increase in kallikrein activity, which is blocked by adhesion molecule inhibitors, which also prevented the associated airway hyperreactivity (Abraham *et al* 1999).

Bradykinin receptors

Bradykinin has many different effects in the airways, some of which are mediated directly by stimulation of B_2 receptors on target cells, others indirectly through the release of other mediators or transmitters.

Two types of bradykinin receptor have been cloned, the B_1 and B_2, which are typical G-protein coupled receptors with seven transmembrane segments (Hess *et al* 1992; Mencke *et al* 1994). Most of the bradykinin receptor-mediated effects in the airways are thought to be due to activation of the B_2 receptor as selective B_1 agonists have little effect (Fuller *et al* 1987). The B_2 receptor is widely distributed in the airways. In [^{3}H]bradykinin binding experiments in human and guinea-pig lung, dense labelling was found in bronchial and pulmonary vessels of all sizes and in the lamina propria immediately below the basal epithelial cells in large airways. Smooth muscle in large airways was scarcely labelled, but increased in smaller airways. Labelling was also detected over submucosal

glands and nerve fibres in human intrapulmonary bronchi and over alveolar walls of both species (Mak and Barnes 1991).

Effect of bradykinin on airway smooth muscle

Bradykinin causes contraction of guinea-pig airway smooth muscle *in vitro* and in guinea pig airway *in vivo* when administered intravenously (Tramontana *et al* 2001). These effects are influenced by the presence of the epithelium and local degrading enzymes including NEP, which is expressed in human airway epithelium (Baraniuk *et al* 1995). Inhibition of NEP by phosphoramidon and of ACE by captopril increases the bronchoconstriction to bradykinin *in vivo* in guinea-pigs (Ichinose and Barnes 1990). Bradykinin causes the release of the bronchodilator PGE_2 from the epithelium (Bramley *et al* 1990) and damage to the epithelium would reduce this functional antagonism. NO is also thought to modulate the action of bradykinin as the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) potentiates bradykinin-induced bronchoconstriction in asthmatic patients (Ricciardolo *et al* 1996).

Effect of bradykinin on nerves

An important effect of bradykinin is the ability to activate C-fiber nociceptive sensory nerve endings (Barnes 1992). The bronchoconstriction to bradykinin in guinea-pigs is thought to involve a cholinergic reflex and release of neuropeptides from sensory nerves as it is inhibited by pre-treatment with atropine and capsaicin (which depletes neuropeptides from sensory nerves) (Ichinose *et al* 1990). Tachykinins are also released

by bradykinin, in guinea pig perfused lung (Saria *et al* 1988), and tachykinin antagonists have an inhibitory effect on bradykinin-induced bronchoconstriction and plasma exudation (Sakamoto *et al* 1993).

Other airway effects of bradykinin

Bradykinin induces microvascular leakage, which is at least in part mediated by platelet activating factor (PAF), as a PAF antagonist inhibits this prolonged leakage (Rogers *et al* 1990). The plasma exudation is also blocked by neurokinin and B_2 receptor antagonists (Sakamoto *et al* 1993; Ichinose and Barnes 1990) and enhanced by inhibition of NEP and ACE (Lötvall *et al* 1991). Bradykinin stimulates mucus secretion via B_2 receptors in human submucosal glands *in vitro* (Nagaki *et al* 1996).

1.8 Virus infection and asthma

Substantial evidence implicates common respiratory viral infections in the pathogenesis of asthma and COPD. Children who experience recurrent virally induced wheezing episodes during infancy are at greater risk of developing asthma. In addition, respiratory viral infections are a major trigger for acute exacerbations of both asthma and COPD (Frick *et al* 1979). Viral infections lead to enhanced airway inflammation and can cause airways hyperresponsiveness. The epithelial cell is the principal site of viral infection in the airways and plays a central role in viral modulation of airway inflammation via release of a variety of cytokines, chemokines, and growth factors. These induce recruitment of inflammatory cells, particularly neutrophils, lymphocytes, and eosinophils to the lungs. The epithelium also contributes to the host innate defense response to viral

infection by releasing products that are antiviral and/or can lead to increased recruitment of dendritic cells and lymphocytes. Some evidence supports a role for the epithelial cell in specific immunity, although the response of more conventional cells of the immune system to viral infections is likely the dominant factor in this regard (Proud and Chow 2006). A significant fraction of the cost and morbidity of asthma derives from acute care for asthma exacerbations. In the United States alone, there are approximately 15 million outpatient visits, 2 million emergency room visits, and 500,000 hospitalizations each year for management of acute asthma. Common respiratory viruses, especially rhinoviruses, cause the majority of exacerbations in children and adults. Having had at least one exacerbation is an important risk factor for recurrent exacerbations suggesting an 'exacerbation-prone' subset of asthmatics. Factors underlying the 'exacerbation-prone' phenotype include extrinsic factors: cigarette smoking, medication non-compliance, psychosocial factors, and co-morbidities such as gastroesophageal reflux disease, rhinosinusitis, obesity, and intolerance to non-steroidal anti-inflammatory drugs; as well as intrinsic factors such as deficient epithelial cell production of the anti-viral type I interferons (IFN-alpha and IFN-beta) (Dougherty and Fahy 2009). Evidence is emerging that viral infections may alter the inflammatory infiltrate present in chronic asthma with a more heterogenous neutrophil/eosinophil composition infiltrate (Wark *et al* 2001). Increased sputum concentrations of cysteinyl leukotrienes have been found in patients with parainfluenza virus type 3 (PIV-3) induced asthma exacerbations (Matsuse *et al* 2005). Among the respiratory viruses implicated in childhood asthma are respiratory syncytial virus (RSV), rhinovirus, PIV-3 and coronovirus.

Steroid resistance has been attributed to viral and bacterial infection, which lead to exacerbations of asthma. Viral infection in guinea-pigs amplifies the inflammatory response and induces steroid resistance (Yamada *et al* 2002). The steroid resistance may be explained by a reduction in HDAC activity (see above) by the viral infection (Barnes *et al* 2005). Infection with virus has been implicated in steroid resistance in children with asthma (Macek *et al* 1994). Mycoplasma pneumoniae and Chlamydia are both prevalent bacterial infections in asthmatics. These infections reduce mast cell numbers. Clarithromycin is used to treat exacerbations.

1.9 Parainfluenza virus

PIV is associated with croup in children. In studies in humans there has been an association between virus-induced laryngotracheobronchitis (croup) in children and increased reactivity in later years. Loughlin and Taussig (1979) showed that children with a history of croup showed hyper-responsiveness after exercise. In a larger study of children with croup in the first three years of life, it was found that children who showed croup with wheezing were more likely to develop wheezing later in life than those with croup without wheezing (Castro-Rodríguez *et al* 2001). In this study it was found that the children who developed croup with wheezing had lower levels of small airway function at birth, age 6 and at age 11 compared to those with croup without wheezing, who had similar levels to control children at all ages. The virus most commonly isolated in the wheezing phenotype was RSV and in croup without wheezing PIV was most common.

PIV infection induces changes in the airways that are similar to the effects seen in asthma, including bronchial hyper-responsiveness, an influx of inflammatory cells, epithelial damage and bronchiolar fibrosis.

Animal studies

There have been several experiments in which PIV infected animals show persistent changes in lung morphology and function. Neonatal rats, which were infected with Sendai virus (PIV-1), and subsequently developed bronchiolitis still showed decreased lung function and hyperreactivity 13-16 weeks later (Sorkness *et al* 1991). Quan *et al* (1999) showed chronic small airways inflammation and altered lung function during growth in canine PIV-2 infected beagle puppies, though there was no long-term hyperreactivity to histamine. These animal models suggest that acute PIV infection in the lower airways can in some cases lead to long-term changes in the lung.

Characteristics of parainfluenza viruses

Parainfluenza viruses were first discovered in the late 1950's. They have been divided into subtypes 1 to 4, with PIV-4 being further divided into types a and b. Types 1 to 3, along with RSV are major causes of lower respiratory disease in infants, the young, the immunocompromised and the elderly (Glezen *et al* 1984; Glezen *et al* 2000; Falsey 1991). PIV infection can cause croup, bronchiolitis, and bronchopneumonia, though the most common presentation is an upper respiratory tract infection (Belshe *et al* 1983). The pathogenesis of PIV infection is still being studied but the respiratory epithelium appears to be the major site of virus binding and subsequent infection. The epithelium is an

extensive source of inflammatory mediators including chemokines and cytokines, which can be released to recruit inflammatory cells after viral infection (Garofalo and Haebere 2000). Little is known about type 4.

PIV belong to the family *Paramyxoviridae*, which also includes measles, mumps and RSV. The virus is spherical and approximately 150-400nm in diameter. They have an envelope composed of host cell lipids and viral glycoproteins derived from the plasma membrane of the host cell during viral budding. The PIV genome is single stranded, negative sense RNA that must be transcribed into message sense RNA before it can be translated into protein. The RNA genome is approximately 15,500 nucleotides in length and is encapsilated by the viral nucleocapsid protein, forming a helical nucleocapsid. (Fig. 1.7) (Lamb *et al* 1976).

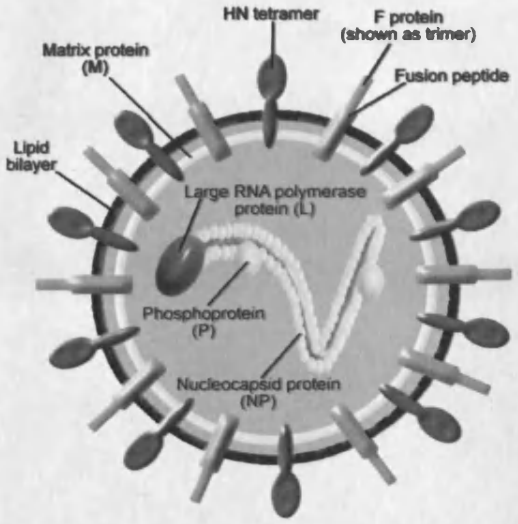


Figure 1.7

Schematic diagram of the parainfluenza virion. (taken from Moscona 2005).

The first step in infection of a cell by PIV is binding to the target cell, via interaction of the viral receptor-binding molecule haemagglutinin-neuraminidase (HN) with sialic acid-containing receptor molecules on the cell surface (Fig. 1.8). The viral envelope is then thought to fuse directly with the plasma membrane of the cell, mediated by the viral fusion (F protein), releasing the nucleocapsid into the cytoplasm (Lamb 1993; Plember *et al* 2003). The nucleocapsid released into the cytoplasm after fusion contains the genome RNA in tight association with the viral nucleocapsid protein, and this RNA-protein complex is the template both for transcription and for replication of the genome RNA that is packaged into progeny virions.

The 6 viral replication genes encode the 2 surface glycoproteins HN and F; the matrix protein, which is involved in assembly and budding; the RNA polymerase proteins and a

protein that encapsulates the RNA; and 1 or more proteins that are expressed only in the infected cell and whose roles include evasion of the host immune response.

Virions are formed with the full-length viral RNA genome along with the polymerase proteins bud out through areas of the plasma membrane that contain the F and HN proteins and the matrix protein. In polarised epithelial cells, the viruses bud from the apical surface of the cell. The matrix protein binds to the nucleocapsid and with the cytoplasmic tails of the HN and F proteins. This aligns the nucleocapsid with the areas of the plasma membrane containing viral glycoproteins in readiness for budding (Ali and Nayak 2000). The neuraminidase or receptor cleaving activity of the HN molecule cleaves sialic acid-containing receptor moieties that would attach the viral HN protein to the cell surface and allows the release of newly budded particles from the cell to begin a new round of infection (Moscona 2005).

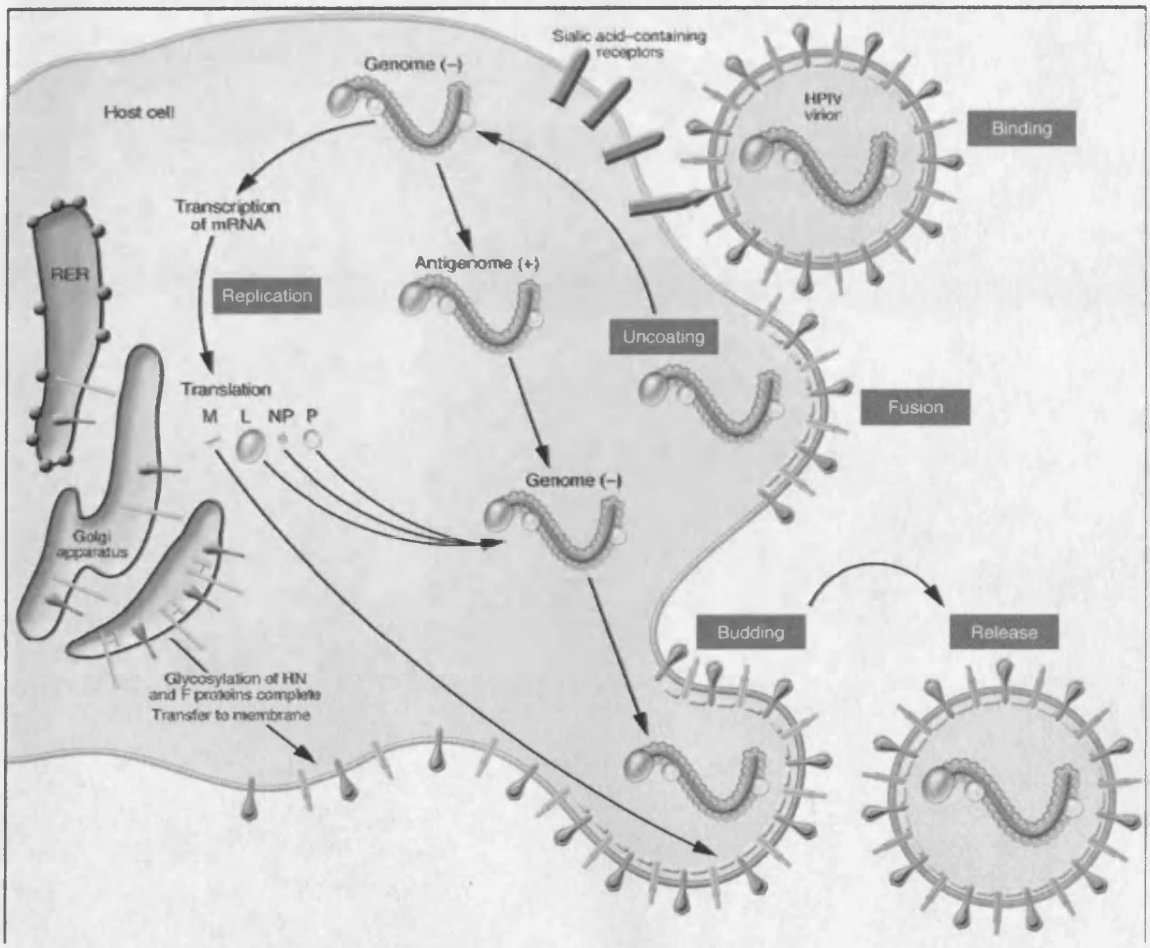


Figure 1.8

Life cycle of the parainfluenza virus (taken from Moscona 2005).

When PIV infects a cell, the first observable morphologic changes may include focal rounding and increase in size of the cytoplasm and nucleus. PIV decreases host cell mitotic activity as soon as 24 h after inoculation. Other changes include single or multilocular cytoplasmic vacuoles, basophilic or eosinophilic inclusions, and the formation of multinucleated giant cells (Craighead and Brennan 1968). These giant cells (fusion cells) usually occur late in infection and contain between two and seven nuclei.

Disease severity has been correlated with PIV shedding in children (Hall *et al* 1977). The parainfluenza viruses generally initiate localised infections in the upper and lower respiratory tracts without causing systemic infection. Local and serum antibodies develop after primary infection. The resulting immunity is not adequate to prevent re-infection, but does not provide some protection against disease.

The parainfluenza viruses replicate in the epithelium of the upper respiratory tract and spread from there to the lower respiratory tract. Epithelial cells of the small airways become infected, and this is followed by the appearance of inflammatory cell influx. The innate immune responses help to clear the virus. Both humoral and cellular components of the immune system appear to contribute to both protection and pathogenesis (Smith *et al* 1966). Infection with PIV in immuno-compromised children (e.g. transplant recipients) is associated with a range of disease, from mild respiratory symptoms to severe disease requiring mechanical ventilation and leading to death (Apalsch *et al* 1995).

Acute infection of guinea-pigs with PIV increased numbers of airways inflammatory cells, histamine levels and airway hyperreactivity (Folkerts *et al* 1993) and airways hyperreactivity of the trachea *in vitro* (Folkerts *et al* 1990a) and decreased bronchoalveolar cellular superoxide production (Folkerts *et al* 1990b). PIV-3 infection of guinea-pigs also enhanced allergic sensitization (Riedel *et al* 1996). In addition, PIV-3 induced peribronchiolar lymphocyte aggregation, bronchiolitis and interstitial pneumonia in cotton rats (Porter *et al* 1991).

Parainfluenza type 1 (PIV-1)

PIV-1 is reported to cause 50% of all croup cases in children younger than 5 years. It can also cause bronchiolitis, pneumonia, febrile and afebrile wheezing during epidemics. The majority of infections occur in children between 7 and 36 months with a peak occurrence in the second and third year (Denny *et al* 1983). PIV-1 can cause lower respiratory tract infections (LRTI) in children younger than 6 months but is rare in infants under 1 month. PIV-1 causes biennial autumn epidemics, resulting in hospitalisation of an estimated 18000 to 35000 children under 5 years in the US (Belshe *et al* 1983).

Parainfluenza type 2

PIV also causes croup as well as all the other LRTI diseases seen with PIV infection. PIV-2 infection is generally less common than with PIV-1 and 3, though in any particular year or location it can be the most prevalent. PIV-2 also causes biennial epidemics, which can be in alternate years, or the same years as PIV-1. There can also be yearly outbreaks of PIV-2. PIV-2 epidemics usually occur in autumn or early winter. 60% of infections occur in children under 5 years with a peak in the second and third years, though many infants in their first year are hospitalised (Murphy *et al* 1980).

Parainfluenza type 3

PIV-3 infection occurs the earliest and is the most common. It can cause bronchiolitis and pneumonia. Around 50% of children are infected in their first year and infants under 6 months are vulnerable to infection. Only RSV causes more infections at this age. PIV-3 causes annual spring and summer epidemics though infections occur all year round, with the immunocompromised and the chronically ill being particularly at risk (Glezen *et al* 1984).

1.10 Influenza

The influenza virus is a negative stranded (-) RNA virus belonging to the family *Orthomyxoviridae*. Influenza types A and B cause seasonal outbreaks whereas type C causes only mild respiratory conditions. Influenza types A and C infect many species, whereas type B only infects humans. Influenza type A viruses are divided according to their surface glycoproteins, termed haemagglutinin (HA or H) and neuraminidase (NA or N), also known as sialidase. Three types of HA (HA1, HA2 and HA3) and two NA (NA1 and NA2) are commonly found in humans, whereas more types are found in avian influenza viruses (Richman *et al* 2002). The first identified influenza virus was H1N1 which caused the "Spanish flu" epidemic of 1918 which killed more than 20 million people (Taubenberger 2006). Genetic rearrangements known as antigenic shift resulted in the appearance of two further human viruses; H2N2 and H3N2, which were responsible for the "Asian" and "Hong Kong" flu pandemics of 1957 and 1968, respectively (Kilbourne 2006). The threat of an influenza pandemic has increased dramatically due to the emergence in Hong Kong of a new, highly pathogenic avian strain of H5N1 (Webster

et al 2007). However, this new strain of influenza is not able to transmit between humans. Only type A influenza viruses undergo antigenic shift and cause epidemic outbreaks. "Swine flu" was first described in the 1918 pandemic and made a recent resurgence in April 2009 in the form of a combination of human, swine, and Eurasian avian strains. Young adults and children aged < 24 years are the population most affected. The pandemic variant influenza A (H1N1) strain is typically susceptible to oseltamivir and resistant to adamantanes, unlike the 2008 to 2009 seasonal influenza A (H1N1). However, 2 cases of oseltamivir-resistant pandemic-variant influenza A (H1N1) were reported in late August 2009 and widespread oseltamivir resistance is a possiblity. The recent development of a vaccine is therefore important for protection (Scalera and Mossad 2009).

The structure and trafficking of the virion is similar to that described for PIV (Whittaker 2001). Influenza types A and B virus bind to the host cell by means of their HAs attaching to cell surface 5-*N*-acetyl neuraminic acids (sialic acids), as for PIV. Human influenza viruses preferentially attach to α-(2,6)-linked sialic acids (Rogers *et al* 1983). The virus is then taken up by endocytosis, followed by fusion of the viral envelope with the host endosomal membrane for release of viral ribonucleoproteins (vRNPs). This process is enhanced by acidification of the interior of the virus via an M2 ion channel. The vRNPs are then imported into the nucleus, where the influenza type A genome is transcripted and replicated by viral RNA-dependent RNA polymerase. The nuclear export of viral mRNA is controlled by the viral non-structural protein, NS1. The viral constituents are trafficked to the apical surface of the epithelial cell under the influence of matrix protein. Budding of the virus occurs at detergent insoluble glycolipid domains of

the host cell plasma membrane. The mature virus detaches because neuraminidase cleaves sialic acid residues from the host cell. After release of virus, the host cell dies.

The influenza virus causes sudden onset of symptoms of fever, aches, fatigue, sore throat and nasal congestion about 24-48 hours after infection. Gastrointestinal symptoms of vomiting, abdominal pain and diarrhoea can also occur. Infection starts when the virus invades the lung epithelial cells. After replication and an innate immune response in these cells, the influenza virus infects alveolar macrophages. Peripheral blood neutrophils, T cells and macrophages are then activated at the site of infection to release large amounts of cytokines. Human airway epithelial cells release RANTES, MCP-1 and IL-8 (Adachi *et al* 1997). IFNα/β and the pro-inflammatory cytokines IL-1β, IL-6 and TNFα are produced poorly by epithelial cells but large amounts are produced by influenza infected monocytes and macrophages (Ronni *et al* 1995, 1997).

Influenza in guinea-pigs

Several studies have shown that guinea-pigs are susceptible to infection by influenza. Azoulau-Dupouis *et al* (1984) inoculated guinea-pigs with influenza A/Hong Kong/68 (H3N2) which caused significant histopathological changes to the whole respiratory tract and Lowen *et al* (2006) demonstrated the spread of influenza between guinea-pigs after infection with A/Panama/2007/99 (H3N2).

1.11 Virus recognition and the host innate immune response

Viral genomes are recognized by the host Toll-like receptors (TLRs) and retinoic acid inducible protein-I (RIG-like receptors, RLRs). Ten Toll-like receptors have been

identified in humans. They are transmembrane proteins structurally similar to the IL-1 receptor which play a crucial role in protecting against viral and bacterial pathogens through release of cytokines. TLR1 and TLR2 heterodimers sense bacterial triacylated lipopeptides, whereas TLR2 and TLR6 recognise diacetylated lipopeptides. TLR4 detects lipopolysaccharide (LPS) or bacterial endotoxin from gram negative bacteria. Polymyxin B (PMB) is a cyclic, cationic peptide antibiotic which neutralizes LPS but induces severe side effects in the process. Efficient neutralization of endotoxin by PMB is not achieved by mere binding to LPS but also requires its sequestration from the membrane (Bhor *et al* 2005). TLR3 is found in the endosome of dentritic cells, epithelial cells and macrophages, and senses single and double stranded viral RNA and activates the pro-inflammatory transcription factor NF-κB (Alexopoulou *et al* 2001) (see also Fig. 1.5). A selective agonist for TLR3 is poly(I-C) (polyinosinic-polycytidylic acid), which is a synthetic analogue of viral double stranded RNA which is synthesized during replication of single stranded RNA viruses. Poly(I-C) elicits an innate immune response through activation of the transcription factors NF-κB or interferon regulatory factor/interferon-sensitive response-element pathways. Ultimately, this signal transduction elicits an epithelial response that includes the secretion of high levels of pro-inflammatory cytokines IL-8, IL-6, RANTES, the antiviral interferon-β and the up-regulation of the major adhesion molecule, intercellular adhesion molecule (ICAM)-1 (Guillot *et al* 2005). TLR7 recognises single stranded RNA viruses, including influenza (Lund *et al* 2004). A cascade of interactions with kinases results in activation of transcription factor NF-κB and type I interferon. Type I interferons (interferon-α/β IFN-α/β) are the key cytokines which elicit antiviral responses through activation of IFN stimulated genes by binding to

IFN-α/β receptor. This ligand-receptor interaction activates a cascade of kinases which in the host cell leads to inhibition of viral and cellular protein synthesis and apoptosis (Taniguchi and Takaoka 2002). TLR5 is a receptor for flagellin from bacteria (Hayashi *et al* 2001) and TLR9 is a receptor for unmethylated CpG-containing DNA motifs which occur in bacterial and viral DNA (Hemmi *et al* 2000).

1.12 Treatment of PIV and influenza infection

Currently there are no licenced antiviral drugs with proven clinical efficacy against PIV infection. Ribavirin has both *in vitro* and *in vivo* activity against PIV and influenza (Browne 1981; Sidwell *et al* 1975). Furthermore, there have been anecdotal reports of decreased viral shedding and clinical improvement when infected immunocompromised patients were treated with aerosolized and oral ribavirin (Chakrabarti *et al* 2000; Malinowski and Hostoffer 2001).

Influenza virus infections can be treated by targeting stages in the life cycle of the virus. Amantadine (Symmetrel) and rimantadine (Flumadine) target the pH-dependent uncoating of the virus once it is inside the host cell. Amantadine targets the M2 ion channel of the virus (Hay *et al* 1985). Export of nucleoprotein from the nucleus in influenza-infected cells is inhibited by the antibiotic leptomycin B (Elton *et al* 2001). Zanamivir (Relenza) and oseltamivir (Tamiflu) are analogues of sialic acid which bind to and block the sialic acid residues of the surface HA. There are now reports of resistance appearing to amantadine and oseltamivir (Aoki *et al* 2007). Currently, the use of inactivated vaccines is the most effective means of reducing influenza infection.

Neutralizing antibodies to the haemagglutinin protein can directly block virus entry, but protective antibodies to the neuraminidase protein are thought to primarily aggregate virus on the cell surface, effectively reducing the amount of virus released from infected cells. The neuraminidase protein can be divided into nine distinct antigenic subtypes, where there is little cross-protection of antibodies between subtypes. All nine subtypes of neuraminidase protein are commonly found in avian influenza viruses, but only selected subtypes are routinely found in mammalian influenza viruses; for example, only the N1 and N2 subtypes are commonly found in both humans and swine. Even within a subtype, the neuraminidase protein can have a high level of antigenic drift, and vaccination has to specifically be targeted to the circulating strain to give optimal protection (Sylte and Suarez 2009). However, vaccine-induced immunity declines with time and annual flu re-vaccination is necessary even if the vaccine antigens have remained unaltered.

The use of corticosteroids to treat infectious diseases is counterintuitive since they would suppress the immune system of the host which could lead to increased viral replication. Meta-analysis of numerous studies has demonstrated that oral or systemic steroids are effective at improving symptoms of croup as early as 6 h after treatment (Somani and Evans 2001). Other studies, have determined that corticosteroids may be beneficial in artificially ventilated patients with severe bronchiolitis, although they had no effect on pneumonia (Van Woensel *et al* 2004). These findings support the idea that corticosteroids may be beneficial in virus infections, although further research is required. Combined immunotherapy and steroids have been demonstrated to decrease pulmonary virus titre and inflammation in a cotton rat model (Prince and Porter 1996).

1.13 Aims of the study

- The aim of this thesis is to examine the role of bradykinin in airways inflammation induced by viral infection in guinea-pigs and the associated functional changes.
- The initial objective was to establish models of viral inflammation in guinea-pigs inoculated with parainfluenza and influenza virus. Comparative studies were undertaken with PIV-2 and PIV-3.
- The effect of inhaled bradykinin in non-inflamed lungs was examined with the use of inhibitors of its breakdown (captopril and phosphoramidon) and selective bradykinin B_2 antagonists (MEN16132 and icatibant).
- The role of the bradykinin in airways inflammation was studied in the PIV-3 model by inhibition of its production by the tissue kallikrein inhibitor EE999024 or with the selective bradykinin B_2 receptor antagonist MEN16132. Dexamethasone was used for comparison.
- Guinea-pigs were sensitized and exposed to ovalbumin to produce the asthma symptoms of AHR, EAR, LAR and inflammatory cell influx
- The effect of bradykinin inhalation in the ovalbumin model was examined before and after ovalbumin exposure to determine whether this model produces AHR to bradykinin.
- The ovalbumin model was combined with the PIV-3 model to produce a viral exacerbation of allergy model. This exacerbation model was compared to

ovalbumin alone. The anti-inflammatory effects of dexamethasone were compared between both models.

2.1 Pharmacological Methods

2.1.1 Animal welfare and ethics

Male Dunkin Hartley guinea-pigs were obtained from Harlan UK Ltd (Oxon UK). The animals were housed at the laboratory animal facilities of Cardiff University. Controlled conditions were imposed on climate and diet and ambient temperature was maintained at $20^0C \pm 2^0C$ with 12 hour alternating light/dark cycles at approximately 50% humidity. The animals were fed on commercial guinea-pig diet pellets (Harlan UK Ltd, Oxon, UK) supplemented with ascorbic acid and water allowed *ad libitum*. The animals were provided with cardboard tubes and received hay every day for environmental enrichment. The animals were acclimatised for at least 1 week before the commencement of any experiments.

Animal welfare was undertaken in accordance with the Animal Scientific Procedures Act 1986 under Home Office personnel and project licenses. The guinea-pigs were without infections of the respiratory airways as evaluated by the health monitoring quality control report by Harlan UK Ltd.

After careful consideration of the ethical issues surrounding use of laboratory animals, it was decided that the potential importance of these experiments to improve our understanding of viral infections of the airways and their treatment, that the use of live animals was justified. In the course of these experiments every care was taken to reduce the stress and discomfort of the animals. This was achieved by handling the animals

regularly and acclimatising them to all the equipment used before starting any experiments.

2.1.2 Measurement of respiratory function

Specific airways conductance

Whole body plethysmography was used to monitor airway function and was recorded as specific airway conductance (sG_{aw}). This was performed in un-anaesthetised spontaneously breathing guinea-pigs based on the methods described previously by Griffiths-Johnson *et al* (1988).

Airway resistance (R_{aw}) is defined as the pressure difference between the alveoli and mouth divided by airflow. R_{aw} is dependent upon the volume of air in the lungs, the thoracic gas volume (TGV). To correct for TGV, sG_{aw} is often used instead of R_{aw}. sG_{aw} is the reciprocal of Raw per unit TGV (Tattersfield and Keeping 1981). The theory behind determination of sG_{aw} is described in Appendix 1.

Guinea-pigs were held in a restraining device consisting of a neckpiece to restrain the animal, which slotted into a base section that was secured by 2 pins. The animals' snout was covered by a mask with a rubber diaphragm creating an airtight seal around the nose. This was attached to a pneumotachograph (Mercury FIL, Glasgow) before the animal and restrainer were placed in the plethysmograph chamber, which was then sealed by a plate secured to the front of the chamber. Prior to each experiment, the guinea-pigs were handled and familiarised with the restrainer and the plethysmograph chamber to reduce stress-related factors (Fig. 2.1).

Pressure transducers (Pioden Type 1, Pioden Controls Ltd. Canterbury, UK), UP1 and UP2, attached to the pneumotachograph and plethysmograph chamber, respectively, measured changes in respiratory flow and box pressure. These transducers were attached to a computerised recording system comprising of AcqKnowledge® software with a Biopac® data acquisition system, as previously used by Danahay and Broadley (1997) which replaced the original oscilloscope and angle resolver as used by Griffiths-Johnson *et al* (1988). The resulting waveforms were analysed by comparing the gradients of the flow and box pressure at a point where flow tended towards zero; - i.e. end tidal volume (at the end of expiration and beginning of inspiration). Each recording period was 5 seconds long, and from this, a minimum of 5 breaths were analysed. Using these values and taking into account air pressure and the weight of each guinea pig, resultant values for sG_{aw} were determined which were averaged for each time point (see appendix). Between recordings, animals were removed from the plethysmograph and placed in a holding cage.

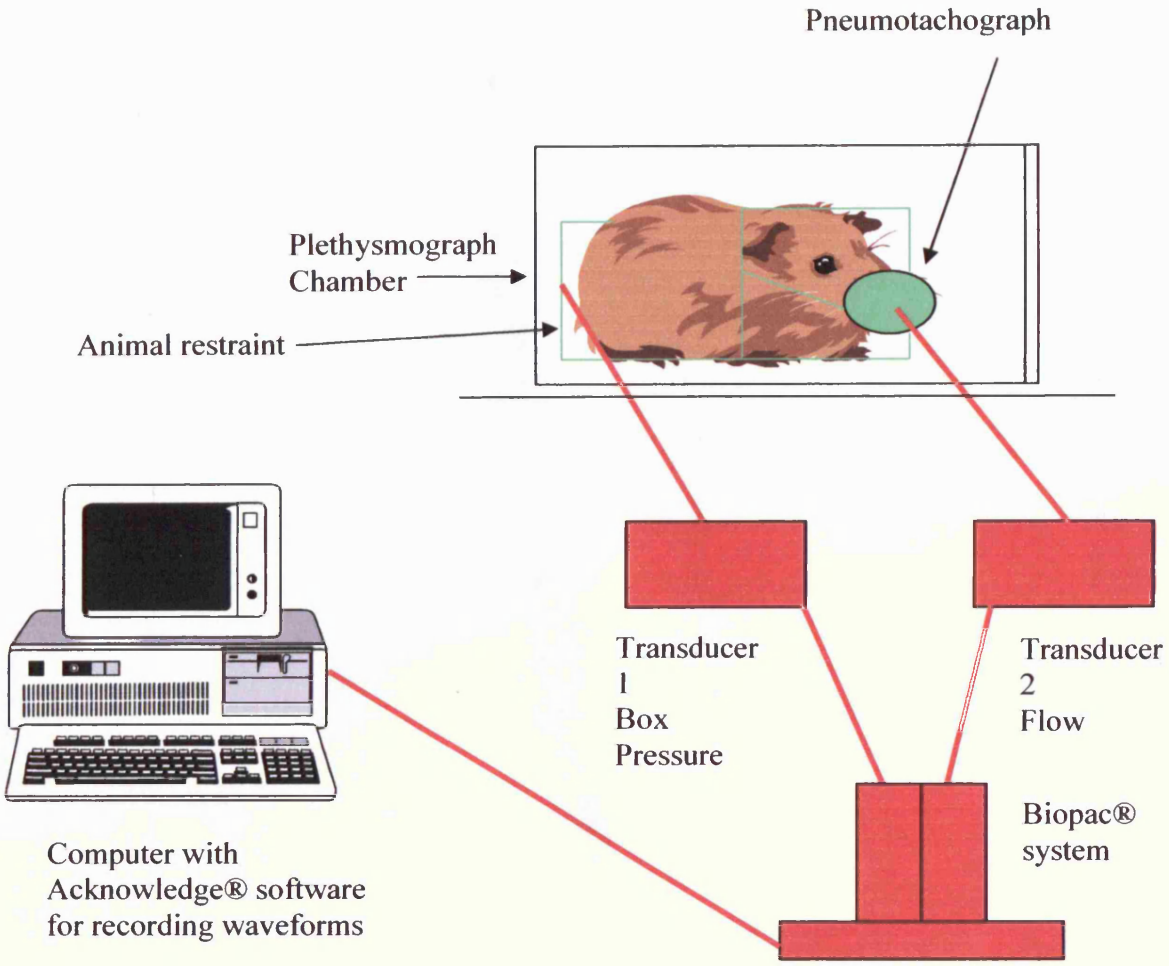


Figure 2.1

A simplified schematic of the whole body plethysmograph and acquisition packs used to measure specific airways conductance sG_{aw}, in conscious, restrained guinea-pigs.

2.1.3 Measurement of airway reactivity

Airway reactivity was measured by testing pulmonary function before and after an aerosolised exposure of the brochospastic agent histamine (in one experiment bradykinin was used).

Airways reactivity was measured by testing pulmonary function before a 20 second, nose only 1mM histamine exposure and at 0, 5 and 10 minutes after exposure by whole body plethysmography. As mentioned in Chapter 1, histamine activates H_1 histamine receptors in the airway smooth muscle causing bronchoconstriction. From previous experience in these laboratories the selected dose (1mM) of histamine causes minimal bronchoconstriction having been found to be a threshold concentration in naïve guinea-pigs (Toward *et al* 2003). The effects of viral inoculation and allergen challenge were examined on reactivity to inhaled histamine. The appearance of a bronchoconstriction in response to the 1mM of histamine would indicate the presence of airways hyperreactivity.

2.1.4 Bronchoalveolar lavage and removal of lungs

Within 30 minutes of the hyper-reactivity test, the guinea-pigs were sacrificed with a lethal overdose of the anaesthetic, pentobarbital sodium (Euthatal 400mg/kg), by bilateral intraperitoneal injection. After examination to ensure cessation of breathing and cardiac activity a ventral incision was made in the neck and any tissue was moved aside thereby exposing the trachea. The trachea was then cannulated by insertion of a nylon intravenous cannula (Sims Portex Ltd, Kent, UK). An incision was made below the level of the diaphragm upwards to expose the ribcage. The ribcage was then removed exposing the lungs and trachea. The trachea and lungs were then removed from the thoracic cavity and the heart and any fat and connective tissue were then removed. The right bronchi was clamped shut with Spencer-Wells forceps so the lavage could be performed on the left lung only.

Saline (0.9%, 100ml/kg) was then injected into the left lung with a syringe, via the tracheal cannula. After 3 minutes the fluid was withdrawn, while gently massaging the lung. This procedure was repeated and the two recovered BALF samples were combined and placed on ice for total and differential cell counts. Only plasticware was used for the collection process to minimise adherence of the cells to the surface of the tube.

2.1.5 Total and differential cell counts

Total cell counts (cells/ml) were determined using a Neubauer haemocytometer (Marienfield, Germany) with a light microscope (X10, Nikon, Tokyo, Japan).

Differential cell counts were then undertaken to determine the levels of alveolar macrophages, eosinophils, lymphocytes and neutrophils. A 100µl sample of the BALF was centrifuged using a cytospin (ThermoShandon Ltd. Cheshire, UK) at 1000 r.p.m. for 7 minutes, onto a glass microscope slide and air-dried. The slides were then stained with 1.5% Leishman's stain (Sigma-Aldrich, Dorset, UK) for 7 minutes. Using a light microscope (X100, Nikon, Tokyo, Japan), a minimum of 200 consecutive cells were examined, using standard morphological criteria (see leukocyte morphology 2.1.6) to determine the cell type and expressed as a percentage. Using the corresponding total cell count, cells/ml for the subtypes of leucocytes in each guinea-pig could then be determined.

The remainder of the BALF was then centrifuged (Mistral 3000, Fisher Scientific, Loughborough, UK) at 2000 r.p.m. for 6 minutes and the cell free supernatant transferred into 1ml aliquots and stored at -80^{0}C for further analysis.

