

**AIRWAY FUNCTION, INFLAMMATION AND  
PULMONARY HISTOPATHOLOGY  
FOLLOWING PARAINFLUENZA-3 VIRUS  
INFECTION**

**A thesis in accordance with the conditions governing candidates for the degree of**

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**Presented by**

**Sharon Michelle Chidgey B.Sc. (Hons)**

**Division of Pharmacology  
Welsh School of Pharmacy  
Cardiff University**

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## ABSTRACT

Human parainfluenza viruses (PIV) 1, 2, 3 and 4 (A and B) cause approximately 39-40% of all acute respiratory infections in infants and children for which there is no effective therapy. Firstly, this thesis was aimed at ascertaining a suitable guinea pig model of PIV-3 infection by determining the most efficient route of virus application. Secondly, to ascertain if a temporal association may be established during the time course of infection (Day 1-40) by measuring the following parameters: body weight, rectal temperature, airway function ( $sG_{aw}$ ), airways reactivity to inhaled histamine, inflammatory cell infiltration to the lungs measured by bronchoalveolar lavage, wet lung weights, inflammatory markers (nitric oxide and total protein levels), lung histological analysis and recovery of the virus from bronchoalveolar lavage fluid and lung tissue. Further to this, experiments were also designed to determine the impact of the glucocorticoid, dexamethasone (*in vivo* and *in vitro*) and the phosphodiesterase inhibitor, rolipram (*in vivo*). Lastly, this study ascertained the effect of pre-sensitisation with antigen on the responses of antigen in PIV-3 infected guinea pigs.

These investigations have shown guinea pigs to be a suitable model for PIV-3 infection and intranasal inoculation is the most efficient route of virus application. In addition, these investigations also established a temporal association between the time course of PIV-3 infection including airway reactivity to histamine, airway function ( $sG_{aw}$ ), airways reactivity to inhaled histamine, inflammatory cell infiltration, wet lung weights, inflammatory markers (nitric oxide and total protein levels), pulmonary histopathology and recovery of the virus from bronchoalveolar lavage fluid and lung tissue. Pre-treatment of PIV-3 infected guinea pigs with the glucocorticoid, dexamethasone and the phosphodiesterase inhibitor, rolipram (*in vitro*) ameliorated the inflammatory response and airway hyperreactivity. Unlike rolipram, dexamethasone also reduced viral titre (*in vivo* and *in vitro*), supporting a role for the anti-viral effects of dexamethasone. The anti-inflammatory effects of dexamethasone are well known and it has been speculated by other authors which maybe caused by decreased viral receptors on the epithelial cells via inhibition of intracellular adhesion molecule-1 expression. Finally, in this study PIV-3 infection, enhanced the effect of pre-allergen sensitisation, which may arise from increased permeability of the airway mucosa to allergens, due to damage of the respiratory epithelium and increased recruitment of dendritic cells. In summary, this thesis has established a clearly defined efficacy of dexamethasone use in the treatment of PIV-3 infection.

# CHAPTER 1

## GENERAL INTRODUCTION

The world health organisation estimates that approximately 14 million people die each year from infections that are transmitted via the respiratory tract, most of which occur in childhood. Respiratory infections are common in both adults and children. Most are fairly mild and self limiting and confined to the upper respiratory tract (URT). However, in infants and children, URT infections may spread downwards and cause more severe infections and even death. Viruses are the most common cause of lower respiratory tract (LRT) infection in infants and young children and are a major public health problem in this age group. Costs attributable to viral LRT infections in both outpatient and inpatient settings are an important burden on national healthcare budgets (Miedema *et al*; 2001). Each year approximately 3% of all children less than 1 year of age need to be admitted to hospital with moderate or severe LRT infection in the United States (Shay *et al*; 2001)

Viruses belonging to the *paramyxovirus* family, particularly respiratory syncytial virus (RSV), and the parainfluenza viruses (PIV's) cause the majority of childhood cases of croup, bronchiolitis, and pneumonia worldwide (Collins *et al*; 1996). PIV's cause approximately 39-40% of all acute respiratory infections in infants and children. Among the *paramyxoviruses* four parainfluenza serotypes are now recognised 1, 2, 3 and 4 (A and B). PIV-3 is the predominant cause of croup in young infants, while PIV types 1 and 2 infect older children and adolescents (Collins *et al*; 1996).

## EPIDEMIOLOGY AND CLINICAL FEATURES

PIV's are community-acquired respiratory pathogens without ethnic, socioeconomic, gender, age, or geographic boundaries. PIV's are common causes of URT infections in infants, children, and adults. Several patient groups are also at high risk of a severe LRT infections including the immunocompromised, those with chronic diseases (e.g. heart and lung disease and asthma) and the elderly (Glezen *et al*; 1984, Whimbey *et al*; 1997, Glezen *et al*; 2000). Immunity to PIV is incomplete, and infections occur throughout life, however less is known about infections in adults.

Biennial autumn epidemics are the hallmark of PIV-1 and occur in both hemispheres (Marx *et al*; 1999). Reports have suggested that a minimum of 50% of croup cases are caused by this virus (Denny & Clyde; 1986, Marx *et al*; 1997). In PIV-1 epidemics, an estimated 18,000 to 35,000 children younger than 5 years are hospitalised in the United States (Glezen *et al*; 1984, Denny & Clyde; 1986). Some of these children have bronchiolitis, tracheobronchitis, pneumonia, and a febrile wheezing. The majority of infections occur in children aged 7 to 36 months, with a peak incidence in the second and third year of life. PIV-1 can cause LRT infections in young infants but is rare in those younger than 1 month.

The full burden of PIV-1 in adults and the elderly has not been determined, but several studies have shown this virus to cause yearly hospitalisations in healthy adults and perhaps play a role in bacterial pneumonias and deaths in nursing home residents (Marx *et al*; 1999). PIV-1, PIV-2 and PIV-3 have all been found to occur at low levels in most months of the year, similar to RSV and influenza virus. PIV-2 has been reported to cause infections biennially with PIV-1 or in alternate years with PIV-1 or to cause yearly outbreaks (Downham *et al*; 1974).

PIV-2 causes all of the typical LRT syndromes (croup, bronchiolitis, pneumonia, tracheobronchitis) but in non-immunocompromised or chronically ill children, croup is the most frequent syndrome brought to medical attention. LRT infections caused by this virus has been reported less frequently than with PIV-1 and PIV-3, which maybe due to difficulties in isolation and detection. As many as 6,000 children younger than 18 years may be hospitalised each year in the United States because of PIV-2 (Glezen *et al*; 1984, Denny & Clyde; 1986). About 60% of all PIV-2 infections occur in children younger than 5 years, and although the peak incidence is between 1 and 2 years of age, significant numbers of infants younger than 1 year are hospitalised each year. PIV-2 is often overshadowed by PIV-1 or PIV-3 infections, yet in any one year or location it can be the most common cause of PIV LRT infections in young children.

Young infants (younger than 6 months) are particularly vulnerable to infection with PIV-3. Unlike the other PIV strains, 40% of PIV-3 infections occur in the first year of life with bronchiolitis and pneumonia being the most common clinical presentations. Only RSV causes more LRT infections in neonates and young infants. Approximately 18,000 infants and children are hospitalised each year in the United States because of LRT infection caused by PIV-3 (Glezen *et al*; 1984, Denny & Clyde; 1986). This virus causes yearly spring and summer epidemics in North America and Europe and is somewhat endemic, especially in the immunocompromised and chronically ill. Although the exact reasons for the different seasonality of the PIV types is unknown, differences in ambient climate conditions maybe responsible (Glezen *et al*; 1984). Only a small number of studies have reported on the isolation or epidemiology of PIV-4 (Gardener; 1969). These cases and data are distributed fairly equally between infants younger than 1 year, preschool children, school age children and adults. Seroprevalence studies have demonstrated that 60 to 84% of infants have significant antibody levels after birth (presumably maternal in origin).

These levels drop to 7-9% by 7-12 months of age and stay low for several years before increasing to about 50% by 3-5 years of age. Antibody levels to PIV-4 continue to rise throughout childhood until approximately 95% of adults have antibody to PIV-4 A and 75% have antibody to PIV-4 B (Gardner; 1969). Interestingly, the majority of PIV-4 clinical isolates appear to be subtype B. All of the different respiratory tract syndromes can be caused by PIV-4. Although, hospitalisation of infants and young children secondary to PIV-4 LRT infections have been reported, serious disease is either rare or difficult to diagnose based on the seroprevalence (Lindquist *et al*; 1997).

## **CLINICAL MANIFESTATIONS**

PIV's have been associated with every kind of upper and lower respiratory tract illness. However, there is a strong relationship between PIV-1, PIV-2, and PIV-3 and specific clinical syndromes, age of child, and time of year. At present epidemiologic data on PIV-4 is limited and has so far prevented a clear understanding of the unique clinical niche of this virus.

### **Croup**

Children present with fever, a hoarse barking cough, laryngeal obstruction, and inspiratory stridor (vibratory sound of variable pitch caused by partial obstruction of the respiratory passages). The incidence peaks between 1 and 2 years of age and is more frequent in boys. Approximately 10 to 25% of cases of LRT infection in children younger than 5 years presents as croup (depending on age). PIV's have made up between 56 and 74% of these cases, with PIV-1 (26 to 74%) being the most frequent subtype (Denny & Clyde; 1986). PIV-2 and PIV-3 average around 10% each as aetiologic agents. In years when PIV-1 is not epidemic, PIV-2 has been found to cause croup outbreaks. Even during PIV-1-epidemic years, PIV-2 has been reported to cause 60% of the croup cases in an individual community (Marx *et al*; 1997).



## **Bronchiolitis**

The diagnosis of bronchiolitis is unique to paediatrics because of the size of an infant's terminal airways. The predominant symptoms include fever, expiratory wheezing, tachypnea (rapid breathing), retractions (visible sinking in of the chest with inspiration in a child with respiratory difficulty), rales (wet, crackly lung noises heard on inspiration which indicate fluid in the air sacs of the lungs), and air trapping. The peak incidence of bronchiolitis is in the first year of life (81% of cases occur during this period) and then dramatically declines until it virtually disappears by school age. This syndrome is diagnosed in approximately 25 to 30% of LRT infections in childhood but makes up a larger percentage in the first year or two of life. At least 90% of cases of bronchiolitis are thought to be viral in origin, and a viral identification rate as high as 83% has been reported (Denny & Clyde; 1986). All four types of PIV can cause bronchiolitis, but PIV-1 and PIV-3 have been reported most commonly. Each of these two groups appears to cause 10 to 15% of cases of bronchiolitis in non-hospitalised children. However, in hospitalised children, PIV-3 causes many more cases than PIV-1 and is second only to RSV as a cause of bronchiolitis and pneumonia in young infants (Denny & Clyde; 1986).

## **Pneumonia**

Pneumonia is classically diagnosed by the presence of fever and rales (wet, crackly lung noises heard on inspiration indicating fluid in the air sacs of the lungs), and evidence of pulmonary consolidation (accumulation of inflammatory cellular exudates in the alveoli and adjoining ducts) on physical examination or x-ray. Pneumonia is diagnosed in 29 to 38% of children hospitalised with LRT infections and in 23% treated as outpatients (Glezen & Denny; 1986). However, since several of these classic LRT syndromes decrease with age, pneumonia causes 83% of hospitalizations of children with LRT infection older than 5 years.

The peak incidence for pneumonia is in the second and third years of life. Recently, at least one third of pneumonia hospitalisations have been in children with chronic diseases (Glezen *et al*; 2000). Viruses have been shown to cause up to 90% of these LRT infections, especially in the first year (Denny & Clyde; 1986), and this percentage decreases to approximately 50% by school age (Denny & Clyde; 1986). PIV-1 and PIV-3 each cause about 10% of outpatient pneumonias, but similar to bronchiolitis, PIV-3 causes a larger percentage of cases in hospitalised patients. Pneumonia can be caused by both PIV-2 and PIV-4, but the incidence of disease is not well described.

### **Tracheobronchitis**

Patients with lower respiratory signs and symptoms who do not fit well into the above three syndromes often receive a diagnosis of tracheobronchitis. The most common symptoms include cough and large-airway noise on auscultation, and patients may also present with fever and URT infection, with 20 to 30% of children with LRT infection receiving this diagnosis. The incidence is lower in the first 5 years of life, but tracheobronchitis is fairly evenly diagnosed throughout school age and adolescence. Viral agents make up the majority of aetiologies in children (Denny & Clyde; 1986). More than 25% of the agents identified to cause tracheobronchitis have been PIVs (PIV-3 is more common than PIV-1 or PIV-2). Several studies have recorded tracheobronchitis as the most common diagnosis in patients with PIV-4 infections (Denny & Clyde; 1986). Any single PIV can cause more than one of these LRT syndromes to occur simultaneously or progressively in the same child. PIV routinely causes otitis media, pharyngitis, conjunctivitis, and coryza (common cold). These URT infections can occur singly or in any combination with the above mentioned LRT infections.

## THE PARAINFLUENZA LIFE CYCLE

PIV's are roughly spherical in shape, 150-400nm in diameter, and have an envelope composed of host cell lipids and viral glycoproteins derived from the plasma membrane of the host cell during viral budding (Figure 1.1). The PIV genome is single stranded, negative sense ribonucleic acid (RNA) that must be transcribed into message sense RNA before it can be translated into protein. Like all negative stranded RNA viruses, the PIV's encode and package an RNA-dependent RNA polymerase in the virion particles (Lamb *et al*; 1976). The RNA genome is approximately 15,500 nucleotides in length and is encapsidated by the viral nucleocapsid by the viral nucleocapsid protein, forming helical nucleocapsid (Lamb *et al*; 1976).

The first step in infection of a cell by PIVs 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 (Figure 1.2). The viral envelope is 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); the matrix protein (F), which is involved in assembly and budding; the RNA polymerase proteins and a protein that encapsidates the RNA; and through alternative reading frames and/or RNA editing, one or more proteins that are expressed only in the infected cell and whose roles include evasion of the host immune response (Moscono; 2005).

Virions are formed, according to the prevailing model for virion assembly. Nucleocapsids containing 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 also interacts with the cytoplasmic tails of the HN and F proteins, in this way mediating the alignment of the nucleocapsid with the areas of the plasma membrane containing viral glycoproteins in order to set the scenario for budding (Ali & 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 (Huberman *et al*; 1995, Porotto *et al*; 2001).

Figure 1.1

A schematic diagram of the parainfluenza virion.  
(Adapted from Breese Hall; 2001)

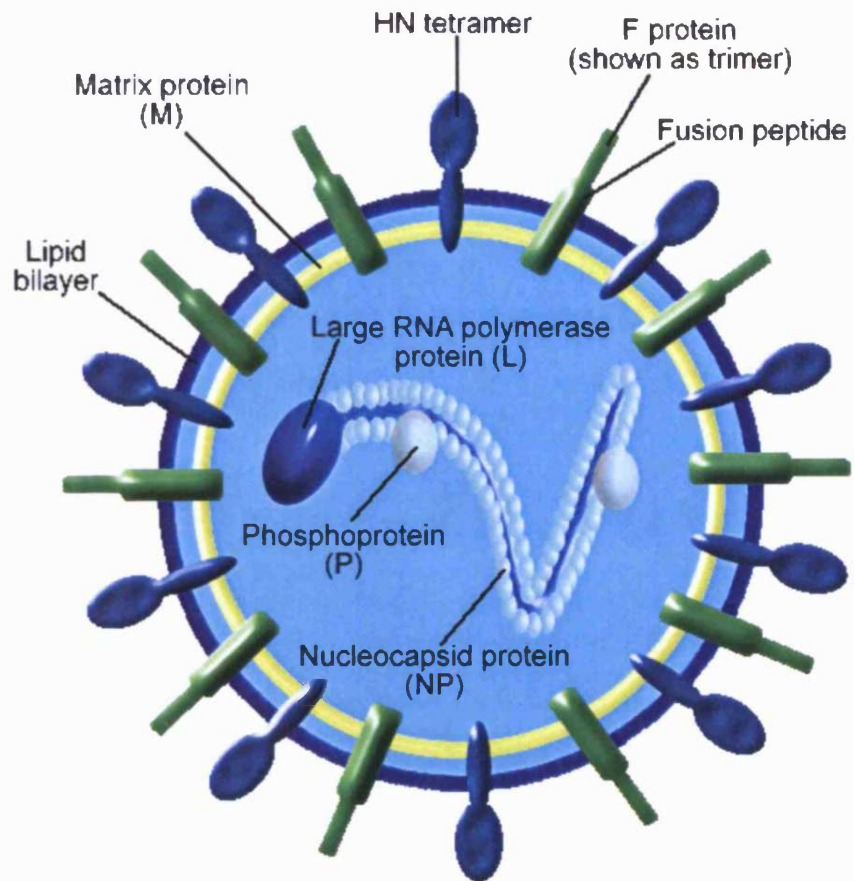
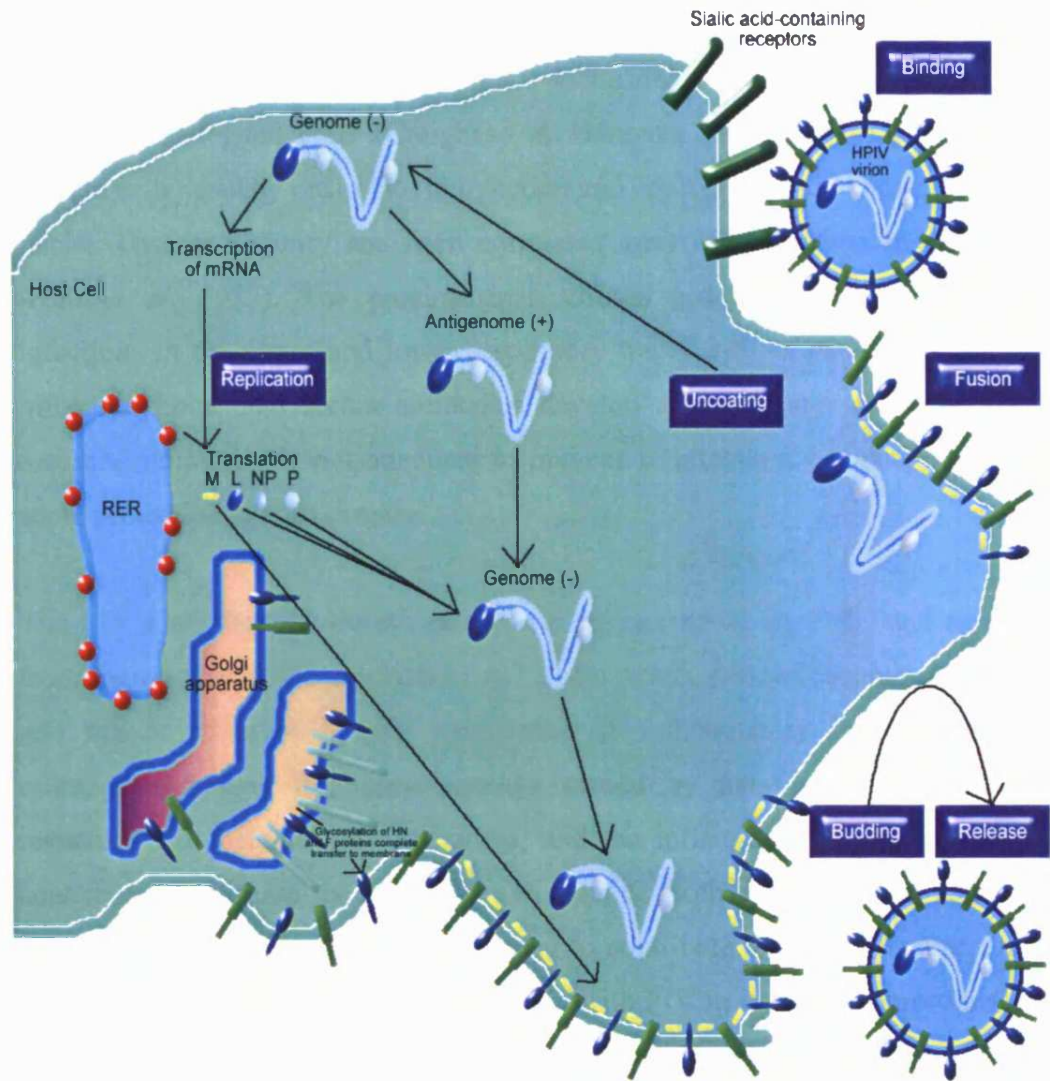


Figure 1.2

A schematic illustration of the parainfluenza viral life cycle.  
(Adapted from Moscono; 2005)



## PATHOGENESIS

When PIV infects a cell, the first observable morphologic changes may include focal rounding and an increase in size of the cytoplasm and nucleus. PIV decreases host cell mitotic activity as soon as 24 hours post inoculation. Other changes that can be observed include single or multilocular cytoplasmic vacuoles, basophilic or eosinophilic inclusions, and the formation of multinucleated giant cells (Craighead & 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 provide some protection against disease.

The PIV's are thought to replicate in the epithelium of the URT and spread from there to the LRT. Epithelial cells of the small airways become infected, and this is followed by the appearance of inflammatory infiltrates. The relationship among the tissue damage caused by the virus, the immune responses that help to clear the virus, and the inflammatory responses that contribute to disease is still quite enigmatic. 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).

During acute infection in guinea pigs, increased numbers of inflammatory cells, airway hyperreactivity and decreased bronchoalveolar cellular superoxide production can be demonstrated (Folkerts *et al*; 1990a, Folkerts *et al*; 1990b). In addition, PIV-3 induced greater peribronchiolar lymphocyte aggregation and histologic change in one species of cotton rat (*Sigmodon hispidus*) compared to another (*Sigmodon fulviventer*) (Porter *et al*; 1991).

## VIRUSES AND ASTHMA

Asthma is a disease of major importance affecting 20 to 33% of children in the United Kingdom (Johnson; 1998). The health costs of this condition are enormous in terms of time off school, general practitioner consultations, hospital admissions and mortality. Asthma is a multifaceted syndrome involving atopy, bronchial hyper-reactivity and IgE- and non-IgE- mediated acute and chronic immune responses. The asthmatic airway is characterised by an infiltrate of eosinophils and of T lymphocytes expressing the type 2 cytokines (IL-4, IL-5 and IL-13).

The role of viral respiratory tract infections in the onset of allergic sensitisation and asthma in children is an area of considerable contemporary interest. In recent years, there has been much debate over whether viral infections in early childhood are pathogenic or protective for the development of asthma and allergy (Frick *et al*; 1979, Martinez, 1994). Clinically, respiratory virus infection has been proposed as a common triggering factor in the development of allergy in children, and could also account for a large number of such episodes in adults (Zweiman *et al*; 1971, Frick *et al*; 1979, Gurwitz *et al*; 1981,). It is well known that asthma symptoms such as wheezing worsen after respiratory viral infection (Frick *et al*; 1979). 80% of wheezing episodes in school children and 76% of asthma exacerbations in adults were associated with respiratory infections (Johnson *et al*; 1995, Johnson; 1998, Wark *et al*; 2002).



Among the respiratory viruses implicated in childhood asthma are RSV, PIV-3, rhinoviruses (RV) and coronavirus (CV) (McIntosh *et al*, 1973). Furthermore, viral infection with RSV, during the first years of life greatly enhances the risk of developing asthma (Sigurs *et al*; 1995, Folkerts *et al*; 1998).

## **PREVENTION AND TREATMENT**

While other causes of respiratory diseases in children, influenza and measles have yielded in part to vaccination programs and anti-viral therapy, children are still virtually unaided in their battle against major causes of croup and bronchiolitis. RSV has been extensively studied, and some effective strategies of prophylaxis have been developed (Groothuis *et al*; 1993), but for the PIV's there are no therapeutic weapons. The hurdle for developing modes of preventing and treating croup and bronchiolitis caused by PIV is due to our lack understanding of fundamental processes of viral biology and of the interaction of these viruses with their hosts during pathogenesis.

### **PREVENTION**

Field trials of formalin-killed whole PIV-1, PIV-2, and PIV-3 vaccines failed to protect children against natural infection in the late 1960s (Chin *et al*; 1969, Fulginiti *et al*; 1969). The majority of children developed antibodies to all three PIV serotypes, but the levels were considerably lower than those seen following natural infection (Fulginiti *et al*; 1969). Current approaches to PIV vaccines include intranasal administration of live attenuated strains, subunit strategies using the HN and F proteins, recombinant bovine/human viruses, and strains engineered using reverse genetics. Human PIV-3 and bovine PIV-3 are antigenically related, and bovine PIV-3 induces resistance in rats and primates to challenge with human PIV-3 (Van Wyke Coelingh; 1987).

In human trials, seropositive adults did not produce significant immune responses to bovine PIV-3, but seronegative volunteers responded quite well to this vaccine (Clements *et al*; 1991, Crowe; 1995). Vaccine studies of young infants and children have demonstrated the same promising results (Karron *et al*; 1996). Further studies evaluating the safety and efficacy of bovine PIV-3 when given as multiple doses and simultaneously with routine childhood immunisations are under way (Lee *et al*; 2001). A new approach using recombinant bovine PIV-3 with human F and HN genes and human RSV G and F genes has induced good antibody titres and protection in hamsters and rhesus monkeys (Haller *et al*; 2000, Schmidt *et al*; 2001).

Antigenically and genetically stable attenuated strains of PIV-3 have been developed by cold adaption (CA). The stability of these CA vaccine strains is enhanced because of multiple markers of attenuation in tissue culture (Belshe *et al*; 1982). The initial work with these CA strains showed poor infectivity in older children and adults but protection in hamsters and chimpanzees (Clements *et al*; 1991). Higher-passage CA PIV-3 strains demonstrated immunogenicity and attenuation in younger infants, but symptoms still developed (Belshe *et al*; 1992). The envelope glycoproteins (HN and F) of PIV-3 have been cleaved from whole virus to create subunit vaccines that have demonstrated efficacy in rats, lambs, mice, and hamsters (Ray *et al*; 1985, Ray *et al*; 1986, Ambrose *et al*; 1991,). Many different routes of immunisation have been tried, including subcutaneous, intramuscular, and intranasal, although no human trials have yet been reported. Reverse genetics are now being used to develop systems able to evaluate current live PIV-3 vaccines in terms of specific mutations and hopefully introduce specific mutations into wild-type strains in the future (Durbin *et al*; 1997). An example of this is the production of a recombinant PIV-3 that contains the HN and F for PIV-1 and the specific CA mutations found in the L gene of PIV-3 (Tao *et al*; 1999).

This vaccine has been found to be protective in hamsters. Sometime over the next decade, one or more of these approaches may produce the first licensed HPIV vaccine.

## TREATMENT

Currently there are no licensed antiviral drugs with proven clinical efficacy against PIV. However, potent inhibitors of PIV syncytium formation in tissue culture have been found by screening hundreds of small synthetic peptides. These peptides, which are to a specific domain on the F protein, may provide a novel approach to the development of antiviral therapies for PIV (Pastey *et al*; 2000). Similarly, ribavirin has both *in vitro* and *in vivo* activity against PIV (Sidwell *et al*; 1975, Browne; 1981). Furthermore, there have been anecdotal reports of decreased PIV shedding and clinical improvement when infected immunocompromised patients were treated with aerosolized and oral ribavirin (Chakrabarti *et al*; 2000, Malinowski & Hostoffer; 2001).

### Glucocorticoids

Glucocorticoids are widely used for the suppression of inflammation in a wide variety of inflammatory diseases, including chronic inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease and autoimmune diseases, all of which are associated with increased expression of inflammatory genes. Glucocorticoids bind to glucocorticoid receptors in the cytoplasm, which then dimerise and translocate to the nucleus, where they bind to glucocorticoid response elements (GRE) on glucocorticoid-responsive genes, resulting in increased transcription (Figure 1.3). Glucocorticoids may increase the transcription of genes coding for anti-inflammatory proteins, including lipocortin-1, IL-10, interleukin-1 receptor antagonist and neutral endopeptidase, but this is unlikely to account for all of the widespread anti-inflammatory actions of glucocorticoids (Barnes; 1998).

Several of the genes that are switched on by glucocorticoids have anti-inflammatory effects, including annexin-1 (lipocortin-1), secretory leukoprotease inhibitor (SLPI), IL-10 and the inhibitor of nuclear factor kappa-beta (NF- $\kappa$ B).

Glucocorticoids also switch on the synthesis of two proteins that affect inflammatory signal transduction pathways, glucocorticoid-induced leucine zipper protein (GILZ), which inhibits both NF- $\kappa$ B and AP-1 (Mittelstadt & Ashwell; 2001) and MAP kinase phosphatase-1 (MKP-1), which inhibits p38 MAP kinase (Lasa *et al*; 2002). However, it seems unlikely that the widespread anti-inflammatory actions of glucocorticoids could be entirely explained by increased transcription of small numbers of anti-inflammatory genes, particularly as high concentrations of glucocorticoids are usually required for this effect, whereas in clinical practice glucocorticoids are able to suppress inflammation at low concentrations (Barnes; 2006). In controlling inflammation, the major effect of corticosteroids is to inhibit the synthesis of multiple inflammatory genes including cytokines, enzymes, receptors and adhesion molecules through suppression of the genes that encode them. This is an important determinant of glucocorticoid responsiveness and is a key mechanism whereby glucocorticoids exert their anti-inflammatory effect (Barnes; 1998). Glucocorticoids have direct inhibitory actions on many inflammatory and structural cells involved in inflammation including macrophages, eosinophils and neutrophils.

The efficacy of glucocorticoids has been evaluated mostly in infants and children with RSV with disappointing results. Glucocorticoids are widely used to treat inflammatory diseases. The four main types which have been investigated to treat viral infections *in vivo* and *in vitro* have included triamcinolone acetonide, (Prince *et al*; 2000, Ottolini *et al*; 2002; Ottolini *et al*; 2003, Eichelberger *et al*; 2004), methylprednisolone (Kimsey *et al*; 1989, Ottolini *et al*; 2002), dexamethasone (Sheth *et al*; 1994, Ottolini *et al*; 2002, Moreno *et al*; 2003, Toward *et al*; 2005) and hydrocortisone (Bonville *et al*; 2001, Domachowske *et al*; 2001). The types and doses of corticosteroids have been sufficiently varied to make interpreting of results extremely difficult.

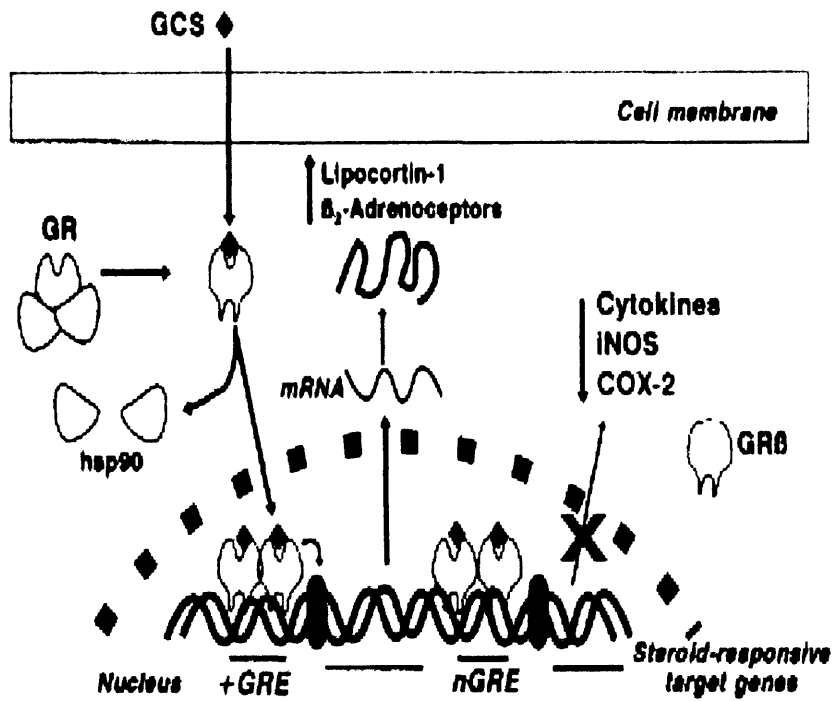
Treatment of tracheobronchitis (predominantly caused by PIV-1)) with corticosteroids has been shown to decrease the severity of symptoms, reduce the need for hospitalisations or time spent in the emergency room, and reduce the need for further pharmacologic intervention (Ausejo *et al*; 1999). In addition, several animal studies have shown the beneficial effect of corticosteroids on the respiratory symptoms of virus infections (Ottolini *et al*; 2002, Moreno *et al*; 2003, Toward *et al*; 2005). In direct contrast, clinical studies suggest that glucocorticoid administration has only marginal, if any, benefit in the treatment of viral infections (Springer *et al*; 1990, Roosevelt *et al*; 1996, De Boeck *et al*; 1997).