2.1.6 Leukocyte morphology after Leishman's staining

Macrophages

Macrophages are around 15 to 20μm in diameter. They have a single, dark bluish purple nucleus near the centre of the cell. The cytoplasm has an irregular appearance and a much lighter bluish purple colour (Fig. 2.2).

Eosinophils

Eosinophils are around 10 to 14 μm in diameter. They have a dark blue bi-lobed nucleus. The cytoplasm is granular and dark red (Fig. 2.2).

Lymphocytes

Lymphocytes vary in size from around 6 to 10μm or more in diameter. They have a large, off centre bluish purple nucleus which almost fills the whole cell, leaving an area of light blue cytoplasm at the perimeter of the cell (Fig. 2.2).

Neutrophils

Neutrophils are around 9 to 12μm in diameter. They have dark a blue multi-lobed nucleus and light pink granular cytoplasm (Fig. 2.2).

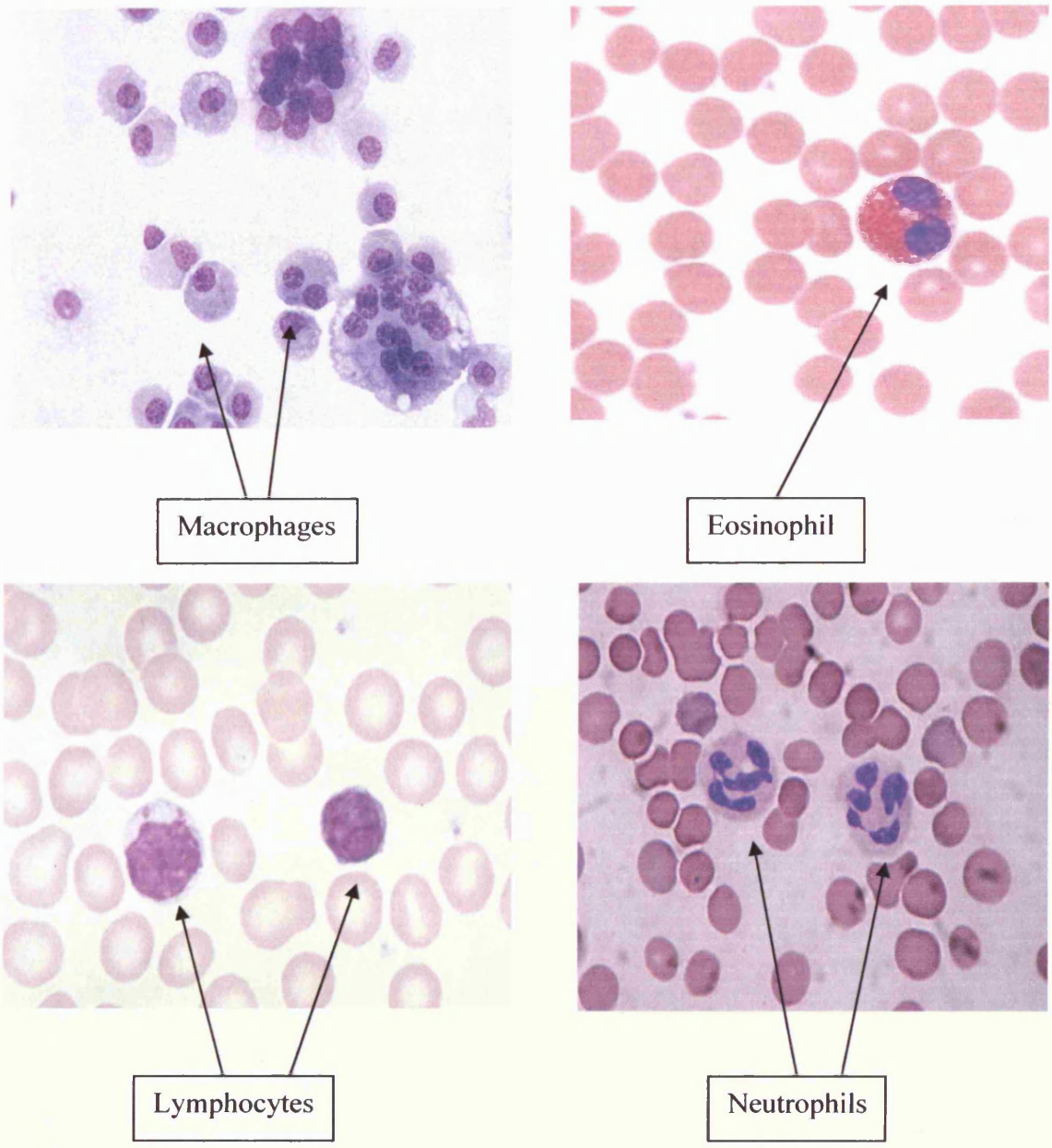


Figure 2.2

Macrophages, eosinophils, lymphocytes and neutrophils after staining with leishman's stain.

2.1.7 Lung preparation

The smallest of the four lobes of the right lung was used to estimate percentage wet weight of the whole lung, while the other three lobes were stored at -80^0C for viral recovery at a later date.

The four lobes were separated, then cleaned and dried thoroughly by blotting. The weight of the whole lung and the weight of the smallest lobe were then measured. The three larger lobes were then immediately frozen in liquid nitrogen and stored at -80^0C for later analysis of viral titre. The smallest lobe was then placed in an oven set at 40^0C until it had completely dried out to constant weight. Its weight was recorded as ratio of wet weight to total weight and recorded as a percentage according to the following equation:

$$\text{Percentage wet weight} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100$$

2.1.8. Data handling and statistical analysis

Baseline sG_{aw} level was determined as the average of two sG_{aw} readings taken before each challenge. Subsequent readings taken after challenge with histamine, bradykinin or ovalbumen were shown as percentage changes to the baseline value. Statistical comparison of histamine or bradykinin responses were determined between the percentage changes from baseline before and after treatment (e.g. viral inoculation, ovalbumen challenge or drug treatment).

The ovalbumen model of allergy shows distinct early and late phases of airway reactivity, however due to natural variation each animal can display these reactions at different time points. The average maximum decrease from baseline in each animal between 0 and 6 hours and between 7 and 12 hours is shown in a separate graph to demonstrate the early and late phase respectively alongside the graph showing the time course (e.g. Fig. 5.9).

Statistical comparisons between mean values were made by a two-tailed Student's t-test. A P value of <0.05 was considered significant. All values are presented as mean ± standard error of the mean (S.E.M.).

2.2 Virus experiments

Inoculation of guinea-pigs with virus

Viral inoculation was performed in a Class II safety cabinet (Captair Madcap 804, Erlab, Wiltshire, UK).

The guinea-pigs were held in a supine position with the head firmly supported. The virus or virus free medium was then pipetted into the nostrils using a Gilson Pipette. Each guinea-pig was given a 250µl inoculation: 125µl in one nostril, which was then repeated after 15 minutes in the other nostril. This procedure was then repeated 24 hours later. In all experiments a fresh vial of virus (or virus free media) was thawed immediately prior to inoculation to ensure no decrease in the viral titre and warmed by hand to 37°C to facilitate delivery.

Of the three established techniques for viral inoculation in guinea-pigs: intranasal (Hegele *et al* 1993), intratracheal (Folkerts *et al* 1992) and aerosolised inhalation (Streckert *et al* 1996), intranasal inoculation was used as it is simple, less invasive and causes minimum stress to the animals. Studies using colloidal carbon as a tracer have shown that the volume used is sufficient to allow the inoculum to enter all lobes of the lower respiratory tract in this weight of guinea-pig used (Dakhama *et al* 1997).

2.3 Effects of inhaled bradykinin

Bradykinin exposure experiments

Airway reactivity to bradykinin was measured by recording pulmonary function using whole body plethysmography and recorded as sGaw (see pharmacological methods 2.2.1). Baseline sGaw recordings were taken and then at 0, 5 and 10 minutes after a 20 second exposure to bradykinin dissolved in saline.

The effects of bradykinin were also examined 1 hour after administration of captopril and/or phosporamidon to inhibit the enzymes ACE and NEP respectively. These enzymes catalyze the breakdown of bradykinin into inactive metabolites (see chapter 1) and therefore inhibitors were used in an attempt to prolong the duration of the inhaled bradykinin in the lung. Captopril was administered by i.p. injection (1mg/kg) and phosphoramidon was administered by i.p. injection (0.1mg/kg) or by inhalation (1mM, 20 minute exposure). The effect of combining captopril (i.p., 1mg/kg) and phosphoramidon (1mM, 20 minute exposure) was also examined.

The effect of the duration of bradykinin exposure was examined by comparing 20, 40, and 60 second, 0.3mM bradykinin exposures after captopril (1mg/kg) treatment.

Bradykinin is thought to produce bronchoconstriction by activation of the B_2 bradykinin receptor on the airway smooth muscle (Fuller *et al*, 1987). The selective B_2 bradykinin antagonists: Icatibant (10μM inhalation exposure) and MEN16132 (30, 100 or 300nM/kg, i.p.) (1 or 10μM, 20 minute inhalation exposure) were administered 1 hour

before inhalation exposure to 1mM bradykinin in guinea-pigs treated with captopril (1mg/kg).

At least 4 days after each experiment with icatibant and MEN16132 the animals were treated with an appropriate saline control (i.p. injection or inhalation exposure) before further exposure to 1mM bradykinin with 1mg/kg captopril. If a significant bronchoconstriction was not produced the animals were left for a further 4 days and the control experiment was repeated until a significant bronchoconstriction was seen. This was to determine the recovery from B_2 receptor blockade.

2.4 Ovalbumen sensitization and challenge

Ovalbumen sensitization

On days 1 and 5 guinea-pigs were sensitized with a bilateral i.p. injection of a suspension containing 100μg of ovalbumen and 100mg of aluminium hydroxide ($Al(OH)^3$) which is an adjuvant and increases the immune response.

0.01g of ovalbumen was dissolved in 10ml sterile saline. 5g of $Al(OH)^3$ was added to 45ml of sterile saline. 5ml of the ovalbumen solution was added to the $Al(OH)^3$ solution and places on a magnetic stirrer for at least 2 hours.

Ovalbumen exposure

On day 15 the animals were placed in a steel exposure chamber (40cm diameter, 15cm height) and given a 60 minute inhalation exposure of ovalbumen (0.01%). Any animals that looked to be in respiratory distress were immediately removed from the exposure chamber and the exposure considered complete. Lung function measurements were taken by whole body plethysmography immediately before challenge and at 0, 15, 30, 45 and 60 minutes after, then hourly up to 12 hours and finally at 24 hours.

Histamine exposure

Animals were given a 20 second, nose only histamine exposure on the day before and the day after ovalbumen exposure (see pharmacological methods 2.1.3).

Dosing with drugs

Animal were dosed twice daily by bilateral subcutaneous injection from day 8 to day 14 and 1 hour before ovalbumen exposure on day 15 with either saline or 10mg/kg deamethasone in saline.

Bronchoalveolar lavage

Within 30 minutes of the histamine exposure the animals were sacrificed with an overdose of anaesthetic, pentobarbital sodium, by bilateral intraperitoneal injection and a bronchoalveolar lavage was performed (see pharmacological methods 2.1.4).

2.5 Cell culture and viral growth

Cell Culture

All cell culture and viral growth and detection procedures were performed in a Class II microbiological safety cabinet (Envair, Lancashire).

African green monkey kidney epithelial (VERO) cells, provided by Dr Joachim Bugert (University of Wales College of Medicine, Medical Microbiology), were obtained from the European Collection of Cell Cultures (ECCAC, Wiltshire). The cells were grown in a T75 culture flask (Fig. 2.3). The medium (10ml per flask) consisted of Dulbecco's modified essential medium (DMEM), 10% heat inactivated foetal bovine serum (FBS) and 1% L-glutamate (the L-glutamate was added to the DMEM). It was decided not to use antibiotics, to ensure complete absence of bacterial contamination, the presence of antibiotics could mask any low level bacterial contamination, which would become easily detectable in the absence of antibiotics. This would avoid the possibility of inoculating guinea-pigs with bacterial debris, which could generate inflammatory responses to obscure the viral actions. The cells were grown to almost total confluence and then split or virally infected. The cells were incubated at 37^0C in a humidified incubator (Sanyo, Osaka, Japan) maintained at 95% with an atmosphere of 95% O_2, 5% CO_2.

Splitting VERO cells

VERO cells were split when they reached almost total confluence in the T75 culture flask (Fig. 2.3). The medium was first removed and the cells were then washed with 1ml of

trypsin, which was then immediately removed. The cells were then incubated with 1ml of trypsin, covering all cells at 37^0C for 7minutes. After removal from the incubator the side of the culture flask was tapped twice on the side of the bench to ensure all cells had detached from the surface of the flask and examined in the light microscope (×10) (Nikon, Tokyo, Japan). 1ml of FBS was then added to inactivate the trypsin. DMEM and FBS were then added as required. The suspension was pippetted up and down to distribute the cells evenly in the medium. 10 ml of the cell suspension was then removed and added to each new T75 culture flask.

Growth and harvesting of virus

Human PIV-3 (Strain:DEL/139/05) and PIV-2 (Strain: Greer) virus provided by Dr Joachim Bugert (University of Wales College of Medicine, Medical Microbiology) was obtained from ECCAC (Wiltshire, UK). VERO cells were considered ready for viral infection when the monolayer of cells reached almost total confluence in the culture flask. The medium was removed and the cells were trypsinized as described above (splitting cells) and 1ml of FBS was added to deactivate the trypsin. The cells were then transferred to a 50ml bluecap centrifuge tube. The cells were pelleted by centrifugation (Jouan CR412, Fisher Scientific, Loughborough, UK) at 1200 r.p.m. for 6 minutes. The supernatant was removed and the cells were resuspended in 2ml of DMEM. A previously frozen vial of virus was then defrosted by hand and the virus was added to the cells and mixed by pipetting up and down three times. The cell/viral suspension was then placed in the incubator for 30mins, and swirled by hand every 5mins to ensure the virus has access

to every cell. 3ml of the cell/viral mix was then added to the T75 flask and topped up with 7ml of DMEM and the flask was replaced in the incubator.

After 4 to 5 days the cytopathic effects (CPE) of viral infection became obvious, with large syncitia (multi-nuclear cells) and dead cells floating in the medium (Fig. 2.3). The virus was then harvested. The cells were frozen at -80^0C for 5minutes and allowed to thaw at room temperature to break open the cell membranes. This freeze-thaw cycle was repeated twice and the resulting suspension was transferred to a 50ml centrifuge tube and centrifuged (Jouan CR412, Fisher Scientific, Loughborough, UK) at 4000 r.p.m. for 5 minutes to remove cellular debris. The supernatant was then transferred into sterile 1ml vials and stored at -80^0C. For control inoculation uninfected infected VERO cells were subjected to the same procedure.

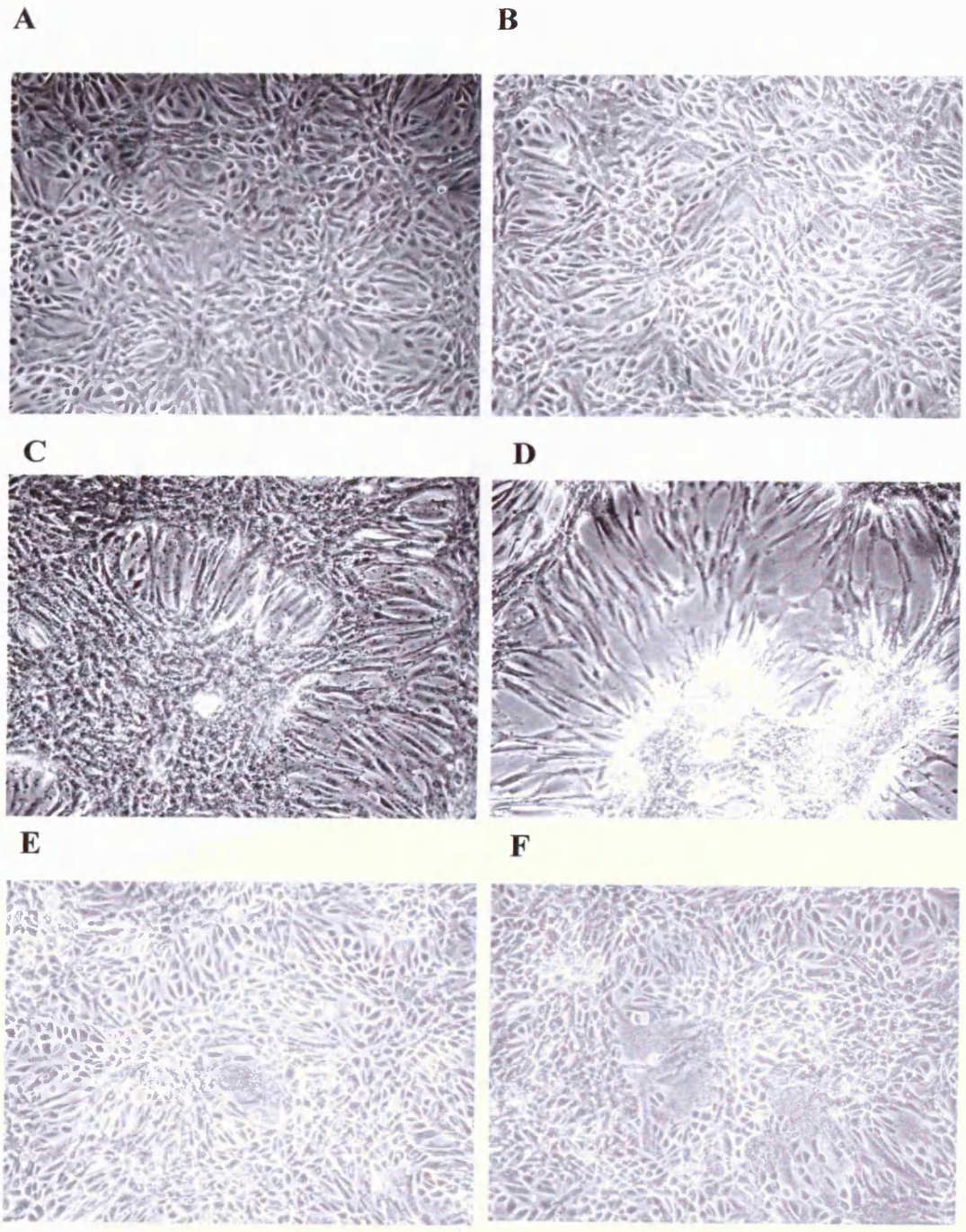


Figure 2.3

Confluent VERO cells (A and B), infected with PIV-3 (C and D) and PIV-2 (E and F).

Influenza

All Influenza A H1N1 viruses were grown and provided by Dr Joachim Bugert.

The WSN33 strain was obtained from ECCAC-NCPV (NPCV No.402). It was isolated from throat washings of a patient with influenza in London in 1933 (Burnett 1951). The virus was grown in Madine-Darby Canine Kidney epithelial cells (MDCK). Media from uninfected MDCK cells was used for control inoculation.

The A/PR/8/34 strain was obtained from ECCAC-NCPV (NPCV No.235). It was isolated from a patient in Puerto Rico (Francis and Magill 1935). The virus was grown in chicken embryos and harvested in the allantoic fluid. Allantoic fluid from uninfected chicken embryos was used for control inoculation.

Despite the increasing use of tissue culture, chicken embryos are still the most sensitive host mechanism for influenza (Clavijo *et al* 2002). The amniotic cavity, allantoic cavity, yolk sac and chorioallantoic membrane are used for growth of virus. The allantoic cavity was used as the yield of virus is greater.

Freshly layed, fertilized specific pathogen free eggs were obtained from the Institute for Animal Health (Compton, Newbury, UK). The eggs were stored in the incubator at 37^0C at relative humidity of 30-40%. The eggs were examined daily with a bright lamp in a darkened room to ensure the eggs were still alive. After seven days a small hole was made with a sterile drill over the egg sack and a needle was inserted and 100µl of virus was inserted through the chorioallantoic membrane into the allantoic cavity. The hole was sealed with candle wax and the eggs were placed in the incubator at 35%, which is

the best temperature for mammalian influenza growth. After three days the eggs were chilled for at 4°C for 18 hours to constrict blood vessels and make harvesting easier. The shell over the air over the air sac was broken with sterile forceps, the chorioallantoic membrane pushed aside and amniotic and allantoic fluids were collected using a needle and syringe. The embryos were then killed by decapitation. The harvested fluid was then centrifuged at 4000 r.p.m. for 5 minutes to remove excess blood and tissues. In these procedures a Home Office project or personal license was not required as all procedures were performed before the halfway point of gestation.

$TCID_{50}$

Tissue culture infective dose ($TCID_{50}$) refers to the quantity of virus that will produce cytopathic effects in 50% of wells (for example in a 96 well plate) infected.

450µl of DMEM was added to 10 wells of a 24 well plate, which were marked –1 to -10. A vial of the previously frozen virus was thawed by hand and 50µl was removed and added to the well marked –1 and pipetted up and down three times. Serial 1 in 10 dilutions were made up to –10 and the plate was placed on ice. A T75 cell culture flask almost confluent with VERO cells was trypsinised as previously described. After the addition of the FBS to deactivate the trypsin, the cells were centrifuged (Jouan CR412, Fisher Scientific, Loughborough, UK) at 1000 r.p.m. for 6 minutes, the supernatant was then removed and the cells were resuspended in 5ml of DMEM. The first 10 columns of a 96 well plate were labelled –1 to –10 and the last two were left for control. 50µl of each viral dilution from the 24 well plate was added to each of the wells of the corresponding

column of the 96 well plate. 50µl of DMEM was added to each of the wells of the last two columns. 50µl of the VERO cell suspension was then added to every well and the plate was placed in the 37^0C incubator (Fig. 2.4). The cells were then checked every day for cytopathic effects. After around 8 to 10 days, when no additional wells had shown any sign of infection for two days, the cells were stained. The media was removed and 50µl of crystal violet was immediately added to each well and left for 30 minutes. The crystal violet was then washed off with water and the plate was left to dry. Each well was then examined for signs of infection, which were easily determined as there was very little staining of the cells that had been destroyed. The uninfected and control wells showed complete staining as the cells were intact (Fig. 2.4).

The viral concentration was then determined using the Karber formula (Reed and Meunch 1938) (Fig. 2.4).

The final viral concentration for inoculation was determined by tissue culture infective dose ($TCID_{50}\,ml^{-1}$). Two concentrations of PIV-3 were used. In the initial experiments, the viral concentration used was 6.32×10^6 infectious units per ml. In the later experiments the viral concentration was 3×10^8 infectious units per ml. The concentration of PIV-2 used was 1×10^8.

2.6 Viral recovery and identification

RNA purification

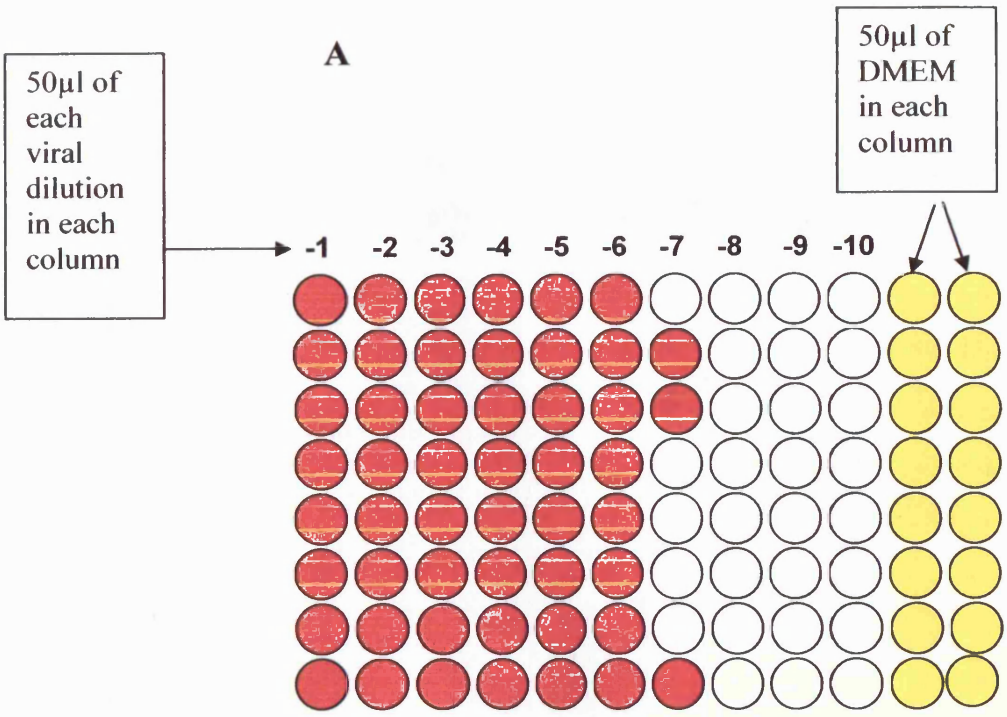
50µl of RNA was purified from 200µl samples of BALF using the RNeasy mini kit (Qiagen, Crawley, West Sussex).

Synthesis of cDNA

8µl of each sample of purified RNA, 1µl of random hexamers and 1ul of a dNTP mix were added to a 0.2ml sterile centrifuge tube and placed in a thermal cycler (PTC-100, Biorad, Hemmel Hemsread, UK) heated to 65^0C to for 5 mins and then cooled to 4^0C. 2µl of 10 × RT buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT and 1µl of RNAse inhibitor were then added to each tube then mixed by pippeting and heated to 25^0C for 5minutes. 1µl (50 units) of reverse transcriptase was then added to each tube and after another 10 minutes at 25^0C they were heated to 42^0C for 50 minutes then to 72^0C for 15 minutes then the experiment terminated at 4^0C.

Amplification of cDNA

The amplification of cDNA was performed in a 2 step process. Fig. 2.5 shows a diagram of the PIV-3 genome with the haemaglutinin-neuraminidase gene and PCR products with the location of the primers used.



B

Dilution	Infected	% Infected
10-1	8/8	100
10-2	8/8	100
10-3	8/8	100
10-4	8/8	100
10-5	8/8	100
10-6	8/8	100
10-7	3/8	37.5
10-8	0/8	0
10-9	0/8	0
10-10	0/8	0

C

50% endpoint titer = $10^{\text{log dilution above 50\% infection- (I} \times \text{Log H)}}$

$$I = \frac{100-50}{100-37.5} = 0.8$$

log Dilution above 50 % Infection $=10^{-6}$
dilution factor = 10, Log dilution factor = 1

50% endpoint titer = $10^{-6-(0.8\times 1)}$
50% endpoint titer = $10^{-6.8}$

$= 10^{6.8}$ TCID50/50µl
$= 6.31 \times 10^{6}$ TCID50/50µl
$= 1.26 \times 10^{8}$ TCID50/ml

Figure 2.4

A: Diagram of a 96 well plate showing the viral dilution or DMEM added to each column. 50µl of VERO cells in DMEM was then added to each well. Red circles show infected wells, white non-infected and yellow control. B: Table showing percentage infection for each dilution. C: Karber formula calculation where I = interpolated value of 50% endpoint (percentage of wells infected above 50%)-50 / percentage of wells infected above 50% - % of wells infected below 50%) and H = dilution factor.

Round 1:

For each sample add to PCR tube

- 15µl of H_2O
- 2.5 µl of PCR buffer P2192
- 2 µl of dNTP
- 0.5 µl of Primer PIV-3 forward
- 0.5 µl of Primer PIV-3 reverse
- 0.2 µl of Taq polymerase
- 4µl of cDNA sample

Placed in thermal cycler for:

- 30 minutes at 58°C
- 15 minutes at 94°C
- 45 cycles of: 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C

Round 2 as before but 1µl of cDNA sample (3µl more H_20), PIV-3 nested forward and reverse primers and only 32 cycles.

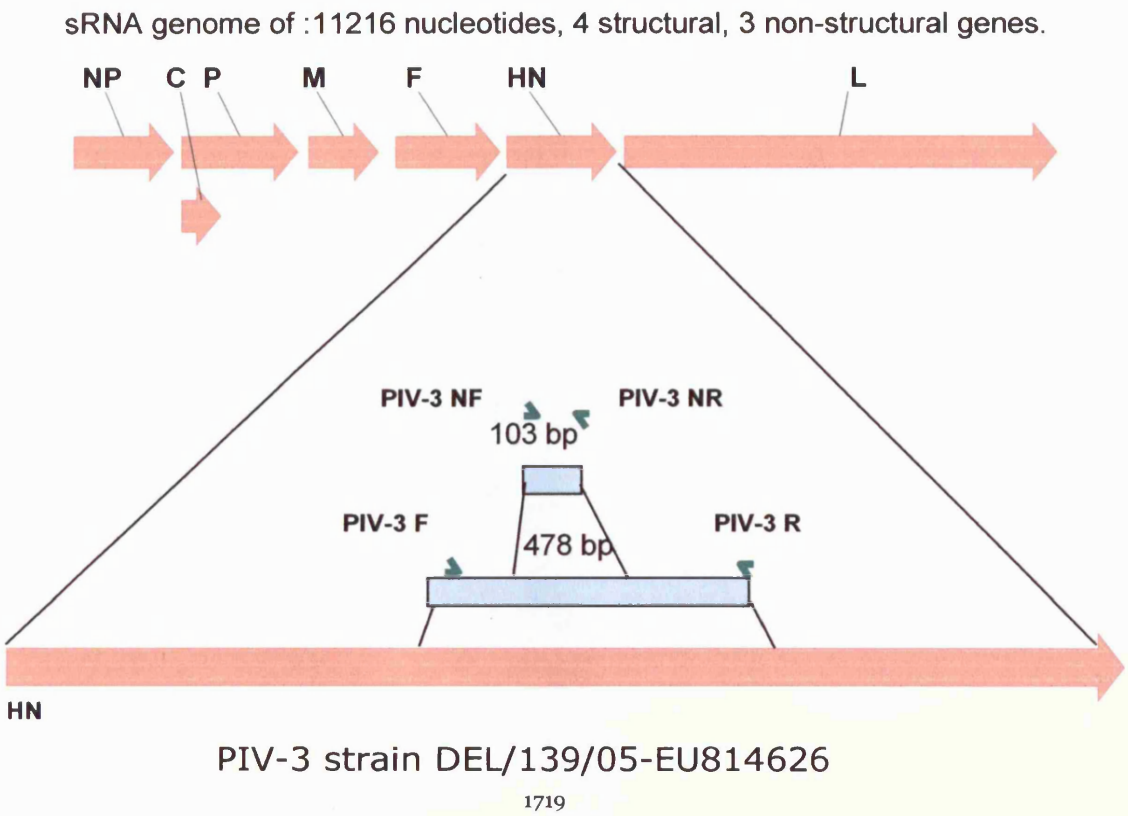


Figure 2.5

Simplified diagram of RNA genome of PIV-3, showing size of Haemaglutinin-neuraminidase gene (HN) and PCR products with primers used (PIV-3 forward and reverse and PIV-3 nested forward and reverse).

Tissue culture

In a class II safety cabinet (Envair, Lancashire) previously frozen guinea-pig lung was thawed and a piece (0.1 – 0.2g) was removed and placed in a tube with ceramic beads in 1ml of sterile PBS and placed in a Precellys24 shaker (Bertin technologies, Montigny-le-Bretonneux, France) for 40 seconds at 5600rpm to homogenization the tissue. After centrifugation (1000 r.p.m., 1 minute) to remove debris serial dilutions of the supernatant were made and used to infect VERO cells in a 96 well plate as explained above (Methods, cell culture and viral growth, $TCID_{50}$).

2.7 Materials

Dulbecco's modified essential medium, 10% heat inactivated foetal bovine serum, histamine diphosphate salt, crystal violet, bradykinin acetate, icatibant, phosphoramidon disodium salt, dexamethasone 21-phosphate disodium salt, captopril, Leishman's, stain, PCR buffer P2192, aluminium hydroxide and DMSO were purchased from Sigma-Aldrich, Dorset, UK.

Random Hexamers (50ng/µl), 10mM dNTP Mix, 5X cDNA synthesis buffer, 25mM $MgCl_2$, 2µl of 0.1M DTT, RNaseOUT RNAse inhibitor and Cloned AMV reverse transcriptase (15 U/µl) were purchased from Invitrogen, London, UK as part of the Cloned AMV First-Strand cDNA Synthesis Kit.

1% L-glutamate and trypsin were purchased from Invitrogen, London, UK.

Taq DNA polymerase purchased from Roche, Basel, Switzerland.

Euthatal was purchased Genus express, Chepstow, UK

MEN16132 was donated by Menarini Research, Pomezia, Italy.

FE999024 was donated by Ferring Research, Chilworth, UK

Ovalbumen was purchased from BDH laboratory supplies, Poole, Dorset, UK.

Unless otherwise stated drugs were dissolved in 0.9% sterile saline (for injections) Baxter Healthcare, Thetford, Norfolk, UK.

Chapter 3

Effects of viral inoculation on guinea-pig airways

3.1 Introduction

Viral infection and asthma

Virus infections, including parainfluenza induce changes in the airways that are similar to the effects seen in asthma including bronchial hyper-responsiveness, an influx of inflammatory cells, epithelial damage and bronchiolar fibrosis (Folkerts *et al* 1992). Viral respiratory infections are the most common cause of asthma exacerbations (Pattemore *et al* 1992). These changes raise the questions of the involvement of PIV in the pathogenesis and/or exacerbations of asthma.

Parainfluenza virus

Parainfluenza viruses (PIV) were first discovered in the late 1950's. They have been divided into subtypes 1 to 4, with PIV-4 being further divided into types a and b. Types 1 to 3, along with Respiratory Syncytial Virus (RSV) are major causes of lower respiratory tract disease in infants, the young, the immunocompromised and the elderly (Glezen *et al* 1984; Glezen *et al* 2000; Falsey 1991). PIV infection can cause croup, bronchiolitis, and bronchopneumonia, though the most common presentation is an upper respiratory tract infection (Belshe *et al* 1983). The pathogenesis of PIV infection is still being studied but respiratory epithelium appears to be the major site of virus binding and subsequent infection. The epithelium is an extensive source of inflammatory mediators including chemokines and cytokines, which can be released to recruit inflammatory cells after viral infection (Garofalo and Haebere 2000). PIV has been shown to cause inflammatory cell influx after instillation intranasally to guinea pigs (Toward *et al* 2005) and rats (McWilliam *et al* 1997). This was accompanied by airway hyperreactivity to histamine in

guinea-pigs (Toward *et al* 2005). In order to examine drug interventions on PIV-induced inflammation and AHR, it was first necessary to examine the relative ability of PIV-2 and PIV-3 to produce these changes and to confirm that there was viral infection of the lungs by recovery of live virus and to identify the virus in the lungs by means of PCR. The effects of different inoculation titres were also examined.

Influenza in guinea-pigs

Mice are known to be susceptible to infection with influenza virus (Schulman and Kilbourne 1963) and several studies have shown that guinea-pigs are susceptible to infection by influenza. Azoulau-Dupouis *et al* (1984) caused significant histopathological changes to the whole respiratory tract after infection with influenza A/Hong Kong/68 (H3N2) and Lowen *et al* (2006) demonstrated the spread of influenza between guinea-pigs after infection with A/Panama/2007/99 (H3N2). Although many studies have shown airway hyperreactivity to inhaled histamine after PIV inoculation (Toward *et al* 2005) no studies have shown airway hyperreactivity after influenza inoculation. This study was therefore undertaken to establish airways inflammation and AHR in guinea-pigs after infection with influenza virus. Type of virus and method of culture were compared to obtain optimum levels of AHR for evaluation of drug interventions.

3.2 Methods

Airway reactivity test

On days 2 and 8, airway reactivity, measured as specific airways conductance (sG_{aw}) was tested by whole body plethysmography as described in chapter 2. Baseline readings were taken and then at 0, 5 and 10 minutes after a nose only 1mM histamine inhalation exposure.

Virus inoculation

On days 3 and 4 the guinea-pigs were inoculated intranasally with 250µl of virus in different known titres or with virus-free medium.

Bronchoalveolar lavage

Within 30 minutes of the second histamine exposure the animals were killed and a bronchoalveolar lavage was performed on the left lung. The total cells in the recovered BALF were counted using a haemocytometer. The cells were then centrifuged on to a slide, stained with Leishman's stain so that a differential cell count could be performed. Macrophages, eosinophils, lymphocytes and neutrophils were counted.

Viral recovery

Following removal of the lungs, the right lung was immediately placed on ice and then stored at -80^{0}C for subsequent viral recovery. A 100µg section of the right lung was homogenized in PBS and after centrifugation the supernatant was used to infect VERO cells in a 96 well plate to determine viral titre.

Experimental Protocol

The experiments performed in this chapter were aimed at establishing the optimum conditions for virus-induced airways inflammation and airways hyperreactivity for future studies with drug interventions. In preparation for drug intervention studies, these studies were performed with vehicle administrations at the proposed drug treatment times. The selected vehicles were saline or dimethyl sulphoxide (DMSO) (25 or 50%) in saline all delivered by the subcutaneous (s.c.) route. The guinea pigs received bilateral s.c. injections every 12 hours of either

- 0.9% sterile saline
- 25 or 50% DMSO in 0.9% sterile saline

The volume used was 1ml\kg

Day 1: Dosing with 0.9% sterile saline or DMSO (25 or 50%) in 0.9% sterile saline

Day 2: Dosing with 0.9% sterile saline or DMSO (25 or 50%) in 0.9% sterile saline and **reactivity test to histamine**

Day 3: Dosing with 0.9% sterile saline or DMSO (25 or 50%) in 0.9% sterile saline and **inoculation 3 hours later with virus or virus free medium**

Day 4: Dosing with 0.9% sterile saline or DMSO (25 or 50%) in 0.9% sterile saline and **inoculation 3 hours later with virus or virus free medium**

Day 5: Dosing with 0.9% sterile saline or DMSO (25 or 50%) in 0.9% sterile saline

Day 6: Dosing with 0.9% sterile saline or DMSO (25 or 50%) in 0.9% sterile saline

Day 7: Dosing with 0.9% sterile saline or DMSO (25 or 50%) in 0.9% sterile saline

Day 8: Dosing with 0.9% sterile saline or DMSO (25 or 50%) in 0.9% sterile saline, **reactivity test to histamine and lavage**

Viral identification

Viral identification was undertaken by PCR on the BAL fluid recovered from the left lung. RNA was purified using the RNeasy mini kit then cDNA was synthesized using the SuperScript™ II reverse transcriptase enzyme. The DNA was then amplified using primers specific for a section of the haemaglutinin-neuraminidase gene of PIV-3.

3.3 Results

3.3.1: Effects of parainfluenza on airway inflammation in guinea-pigs

Airway reactivity to histamine

Prior to inoculation with virus there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Figs. 3.1 and 3.2).

After inoculation with 3 x 10^8 infectious units per ml of PIV-3 there was evidence of airway hyperreactivity as there was a 31.5 ± 6.3% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to the baseline value. There was a recovery after 5 and 10 minutes but not to baseline (Fig. 3.1 and 3.2).

There were also significant decreases in sG_{aw} after histamine exposure after inoculation with PIV-2 (1 x 10^8 infectious units per ml) and PIV-3 (6.32 x 10^6 and 3 x 10^8 infectious units per ml) (Fig. 3.2).