Numerous studies, as summarized in a recent meta-analysis, have demonstrated that oral or systemic steroids are effective at improving symptoms of croup as early as 6 hours after treatment (Somani *et al*; 2001). Other studies, have determined that glucocorticoids maybe beneficial in artificially ventilated patients with severe bronchiolitis, although they had no effect on pneumonia (Van Woensel *et al*; 2003). These findings support the idea that glucocorticoids 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 & Porter; 1996). Immunotherapy may offer a possible therapeutic option in patients with severe disease until safe and effective vaccines and anti-virals are available.

Figure 1.3

Classical model of glucocorticoid action.

(Adapted from Barnes; 1998)



## **Phosphodiesterase isoenzymes**

A phosphodiesterase inhibitor is a drug, which blocks one or more of the five subtypes of the enzyme phosphodiesterase (PDE), therefore preventing the inactivation of the intracellular second messengers, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), by the respective PDE subtypes. The PDE isoenzymes are widely distributed throughout the body (Table 1.1), therefore by inhibiting PDEs, intracellular levels of cyclic nucleotides rise (Raeburn *et al*; 1993). This rise in cAMP and cGMP, following PDE inhibition, is associated with airway smooth muscle relaxation (Torphy, 1994). The major limitation of using these compounds is their narrow therapeutic window. Doses above this therapeutic index cause adverse effects relating to their penetration across the blood brain barrier. These include nausea, vomiting, gastric upset, central nervous stimulation and cardiac arrhythmia.

Recently reviewed interest has developed in the concept of using PDE inhibitors to treat respiratory diseases. To date eleven distinct PDEs have been identified, and several variants within the PDE subsets exist, although their functions and relevance have not been confirmed (Torphy & Page; 2000) and attention has focused on the PDE-4 isoenzyme. PDE-4 is the major isotype in human (Dent *et al*; 1994; Hatzelmann *et al*; 1995) and guinea pig (Raeburn *et al*; 1994; Souness *et al*; 1991) eosinophils, and therefore PDE-4 was an obvious target. PDE-4 inhibitors (e.g. roflumilast) have proven potential as anti-inflammatory drugs especially in airway diseases. They suppress the release of inflammatory signals e.g. cytokines, and inhibit the production of reactive oxygen species. There have been several reports demonstrating inhibitory effects of PDE-4 inhibitors on airway hyperreactivity (AHR) and lung eosinophilia in allergen induced airway obstruction in guinea pigs (Raeburn *et al*; 1994, Santing *et al*; 1995, Danahay & Broadley; 1997, Manabe *et al*; 1997). Other studies involving the PDE-3 inhibitor, milrinone have shown poor inhibitory activity (Ikemura *et al*; 2001).

Studies by Toward *et al* (2005) have also indicated a potential for the PDE-4 inhibitor in the treatment of PIV-3 infected guinea pigs.

**Table 1.1 Tissue distribution of the eleven families of phosphodiesterases.**  
Data adapted from: Raeburn *et al*; 1993, Fuhrmann *et al*; 1999, Torphy & Page; 2000.

PDE isozyme	Tissue distribution
<b>PDE 1</b>	Heart, liver, brain, trachea, mast cell, macrophage, T-lymphocyte.
<b>PDE 2</b>	Heart, liver, brain, trachea, T-lymphocyte, platelet, endothelial cell.
<b>PDE 3</b>	Heart, liver, brain, trachea, lung, bronchi, T-lymphocyte, platelet, basophil.
<b>PDE 4</b>	Heart, liver, brain, trachea, lung, bronchi, T & B lymphocyte, eosinophil, mast cell, basophil, neutrophil, macrophage, monocyte, endothelial, epithelial cell.
<b>PDE 5</b>	Trachea, aorta, lung, bronchi, mast cell, T-lymphocyte, macrophage, platelet, epithelial cell.
<b>PDE 6</b>	Retina
<b>PDE 7</b>	Brain, kidney, testis, liver, adrenal gland, thymus spleen, airway epithelial cells, T & B lymphocytes
<b>PDE 8</b>	Testis, ovary, small intestine, colon.
<b>PDE 9</b>	Spleen, small intestine, brain
<b>PDE 10</b>	Not fully determined
<b>PDE 11</b>	Skeletal muscle, prostate, kidney, liver, pituitary, salivary gland, testis.



## **MODELS**

From early times, humans have subjected animals to unknown foods, chemicals and atmospheric conditions, before themselves. An animal model provides a representation of the human disease, to allow investigation into the pathology of the disease or to evaluate the therapeutic effects of new treatments prior to being introduced into man. Although it is uncommon to find in nature animals that have exactly the same disorder as in man, parallels can often be drawn. An ideal animal model should possess certain key features, including allowing detailed examination and measurement of pharmacokinetics and pharmacodynamics, permitting the testing of small quantities of compounds, being reproducible and mimicking in some form the relevant disease. Furthermore, for the model to be a useful screening tool in the pharmaceutical industry, it should also be economically practical to evaluate a number of compounds within a short period of time. At present there is a current need for models of inflammatory lung disease to further understand the pathophysiology in viral infection and to allow evaluation of novel therapeutic compounds.

### **VIRAL INFECTION AND THE GUINEA PIG**

Historically, the guinea pig has been widely used as an experimental tool to model and understand the mechanics and pathophysiology relating to human lungs, for a number of reasons. Guinea pigs are small, easy to manage mammals, with a number of histological similarities to the human bronchial tree. Genetically, the guinea pig has fairly pure lines of breeding, allowing repeatability of data, and their husbandry is economical. The processes of inflammation within the lungs in response to PIV infection of these animals can lead to the development of several pathological characteristics analogous to human PIV infection.

Unlike rats and mice the main mast cell mediators of inflammatory lung disease in guinea pigs is histamine, which is common with humans. As with any model, using guinea pigs to interpret data relating to a potential outcome in humans is not without limitations. Unlike humans, guinea pigs fail to present with any clinical signs of infection (e.g. sneezing) and are asymptomatic. It is also well documented that guinea pigs are more resistant to the anti-inflammatory activities of steroids, and consequently require larger doses than humans (Whelan *et al*; 1995). Guinea pigs are obligatory nasal breathers, which is a further consideration for these models where airway function is measured and where spasmogens, antigens and other inducers of inflammation have been assumed to be deposited in the lower airways. This is discussed in more detail in chapter 2.

The next chapter describes the common techniques and methods in this thesis for each of the experimental chapters. Further details relating to chapter specific methods are described in the experimental chapters. The experimental chapters are written as self-contained studies with each chapter introduced and concluded as a self-supporting article. The conclusions of each study are discussed in detail at the end of each chapter and consequently the final chapter discusses only the general conclusions arising from this thesis.

## AIMS OF THESIS

The ultimate aims of this thesis are as follows:

1. Establish a guinea pig model of viral infection using PIV-3, which displays inflammatory features of infection, airway function changes to inhaled histamine and pulmonary histopathology. Ascertain the most efficient mode (intranasal / nebulisation) of virus delivery in the guinea pig.
2. Based on previous findings, establish a time course of PIV-3 infection and ascertain the peak response to infection by assessing the following parameters airway function, airway reactivity to histamine, cellular infiltration, wet lung weight, nitric oxide and protein levels in bronchoalveolar lavage fluid, pulmonary histology and recovery of virus from the lung tissue. Therefore, it was hypothesised whether a temporal association could be established between these parameters.
3. Despite decades of research, there are no licensed agents with proven clinical efficacy against parainfluenza infections. Therefore, it was hypothesised whether the glucocorticoid, dexamethasone, and the phosphodiesterase inhibitor, rolipram could reduce viral infection, and therefore provide a potential treatment in PIV-3 infection.
4. Overall, there is considerable controversy with regard to the potential role of viral respiratory tract infections, in triggering allergic sensitisation. Thus, interactions of respiratory viruses with allergen sensitisation and allergic inflammation of the airways are of particular interest, since these constitute the underlying pathomechanisms of asthma. In view of this it was hypothesised, whether pre-existing allergen sensitisation could exacerbate the development of respiratory allergen sensitisation during the acute phase of PIV-3 infection.

# **CHAPTER 2**

## **GENERAL METHODS**

### **INTRODUCTION**

This chapter describes the general methods undertaken throughout the entire investigation. Procedures used and further experimental details are also referred to in each experimental chapter.

### **ANIMAL AND ANIMAL HUSBANDRY**

Male Dunkin Hartley guinea pigs (200-250g) were obtained from Harlan UK Ltd (Oxon, UK). For the viral infection protocol, juvenile guinea pigs 4 weeks of age were selected, since in PIV-3 infection usually takes places in the first two years of life being equivalent to the first weeks of life in guinea pigs (Glezen & Denny; 1973). Animals were housed at the laboratory animal facilities at Cardiff University in flat-bottomed cages with cage liners and placed inside a flexible film isolator (Moredun Scientific Ltd, Midlothian, UK). Virus or virus-free medium inoculated animals were placed in close contact groups to allow transmission of the virus between animals. During experimental procedures animals were removed from the isolator and precautions to avoid transmission of infection to the user and other animals within the facility were implemented. These included using designated rooms equipped with air filters, investigators wearing disposable laboratory coats, respiratory masks, gloves and equipment swabbed with 5% virkon.

Virus and virus-free medium infected animals were housed in separate rooms, but maintained under identical conditions. Controlled conditions were imposed on climate and diet and ambient temperature was maintained at  $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$  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 three times a week for environmental enrichment. The animals were acclimatised for 1 week before the commencement of viral inoculation. Animal welfare was undertaken in accordance with the Animal Scientific Procedures Act 1986 under home office personal 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.

#### **PREPARATION OF VERO CELL CULTURE**

African green monkey kidney epithelial (Vero) cells were obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Aliquots of frozen cells were removed from liquid nitrogen and rapidly thawed at  $37^{\circ}\text{C}$  to maintain the integrity of the cells. Once thawed, the cells were spun in a microfuge at 2000 r.p.m for 3 minutes. The supernatant was removed and 1ml of phosphate buffered saline (PBS) added to wash the cells and spun at 2000 r.p.m (Jouan CR412, Jouan Ltd, Ilkeston, UK) for 3 minutes. The supernatant was removed and the washing procedure repeated. The cells were suspended in 1ml of PBS and 500  $\mu\text{l}$  of cells were added to 15mls of cell culture medium (Medium A) in a  $80\text{cm}^3$  culture flask. Medium A consisted of Dulbecco's modified essential medium (DMEM) (Sigma-Aldrich, Dorset, UK) supplemented with 10% heat inactivated foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK), 1% L-glutamine (Sigma-Aldrich, Dorset, UK), 1% penicillin/streptomycin (Sigma-Aldrich, Dorset, UK).

L-glutamine is required as an energy source for the Vero cells and antibiotics penicillin/streptomycin were added to prevent bacterial contamination. Cell lines were split every 3-5 days or as required. Cells were incubated at 37<sup>0</sup>C in a humidified incubator containing 5% CO<sub>2</sub> (Sanyo, Loughborough, UK) until semi-confluent monolayers were obtained. All procedures were performed in a Class II Microbiological safety cabinet (Envair Ltd, Lancashire, UK).

### **PREPARATION OF PIV-3 SUSPENSION**

Human PIV-3 used in this study was kindly donated by Dr J. Buggert (University of Wales College of Medicine (UWCM), Medical Microbiology, Cardiff, UK) obtained from the ECACC (Wiltshire, UK). Aliquots of frozen PIV-3 were removed from liquid nitrogen and rapidly thawed at 37<sup>0</sup>C to maintain the integrity of the cells. Once thawed the cells were spun in a microfuge at 2000 r.p.m (Jouan CR412, Jouan Ltd, Ilkeston, UK) for 3 minutes. The supernatant was removed and 1ml of PBS was added to wash the cells and spun at 2000 r.p.m for 3 minutes. The supernatant was removed and the washing procedure repeated. The cells were suspended in 1ml of PBS and 500 µl of PIV-3 were used to inoculate 80cm<sup>2</sup> flasks containing semi-confluent monolayers of Vero cells containing medium A. Cells were incubated at 37<sup>0</sup>C in a humidified incubator (Sanyo, Loughborough, UK) containing 5% CO<sub>2</sub>. At 5-6 days later after the initial inoculation when cytopathic effect (CPE) reached ~ 80% the PIV-3 infected cells were harvested observed by the formation of syncytia appearing as large multi-nucleated refractile cells. PIV-3 was harvested by scraping the culture flasks with a cell scraper to remove the adherent cells, transferred to a 50ml centrifuge tube and centrifuged at 5000 r.p.m (Jouan CR412, Jouan Ltd, Ilkeston, UK) for 15 minutes to remove cellular debris. The resulting PIV-3 supernatant was transferred into 1ml sterile vials and stored at -80<sup>0</sup>C.

### **Calculation of viral titre**

The PIV-3 titre was determined by tissue culture infective dose (TCID<sub>50</sub> ml<sup>-1</sup>). TCID<sub>50</sub> is defined as the amount of virus required to infect 50% of monolayers of Vero cells (Reed & Muench; 1938). The final PIV-3 concentration was determined to be 5x10<sup>6</sup> TCID<sub>50</sub> ml<sup>-1</sup>.

### **Ultra violet light inactivation of PIV-3**

In order to attempt to clarify the true contribution of PIV-3 infection it was decided that uninfected Vero cell supernatant (virus-free medium) would be prepared in an identical manner for inoculation into control animals. All procedures were performed in a Class II Microbiological safety cabinet (Envair, Lancashire, UK). During the initial experiments another control was also implemented using ultra violet (UV) light inactivated PIV-3 (UV-PIV-3) in order to account for the cytokines which may have been generated by PIV-3 infected cells. To inactivate the virus, a small sample of the virus was spread onto a petri dish and exposed to short wavelength UV light for 20 minutes at a distance of 20 metres. After radiation, no evidence of viable PIV-3 could be detected using the TCID<sub>50</sub> assay.

## **MEASUREMENT OF GUINEA PIG BODY WEIGHT**

Measurement of guinea pig body weight was used to determine the severity of illness and for calculation of specific airways conductance (sG<sub>aw</sub>). Guinea pigs were weighed before inoculation and at regular intervals after inoculation for the duration of the study.

## **MEASUREMENT OF GUINEA PIG RECTAL TEMPERATURE**

Measurement of guinea pig rectal temperature was used to determine if any fever was experienced during the study as an indicator of PIV-3 induced effects. Guinea pig temperatures were recorded before inoculation and at regular intervals after inoculation for the duration of the study.

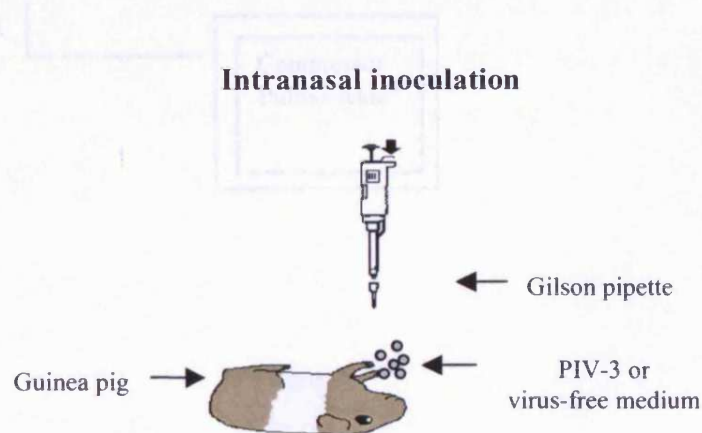
The temperature reading was taken by inserting a rectal thermometer probe (Hanna Instruments, Bedfordshire, UK) into the anus of the guinea pig to a depth of 1cm, while held by a colleague.

## VIRUS INOCULATION

### Intranasal Inoculation

On the day of inoculation guinea pigs were held in a supine position with the head firmly supported. For all experiments, a new vial of PIV-3 was rapidly thawed at 37°C in a water bath, gently vortexed and retained on ice. Each guinea pig received either 500µl of PIV-3 or virus-free medium introduced by intranasal instillation using a Gilson pipette into each of the nostrils (Figure 2.1). Studies have shown using colloidal carbon as a tracer substance that this volume is sufficient to allow the inoculum to enter all lobes of the lung in the LRT in this weight of guinea pig (Dakama *et al*; 1997). Following intranasal inoculation into the nostrils the guinea pigs were held in a supine position for several minutes to facilitate delivery into the airways. A resting period of 15 minutes was allowed between inoculation into the left and right nostril. Intranasal inoculation of PIV-3 or virus-free medium into the guinea pigs was performed in a class II safety cabinet (Captair Madcap 804, Erlab, Wiltshire, UK) to contain the virus and avoid undesired transmission of infections to other animals not included in the study.

Figure 2.1

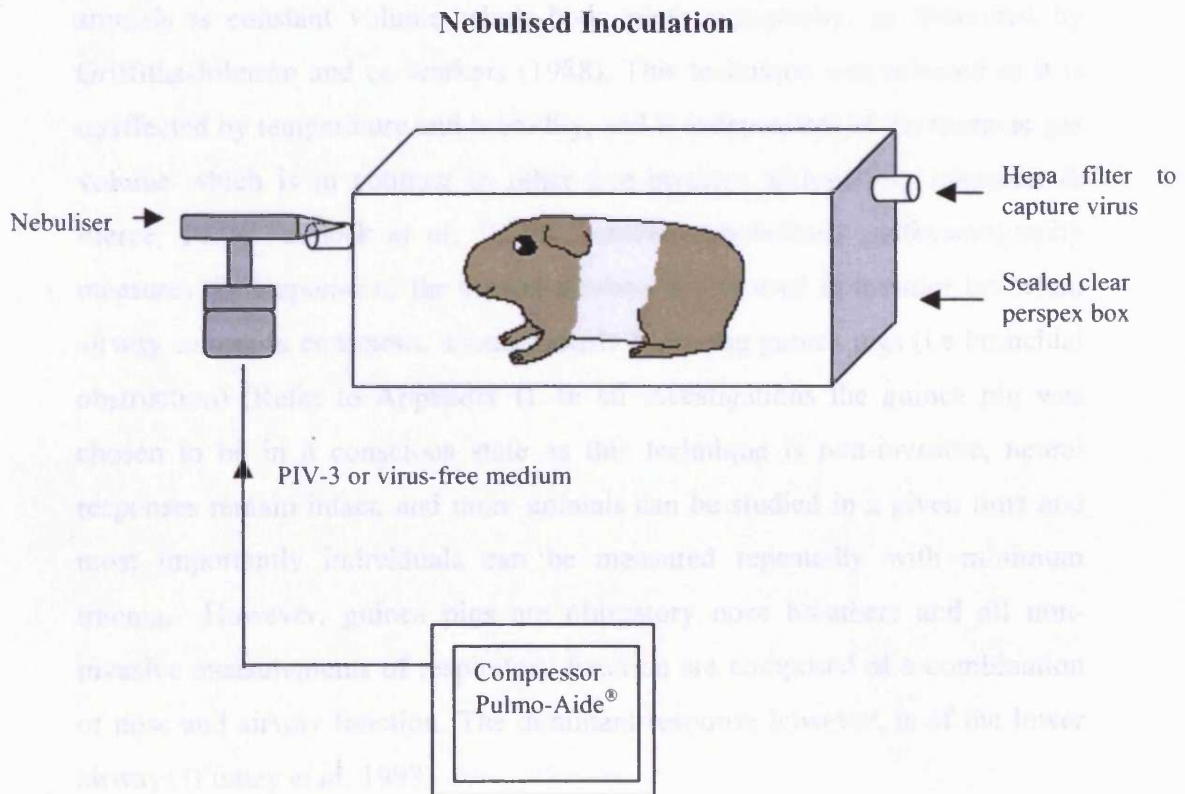




### Nebulisation inoculation

The guinea pigs were infected with a virus-free medium (Vero cell supernatant) or PIV-3 suspension ( $5 \times 10^6 \text{ ml}^{-1}$ ) supplied as an aerosol, for 45 minutes daily on three consecutive days according to studies performed by Streckert *et al* (1997). The aerosol was administered to the guinea pigs in an incubation box (Volume  $32 \times 16 \times 32 \times 16 \text{ cm}$ ), which contained more than 60% particles smaller than  $3 \mu\text{m}$  (Figure 2.2) using a compressor nebuliser (Pulmo-Aide® 561 series) (Devilbiss®, Middlesex, UK). The aerosol inoculation process was performed in a class II safety cabinet (Naire, Triple Red LTD, Oxfordshire, UK).

Figure 2.2



## MEASUREMENT OF PULMONARY FUNCTION

Pulmonary function is measured using a technique known as plethysmography, which has been specifically developed for pulmonary physiology research. Plethysmography is derived from the Greek *πληθυσμοζ* (*plethysmos*), meaning enlargement and is a reliable, reproducible and rapid technique (Finney *et al*; 1993). Plethysmography has been widely used for pulmonary studies in man and is considered appropriate to apply this approach to measurements in small animals including guinea pigs (Griffiths-Johnson *et al*, 1988).

The plethysmographic technique adopted in these investigations for small animals is constant volume whole-body plethysmography, as described by Griffiths-Johnson and co-workers (1988). This technique was selected as it is unaffected by temperature and humidity, and is independent of the thoracic gas volume which is in contrast to other non-invasive techniques (Johanson & Pierce; 1971, Pennock *et al*; 1979). Constant whole-body plethysmography measures the response of the central airways and is used to monitor bronchial airway calibre in conscious, spontaneously breathing guinea pigs (i.e bronchial obstruction) (Refer to Appendix I). In all investigations the guinea pig was chosen to be in a conscious state as this technique is non-invasive, neural responses remain intact, and more animals can be studied in a given time and most importantly individuals can be measured repeatedly with minimum trauma. However, guinea pigs are obligatory nose breathers and all non-invasive measurements of respiratory function are composed of a combination of nose and airway function. The dominant response however, is of the lower airways (Finney *et al*; 1993).

The plethysmograph apparatus used in these investigations consisted of a cuboidal transparent perspex chamber (12x40x12cm) with a built in flat floor and a removable perspex endplate to allow entry and exit into the chamber, which has a total box volume of 5.5 litres. An airtight seal is ensured by the use of high-density foam separators between the chamber and the end plate. The box has four ports, one to record box pressure, two to measure airflow at the mask, and one to open the box to atmosphere with a simple tap (Figure 2.3). The technique utilises a specially designed two-part animal-restraining device consisting of a base unit and neckpiece (Figure 2.4). The neckpiece consists of two flexible bars held together by a metal spring, with a minimum amount of pressure to allow the bars to restrain the animal at the neck. This section slots into the base unit, which has high sides to give the animal a sense of security, and is secured by two pins.

The conscious guinea pigs were placed in the two-part restraining device and a close fitting face-mask placed over the animals snout creating an airtight seal around the nose. The mask is constructed of lightweight perspex and internally shaped to fit a wide range of animals (200-750g). An airtight seal at the end of the mask is achieved by stretching a cut section from the neck of a rubber balloon over the mask opening, so that it forms an aperture into which the animals' snout is placed. The mask was secured with two low-tension springs hooking onto eyes located on the base unit. A pneumotograph was attached to the facemask, a resistor, which measures instantaneous nasal flow of respiratory gases at the mask. The animal and restrainer were placed inside the plethysmography chamber, which was then sealed with the end plate.

The plethysmograph equipment also involves two differential pressures transducers UP1 and UP2 (Pioden controls LTD, Kent, UK) attached to the pneumotach and plethysmograph chamber respectively, which measure changes in respiratory flow and box pressure respectively, as the animal spontaneously breathes.

This allows the collection of data regarding the animal's breathing patterns. The nasal airflow is measured in the front of the chamber and is separate from the box pressure measured in the rear of the chamber. The guinea pig breathing in the plethysmograph withdraws air from the chamber during inspiration leading to an expansion of the thorax and returns it during expiration. Thus, withdrawing air from the plethysmograph lowers the box pressure and although very small, is directly proportional to flow measured across a Fleisch pneumotachograph. During a breathing cycle there are 2 parts of zero flow, at the onset and end of breath as well as the end of inspiration.

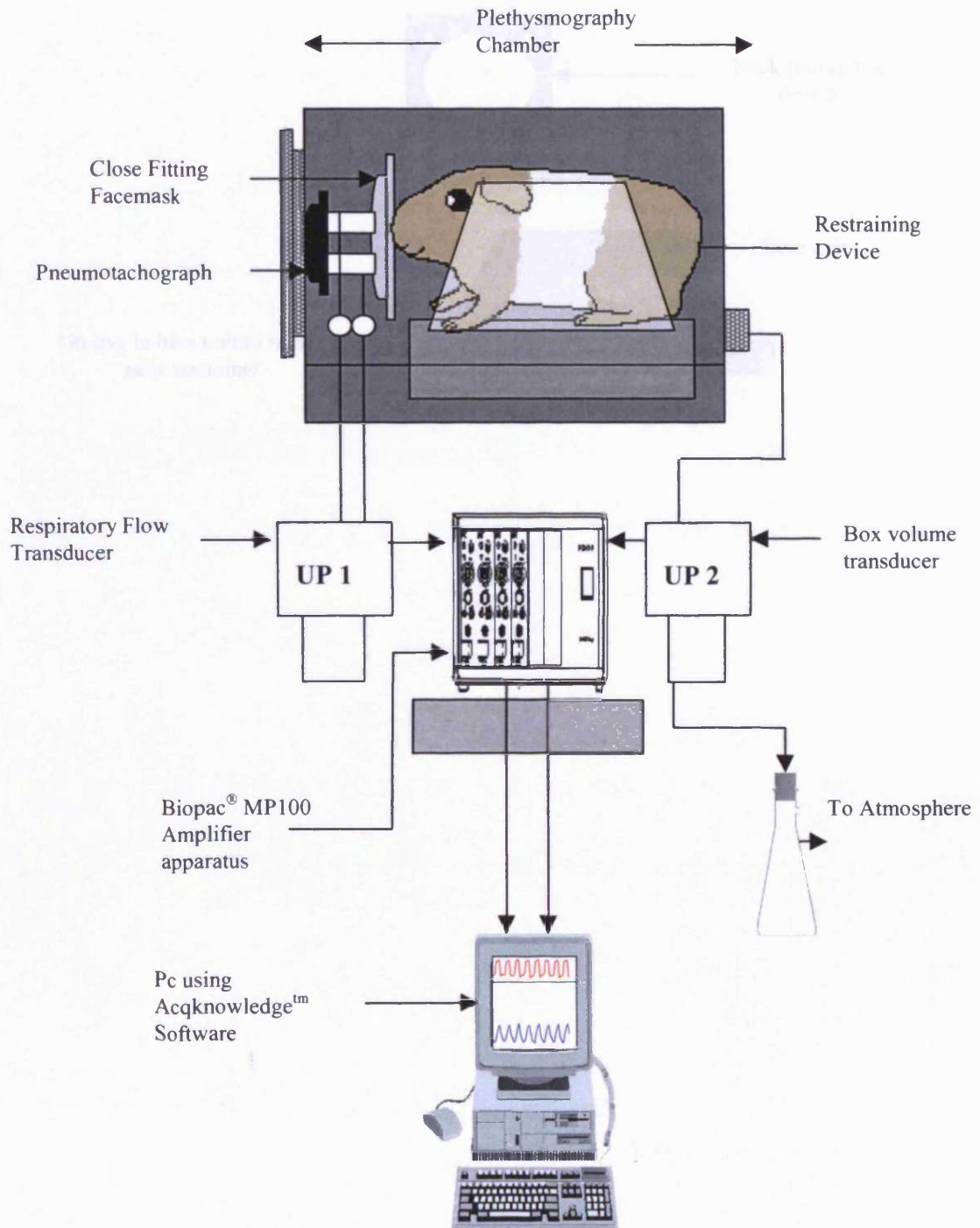
The output from the transducers is applied to an electronic amplification device, which converts the pressure fluctuations experienced by the transducers into a voltage range. This is attached to a computerised recording system comprising of Acqknowledge™ software with a Biopac® data acquisition system, as previously described by Danahay and Broadley (1997) which replaces the original oscilloscope and angle resolver as used by Griffiths-Johnson *et al* (1988). The data are then acquired and analysed by BIOPAC Acqknowledge™ software running on a PC with an A/D card. The pressure changes from the transducers are recorded and plotted against each other using an XY detector. A waveform of respiration for the guinea pig is produced (Figure 2.5) and converted into specific airway conductance ( $sG_{aw}$ ) measured in  $\text{cm H}_2\text{O}^{-1} \text{Sec}^{-1}$ .  $sG_{aw}$  is determined from the phase displacement between the nasal flow and box pressure. The phase displacement is measured at the end of inspiration and the steep changeover to the expiratory phase. Each recording period was 5 seconds long, and from this, a minimum of 5 breaths were analysed and a total of 5 measurements were taken for each time point. In all investigations, the experimental protocol was standardised by measuring  $sG_{aw}$  values at the same time each day. 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.

Occasionally, animals exhibited irregular breathing patterns, which were easily discernable on the respiratory trace, and another breath was recorded. Between recordings the animals were removed from the plethysmograph and placed in a holding cage with food and water. Baseline  $sG_{aw}$  measurements were taken for each animal to provide an assessment of normal respiratory rate. The plethysmograph apparatus was calibrated daily using compressed air of defined volumes. Flow was calibrated at a flow rate of  $6.2 \text{ litres air min}^{-1}$  obtained from an oxygen cylinder and box pressure was calibrated with a fixed volume of air (5ml). All experiments were conducted at room temperature between  $18\text{-}22^{\circ}\text{C}$  and humidity between 45-55% as both of these factors affect flow and box pressure parameters.

Several investigators have suggested that using plethysmography in conscious guinea pigs may cause stress and affect pulmonary parameters. Other investigators, however, suggest that no effect is observed on  $sG_{aw}$  with repeated measurements (Finney *et al*; 1993). The true extent of this on the respiratory function is unknown and further studies are warranted. To compensate for this problem, animals are frequently handled and trained to acclimatise them to the measurement of respiratory function prior to each experiment.