The highest concentration of PIV-3 produced the greatest decrease in sG_{aw} after histamine exposure but there was no significant difference in the decrease in sG_{aw} seen compared to histamine exposure after inoculation with PIV-2 or the lower concentration of PIV-3 (Fig 3.2).

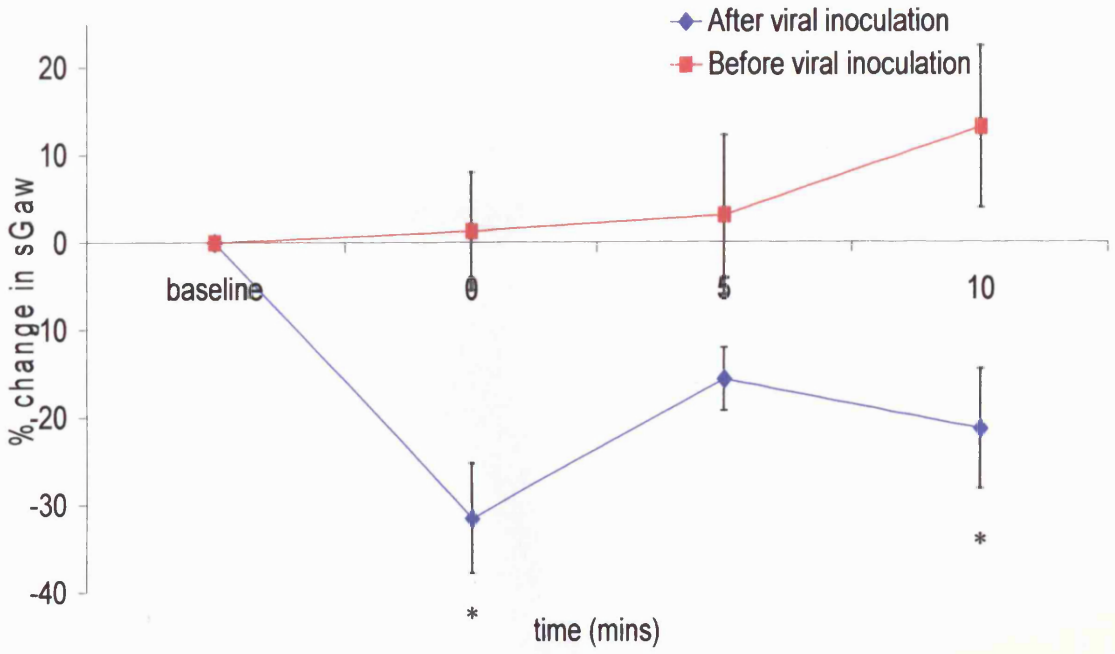


Figure 3.1

Airway function before and after PIV-3 (3 x 10^8 infectious units per ml) virus infection in saline treated guinea-pigs. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after virus inoculation as determined by a Student's *t* test (paired).

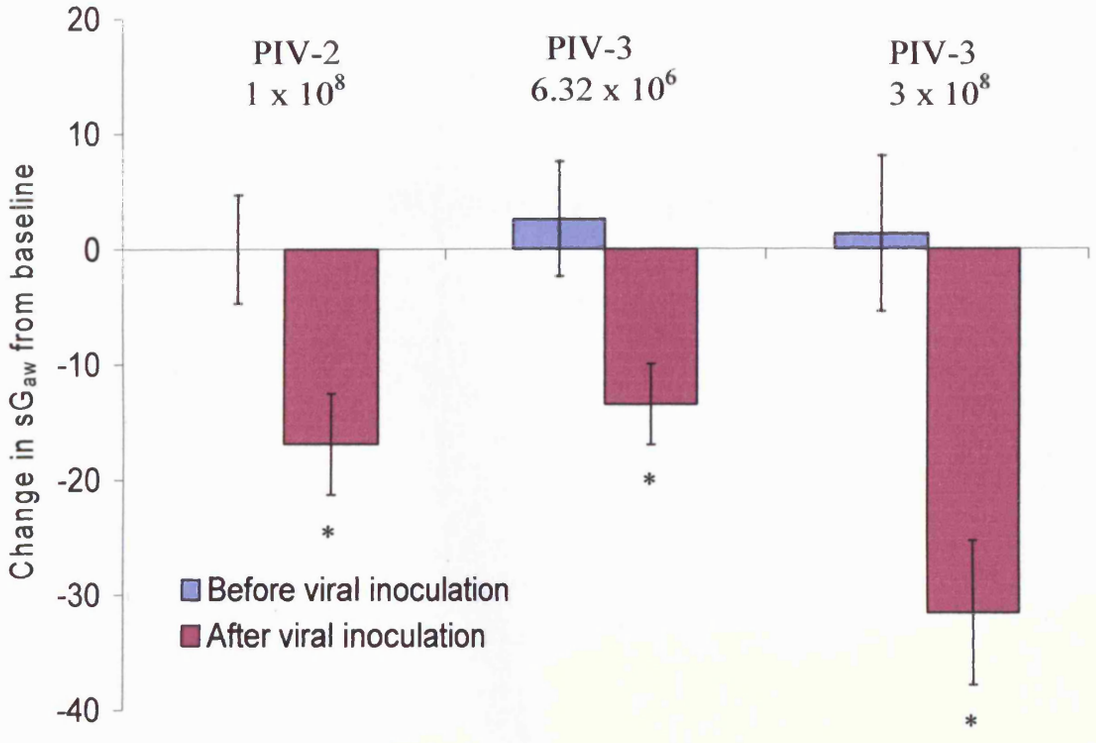


Figure 3.2

Maximum change in sG_{aw} from baseline in saline treated guinea-pigs after 1mM histamine exposure recorded at the corresponding time before and after inoculation with PIV-2 (1 x 10^8 infectious units per ml) or PIV-3 (6.32 x 10^6 or 3 x 10^8 infectious units per ml). Each point represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference in the maximum decrease in sG_{aw} from baseline before and after viral inoculation, as determined by a Student's *t* test (paired).

Bronchoalveolar lavage cell counts

In animals inoculated with medium and treated with saline, the numbers of macrophages, lymphocytes, eosinophils and neutrophils in the BALF were as follows; total cells: 1.54 ± 0.17 ($\times 10^6$/ml), macrophages: 1.37 ± 0.15 ($\times 10^6$/ml), eosinophils: 0.12 ± 0.01 ($\times 10^6$/ml), lymphocytes: 0.06 ± 0.02 ($\times 10^6$/ml) and neutrophils 0.03 ± 0.01 ($\times 10^6$/ml) (Fig. 3.3).

In PIV-2 (1 x 10^8) inoculated guinea pigs treated with saline, there were significant increases in total cells, macrophages, eosinophils and neutrophils compared to the no virus control. There was also a modest increase in lymphocytes (Fig. 3.3).

In PIV-3 (6.32 x 10^6) inoculated guinea pigs treated with saline, there were significant increases in total cells, macrophages, eosinophils and lymphocytes compared to the no virus control. There was also a modest increase in neutrophils. There was no significant difference compared to the PIV-2 (3 x 10^8) inoculated group but modest increases in total cells, macrophages, eosinophils and lymphocytes but slightly fewer neutrophils (Fig. 3.3).

In PIV-3 (3 x 10^8) inoculated guinea-pigs treated with saline, there were significant increases in total cells, macrophages, eosinophils, lymphocytes and neutrophils compared to the no virus control. There was a significant increase in total cells compared to the PIV-2 (1 x 10^8) inoculated group but only modest increases in each individual cell type.

There were modest but insignificant increases in all cell types compared to the PIV-3 (6.32×10^6) inoculated group (Fig. 3.3).

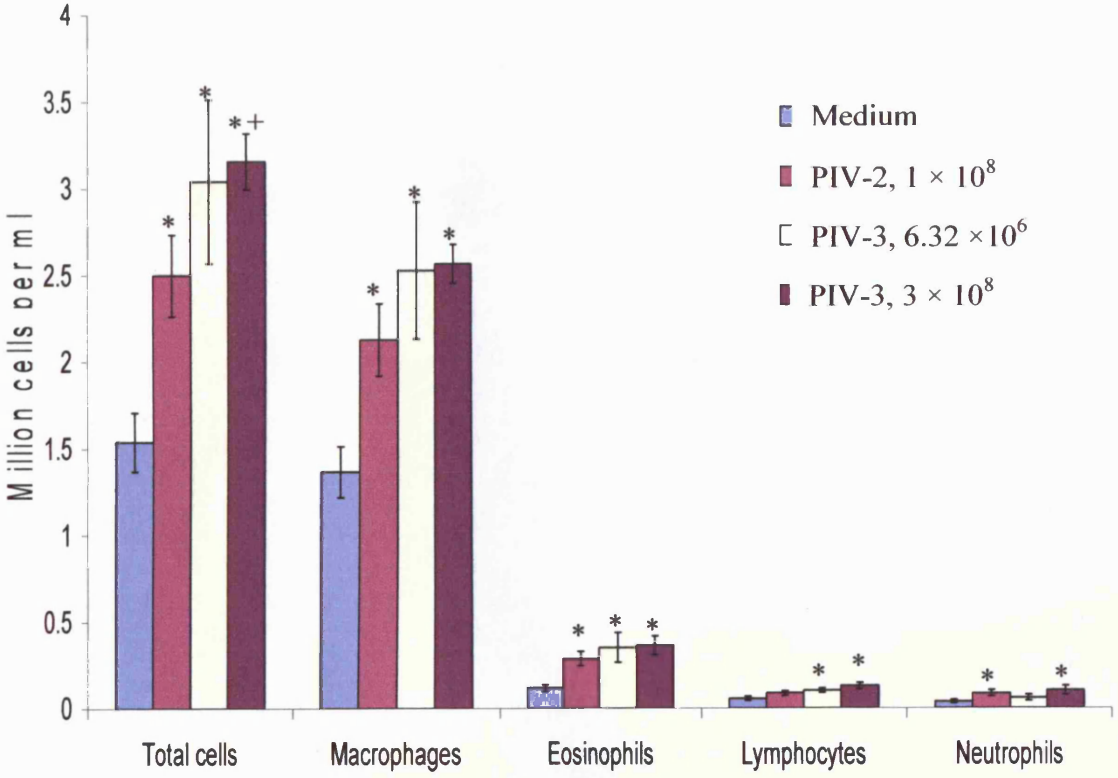


Figure 3.3

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from virus free medium, PIV-2 (1 x 10^8 infectious units per ml) or PIV-3 (6.32 x 10^6 or 3 x 10^8 infectious units per ml) inoculated guinea-pigs. Each bar represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding no virus control group and + denotes the significance ($P<0.05$) of difference compared to the corresponding PIV-2 group as determined by a Student's *t* test (paired).

Percentage lung wet weight

In animals receiving no virus and treated with saline, the percentage wet weight of the lung tissue was 79.0 ± 0.4 (Fig. 3.4).

In the PIV-2 (1×10^8) inoculated guinea-pigs treated with saline the percentage wet weight had increased significantly compared to the media control to 81.7 ± 0.3%. (Fig. 3.4).

In the PIV-3 (6.32×10^6) inoculated guinea-pigs treated with saline the percentage wet weight had increased significantly compared to the media control to 81.6 ± 0.5%. (Fig. 3.4).

In the PIV-3 (3×10^8) inoculated guinea-pigs treated with saline the percentage wet weight had increased significantly compared to the media control to 82.4 ± 0.8%. (Fig. 3.4).

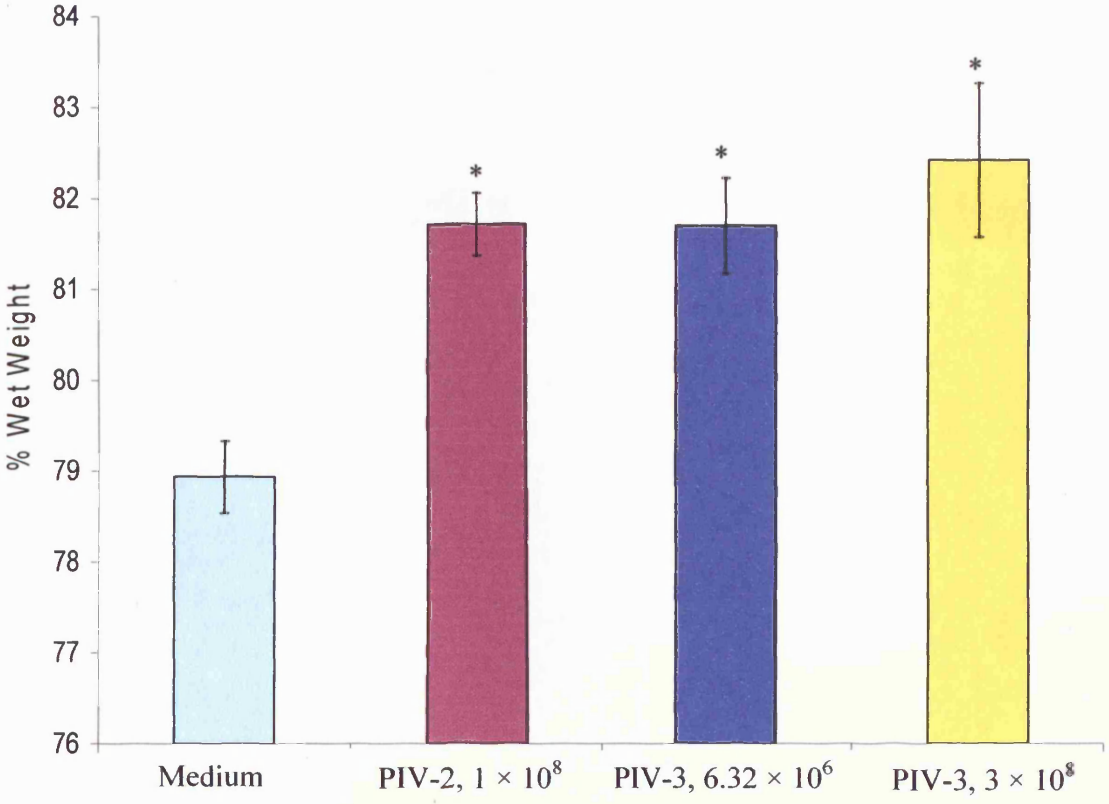


Figure 3.4

Effect on percentage wet lung weight of inoculation with virus free medium, PIV-2 (1 x 10^8 infectious units per ml) or PIV-3 (6.32 x 10^6 or 3 x 10^8 infectious units per ml) in saline treated guinea-pigs. Each bar represents the mean ± standard error of mean (n=6). * denotes the significant ($P<0.05$) difference between the viral inoculated groups and their corresponding no virus controls as determined by a Student's *t* test (paired).

Viral recovery

After 25% DMSO treatment and inoculation with 3×10^8 of PIV-3 the average amount of virus recovered per gram of lung tissue and grown on VERO cells was $3.62 \times 10^4 \pm 2 \times 10^4$ (Fig. 3.5).

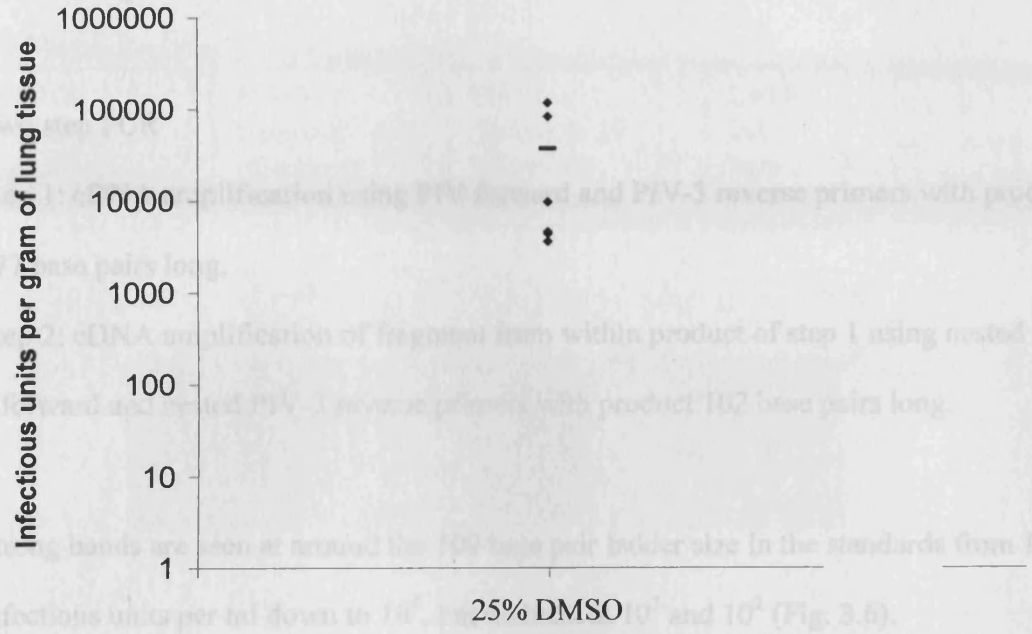


Figure 3.5

Effect of 25% DMSO on PIV-3 recovered from guinea-pig lung tissue after inoculation with 3 x 10^8 of PIV-3. Each point shows the amount of PIV-3 recovered per gram of lung tissue and grown in VERO cells and – shows the average (n = 6).

Virus identification

Two step PCR

Step 1: cDNA amplification using PIV forward and PIV-3 reverse primers with product 477 base pairs long.

Step 2: cDNA amplification of fragment from within product of step 1 using nested PIV-3 forward and nested PIV-3 reverse primers with product 102 base pairs long.

Strong bands are seen at around the 100 base pair ladder size in the standards from 10^7 infectious units per ml down to 10^4, but no band at 10^3 and 10^2 (Fig. 3.6).

A strong band was seen at the 100 base pair ladder size in the BALF from the PIV-3 infected guinea-pig treated with 25% DMSO sample but only a faint band in the guinea-pig treated with saline. Comparison with the standards indicates that the BALF samples had at least 10^3 copies of the viral RNA as this was below the detection limit in the standards (Fig. 3.6).

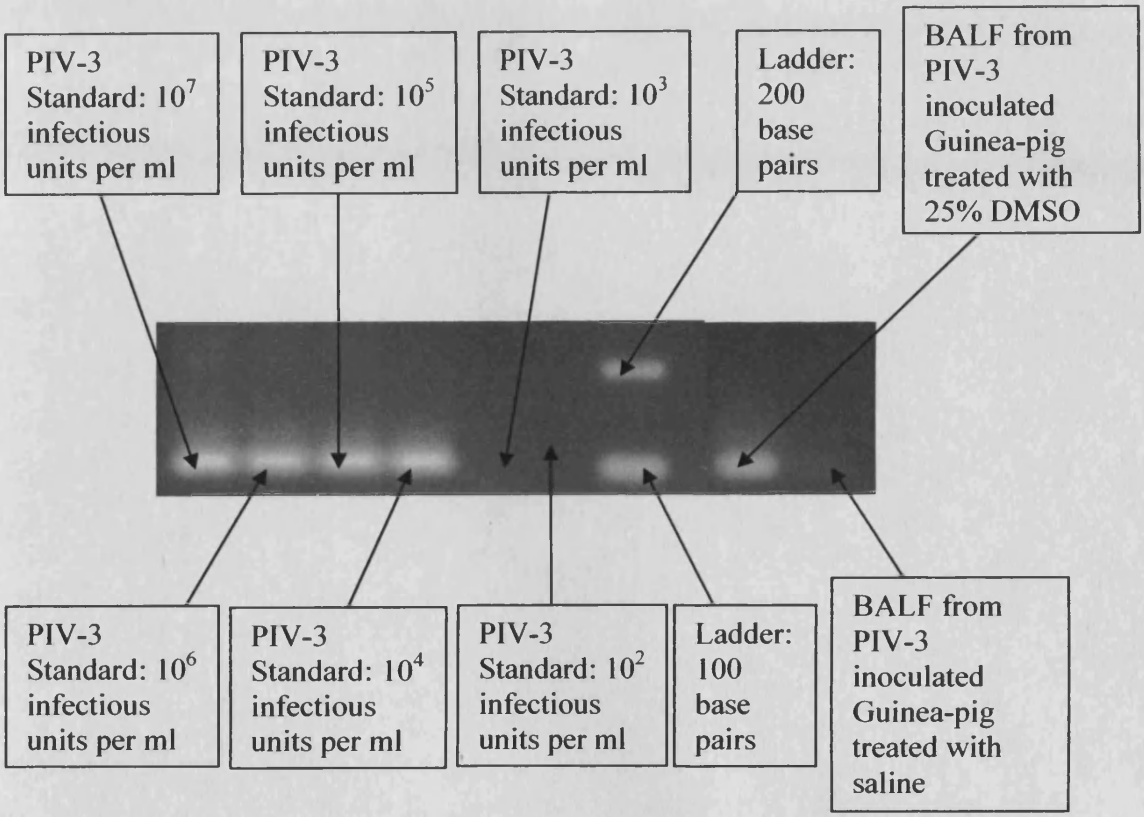


Figure 3.6

PCR with 100 base pair ladder (100 and 200 base pairs shown), PIV-3 standards (10^7 to 10^2 infectious units per ml) and BALF from PIV-3 (3×10^8 infectious units per ml) inoculated guinea-pigs treated with 25% DMSO or saline. Strong band are seen at around the 100 base pair ladder size in the standards from 10^7 infectious units per ml down to 10^4, but no band at 10^3 and 10^2. There is also a strong band at the 100 base pair ladder size in the BALF from PIV-3 infected guinea-pig treated with 25% DMSO sample but only a faint band in the guinea-pig treated with saline.

3.3.2: Effects of influenza on airway inflammation in guinea-pigs

Airway reactivity to histamine

Prior to inoculation with virus there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Figs. 3.7 and 3.8), since this was a sub-threshold dose of histamine.

Effect of inoculation with WSN33 (9.35×10^5) grown in MDCK cells

In 25% DMSO treated guinea-pigs inoculated with influenza WSN33 virus, there was no evidence of airway hyperereactivity as there was only a small -3.1 ± 2.6% decrease in sGaw after viral inoculation (Fig. 3.8).

Effect of inoculation with A/PR/8/34 (1.1×10^5) grown in chicken embryos

In 25% DMSO treated guinea-pigs inoculated with influenza A/PR/8/34 virus, there was no evidence of airway hyperereactivity as there was no decrease in sG_{aw} after viral inoculation (Fig. 3.8).

Effect of inoculation with A/PR/8/34 (1.1×10^6) grown in chicken embryos

In 25% DMSO treated guinea-pigs inoculated with a higher titre of A/PR/8/34 virus there was evidence of airway hyperreactivity after viral inoculation as there was a -20.4 ± 3.7% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to the baseline value. sG_{aw} values recovered to –9.7 ± 4.9% after 10 minutes (Fig. 3.7 and 3.8).

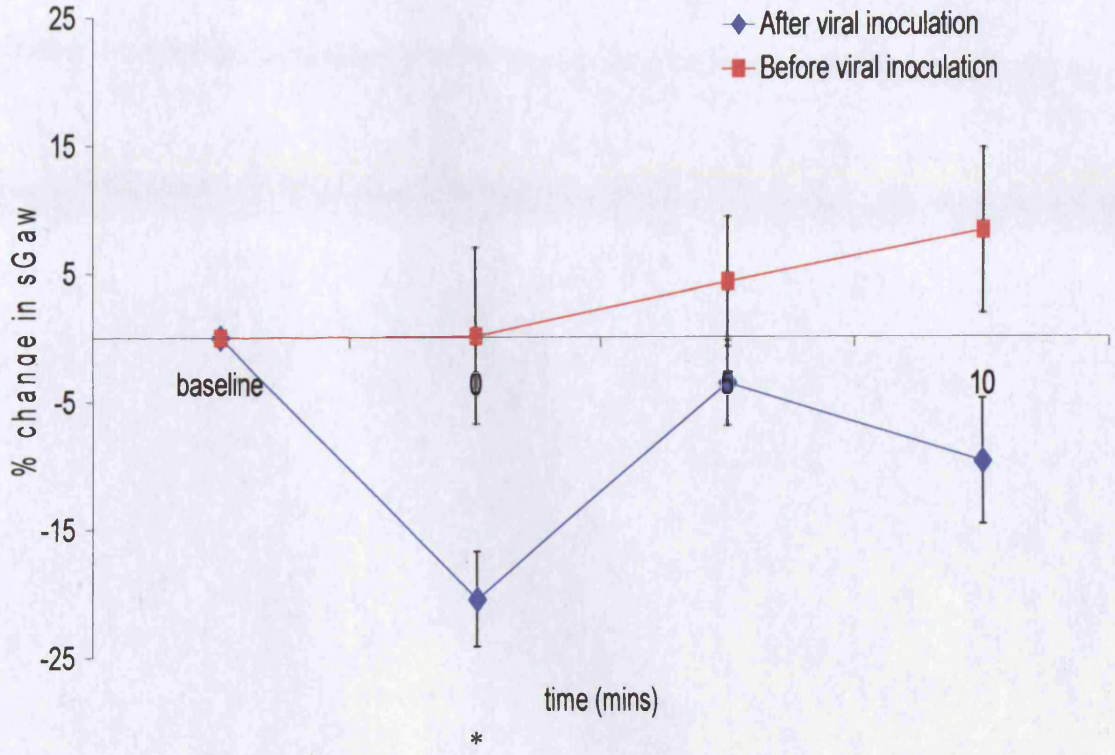


Figure 3.7

Airway response to histamine before and after influenza H1N1 A/PR/8/34 (1.1×10^6, grown in chicken embryos) virus infection in 25% DMSO treated guinea-pigs. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. . * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after virus inoculation as determined by a Student's *t* test (paired).

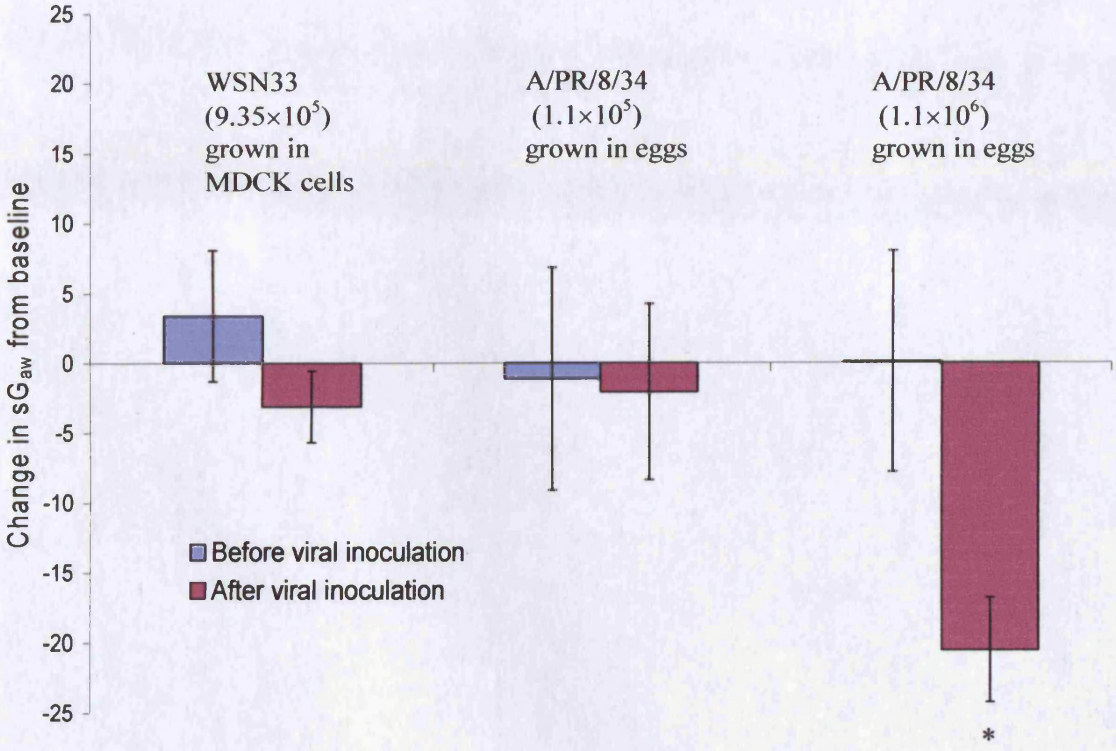


Figure 3.8

Maximum decrease in sG_{aw} from baseline in GPs after 1mM histamine exposure before and after inoculation with H1N1:WSN33 (9.35×10^5) grown in MDCK cells, A/PR/8/34 (1.1×10^5) and (1.1×10^6) grown in chicken embryos and all were treated with 25% DMSO. Each bar represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference in the maximum decrease in sG_{aw} from baseline before and after viral inoculation, as determined by a Student's *t* test (paired).

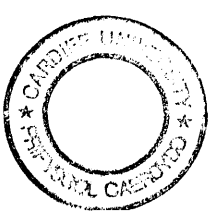
Bronchoalveolar lavage cell counts

In animals inoculated with media, the numbers of macrophages, eosinophils, lymphocytes and neutrophils in the BALF were as follows; total cells: 1.96 ± 0.11 ($\times 10^6$/ml), macrophages: 1.47 ± 0.08 ($\times 10^6$/ml), eosinophils: 0.28 ± 0.04 ($\times 10^6$/ml) lymphocytes: 0.07 ± 0.01 ($\times 10^6$/ml) and neutrophils 0.14 ± 0.02 ($\times 10^6$/ml) (Fig. 3.9).

In the animals inoculated with H1N1 WSN33 (9.35×10^5) grown in MDCK cells there was a significant increase in macrophages and smaller non-significant rises in eosinophils, lymphocytes and neutrophils (Fig 3.9).

In animals inoculated with allantoic fluid from chicken embryo, the numbers of macrophages, eosinophils, lymphocytes and neutrophils in the BALF were as follows; total cells: 2.39 ± 0.1 ($\times 10^6$/ml), macrophages: 1.95 ± 0.11 ($\times 10^6$/ml), eosinophils: 0.28 ± 0.08 ($\times 10^6$/ml) lymphocytes: 0.08 ± 0.02 ($\times 10^6$/ml) and neutrophils 0.08 ± 0.02 ($\times 10^6$/ml). Compared to the media control, the macrophages were significantly increased, but there were no increases in eosinophils, lymphocytes or neutrophils (Fig. 3.9).

In the animals inoculated with H1N1 A/PR/8/34 1.1×10^5 grown in a chicken embryo there were significant increases in macrophages, eosinophils and lymphocytes and a smaller but insignificant increase in neutrophils. In the animals inoculated with the higher concentration (1.1×10^6) of the same virus compared to the allantoic fluid no virus control,



there was a significant increase in macrophages and a smaller increase in eosinophils. There was no increase in lymphocytes or neutrophils (Fig. 3.9).

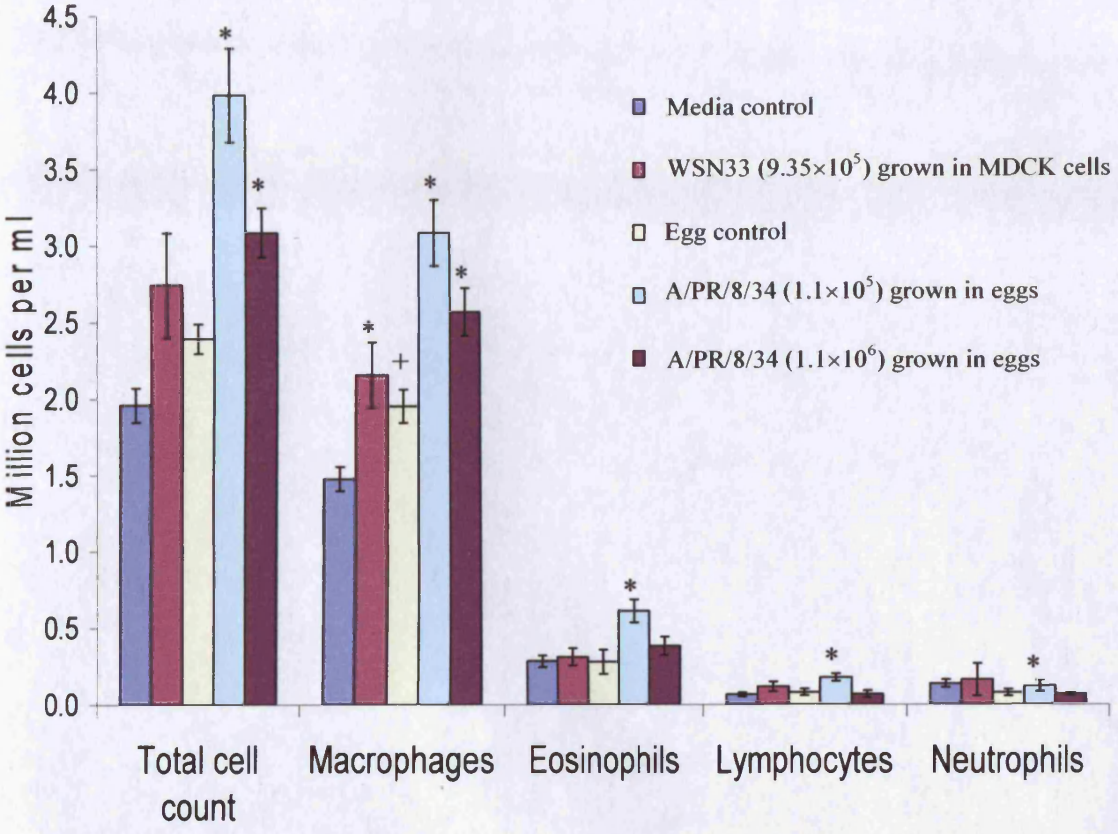


Figure 3.9

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from virus free medium, H1N1 WSN33 ($9.35*10^5$) grown in MDCK cells, allantoic fluid from chicken embryo, and H1N1 A/PR/8/34 grown in chicken embryo (1.1×10^5 and 1.1×10^6) all treated with 25% DMSO. The final group were inoculated with H1N1 A/PR/8/34 grown in chicken embryo (1.1×10^6) and treated with dexamethasone (10 mg/kg in 25% DMSO. Each bar represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding no virus control and + denotes the significance ($P<0.05$) of difference compared to the egg control group as determined by a Student's *t* test (paired).

Percentage wet lung weight

In animals receiving medium only with no virus, the percentage wet weight of the lung tissue was 81.0 ± 0.3 (Fig. 3.10).

In the animals inoculated with H1N1 WSN33 (9.35×10^5) grown in MDCK cells there was no increase in wet weight compared to the no virus control.

In animals inoculated with allantoic fluid from chicken embryo there was no significant increase in wet weight compared to the no virus control; in fact there was a reduction.

In the animals inoculated with H1N1 A/PR/8/34 1.1×10^5 grown in a chicken embryo there was a modest increase in wet weight compared to the allantoic fluid control.
In the animals inoculated with the higher concentration (1.1×10^6) of the same virus there was a further increase in wet weight but not a significant increase compared to the allantoic fluid control (Fig. 3.10).

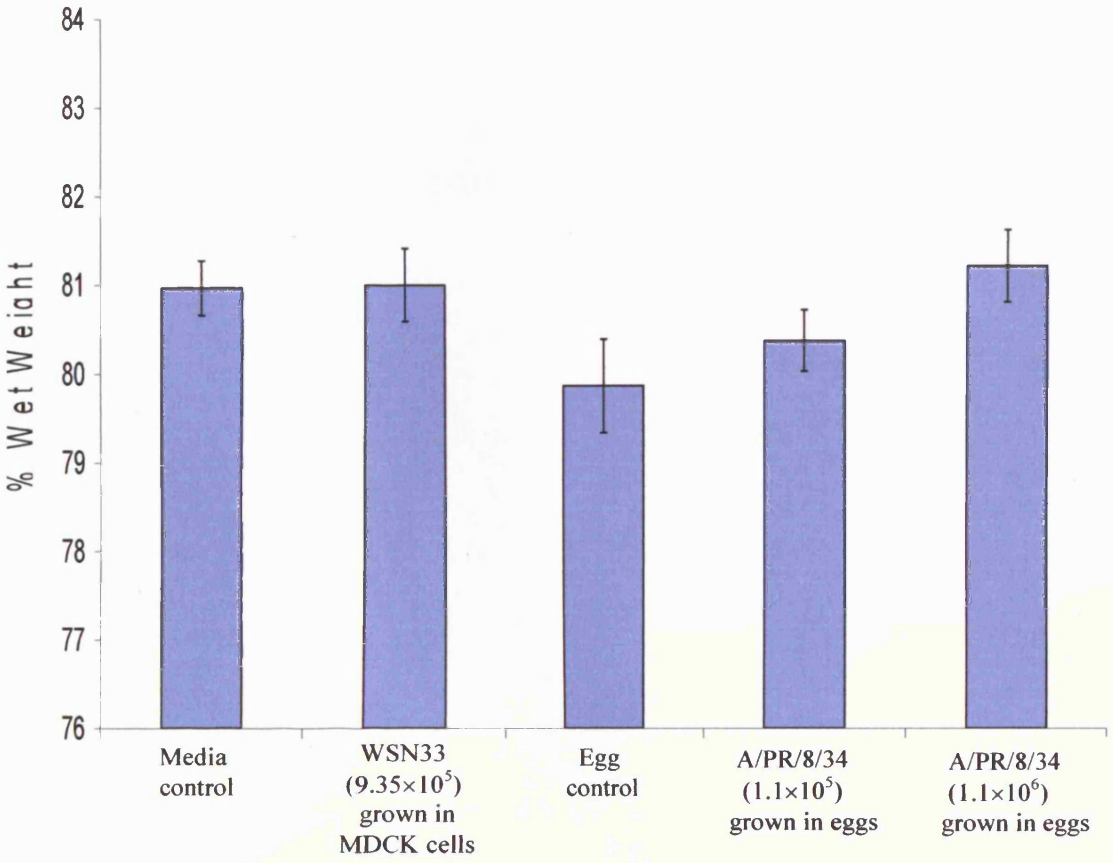


Figure 3.10

Effect on percentage wet lung weight of inoculation with: virus free medium, H1N1 WSN33 (9.35×10^5) grown in MDCK cells, allantoic fluid from chicken embryo, and H1N1 A/PR/8/34 (1.1×10^5 and 1.1×10^6), all treated with 25% DMSO. Each bar represents the mean ± standard error of mean (n=6). There was no significant ($P<0.05$) difference between the viral inoculated groups and their corresponding no virus controls as determined by a Student's *t* test (paired).

3.4 Discussion

Effects of PIV inoculation on airway hyperreactivity

PIV infection resulted in bronchoconstriction to a dose of inhaled histamine that prior to inoculation produced no response. This indicates airway hyperreactivity 4 days after the viral infection. Inoculation with a higher concentration of PIV-3 resulted in a greater bronchoconstriction than with the lower concentration. Inoculation with a high concentration of PIV-2 produced a bronchoconstriction similar to that seen with the lower concentration of PIV-3 but much lower than the higher concentration of PIV-3. The respiratory epithelium is thought to be the major site of binding and replication of PIV (Garofalo and Haebere 2000). The subsequent damage leads to epithelial dysfunction and can cause AHR by a variety of mechanisms including increased exposure of airway smooth muscle to histamine mediated bronchoconstriction via H_1 receptors.