Figure 2.3

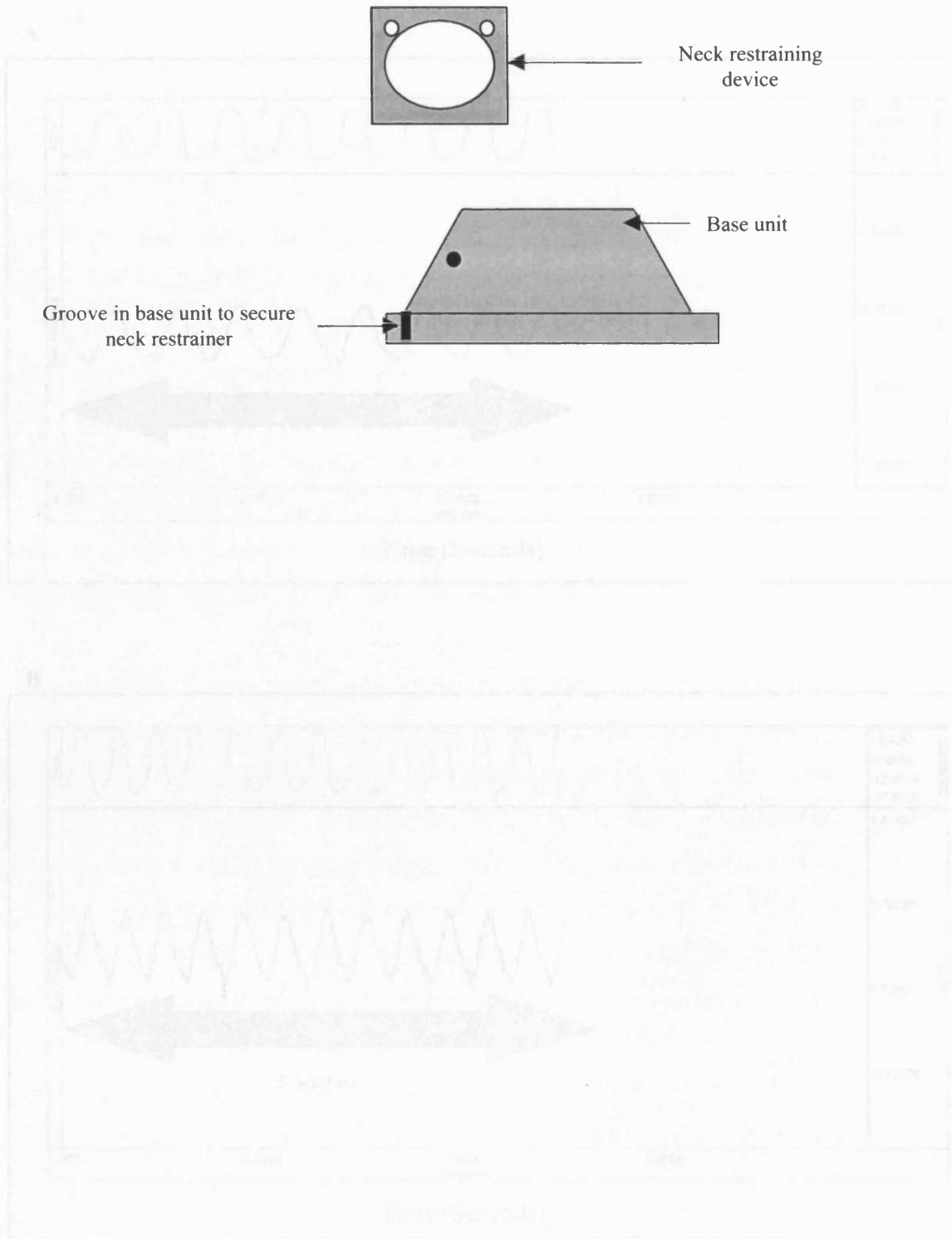
Constant Whole-body plethysmography



**Figure 2.4**

**Guinea Pig Two-Part Restraining Device**

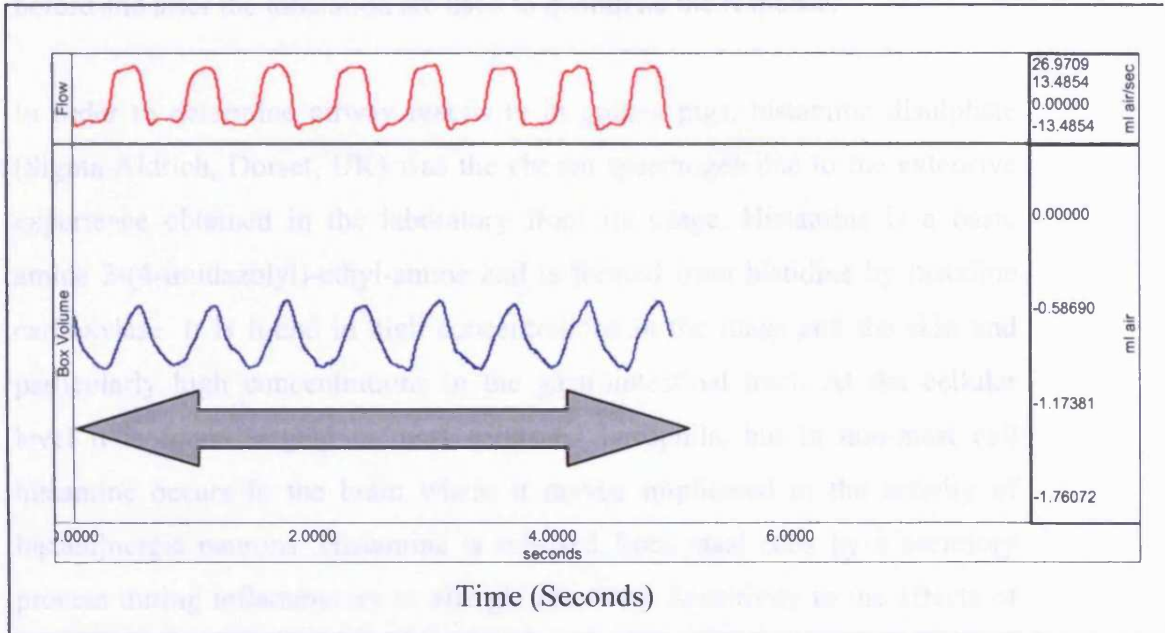
The guinea pig is restrained by the neck and the body. The neck is restrained by the neck restraining device. The body is restrained by the base unit. The neck restraining device is placed around the neck of the guinea pig. The base unit is placed around the body of the guinea pig. The neck restraining device is placed around the neck of the guinea pig. The base unit is placed around the body of the guinea pig.



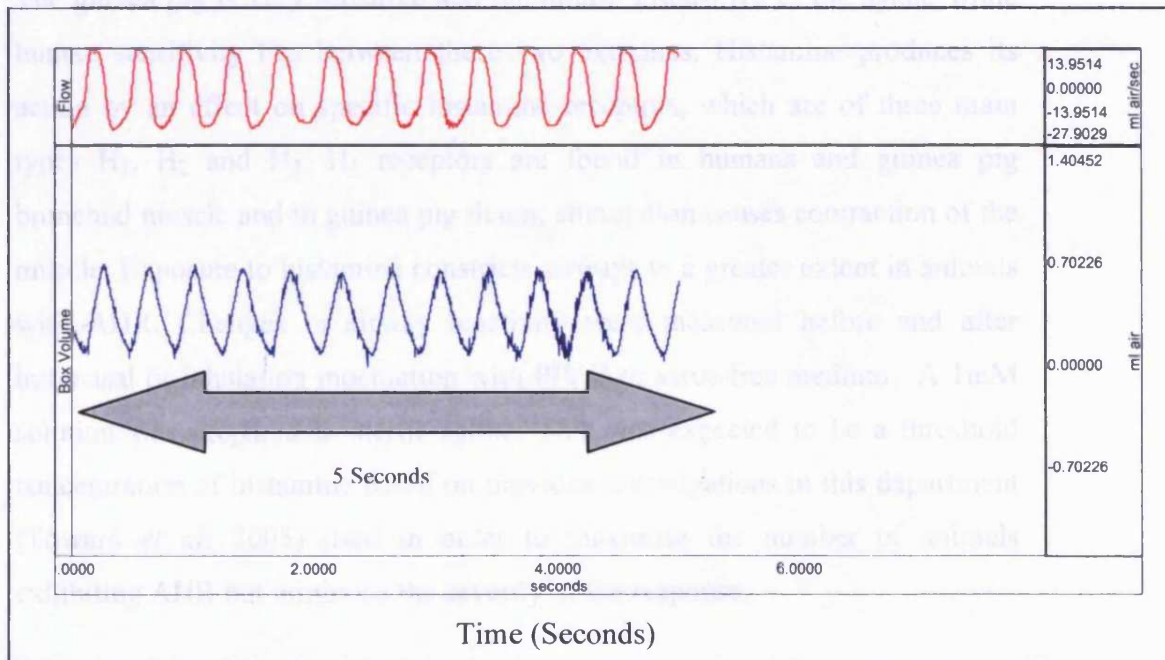
**Figure 2.5**

The patterns are signals produced by a guinea pig with **A)** normal respiratory rate, **B)** airway hyperreactivity and represents 5 seconds of respiratory data. The upper trace is the respiratory flow pattern and the bottom trace is the box volume, both plotted against time.

**A**



**B**





## MEASUREMENT OF AIRWAY REACTIVITY

Airway reactivity testing identifies and characterises hyperresponsive airways by the subject inhaling an aerosolised bronchospastic agonist such as methacholine or histamine. Results of pulmonary function tests performed before and after the inhalation are used to quantitate the response.

In order to determine airway reactivity in guinea pigs, histamine disulphate (Sigma-Aldrich, Dorset, UK) was the chosen spasmogen due to the extensive experience obtained in the laboratory from its usage. Histamine is a basic amine 2-(4-imidazolyl)-ethyl-amine and is formed from histidine by histidine carboxylase. It is found in high concentrations in the lungs and the skin and particularly high concentrations in the gastrointestinal tract. At the cellular level it is found largely in mast cells and basophils, but in non-mast cell histamine occurs in the brain where it maybe implicated in the activity of histaminergic neurons. Histamine is released from mast cells by a secretory process during inflammatory or allergic reactions. Sensitivity to the effects of histamine varies between tissues and between species.

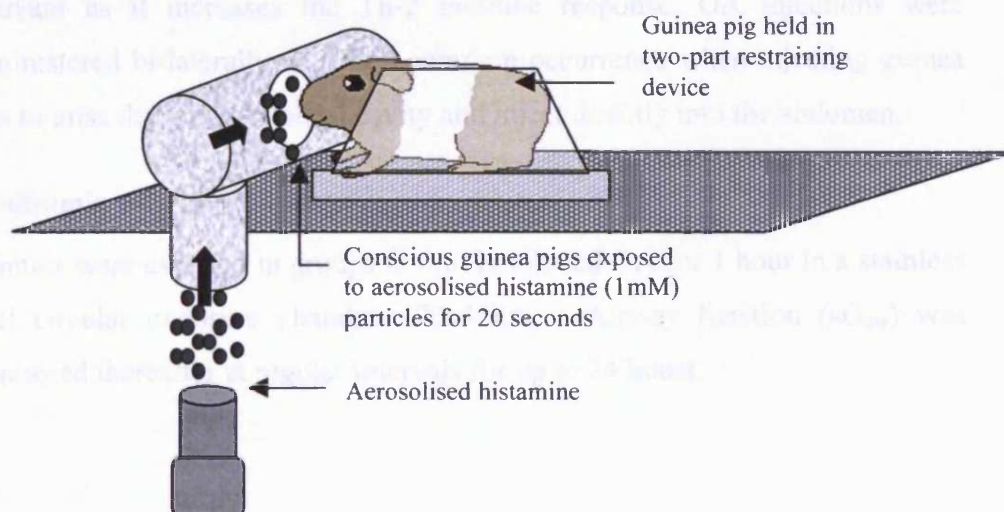
The guinea pig is very sensitive and the mouse insensitive to the agent, while human sensitivity lies between these two extremes. Histamine produces its action by an effect on specific histamine receptors, which are of three main types H<sub>1</sub>, H<sub>2</sub> and H<sub>3</sub>. H<sub>1</sub> receptors are found in humans and guinea pig bronchial muscle and in guinea pig ileum, stimulation causes contraction of the muscle. Exposure to histamine constricts airways to a greater extent in animals with AHR. Changes in airway reactivity were measured before and after intranasal or inhalation inoculation with PIV-3 or virus-free medium. A 1mM solution was prepared in sterile saline. This was expected to be a threshold concentration of histamine based on previous investigations in this department (Toward *et al*; 2005) used in order to maximise the number of animals exhibiting AHR but minimise the severity of the response.

A Pulmo-Aide® 561 series Wright nebuliser supplied with air (20 psi, 10 litres air min<sup>-1</sup>) from a compressor (Devilbiss®, Middlesex, UK) was used to generate and deliver aerosolised particles with a mean droplet diameter of 5µm, at a rate of 0.5ml min<sup>-1</sup> for 20 seconds via the apparatus shown in (Figure 2.6).

In order to expose the animal to histamine, the animal was placed in the two-part restraining device consisting of a base unit and neckpiece used for sG<sub>aw</sub> measurements. With the animal restrained, the animals' snout was placed in an opening in the exposure cylinder. An airtight seal around the animals' snout was achieved by stretching a cut section from the neck of a rubber balloon over the opening of the exposure cylinder. An initial (basal) value of sG<sub>aw</sub> was recorded prior to nebulised inhalation of histamine under steady state conditions. Directly after nebulisation breathing patterns (sG<sub>aw</sub>) were recorded at 0, 5 and 10 minutes post histamine exposure. These time intervals were selected as peak responses following histamine challenges, which generally occur within the first 5 minutes from previous studies in our laboratory.

**Figure 2.6**

**Nebulised histamine apparatus**



## **SENSITISATION AND EXPOSURE TO OVALBUMIN**

In these studies ovalbumin (OA) was the chosen antigen for sensitisation due to the extensive experience obtained in the laboratory from its usage. Chicken egg is a major source of ovalbumin protein. Two methods were employed for allergic sensitisation of the guinea pigs, including inhalation and intraperitoneal injection.

### **Ovalbumin administration by inhalation**

Groups of animals were sensitised together by a single inhalation of 0.5% OA in commercial saline (0.9%) for 3 minutes in a stainless steel circular exposure chamber (70x16cm). OA solution was nebulised by an air driven Wright nebuliser at a pressure of 20 psi at 0.3mls min<sup>-1</sup>. Any animal appearing in distress was immediately removed and the exposure considered complete. After 2 weeks, OA sensitisation by a single inhalation was repeated in the same manner (booster inhalation).

### **Ovalbumin administration by intraperitoneal injection**

Groups of animals were sensitised by bilateral intraperitoneal injection of OA (100mg) and aluminium hydroxide (100mg in 1ml of sterile saline) on day 1, with a booster injection administered on day 5. All subsequent procedures commenced day 10-17. Aluminium hydroxide (Al OH<sub>3</sub>) was used as an adjuvant as it increases the Th-2 immune response. OA injections were administered bi-laterally as it is a common occurrence when injecting guinea pigs to miss the intraperitoneal cavity and inject directly into the abdomen.

### **Ovalbumin allergic challenge**

Animals were exposed in groups to 0.01% inhaled OA for 1 hour in a stainless steel circular exposure chamber (70x16cm). Airway function (sG<sub>aw</sub>) was monitored thereafter at regular intervals for up to 24 hours.

## **ADMINISTRATION OF ANTI-INFLAMMATORY COMPOUNDS**

In this study the effects of the glucocorticoid, dexamethasone ( $20 \text{ mg kg}^{-1}$ ) and the phosphodiesterase isoenzyme-4 (PDE-4) inhibitor, ( $1 \text{ mg kg}^{-1}$ ) rolipram were evaluated against the parameters measured after viral infection. These doses of dexamethasone (Whelan *et al*; 1995) and rolipram (Danahay and Broadley; 1997, Toward *et al*; 2005) were selected based upon data from other studies using models of inflammation that were without adverse effects. Pre-treatments of dexamethasone and rolipram were administered bilaterally by intraperitoneal injection. In all studies the guinea pigs were dosed 24 hours and 30 minutes prior to intranasal instillation of PIV-3 or virus free medium and daily injections thereafter until the end of the study (day 4). The guinea pigs did not exhibit any obvious adverse effects from the treatment with dexamethasone or rolipram or their appropriate vehicles. The dexamethasone vehicle (50% DMSO (dimethyl sulfoxide) and 50% saline) and rolipram vehicle (saline) has been previously shown to have no affect in naïve animals or PIV-3 inoculated guinea pigs (Toward *et al*; 2005)

## **CYTOLOGICAL EXAMINATION OF BAL FLUID**

Bronchoalveolar lavage (BAL) is a diagnostic procedure for sampling cells and secretions from the alveolar and bronchial air spaces in the lower respiratory tract, for detection of infectious agents and the analysis of markers of lung inflammation.

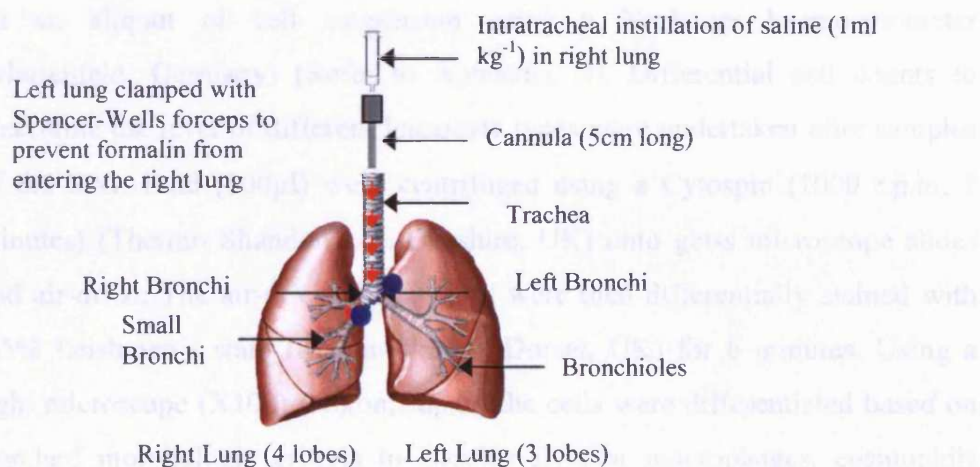
### **Lavage Procedure**

Within 20 minutes of assessing airway reactivity to histamine guinea pigs were sacrificed with a lethal dose of pentobarbitone sodium (Euthatal<sup>®</sup>  $200 \text{ mg ml}^{-1}$ ) ( $400 \text{ mg kg}^{-1}$ ) (Genusexpress, Chepstow, UK) injected bilaterally via the intraperitoneal cavity. After termination the animals were examined for cessation and placed in the supine position ready for dissection.

A small incision was made in the neck region of the animal and the trachea cannulated using a nylon intravenous cannula (Sims Portex Ltd, Kent, UK) wedged into the bronchus (Figure 2.7). In each guinea pig, BAL was performed *in situ* by intratracheal instillation using commercial sterile saline (0.9% 1 ml 100g<sup>-1</sup> body weight) at room temperature into the right lung via a syringe. The instilled fluid fills the airspaces, replacing the air and a portion of the instilled volume remains to be absorbed or expectorated after the procedure. After 3 minutes, the fluid was immediately withdrawn, recovered by gentle aspiration from massaging the thoracic cavity. A second lavage was performed and the two fluid collections were combined and retained on ice until further processed. Only plasticware was used throughout the collection process to minimise adherence of the cells to the walls of the tubes. Removal of BAL fluid from virus and virus-free medium infected guinea pigs was performed in a class II safety cabinet.

**Figure 2.7**

**Bronchoalveolar lavage technique**



In experiments, to maximise the use of the guinea pig lungs, left and right lungs were processed as follows:

#### **Left Lung**

Histological analysis

Wet lung weight

#### **Right Lung**

Bronchoalveolar lavage

Viral determination



#### **Morphometric cell analysis**

Routine cytological examination of BAL fluid included the determination of total and differential cell counts. Total cell counts (cells ml<sup>-1</sup>) were undertaken on an aliquot of cell suspension using a Neubauer haemocytometer (Marienfeld, Germany) (Refer to Appendix II). Differential cell counts to determine the level of different leucocyte types were undertaken after samples of the BAL fluid (100µl) were centrifuged using a Cytospin (1000 r.p.m, 7 minutes) (Thermo Shandon Ltd, Cheshire, UK) onto glass microscope slides and air-dried. The air-dried preparations were then differentially stained with 1.5% Leishman's stain (Sigma-Aldrich, Dorset, UK) for 6 minutes. Using a light microscope (X100) (Nikon, Japan) the cells were differentiated based on standard morphologic criteria to classify alveolar macrophages, eosinophils and neutrophils (Table 2.1). A minimum of 200 consecutive cells were counted and expressed as percentages (%) of the total cell preparation to give individual cell counts for each cell type (cells ml<sup>-1</sup>) in the BAL fluid to assess the presence of inflammatory responses.

The remaining BAL fluid was centrifuged (Mistral 3000, Fisher Scientific, Loughborough, UK) at 2000 r.p.m for 6 minutes and the cell free supernatant transferred into 500µl aliquots in sterile microfuge tubes and stored at  $-80^{\circ}\text{C}$  for determination of inflammatory markers of inflammation including NO and total protein levels.

**Table 2.1**

**Cell Characteristics**

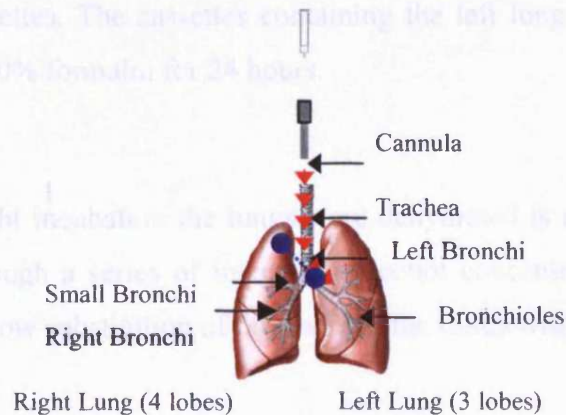
<b>Cell Type</b>	<b>Characteristics</b>	
<b>Macrophage</b> (12-18µm)	<b>Nucleus:</b>	Deep Blue
	<b>Cytoplasm:</b>	Purple
	<b>Appearance:</b>	Typically circular or oval shape
	<b>Activated appearance:</b>	Enlarged with phagocytic vesicles
<b>Neutrophil</b> (10-12µm)	<b>Nucleus:</b>	Blue
	<b>Cytoplasm:</b>	Light pink and faint granules
	<b>Appearance:</b>	Typically 2-4 inter connected nuclei
	<b>Activated appearance:</b>	Enlarged with dispersed granulation
<b>Eosinophil</b> (10-12µm)	<b>Nucleus:</b>	Blue
	<b>Cytoplasm:</b>	Orange/pink and large dense granules
	<b>Appearance:</b>	Typically bi-lobed nuclei
	<b>Activated appearance:</b>	Enlarged with dispersed granulation

## LUNG PREPARATION FOR VIRAL DETERMINATION AND HISTOLOGICAL LUNG ANALYSIS

On completion of the right lung BAL, the cannula remained in the trachea and the lungs were removed from the thoracic cavity as follows. The trachea, mainstem bronchi, and lungs were exposed by an incision beginning at or below the level of the diaphragm. The ribcage was opened by cutting through the camina and continuing to the thyroid gland. After removing the ribs, the trachea and lungs are readily apparent. The trachea and lungs were removed from the animal and placed in Krebs solution in a sterile petri dish to prevent from drying out and adherent fat, connective tissue and blood vessels were removed. The right main stem bronchus was ligated using Spencer-Wells forceps and the right lung (4 lobes) was removed, separated and immediately frozen in liquid nitrogen, placed in a sterile vessel and stored at  $-80^{\circ}\text{C}$  for viral titre determinaton. The remaining left lung (3 lobes) were blotted dry and the weight recorded using a balance (Precisa balances Ltd), the lungs were them fully immersed in 100 ml of 10% neutral buffered formalin (Fisher Scientific, Leicestershire, UK) and further fixed by inflation of the airways via intratracheal instillation of formalin ( $1\text{ml } 100\text{g}^{-1}$  body weight) (Figure 2.8).

**Figure 2.8**

### Guinea pig lung preparation for viral determination and histological lung analysis





## **DETERMINATION OF WET LUNG WEIGHTS**

Pulmonary oedema is a condition in which fluid accumulates in the lungs, caused by back pressure in the pulmonary veins and by increased capillary permeability due to locally released inflammatory mediators. It was determined by measured changes in the wet left lung weights. Changes in lung oedematous fluid after exposure to PIV-3 were determined at regular intervals post inoculation and after treatment with dexamethasone and rolipram. For each guinea pig, wet weight on the left lung were determined immediately after removal from the thorax

## **HISTOLOGICAL ANALYSIS**

### **Fixation**

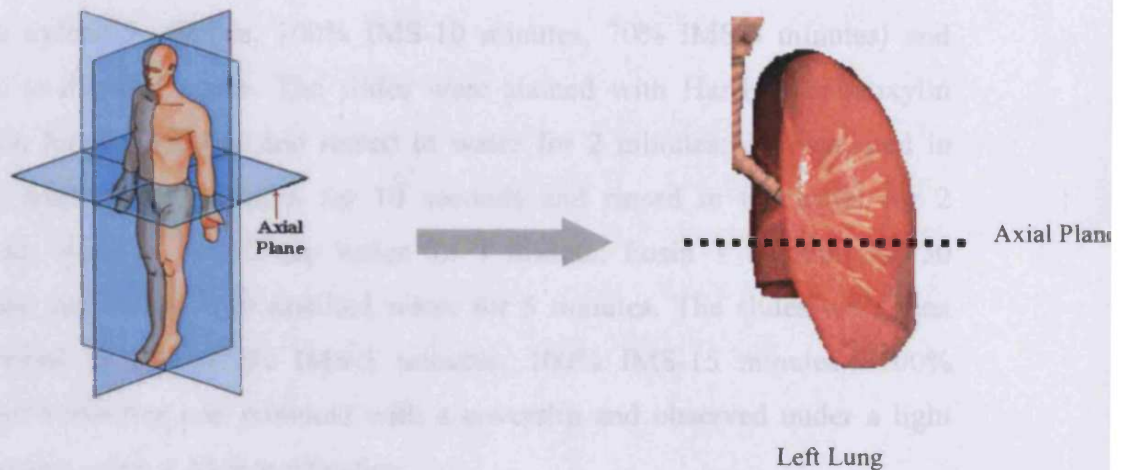
Following immediate removal from the thoracic cavity the left lung was weighed, placed in a sterile container and fully immersed in 100 ml of 10% neutral buffered formalin (Sigma-Aldrich, Dorset, UK) for 24 hours. This process is known as fixation and is required to prevent structural alterations through decomposition. Fixation involves the chemical cross-linking of proteins (to prevent enzyme action and digestion) and the removal of water to further denature the proteins of the cell. After fixation, the left lung was serially sectioned with alternating parasagittal slices (Fig 2.9). 3-5 mm thick sections were cut through the large bronchi of the left lung (medial lobe) and placed in cassettes. The cassettes containing the left lung sample were again immersed in 10% formalin for 24 hours.

### **Dehydration**

After, overnight incubation the lungs were dehydrated is achieved by passing the tissue through a series of increasing alcohol concentrations. Dehydration involves the slow substitution of the water in the tissue with an organic solvent.

**Figure 2.9**

**Axial Plane of left lung**



The cassettes were removed from 10% formalin, and placed in a series of industrial methylated spirit (IMS) solutions (50%-1 hour, 70%-1hour, 90%-1hour, 100%-1.5 hours) each time blotting the cassette to remove the excess liquid. Finally, the cassettes were placed in 50:50 IMS/chloroform solution overnight. The next day, the cassettes were transferred to 100% chloroform (intermediate fluid that is miscible with ethanol and paraffin) for 3 hours.

**Embedding**

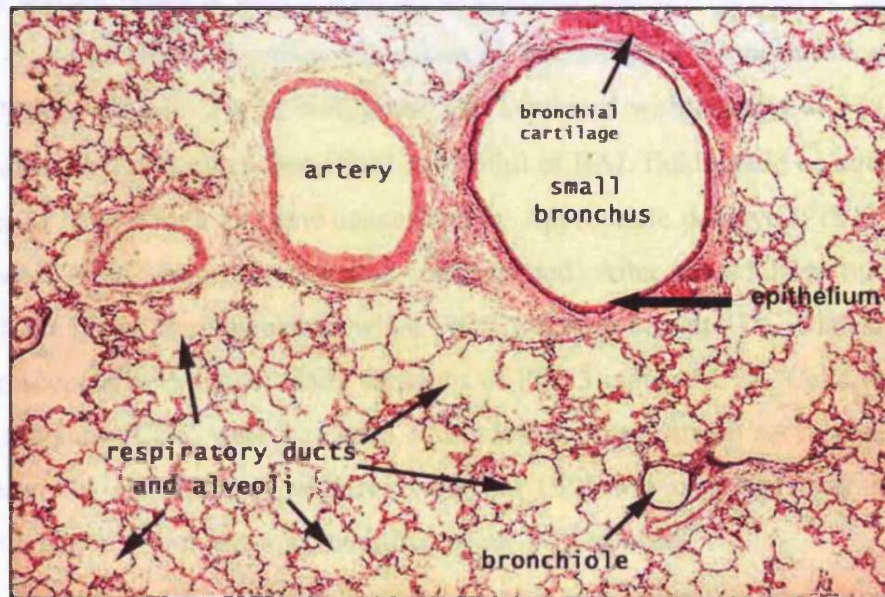
The cassettes were drained and immersed in paraffin wax for 6 hours and placed in the oven (wax was changed every 2 hours). During this time the tissue block is completely infiltrated with melted paraffin. Subsequent to infiltration, the tissue is placed into an embedding mould and melted paraffin is poured onto the mould to form a block. The blocks were allowed to cool and are then ready for sectioning. Using a microtome, 2 x 3 $\mu$ m thick sections were cut from the paraffin block and placed in a water bath at 50<sup>0</sup>C and mounted onto a glass slide.

### Haematoxylin and Eosin staining

The lungs were stained with haematoxylin and eosin for general morphology (Figure 2.10). Before staining, the slides were de-paraffinised as follows: (100% xylene-5 minutes, 100% IMS-10 minutes, 70% IMS-5 minutes) and placed in distilled water. The slides were stained with Harris haematoxylin solution for 2.5 minutes and rinsed in water for 2 minutes; differentiated in 0.25% acid-alcohol solution for 10 seconds and rinsed in tap water for 2 minutes; blued in Scott's tap water for 1 minute; Eosin Y solution for 30 seconds; and rinsed with distilled water for 5 minutes. The slides were then dehydrated in IMS (70% IMS-5 minutes, 100% IMS-15 minutes), 100% xylene-10 minutes and mounted with a coverslip and observed under a light microscope using x 40 magnification.

Figure 2.10

#### General lung histology



Slides were evaluated for three indices of pulmonary inflammatory changes. The lungs were scored in each lung section: peribronchiolitis (inflammatory cells clustered around the periphery of the small airways), interstitial pneumonitis (inflammatory cell infiltration and thickening of the alveolar walls), and alveolitis (inflammatory cells within the alveolar air spaces). Each of the parameters were scored separately for each histologic section on a semi-quantitative scale as absent (0), minimal (1), slight (2), moderate (3), marked (4), or severe (5) (Barends *et al*; 2002). Although each was scored using the same scale, the scores are relative and are only valid for comparing the same parameter in different sections and not for comparing different parameters within a section.

#### **DETERMINATION OF PIV-3 IN GUINEA PIG BAL FLUID**

The presence of PIV-3 in BAL fluid and guinea pig right lung tissue was evaluated by determining the CPE of the lavage fluid on monolayers of Vero cells and by reaction with a specific immunofluorescence (IF) probe against PIV-3. Vero cells were grown in medium A, and seeded at a concentration of  $1 \times 10^5$  per well onto 2 x 12 well plates and incubated with 5% CO<sub>2</sub> at 37<sup>0</sup>C for 24 hours. It was decided that 100µl and 350µl of BAL fluid would be added in order to have a high and low concentration. Appropriate positive (PIV-3) and negative (PBS) controls were also implemented. After the addition of BAL fluid, the plates were incubated win a 5% CO<sub>2</sub> atmosphere at 37<sup>0</sup>C. Plates were microscopically examined daily for signs of PIV-3 induced CPE. Cells, which did not show CPE, were passaged at weekly intervals for up to 1 month. A culture was classified as negative when no CPE was observed over a one month period. Viral titre was measured using TCID<sub>50</sub> assay.

## **DETERMINATION OF PIV-3 IN GUINEA PIG LEFT LUNG**

Guinea pig left lungs stored at  $-80^{\circ}\text{C}$  were transferred to a sterile 50 ml centrifuge tubes and rapidly thawed in an incubator at  $37^{\circ}\text{C}$  to maintain the integrity of the cells. The tissue preparation was carried out on ice and all solutions and centrifuge were pre-cooled to  $4^{\circ}\text{C}$ . Once thawed, the right lung tissue was transferred through a series of washes x 3 using medium A.

After washing the left lung was transferred to a sterile petri dish with 5mls of medium A. The left lung tissue was then finely minced using a sterile scalpel into  $1\text{mm}^3$  pieces and the dissected sample was then resuspended in 30mls of growing DMEM and filtered through a cell strainer ( $100\mu\text{m}$  Falcon, UK). The plunger from a 5ml syringe was used to apply pressure to the upper surface of the strainer, containing the cells to be filtered. This process was repeated twice, each time adding 5-10mls of medium A to rinse the strainer. The cell strained medium (50mls) was then pelleted by centrifugation at 2000 r.p.m for 10 minutes at  $4^{\circ}\text{C}$ . Once centrifuged, the supernatant was aspirated and the pellet discarded and 6-7mls of medium A was added to create a thick suspension.

Vero cells ( $2 \times 10^5$ ) were seeded onto the bottom of a 6 well cell culture plate in medium A. Using a 6 well plate, 2mls of lung tissue cell homogenate was transferred to 3 wells and incubated with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$  for 24 hours. Appropriate positive (PIV-3) and negative control (PBS) samples were also implemented. Plates were microscopically examined daily for signs of PIV-3 induced CPE. Cells, which did not show CPE, were passaged at weekly intervals for up to 1 month. A culture was classified as negative when no CPE was observed over a one month period. Viral titre was measured using  $\text{TCID}_{50}$  assay.

Lung tissue homogenate supernatants were inoculated in serial 10-fold dilutions into Vero cells monolayers. Titres of intrapulmonary replicating PIV-3 were determined in right lung tissue samples on various days post infection were normalised to left lung tissue weight (TCID<sub>50</sub> ml<sup>-1</sup>g<sup>-1</sup>). This normalisation was necessary due to the varying weights of the guinea pigs and only the left lung was used to determine viral titre.

### **DETECTION OF PIV-3 IN BAL FLUID AND RIGHT LUNG TISSUE USING INDIRECT IMMUNOFLUORESCENCE**

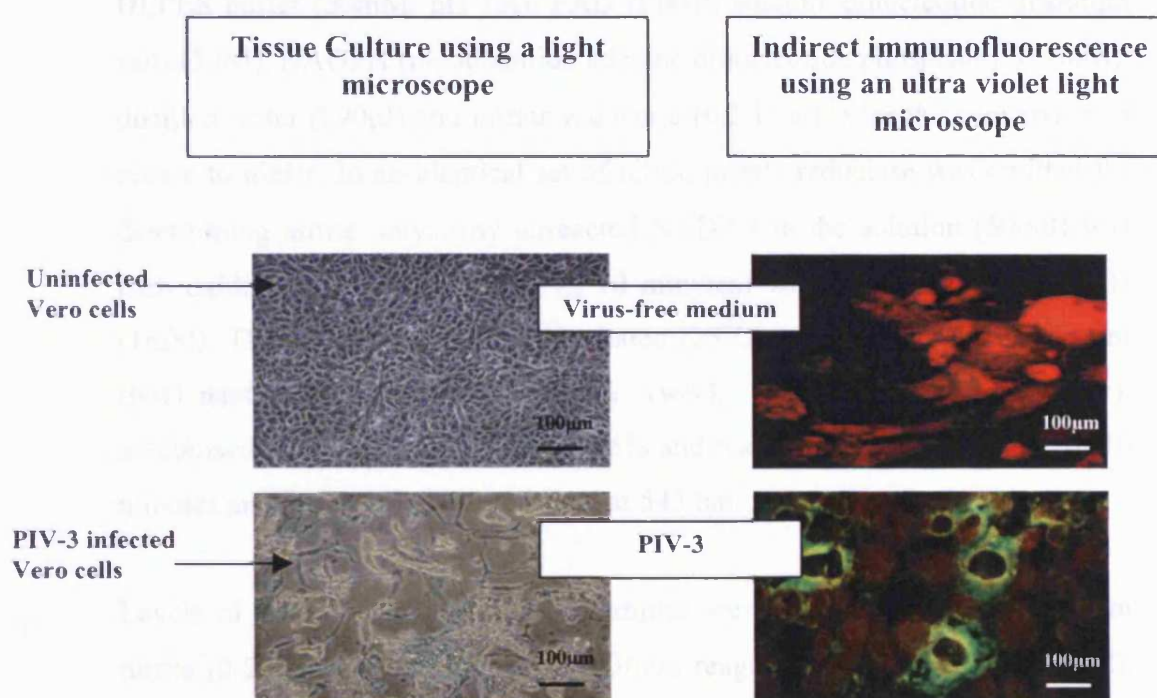
Indirect immunofluorescence (IF) is a technique used to detect infected cells in exudates and secretions by laying a known antiserum onto cell spots fixed to glass slides. This study used an indirect immunofluorescence technique in which antiserum was applied to the wells and then a second labelled antibody (antiIg) directed against the unlabelled immunoglobulins of the first antiserum is used. Indirect immunofluorescence was used to test guinea pig BAL fluid and guinea pig homogenised right lung samples for the detection of PIV-3. To further confirm the presence or absence of PIV-3 the appropriate well was scraped with a sterile scraper from BAL fluid and lung tissue plates, and the solution transferred into a 15ml centrifuge tube. The medium was centrifuged at 1500 r.p.m for 5 minutes and the supernatant discarded. The cell pellet was disrupted and resuspended in 15mls of PBS, mixed by inversion and centrifuged.

The supernatant was discarded and the washing procedure was repeated. Once the cells were washed for a second time, the supernatant was discarded and the cells suspended in enough PBS to produce a milky suspension. Using a Pasteur pipette, 40µl of cell suspension was dispensed onto multi-spot teflon coated glass slides according to the designed template. Two spots are required for each sample, one for the test sample and the other for a negative control. The preparation was then air dried in a stream of cool air from a small fan heater in a class II cabinet.

Once dry, the slides were fixed in reagent grade acetone for 5 minutes at 4<sup>0</sup>C to permeabilise the cell walls and to render the virus inactive. The slides were removed and allowed to dry and stored in moisture tight boxes at -20<sup>0</sup>C. The slides were removed from -20<sup>0</sup>C storage and hydrated by placing in a moist chamber at 37<sup>0</sup>C for 15-30 minutes. This was necessary to reduce non-specific staining in the sample. Primary antisera (PIV-3 Mab) (Chemicon International, Inc. Temecula, CA) or mouse immunoglobulin was added to the cells spots and the slides were incubated in a moist chamber at 37<sup>0</sup>C for 30 minutes. After incubation, the slides were removed and gently washed twice (5 minutes each) by immersion in PBS using a slow rocking motion to remove residual unattached antibody. The slides were turned on their edge and gently tapped on tissue paper to drain excess PBS from the slide surface without touching the surface of the wells.

Next, 25µl of 1% fluorescein isothiocyanate (FITC) labelled goat antibody in 1% Evans blue stain was applied to each spot and the slides incubated for a further 30 minutes at 37<sup>0</sup>C in a moist chamber. FITC binds to the antigen antibody complex and exhibits an apple green fluorescence when excited by ultra violet light allowing visualisation of the complex by microscopy. Evans blue is a counterstain that fluoresces red and blankets non-specific cell staining by the fluorescein conjugate. After two further washes to remove unbound conjugated antiglobulins, the slide was turned on its edge and gently tapped on a tissue to remove any residual PBS off the surface. A tissue was then used to dry the surface without touching the well contents. The slide was overlaid with 50% glycerol in PBS and a large cover slip placed over the whole surface. The slides were examined using an ultra violet light microscope with an oil immersion lens and cells that fluoresce indicate a positive specimen. PIV-3 presents in cells as a globular, speckled cytoplasmic pattern. Uninfected cells stain a dull red due to the presence of Evans blue counterstain in the FITC labelled secondary antibody (Figure 2.11 ).

**Figure 2.11**



## MEASUREMENT OF INFLAMMATORY MARKERS IN BAL FLUID

### Nitric oxide

A spectrophotometric assay was used to determine the stable decomposition products of nitric oxide (NO) including nitrite ( $\text{NO}_2$ ) and nitrate ( $\text{NO}_3$ ) based on the Griess reaction, as described by Grisham and Co-workers (1996). This method requires that  $\text{NO}_3$  first be reduced to  $\text{NO}_2$  using *Aspergillus* nitrate reductase, and then  $\text{NO}_2$  determined by the Griess reaction. Nitrate rich foods such as lettuce and cabbage have been shown to affect the determinations of nitrate and so were not given to the guinea pigs to supplement their diet (Grisham *et al*; 1996). This method allows the determination of NO in BAL fluid and unlike the use of other methods that rely on exhaled NO, provides measurements of lower airways NO levels, without contamination from the excess amounts of NO produced from the nasal sinus cavities in the upper airways.



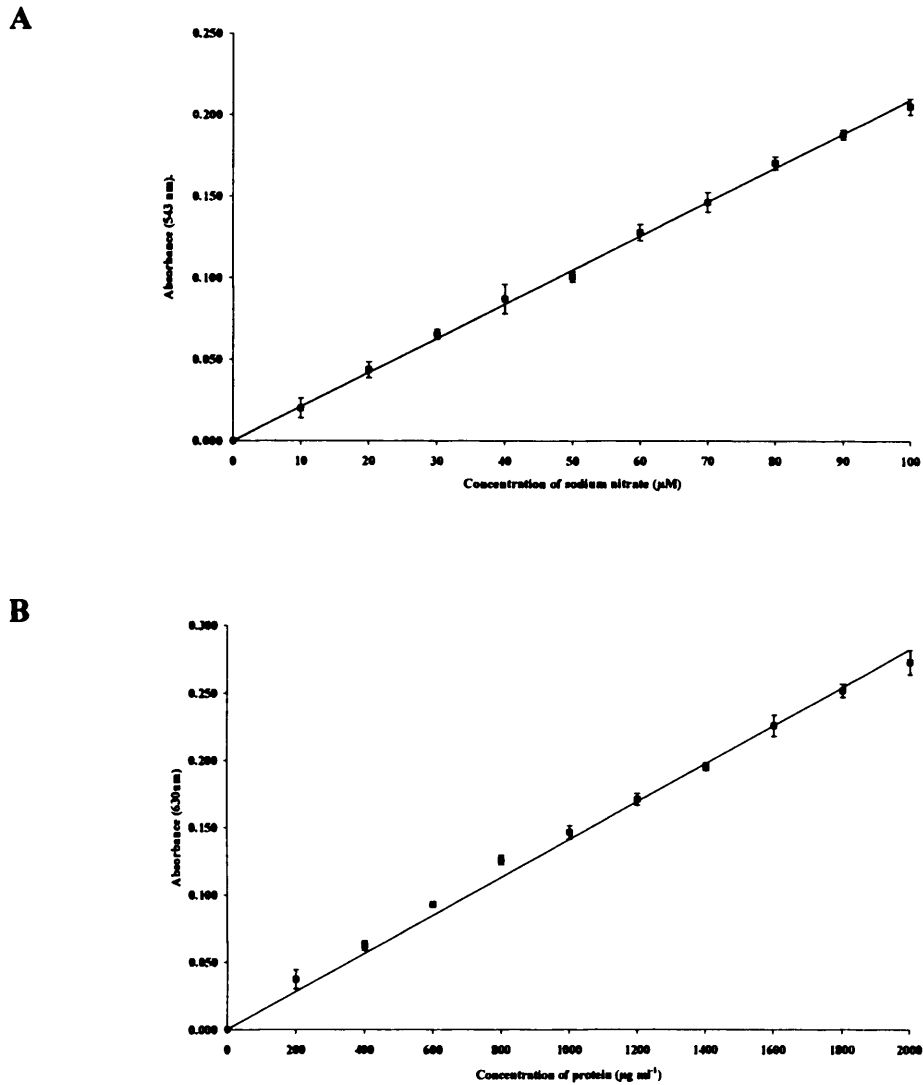
The BAL fluid (100µl) samples were incubated at 37<sup>0</sup>C for 30 minutes with HEPES buffer (50mM, pH 7.4), FAD (Flavin adenine dinucleotide disodium salt) (5µM), NADPH (nicotinamide adenine dinucleotide phosphate) (0.1mM), distilled water (290µl) and nitrate reductase (0.2 U ml<sup>-1</sup>) for the conversion of nitrate to nitrite. In an identical set of tubes, nitrate reductase was omitted for determining nitrite only. Any unreacted NADPH in the solution (500µl) was then oxidised by incubating (25<sup>0</sup>C, 10 minutes) with potassium ferricyanide (1mM). The samples were then incubated (25<sup>0</sup>C) with 1ml of Griess reagent (N-(1-naphthyl)ethylenediamine: 0.2% (w/v), sulphanilamide: 2% (w/v), solubilised in double distilled water: 95% and phosphoric acid: 5% (v/v) for 10 minutes and the absorbance measured at 543 nm.

Levels of nitrite in the BAL fluid samples were determined from a sodium nitrite (0-200µM) standard curve of Griess reagent absorbance (Figure 2.12). Saline replaced BAL fluid in the above studies to determine the background level of Griess reagent absorbance in this procedure. The background level of absorbance was subtracted from that obtained for each BAL fluid sample, to determine the true value of nitrate or nitrite. The maximum linear limit of detection for the assay was 1mM. In all cases, all the samples to be compared were assayed at the same time.

### **Total protein**

The levels of total protein in the supernatant from the BAL fluid were determined as an index of increased plasma exudation (Persson *et al*; 1998) according to previously described methodology (Bradford; 1976, Banner *et al*; 1996). Coomassie blue reagent (100mg) was dissolved in 50ml of 95% ethanol. To this solution 100ml of 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. This was left to stand for 24-48 hours and then filtered to remove the natural precipitate that forms.

100µl of six known concentrations of bovine serum albumin (0-900 µg ml<sup>-1</sup>) were added to 1ml of Coomassie blue reagent. After 1 hour at 24<sup>0</sup>C, the optical density at 630nm was evaluated (MRX TC revelation, Dynex technologies) and a line drawn by linear regression (Figure 2.12). When assaying BAL fluid, 30 µl of supernatant was added to 70µl of normal saline together with 1ml Coomassie blue reagent. Each sample was assayed in duplicate.



**Figure 2.12**

Typical standard curves for NO and total protein assays. **A** Greiss reagent absorbance (543nm) to standards of sodium nitrate (0-1000µm) in saline, or **B** Coomassie blue absorbance (630nm) in standards of bovine serum albumin (0-2000 µg ml<sup>-1</sup>) in saline. In the absence of standard, the background level of absorbance was subtracted from that obtained from each sample, to determine the true Greiss reagent or Coomassie brilliant blue absorbance. Each point (n=6) represents the mean ± S.E.M of the standard concentration.

## EXPRESSION OF RESULTS AND STATISTICAL ANALYSIS

Due to intersubject variability,  $sG_{aw}$  values were expressed as the percentage change from a baseline value of  $sG_{aw}$  taken immediately prior to the start of a procedure. Exposure to OA in sensitised animals caused a late phase antigen-induced bronchoconstrictor response (LAR) at different times in individual animals, but consistently between 7 and 11 hours after OA exposure. The means of the individual peak % fall from baseline  $sG_{aw}$  between 7 and 11 hours are reported  $\pm$  standard error of mean (SEM). This was considered a better representation of airway function during the LAR, as reporting mean changes in  $sG_{aw}$  from baseline values, or area under the curve would not indicate the severity of the bronchoconstriction, or would be compromised by the duration of the LAR (i.e. a long shallow bronchoconstriction would be considered equivalent to a transient but severe bronchoconstriction). Similarly, the mean of the individual peak % falls from baseline  $sG_{aw}$  between 0 and 10 minutes following assessment of airway reactivity to histamine, are also reported for the groups.

For all parameters investigated, a two-way analysis of variance (ANOVA) followed by the Dunnett's test was used for statistical comparison. All values are presented as mean  $\pm$  standard error of means. All statistics were performed using SPSS (Statistical Package for the Social Sciences).

## MATERIALS

Coomassie blue reagent, dexamethasone-21-phosphate disodium salt, dimethylsulphoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum, Greiss reagent, Haematoxylin & Eosin stain, histamine disphosphate salt, Leishman's stain, L- glutamine, N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonicacid-4-(2hydroxethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, ovalbumin, penicillin, phosphoric acid, rolipram, sodium nitrite and streptomycin were purchased from Sigma (Poole, Dorset, UK). Acetone, ethanol, 10% neutral buffered formalin and xylene were purchased from BDH (Poole Dorset, UK). Aspergillus nitrate reductase (NADPH:nitrate oxidoreductase) was purchased from Boehringer Mannheim (Indianapolis, USA). Primary antisera (PIV-3 Mab) was purchased from Chemicon International Inc (Temecuta, CA). Sterile saline (0.9% NaCl) was purchased from Baxter Healthcare (Thetford, Norfolk, UK).

## **CHAPTER 3**

# **DEVELOPMENT OF A GUINEA PIG MODEL OF PIV-3 INFECTION**

### **INTRODUCTION**

PIV-3 is second only to human RSV as the most common cause of serious respiratory tract disease in infants and children. The guinea pig has previously been used as an inflammatory model of acute bronchiolitis and airway hyperresponsiveness to PIV-3 in several studies (Folkerts & Nijkamp 1993, Kudlacz *et al*; 1993). The aim of this chapter was to develop a guinea pig model of PIV-3 infection that is useful in investigating the pathogenesis of acute bronchiolitis and its sequelae. In order to develop a suitable model of PIV-3 infection for these studies it was necessary to determine the most efficient route of viral inoculation in order to produce sufficient infection. Investigations have established the use of three techniques including intra-nasal (Hegele *et al*; 1993), intra-tracheal instillation (Folkerts *et al*; 1993) and aerosolised inhalation (Streckert *et al*; 1996). Intranasal instillation involves pipetting the inoculum medium into the nasal cavity of the guinea pigs in the conscious or anaesthetised state (Kudlacz *et al*; 1993). Intratracheal instillation can be performed under anaesthesia where the animals are laid on their backs. The mandibles are kept open by two rubber elastic rings and a needle placed behind the glottis and the inoculum gently injected into the trachea (Folkert *et al*; 1992). Aerosolised virus inhalation involves placing the guinea pigs in an incubation box and a compressor is used to nebulise the virus which the guinea pig inhales (Streckert *et al*; 1996).

In these studies it was decided that a comparison between intranasal and nebulisation modes of virus application would be investigated in un-anaesthetised animals. These techniques were chosen as they are less invasive than intratracheal inoculation and do not require the animal to be anaesthetised. In order to assess sufficient infection in the guinea pigs, inflammatory parameters were investigated including airway reactivity to histamine, cellular infiltration, and pulmonary histopathology

### **EXPERIMENTAL PROTOCOL**

The following procedures used to perform this study have all been previously described in chapter 2.

The experimental groups were as follows.

	<b>GROUP (n=6)</b>
<b>1</b>	Virus-free medium (Intranasal) infected animals assessed post inoculation day 4
<b>2</b>	Virus-free medium (Intranasal) infected animals assessed post inoculation day 7
<b>3</b>	Virus-free medium (4Nebulised) infected animals assessed post inoculation day 4
<b>4</b>	Virus free medium (Nebulised) infected animals assessed post inoculation day 7,
<b>5</b>	PIV-3 (Intranasal) infected animals assessed post inoculation day 4
<b>6</b>	PIV-3 (Intranasal) infected animals assessed post inoculation day 7
<b>7</b>	PIV-3 (Nebulised) infected animals assessed post inoculation day 4
<b>8</b>	PIV-3 (Nebulised) infected animals assessed post inoculation day 7
<b>9</b>	UV inactivated PIV-3 (Intranasal) inoculated animals assessed post inoculation day 4

These study days were selected as research by other investigators using PIV-3 demonstrated maximal inflammatory changes, indicating infection between, 4 and 7 days post inoculation.

On day -1 guinea pigs were assessed for airway reactivity to histamine 24 hours before inoculation. On study day 0 guinea pigs were inoculated with PIV-3 or virus-free medium by intranasal instillation or nebulisation techniques. Guinea pigs were assessed 4, and 7 days post inoculation. At the end of each study the animals were reassessed for airway reactivity to histamine and within 20 minutes were sacrificed and BAL performed and lung tissue removed. BAL fluid specimens were analysed for virus-induced changes by comparison of total and differential cell counts. Lung tissue specimens were used for histological analysis. Animals were also examined daily for general signs of illness including hair loss and decreased locomotor activity.

## RESULTS

### *Effect of PIV-3 on airway reactivity to inhaled histamine comparing intranasal and nebulisation methods of application.*

Inhalation of histamine (1mM) for 20 seconds, 24 hours before intranasal or inhalation inoculation of virus-free medium or PIV-3 did not produce any significant ( $P>0.05$ ) bronchoconstriction and failed to cause significant deviation from baseline  $sG_{aw}$  values in any of the groups studied (Figure 3.1). Thus indicating that this was a sub-threshold dose of histamine.

Exposure to histamine produced a significant bronchoconstriction ( $P<0.05$ ) occurred ( $-40.5\pm 3.3\%$ ,  $-32.8\pm 3.9\%$   $H_2O^{-1}sec^{-1}$ ) in guinea pigs receiving PIV-3 intranasally post inoculation day 4 and 7 respectively in comparison to the appropriate virus-free medium guinea pigs ( $-1.0\pm 0.9\%$ ,  $-1.6\pm 1.1\%$   $H_2O^{-1}sec^{-1}$ ).



In addition, a significant bronchoconstriction ( $P < 0.05$ ) occurred ( $-15.8 \pm 2.3\%$ ,  $-9.0 \pm 2.5\%$   $\text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ) in PIV-3 infected guinea pigs post inoculation days 4 and 7 using the nebulisation inoculation procedure compared to corresponding virus-free medium guinea pigs ( $1.0 \pm 0.2\%$ ,  $-1.3 \pm 1.0\%$   $\text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ). No significance difference ( $P > 0.05$ ) was observed in AHR between UV inactivated PIV-3 compared to virus-free medium infected guinea pigs using intranasal inoculation post inoculation day 4.

***Effect of PIV-3 on airways infiltration of leucocytes comparing intranasal and nebulisation methods of application.***

On days 4 and 7 days after PIV-3 inoculation, a significant increase ( $P < 0.05$ ) in total cell counts ( $8.1 \pm 0.3$ ,  $5.9 \pm 0.3$  cells  $\text{ml}^{-1}$ ) occurred in BAL fluid compared to corresponding virus-free medium infected guinea pigs ( $2.0 \pm 0.2$ ,  $2.0 \pm 1.0$  cells  $\text{ml}^{-1}$ ). In addition, a significant increase ( $P < 0.05$ ) in differential cell counts occurred in BAL fluid including macrophages ( $6.5 \pm 0.3$ ,  $4.9 \pm 0.2$  cells  $\text{ml}^{-1}$ ) eosinophils ( $1.4 \pm 0.1$ ,  $0.8 \pm 0.1$  cells  $\text{ml}^{-1}$ ) and neutrophils ( $0.3 \pm 0.1$ ,  $0.2 \pm 0.1$  cells  $\text{ml}^{-1}$ ) respectively to virus-free medium infected guinea pigs (Macrophages  $1.8 \pm 0.1$ ,  $1.8 \pm 1.0$  cells  $\text{ml}^{-1}$ ), eosinophils ( $0.2 \pm 0.03$ ,  $0.2 \pm 0.01$  cells  $\text{ml}^{-1}$ ), neutrophils  $0.03 \pm 0.01$ ,  $0.02 \pm 0.01$  cells  $\text{ml}^{-1}$ ) (Figure 3.2).

A significant increase ( $P < 0.05$ ) in total cell influx into the lungs was also observed 4 and 7 days post inoculation in PIV-3 infected guinea pigs using nebulisation as a mode of application ( $3.5 \pm 0.2$ ,  $2.8 \pm 0.2$  cells  $\text{ml}^{-1}$ ) compared to corresponding cells in virus-free medium guinea pigs. In addition, a significant increase ( $P < 0.05$ ) in differential cell counts occurred in BAL fluid including macrophages ( $3.0 \pm 0.2$ ,  $2.4 \pm 0.2$  cells  $\text{ml}^{-1}$ ) eosinophils ( $0.5 \pm 0.1$ ,  $0.3 \pm 1.0$  cells  $\text{ml}^{-1}$ ) and neutrophils ( $0.10 \pm 0.03$ ,  $0.07 \pm 0.03$  cells  $\text{ml}^{-1}$ ) respectively compared to virus-free medium infected guinea pigs (Macrophages  $2.0 \pm 0.1$ ,  $2.0 \pm 0.1$  cells  $\text{ml}^{-1}$ , eosinophils ( $0.19 \pm 0.02$ ,  $0.20 \pm 0.01$  cells  $\text{ml}^{-1}$ ), neutrophils  $0.02 \pm 0.01$ ,  $0.02 \pm 0.01$  cells  $\text{ml}^{-1}$ ) (Figure 3.2).

No significance differences ( $P>0.05$ ) was observed in airway infiltration of leucocytes between UV PIV-3 compared to virus-free medium infected guinea pigs using intranasal inoculation post inoculation day 4.

***Effect of PIV-3 on pulmonary histopathology and mean pulmonary score comparing intranasal and nebulisation methods of application.***

The histological analysis of the lungs in this study was used to assess the virus-induced changes in order to determine the most efficient route of viral application. The lungs were assessed for peribronchiolitis, interstitial pneumonitis, alveolitis by using a scoring system (Figure 3.3). Histological analysis of lungs from PIV-3 infected guinea pigs using intranasal inoculation revealed a significant increase ( $P<0.05$ ) in mean composite pulmonary pathology ( $4.0\pm 0.3$ ;  $3.0\pm 0.3$ ) compared to virus-free medium infected guinea pigs of the corresponding group ( $0.1\pm 0.1$ ;  $0.1\pm 0.1$ ) (Figure 3.4).

Histological analysis in guinea pig lungs from PIV-3 using nebulised inoculation post inoculation day 4 and 7 also revealed a significant increase ( $P<0.05$ ) in mean composite pulmonary pathology ( $1.4\pm 0.3$ ;  $1.0\pm 0.2$ ) compared to virus-free medium infected guinea pigs of the corresponding group ( $0.1\pm 0.1$ ;  $0.1\pm 0.1$ ) (Figure 3.4). However, a significant difference ( $P<0.05$ ) was observed between intranasal and nebulisation modes of viral application on both 4 and 7 days post inoculation.

No significance difference ( $P>0.05$ ) in pulmonary histopathology and mean pulmonary score was observed between UV inactivated PIV-3 compared to virus-free medium using intranasal inoculation post inoculation day 4.

The data for all parameters recorded in virus-free medium and PIV-3 using intranasal and nebulised modes of application on day 4 and day 7 are summarised in Table 3.1.

## DISCUSSION

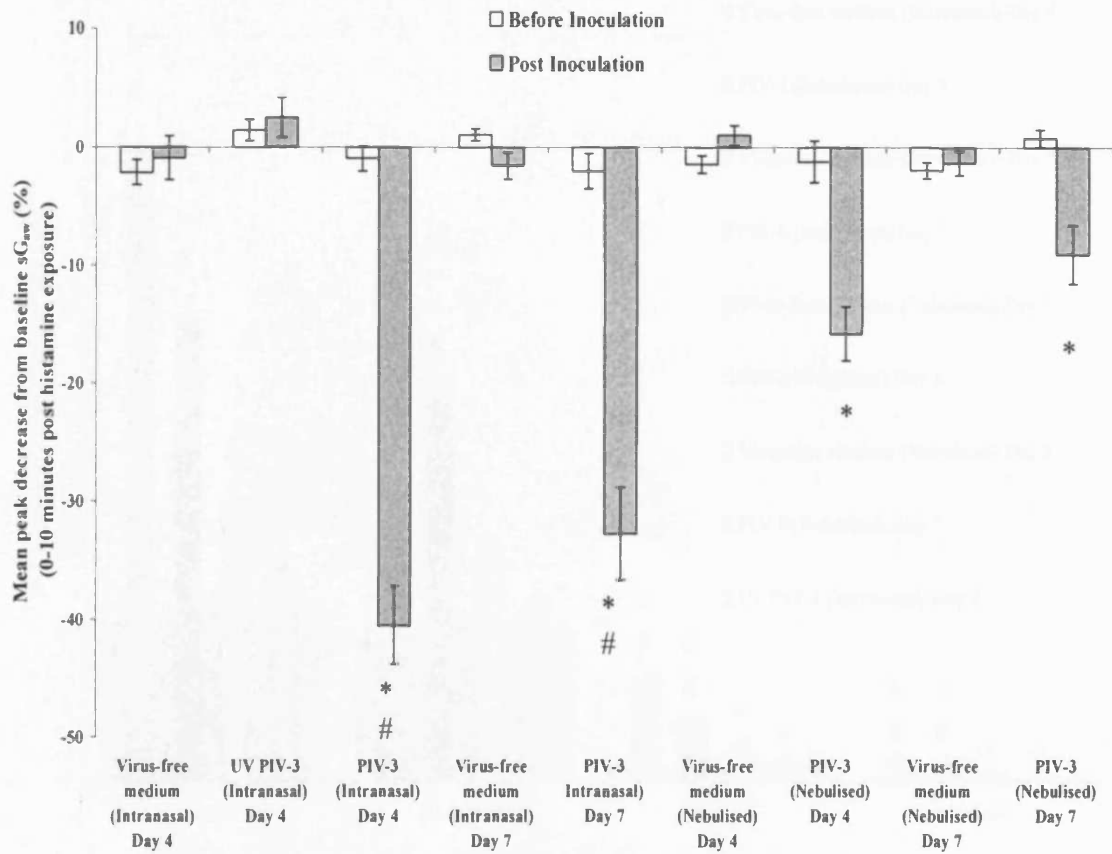
The objective of this study was to determine the most efficient route of viral application to produce sufficient PIV-3 infection in guinea pigs. This was evaluated by airway reactivity to histamine, infiltration of inflammatory cells and pulmonary histology.

In these studies inoculation of guinea pigs with UV inactivated PIV-3 virus did not lead to airway hyperreactivity, airway infiltration of inflammatory cells or enhanced pulmonary pathology. Thus, indicating that infectious PIV-3 is required to trigger the changes that persist beyond the effect of acute infection. According to the parameters measured in this investigation, the results suggest that intranasal application of the virus provides a more efficient transfer of virus into the guinea pigs resulting in the subsequent development of acute infection. This observation is in concordance with other investigators using intranasal inoculation (Kudlacz *et al*; 1993, Dakhama *et al*; 1997).

Application of PIV-3 as an aerosol failed to cause sufficient disease as assessed by consequences of PIV-3 infection. This may be due to the virus being destroyed during the nebulisation process or it may be due to particle size of the virus solution limiting penetration into the airways.

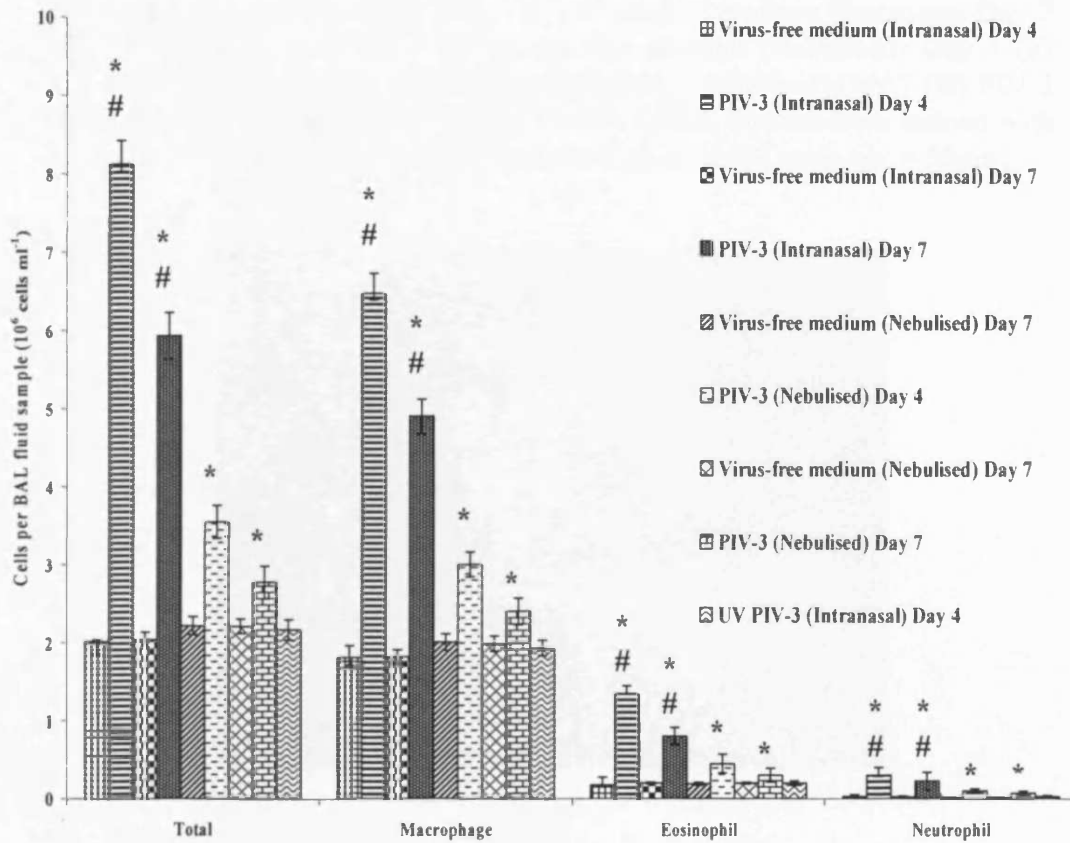
However, in contrast, the nebulisation method caused significant signs of acute bronchiolitis distributed in the lung infecting guinea pigs with RSV (Streckert *et al*; 1996) these investigators also demonstrated intranasal inoculation failed to cause significant lung pathology. The direct effects on the parameters of airway reactivity to histamine, total and differential cell counts and pulmonary histology are discussed more extensively in chapter 4.