The respiratory epithelium is a source of NO which increases cGMP levels and induces bronchodilatation. Damage to the epithelium would reduce the levels of NO and could cause AHR in much the same way as NO synthase inhibitors cause AHR in guinea-pigs (Nijkamp *et al* 1993).

Damage to the epithelium can also expose sensory C-fibres, activation of which can cause release of tachykinins including NKA and SP which act predominantly on NK_1 and NK_2 receptors respectively (Nakanishi 1991). Activation of NK_2 receptors mediates contraction of human airways smooth muscle *in vitro* (Frossard and Barnes 1991). The most important effect of tachykinins is possibly the effect on airways blood vessels. SP is

the tachykinin most potent in causing microvascular leakage in guinea-pigs indicating a NK_1 mediated effect (Lotvall *et al* 1990).

Tachykinins can also cause the release of acetylcholine (ACh) which causes bronchoconstriction by activation of M_3 muscarinic receptors. PIV-1 has been shown to alter the function of the inhibitory M_2 muscarinic receptor in guinea pigs. Release of ACh from parasympathetic nerves causes contraction of post-junctional M_3 muscarinic receptors. Ach also binds to pre-junctional M_2 muscarinic receptors, which reduces ACh release. Damage to this negative feedback system could lead to increased release of ACh (Fryer *et al* 1990). Thus, viral infection by damaging the airways epithelium could cause increased reactivity to inhaled histamine through release of tachykinins and reduced levels of NO.

Effects of PIV inoculation on inflammatory cell influx

PIV-3 infection produced an increase in inflammatory cell influx. The increases seen after PIV-3 inoculation were greater than those seen after PIV-2 inoculation. There was a small increase after increasing the concentration of PIV-3 but the change was not as great as the effect on the airway hyperreactivity. The pulmonary epithelium is the first site where interaction with the virus occurs. The epithelium synthesizes and releases a wide range of pro-inflammatory mediators in response to PIV-3 including interleukin (IL)-6, IL-8 , IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF) and regulated-upon-activation normal-T cell-expressed and secreted (RANTES) (Barnes *et al* 1998). The release of these mediators from the epithelium would lead to a complex series

of events including, activation of resident leukocytes, further release of cytokines and chemokines and the migration to the lungs of additional leukocytes as seen in this study.

Effect of PIV-3 inoculation on pulmonary oedema

PIV infection appears to cause pulmonary oedema as there was an increase in the percentage wet weight of the lung tissue. There was an increase in wet weight after increasing the concentration of PIV-3. PIV-2 inoculation produced a similar increase to that seen with the lower dose of PIV-3. The increase in wet weight may be due to an increase in mucus production but the most likely cause is pulmonary oedema (Hwang *et al* 2001). Pulmonary oedema can be caused by increased blood pressure in the pulmonary capillaries or increased permeability of the capillary wall. PIV-3 infection can lead to a release in pro-inflammatory mediators which increase blood pressure in pulmonary capilliaries (thromboxane, 5-lipoxygenase and leukotrienes C4, D4 and E4) and increase permeability of the capillary wall (tachykinins, reactive oxygen species (ROS), mast cell-derived histamine, excess NO and ROS-activated proteolytic enzymes) (Barnes *et al* 1999).

Viral recovery and identification of PIV-3

Recovery of live virus 4 days after inoculation confirms infection. As the respiratory epithelium is the main site of adhesion and replication of PIV-3 this would be the most likely location of the virus in the lung tissue. The most likely binding site is the ICAM receptors on the respiratory epithelium, ICAM-1 is the receptor for most rhinoviruses (Suzuki *et al* 2000). Viruses can also activate toll-like receptors (TLR) which leads to

secretion of type 1 interferons (Samuel 2001). The inflammation seen in mice after exposure to inactivated H5N1 influenza virus was reduced in TLR-4 mutant mice (Imai *et al* 2008). The virus recovered was identified as PIV-3 by PCR.

Effect of influenza on airway hyperreactivity

There was no evidence of airway hyperreactivity after inoculation with H1N1 WSN33 (9.35×10^5) grown in MDCK cells as there was no bronchoconstriction after histamine exposure. There was no increase in wet lung weight. There was no evidence of airway hyperreactivity after inoculation with H1N1 A/PR/8/34 (1.1×10^5) grown in a chicken embryo as there was no bronchoconstriction after histamine exposure. The increased concentration of this virus (1.1×10^6) did produce a bronchoconstriction indicating airway hyper-reactivity. This would appear to show that this virus can cause airway reactivity in guinea-pigs dependant on the concentration of virus in the inoculum. This concentration-dependant effect has also been seen in other studies. Lowen *et al* (2006) saw an increase in infection of guinea-pigs by increasing the concentration of influenza A/Panama/2007/99 (Pan/99) (H3N2) in the inoculum and an increase in the concentration of the 1918 influenza strain increased the severity of the symptoms in mice.

Effect of influenza on inflammatory cell influx

There was only a small rise in inflammatory cells after inoculation with H1N1 WSN33 (9.35×10^5) grown in MDCK cells. After inoculation with H1N1 A/PR/8/34 (1.1×10^5)

grown in chicken embryos there was a large rise in inflammatory cells compared to the no virus control. There was also a rise in inflammatory cells after inoculation with the increased concentration, compared to control, though not to the same level as the lower concentration of the virus. This is contrary to expectations and the reason for this is unknown. It is notable that the lower concentration of H1N1 A/PR/8/34 produced an increase in inflammatory cells but there was no AHR. It is possible that different mediators are involved in these two effects or that the respiratory epithelium was still largely intact and therefore the sensory C-fibers would not be exposed to activation causing bronchoconstriction.

Effect of influenza on pulmonary oedema

There was no increase in wet lung weight after inoculation with H1N1 WSN33 (9.35×10^5) grown in MDCK cells. The wet lung weight increased after inoculation with H1N1 A/PR/8/34 (1.1×10^5) grown in a chicken embryo compared to control. In the group inoculated with the higher concentration of the virus there was an additional rise in wet lung weight but not significantly compared to control.

Inoculation with H1N1 A/PR/8/34 grown in a chicken embryo induces AHR, inflammatory cell influx and a small rise in wet lung weight in the guinea- pig. Inoculation with H1N1 WSN33 grown in MDCK cells only induces a small rise in inflammatory cells. Of the influenza viruses tested H1N1 A/PR/8/34 grown in a chicken embryo appears to be more pathogenic in guinea-pigs. The virulence of influenza is

influenced by the heamagglutinin (HA) molecule, which is involved in entry of the virus into the host cell (White *et al* 1982). In cell culture altered posttranslational modification of the HA molecule can lead to a lack of pathogenicity compared to *in vivo* growth (Li *et al* 1990). This may account for the differences seen in this study although it must be noted that we were comparing two different influenza virus strains.

Chapter 4

Drug interventions

4.1 Introduction

The role of Bradykinin in airway inflammation

Bradykinin is thought to be an important mediator of asthma. Inhalation of bradykinin causes bronchoconstriction in asthmatic patients, but has little or no effect in non-asthmatics (Fuller *et al* 1987). Kinin levels are increased in BALF of asthmatics after allergen challenge (Christiansen *et al* 1992). Most of the bradykinin receptor-mediated effects in the airways are thought to be due to activation of the bradykinin B_2 receptor as selective B_1 agonists have little effect (Fuller *et al* 1987). The selective bradykinin B_2 receptor antagonist icatibant (also known as HOE140) blocked PIV-3 induced AHR but not the inflammatory cell influx (Folkerts *et al* 2000). The non peptide, selective bradykinin B_2 receptor antagonist MEN16132 prevented the bronchoconstriction and plasma extravasation produced by dextran sulfate-induced contact activation of the kinin–kallikrein cascade, whereas icatibant had a 3-30 less potent inhibitory effect (Valenti *et al* 2007).

Despite the lack of effect of bradykinin B_2 antagonist icatibant on inflammatory cell influx seen by Folkerts *et al* (2000), bradykinin is thought to play a role in the recruitment of inflammatory cells to the airways. Selective bradykinin B_2 antagonists have been shown to inhibit the antigen-induced eosinophilia and neutrophilia as well as the airway hyperresponsiveness in rats (Bandeira-Melo *et al* 1999) and guinea-pigs (Farmer *et al* 1992).

Bradykinin is a nonapeptide formed by the cleavage of its precursor, high molecular weight kinninogen (HMWK) by the kinninogenase enzymes, which include tissue and plasma kallikrein (Bhoola *et al* 1992). In this study we used the tissue kallikrein inhibitor FE999024 (Evans *et al* 1996) in an attempt to reduce bradykinin levels in the lung and therefore inhibit the bradykinin-mediated effects of the PIV induced airway inflammation in guinea-pigs.

Dexamethasone is a corticosteroid which binds to cytosolic glucocorticoid receptors, this dimer then migrates to the nucleus, binds to target DNA and reduces the transcription and expression of a wide range of pro-inflammatory mediators (Barnes and Adcock 1993). Dexamethasone has been shown to inhibit AHR and leukocyte influx in rats after sendai virus (PIV-1) inoculation (Mehta *et al* 1997) and in guinea-pigs after PIV-3 inoculation (Toward *et al* 2005). In this study we tried to recreate the inhibitory effect of dexamethasone on PIV-3 induced AHR and inflammatory cell influx. We also looked at the effect of dexamethasone on increased lung wet weight.

Various studies have shown contradictory results on the ability of corticosteroids to inhibit the replication of viruses. Domachowske *et al* (2001) increased titres of pneumonia virus in mice with hydrocortisone but Moreno *et al* (2003) have shown that dexamethasone reduced the titres of virus recovered from the lung after sendai virus (PIV-1) infection in guinea-pigs.

4.2 Methods

The methods in this section are the same as in chapter 3.2 with the exception of the treatment with FE999024 and dexamethasone. The guinea-pigs received bilateral sub-cutaneous injections every 12 hours of either

- 0.9% sterile saline (vehicle for FE999024)
- FE999024 (1, 3 or 10mg/kg) in 0.9% sterile saline
- 25 or 50% DMSO in 0.9% sterile saline (vehicle for dexamethasone)
- Dexamethasone (10mg/kg) in 25 or 50% DMSO in 0.9% sterile saline

The volume used was 1ml\kg

4.3 Results

4.3.1 Effects of FE999024 and dexamethasone on inoculation of guinea-pigs with a viral concentration of 6.32 x 10^6 infectious units per ml of PIV-3

Airway reactivity to histamine

Prior to inoculation with virus there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Figs. 4.1 and 4.2)

In the saline control there was evidence of airway hyperreactivity after viral inoculation with significant reductions in sG_{aw} at 5 (-10.0 ± 2.7%) and 10 (-13.5 ± 3.5%) minutes after histamine exposure compared to the baseline values (Figs. 4.1 and 4.2).

After treatment with FE999024 (1mg/kg) there was evidence of airway hyperreactivity after viral inoculation with a -19.5 ± 4.2% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to the baseline sG_{aw} values (Fig. 4.2). There was a gradual recovery to –12.4 ± 8.8% after 10 minutes

After treatment with FE999024 (3mg/kg) there was still some evidence of airway hyperreactivity after viral inoculation as there was a -4.8 ± 4.6% reduction in sG_{aw} value at 0 minutes, which peaks at -7.5 ± 3.2% at 5 minutes after histamine exposure compared to the baseline sG_{aw} values. sG_{aw} values recovered after 10 minutes (Fig. 4.2).

After treatment with FE999024 (10mg/kg) there was no evidence of airway hyperreactivity after viral inoculation as there was no decrease in sG_{aw} values compared to baseline (Fig. 4.2).

After treatment with 50% DMSO (dexamethasone vehicle) there was no evidence of airway hyperreactivity after viral inoculation as the -8.2 ± 6.6% reduction in sG_{aw} value at 0 minutes after histamine exposure was not significantly different compared to baseline. There was complete recovery after 5 minutes (Fig. 4.2).

After treatment with dexamethasone (10mg/kg) in 50% DMSO there was no evidence of airway hyperreactivity after viral inoculation as there is no reduction in sG_{aw} values after histamine exposure compare to baseline. In fact there was an increase in sG_{aw} reaching a peak of 19.2 ± 10.4%, indicating a bronchodilator response. Before viral inoculation there was a small decrease in sG_{aw} to -5.6 ± 8.8% at 5 minutes after histamine exposure, which returned to baseline value after 5 minutes (Fig. 4.2).

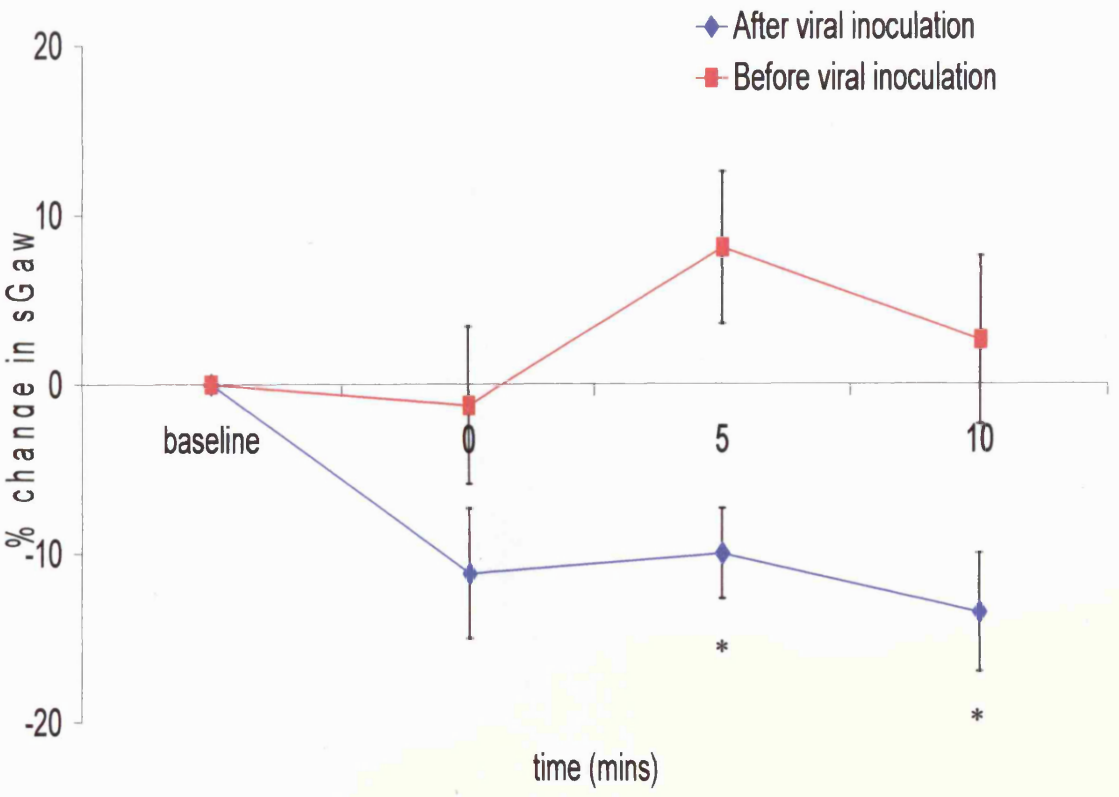


Figure 4.1

Effect of saline treatment on airway function before and after PIV-3 (6.32×10^6) infection. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. ($P<0.05$) difference between the changes to baseline sG_{aw} values at each time point, before and after virus inoculation as determined by a Student's *t* test (paired).

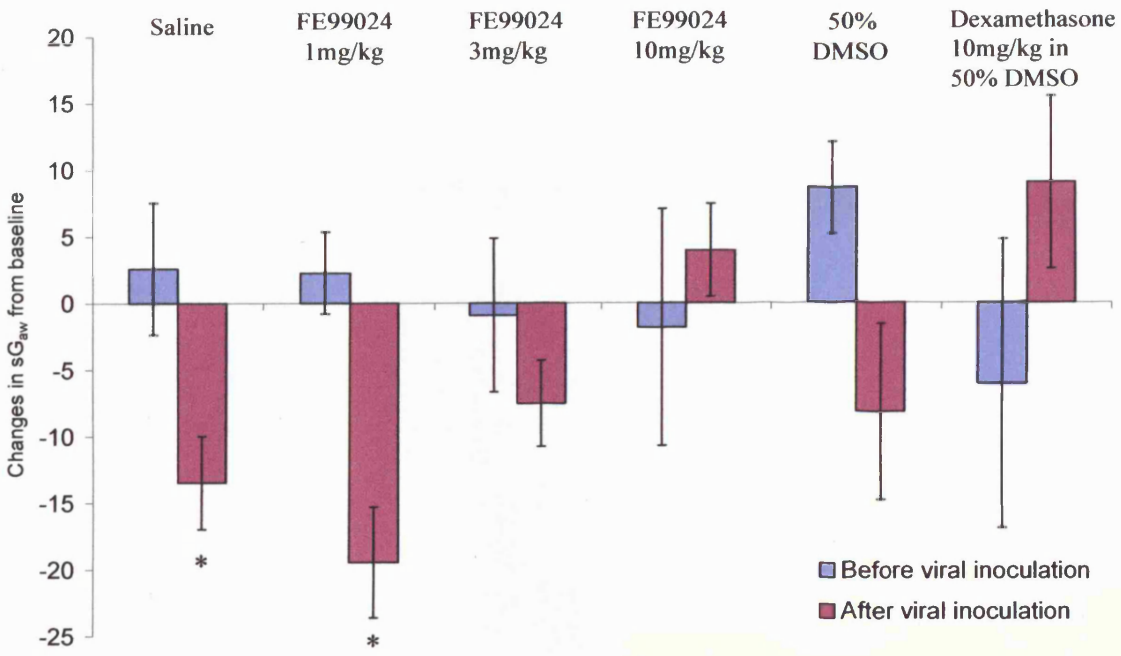


Figure 4.2

Maximum change in sG_{aw} from baseline in GPs after 1mM histamine exposure after inoculation with 6.32 x 10^6 infectious units per ml of PIV-3 compared with the corresponding time after histamine obtained before inoculation. Guinea-pigs were treated with either saline, FE999024 (1, 3, or 10 mg/kg), 50% DMSO or dexamethasone (10mg/kg) in 50% DMSO. Each point represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference in the maximum decrease in sG_{aw} from baseline before and after viral inoculation, as determined by a Student's *t* test (paired).

Bronchoalveolar lavage cell counts

In animals receiving no virus (medium controls), the numbers of macrophages, lymphocytes, eosinophils and neutrophils in the BALF were as follows; total cells: 1.54 ± 0.17 ($\times 10^6$/ml), macrophages: 1.37 ± 0.15 ($\times 10^6$/ml), eosinophils: 0.12 ± 0.01 ($\times 10^6$/ml), lymphocytes: 0.06 ± 0.02 ($\times 10^6$/ml) and neutrophils 0.03 ± 0.01 ($\times 10^6$/ml) (Fig. 4.3).

In PIV-3 virus inoculated guinea-pigs treated with saline vehicle, there were significant increases in total cells, macrophages eosinophils and lymphocytes. There was also an increase in neutrophils (Fig. 4.3).

The increases in macrophages, eosinophils and lymphocytes were inhibited in a dose-dependant manner by FE999024. The increase in neutrophils was inhibited by the maximum dose of FE999024 (Fig. 4.3).

In 50% DMSO treated guinea-pigs inoculated with PIV-3, there were modest increases in total cells, macrophages and eosinophils compared with no virus controls but significantly less than the virus and saline group in the case of total cells and macrophages.

The increases in cell numbers were restored to baseline values by dexamethasone treatment in the case of total cells, macrophages and lymphocytes. Total cell numbers and macrophages were significantly less than the 50% DMSO controls (Fig. 4.3).

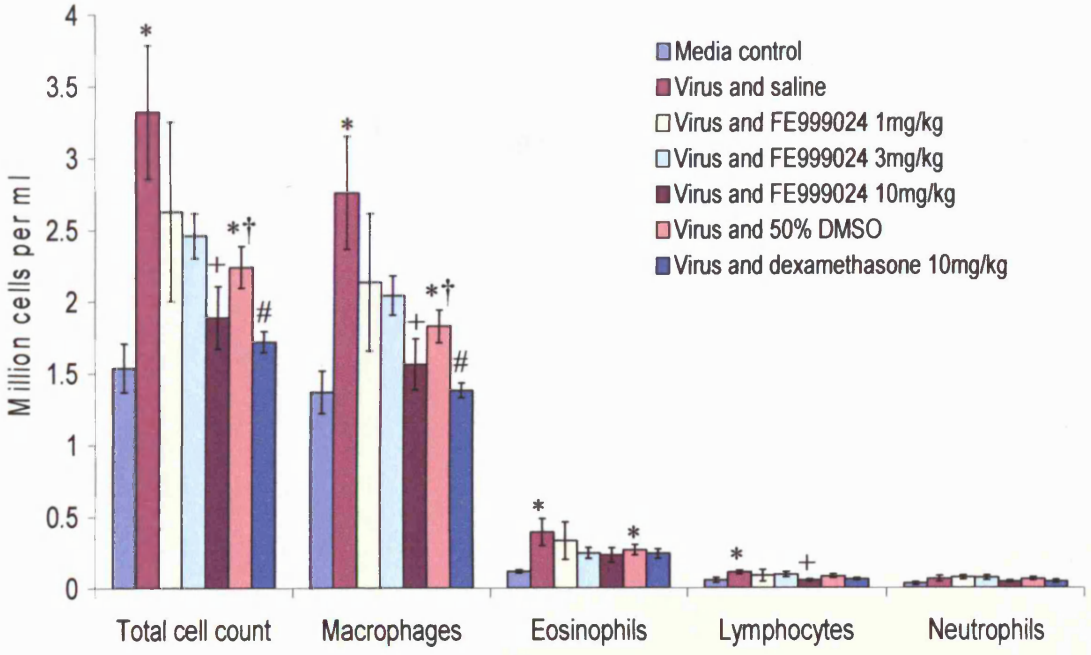


Figure 4.3

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from PIV-3 or virus free medium inoculated GPs treated with saline (PIV-3 and virus free medium inoculated), FE999024 (1, 3, and 10 mg/kg) 50% DMSO and dexamethasone (10mg/kg) in 50% DMSO. Each point represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding no virus control, + denotes the significance ($P<0.05$) of difference as compared to the corresponding saline control, † denotes the significance ($P<0.05$) of difference in the 50% DMSO group as compared to the corresponding saline group and # denotes the significance ($P<0.05$) of difference in the dexamethasone group as compared to the corresponding 50% DMSO control as determined by a Student's *t* test (paired).

Percentage wet lung weight

In animals receiving no virus, the percentage wet weight of the lung tissue was 79.0 ± 0.4 (Fig. 4.4).

In the PIV-3 inoculated guinea-pigs treated with saline the percentage wet weight had increased significantly to 81.4 ± 0.5. In the FE999024 1mg/kg and 3mg/kg treated groups there was no significant decrease in the percentage wet weight, in fact there is a small increase in the FE999024 1mg/kg group. The increase seen in the saline group was significantly reduced to 80.0 ± 0.4% by the 10mg/kg dose of FE999024 (Fig.4.4).

In the PIV-3 inoculated guinea-pigs treated with 50% DMSO the percentage wet weight had increased significantly to 81.6 ± 0.4 compared to the media control. In the dexamethasone (10mg/kg) treated group there was no significant decrease (Fig. 4.4).

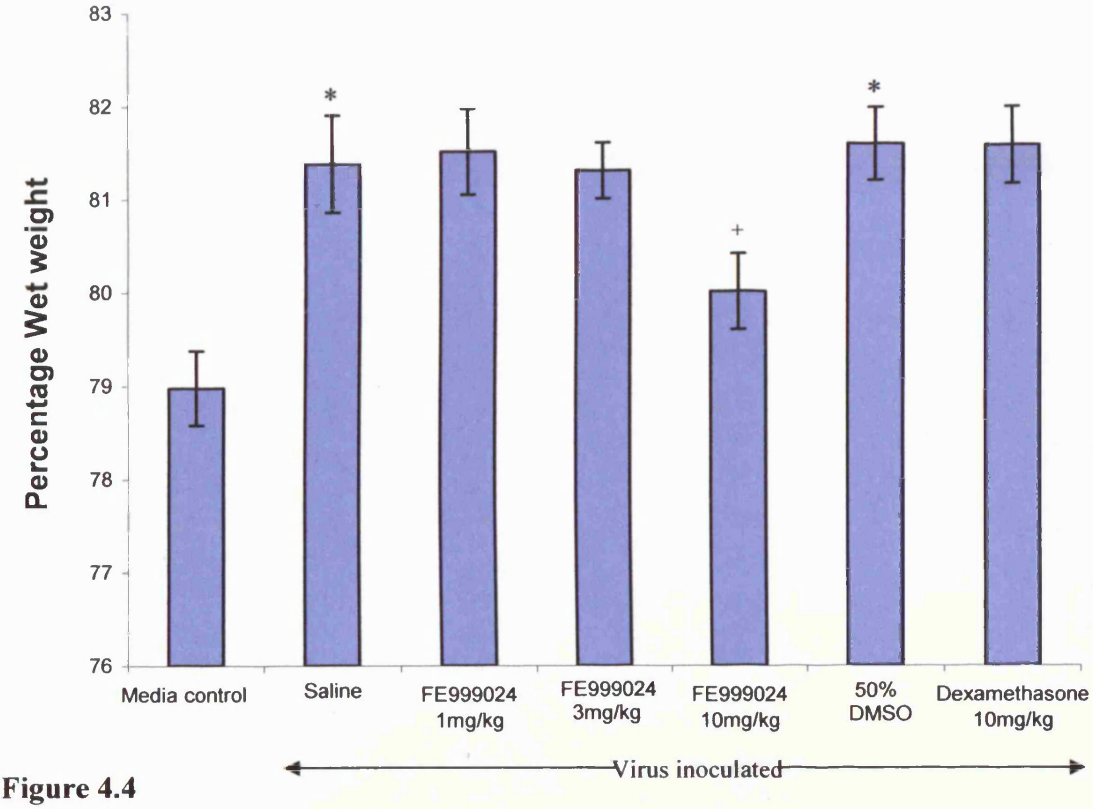


Figure 4.4

Effect of PIV-3 infection on percentage wet weight after treatment with saline, FE999024, 50% DMSO (dexamethasone vehicle) and dexamethasone. Each point represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference compared to the media control, + denotes the significance ($P<0.05$) in difference compared to the saline group as determined by a Student's *t* test (paired).

4.3.2: Effects of increasing PIV-3 viral concentration to 3 x 10^8 infectious units per ml

Airway reactivity to histamine

Prior to inoculation with virus there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Figs. 4.5. and 4.6)

In the saline control there was evidence of airway hyperreactivity after viral inoculation as there was a -31.5 ± 6.3% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to the baseline value. There was a recovery after 5 and 10 minutes but not to baseline (Figs. 4.5 and 4.6).

After treatment with FE999024 (10mg/kg) there was no evidence of airway hyperreactivity after viral inoculation as there was only a -2.4 ± 6.2% decrease in sG_{aw} values compared to baseline (Fig. 4.6).

Because of the effects of 50% DMSO on cells seen previously, further experiments were conducted with 25% DMSO.

After treatment with 25% DMSO there was evidence of airway hyperreactivity after viral inoculation as there was a -22.5 ± 6.1% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to baseline. There was complete recovery after 5 minutes (Fig.4.6).

After treatment with dexamethasone (10mg/kg) there was no evidence of airway hyperreactivity after viral inoculation as there was only a $1.2 \pm 6.9\%$ reduction in sG_{aw} values after histamine exposure compared to baseline (Fig.4.6).

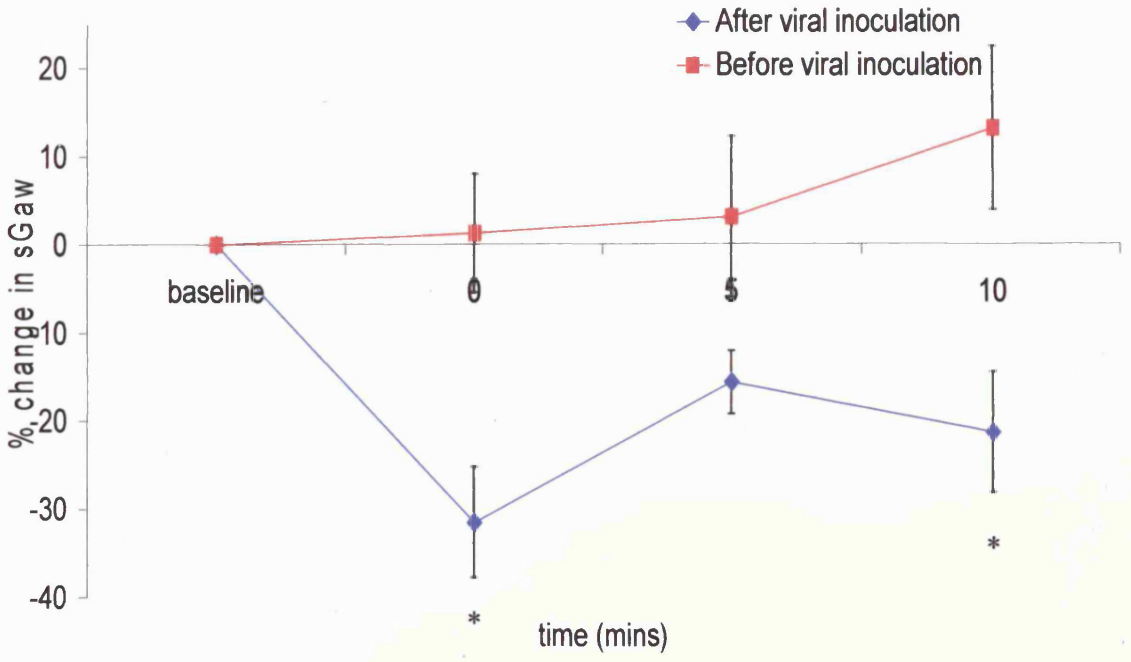


Figure 4.5

Effect of saline treatment on airway function before and after PIV-3 virus infection. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after virus inoculation as determined by a Student's *t* test (paired).

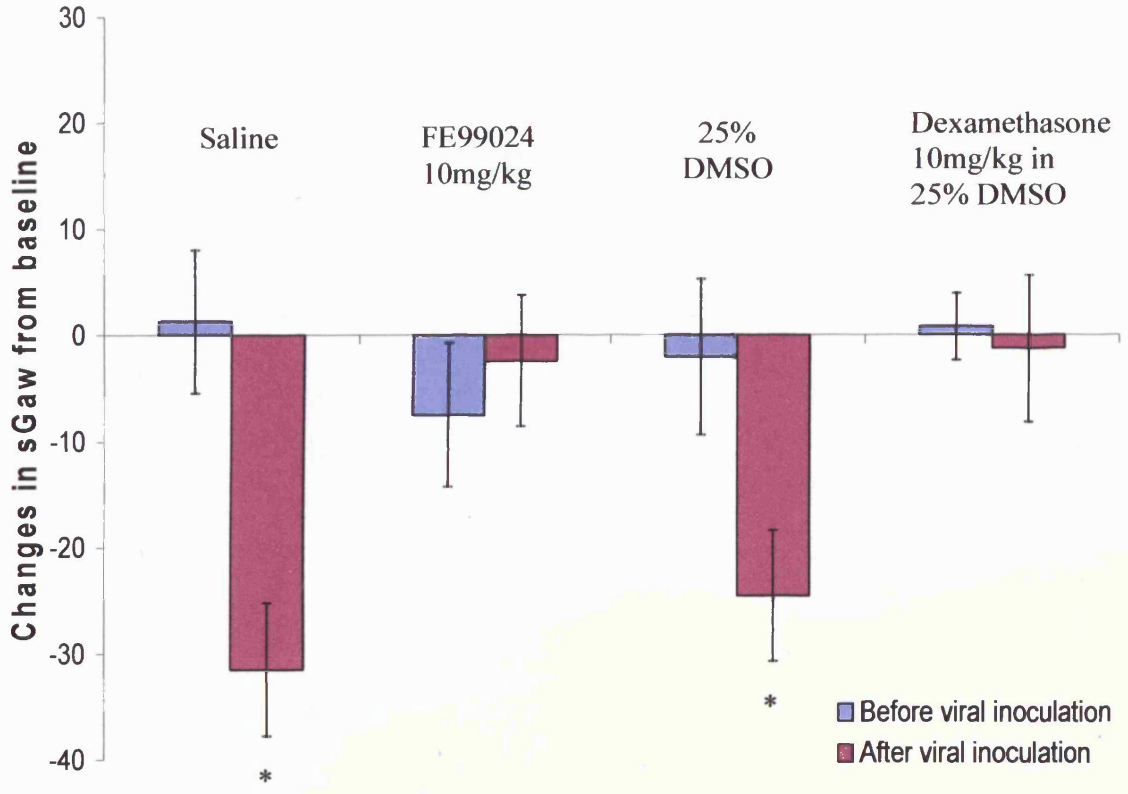


Figure 4.6

Maximum change in sG_{aw} from baseline in guinea-pigs after 1mM histamine exposure after inoculation with 3 x 10^8 infectious units per ml of PIV-3 compared with the corresponding time after histamine obtained before inoculation. Guinea-pigs were treated with either saline, FE999024 (10 mg/kg), 25% DMSO or dexamethasone (10mg/kg) in 25% DMSO. Each point represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference in the maximum decrease in sG_{aw} from baseline before and after viral inoculation, as determined by a Student's *t* test (paired).

Bronchoalveolar lavage cell counts

In animals receiving no virus, the numbers of macrophages, lymphocytes, eosinophils and neutrophils in the BALF were as follows; total cells: 1.54 ± 0.17 ($\times 10^6$/ml), macrophages: 1.37 ± 0.15 ($\times 10^6$/ml), eosinophils: 0.12 ± 0.01 ($\times 10^6$/ml), lymphocytes: 0.06 ± 0.02 ($\times 10^6$/ml) and neutrophils 0.03 ± 0.01 ($\times 10^6$/ml) (Fig 4.7).

In PIV-3 virus inoculated guinea-pigs treated with saline, there were significant increases in total cells, macrophages, eosinophils, lymphocytes and neutrophils. FE999024 (10mg/kg) had little effect on the cell counts compared to the saline control (Fig. 4.7).

In 25% DMSO treated guinea pigs inoculated with PIV-3, there were significant increases in total cells, macrophages, eosinophils, lymphocytes and neutrophils compared with the no virus control. They were not significantly different from the saline controls. The increases in cell numbers were significantly reduced, but not to baseline values by dexamethasone treatment in the case of total cells, macrophages and neutrophils (Fig. 4.7).

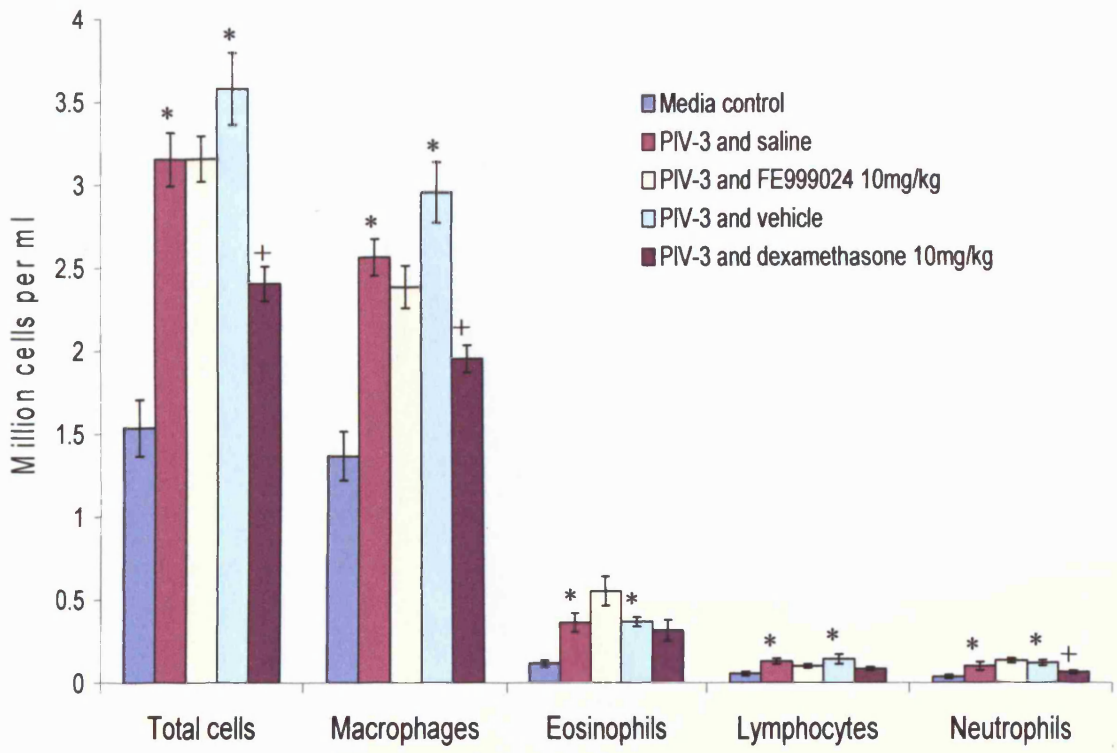


Figure 4.7

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from virus free medium inoculated animals or PIV-3 inoculated animals treated with saline, FE999024 10 (mg/kg), 25 % DMSO or dexamethasone (10mg/kg). Each point represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding no virus control. + denotes the significance ($P<0.05$) of difference compared to the corresponding DMSO group, as determined by a Student's *t* test (paired).

Percentage wet lung weight

In animals receiving no virus, the percentage wet weight of the lung tissue was 79.0 ± 0.4 (Fig. 4.8).

In the PIV-3 inoculated guinea-pigs treated with vehicle (saline) the percentage wet weight had increased significantly to 82.8 ± 0.8. In the FE999024 (10mg/kg) treated group this increase was significantly reduced to 80.1 ± 0.5% (Fig. 4.8).

In the PIV-3 inoculated guinea-pigs treated with 25% DMSO the percentage wet weight had increased significantly to 83.3 ± 0.3. In the dexamethasone (10mg/kg) treated group this increase was significantly reduced to 79.9 ± 0.5 (Fig. 4.8).