In conclusion, PIV-3 produces a suitable model of virus infection in the guinea pig and intranasal inoculation is the most efficient route of viral application.



**Figure 3.1**

Peak percentage changes in the airway reactivity responses of conscious guinea pigs to a nose-only challenge with histamine (1mM, 20 seconds), 24 hours before the commencement of virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) using intranasal and nebulisation inoculation 4 and 7 days post inoculation. Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of the peak change from baseline  $sG_{aw}$  ( $S^{-1} \text{cm}$  of  $\text{H}_2\text{O}^{-1}$ ), 0-10 minutes post histamine exposure. Negative values represent bronchoconstriction. \*  $P < 0.05$  denotes the significance of differences between 24 hours before inoculation and 4 and 7 days post inoculation with virus-free medium or PIV-3 in response to inhaled histamine. #  $P < 0.05$  denotes the significance of differences between intranasal and nebulised methods of viral application. All statistical analysis was determined by ANOVA (single factor), followed by a Dunnet's test.

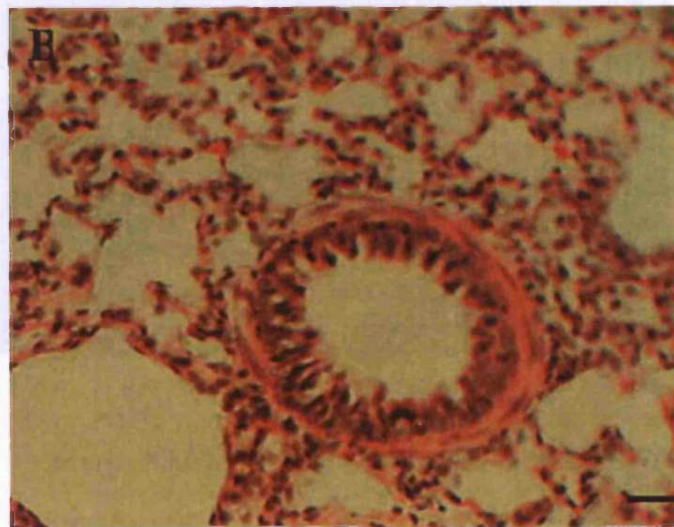
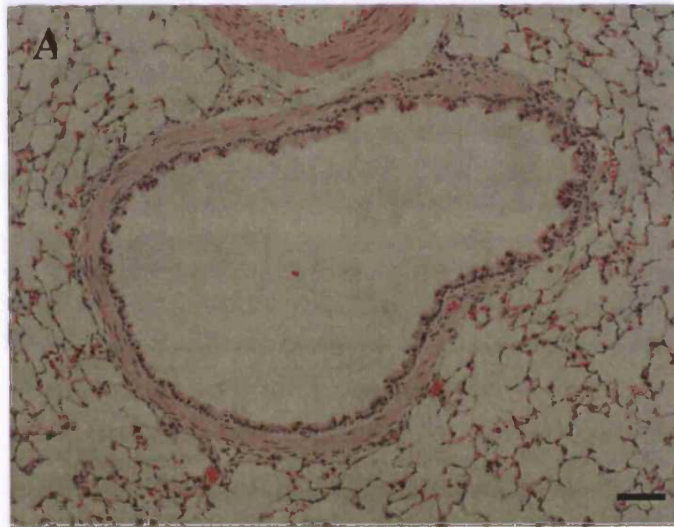


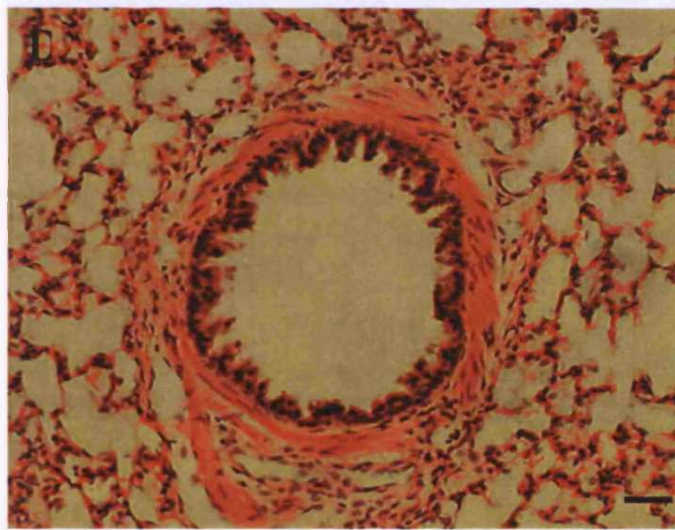
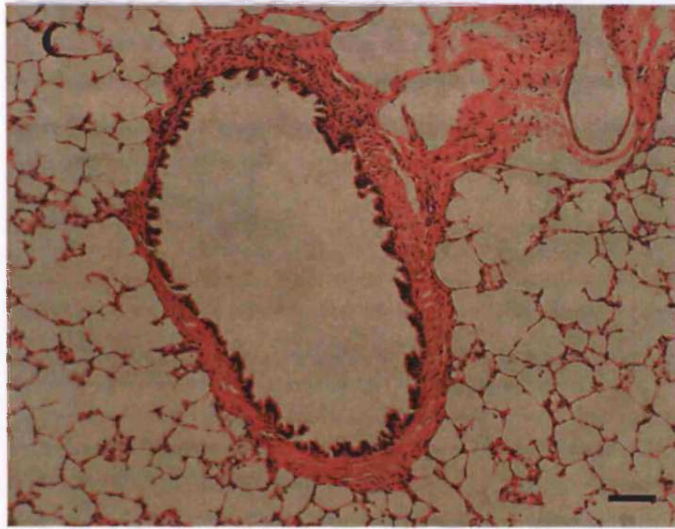
**Figure 3.2**

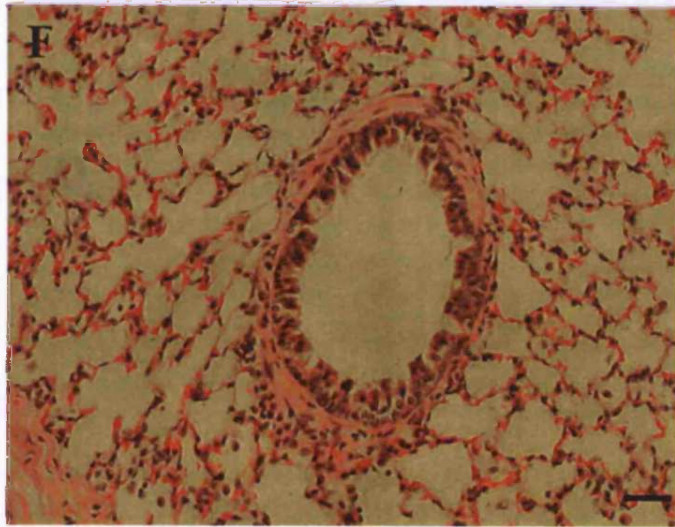
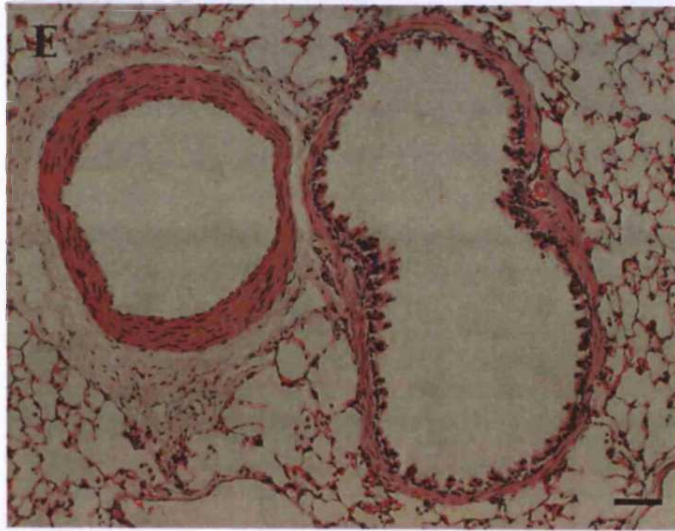
Total and differential cell (macrophage, eosinophil and neutrophil) counts of BAL fluid removed from virus-free medium (vero supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) post inoculation day 4 and 7 using intranasal and nebulisation methods of inoculation. Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of the differential cells per BAL fluid sample ( $\times 10^6$ ). \*  $P < 0.05$  denotes the significance of differences in airway cell influx removed from PIV-3 infected guinea pigs compared to virus-free medium infected guinea pigs 4 and 7 days post inoculation. #  $P < 0.05$  denotes the significance of differences in airway cell influx removed in intranasal and nebulised methods of viral application. All statistical analysis was determined by ANOVA, followed by Dunnet's test.

**Figure 3.3**

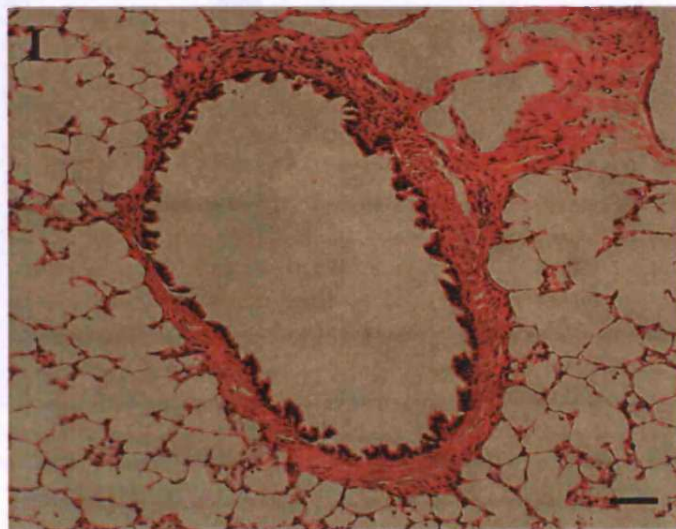
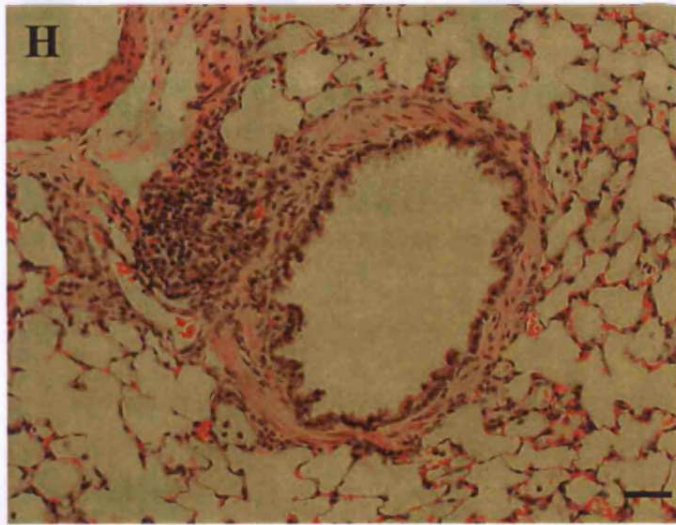
Histological appearance of lung tissue from (A) Virus-free medium (Intranasal) Day 4 (B) PIV-3 (Intranasal) Day 4 (C) Virus-free medium (Intranasal) Day 7 (D) PIV-3 (Intranasal) Day 7 (E) Virus-free medium (Nebulised) Day 4 (F) PIV-3 (Nebulised) Day 4 (G) Virus-free medium (Nebulised) Day 7 (H) PIV-3 (Nebulised) Day 7 (I) UV PIV-3 (Intranasal) Day 4. Tissues were stained with haematoxylin and eosin for general morphology (Internal scale bar = 50µm).

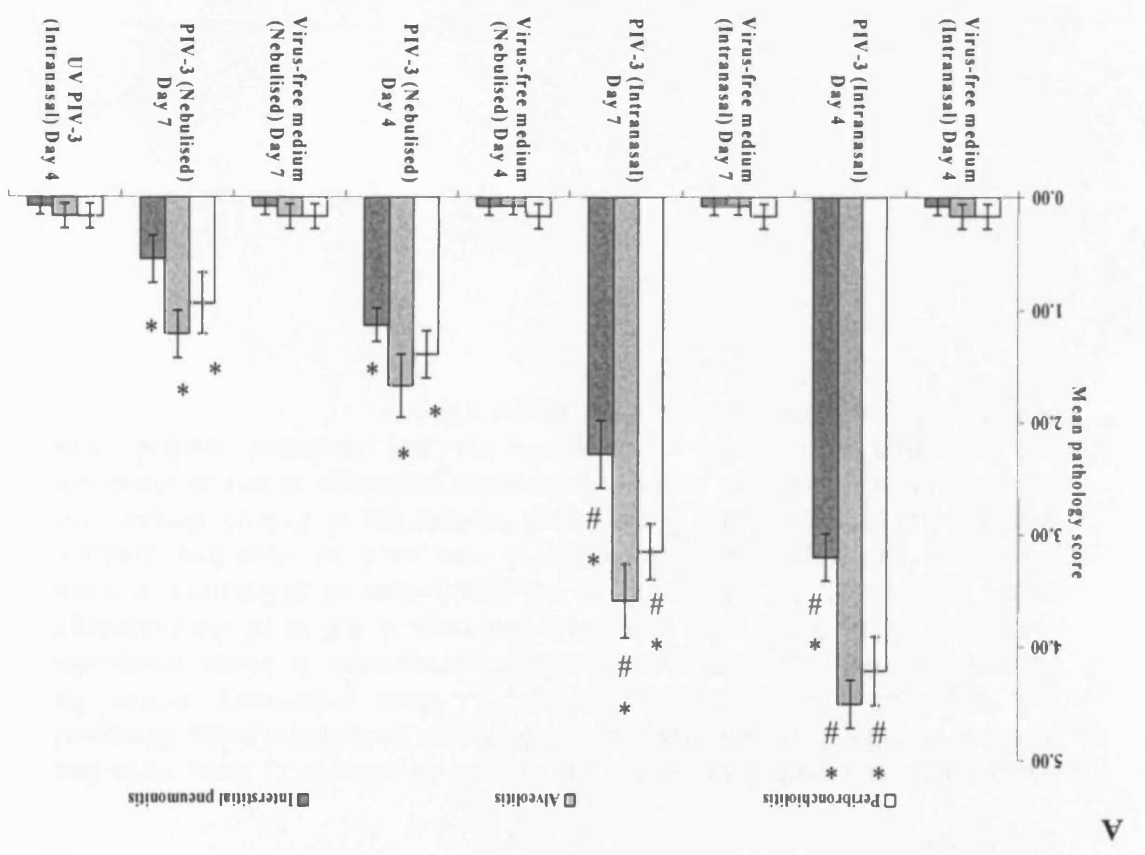
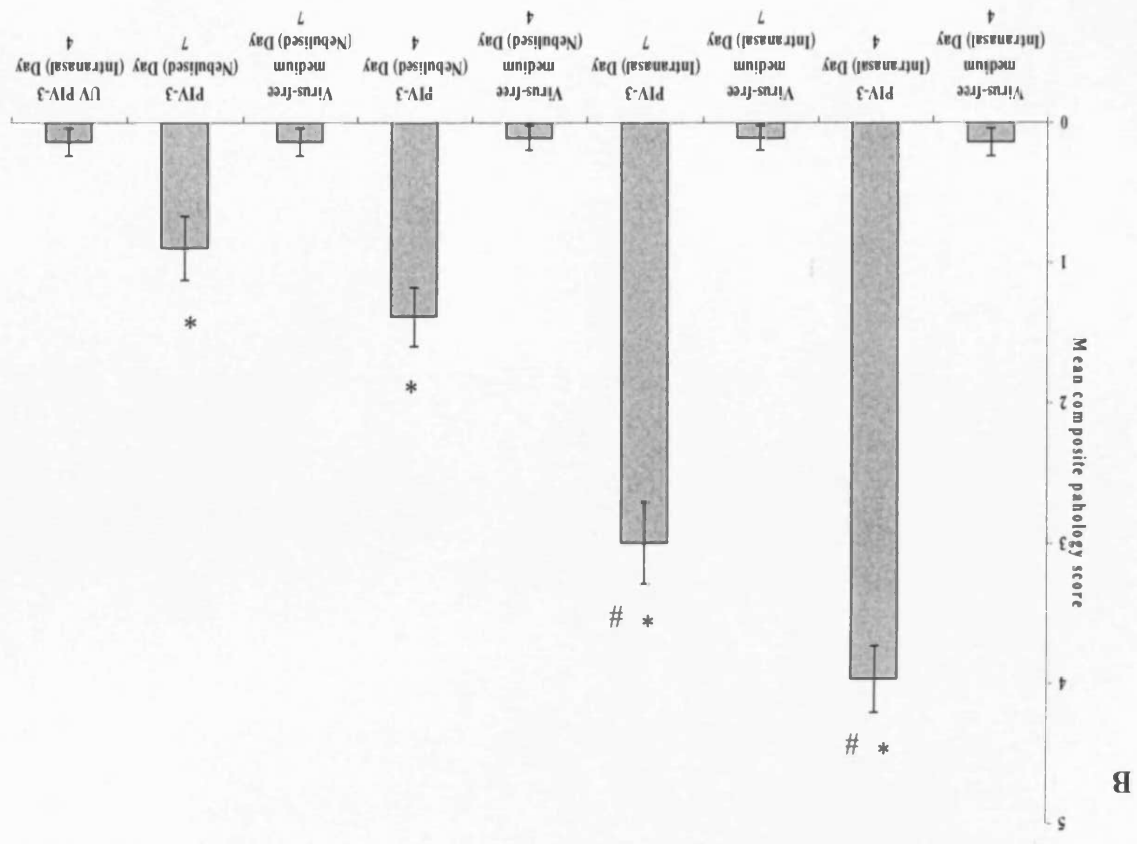












### **Figure 3.4**

Mean pulmonary pathology scores (as defined in chapter 2) from virus-free medium and PIV-3 guinea pigs 4 and 7 days post inoculation using intranasal and nebulised methods of application. **A** Mean pulmonary scores for peribronchiolitis, alveolitis and interstitial pneumonitis **B** Mean composite pulmonary score. Each point represents the mean  $\pm$  S.E.M of the pathology scores (range 0-5). \*  $P < 0.05$  denotes the significance of differences in mean pulmonary pathology scores from PIV-3 compared to virus-free medium infected guinea pigs 4 and 7 days post inoculation. #  $P < 0.05$  denotes the significance of differences in mean pulmonary pathology scores in intranasal and nebulised methods of viral application. All statistical analysis was determined by ANOVA, followed by Dunnet's test.

A

	Virus-free medium (Intranasal) Day 4 (n=6)	Virus-free medium (Nebulised) Day 4 (n=6)	Virus-free medium (Intranasal) Day 7 (n=6)	Virus-free medium (Nebulised) Day 7 (n=6)	UV PIV-3 (Intranasal) Day 4 (n=6)
Lung function before histamine exposure ( $s^{-1}cm$ of $H_2O^{-1}$ )	-2.2±1.1	-1.5±0.8	1.0±0.5	-2.0±0.7	1.4±0.9
Lung function after histamine exposure ( $s^{-1}cm$ of $H_2O^{-1}$ )	-1.0±0.9	1.06±0.2	-1.6±1.1	-1.3±1.01	2.5±1.7
Total Cells ( $10^6$ cells $ml^{-1}$ )	2.0±0.2	2.2±0.1	2.0±0.1	2.2±0.1	2.2±0.1
Macrophages ( $10^6$ cells $ml^{-1}$ )	1.8±0.1	2.0±0.1	1.8±0.1	2.0±0.1	1.9±0.1
Eosinophils ( $10^6$ cells $ml^{-1}$ )	0.18±0.03	0.19±0.02	0.20±0.01	0.20±0.01	0.2±0.1
Neutrophils ( $10^6$ cells $ml^{-1}$ )	0.03±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.03±0.01
Mean pulmonary score	Peribronchiolitis	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
	Alveolitis	0.2±0.1	0.08±0.08	0.08±0.08	0.2±0.1
	Interstitial pneumonia	0.08±0.08	0.08±0.08	0.08±0.08	0.08±0.08
Mean composite pulmonary score	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1

B

	PIV-3 (Intranasal) Day 4 (n=6)	PIV-3 (Nebulised) Day 4 (n=6)	PIV-3 (Intranasal) Day 7 (n=6)	PIV-3 (Nebulised) day 7 (n=6)	
Lung function before histamine exposure ( $s^{-1}cm$ of $H_2O^{-1}$ )	-1.0±1.1	-1.2±1.8	-2.0±1.5	0.8±0.7	
Lung function after histamine exposure ( $s^{-1}cm$ of $H_2O^{-1}$ )	-40.5±3.3	-15.8±2.28	-32.8±3.9	-9.0±2.5	
Total Cells ( $10^6$ cells $ml^{-1}$ )	8.1±0.3	3.5±0.2	5.9±0.3	2.8±0.2	
Macrophages ( $10^6$ cells $ml^{-1}$ )	6.5±0.3	3.0±0.2	4.9±0.2	2.4±0.2	
Eosinophils ( $10^6$ cells $ml^{-1}$ )	1.4±0.1	0.5±0.1	0.8±0.1	0.3±0.09	
Neutrophils ( $10^6$ cells $ml^{-1}$ )	0.3±0.1	0.1±0.03	0.2±0.1	0.07±0.03	
Mean pulmonary score	Peribronchiolitis	4.2±0.3	1.4±0.2	3.6±0.3	0.9±0.3
	Alveolitis	4.5±0.2	1.7±0.3	3.6±0.3	1.2±0.2
	Interstitial pneumonia	3.2±0.2	1.1±0.6	2.3±0.3	0.5±0.2
Mean composite pulmonary score	4.0±0.3	1.4±0.3	3.0±0.3	0.10±0.2	

**Table 3.1**

Summary of results in virus-free medium (A) and PIV-3 (B). For each parameter the value given is that recorded or determined on the final day in groups of animals that were examined 4 or 7 days post inoculation post 7 using intranasal and nebulisation methods of inoculation

## CHAPTER 4

### A TIME COURSE FOR EFFECTS OF PIV-3 INFECTION IN GUINEA PIGS

#### INTRODUCTION

Respiratory viral infections can cause AHR, characterised by increased airway sensitivity to stimuli (e.g. histamine) (Busse; 1988; Empey *et al*; 1976). Previous investigations using PIV-3 infections have included both *in vitro* (Folkerts *et al*; 1992) and *in vivo* studies (Folkerts *et al*; 1993, Kudlacz *et al*; 1993). Clinical studies suggest that eosinophils play a role in triggering and sustaining lung inflammation following PIV-3 infection (Glezen *et al*; 1982). BAL removed from PIV-3 infected guinea pigs exhibit elevated eosinophils and macrophage levels and airway histology reveals pitted epithelial lining (Folkerts *et al*; 1995). Although some of the respiratory effects of PIV-3 infection have been examined by other investigators, the temporal relationships between virus-induced changes both physiological and histological have not been determined. In order to obtain a better understanding of the PIV-3 induced changes, these experiments have been designed to explore effects of PIV-3 infection in guinea pigs over a period of several weeks after infection. The parameters investigated included airway function, airway reactivity to histamine, cellular infiltration, wet lung weight, NO and total protein levels in BAL fluid, pulmonary histology and recovery of virus from the lung tissue. A time course of PIV-3 infection was necessary to determine if there is a temporal relationship between the various parameters and if a causal relationship could be established.

## EXPERIMENTAL PROTOCOL

The following protocols used to perform this study have all been previously described in chapter 2.

To determine a time course in PIV-3 infected guinea pigs, the following experimental groups were as follows.

	<b>GROUPS (n=6)</b>
1	Virus-free medium infected animals assessed post inoculation day 1,
2	Virus-free medium infected animals assessed post inoculation day 2,
3	Virus-free medium infected animals assessed post inoculation day 4,
4	Virus-free medium infected animals assessed post inoculation day 7,
5	Virus-free medium infected animals assessed post inoculation day 25,
6	Virus-free medium infected animals assessed post inoculation day 40,
7	PIV-3-infected animals assessed post inoculation day 1,
8	PIV-3 infected animals assessed post inoculation day 2,
9	PIV-3 infected animals assessed post inoculation day 4,
10	PIV-3 infected animals post inoculation day 7,
11	PIV-3 infected animals post inoculation day 25,
12	PIV-3 infected animals (n=6) post inoculation day 40.

These study days were selected to determine a time course to correspond to the maximal intrapulmonary replication (day 4), maximal bronchiolar inflammation (Day 7), and persistent infection (day 25). On day -1 guinea pigs were assessed for airway reactivity to histamine 24 hours before inoculation. On study day 0, guinea pigs were inoculated with virus-free medium or PIV-3 by intranasal instillation. Guinea pigs were assessed 1, 2, 4, 7, 25 and 40 days post inoculation.

At the end of each study the animals were reassessed for airway reactivity to histamine and within 20 minutes were sacrificed and BAL performed and lung tissue removed. BAL fluid specimens were analysed for virus-induced changes by comparison of total and differential cell counts and viral determination. Lung tissue specimens were used for total viral content per gram of lung and pulmonary histology. PIV-3 was detected in BAL fluid and lung tissue samples by using standard culture techniques and indirect immunofluorescence as described in chapter 2. Inflammatory markers NO and protein levels were also measured in BAL fluid. Throughout the study each animal was weighed and rectal temperature recorded on selected days throughout the study. Animals were also examined daily for general signs of illness including hair loss and decreased activity.

## RESULTS

### ***Effect of PIV-3 on body weight gain 1, 2, 4, 7, 25 and 40 days post inoculation.***

Intranasal inoculation of guinea pigs with PIV-3 failed to significantly ( $P>0.05$ ) alter body weight gain during any time point investigated throughout the study compared with virus-free medium infected guinea pigs (Figure 4.1).

### ***Effect of PIV-3 on rectal temperature 1, 2, 4, 7, 25 and 40 days post inoculation.***

Intranasal inoculation of guinea pigs with virus-free medium or PIV-3 failed to significantly ( $P>0.05$ ) alter rectal temperature at any time point investigated throughout the study (Figure 4.2).

***Effect of PIV-3 on airway function 1, 2, 4, 7, 25 and 40 days post inoculation.***

Intranasal inoculation of guinea pigs with virus-free medium and PIV-3 failed to significantly ( $P>0.05$ ) alter airway function from baseline  $sG_{aw}$  values at any time point investigated throughout the study (Figure 4.3).

***Effect of PIV-3 on airway reactivity to inhaled histamine 1, 2, 4, 7, 25 and 40 days post inoculation.***

Inhalation of histamine (1mM) for 20 seconds, 24 hours before intranasal inoculation of virus-free medium or PIV-3 did not produce any significant ( $P>0.05$ ) bronchoconstriction and failed to cause significant deviation from baseline  $sG_{aw}$  values (Figure 4.4). This indicates that this was a sub-threshold dose of histamine.

Airway reactivity to histamine in virus-free medium guinea pigs post inoculation day 1, 2, 4, 7, 25 and 40 did not produce any significant bronchoconstriction ( $P>0.05$ ) and failed to cause any significant deviation from baseline  $sG_{aw}$  values (Before:  $-2.2 \pm 2.1\%$ ,  $-1.5 \pm 0.7\%$ ,  $1.0 \pm 0.5\%$ ,  $-2.0 \pm 0.7\%$ ,  $-2.4 \pm 0.3\%$ ,  $1.9 \pm 1.3\%$ ; After:  $-1.0 \pm 1.9\%$ ,  $1.0 \pm 0.8\%$ ,  $-2.6 \pm 1.1\%$ ,  $-1.3 \pm 2.2\%$ ,  $-1.5 \pm 0.7\%$ ,  $0.7 \pm 1.1\%$   $H_2O^{-1}sec^{-1}$  respectively). This is in contrast to PIV-3 infected guinea pigs which produced a significant bronchoconstriction ( $P<0.05$ ) on days 1, 2, 4, 7, 25 and 40 days post inoculation with peak reductions in  $sG_{aw}$  (Before:  $1.0 \pm 2.1\%$ ,  $-1.2 \pm 1.8\%$ ,  $-2.0 \pm 1.5\%$ ,  $0.8 \pm 0.7\%$ ,  $-2.5 \pm 1.8\%$ ,  $-0.7 \pm 1.7\%$ ; After:  $-9.4 \pm 3.2\%$ ,  $-15.8 \pm 2.3\%$ ,  $-40.5 \pm 5.4\%$ ,  $-33.0 \pm 2.5\%$ ,  $-22.4 \pm 2.8\%$ ,  $-8.8 \pm 3.3\%$   $H_2O^{-1}sec^{-1}$  respectively). This indicates airway hyperreactivity to histamine. Therefore, significant bronchoconstriction to inhaled histamine appeared at day 1, 2, peaking on day 4 and gradually decreased from day 7 post inoculation. Airway hyperreactivity was still evident at 40 days post inoculation.



***Effect of PIV-3 on airways infiltration of leucocytes 1, 2, 4, 7, 25 and 40 days post inoculation.***

On days 1, 2, 4, 7, and 25 days after PIV-3 inoculation, a significant increase ( $P<0.05$ ) in total cell counts ( $3.0\pm 0.2$ ,  $3.5\pm 0.2$ ,  $8.1\pm 0.3$ ,  $6.1\pm 0.2$ ,  $4.0\pm 0.2$  cells  $\text{ml}^{-1}$ ) occurred in BAL fluid compared to virus-free medium infected guinea pigs ( $2.0\pm 0.1$ ,  $2.2\pm 0.1$ ,  $2.0\pm 0.1$ ,  $2.1\pm 0.1$ ,  $2.2\pm 0.1$  cells  $\text{ml}^{-1}$ ). In addition, a significant increase ( $P<0.05$ ) in differential cell counts occurred in BAL fluid 1, 2, 4, 7, 25 days after PIV-3 inoculation, including macrophages ( $2.6\pm 0.1$ ,  $2.9\pm 0.2$ ,  $6.4\pm 0.2$ ,  $5.2\pm 0.2$ ,  $4.0\pm 0.2$  cells  $\text{ml}^{-1}$ ) eosinophils ( $0.3\pm 0.03$ ,  $0.5\pm 0.02$ ,  $1.4\pm 0.1$ ,  $0.7\pm 0.03$ ,  $0.4\pm 0.03$  cells  $\text{ml}^{-1}$ ) and neutrophils ( $0.09\pm 0.01$ ,  $0.1\pm 0.01$ ,  $0.3\pm 0.05$ ,  $0.3\pm 0.02$ ,  $0.15\pm 0.01$  cells  $\text{ml}^{-1}$  respectively) occurred in BAL fluid compared to virus-free medium infected guinea pigs including macrophages ( $1.8\pm 0.10$ ,  $2.0\pm 0.1$ ,  $1.8\pm 0.1$ ,  $1.9\pm 0.1$ ,  $2.0\pm 0.1$ , cells  $\text{ml}^{-1}$ ), eosinophils ( $0.2\pm 0.03$ ,  $0.2\pm 0.01$ ,  $0.2\pm 0.01$ ,  $0.2\pm 0.02$ ,  $0.2\pm 0.02$  cells  $\text{ml}^{-1}$ ), neutrophils ( $0.03\pm 0.01$ ,  $0.02\pm 0.01$ ,  $0.02\pm 0.01$ ,  $0.02\pm 0.01$ ,  $0.02\pm 0.01$  cells  $\text{ml}^{-1}$ ) (Figure 4.5). No significant differences was observed on day 40 post inoculation between PIV-3 infected guinea pigs compared to virus-free medium infected guinea pigs. Therefore, an increase in total and differential cell counts appeared at day 1, peaking on day 4 and gradually decreased thereafter, returning to basal levels by day 40 post inoculation.

***Effect of PIV-3 on respiratory rate 1, 2, 4, 7, 25 and 40 days post inoculation.***

Respiratory rates were determined at regular intervals in each of the groups studied for 1, 2, 4, 7, 25 and 40 days post inoculation. In PIV-3 infected guinea pigs a significant increase ( $P<0.05$ ) in respiratory rates 1, 2, 4, 7 and 25 days post inoculation ( $109.0\pm 5.5$ ,  $126.0\pm 6.8$ ,  $160.0\pm 6.0$ ,  $132.0\pm 3.5$ ,  $114.0\pm 4.9$  breaths  $\text{min}^{-1}$ ) compared to virus-free medium infected guinea pigs for the corresponding study days ( $77.0\pm 5.5$ ,  $72.0\pm 7.0$ ,  $72.0\pm 7.0$ ,  $62.0\pm 6.5$ ,  $82.0\pm 5.7$  breaths  $\text{min}^{-1}$ ) (Figure 4.6).

By day 40 post inoculation, respiratory rates of PIV-3 infected guinea pigs had returned to baseline values ( $75.0 \pm 2.8$  breaths  $\text{min}^{-1}$ ) in comparison to virus-free medium infected guinea pigs ( $63.0 \pm 4.3$  breaths  $\text{min}^{-1}$ ). Therefore, an increase in respiratory rates appeared at day 1, peaking on day 4 and gradually decreased thereafter on days 7, 25 returning to baseline levels by day 40.

***Effects of PIV-3 on wet left lung weights 1, 2, 4, 7, 25 and 40 days post inoculation.***

A significance increase ( $P < 0.05$ ) was observed in wet left lung weight was observed 2, 4, and 7 days post infection ( $11.1 \pm 0.3$ ,  $11.8 \pm 0.3$ ,  $9.2 \pm 0.2$  g  $\text{Kg}^{-1}$  respectively) compared to virus-free medium inoculated animals ( $7.7 \pm 0.3$ ,  $6.7 \pm 0.3$ ,  $6.4 \pm 0.1$  g  $\text{kg}^{-1}$ ) respectively (Figure 4.7). No significant differences ( $P > 0.05$ ) were observed post inoculation day 1 or day 40 between virus-free medium and PIV-3 infected groups. Therefore, an increase in wet right lung weights appeared at day 2, peaking on day 4 and gradually decreased thereafter on days 7, returning to basal weights by day 25 post inoculation.

***Effect of PIV-3 on airways NO levels 1 2, 4, 7, 25 and 40 days post inoculation.***

In PIV-3 infected animals a significance increase ( $P < 0.05$ ) in combined NO levels, 2, 4, and 7 days post infection ( $71.3 \pm 2.8$ ,  $93.3 \pm 3.3$ ,  $80.0 \pm 2.0$   $\mu\text{M}$ ) was observed compared to virus-free medium infected animals at the corresponding study days ( $48.4 \pm 2.0$ ,  $44.2 \pm 2.6$ ,  $53.3 \pm 5.6$   $\mu\text{M}$ ) (Figure 4.8). No significant differences ( $P > 0.05$ ) were observed post inoculation day 1 or day 40 between virus-free medium and PIV-3 infected groups. Therefore, an increase in combined NO appeared at day 2, peaking on day 4 and gradually decreased thereafter on days 7 returning to baseline levels by day 25.

***Effect of PIV-3 on total protein 1 2, 4, 7, 25 and 40 days post inoculation.***

A significant increase ( $P < 0.05$ ) in total protein levels in BAL fluid was observed in PIV-3 infected guinea pigs 2, 4, 7 and 25 days post inoculation ( $1314.7 \pm 48.9$ ,  $1793.6 \pm 50.9$ ,  $1483.4 \pm 55.2$ ,  $1243.9 \pm 36.5 \mu\text{g ml}^{-1}$ ) compared to virus-free medium infected animals at the corresponding study days ( $1121.8 \pm 36.6$ ,  $992.7 \pm 35.9$ ,  $1143.7 \pm 31.3$ ,  $1011.5 \pm 35.3 \mu\text{g ml}^{-1}$ ) (Figure 4.9). No significant differences ( $P > 0.05$ ) were observed post inoculation day 1 or day 40 between virus-free medium and PIV-3 infected groups. Therefore, an increase in total protein levels appeared at day 2, peaking on day 4 and gradually decreased thereafter on days 7 and 25 returning to baseline levels by day 40.

***Isolation of PIV-3 in BAL fluid and lung tissue samples 1, 2, 4, 7, 25 and 40 days post inoculation.***

Replicating virus was isolated in PIV-3 infected animals in BAL fluid 2, 4, 7 and 25 days post inoculation (Table 4.1) as measured by CPE of BAL fluid and homogenised left lung tissue on Vero cells and a positive reaction by indirect immunofluorescence using an antibody against PIV-3. Titres of intrapulmonary replicating PIV-3 were determined 1, 2, 4, 7, 25 and 40 days post inoculation normalised to left lung tissue weight ( $\text{TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ) (Figure 4.10). Titres of PIV-3 virus were significantly increased ( $P < 0.05$ ) by day 1 ( $197 \pm 95 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ), day 2 ( $3.5 \times 10^4 \pm 1.3 \times 10^4 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ), reaching a peak titre by day 4 ( $4.2 \times 10^7 \pm 7.8 \times 10^6 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ). The viral titre gradually decreased, thereafter on day 7 ( $6.6 \times 10^5 \pm 2.5 \times 10^5 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ), by day 25 ( $6.3 \times 10^2 \pm 2 \times 10^2 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ), reaching minimal levels by day 40 ( $2.4 \pm 1.6 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ).

***Effect of PIV-3 on pulmonary histology and mean pulmonary score 1, 2, 4, 7, 25 and 40 days post inoculation.***

The histological analysis of the lungs in this study was used to evaluate virus-induced pulmonary inflammatory changes (Figure 4.11 - 4.16). The lungs were assessed for three peribronchiolitis, interstitial pneumonitis, alveolitis by using a scoring system. Histological analysis of the lungs from PIV-3 infected guinea pigs post inoculation day 1 revealed a significant increase ( $P < 0.05$ ) mean composite pathology scores ( $0.9 \pm 0.2$ ) compared to virus-free medium infected guinea pigs of the corresponding group ( $0.1 \pm 0.1$ ). This increase continued to increase by day 2 ( $1.3 \pm 0.3$ ) with peak pulmonary pathology scores occurring 4 days post inoculation ( $4.0 \pm 0.2$ ) compared to virus-free medium groups ( $0.1 \pm 0.1$ ,  $0.2 \pm 0.1$ ). Pulmonary pathology scores remained significantly increased although steadily declined on days 7 ( $3.1 \pm 0.2$ ) and 25 days ( $1.4 \pm 0.2$ ) post inoculation, reaching baseline levels by day 40 ( $0.1 \pm 0.1$ ) in comparison to the corresponding virus-free medium infected control groups ( $0.2 \pm 0.1$ ,  $0.1 \pm 0.1$ ,  $0.2 \pm 0.1$ ) (Figure 4.17)

The data for all parameters recorded on the final day of each experimental groups is summarised in Table 4.2.

## **DISCUSSION**

This study was used to determine a time course for a comprehensive range of parameters recorded in conscious PIV-3 inoculated guinea pigs from day 1 until day 40 post inoculation. The parameters investigated were body weight gain, rectal temperature, baseline  $sG_{aw}$ , airway reactivity to histamine, respiratory rate, infiltration of inflammatory cells, wet lung weights, viral titre, nitric oxide levels, protein levels and histological analysis.

During the investigation, no significant differences were observed in rectal temperature or body weight gain following PIV-3 inoculation compared to virus-free medium inoculated guinea pigs on any of the study days investigated. Throughout the study, no signs of clinical infection (e.g. sneezing, panting, nasal discharge) were evident in the guinea pigs. This is in concordance with several animal studies involving inoculation with live virus (Dakhama *et al*; 1997). However, studies in guinea pigs using PIV-3 have reported periodic panting in 20% of animals (Kudlacz *et al*; 1993) within 48 hours after administration of the virus.

Airway function was measured at regular intervals in all of the study groups investigated. PIV-3 infected guinea pigs failed to display any decline in airway function during any of the study days compared to virus-free medium infected guinea pigs. This observation is in concordance with similar studies by Toward *et al* (2005). However, studies by Sorkness *et al*; (1994) have previously demonstrated a decline in airway function after several weeks in PIV-3 infected rats, however this difference may be species dependent.

Respiratory rates were also determined in PIV-3 infected guinea pigs during the study. Respiratory rate was significantly increased by post inoculation day 1 compared to the corresponding virus-free medium infected animals. This steadily increased, peaking 4 days post inoculation and gradually declining thereafter by day 40, although remaining significantly increased. Interestingly PIV-3 failed to produced any effect on lung function ( $sG_{aw}$ ) but produced a significant increase in respiratory rate. Therefore, inflammation of the airways must have had some detrimental effect on respiratory function although this was not evident from conductance or observed as panting. This is in concordance with similar studies by Kudlacz *et al* (1993) who also observed similar increases in respiratory rates in PIV-3 infected guinea pigs as a result of inflammatory mediators released from infiltrating immune cells.

Following inoculation guinea pigs experienced significant changes in airway reactivity to histamine by day 1 post inoculation compared to the corresponding virus-free medium infected group. AHR continued to increase, reaching a maximal response by day 4 post inoculation, and steadily declined thereafter, although not returning to basal levels by the end of the study (day 40). Other investigators (Buckner *et al*; 1985) have also reported this maximal increase in AHR 4 days after PIV-3 inoculation. This data is also consistent with clinical findings in patients, where hyperresponsiveness was observed in patients with upper respiratory viral infections, during the infection and for several weeks after recovery (Empey *et al*; 1976).

The airway epithelium is the physical barrier that protects sensory nerves and smooth muscle from stimulation by inhaled irritants (Folkerts & Nijkamp; 1998). Epithelial cells are the principal hosts for PIV-3 replication causing damage to the airway epithelium in the guinea pigs. Such damage would be fundamental in causing many deleterious PIV-3 induced effects to airway homeostasis. Damage to the airway epithelium would contribute to the airway hyperreactivity in this study, as exposed sensory nerves and airway smooth muscle (ASM) would facilitate increased histamine H<sub>1</sub> receptor, mediated ASM contraction, and stimulate tachykinin-induced reflex bronchoconstriction from sub-epithelial sensory neurones (Folkerts & Nijkamp; 1998). The epithelial layer also metabolises histamine, tachykinins and acetylcholine via intra-cellular diamine oxidase, neutral endopeptidase (NEP) and cholinesterase, respectively (Folkerts & Nijkamp; 1998). Consequently, cytotoxic damage would impair epithelial cell metabolic activity and in these PIV-3 infected guinea pigs, lead to an increase in the airways concentration of bronchoconstrictor mediators, and potentiate contractility to inhaled histamine (i.e. airway hyperreactivity).

Epithelial cells also have a secretory function, synthesising and releasing a wide range of pro-inflammatory cytokines and chemokines in response to PIV-3. These include IL-6, IL-8, IL-11, granulocyte-macrophage colony stimulating factor (GM-CSF), and regulated-upon-activation-normal-T-cell-expressed and secreted (RANTES) (Folkerts *et al*; 1998, Barnes *et al*; 1999). The release of such cytokines, abuminally onto ASM and into the airway lumen, would induce a complex release of pro-inflammatory mediators and activation of airway resident macrophages, lymphocytes, granulocytes, mast cells and basophils (Folkerts *et al*; 1998).

In this study, a significant increase in the number of inflammatory cells types (macrophages, eosinophils, neutrophils) was observed in BAL fluid from PIV-3 infected guinea pigs post inoculation day 1 compared to corresponding virus-free medium infected guinea pigs. This inflammatory cell influx continues to increase, producing a maximal response 4 days post inoculation and slowly declining thereafter by day 40. The majority of the inflammatory cells were found to be alveolar macrophages and are likely to be involved in the early immune response to PIV-3 infection. Macrophages release amongst other pro-inflammatory mediators, IL-1 and tumour necrosis factor-alpha (TNF-  $\alpha$ ), which facilitate cell recruitment into the airways by increasing expression of endothelial cell adhesion molecules (Folkerts *et al*; 1998, Barnes *et al*; 1999). Upon activation of these cells, an array of different phospholipid mediators could be released including prostaglandins, leukotrienes and platelet activating factor, which may interfere with the responsiveness of airways.

In the present study, this inflammatory response is further supported by the presence of classical features of PIV-3 infection including interstitial pneumonia, alveolitis and perbronchiolitis. In addition, many of the small airways were occluded with inflammatory cells and several airways were constricted. In concordance, Folkerts *et al* (1993) also demonstrated pronounced morphologic changes in the respiratory tract of PIV-3 infected guinea pigs 4 days post inoculation.

These occlusions of bronchioli may contribute to airway obstruction during viral infections. Interestingly, when histology is compared to physiological data, it is observed that at 40 days post inoculation complete repair of the airways was evident, although a significantly increased AHR remained present. Therefore, the morphological changes may contribute but are not directly responsible for the increased responsiveness of the airways.

Wet lung weights were measured in this study as an index of assessing oedema in the lungs. Wet lung weights were significantly increased by day 2 post inoculation, reaching a peak weight by day 4 and slowly declining by day 7, and returning to basal weights by day 25 and 40. This increase in wet lung weights, indicating oedema, may also contribute to the AHR and the increase in respiratory rates observed in these animals and supports the findings of the increased protein levels in this study.

In this study the effects of PIV-3 infection on production of NO in the guinea pig model was investigated. Recent evidence suggests that NO is a key mediator of inflammation in the airway, potentially facilitating the complex initiation of multiple resident cell types and migrating inflammatory cells into the airway (Ricciardoid; 2003). Its formation is catalysed by NO synthase (NOS), which exists in 3 distinct isoforms that have characteristic patterns of tissue-specific expression—constitutively expressed neural NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Ricciardoid; 2003).

Expression of iNOS in macrophages, neutrophils, endothelial and smooth muscle cells, and the respiratory epithelium has been described and is regulated by a number of protein inflammatory cytokines including TNF- $\alpha$ , interferon (IFN)- $\gamma$ , and interleukin 1-beta (IL-1 $\beta$ ) at least partially through activation of nuclear factor-kappaB (NF- $\kappa$ B) (Xie *et al*; 1994) and are therefore probable contributors to the airways NO in this study.



The effects of viral infection on NO have been studied in several animal models, with variable effects depending upon the virus and the system studied (Kurupiah *et al*; 1998, Xiong *et al*; 1999, Domachowske *et al*; 2001, Shanley *et al*; 2002). A number of other respiratory viruses have been found to induce production of NO by respiratory epithelium, both *in vitro* and *in vivo*, including influenza, adenovirus (Zsengeller *et al*; 2001), RV (Sander *et al*; 2001), sendai virus and RSV (Kao *et al*; 2001). However, production of NO *in vivo* has been demonstrated to significantly contribute to clearance of some viruses (Croen; 1993, Saura *et al*; 1999, Sander *et al*; 2001).

The BAL fluid removed from virus-free medium guinea pigs produced a basal level of the combined NO metabolites, NO<sub>3</sub> and NO<sub>2</sub>. The basal level of NO is most probably responsible for maintaining bronchodilator tone and suppressing leukocyte activation, microvascular leakage and excitatory neurotransmission (Barnes *et al*; 1999, Colasanti & Suzuki; 2000). However, on day 1 post inoculation levels of NO were significantly increased in BAL fluid in PIV-3 infected guinea pigs compared to control virus-free guinea pigs on the corresponding day. The levels of NO remained increased, peaking at 4 days post inoculation and steadily declined to basal levels by day 40 post inoculation. These results are in concordance with studies by Toward *et al* (2005) who also demonstrated significantly increased NO levels 4 days post inoculation with PIV-3 in the guinea pig model.

Studies by Stark *et al* (2005) have also demonstrated a significantly increased production of NO in RSV infected mice and immunohistochemical analysis identified iNOS in the respiratory epithelium as the major NOS enzyme altered during acute infection. Prevention of the formation of NO significantly impaired the ability of mice to clear RSV from the lungs (Stark *et al*; 2005). Interestingly, in this study inhibition of production of NO was associated with decreased recruitment of inflammatory cells into the lungs and reduced airway hyperresponsiveness to methacholine (Stark *et al*; 2005).

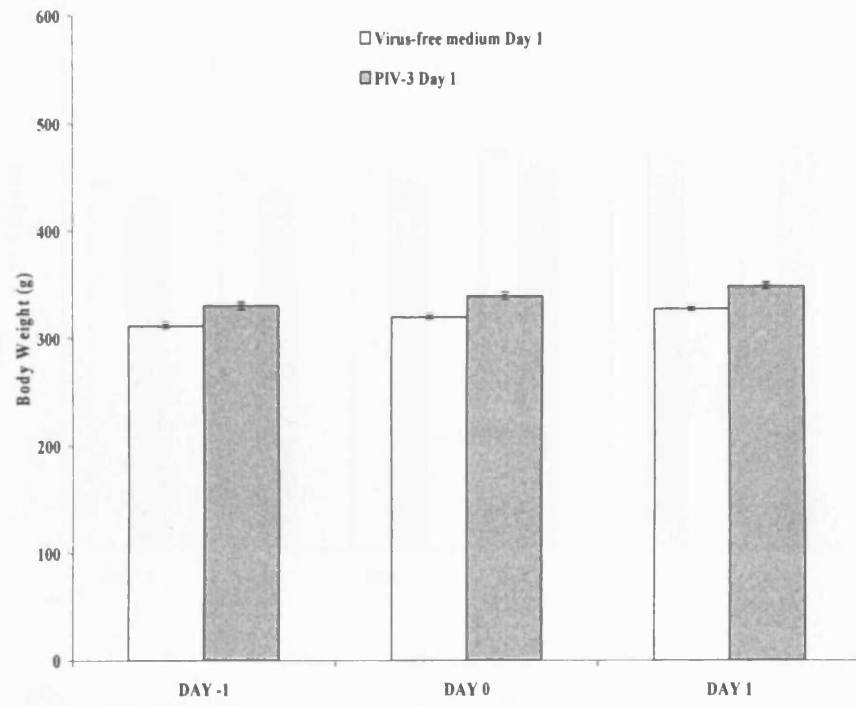
Studies in cell culture models indicate that NO inhibits production of cytokines (Sander *et al*; 1998, Trifilieff *et al*; 2000) implying that inhibition of NO decreases lung inflammation by other mechanisms such as decreasing interactions with reactive oxygen species to produce peroxynitrite and 8-nitroguanosine (Zsengeller *et al*; 2001). This data is also supported by studies in mice, which clearly demonstrate the contribution of NO to the development of acute lung inflammation (Karupiah *et al*; 1998, Xiong *et al*; 1999, Domachowske *et al*; 2001, Shanley *et al*; 2002).

In contrast, studies by Folkert & Nijkamp (1995) in isolated trachea removed from PIV-3 inoculated guinea pigs demonstrated a deficiency in NO. This discrepancy in the levels of NO after PIV-3 infection is likely to be related to the source of NO generation. In addition to synthesising and secreting cytokines and proinflammatory mediators, epithelial cells also generate NO, via constitutive NOS (Folkert & Nijkamp; 1998). Damage to the epithelial cells in viral replication would impair the generation of constitutive NOS, causing NO deficiency. The presence of acute lung injury and acute respiratory failure has led to clinical trials using NO in ventilated patients (Hoehn *et al*; 1998, Leclerc *et al*; 2001). The effects of therapy with NO on the actual viral titres have not been reported for these patients.

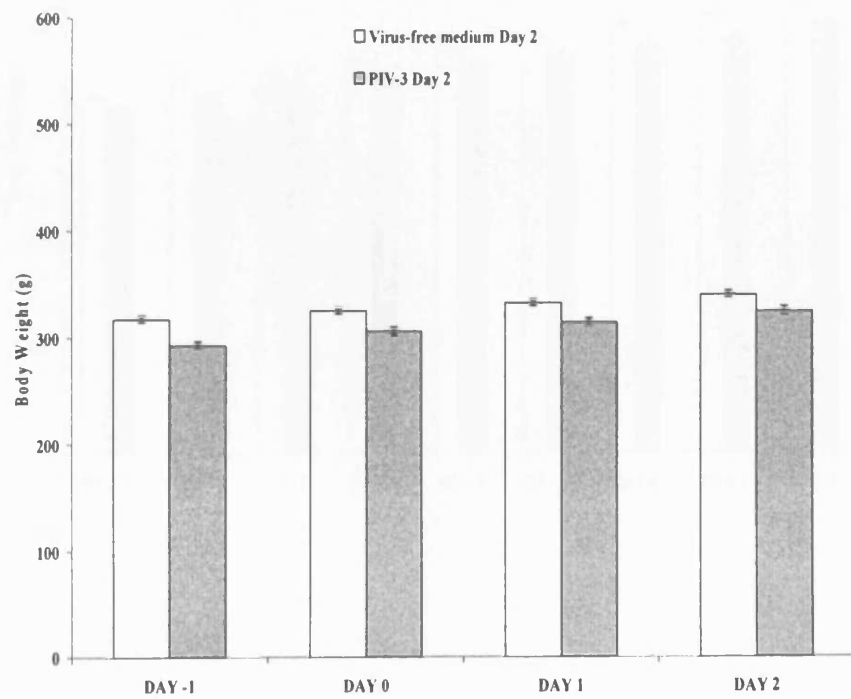
PIV-3 infection in the guinea pig was confirmed by culturing PIV-3 from infected lungs as well as from cells obtained from BAL fluid of infected animals during each of the time points studied. Viral titres in the lung began to appear on day 1 post inoculation, reaching a maximal titre 4 days post inoculation. Levels of virus declined thereafter, although very low levels were still present at day 40. This data suggests that the virus entered the lungs after intranasal inoculation was replicating, as levels retrieved were higher than the initial titre instilled into the nasal cavity. This confirms that positive cultures determined in the BAL fluid and right lung were not solely attributable to free virus remaining from the inoculation procedure.

This aim of this study was to explore the effects of PIV-3 infection in guinea pigs over a period of several weeks after infection using a variety of measured parameters. In conclusion, this study has successfully determined the time course of PIV-3. Evidence of PIV-3 induced changes can be observed as early as day 1 post inoculation, reaching a peak response by day 4 and thereafter declining by day 40 where evidence of the viral infection is minimal. Overall, this suggests a temporal association between the investigated parameters, which is unique to this study.

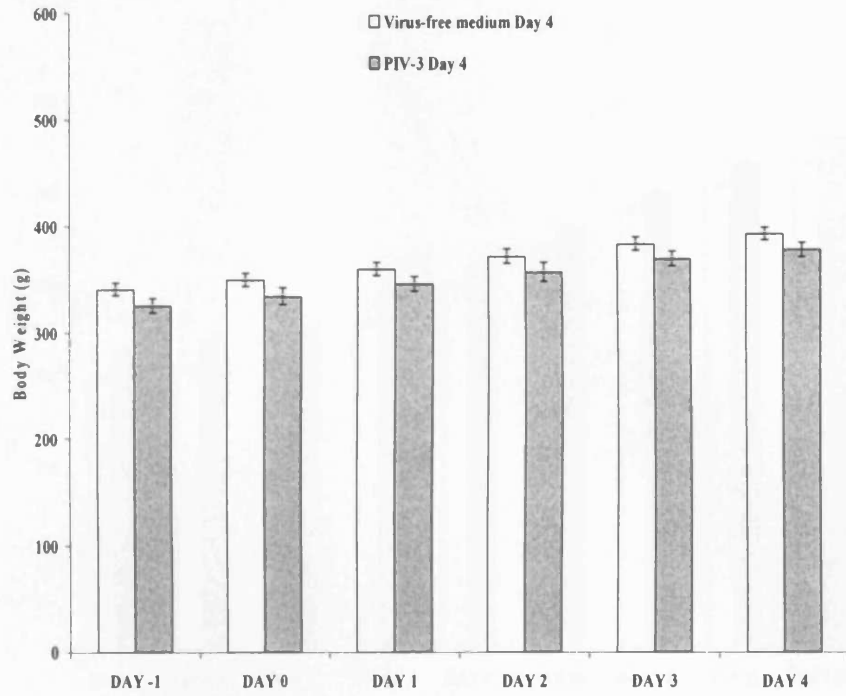
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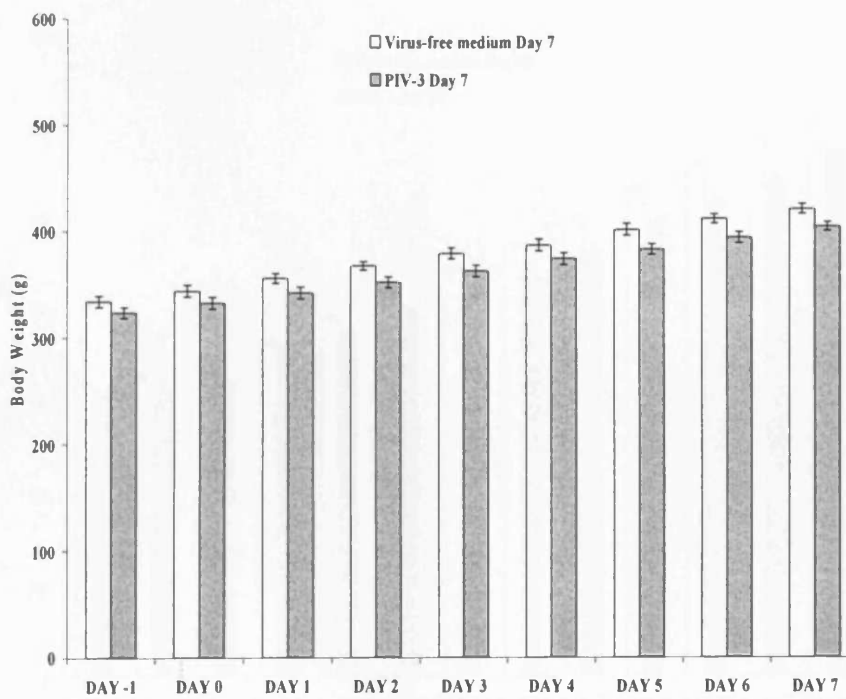
**B**



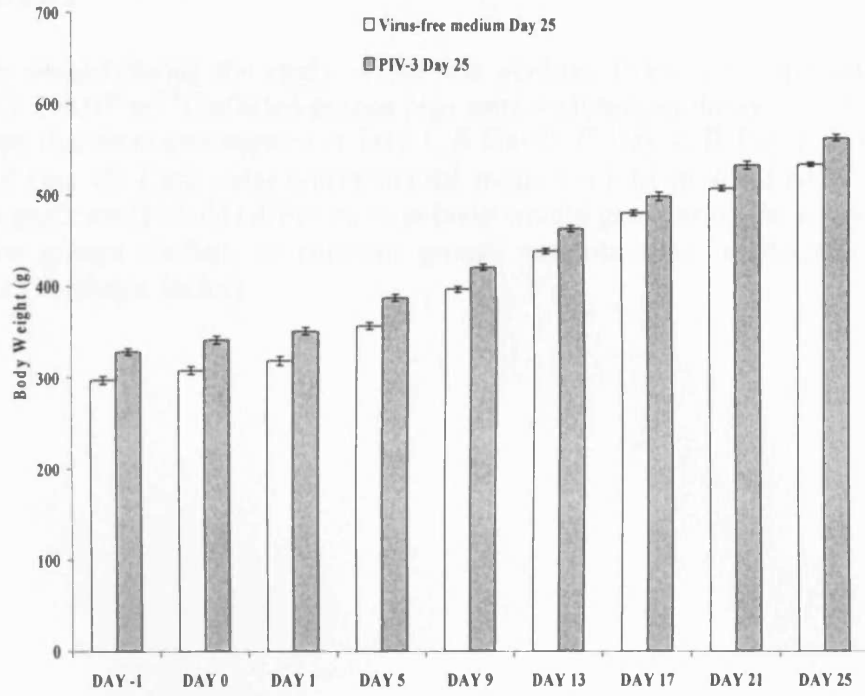
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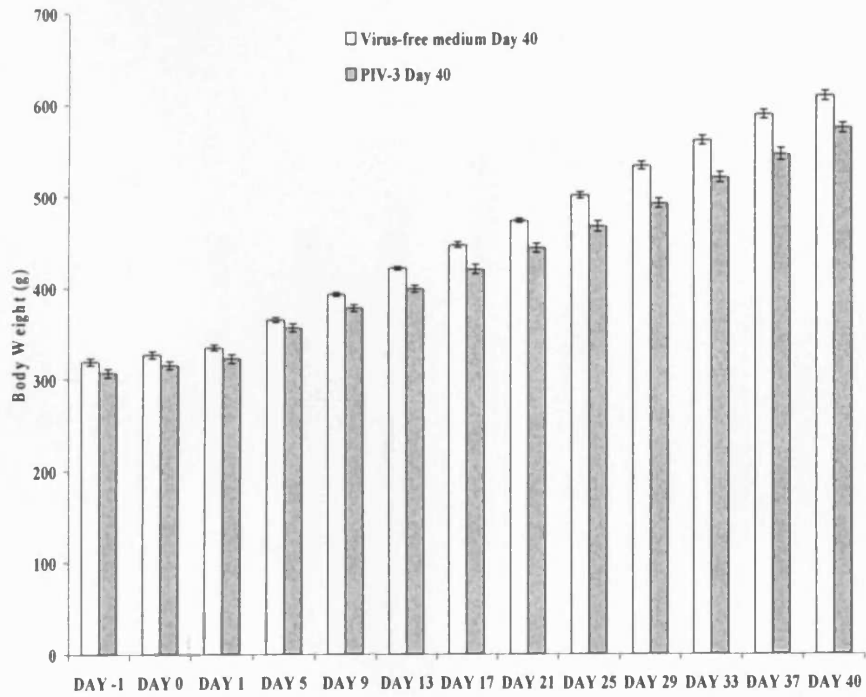
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**E**