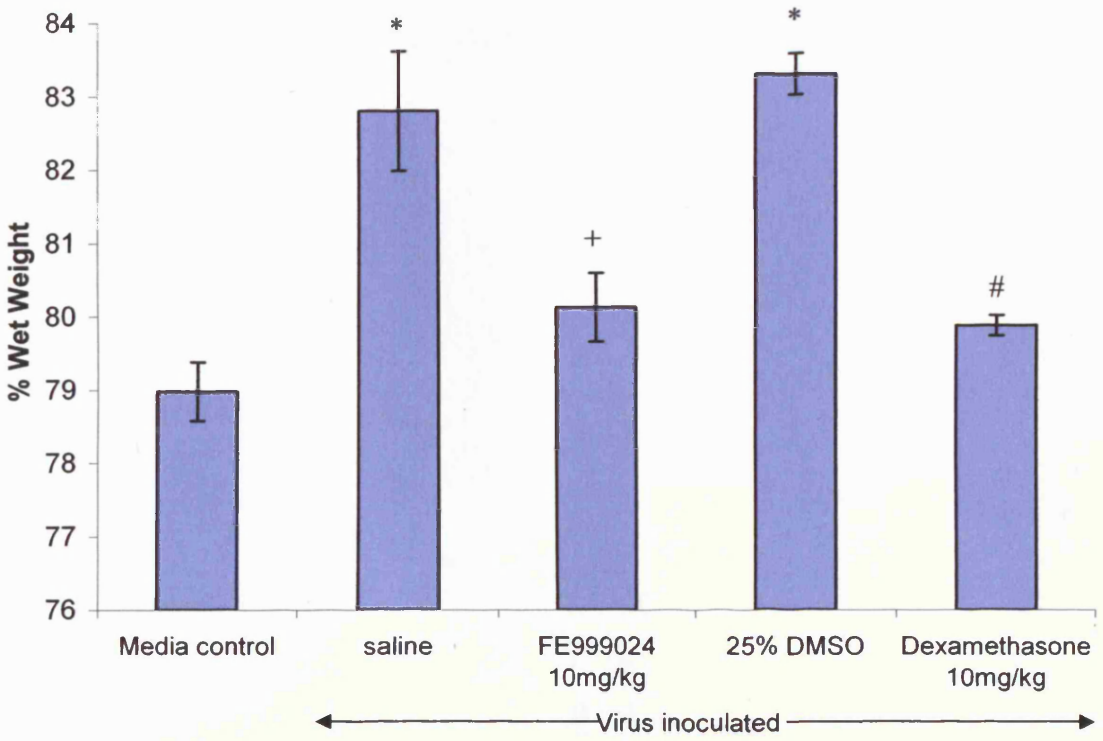


Figure 4.8

Effect on percentage wet lung weight of PIV-3 infection after treatment with saline, FE999024 (10mg/kg), 25% DMSO and dexamethasone (10mg/kg). Each bar represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference compared to the media control, + denotes the significance ($P<0.05$) in difference compared to the saline group and # denotes the significance ($P<0.05$) in difference compared to the DMSO group as determined by a Student's *t* test (paired).

4.3.3 Effects of FE999024 and dexamethasone on PIV-2 (1 x 10^8) inoculation in guinea-pigs

Airway reactivity to histamine

Prior to inoculation with virus there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Figs. 4.9 and 4.10).

In the saline control there was evidence of airway hyperreactivity after viral inoculation as there was a significant -16.9 ± 4.4% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to the baseline value. There was no recovery after 5 minutes and only a small recovery after 10 minutes (Figs. 4.9 and 4.10).

After treatment with FE999024 (1mg/kg), there was still evidence of airway hyperreactivity after viral inoculation as there was a -23.1 ± 7.6% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to the baseline sG_{aw} values. There was a recovery to –9.7 ± 4.6% after 10 minutes (Fig. 4.10)

After treatment with FE999024 (3mg/kg), there was no evidence of airway hyperreactivity after viral inoculation as there was almost no decrease in sG_{aw} values at 0 and 5 minutes although a non significant reduction of -13 ± 4.6% in sG_{aw} occurred 10 minutes after histamine exposure (Fig. 4.10).

After treatment with FE999024 (10mg/kg), there was no evidence of airway hyperreactivity after viral inoculation as there was no decrease in sG_{aw} values compared to baseline (Fig. 4.10).

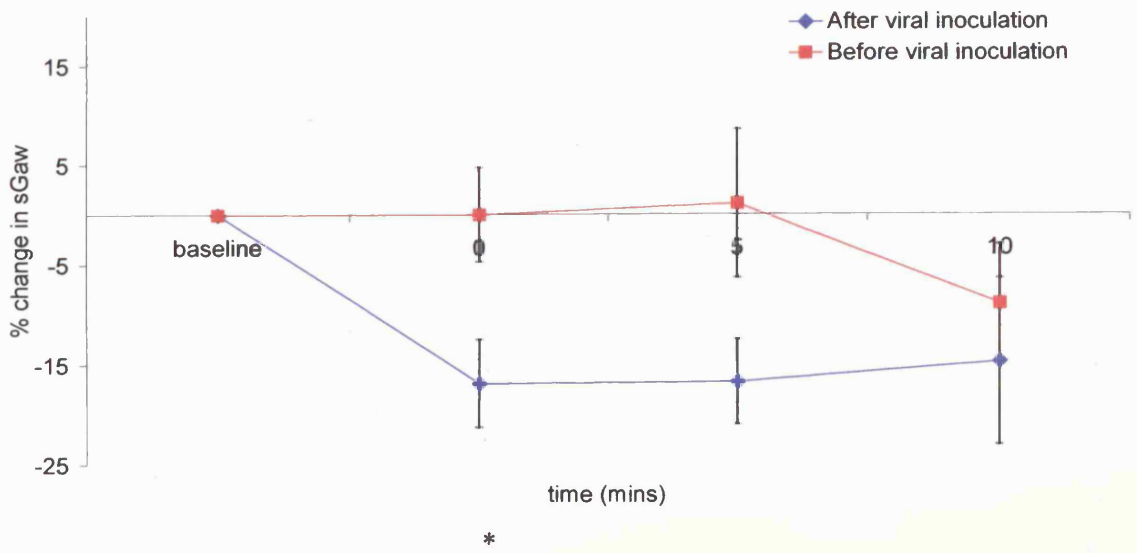


Figure 4.9

Effect of saline treatment on airway function before and after PIV-2 virus infection. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after virus inoculation as determined by a Student's *t* test (paired).

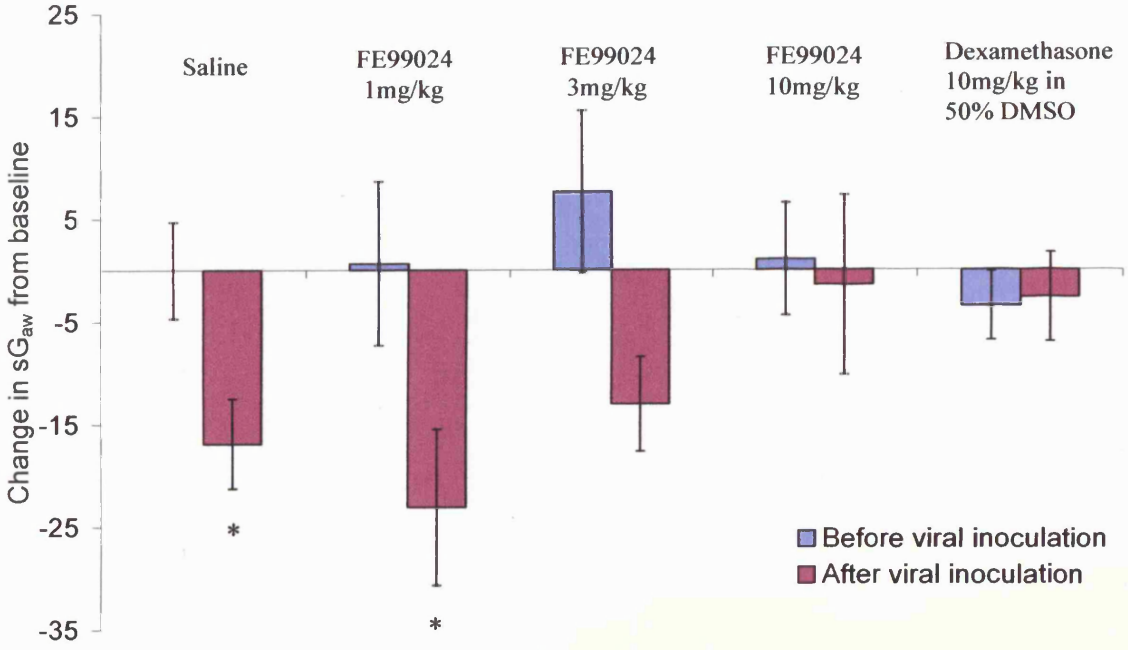


Figure 4.10

Maximum change in sG_{aw} from baseline in guinea-pigs after 1mM histamine exposure after inoculation with 1 x 10^8 infectious units per ml of PIV-2 compared with the corresponding time after histamine obtained before inoculation. Guinea pigs were treated with either saline, FE999024 (1, 3, or 10 mg/kg), or dexamethasone (10mg/kg) in 50% DMSO. Each bar represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference in the maximum decrease in sG_{aw} from baseline before and after viral inoculation, as determined by a Student's *t* test (paired).

Bronchoalveolar lavage

In animals receiving no virus, the numbers of macrophages, lymphocytes, eosinophils and neutrophils in the BALF were as follows; total cells: 1.54 ± 0.17 ($\times 10^6$/ml), macrophages: 1.37 ± 0.15 ($\times 10^6$/ml), eosinophils: 0.12 ± 0.01 ($\times 10^6$/ml), lymphocytes: 0.06 ± 0.02 ($\times 10^6$/ml) and neutrophils 0.03 ± 0.01 ($\times 10^6$/ml) (Fig. 4.11).

In PIV-2 virus inoculated guinea-pigs treated with saline, there were significant increases in total cells, macrophages, eosinophils and neutrophils. The increases in macrophages were inhibited in a dose-dependant manner by FE999024. Similar trends were observed for lymphocytes, eosinophils and neutrophils (Fig. 4.11).

In 50% DMSO treated guinea-pigs inoculated with PIV-2, there was a significant increase in eosinophils and modest increases in total cells and macrophages, lymphocytes and neutrophils compared with the no virus control. The increases seen were reduced as compared to the saline control though not significantly. The increases in cell numbers were restored to baseline values by dexamethasone treatment in the case of total cells, macrophages, eosinophils, lymphocytes and neutrophils (Fig. 4.11).

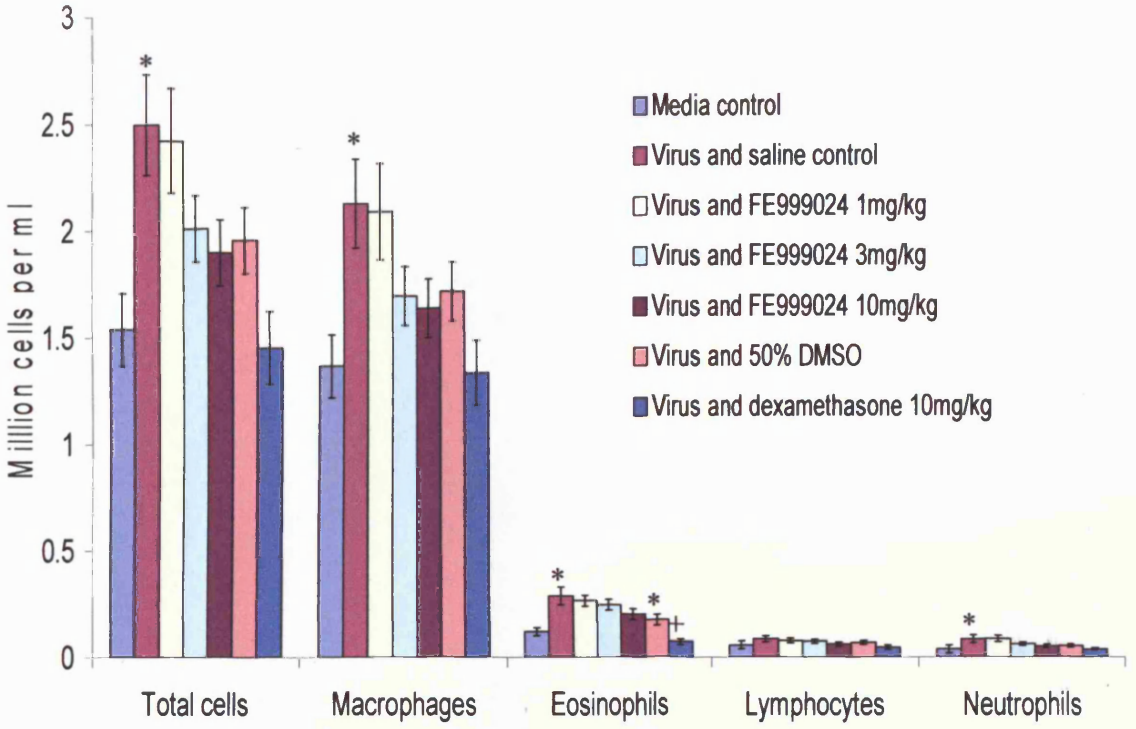


Figure 4.11

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from virus free medium inoculated animals or PIV-2 inoculated animals treated with saline, FE999024, 50 % DMSO or dexamethasone (10mg/kg). Each bar represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding no virus control. + denotes the significance ($P<0.05$) of difference compared to the corresponding DMSO group, as determined by a Student's *t* test (paired).

Percentage wet lung weight

In animals receiving no virus, the percentage wet weight of the lung tissue was 79.0 ± 0.4 (Fig. 4.12).

In the PIV-2 inoculated guinea-pigs treated with saline the percentage wet weight had increased significantly compared to the media control to 82.5 ± 0.8%. In the FE999024 treated groups there was a decrease in wet weight though not significantly and not dose dependently (Fig. 4.12).

In the PIV-2 inoculated guinea-pigs treated with 50% DMSO the percentage wet weight had increased significantly compared to the media control. In the dexamethasone treated group the percentage wet weight had significantly decreased (Fig. 4.12).

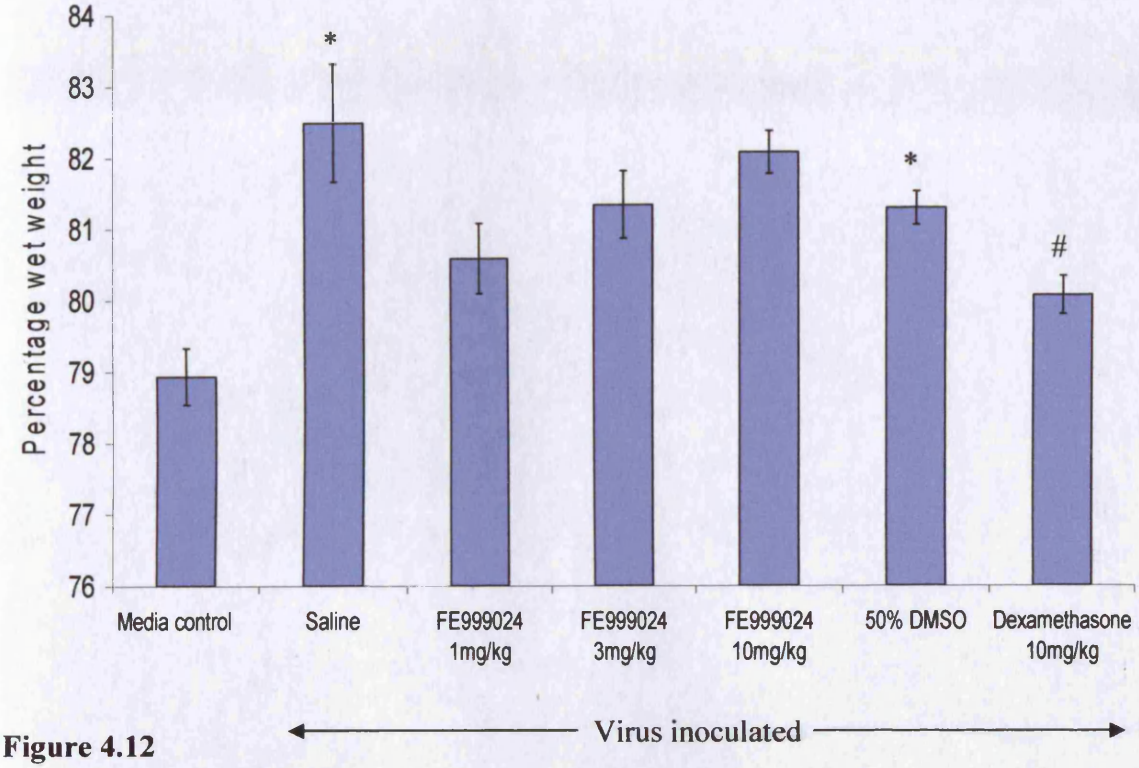


Figure 4.12

Effect on percentage wet lung weight of PIV-2 infection after treatment with saline, FE999024, 50% DMSO and dexamethasone (10mg/kg). Each bar represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference compared to the media control and # denotes the significance ($P<0.05$) in difference compared to the DMSO group as determined by Student's *t* test (paired).

4.3.4 Effect of dexamethasone on viral recovery

After 25% treatment and inoculation with 3 x 10^8 of PIV-3, the average amount of virus recovered per gram of lung tissue and grown on VERO cells was 3.62 x 10^4 ± 2 x 10^4 (Fig. 4.13).

After treatment with dexamethasone (10mg/kg) in 25% DMSO there was a small but insignificant reduction in virus recovered (Fig. 4.13).

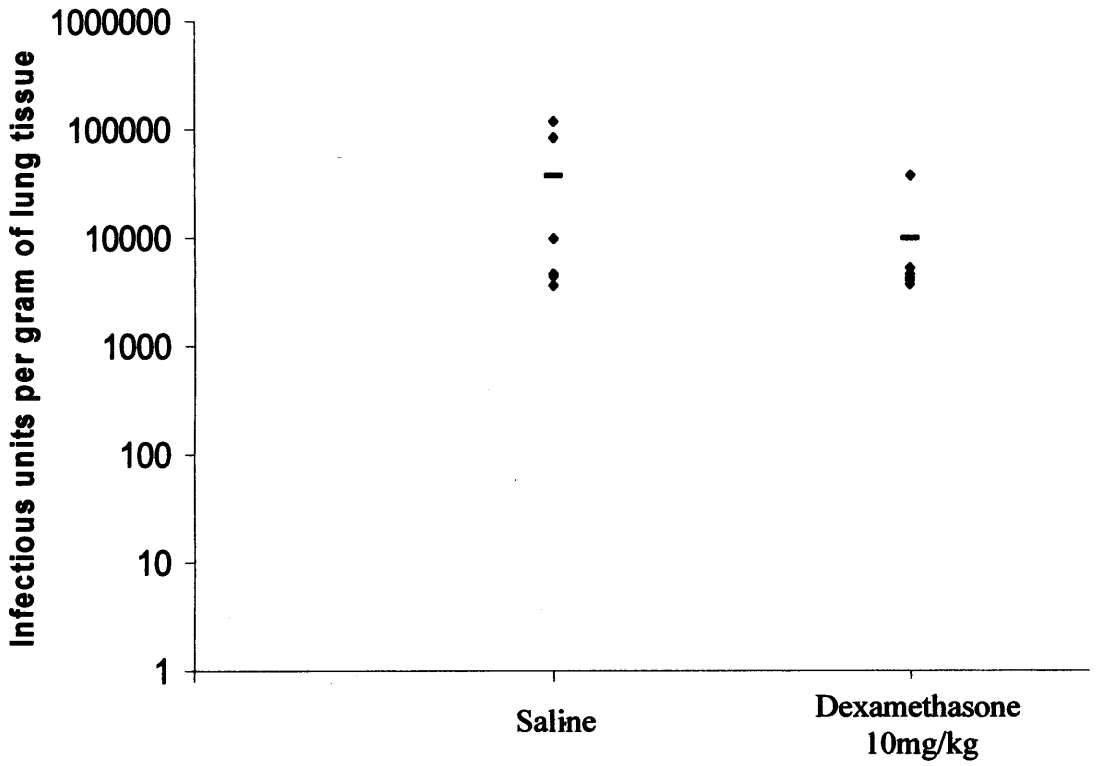


Figure 4.13

Effect of 25% DMSO and dexamethasone (10mg/kg) on PIV-3 recovery in guinea-pig lung tissue after inoculation with 3 x 10^8 of PIV-3. Each point shows the amount of PIV-3 recovered per gram of lung tissue and grown in VERO cells and – shows the average (n = 6). There was no significant ($P<0.05$) difference between the saline and dexamethasone treated groups as determined by a Student's *t* test (paired).

4.3.5 Effects of dexamethasone on influenza-induced airway inflammation in guinea-pigs

Airway reactivity to histamine

Prior to inoculation with virus there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Fig 4.14)

Effect of inoculation with A/PR/8/34 (1.1×10^6) grown in chicken embryos

In 25% DMSO treated guinea-pigs inoculated with influenza type A/PR/8/34 there was evidence of airway hyperreactivity after viral inoculation as there was a -20.4 ± 3.7% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to the baseline value (Fig.4.14). sG_{aw} values recovered to –9.7 ± 4.9% after 10 minutes (Fig.4.14).

After 10mg/kg dexamethasone in 25% DMSO treatment the decrease in sG_{aw} seen at 0 minutes was reduced to -4.6% but peaked at -8.3% after 5 minutes, which was not significantly different from pre-histamine baseline (Fig. 4.14).

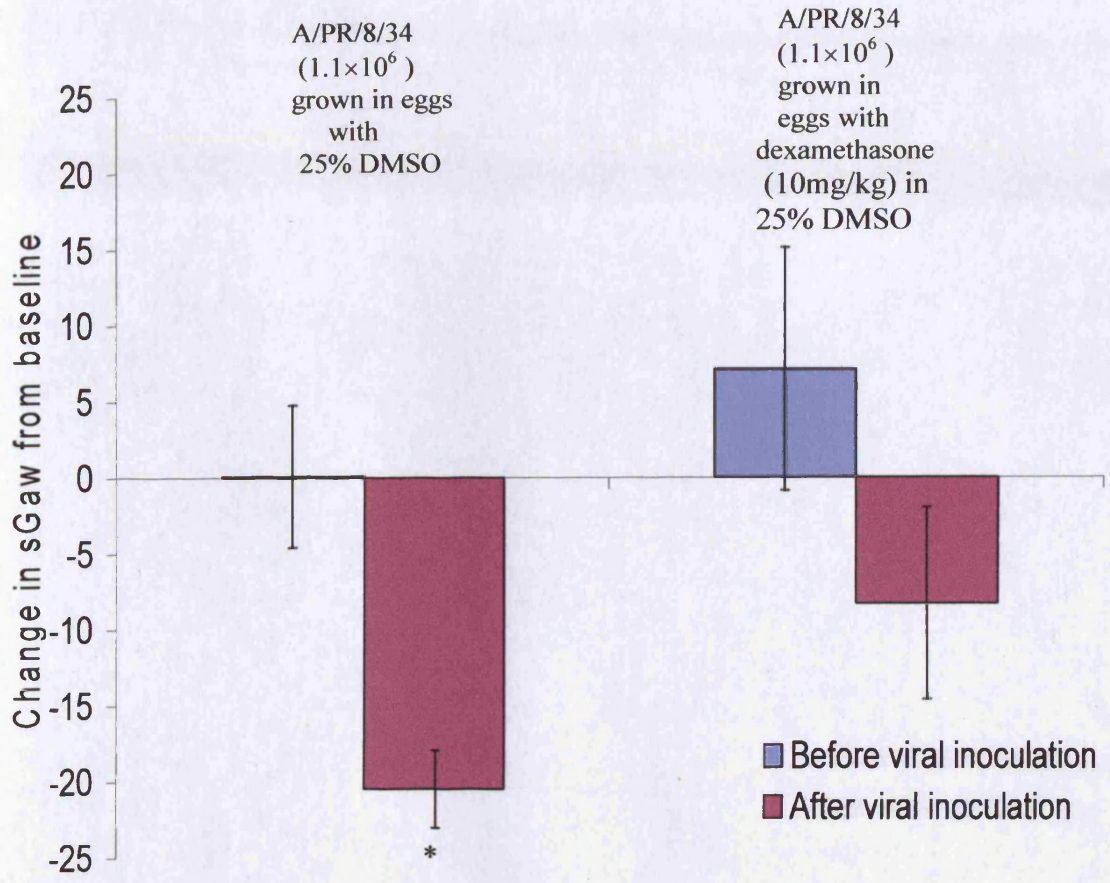


Figure 4.14

Maximum decrease in sG_{aw} from baseline in guinea-pigs after 1mM histamine exposure before and after inoculation with (1.1×10^6) grown in chicken embryos and treated with 25% DMSO or dexamethasone (10mg/kg in) 25% DMSO. Each bar represents the mean ± standard error of mean (n=6). * denotes the significance ($P<0.05$) in difference in the maximum decrease in sG_{aw} from baseline before and after viral inoculation, as determined by a Student's *t* test (paired).

Bronchoalveolar lavage cell counts

In animals inoculated with allantoic fluid from chicken embryo and treated with 25% DMSO, the numbers of macrophages, eosinophils, lymphocytes and neutrophils in the BALF were as follows; total cells: 2.39 ± 0.1 ($\times 10^6$/ml), macrophages: 1.95 ± 0.11 ($\times 10^6$/ml), eosinophils: 0.28 ± 0.08 ($\times 10^6$/ml) lymphocytes: 0.08 ± 0.02 ($\times 10^6$/ml) and neutrophils 0.08 ± 0.02 ($\times 10^6$/ml) (Fig. 4.15).

In the animals inoculated with H1N1 A/PR/8/34 1.1×10^6 grown in a chicken embryo and treated with 25% DMSO, there were significant increases in macrophages and a smaller increase in eosinophils. There was no increase in lymphocytes or neutrophils (Fig. 4.15).

In dexamethasone (10mg/kg) in 25% DMSO treated animals inoculated with H1N1 A/PR/8/34 1.1×10^6 grown in a chicken embryo there were significant reductions total cells, macrophages, eosinophils and neutrophils and a modest decrease in lymphocytes (Fig 4.15).

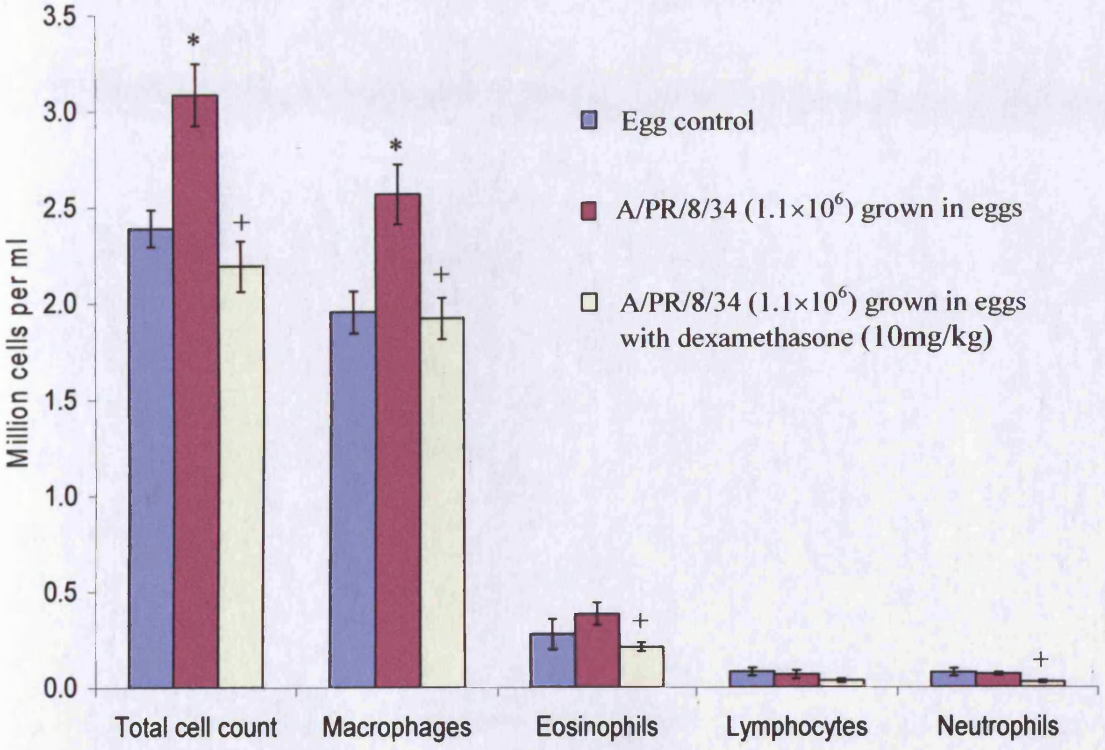


Figure 4.15

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from guinea-pigs inoculated with allantoic fluid from chicken embryos and treated with 25% DMSO or inoculated with H1N1 A/PR/8/34 grown in chicken embryo (1.1×10^6) and treated with 25% DMSO or dexamethasone (10mg/kg in 25% DMSO). Each bar represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding no virus control and + denotes the significance ($P<0.05$) of difference compared to the H1N1 A/PR/8/34 grown in chicken embryo (1.1×10^6) group as determined by a Student's *t* test (paired).

Percentage wet lung weight

In animals inoculated with allantoic fluid from chicken embryo and treated with 25% DMSO the percentage wet lung weight was 79.9 ± 0.5 (Fig. 4.16).

In the animals inoculated with H1N1 A/PR/8/34 1.1×10^6 grown in a chicken embryo and treated with 25% DMSO there was an increase in wet weight but not significantly compared to the allantoic fluid control (Fig. 4.16).

In dexamethasone (10mg/kg in 25% DMSO) treated animals inoculated with H1N1 A/PR/8/34 1.1×10^6 grown in a chicken embryo there was a non significant reduction in wet weight (Fig. 4.16).

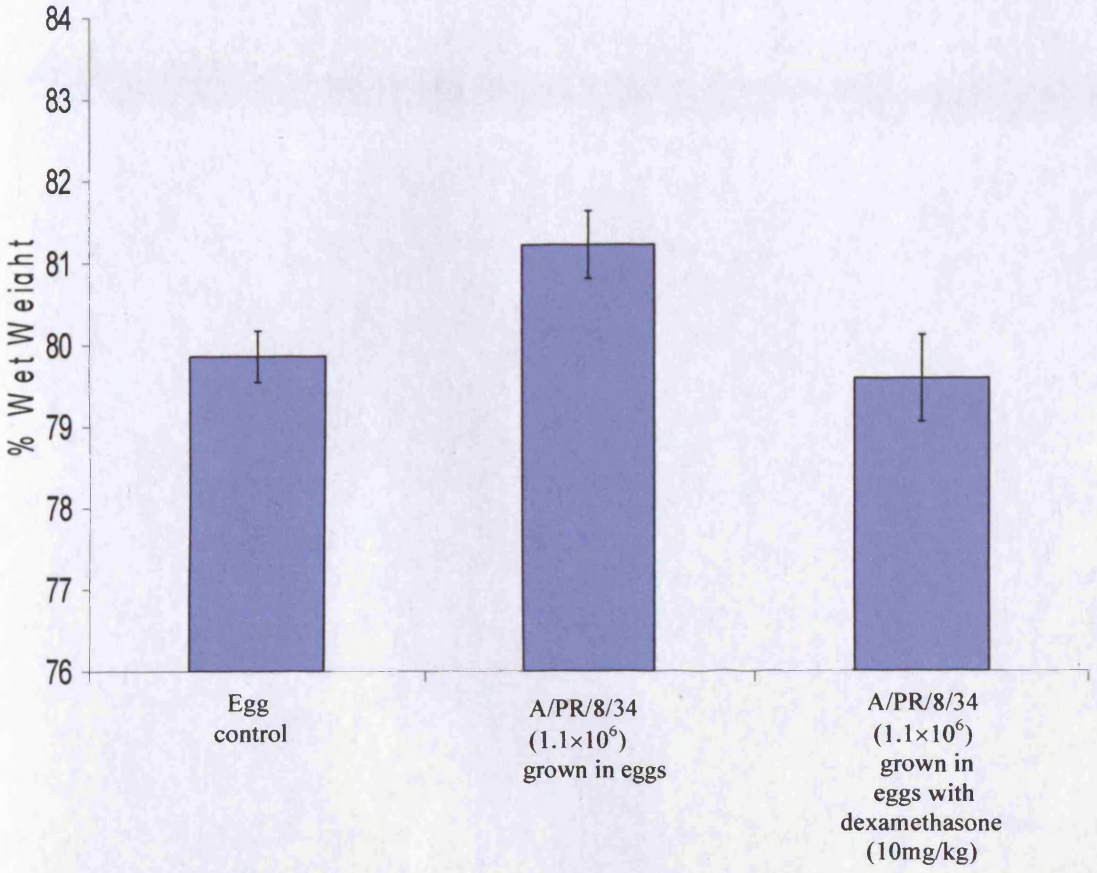


Figure 4.16

Effect on percentage wet lung weight guinea-pigs inoculated with allantoic fluid from chicken embryos and treated with 25% DMSO or inoculated with H1N1 A/PR/8/34 grown in chicken embryo (1.1×10^6) and treated with 25% DMSO or dexamethasone (10mg/kg in 25% DMSO). Each bar represents the mean ± standard error of mean (n=6). There was no significant ($P<0.05$) difference between any of the groups as determined by a Student's *t* test (paired).

4.4 Discussion

Effects of FE999024 on PIV-3 and PIV-2 induced airway inflammation

PIV-3 and PIV-2 infection resulted in bronchoconstriction to an inhaled dose of histamine that before inoculation produced no response. This indicates airway hyperreactivity 4 days after the viral infection. The lowest dose of FE999024 used (1mg/kg) had no inhibitory effect, compared to control, as there was no decrease in the post-viral bronchoconstriction, in fact there was an increase in brochoconstriction. The 3mg/kg dose appeared to have had some effect as there was a small, but incomplete decrease in the histamine-induced bronchoconstriction. The maximum dose of FE999024 (10mg/kg) completely inhibited the airway hyper-reactivity to inhaled histamine seen in the saline control.

Bradykinin relaxes guinea-pig trachea *in vitro* but if the epithelium is removed it causes bronchoconstriction (Bramley *et al* 1990). Similar effects are seen in humans with inhaled bradykinin causing little or no effect in normal patients but causing bronchoconstriction in asthmatics (Simonsson *et al* 1973). This indicates that the presence of an intact epithelium would have an inhibitory effect on the ability of bradykinin to cause bronchoconstriction in the airways.

Damage to the epithelium would enable bradykinin to cause bronchoconstriction via several different possible mechanisms. NEP is the principal enzyme which metabolizes bradykinin in the airways. NEP is strongly expressed in the airway epithelium, so damage

to the epithelium would therefore prolong the presence of bradykinin in the airways (Nadel 1991). Bradykinin causes the release of the bronchodilator PGE_2 from the epithelium therefore damage to the epithelium would reduce this inhibitory effect on the ability of bradykinin to cause bronchoconstriction (Bramley *et al* 1990). NOS inhibitors potentiate the bronchoconstrictor effect of inhaled bradykinin in guinea-pigs (Ricciardolo *et al* 1994) and in asthmatic patients (Ricciardolo *et al* 1996). The likely source of the NO is the epithelium which expresses both constitutive (Robbins *et al* 1994) and inducible NOS (Asano *et al* 1994). This potentiating effect of NOS inhibitors is not seen in more severe asthma, possibly due to more extensive damage to the epithelium and the loss of this source of NO (Ricciardolo *et al* 1997).

Bradykinin can also cause bronchoconstriction through activation of bradykinin B_2 receptors on airway smooth muscle and through the release of tachykinins by the activation of C fibre nociceptive sensory nerve endings. Tachykinins can also cause bronchoconstriction through cholinergic nerve activation (C fibre and cholinergic mediated bronchoconstriction explained in chapter 3.4.2).

The effects of this reduced availability of bradykinin due to inhibition of tissue kallikrein by FE999024 would appear to complement previous studies which show that inhaled bradykinin causes bronchoconstriction in inflamed airways (Fuller *et al* 1987).

After inoculation with PIV-2 and the lower concentration of PIV-3, FE999024 appears to reduce influx of macrophages, lymphocytes, eosinophils and neutrophils in a

concentration-dependent manner. At the higher concentration of PIV-3, FE999024 (10mg/kg) was unable to reduce the influx of inflammatory cells.

Although bradykinin is not thought to have much direct effect on activation and recruitment of inflammatory cells, it is thought to work indirectly through the release of monocyte and neutrophil chemotactic activity from the airway epithelial cells (Koyama *et al* 1995), to cause release of neutrophil, monocyte and eosinophilic factors from alveolar macrophages (Sato *et al* 1996) and also to stimulate eotaxin release from human lung fibroblasts (Sato *et al* 2000). This is reflected in this study as we have seen reduced levels of macrophages, eosinophils and neutrophils by the kallikrein inhibitor FE999024. This supports a role for bradykinin in the inflammatory response and subsequent airway hyper-reactivity following viral infection of the airways with parainfluenza virus.

The highest dose of FE999024 significantly reduced the increase in wet weight after PIV inoculation. The bradykinin induced microvascular leakage, which could contribute to the increased lung wet weight in guinea-pigs can be inhibited by a PAF inhibitor (Rogers *et al* 1990) and a thromboxane A_2 antagonist (Kawikova *et al* 1993) indicating an indirect effect.

Effect of dexamethasone on PIV induced airway inflammation

Dexamethasone completely inhibited the AHR seen after PIV-3 and PIV-2 inoculation. Dexamethasone works through the decreased transcription and translation of a wide

range of pro-inflammatory mediators including those released by the pulmonary epithelium in response to PIV infection such as IL-6, IL-8, IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF) and RANTES (Barnes and Adcock 1993). Suppression of the release of these inflammatory mediators by dexamethasone would also reduce the release of further mediators and the activation of resident macrophages, lymphocytes, granulocytes, mast cells and basophils (Folkerts and Nijkamp 1995) and therefore reduce inflammation and further damage to the epithelium and the subsequent AHR. PIV has been shown to alter the function of the inhibitory M_2 muscarinic receptor in guinea pigs which leads to increased ACh release and bronchoconstriction (Fryer *et al* 1990). Moreno *et al* (2003) have shown that dexamethasone can reverse the PIV-induced M_2 receptor dysfunction in guinea-pigs.

Dexamethasone reduced the PIV induced inflammatory cell influx, in some cases to baseline levels. Dexamethasone reduces the transcription and translation of IL-3, IL-4, IL-5, IL-6, IL-8, TNF-a, RANTES and GMCSF, which are responsible for the PIV induced migration of inflammatory cells to the airways (Barnes and Adcock 1993).

Dexamethasone reduced the PIV-induced increase in lung wet weight. The increase in wet weight may be due to an increase in mucus production but the most likely cause is pulmonary oedema (Hwang *et al* 2001). Dexamethasone may reduce pulmonary oedema by decreasing expression of pro-inflammatory mediators which increase blood pressure in pulmonary capilliaries (thromboxane, 5-lipoxygenase and leukotrienes C4, D4 and E4) or increase permeability of the capillary wall (tachykinins, reactive oxygen species

(ROS), mast cell-derived histamine, excess NO and ROS-activated proteolytic enzymes (Barnes *et al* 1999).

Effect of dexamethasone on viral recovery

Dexamethasone reduced the amount of virus recovered from the lung tissue though not significantly. The reason for this reduction could be decreased viral receptors on the pulmonary epithelium as dexamethasone reduced the infectability of rhinovirus on cultured epithelial cells by decreasing expression of ICAM-1 (Suzuki *et al* 2000).

Effect of dexamethasone on influenza induced airway inflammation

Dexamethasone reduced the AHR and abolished the leukocyte influx and increased wet weight seen after inoculation with H1N1 A/PR/8/34. The mechanism for the reduced influenza-induced inflammation is likely to be similar to the inhibition of the PIV-induced inflammation by dexamethasone explained above.

Chapter 5

The role of bradykinin in airway function

5.1 Introduction

Bradykinin-induced bronchoconstriction

In healthy humans, inhalation of bradykinin has little or no effect, but in asthmatics it produces a bronchoconstriction (Fuller *et al* 1987; Polosa *et al* 1990). Asthmatic subjects show a greater degree of airways hyperreactivity to bradykinin than to methacholine after allergen challenge (Berman *et al* 1995). Kinins exert their pharmacological effects through two main bradykinin receptor subtypes, bradykinin B_1 and B_2 receptors (Hall 1992). B_1 receptors, which are characterized by binding of [des-Arg^9]-bradykinin, are absent in the lungs under normal conditions but their expression is induced by inflammation (Regoli *et al* 1978; Christiansen *et al* 2002). Bradykinin B_2 receptors show high affinity for bradykinin and are constitutively expressed in the airways (Hall 1992).

In guinea-pigs, inhaled bradykinin has been shown to produce bronchoconstriction which can be blocked by the bradykinin B_2 receptor antagonist icatibant (Sakamoto *et al* 1994; Wirth *et al* 1993). To date the effects of bradykinin on the airways have been largely examined after intravenous, inhalation or intratracheal administration to anaesthetized animals such as guinea-pigs (Wirth *et al* 1993; Miura *et al* 1994; Sakamoto *et al* 1994; Valenti *et al* 2005) and rats (Ellis *et al* 2004). In these earlier studies there are a number of discrepancies in the role of bradykinin in allergic responses (Ricciardolo *et al* 1994; Sakamoto *et al* 1996). A possible reason for the variable effects of bradykinin in the lung is its rapid breakdown by NEP and/or ACE. There do not appear to have been any studies on the airways effects of bradykinin in conscious guinea-pigs or rats, where the effect of anaesthetic is eliminated. In this study, we therefore attempted to identify a

bronchoconstriction with inhaled bradykinin and to examine the effects of phosphoramidon and captopril, inhibitors of NEP and ACE, respectively. As bradykinin is thought to act on B_2 receptors in the airways (Ellis and Fozard 2002), we also examined blockade of the bradykinin-induced bronchoconstriction with the bradykinin B_2 receptor antagonists icatibant and MEN16132. Icatibant is a peptide antagonist of B_2 receptors (Leeb-Lundberg *et al* 2005). Intravenous, intratracheal or aerosol administered icatibant has been shown to inhibit the bronchoconstriction to intravenously administered bradykinin in anaesthetized guinea-pigs (Tramonana *et al* 2001; Valenti *et al* 2005). Inhaled icatibant also inhibited the bronchoconstriction and microvascular leakage after inhaled bradykinin in anaesthetized guinea-pigs (Sakamoto *et al* 1994). MEN16132 is a nonpeptide selective antagonist of bradykinin B_2 receptors which has been shown to block bronchoconstriction to intravenous bradykinin (Valenti *et al* 2005) and endogenous Bradykinin released by dextran sulphate infusion (Valenti *et al* 2007) in anaesthetized guinea-pigs.

Airway hyperreactivity to bradykinin

Kinin levels are increased in BALF of asthmatics after allergen challenge (Christiansen *et al* 1992) and inhalation of bradykinin causes bronchoconstriction in asthmatic patients, but has little or no effect in non-asthmatics (Fuller *et al* 1987; Polosa and Holgate 1990). Bradykinin receptor expression is up-regulated in sensitized rat lungs (Huang *et al* 1999), in asthmatic airways inflammation (Christiansen *et al* 2002) and in murine airways under interleukin-4 (IL-4) stimulation (Bryborn *et al* 2004). IL-1β and TNF-α induced transcriptional up-regulation of bradykinin B_1 and B_2 receptors *in vitro* in murine airways

(Zhang *et al* 2004, 2007), which was inhibited by the corticosteroid, dexamethasone (Zhang *et al* 2005). Allergen sensitization and challenge has been repeatedly shown to cause airways hyperreactivity in guinea-pigs to a range of spasmogens, including histamine and methacholine (Toward and Broadley 2004; Smith and Broadley 2007). AHR to intravenous bradykinin has been demonstrated in anaesthetized rats after allergen challenge (Ellis *et al* 2004), but there are few studies examining AHR to inhaled bradykinin in conscious animals. In this study, we therefore determined whether ovalbumen exposure in ovalbumen-sensitized guinea-pigs produced airways hyperreactivity to inhaled bradsykinin.

Bradykinin in PIV-3-induced airway inflammation

In previous experiments we have show that the tissue kallikrein inhibitor F999024 inhibits PIV-induced airway inflammation by reducing the levels of bradykinin in the lung (Chapter 4). Previous studies have shown contradictory effects of bradykinin B_2 antagonists in airway inflammation. Folkerts *et al* (2000) have shown that the bradykinin B_2 antagonist icatibant can inhibit the PIV-3 induced AHR but not the inflammatory cell influx. Bandeira-Melo *et al* (1999) were able to inhibit the ovalbumen-induced eosinophilia and neutrophilia in rats with icatibant. We attempted to determine whether bradykinin was involved in the PIV-3-induced AHR and inflammatory cell influx and whether it was mediated by the bradykinin B_2 receptor by treating PIV-3 inoculated guinea-pigs with the bradykinin B_2 receptor antagonist MEN16132.

5.2 Methods

Bradykinin exposure experiments

Airway reactivity to bradykinin was measured by recording pulmonary function using whole body plethysmography and recorded as sGaw. Baseline sGaw recordings were taken and then at 0, 5 and 10 minutes after a 20 second box exposure to bradykinin dissolved in saline.

The effects of bradykinin were also examined 1 hour after administration of captopril (1mg/kg i.p.) and/or phosporamidon (0.1mg/kg i.p. or 1mM, 20 minute inhalation exposure) to inhibit the enzymes ACE and NEP respectively. These enzymes catalyze the breakdown of bradykinin into inactive metabolites (see Chapter 1) and therefore inhibitors were used in an attempt to prolong the duration of the inhaled bradykinin in the lung.

The effect of the duration of bradykinin exposure was examined by comparing 20, 40, and 60 second, 0.3mM bradykinin exposures after captopril (1mg/kg) treatment.

The selective B_2 bradykinin antagonists: icatibant (10μM inhalation exposure) and MEN16132 (30, 100 or 300nM/kg, i.p.) (1 or 10μM, 20 minute inhalation exposure) were administered 1 hour before inhalation exposure to 1mM bradykinin in guinea-pigs treated with captopril (1mg/kg).

At least 4 days after each experiment with icatibant and MEN16132, the animals were treated with an appropriate saline control (i.p. injection or inhalation exposure) before further exposure to 1mM bradykinin with 1mg/kg captopril to determine the recovery from B_2 receptor blockade.

Ovalbumen sensitization/exposure experiments

The methods in this section are the same as in 2.4 Ovalbumen methods with the exception that in one experiment AHR was tested with bradykinin (1mM, 20 second inhalation exposure) rather than histamine. AHR was tested 24 hours before and after ovabumen challenge.

Virus experiments

The methods in this section are described in detail in Chapter 2 (General Methods, 2.1. and 2.2 Virus experiments), with the exception of the drug treatments. Guinea-pigs were treated with a daily 20 minute inhalation exposure of the B_2 bradykinin receptor antagonist, MEN16132 (10μM in saline) or control (saline).

Protocol for virus experiments

Day 1: Dosing with 0.9% sterile saline or MEN16132 (10μM) in 0.9% sterile saline

Day 2: Dosing with 0.9% sterile saline or MEN16132 (10μM) in 0.9% sterile saline and **reactivity test to histamine**

Day 3: Dosing with 0.9% sterile saline or MEN16132 (10μM) in 0.9% sterile saline and **inoculation 3 hours later with virus or virus free medium**

Day 4: Dosing with 0.9% sterile saline or MEN16132 (10μM) in 0.9% sterile saline and **inoculation 3 hours later with virus or virus free medium**

Day 5: Dosing with 0.9% sterile saline or MEN16132 (10μM) in 0.9% sterile saline

Day 6: Dosing with 0.9% sterile saline or MEN16132 (10μM) in 0.9% sterile saline

Day 7: Dosing with 0.9% sterile saline or MEN16132 (10μM) in 0.9% sterile saline

Day 8: Dosing with 0.9% sterile saline or MEN16132 (10μM) in 0.9% sterile saline, **reactivity test to histamine and lavage**

5.3 Results

5.3.1 Bradykinin exposures

Bronchoconstrictor effect

After 20 second, nose only 0.01, 0.1 and 1mM bradykinin exposures in guinea-pigs there was no significant decrease in sG_{aw} from baseline (Fig. 5.1).

After captopril (1mg/kg, i.p.) treatment there was no significant reduction in sG_{aw} after 0.01 and 0.1mM bradykinin exposure but a significant decrease after a 1mM exposure (Fig. 5.2).

After captopril (1mg/kg) treatment, a 0.3mM bradykinin exposure produced modest decreases in sG_{aw} after 20 (-8.7 ± 7.0 %) and 40 (-5.9 ± 6.3 %) second exposures and a significant decrease after a 60 second (-17.9 ± 6.4 %) exposure (Fig. 5.3).

After phosphoramidon (0.1mg/kg, i.p.) there was no significant decrease (-3.8 ± 4.4%) in sG_{aw} after a 1mM bradykinin exposure, but after a 10mM, 20 minute inhalation exposure there was a significant decrease (21.7 ± 4.4%) after a 1mM bradykinin exposure (Fig. 5.4).

After combined phosphoramidon (10mM, 20 minute inhalation exposure) and captopril (1mg/kg i.p.) there was a significant 22.3 ± 3.2% decrease in sG_{aw} after a 1mM bradykinin exposure. However, this response was no greater than with the same doses of phosphoramidon or captopril alone (Fig. 5.4).

After i.p.injections of MEN16132 (30, 100 and 300nM/kg) there was no blockade of the decrease in sG_{aw} seen after a 1mM bradykinin exposure after captopril (1mg/kg, i.p.) treatment (Fig. 5.5). The bronchoconstrictor responses (-11.1 ± 4.3, -13.6 ± 4.3 and -11.2 ± 7.1%) were not significantly different from baseline or from the saline control (-17.3 ± 2.4%).

After MEN16132 (1 and 10μM) by a 20 minute inhalation exposure, there was complete blockade of the decrease in sG_{aw} seen after a 1mM bradykinin exposure with captopril (1mg/kg, i.p.) treatment (Fig. 5.6).

Icatibant (10μM) by 20 minute inhalation exposure had no effect on the decrease in sG_{aw} seen after a 1mM bradykinin exposure with captopril (1mg/kg, i.p.) (Fig. 5.7).

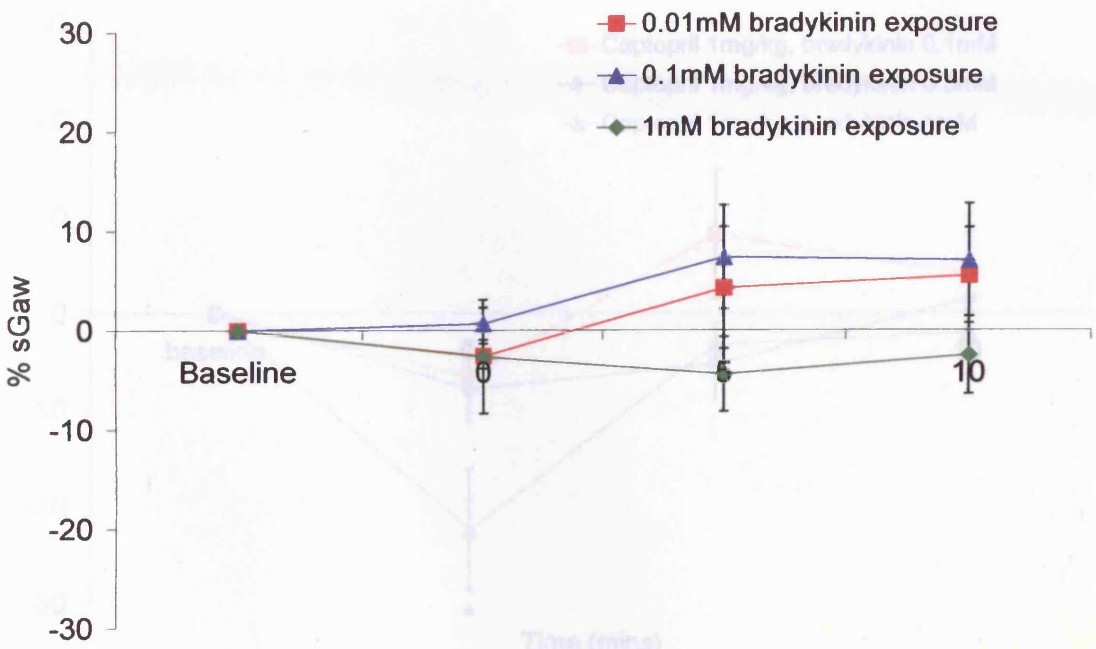


Figure 5.1

Effect of 0.01, 0.1 and 1mM bradykinin exposures (20 seconds). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes. Negative values represent bronchoconstriction. There was no significance ($P<0.05$) in difference between the baseline sG_{aw} values at each time point after bradykinin exposure as determined by a Student's *t* test (paired).

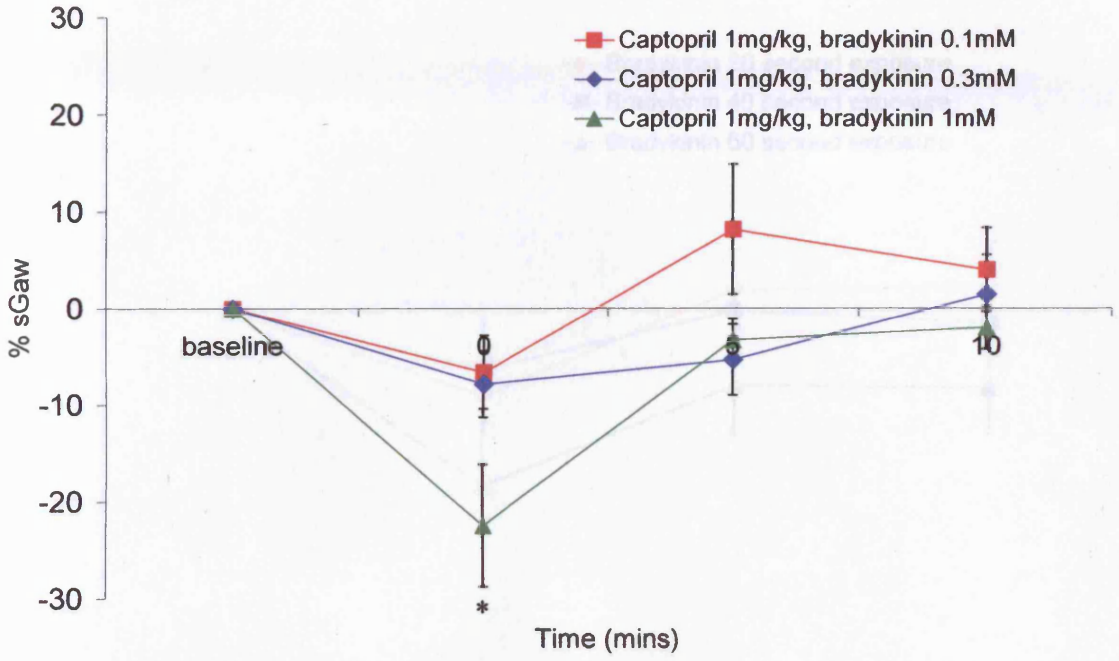


Figure 5.2

Effect of 0.1, 0.3 and 1mM bradykinin exposures (20 seconds) after captopril treatment. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes. Negative values represent bronchoconstriction. * indicates the significance ($P<0.05$) in difference between the changes from baseline sG_{aw} after bradykinin exposure as determined by a Student's *t* test (paired).

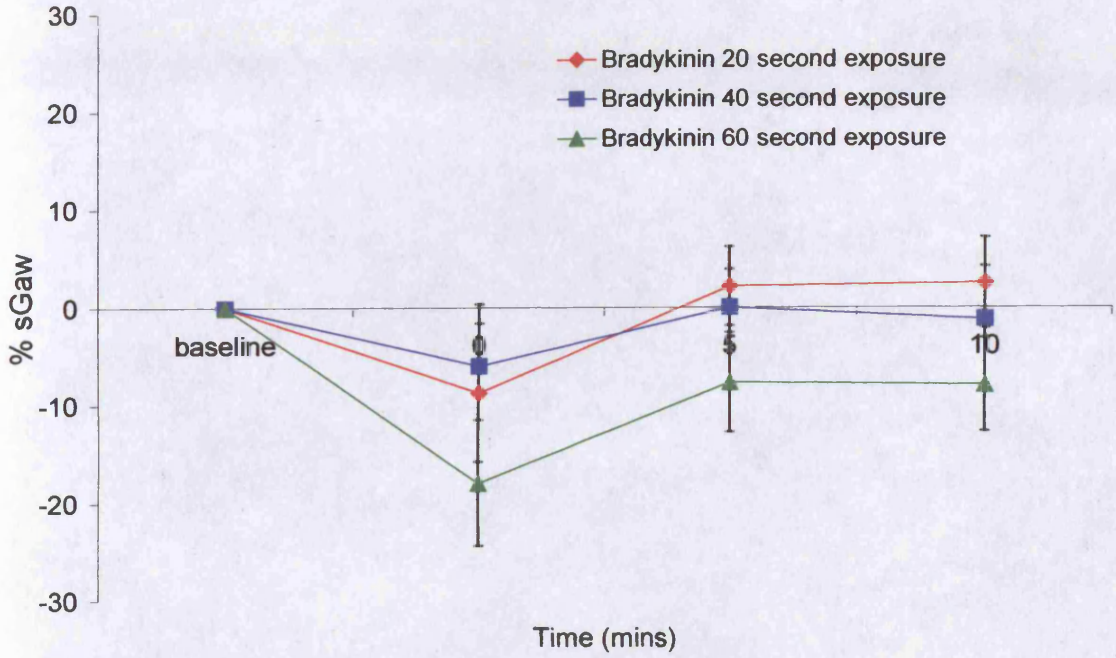


Figure 5.3

Effect of 20, 40 and 60 second, 0.3mM bradykinin exposures after captopril (1mg/kg, i.p.) treatment. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes. Negative values represent bronchoconstriction. There was no significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after bradykinin exposure as determined by a Student's *t* test (paired).

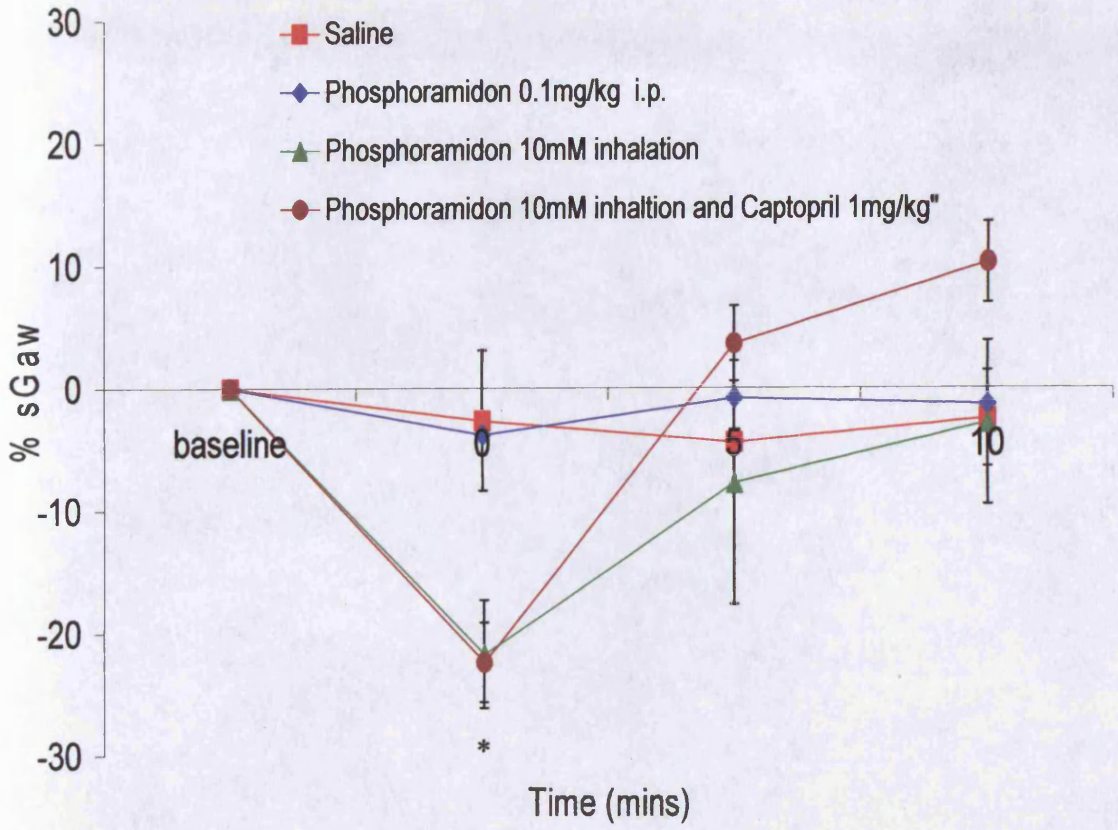


Figure 5.4

Effect of phosphoramidon (10μM, 20 minute exposure or 0.1mg/kg) and combining phosphoramidon (10μM, 20 minute exposure) with captopril (1mg/kg, i.p.) on bradykinin (1mM, 20 second exposure). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, with and without phosphoramidon or captopril treatment as determined by a Student's *t* test (paired).

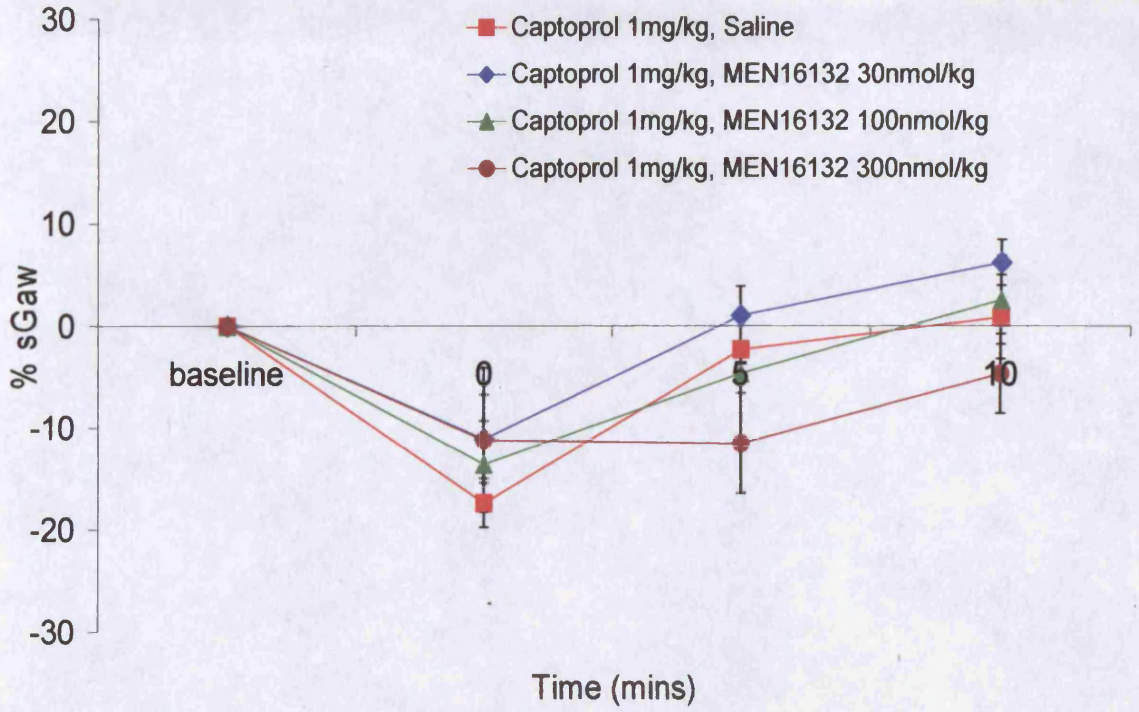


Figure 5.5

Effect of MEN6132 (30, 100 and 300nmol/kg, i.p.) and saline on bradykinin (1mM, 20 second) exposure with captopril (1mg/kg, i.p.). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes. Negative values represent bronchoconstriction. . There was no significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, between the saline and MEN16132 treated groups as determined by a Student's *t* test (paired).

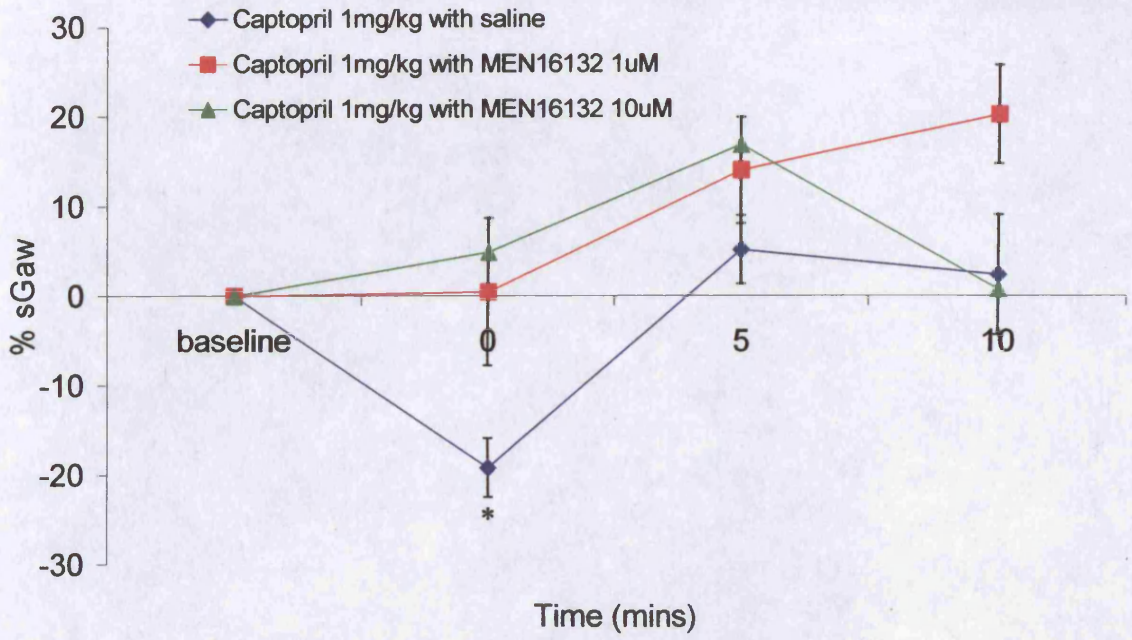


Figure 5.6

Effects of 20 minute inhalation exposures of MEN6132 (1 and 10μM) and saline on bradykinin (1mM, 20 second) exposure with captopril (1mg/kg, i.p.). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes. Negative values represent bronchoconstriction. . * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, with and without MEN16132 treatment as determined by a Student's *t* test (paired).

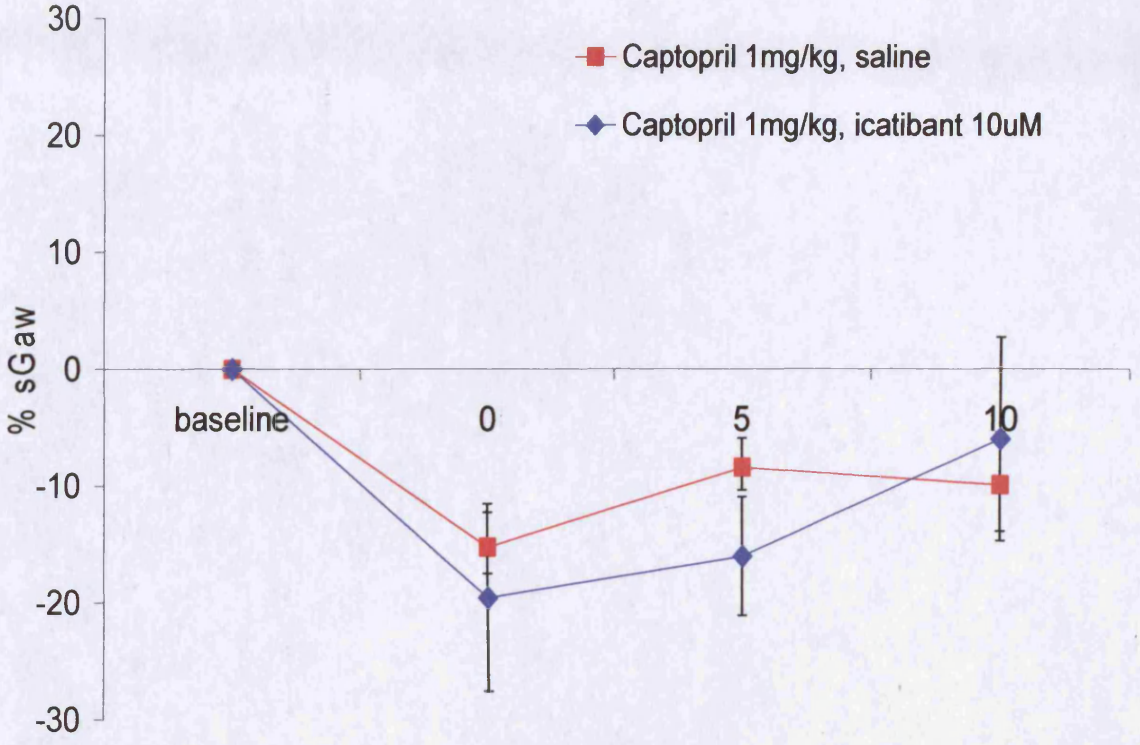


Figure 5.7

Effects of 20 minute exposures of icatibant (10μM) and saline on bradykinin (1mM, 20 second) exposures with captopril (1mg/kg, i.p.). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes. Negative values represent bronchoconstriction. There was no significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, between the saline and icatibant treated groups as determined by a Student's *t* test (paired).

Bronchoalveolar lavage

In animals receiving no treatment, the numbers of macrophages, eosinophils, neutrophils and lymphocytes in the BALF were as follows; total cells: 1.95 ± 0.09 ($\times 10^6$/ml), macrophages: 1.68 ± 0.09 ($\times 10^6$/ml), eosinophils: 0.20 ± 0.04 ($\times 10^6$/ml), neutrophils 0.04 ± 0.03 ($\times 10^6$/ml) and lymphocytes: 0.03 ± 0.01 ($\times 10^6$/ml) (Fig. 5.8).

In animals which received 1mM bradykinin with captopril (1mg/kg, i.p.) prior to lavage there was no significant increase to any of the cell types (Fig.5.8).

In animals which received a 1mM bradykinin, captopril (1mg/kg, i.p.) and phosphoramidon (10mM, 20 minute inhalation exposure) prior to lavage there were significant increases in total cells, macrophages, eosinophils and neutrophils compared to the same experiment without phosphoramidon. There was no increase in lymphocytes (Fig. 5.8).

In animals which received a 1mM bradykinin, captopril (1mg/kg, i.p.), phosphoramidon (10mM, 20 minute inhalation exposure) and MEN16132 (10μM, 20 minute inhalation exposure) prior to lavage, there were significant decreases in total cells, eosinophils, neutrophils and lymphocytes and a modest decrease in macrophages compared to the same experiment without MEN16132 (Fig. 5.8).

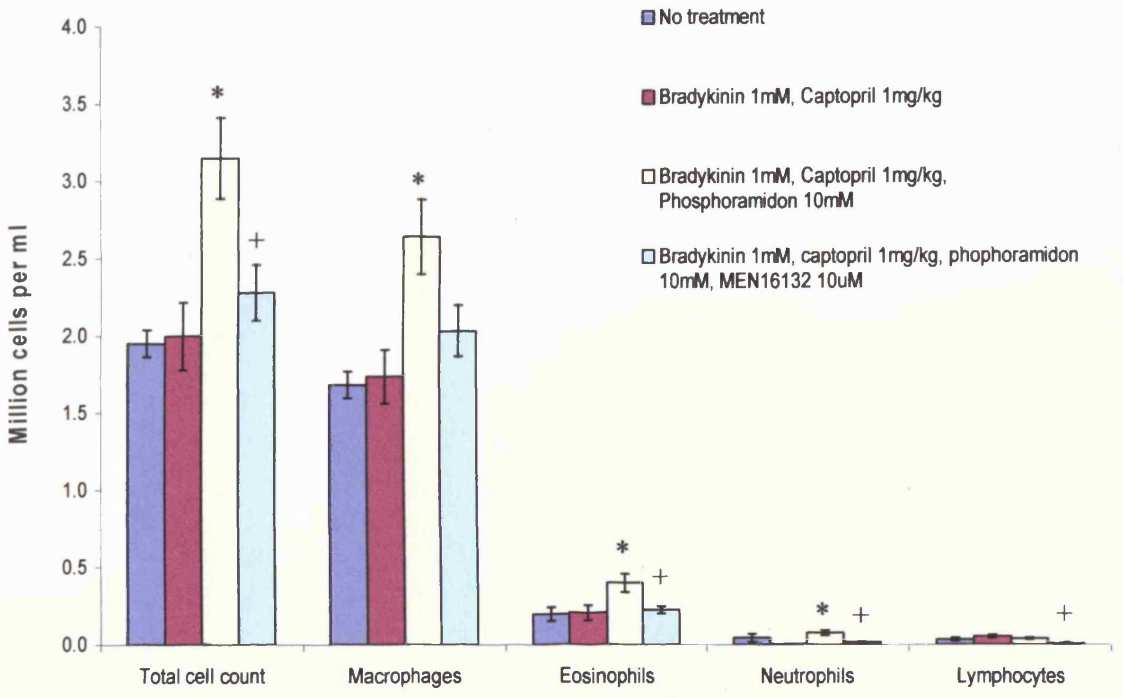


Figure 5.8

Total and differential cell (macrophage, eosinophil, neutrophil and lymphocyte) count of BALF removed from animals receiving no treatment, all other groups received 1mM bradykinin exposure with captopril (1mg/kg, i.p.) with or without phosphoramidon. The final group received phosphoramidon (10mM inhalation exposure) and MEN16132 (10μM inhalation exposure). Each bar represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding group without phosphoramidon and + denotes the significance ($P<0.05$) of difference compared to the group without MEN16132 as determined by a Student's *t* test (paired).

5.3.2 Ovalbumen sensitization experiments

Effect of ovalbumen exposure

In the 50% DMSO (i.p.) treated group there was an immediate 25.5 ± 4.5% decrease in sG_{aw} from baseline following ovalbumen exposure. After 4 hours there was a recovery but not to baseline levels. This was followed by a secondary 19.6 ± 8.3% decrease in sG_{aw} at 8 hours (Fig. 5.9).

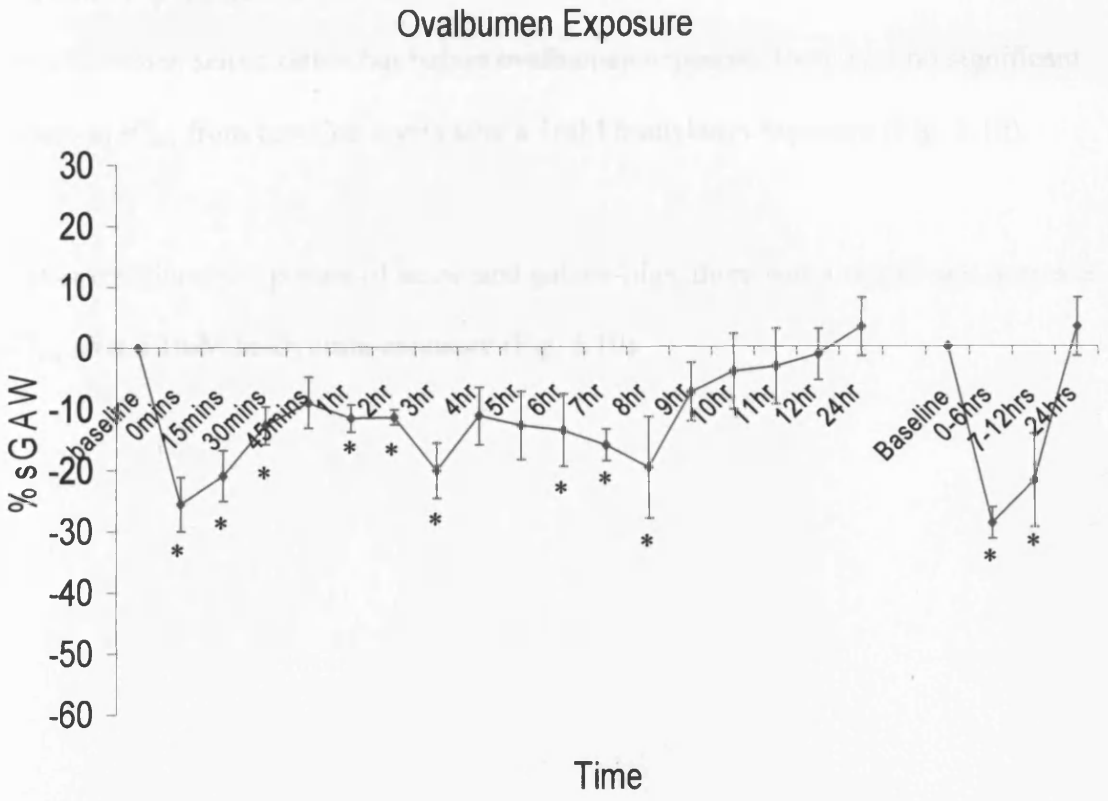


Figure 5.9

Effect of ovalbumen sensitization and exposure on airway function after treatment with 50% DMSO (i.p.). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown up to 24 hours after ovalbumen exposure. Negative values represent bronchoconstriction.
* denotes the significance ($P<0.05$) in difference between the changes from baseline sG_{aw} values at each time point as determined by a Student's *t* test (paired).

Bradykinin exposures

After ovalbumen sensitization but before ovalbumen exposure, there was no significant decrease in sG_{aw} from baseline levels after a 1mM bradykinin exposure (Fig. 5.10).

24h after ovalbumen exposure of sensitized guinea-pigs, there was a significant decrease in sG_{aw} after a 1mM bradykinin exposure (Fig. 5.10).