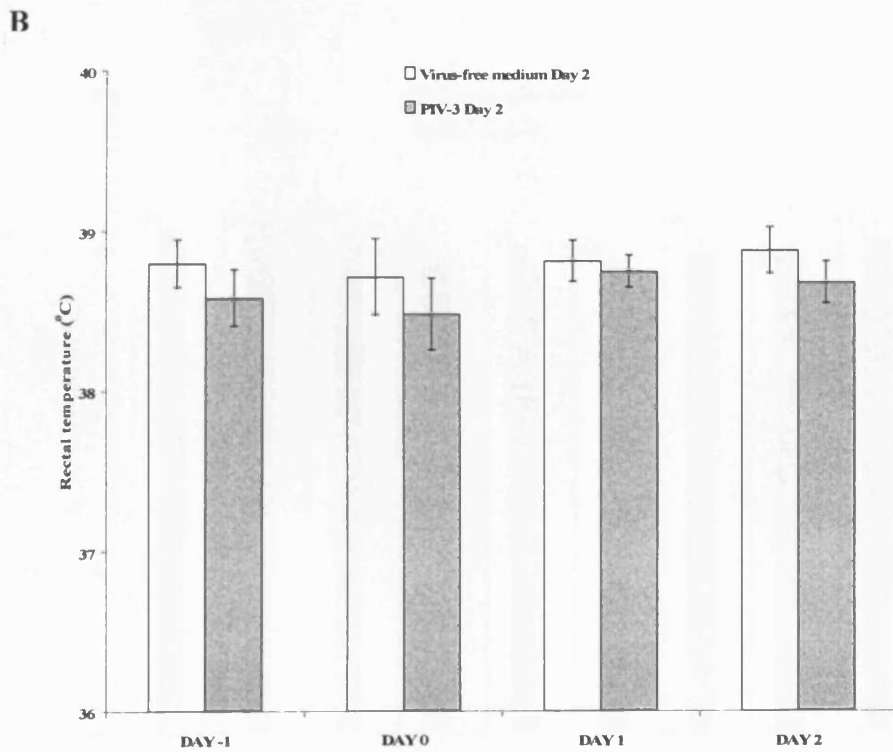
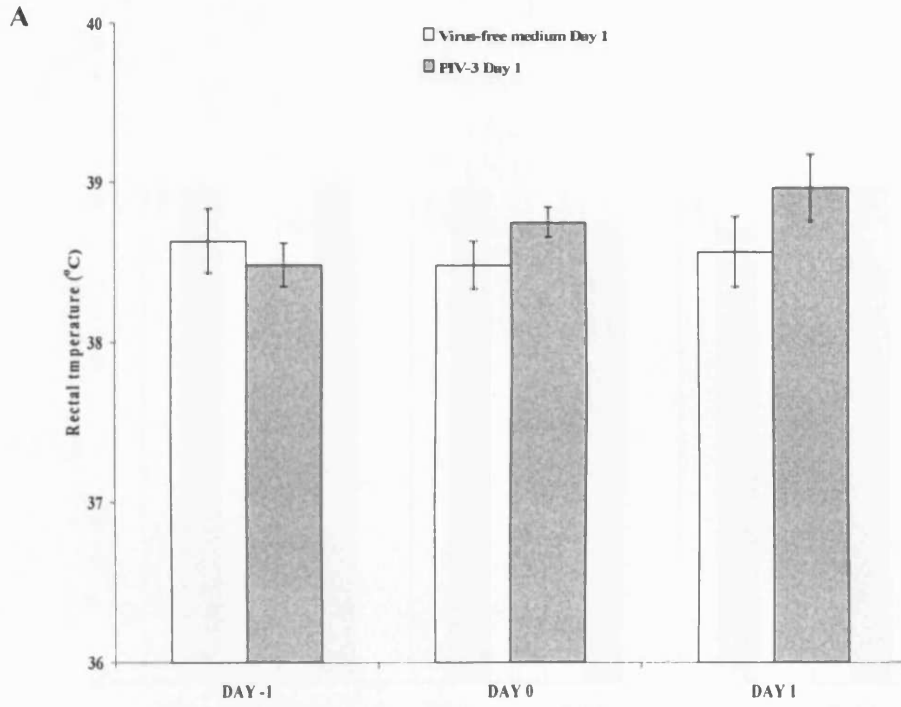


**F**

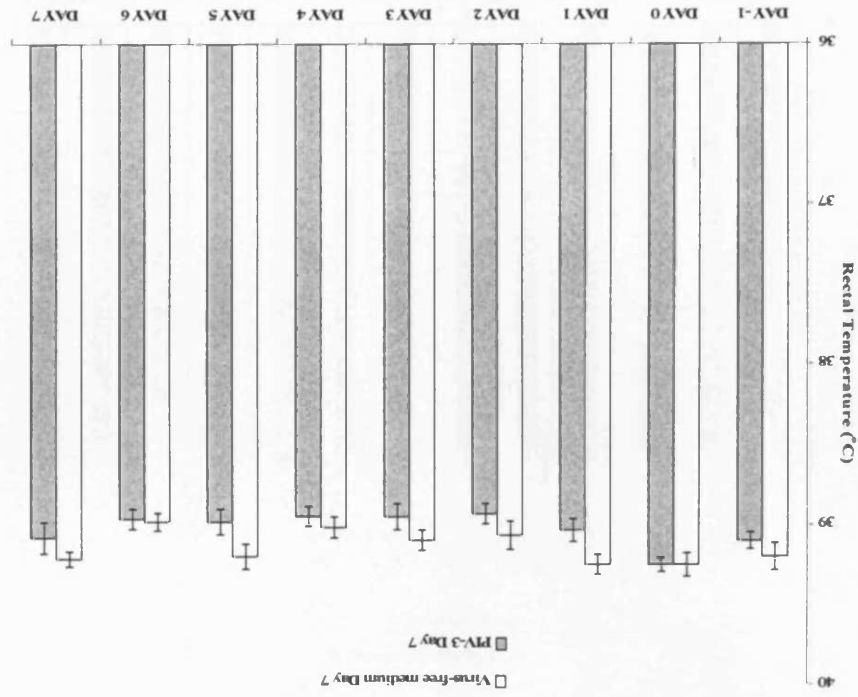


#### **Figure 4.1**

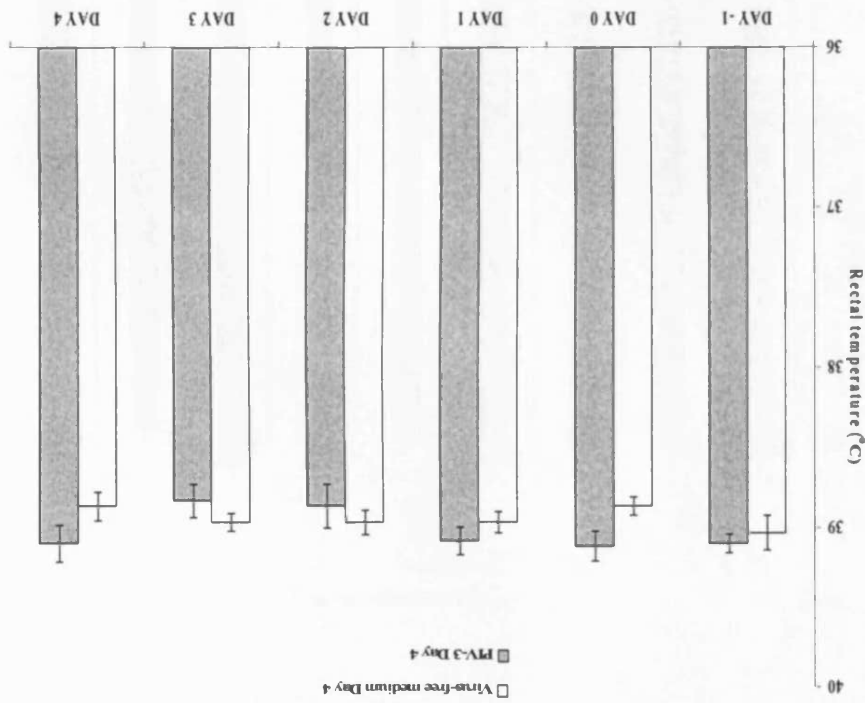
Body weight during the study. Virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs were weighed on the specified days in groups that were investigated **A** Day 1, **B** Day 2, **C** Day 4, **D** Day 5, **E** Day 25 and **F** Day 40. Each point represents the mean  $\pm$  S.E.M (n=6) of body weight. No significant ( $P > 0.05$ ) differences in body weight gain during the study in any of the groups studied, or between groups was observed, as determined by ANOVA (single factor).





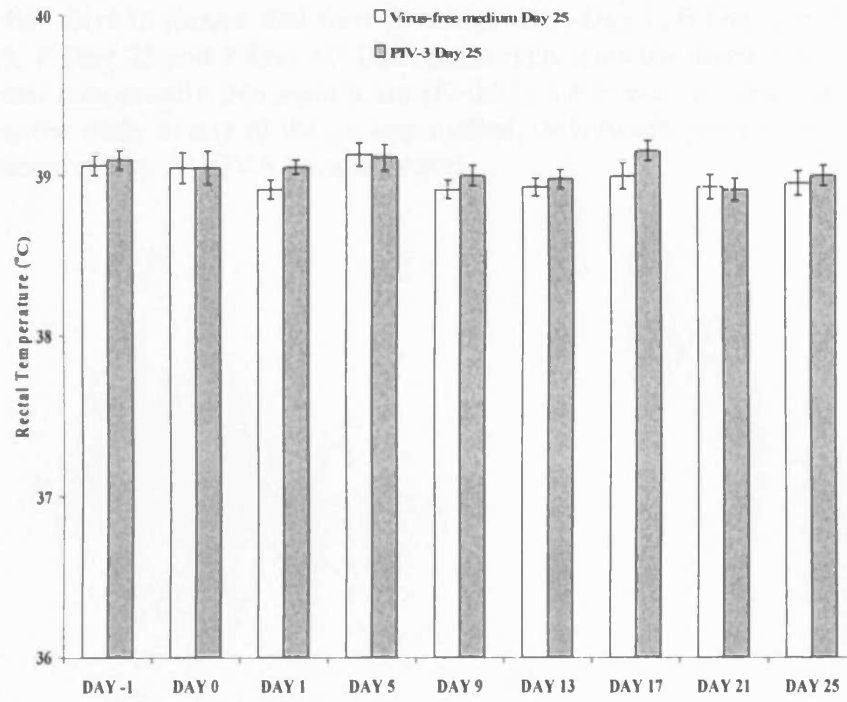


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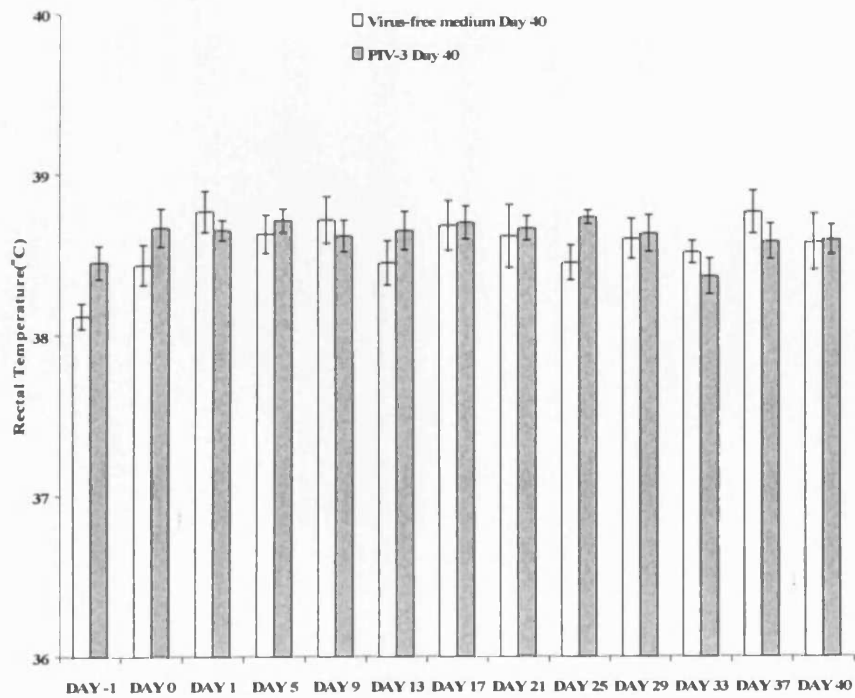


C

**E**



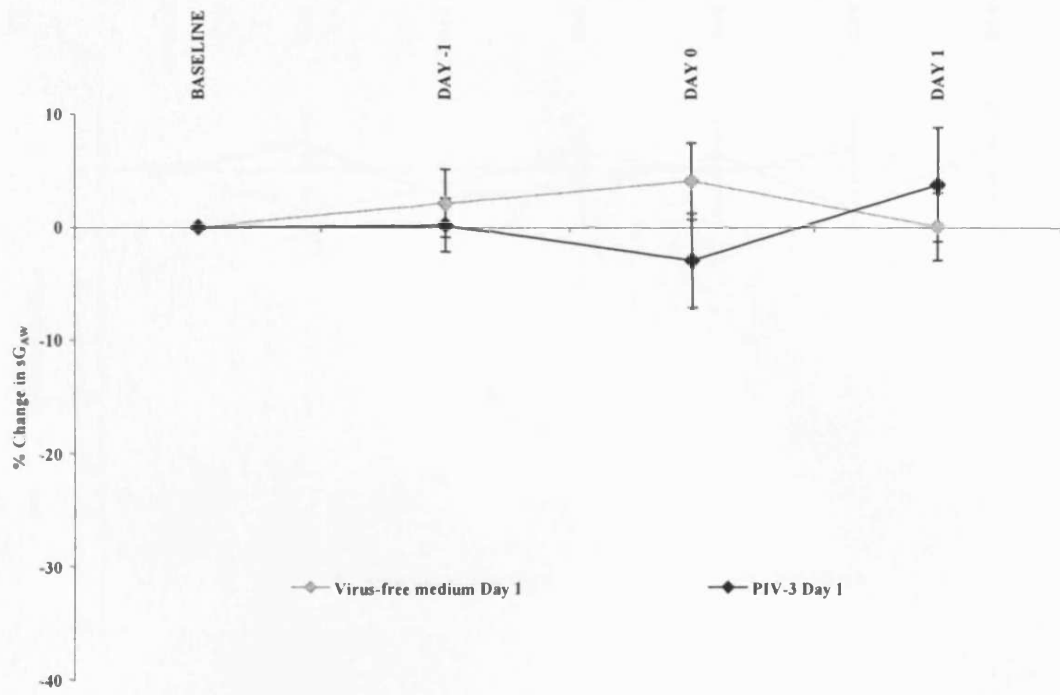
**F**



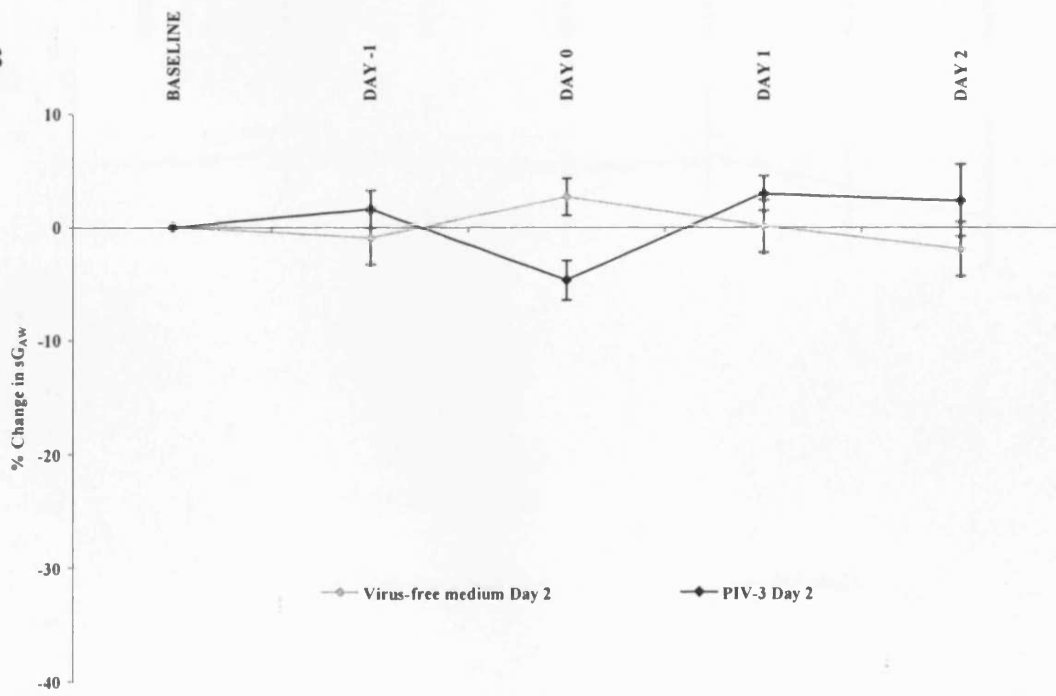
#### **Figure 4.2**

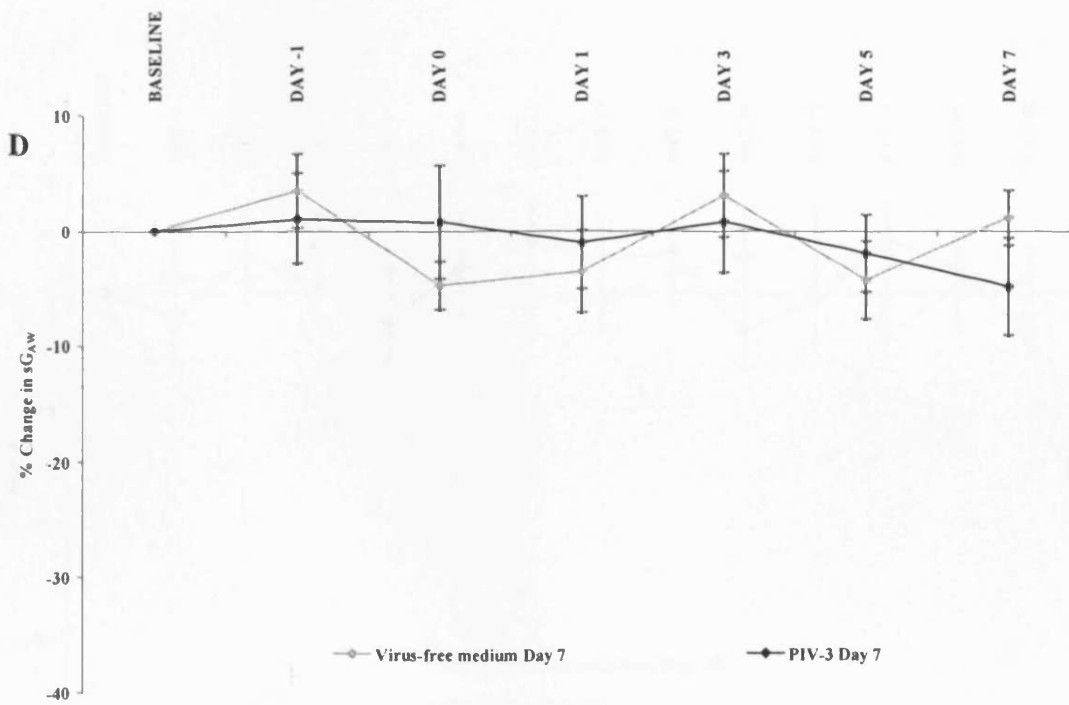
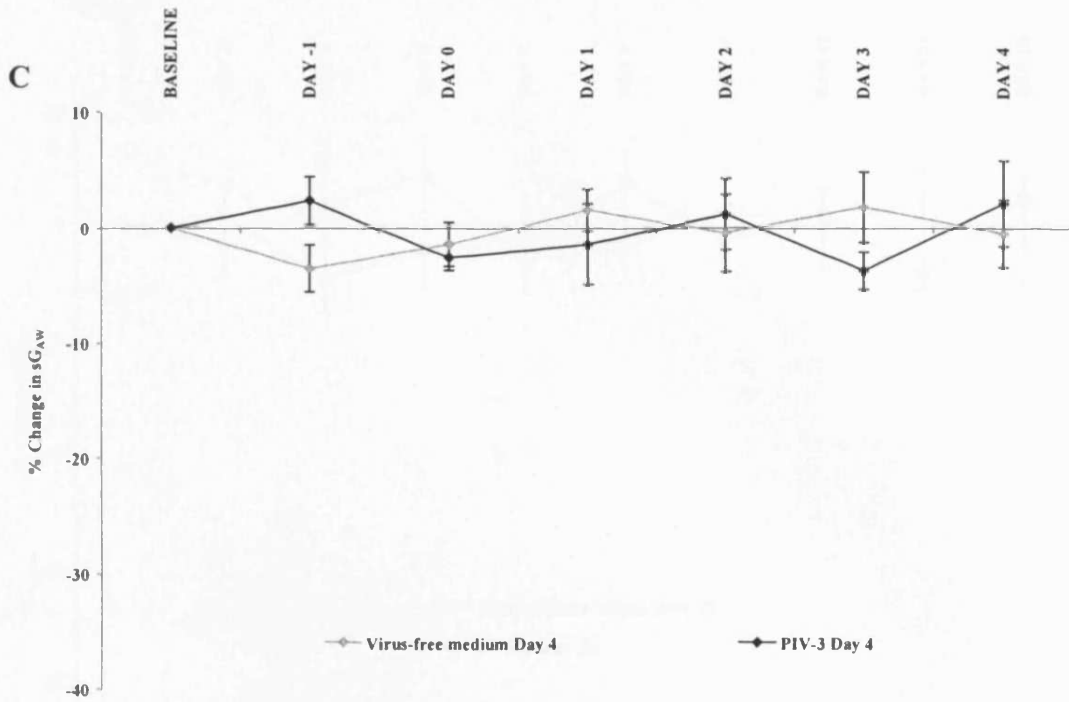
Rectal temperature during the study. Virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs were weighed on the specified days in groups that were investigated **A** Day 1, **B** Day 2, **C** Day 4, **D** Day 5, **E** Day 25 and **F** Day 40. Each point represents the mean  $\pm$  S.E.M (n=6) of rectal temperature. No significant ( $P > 0.05$ ) difference in rectal temperature during the study in any of the groups studied, or between groups was observed as determined by ANOVA (single factor).

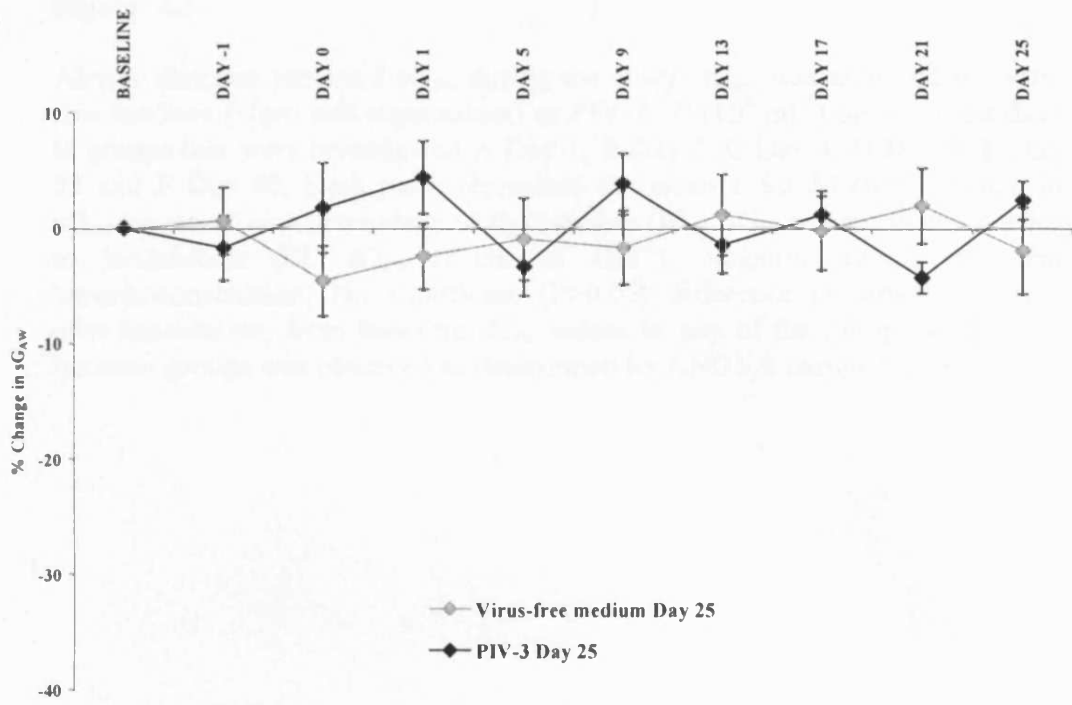
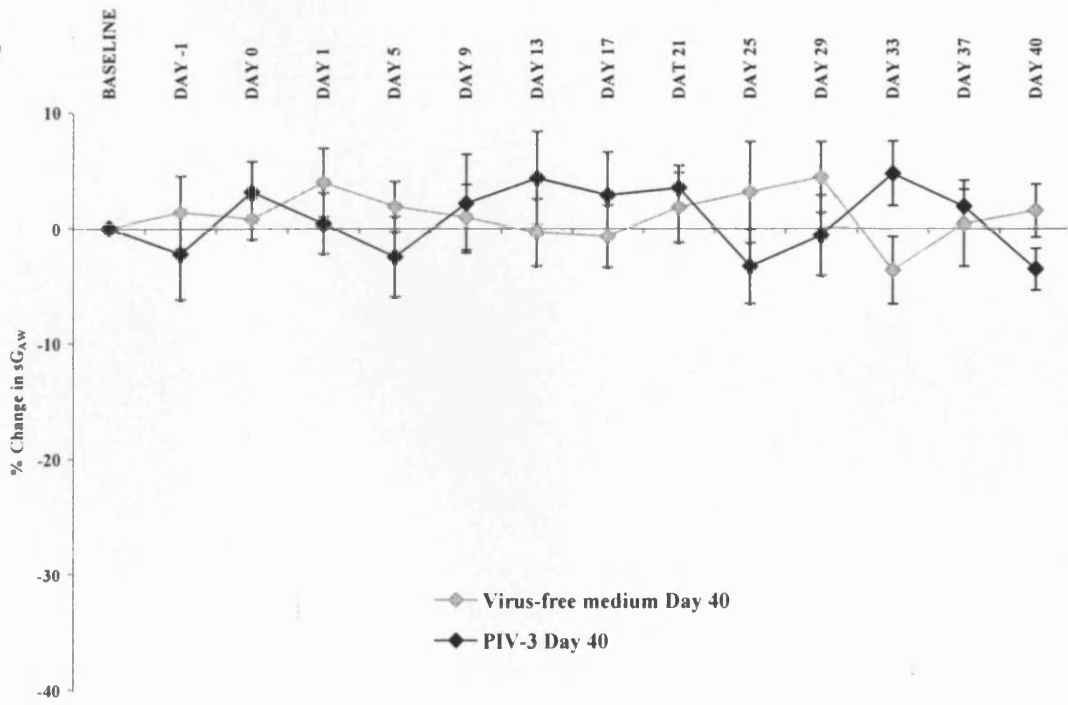
**A**



**B**

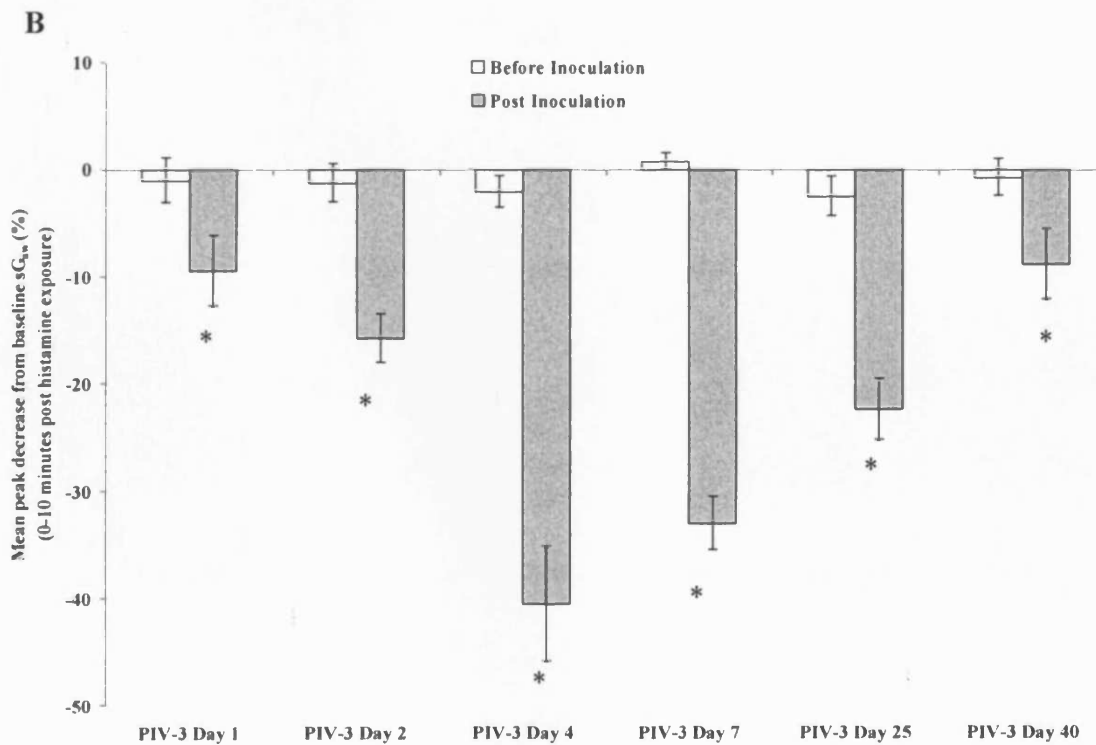
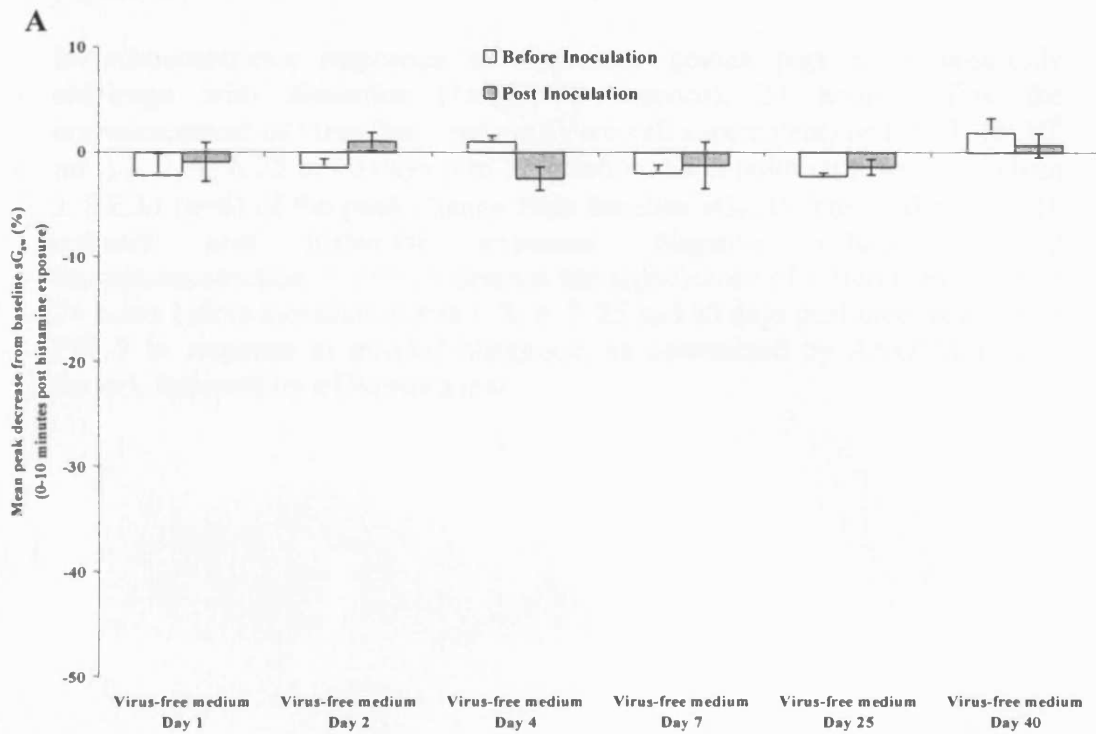




**E****F**

### Figure 4.3

Airway function recorded  $sG_{aw}$  during the study.  $sG_{aw}$  was recorded in virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) on specified days in groups that were investigated **A** Day 1, **B** Day 2, **C** Day 4, **D** Day 5, **E** Day 25 and **F** Day 40. Each point represents the mean  $\pm$  S.E.M (n=6) change in  $sG_{aw}$  expressed as a percentage of the baseline (BL).  $sG_{aw}$  values recorded prior to inoculation (BL  $sG_{aw}$  ( $\text{s}^{-1}\text{cm}$  of  $\text{H}_2\text{O}^{-1}$ )). Negative values represent bronchoconstriction. No significant ( $P > 0.05$ ) difference in airway function after inoculation, from baseline  $sG_{aw}$  values in any of the groups studied, or between groups was observed as determined by ANOVA (single factor).