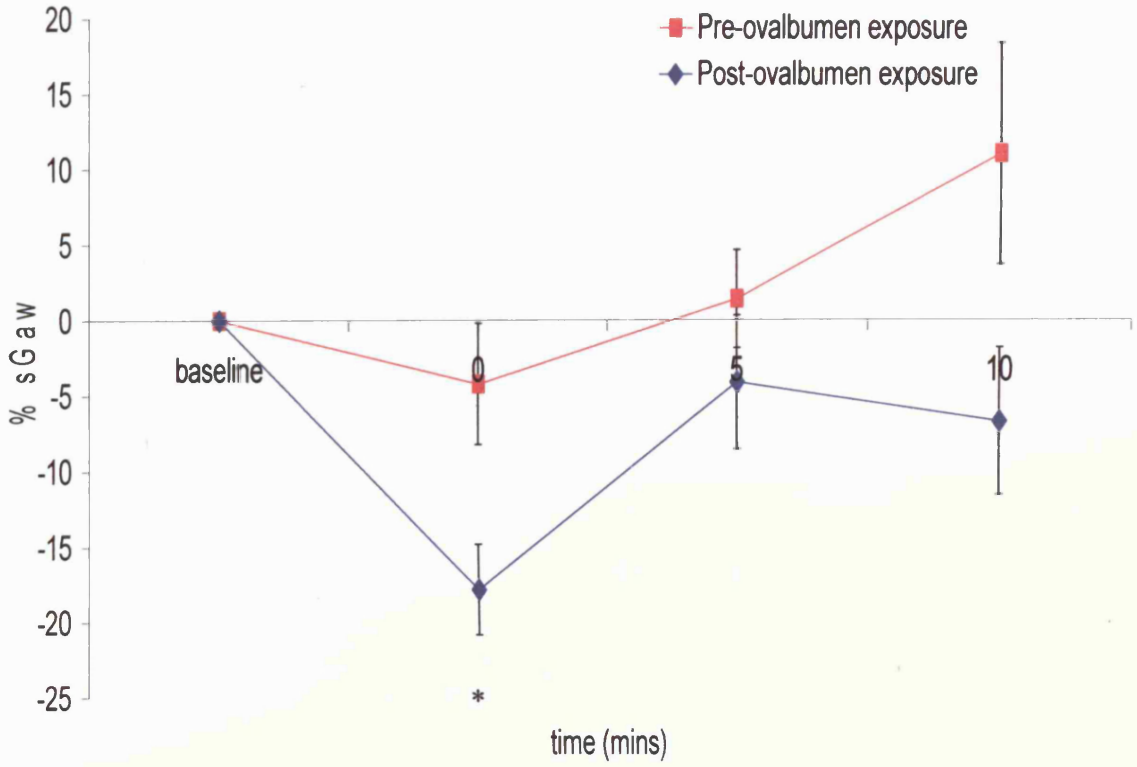


Figure 5.10

Effect of 1mM bradykinin exposure on ovalbumen sensitized guinea-pigs treated with 50% DMS0 (i.p.) before and after ovalbumen exposure. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after ovalbumen exposure as determined by a Student's *t* test (paired).

Bronchoalveolar lavage

In ovalbumen sensitized, saline challenged, histamine exposed and 50% DMSO treated guinea-pigs the numbers of macrophages, eosinophils, neutrophils and lymphocytes in the BALF were as follows; total cells: 4.38 ± 0.42 ($\times 10^6$/ml), macrophages: 2.31 ± 0.22 ($\times 10^6$/ml), eosinophils: 1.95 ± 0.34 ($\times 10^6$/ml), neutrophils 0.03 ± 0.01 ($\times 10^6$/ml) and lymphocytes: 0.1 ± 0.02 ($\times 10^6$/ml) (Fig. 5.11).

In the ovalubumen sensitized and challenged, histamine exposed group treated with 50% DMSO there were significant increases in total cells, macrophages, eosinophils and neutrophils and a modest in lymphocytes (Fig. 5.11).

In the ovalubumen sensitized and challenged, bradykinin exposed group treated with 50% DMSO there were significant increases in total cells, macrophages, eosinophils, neutrophils and lymphocytes compared to the saline challenged group. Compared to the ovalbumen sensitized and challenged, histamine exposed group there was a significant decrease in macrophages, modest decreases in total cells and eosinophils and small increases in neutrophils and lymphocytes (Fig. 5.11).

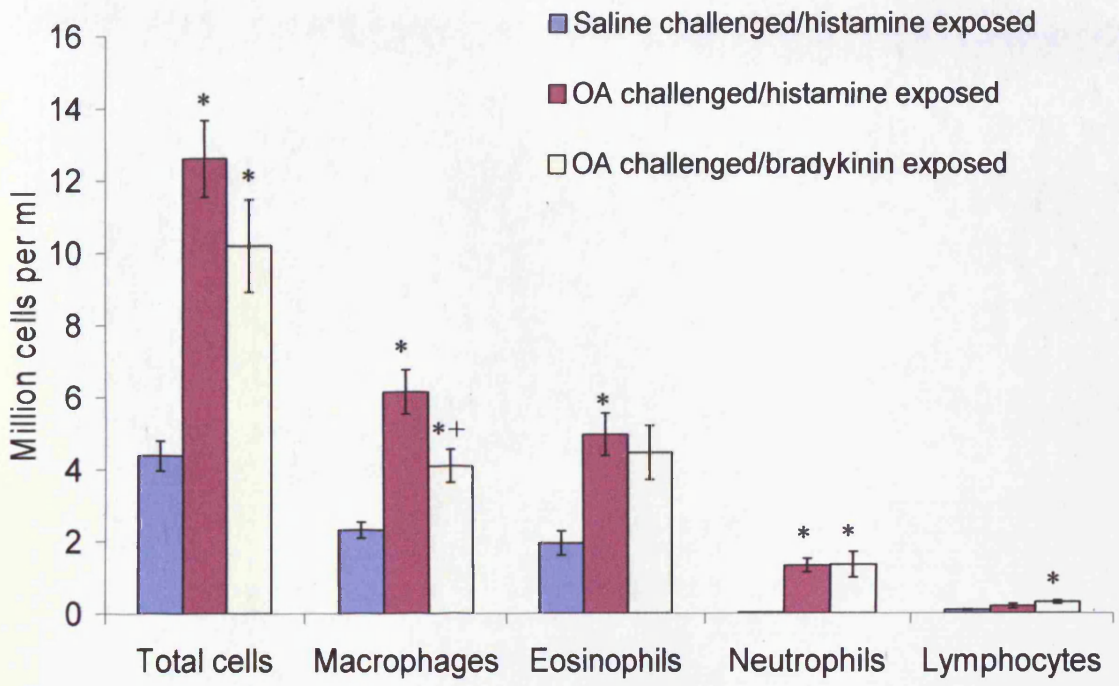


Figure 5.11

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from ovalbumen sensitized animals treated with 50% DMSO (i.p.), challenged with saline and exposed to histamine, challenged with ovalbumen and exposed to histamine or challenged with ovalbumen and exposed to bradykinin. Each bar represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding saline challenged and histamine exposed group and + denotes the significance ($P<0.05$) of difference compared to the ovalbumen challenged and histamine exposed group as determined by a Student's *t* test (paired).

5.3.3 PIV-3 (3×10^8) inoculated animals

Airway reactivity to histamine

Prior to inoculation with virus there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Figs. 5.12 and 5.13)

In the saline control there was evidence of airway hyperreactivity after viral inoculation as there was a 31.5 ± 6.3% reduction in sG_{aw} value at 0 minutes after histamine exposure compared to the baseline value. There was a recovery after 5 and 10 minutes (Fig. 5.12).

After treatment with MEN16132 (10μM, 20 minute inhalation exposure) there was no evidence of airway hyperreactivity after viral inoculation as there was only a 5.6 ± 4.6% reduction in sG_{aw} after histamine exposure (Fig. 5.13).

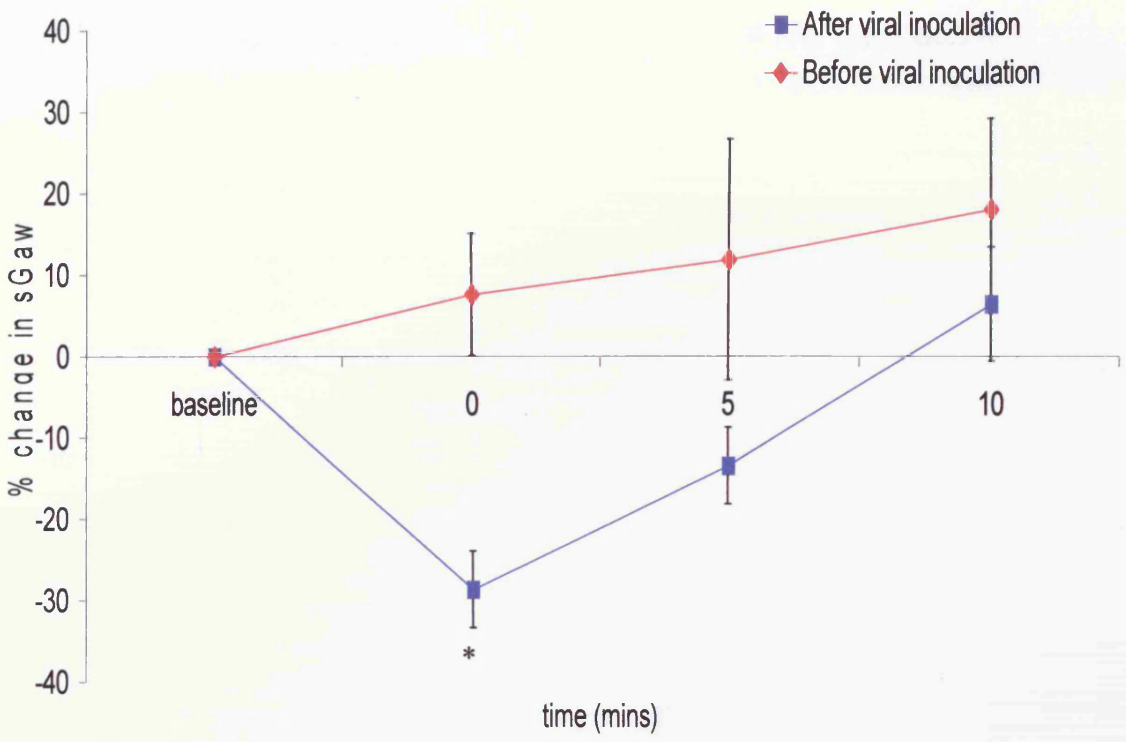


Figure 5.12

Effect of saline (20 minute inhalation exposure) treatment on airway function before and after PIV-3 virus infection. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after virus inoculation as determined by a Student's *t* test (paired).

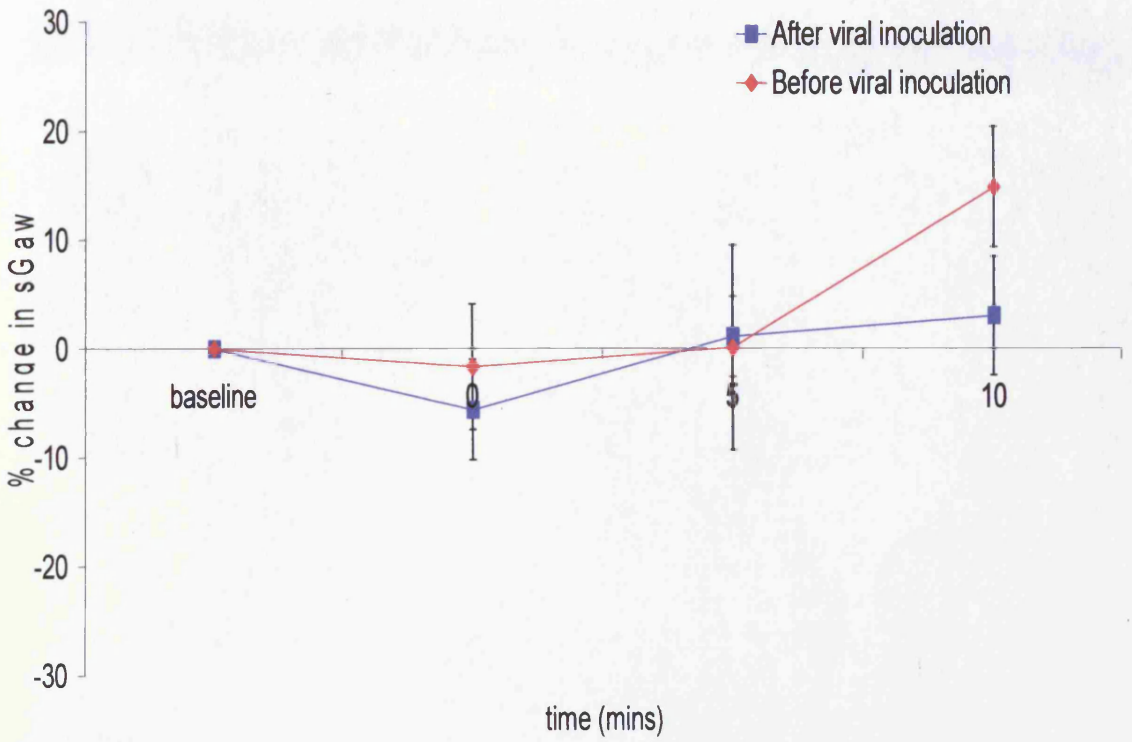


Figure 5.13

Effect of MEN16132 (10μM, 20 minute inhalation exposure) treatment on airway function before and after PIV-3 virus inoculation. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. There was no significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after virus inoculation as determined by a Student's *t* test (paired).

Bronchoalveolar lavage cell counts

In animals receiving no virus, but the medium only, the numbers of macrophages, lymphocytes, eosinophils and neutrophils in the BALF were as follows; total cells: 1.54 ± 0.17 ($\times 10^6$/ml), macrophages: 1.37 ± 0.15 ($\times 10^6$/ml), eosinophils: 0.12 ± 0.01 ($\times 10^6$/ml), lymphocytes: 0.06 ± 0.02 ($\times 10^6$/ml) and neutrophils 0.03 ± 0.01 ($\times 10^6$/ml) (Fig. 5.14).

In PIV-3 virus inoculated guinea-pigs treated with saline (20 minute exposure) there were significant increases in total cells, macrophages, eosinophils and neutrophils and a modest increase in lymphocytes. After MEN16132 (10μM, 20 minute exposure) there were significant decreases in total cells, macrophages, neutrophils and eosinophils and a modest decrease in eosinophils (Fig. 5.14).

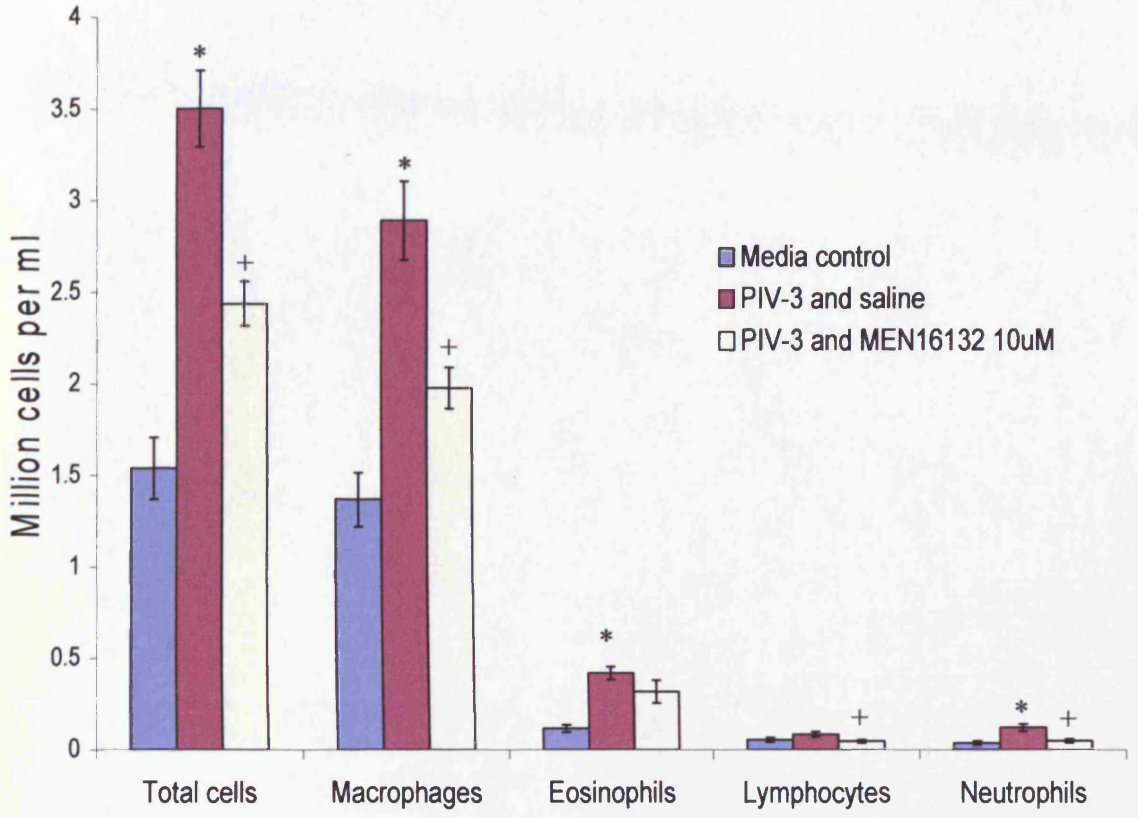


Figure 5.14

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from virus free medium inoculated animals or PIV-3 inoculated animals treated with saline or MEN16132 (10μM, 20 minute inhalation exposure). Each point represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding no virus control. + denotes the significance ($P<0.05$) of difference compared to the corresponding saline group, as determined by a Student's *t* test (paired).

5.4 Discussion

Bradykinin exposures

After bradykinin exposures up to 1mM there was no significant bronchoconstriction in guinea-pigs. This contrasts with observations made in anaesthetized guinea-pigs where intravenous (Wirth *et al* 1993; Tramontana *et al* 2001; Ichinose and Barnes 1990b), intratracheal (Ichinose and Barnes 1990a) or inhaled (Sakamoto *et al* 1994) bradykinin exerted bronchoconstriction. The lack of effect of inhaled bradykinin in conscious guinea-pigs appears to be due to the rapid breakdown of bradykinin as there was a significant bronchoconstriction after treatment with captopril, an inhibitor of ACE. ACE (also known as kininase II) and NEP catalyse the breakdown of bradykinin into inactive metabolites, while kininase I produces the active metabolite [des-Arg^9] bradykinin (Decarie *et al* 1996). [des-Arg9]-bradykinin binds to B_1 receptors (Leeb-Lundberg *et al* 2005) and but does not cause any bronchoconstriction in asthmatic or normal subjects (Polosa and Holgate 1990), suggesting that B_1 receptors are not involved in the bronchoconstriction. Contrary to this clinical finding, isolate murine tracheae have been shown to express both B_1 and B_2 receptors and display contractile responses to both bradykinin and [des-Arg^9]-bradykinin which are upregulated by culture with TNF-α (Zhang *et al* 2004). ACE mediates the breakdown of bradykinin in the circulation but may also be present in the lungs (Dusser *et al* 1988). bradykinin-induced sensitization of airways sensory nerves is thought to be responsible for cough which is a major side effect of ACE inhibitors (Fox *et al* 1996). Bradykinin increases the excitatory nonadrenergic noncholinergic neural bronchoconstriction in anaesthetized guinea-pigs via B_2 receptors

(Miura *et al* 1994). Chronic ACE inhibitor treatment causes spontaneous coughing which can be inhibited with the bradykinin B_2 antagonist icatibant (Fox *et al* 1996). Phosphoramidon (i.p.) treatment had no effect but after inhalation exposure there was a bronchoconstriction similar to that seen after captopril treatment. NEP is expressed in the respiratory epithelium (Baraniuk *et al* 1995), so inhalation exposure would enable phosphoramidon to have an instant effect, whereas after i.p. injection the drug may not be reaching the lung in a concentration sufficient to have an effect. Captopril and phosphoramidon, both by instillation into the lungs, were shown to potentiate the bronchoconstriction by airways-instilled bradykinin in aneasthetized guinea-pigs, although the kininase 1 inhibitor, DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid, failed to alter the bronchoconstriction (Ichinose and Barnes 1990a). This suggested that both ACE and NEP degrade bradykinin but kininase 1 is not involved. In the present study, when capropril and phosphoramidon treatment were combined, the boronchoconstriction by bradykinin was similar to that seen with treatment with each drug individually. A similar observation was made by Ichinose *et al* (1990). This could be because this was the maximum bronchoconstriction to bradykinin. It was interesting, however, that there was also no prolongation of the bronchoconstriction when the two inhibitors were combined.

Thus, either ACE or NEP had to be inhibited to prevent its breakdown before a bronchoconstriction to inhaled bradykinin could be observed in conscious guinea-pigs. Kininase I is probably not involved in the metabolism of inhaled bradykinin as this enzyme is localized to the blood (Proud and Kaplan 1988). The mechanism of this

bronchoconstriction by the inhaled route is probably via a neural mechanism. Atropine, to block parasympathetic muscarinic pathways and capsaicin, to deplete sensory neurone tachykinins, blocked the responses in anaesthetized guinea-pigs to instilled bradykinin but not intravenous bradykinin (Ichinose *et al* 1990). Intravenous bradykinin mediates bronchoconstriction mainly by release of cyclooxygenase products, since it was attenuated by indomethacin (Ichinose *et al* 1990).

The bradykinin B_2 receptor antagonist MEN16132 (1 and 10μM inhalation exposure) blocked the bronchoconstriction to 1mM bradykinin exposure but icatibant (10μM inhalation exposure) had no effect. These results are similar to those by Valenti *et al* (2005), who have shown that MEN16132 is more potent and longer lasing than icatibant in inhibiting the bradykinin induced bronchoconstriction and microvascular leakage in anaesthetized guinea-pigs. MEN16132 administered i.p. had no effect in our study, possibly because the drug was not absorbed by this route and did not reach the lungs in enough concentration to have an effect, although in the study of Valenti *et al* (2005) it was effective by intravenous administration.

After bradykinin inhalation exposure with captopril treatment there was no significant increase in inflammatory cells in the lung but after inhalation exposure with both captopril and phosphoramidon there was significant increases in total cells, macrophages, eosinophils and neutrophils. This suggests that the threshold for leukocyte influx was higher than for bronchoconstriction, since both degradative enzymes were required to be blocked. After treatment with the B_2 receptor antagonist MEN16132 (10μM inhalation

exposure) the increases in total cells, eosinophils, neutrophils and lymphocytes were significantly inhibited and there was a modest inhibition of the macrophage increase. This study shows that bradykinin can produce an influx of inflammatory cell influx but only by prolonging the duration of bradykinin in the lung by inhibition of both ACE and NEP. Although bradykinin is not thought to have much direct effect on activation and recruitment of inflammatory cells, it is thought to work indirectly through the release of monocyte and neutrophil chemotactic activity from the airway epithelial cells (Koyama *et al* 1995), to cause release of neutrophil, monocyte and eosinophilic factors from alveolar macrophages (Sato *et al* 1996) and also to stimulate eotaxin release from human lung fibroblasts (Sato *et al* 2000). The inhibition of the bradykinin-induced inflammatory cell influx by MEN161 indicates that it is mediated through the bradykinin B_2 receptor.

Airway hyperreactivity to inhaled bradykinin

Bradykinin exposure had no effect in sensitized guinea-pigs in the absence of enzyme inhibitors. Thus, sensitization alone is not sufficient to induce a bronchoconstriction to bradykinin. This contrasts with another indirect bronchoconstrictor adenosine, which shows no bronchoconstriction in normal subjects (Cushley *et al* 1983) and unsensitized guinea pigs (Smith and Broadley 2008) but produces a bronchoconstriction in asthmatic subjects and sensitized guinea-pigs. However, in sensitized guinea-pigs, 24 hours after ovalbumen exposure there was a significant bronchoconstrictor response to bradykinin. This was achieved without inhibition of ACE or NEP. This indicates that ovalbumen induces airway hyperreactivity to bradykinin. Since ovalbumen challenge causes airway hyperreactivity to other directly acting spasmogens, including histamine and

methacholine (Toward and Broadley 2004; Smith and Broadley 2007), these results demonstrate that the hyperreactivity is extended to the indirect spasmogen bradykinin. Whether this is because of a common mechanism cannot be deduced from the present study. It is likely due to epithelial damage caused by the ovalbumen challenge. This would expose the sensory nerves through which bradykinin acts after inhalation (Ichinose *et al* 1990) or result in loss of the epithelium-derived NEP. Allergen-induced airways hyperreactivity to bradykinin does not appear to have been demonstrated previously in guinea-pigs and these results provide a basis for the appearance of bronchoconstriction in asthmatics but not in normal subjects after bradykinin inhalation (Fuller *et al* 1987; Polosa and Holgate 1990).

Effect of MEN16132 on PIV-3 induced airway inflammation

PIV-3 inoculation of guinea-pigs caused AHR to inhaled histamine and inflammatory cell influx in the BALF. The bradykinin B_2 antagonist abolished these effects after PIV-3 inoculation. Previously we have shown that the PIV induced inflammation can be reduced by decreasing bradykinin levels by inhibition of the enzyme tissue kallikrein with FE999024 (chapter 4). The inhibition of the PIV-3 induced inflammation with MEN16132 shows that the bradykinin-induced inflammation is mediated through the bradykinin B_2 receptor. Folkerts *et al* (2000) also showed that the B_2 receptor antagonist, icatibant, administered subcutaneously inhibited the AHR to intravenously administered histamine in anaesthetized guinea-pigs treated with PIV-3. However, they did not show any reduction in cell influx after icatibant. In their study, there was a tendency for an

increase in bradykinin levels in BALF after PIV-3 infection, which supports the idea that bradykinin is involved in the AHR and cell influx caused by PIV-3 infection.

Chapter 6

Viral exacerbations in ovalbumen allergic model

6.1 Introduction

Asthma is a chronic inflammatory disease of the airways which is characterized by bronchial hyper-responsiveness, an influx of inflammatory cells, epithelial damage and bronchiolar fibrosis (Cohn *et al* 2005). Inhalation of antigen in patients with atopic asthma results in an early and late phase brochoconstrictions and AHR (Payne and DeNucci 1987). Various groups have reproduced these asthmatic effects in animal studies. Smith and Broadley (2007) have shown that in ovalbumen sensitized guinea-pigs, ovalbumen exposure results in early and late phase bronchoconstrictions, AHR to inhaled histamine and an influx of inflammatory cells.

Viral respiratory infections are the most common cause of asthma exacerbations. Along with respiratory syncytial virus and rhinovirus, PIV has been implicated in the pathogenesis and exacerbations of asthma (Frick *et al* 1979). In this study we attempted to produce a model of viral exacerbation of asthma by combining the ovalbumen guinea-pig model of allergy with the PIV-3 inoculation model (chapter3).

Glucocorticoids are widely used for the management of asthma (Spahn and Leung 1996).Glucocorticoids have many anti-inflammatory actions including inhibition of cytokine production and release, reduction of circulating inflammatory cells and inhibition of the production and release of arachidonic acid metabolites (Schleimer 1988). Toward and Broadley (2004) have shown that dexamethasone inhibits the late bronchoconstriction, AHR, and leukocyte influx induced by ovalbumen exposure in ovalbumen sensitized guinea-pigs. In this study we attempted to confirm this

dexamethasone induced inhibition of the inflammation seen in the ovalbumen allergic model of allergy in the guinea-pig. We also attempted to establish an exacerbation of the ovalbumen-induced responses by PIV-3 virus inoculation in the guinea-pig and to determine the effectiveness of dexamethasone in this model.

6.2 Methods

Ovalbumen sensitization and exposure

On days 1 and 5 guinea-pigs were sensitized with a bilateral i.p. injection of a suspension containing 100μg of ovalbumen and 100mg of aluminium hydroxide, which is an adjuvant and increases the immune response. On day 15 the animals were placed in a steel exposure chamber and given a 60 minute inhalation exposure of ovalbumen (0.01%). Lung function measurements were taken by whole body plethysmography immediately before challenge and at 0, 15, 30, 45 and 60 minutes after, then hourly up to 12 hours and finally at 24 hours.

Histamine exposure

Animals were given a 20 second, nose only histamine exposure on the day before and the day after ovalbumen exposure (see pharmacological methods: measurement of airway reactivity).

Treatment with dexamethasone

Animal were treated twice daily with dexamethasone (10mg/kg in saline) or saline control by bilateral subcutaneous injection from day 8 to day 14 and 1 hour before ovalbumen exposure on day 15.

Bronchoalveolar lavage

Within 30 minutes of the final histamine exposure the animals were sacrificed with an overdose of anaesthetic, pentobarbital sodium, by bilateral intraperitoneal injection and a bronchoalveolar lavage was performed (see general methods 2.1.4).

6.3 Results

6.3.1 Ovalbumen exposure without virus

Effect of ovalbumen exposure

In the saline treated group there was a 68.3 ± 5.3 % decrease in sG_{aw} from baseline following ovalbumen exposure. After 6 hours sG_{aw} had returned to baseline levels. This was followed by a secondary 26.8 ± 3.3% decrease in sG_{aw} which began at 8 hours and peaked at 10 hours (Fig 6.1).

In the dexamethasone treated group there was also an immediate decrease in sG_{aw} from baseline (45.4 ± 6.6%) following ovalbumen exposure. This decrease was less than seen in the saline treated group and sG_{aw} levels returned to baseline levels more quickly (3 hours). There was no secondary decrease in sG_{aw} levels (Fig 6.1).

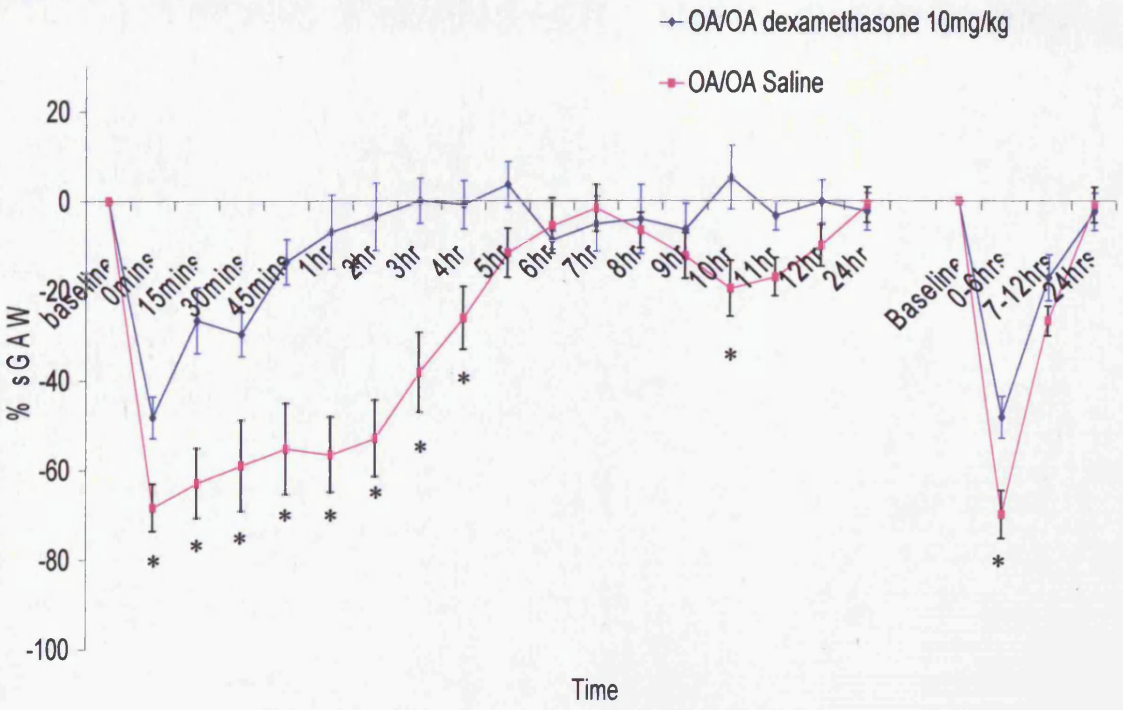


Figure 6.1

Effect of ovalbumen sensitization and exposure on airway function after treatment with saline or dexamethasone (10mg/kg, s.c.). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown up to 24 hours after ovalbumen exposure. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point between the saline and dexamethasone treated groups as determined by a Student's *t* test (paired).

Airway reactivity to histamine

After ovalbumen sensitization but before ovalbumen exposure, there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Fig. 6.2 and 6.3).

24 hours after ovalbumen exposure in the saline treated group there was a significant 16.5% reduction in sG_{aw} from baseline levels (Fig 6.2).

In the dexamethasone (10mg/kg) treated group there was no reduction in sG_{aw} after ovalbumen exposure (Fig 6.3).

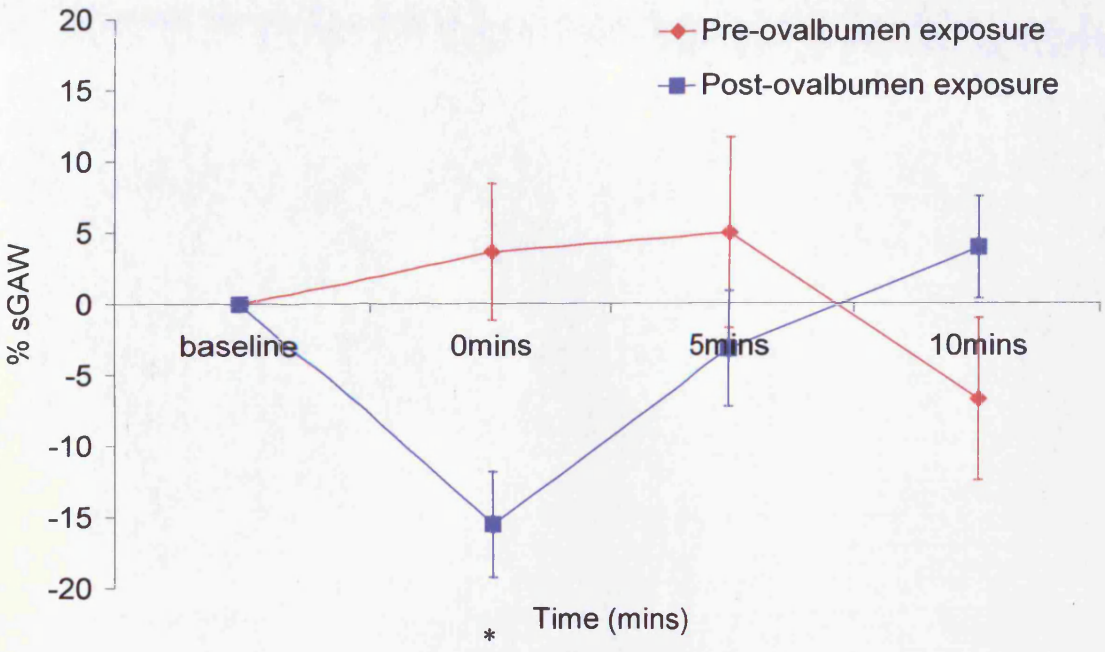


Figure 6.2

Effect of saline treatment on airway function in ovalbumen sensitized (i.p., 0.1 mg/kg) guinea-pigs before and after ovalbumen exposure (0.01% nebulised, 1 hour). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after ovalbumen exposure as determined by a Student's *t* test (paired).

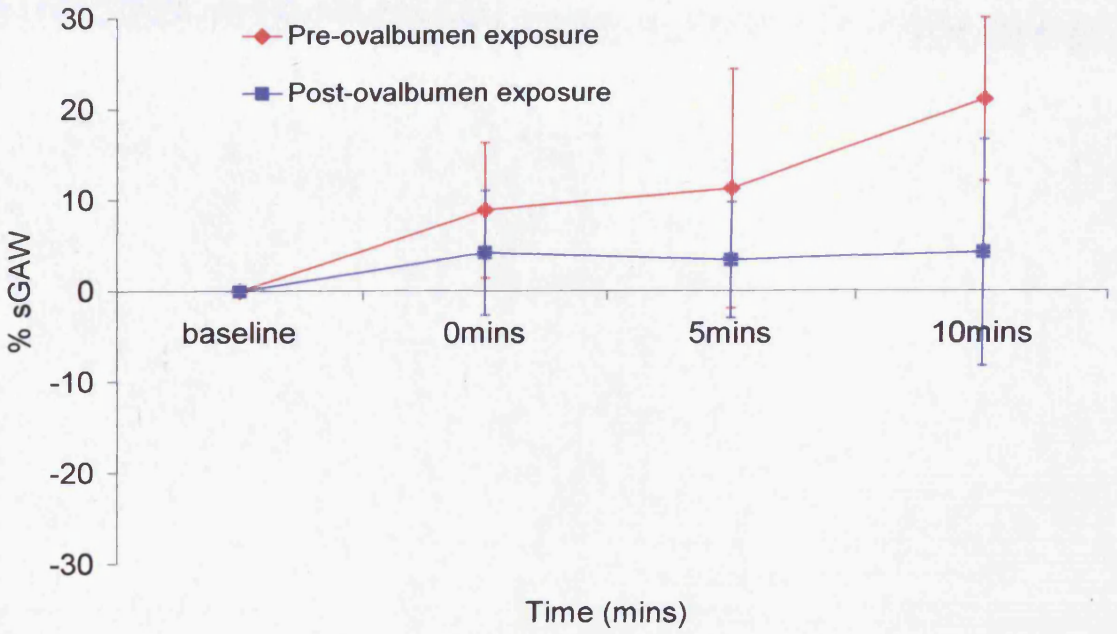


Figure 6.3

Effect of dexamethasone (10mg/kg) treatment on airway function in ovalbumen sensitized (i.p., 0.1 mg/kg) guinea-pigs before and after ovalbumen exposure (0.01% nebulised, 1 hour). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. There was no significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after ovalbumen exposure as determined by a Student's *t* test (paired).

Bronchoalveolar lavage

In ovalbumen sensitized, saline challenged and saline treated guinea pigs the numbers of macrophages, eosinophils, neutrophils and lymphocytes in the BALF were as follows; total cells: 4.38 ± 0.42 ($\times 10^6$/ml), macrophages: 2.31 ± 0.22 ($\times 10^6$/ml), eosinophils: 1.95 ± 0.34 ($\times 10^6$/ml), neutrophils 0.03 ± 0.01 ($\times 10^6$/ml) and lymphocytes: 0.1 ± 0.02 ($\times 10^6$/ml) (Fig. 6.4).

In the ovalubumen sensitized and challenged group treated with saline there were significant increases in total cells (12.60 ± 1.07 $\times 10^6$/ml), macrophages (6.13 ± 0.61 $\times 10^6$/ml), eosinophils (4.95 ± 0.58$\times 10^6$/ml) and neutrophils (1.32 ± 0.19$\times 10^6$/ml) and a modest in lymphocytes (0.20 ± 0.06$\times 10^6$/ml). After treatment with dexamethasone (10mg/kg, s.c.) these increases in total cells, macrophages, eosinophils and neutrophils were significantly reduced though not to baseline levels (5.11 ± 0.27, 2.74 ± 0.14, 2.05 ± 0.12 and 0.24 ± 0.04 $\times 10^6$/ml respectively). There was also a modest decrease in lymphocytes (0.07 ± 0.01 $\times 10^6$/ml) (Fig. 6.4).

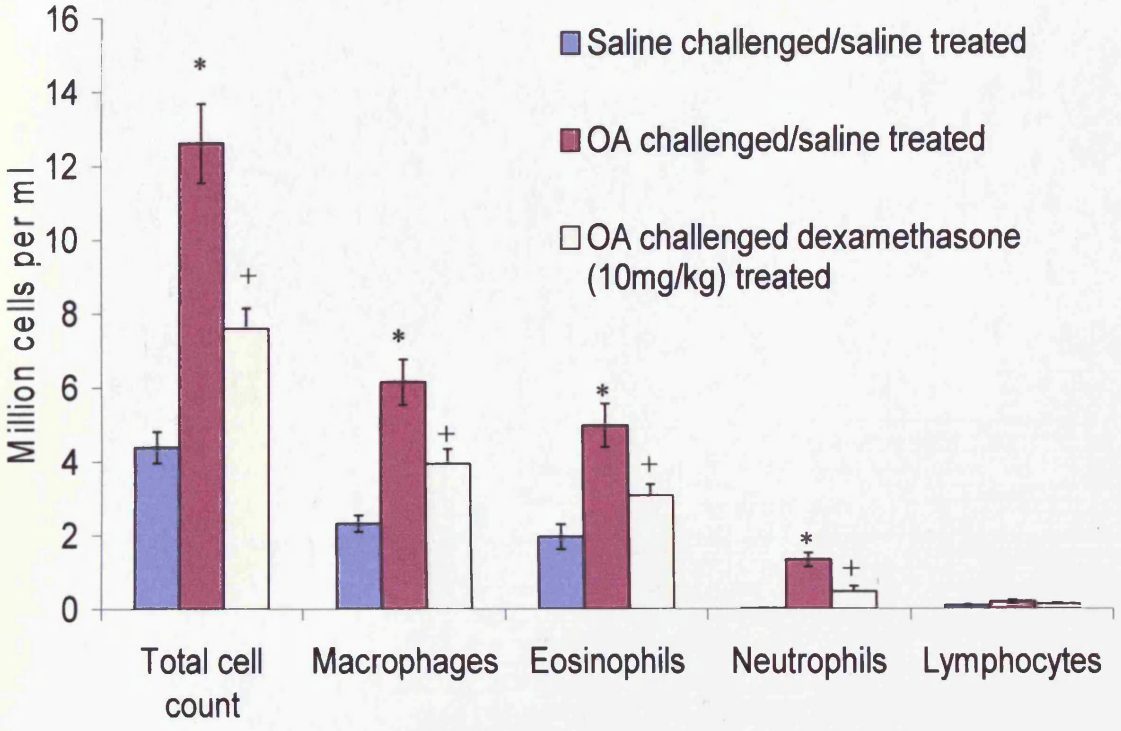


Figure 6.4

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from ovalbumen sensitized animals challenged and treated with saline, challenged with ovalbumen and treated with saline or dexamethasone (10 mg/kg). Each bar represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding saline challenged/ saline treated group and + denotes the significance ($P<0.05$) of difference compared to the OA challenged/saline treated group as determined by a Student's *t* test (paired).

6.3.2 Effect of combining PIV-3 (3.16×10^8) inoculation with ovalbumen sensitization and exposure in guinea-pigs

Effect of ovalbumen exposure

Compared to the ovalbumen sensitized and exposed group without viral inoculation there was a significantly smaller reduction in sG_{aw} at 0, 15 and 30 minutes after ovalbumen exposure compared to the PIV-3 inoculated group. However the virus free group recovered more quickly, significantly so at 7 and 8 hours. The secondary reduction in sG_{aw} in the virus free group became significantly different at 11 hours (Fig.6.5).

In the saline treated group with PIV-3 inoculation there was a 28.3 ± 4.8 % decrease in sG_{aw} from baseline immediately following ovalbumen exposure. The average maximum decrease in sG_{aw} from baseline in the first 6 hours was 44.1 ± 6.5%. There was no clear secondary decrease as sG_{aw} levels did not completely return to baseline levels until 11 hours after exposure (Fig. 6.6).

In the dexamethasone treated group there was also an immediate decrease in sG_{aw} from baseline (27.5 ± 5.2%) following ovalbumen exposure. The average maximum decrease in sG_{aw} from baseline in the first 6 hours was 36.2 ± 63.3%. sG_{aw} values did not recover completely until 24 hours. The only significant difference from the saline group was a better recovery at 8 hours (Fig 6.6).

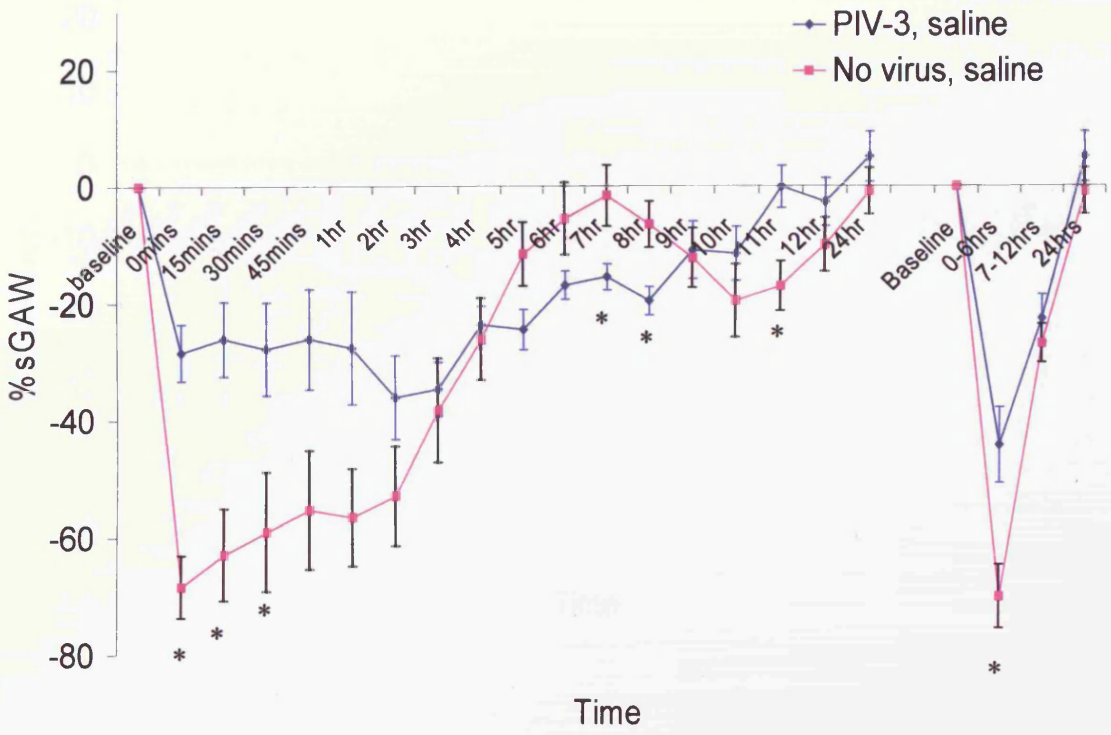


Figure 6.5
The effect on airway function of saline treatment and ovalbumen sensitization and challenge with and without PIV-3 inoculation. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown up to 24 hours after ovalbumen exposure. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point with and without virus as determined by a Student's *t* test (paired).

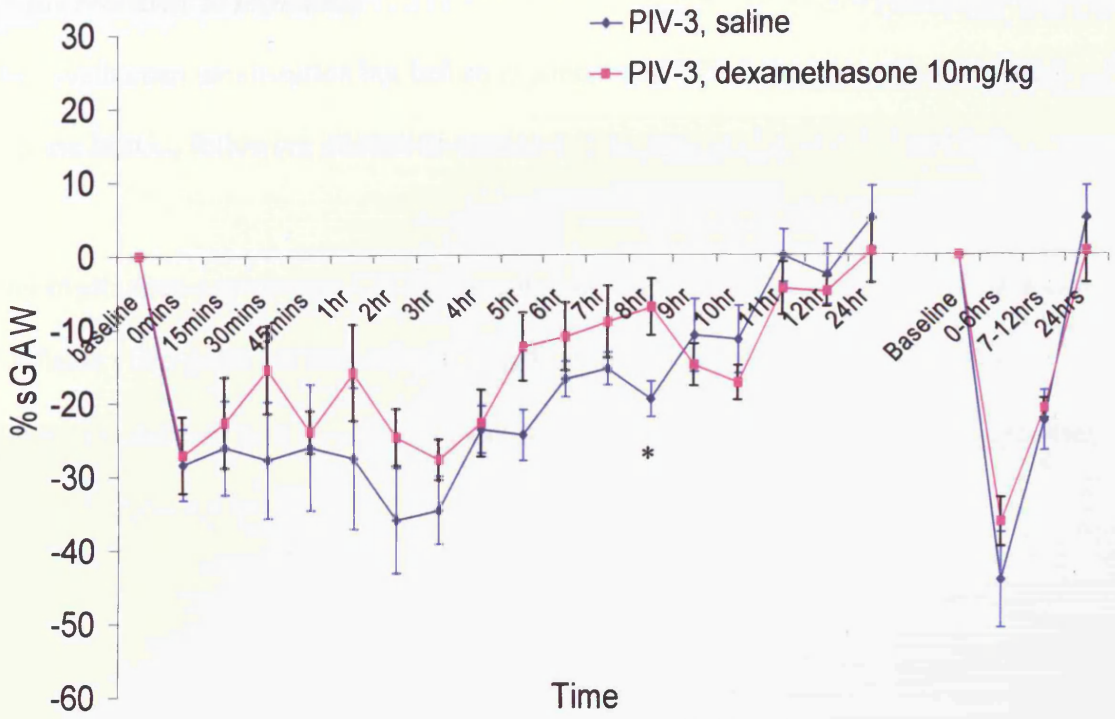


Figure 6.6

Effect of combining PIV-3 inoculation with ovalbumen sensitization and exposure on airway function after treatment with saline or dexamethasone (10mg/kg). Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown up to 24 hours after ovalbumen exposure. Negative values represent bronchoconstriction. * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point between the saline and dexamethasone treated groups as determined by a Student's *t* test (paired).

Airway reactivity to histamine

After ovalbumen sensitization but before exposure and PIV-3 there was no significant decrease in sG_{aw} following inhalation exposure to histamine (Figures 6.7 and 6.8).

After ovalbumen exposure and PIV-3 inoculation in the saline treated group there was a significant (16.9 ± 4.6 %) reduction in sG_{aw} from baseline levels. Dexamethasone (10mg/kg) treatment had no effect as there was still a significant (15.0 ± 4.8%) decrease in sG_{aw} (Figures 6.7 and 6.8).

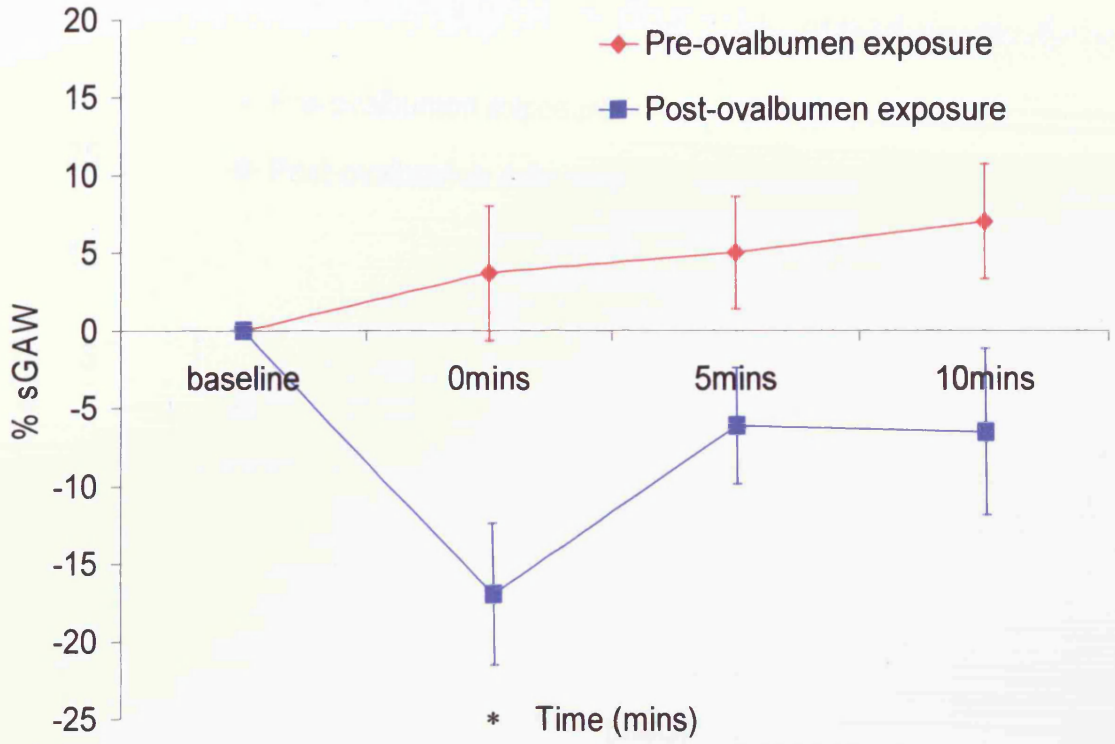


Figure 6.7

Effect of saline treatment on airway function in ovalbumen sensitized guinea-pigs before and after PIV-3 inoculation and ovalbumen exposure. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. . * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after ovalbumen exposure as determined by a Student's *t* test (paired).

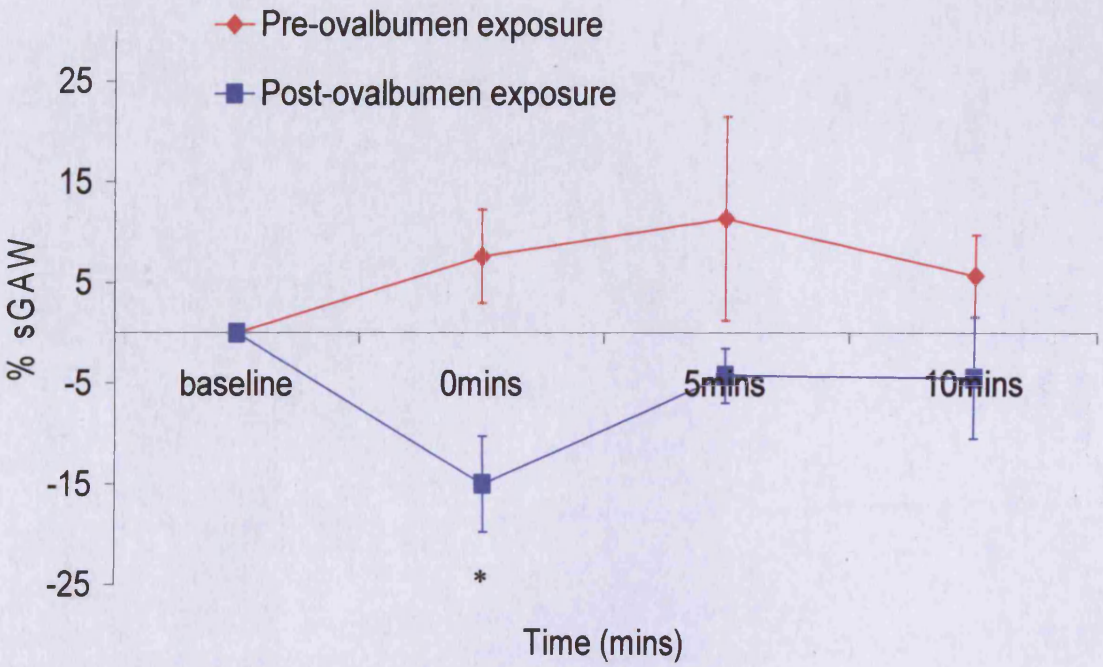


Figure 6.8

Effect of dexamethasone treatment on airway function in ovalbumen sensitized guinea-pigs before and after PIV-3 inoculation and ovalbumen exposure. Each point represents the mean ± standard error of mean (n=6) change in sG_{aw} expressed as a percentage of the baseline sG_{aw} values, shown at 0, 5 and 10 minutes after a nose only 1mM histamine exposure. Negative values represent bronchoconstriction. . * denotes the significance ($P<0.05$) in difference between the changes to baseline sG_{aw} values at each time point, before and after ovalbumen exposure as determined by a Student's *t* test (paired).

Bronchoalveolar lavage

In animals receiving no virus (medium controls), the numbers of macrophages, lymphocytes, eosinophils and neutrophils in the BALF were as follows; total cells: 1.54 ± 0.17 ($\times 10^6$/ml), macrophages: 1.37 ± 0.15 ($\times 10^6$/ml), eosinophils: 0.12 ± 0.01 ($\times 10^6$/ml), lymphocytes: 0.06 ± 0.02 ($\times 10^6$/ml) and neutrophils 0.03 ± 0.01 ($\times 10^6$/ml) (Fig. 6.9).

In the PIV-3 inoculated and ovalubumen sensitized and challenged group treated with saline there were significant increases to all cell types as follows: total cells 10.39 ± 1.00, macrophages: 4.96 ± 0.44 ($\times 10^6$/ml), eosinophils: 4.36 ± 0.43 ($\times 10^6$/ml), lymphocytes: 0.39 ± 0.13 ($\times 10^6$/ml) and neutrophils 0.69 ± 0.21 ($\times 10^6$/ml). After treatment with dexamethasone (10 mg/kg) all these increases were reduced significantly to the following levels: total cells 6.29 ± 0.99, macrophages: 3.69 ± 0.49 ($\times 10^6$/ml), eosinophils: 2.23 ± 0.46 ($\times 10^6$/ml), lymphocytes: 0.14 ± 0.05 ($\times 10^6$/ml) and neutrophils 0.22 ± 0.08 ($\times 10^6$/ml) but not to baseline levels (Fig. 6.9).

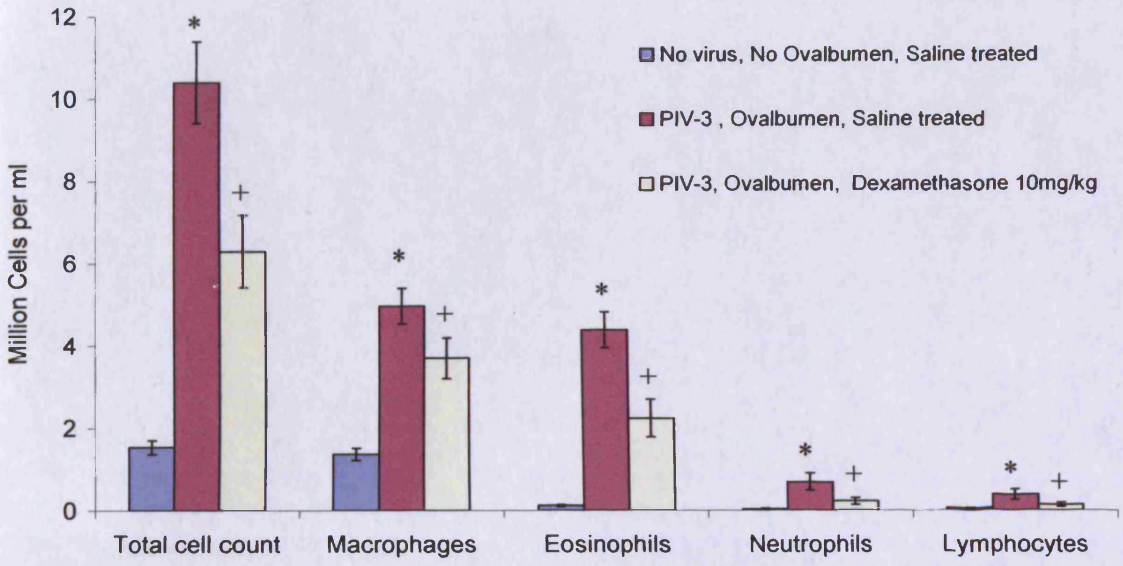


Figure 6.9

Total and differential cell (macrophage, eosinophil, lymphocyte and neutrophil) count of BALF removed from media inoculated animals treated with saline and also PIV-3 inoculated animals, sensitized and challenged with ovalbumen and treated with saline or dexamethasone (10 mg/kg). Each bar represents the mean ± standard error of mean (n=6) of total or differential count of cells per ml/10^6. * denotes the significance ($P<0.05$) of difference compared to the corresponding media inoculated/saline treated group and + denotes the significance ($P<0.05$) of difference compared to the PIV-3/ovalbumen saline treated group as determined by a Student's *t* test (paired).

6.4 Discussion

Ovalbumen sensitization and exposure without virus

Immediately following ovalbumen exposure there was a significant bronchoconstriction in ovalbumen sensitized and saline treated guinea-pigs. After 6 hours there was a recovery to baseline levels then a secondary bronchoconstriction at around 9 hours. These are the early and late asthmatic responses. These responses are also seen in asthmatics (Inman *et al* 1995). Antigen challenge in sensitized animals causes the release of histamine, leukotrienes, prostaglandin and PAF from mast cells and macrophages causing bronchoconstriction via airways smooth muscle (Brocklehurst, 1960; Piper and Vane 1969). The EAR is thought to be mediated by mast cells as mast cell derived mediators including histamine, cysteine-leukotriens PGD_2, and tryptase are found in the lavage fluid of asthmatics at this time (Lui *et al* 1991, Casale *et al* 1987). After dexamethasone treatment there was also an immediate bronchoconstriction after ovalbumen exposure in ovalbumen sensitized animals. Dexamethasone treatment reduced the immediate bronchoconstriction and also the duration as there was complete recovery after 3 hours. Corticosteroids do not usually inhibit the EAR in guinea-pig experimental models (Toward and Broadley 2004) or clinically (Cockcroft and Murdock 1987). Dexamethasone treatment abolished the secondary bronchoconstriction. The LAR is usually susceptible to corticosteroids clinically (Paggiaro *et al* 1994) and in guinea-pigs (Toward and Broadley 2004). Eosinophils are thought to play an important role in the LAR. Katoh *et al* (1991) have shown that eosinophil infiltration in the lungs is required for the development of the LAR in passively sensitized guinea pigs. In patients who

developed a LAR increased eosinophilia was found compared to patients who did not show this late response (Durham and Kay 1985).

In ovalbumen sensitized and exposed guinea-pigs there were significant increases in total cells, macrophages, eosinophils and neutrophils and a modest increase in lymphocytes after saline treatment. After dexamethasone treatment the increases in total cells, macrophages, eosinophils and neutrophils were significantly reduced though not to baseline levels. There was also a modest decrease in lymphocytes. Allergen can cause the release of cytokines including IL-3, IL-5, IL-8, GM-CSF, TNF and eotaxin from T cells causing the migration of eosinophils into the airways and their activation (Corrigan and Kay 1992).

After ovalbumen sensitization but before ovalbumen exposure there was no bronchoconstriction to a 1mM exposure to histamine. After ovalbumen sensitization and exposure there was a significant bronchoconstriction after histamine exposure indicating AHR. After dexamethasone treatment the AHR was abolished as there was no bronchoconstriction after histamine exposure in ovalbumen sensitized and exposed animals. Eosinophil accumulation in the bronchial wall is a characteristic feature of asthma (Gleich *et al* 2000). Release of eosiniphilic products including major basic protein, eosinophilic cationic protein and eosinophilic peroxidase can lead to tissue damage and are thought to play a role in the AHR seen in asthma (Pease and Williams 2001).

Ovalbumen sensitization and exposure in PIV-3 inoculated guinea-pigs

Immediately following ovalbumen exposure there was a significant bronchoconstriction in ovalbumen sensitized and saline treated guinea-pigs inoculated with PIV-3. The secondary bronchoconstriction seen after ovalbumen exposure in ovalbumen sensitized animals without virus was absent as there was a prolonged bronchoconstiction which did not recover fully for 11 hours. This represents an exacerbation of the bronchoconstrictor response to ovalbumen. This model could help with the understanding of asthma as viral respiratory infections are the most common cause of asthma exacerbations (Pattemore *et al* 1992). Dexamethasone treatment had little effect on the initial bronchoconstriction or the rate of recovery. This represents steroid resistance as this dose of dexamethasone was enough to inhibit the LAR, AHR and cell influx without virus and the cell influx in this experiment. Steroid resistance is important clinically as a small proportion of asthmatics fail to respond to even high doses of oral glucocorticocoids (Cypcar and Busse 1993). Steroid resistance can be caused by abnormalities of the steroid receptor (Lamberts *et al* 1992) or a resistance to the anti-inflammatory actions of steroids (Barnes and Adcock 1995). In COPD, HDAC2 activity and expression are reduced in the peripheral lung. Corticosteroid resistance in COPD occurs because corticosteroids use HDAC2 to switch off activated inflammatory genes (Barnes 2009).

After ovalbumen sensitization but before ovalbumen exposure and PIV-3 inoculation there was no bronchoconstriction to a 1mM exposure of histamine in saline treated guinea-pigs. After ovalbumen sensitization and exposure there was a significant bronchoconstriction after histamine exposure in PIV-3 inoculated animals indicating

AHR. Dexamethasone treatment had no effect on AHR as there was still a significant bronchoconstriction after histamine exposure.

In ovalbumen sensitized and exposed guinea-pigs inoculated with PIV-3 there were significant increases in total cells, macrophages, eosinophils, neutrophils and lymphocytes after saline treatment. After dexamethasone treatment the increases in total cells, macrophages, eosinophils, neutrophils and lymphocytes were significantly reduced though not to baseline levels. So the cellular component is not steroid resistant. The other responses (EAR, LAR and AHR) are therefore independent of cell influx.

The addition of PIV-3 to the ovalbumen model of allergy in the guinea-pig produced, in addition to the AHR and inflammatory cell influx, a prolonged bronchoconstriction as opposed to the distinct early and late phases seen without virus. The allergen induced bronchoconstriction and the AHR appear to be resistant to dexamethasone. It seems that the allergen induced bronchconstriction and the AHR are not mediated by inflammatory cells as dexamethasone inhibited the leukocyte influx but not the bronchonstriction or the AHR. It should be noted that although dexamethasone did inhibit the cell influx it was not to baseline levels.

Chapter 7

General discussion

Viral model of inflammation

One of the primary aims of this study was to develop a model of virus-induced airway inflammation in the guinea-pig which demonstrated airway hyperreactivity, inflammatory cell influx and pulmonary oedema in the form of increased wet lung weight. We used a number of different virus types, strains and titres in order to compare the effects of these different inoculums on airway inflammation and to produce sufficient inflammation to enable us to show significant inhibition after later drug intervention experiments.

A concentration of 1 x 10^8 infectious units per ml of PIV-2 produced significant inflammation in the form of airway hyperreactivity, inflammatory cell influx and increased wet lung weight. A smaller concentration of PIV-3 (6.32 x 10^6 infectious units per ml) produced a similar or slight increase in the level of inflammation. After inoculation with a higher concentration of the same strain of PIV-3 (3 x 10^8 infectious units per ml) there was an increase in airway hyperreactivity and wet lung weight but similar levels of inflammatory cell influx. PIV-3 appears to be more pathogenic than PIV-2 in the guinea-pig and the effects of PIV-3 appear to be concentration-dependent with the exception of inflammatory cell influx. It must be noted that in this experiment we were comparing two specific strains of PIV-2 and PIV-3 so the conclusions reached here may not apply to all strains of these viruses. The reason for the lack of increase in inflammatory cell influx after increasing the concentration of PIV-3 is unknown but could be due to reaching the maximum effect with this virus.

Inoculation with the WSN33 strain of influenza H1N1 grown in MDCK cells produced a small increase in inflammatory cells but no airway hyperreactivity or increased wet lung weight. After inoculation with a similar concentration of the A/PR/8/34 strain of H1N1 influenza grown in chicken embryos there was a significant increase in inflammatory cells, a small increase in wet lung weight but still no airway hyperreactivity. Inoculation with an increased concentration of the A/PR/8/34 strain did produce airway hyperreactivity and a further small increase in wet lung weight. The inflammatory cells were increased compared to control but decreased compared to the lower concentration of the same virus. The reason for these contradictory results on inflammatory cell influx is unknown but it is interesting to note that there was a similar lack of effect with the increased concentration of PIV-3. In this experiment the virus grown in chicken embryos appears to be more pathogenic in guinea-pigs but once more it must be noted that we were comparing two different strains of virus. An interesting additional experiment would be to further increase the concentration in the inoculum of the A/PR/8/34 strain of H1N1 influenza grown in chicken embryos to determine whether the level of airway hyperreactivity increased and clarify the effects on inflammatory cell influx.

Steroid intervention

Dexamethasone (10mg/kg in all experiments) abolished the airway hyperreactivity after PIV inoculation in all experiments. Dexamethasone reduced the inflammatory cell influx after all PIV inoculation, significantly with PIV-3 but to a lesser extent after PIV-2. The reason for the lack of significance in the PIV-2 experiment is likely due to the smaller inflammatory cell influx seen in the 50% DMSO control. Dexamethasone also reduced

the increase in wet weight seen after PIV inoculation. In the PIV-2 and PIV-3 (lower concentration) experiments dexamethasone was administered in 50% DMSO so the control was also 50% DMSO. In most of these control experiments DMSO appeared to have an inhibitory effect on the PIV induced inflammation compared to the saline control (for the FE999024 experiments). Due to this effect we reduced the DMSO concentration in the control and dexamethasone experiments to 25%. In subsequent experiments this inhibitory effect of DMSO effect was not seen. Anti-inflammatory effects of DMSO have been reported previously (Santos *et al* 2003). It has been shown to inhibit IL-8 production by human whole blood and decreases NF-κB activation in a macrophage cell line and TNF-α activity (Kelly *et al* 1994).

Dexamethasone reduced the airway hyperreactivity seen after inoculation with the A/PR/8/34 strain of influenza H1N1 grown in chicken embryo. Dexamethasone also significantly reduced the inflammatory cell influx and reduced the small increase in wet lung weight to baseline levels. These actions of dexamethasone can be attributed to its inhibitory effects on transcription of pro-inflammatory cytokines (Barnes and Adcock 1993).

Dexamethasone also reduced the amount of live PIV-3 recovered from the lung tissue and subsequently re-grown in VERO cells. Dexamethasone did not have a significant effect due to the large amount of variation in virus recovered from each animal. This is confirmed by a previous study which shows that dexamethasone reduced the titres of virus recovered from the lung after sendai virus (PIV-1) infection in guinea pigs (Moreno

et al 2003). The mechanism of this antiviral action of dexamethasone may be on the epithelial cells which are the primary target for viral entry to the lungs. Dexamethasone may inhibit key molecules involved in viral entry and replication (Moscona 2005).

Effects of bradykinin on the airways

Bradykinin exposure (1mM) with inhibition of ACE by captopril and/or NEP with phophoramidon (inhalation but not i.p.) produced a significant bronchoconstriction in conscious guinea-pigs. Captopril alone, phosphoramidon alone and both phophoramidon together all produced similar sized bronchoconstrictions after bradykinin exposure. This bronchoconstriction was blocked with the selective bradykinin B_2 antagonist MEN16132 (inhalation but not i.p.) but not by icatibant (inhalation). This confirms previous reports that showed bronchoconstriction was mediated via bradykinin B_2 receptors (Fuller *et al* 1987).

There was no significant increase in inflammatory cells after bradykinin exposure after captopril treatment alone but after both captopril and phosphoramidon there was a significant increase. This appears to indicate that inhibition of both ACE and NEP are required to prolong the duration of bradykinin in the lung long enough to have an effect on inflammatory cell influx. To confirm this conclusion an additional experiment would be required with phosphoramidon but without captopril as phosphoramidon may be having the dominant effect. Therefore under the right conditions, inhaled bradykinin increases cell influx and bronchoconstriction in conscious guinea-pigs. This finding of inflammatory cell influx does not appear to have been made before. The mechanism may

involve release of neurokinins from sensory nerve endings since SP and NKA are known to cause leakage of inflammatory cells (Nénana *et al* 2001). It would be interesting to examine whether neurokinin inhibitors block the inflammatory cell influx induced by bradykinin.

The role of bradykinin in airway inflammation

The lowest dose (1mg/kg) of tissue kallikrein inhibitor FE999024 had little effect on the PIV-3 induced airway inflammation in the guinea pigs. In fact the level of airway hyperreactivity was increased slightly. The 3mg/kg dose generally had a small but incomplete inhibitory effect on the PIV induced inflammation. The highest dose (10mg/kg) completely abolished the airway hyperreactivity but had variable effects on inflammatory cell influx and wet lung weight. There was inhibition of the cell influx in the PIV-2 and the lower concentration of PIV-3 by the 10mg/kg dose but no effect after inoculation with the higher concentration of PIV-3. This lack of effect could be due to the effect of the increased inflammation induced by the higher concentration of PIV-3 overcoming the inhibitory effect of FE999024 even at the 10 mg/kg dose. In the PIV-3 experiments only the highest dose of FE999024 had a significant inhibitory effect on the increase in wet lung weight. In the PIV-2 experiments no dose of FE999024 had a significant effect on wet lung weight. In these experiments the lowest dose of FE999024 had the strongest inhibitory effect and the highest dose had the weakest effect. The reason for this is unknown. Inhibition suggests that bradykinin has a role in inflammation arising from viral infection. Christiansen *et al* (1992) have shown that kinin levels are increased in BALF of asthmatics after allergen challenge.

In PIV-3 (high concentration) inoculated guinea-pigs the bradykinin B_2 antagonist MEN16132 abolished the airway hyperreactivity and reduced the inflammatory cell influx seen in the control. Previous studies have shown that icatibant can inhibit PIV-3 induced inflammation (Folkerts *et al* 1997) but this may be the first study to use MEN16132. The inhibition of the PIV-3 induced inflammatory cell influx with MEN16132 appears to contradict the previous result in this study where even at the highest dose used the tissue kallikrein inhibitor FE999024 failed to block the cell influx induced by inoculation with the same concentration of PIV-3. FE999024 has its effect by reducing bradykinin levels and MEN16132 acts through blocking bradykinin B_2 receptors so it is interesting that only MEN16132 can block this bradykinin mediated cell influx. This indicates that even after treatment with FE999024 there may still be some residual bradykinin activity at the B_2 receptors. B_2 receptor expression can be up-regulated in airway inflammation (Huang *et al* 1999; Christiansen *et al* 2002) and this could be an important factor.

Ovalbumen allergic model

After ovalbumen exposure in ovalbumen sensitized guinea-pigs there was an immediate bronchoconstriction followed by recovery and a secondary bronchoconstriction peaking at 8 hours which mimics the EAR and LAR seen in asthma. These are the well established responses of conscious guinea-pigs to ovalbumen challenge (Smith and Broadley 2007). There was also airway hyperreactivity to histamine and inflammatory cell influx. Bradykinin also displayed airway hyperreactivity to its bronchoconstrictor

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effect which represents a novel finding. This was achieved in the absence of any metabolism inhibitors. This is similar to the effect seen in asthmatics where inhalation of bradykinin causes bronchoconstriction, but has little or no effect in non-asthmatics (Fuller *et al* 1987). The LAR and the airway hyperreactivity were blocked with dexamethasone and the inflammatory cell influx was also reduced.

Viral exacerbations in ovalbumin allergic model

After ovalbumin exposure in ovalbumin sensitized guinea-pigs inoculated with PIV-3 there was an immediate prolonged bronchoconstriction which did not fully recover until 11 hours. There was airway hyperreactivity and inflammatory cell influx. This represents an exacerbation of the response to allergen exposure and mimics the exacerbations of asthma that occur with viral infection (Frick *et al* 1979). Dexamethasone had little effect on the immediate prolonged bronchconstriction, or the airway hyperreactivity but did reduce the inflammatory cell influx. This indicates steroid resistance in a guinea-pig model of viral exacerbations of allergy and is novel to this study. This is therefore a model for steroid resistance in humans which is known to be brought about by viral infections (Macek *et al* 1994).

Future experiments

In this study we inhibited PIV-induced inflammation with the bradykinin inhibitors FE999024 and MEN16132 and also dexamethasone and treated the influenza-induced inflammation with dexamethasone. We have also shown a role for bradykinin in virus induced inflammation. It would be interesting to attempt to treat the ovalbumin and

influenza induced inflammation with the bradykinin inhibitors. If successful in treating the allergic model with either of the bradykinin inhibitors then a further experiment would be to treat the viral exacerbation of allergy model with this drug. Having established the model of steroid resistance it would be beneficial to determine the underlying mechanisms of steroid resistance in human asthma. For example, are the levels of HDAC-2 altered and does this serve as a marker of steroid resistance. The present studies therefore provide the first steps in establishing the underlying mechanisms for steroid resistant asthma and possible ways in the future for developing new treatments for overcoming this major problem in asthma management.

Conclusions

This study confirms the role of bradykinin in viral inflammation. In this study we used virus models but B_2 antagonists and kallikrein inhibitors may also have application in other forms of airway inflammation such as asthma.

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Appendix

Poiseulle's Law:

(1) $\Delta P = 8l\mu v / \pi r$ where ΔP = drop in pressure due to friction

L = length of tube

μ = viscosity

v = flow rate

r = radius

Poiseulle's law describes laminar flow in smooth walled vessels where there is no appreciable turbulence. This equation can also be used to describe the changes in pressure in the airways in response to changes in airway length and radius.

Flow through the airway is driven by the pressure difference (P_d) between the upper airway pressure at the mouth (P_m) and the peripheral airway pressure at the alveoli (P_{alv}).

$P_d = P_m - P_{alv}$

Airway resistance (R_{aw}) is defined as the relationship between instantaneous flow (v) and the pressure difference (P_d) between the mouth and the alveoli

. $R_{aw} = P_d/v$

Airway conductance (G_{aw}) is describes as the reciprocal of resistance

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

To allow comparison between differences in thoracic gas volume (TGV) in individuals sG_{aw} is often used.

(2) $sG_{aw} = v/P_d \times TGV$

Boyle's law states that in a sealed box, at constant temperature, changes in pressure are inversely related to changes in gas volume. Therefore at constant temperature:

$$P1 \times V1 = P2 \times V2$$

or

$$P \times V + (P + \delta P) \times (V - \delta V)$$

By multiplying the two brackets:

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

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

$V \times \delta P = P \times \delta V$

In a sealed plethysmography chamber, changes in box pressure result from the difference in chest volume (V_c) and respired air volume changes (V_r) at atmospheric pressure (P_{atm}), corrected for saturated water vapour pressure (P_{svp}).

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

For Poiseulle's law to be true, flow must be laminar and so v must tend towards zero. This occurs at end during end expiration and end inspiration. In this study measurements were taken at the end of expiration

By substitution equation **(2)** into **(3)** gives

$sG_{aw} = \delta v / \delta(V_{chest} - V_{respir}) \times (P_{atm} - P_{svp})$

Use of the Biopac data acquisition system allows measurement of $\delta(V_{chest} - V_{respir})$ 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.

Atmospheric pressure (P_{atm}) = 1010 cm H_20

Saturated vapour pressure (P_{svp}) = 63 cm H_20

Therefore sG_{aw} can be calculated from the following equation

$sG_{aw} = \delta v / \delta(V_{box}) \times 947 \times cf\ s^{-1}$ cm H_20

cf is the correction factor to allow for the displacement of air by each guinea pig.

The net volume of the plethysmography chamber after displacement by the guinea pig is:

V_n (litres) = $V_{box} - V_{gp}$

Therefore:

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

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

$cf = 1 - W_{gp} / P_{gp} \times P_{box}$

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

Therefore sG_{aw} can be calculated by the following equation:

$$sG_{aw} = \delta(\text{flow})/\ \delta(\text{box volume}) \times 947 \times (1 - W_{gp}/5.885)\ s^{-1}\ \text{cm}\ H_20$$