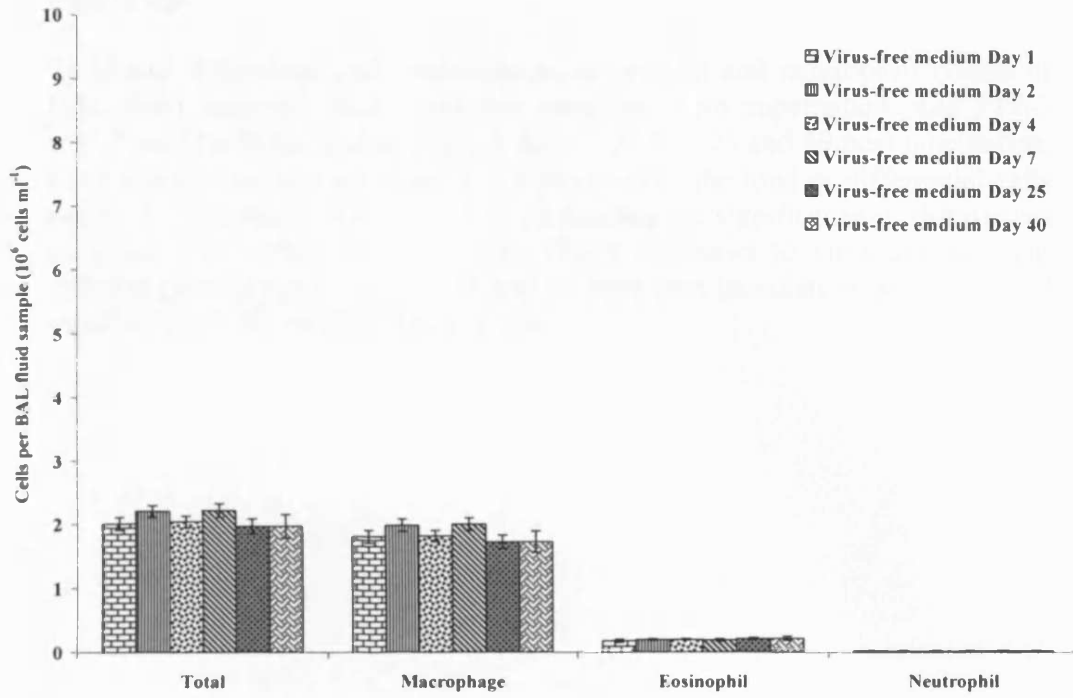




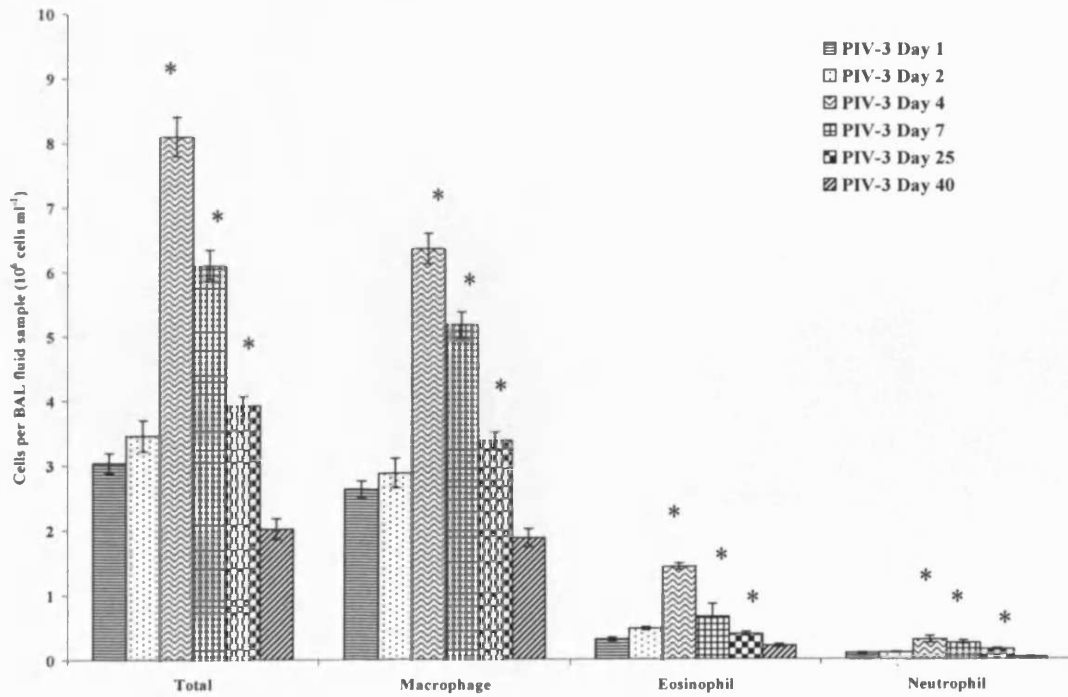
#### Figure 4.4

Bronchoconstrictor responses of conscious guinea pigs to a nose-only challenge with histamine (1mM, 20 seconds), 24 hours before the commencement of virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) 1, 2, 4, 7, 25 or 40 days post inoculation. Each point represents the mean  $\pm$  S.E.M (n=6) of the peak change from baseline  $sG_{aw}$  ( $\text{S}^{-1}\text{cm}$  of  $\text{H}_2\text{O}^{-1}$ ), 0-10 minutes post histamine exposure. Negative values represent bronchoconstriction. \*  $P < 0.05$  denotes the significance of differences between 24 hours before inoculation and 1, 2, 4, 7, 25 and 40 days post inoculation with PIV-3 in response to inhaled histamine, as determined by ANOVA (single factor), followed by a Dunnet's test.

A



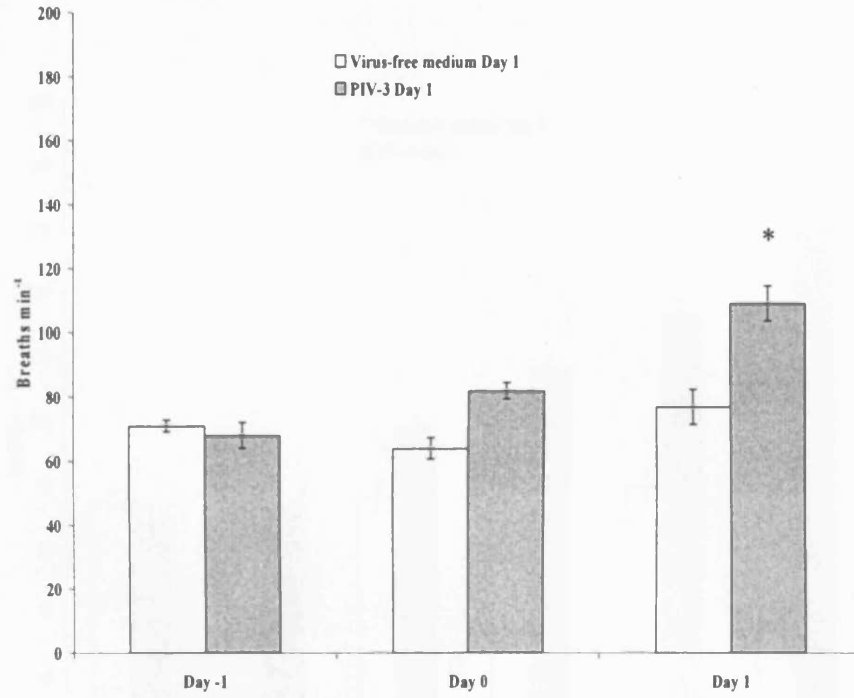
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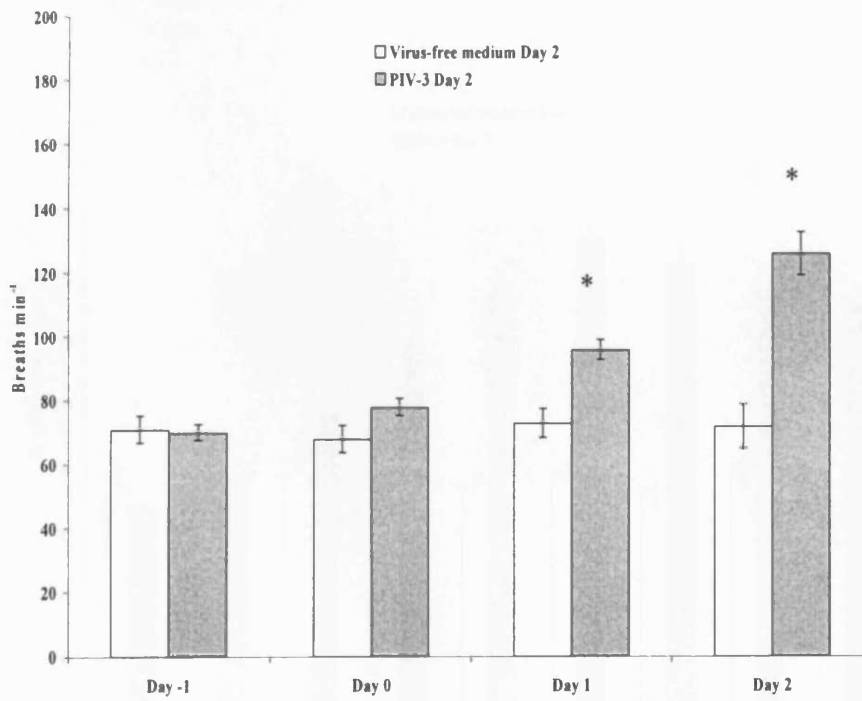
#### **Figure 4.5**

Total and differential cell (macrophage, eosinophil and neutrophil) counts of BAL fluid removed from virus-free medium (Vero supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs on days 1, 2, 4, 7, 25 and 40 post inoculation. Each point represents the mean  $\pm$  S.E.M (n=6) of the total or differential cells per BAL fluid sample ( $\times 10^6$ ). \*  $P < 0.05$  denotes the significance of differences in airway cell influx removed from PIV-3 compared to virus-free medium infected guinea pigs 1, 2, 4, 7, 25 and 40 days post inoculation, as determined by ANOVA, followed by Dunnet's test.

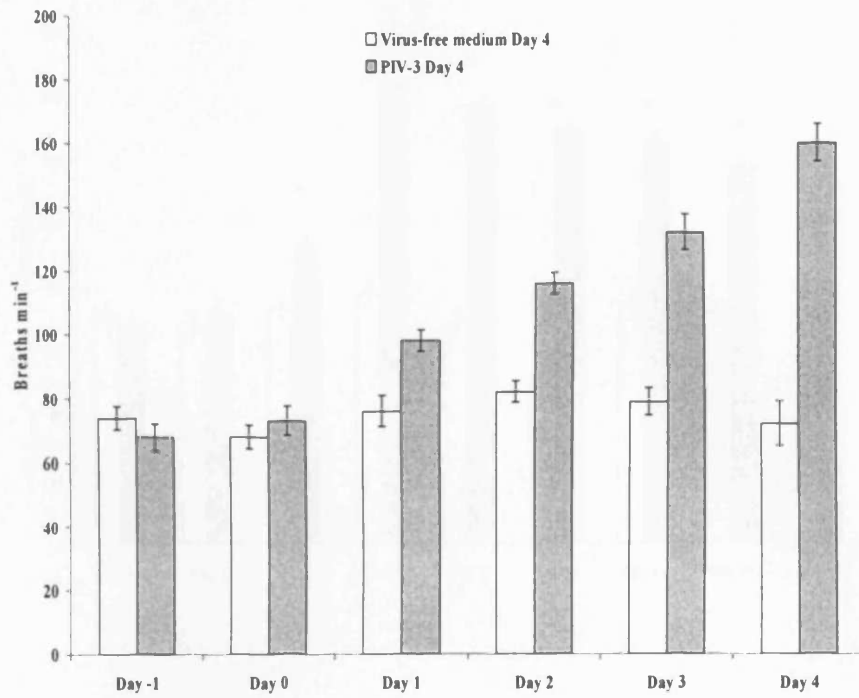
**A**



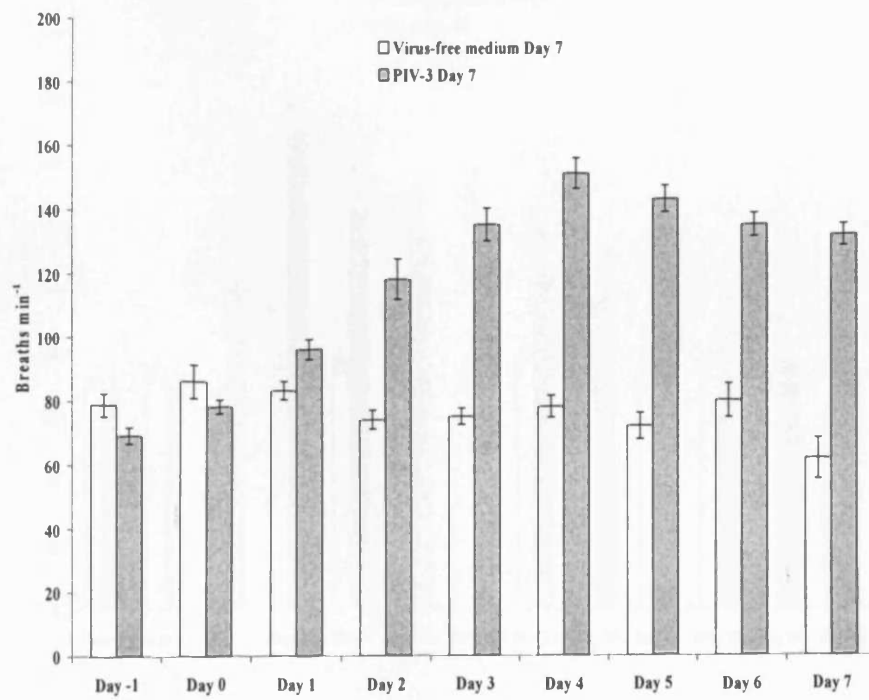
**B**

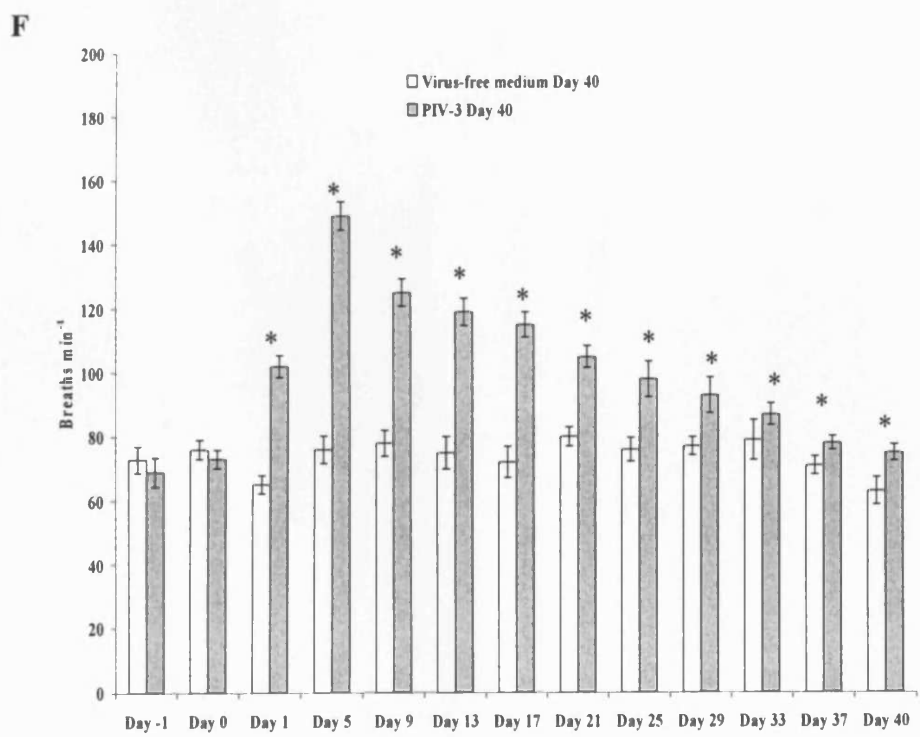
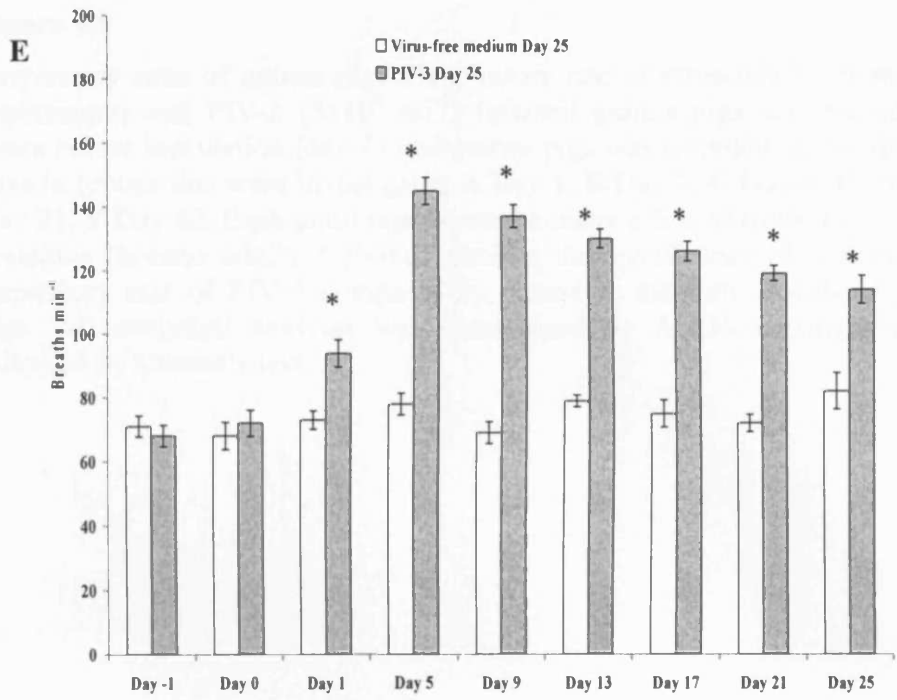


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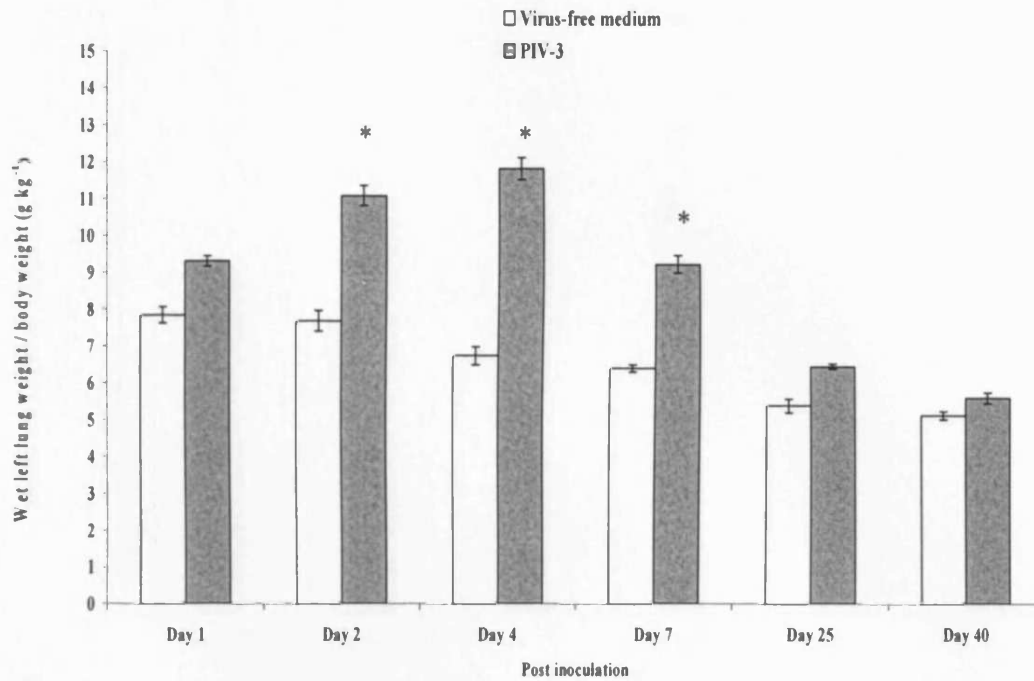
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#### **Figure 4.6**

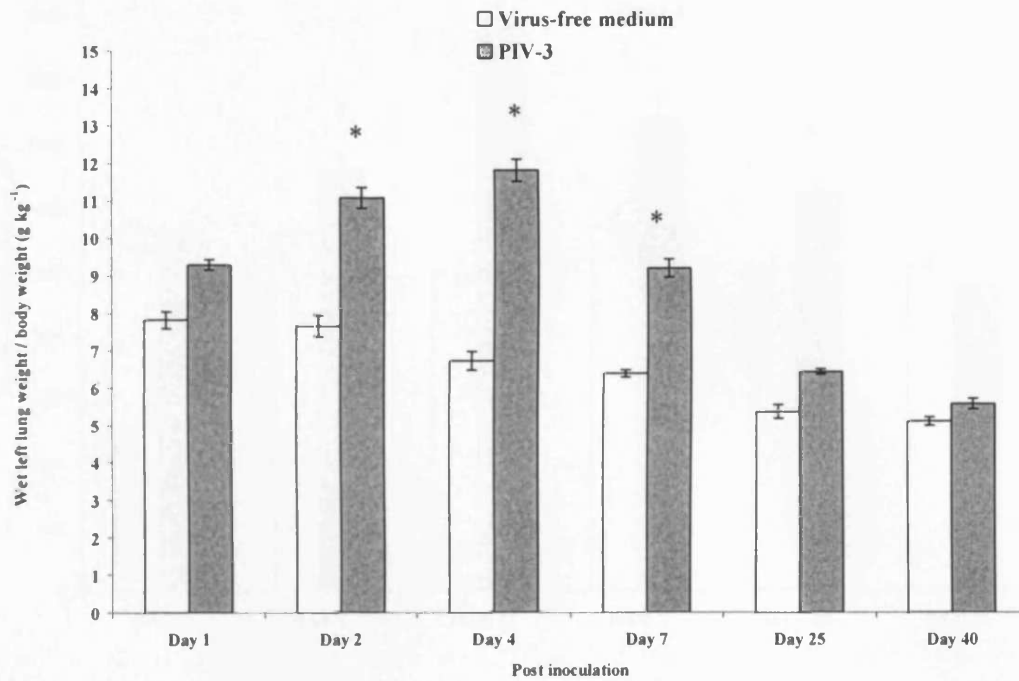
Respiratory rates of guinea pigs. Respiratory rate of virus-free medium (Vero supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs was recorded 24 hours before inoculation (day-1) and guinea pigs was recorded on the specified days in groups that were investigated **A** Day 1, **B** Day 2, **C** Day 4, **D** Day 7, **E** Day 25, **F** Day 40. Each point represents the mean  $\pm$  S.E.M (n=6) frequency of breathing (breaths  $\text{min}^{-1}$ ). \*  $P < 0.05$  denotes the significance of differences in respiratory rate of PIV-3 compared to virus-free medium inoculated guinea pigs. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



**Figure 4.7**

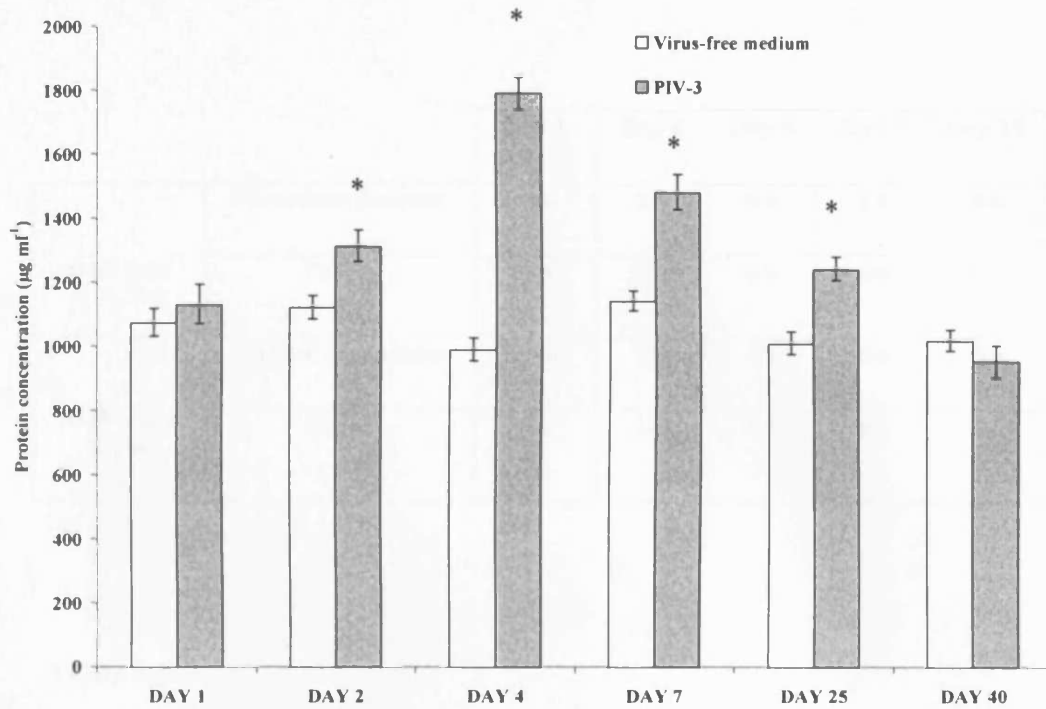
Wet left lung weights from virus-free medium and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs on days 1, 2, 4, 7, 25 and 40 post inoculation. Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of wet left lung weights normalised to body weight ( $\text{g kg}^{-1}$ ). \*  $P < 0.05$  denotes the significance of differences in wet left lung weights from PIV-3 compared to virus-free medium infected guinea pigs as determined by ANOVA (single factor), followed by Dunnet's test.





**Figure 4.8**

Total nitric oxide levels in BAL fluid removed from guinea pigs inoculated with virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) assessed 1, 2, 4, 7, 25 and 40 days post inoculation. Each point represents the mean  $\pm$  S.E.M (n=6) of nitric oxide concentration per sample ( $\mu$ M per 100 $\mu$ l). \* P<0.05 denotes the significant differences in nitric oxide concentrations in PIV-3 compared to virus-free infected guinea pigs. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



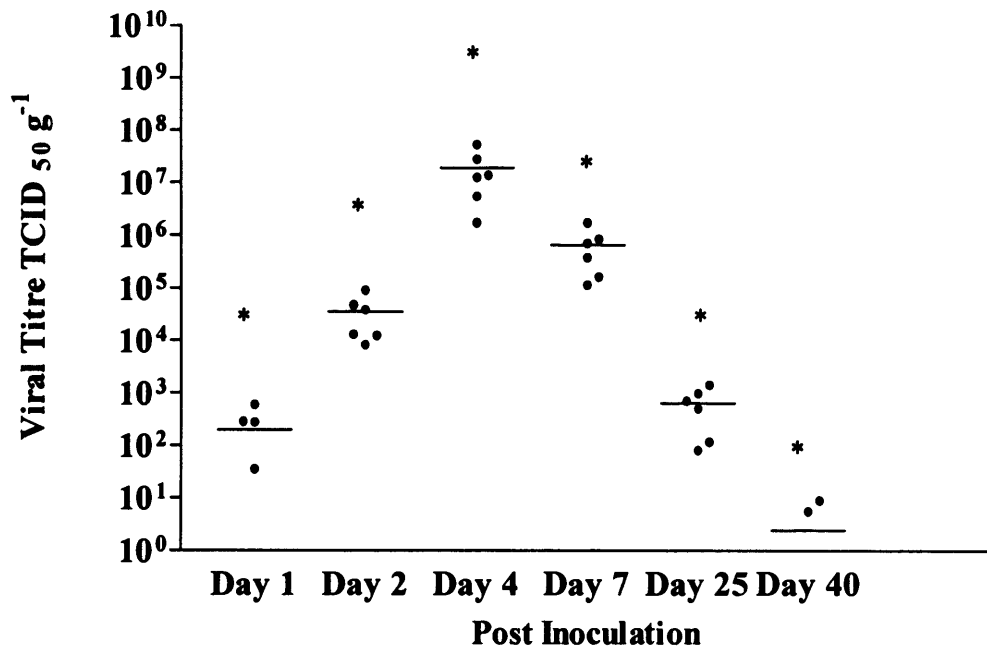
**Figure 4.9**

Total protein in BAL fluid removed from guinea pigs inoculated with virus-free medium or PIV-3 ( $5 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) assessed 1, 2, 4, 7, 25 and 40 days post inoculation. Each point represents the mean  $\pm$  S.E.M (n=6) of the protein concentration per sample ( $\mu\text{g ml}^{-1}$ ). \* P<0.05 denotes the significant differences in protein concentrations in PIV-3 compared with virus-free medium infected guinea pigs. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

		Day 1	Day 2	Day 4	Day 7	Day 25	Day 40
<b>BAL fluid</b>	<b>Virus-free medium</b>	0/6	0/6	0/6	0/6	0/6	0/6
	<b>PIV-3</b>	0/6	6/6	6/6	6/6	6/6	0/6
<b>Right lung tissue</b>	<b>Virus-free medium</b>	0/6	0/6	0/6	0/6	0/6	0/6
	<b>PIV-3</b>	4/6	6/6	6/6	6/6	6/6	2/6

**Table 4.1**

Number of guinea pigs out of each group of six, which displayed evidence of PIV-3 growth on Vero cells using standard culture techniques and positive immunofluorescence in BAL fluid and right lung tissue samples from virus-free medium and PIV-3 infected guinea pigs 1, 2, 4, 7, 25 and 40 days post inoculation.

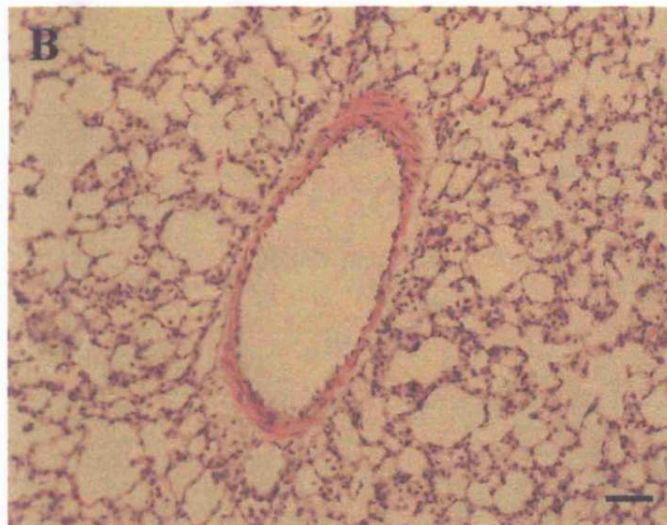
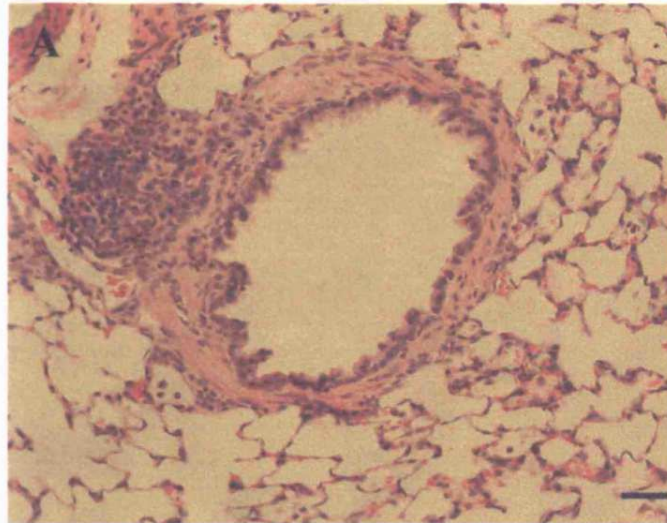


**Figure 4.10**

Titres of intrapulmonary replicating PIV-3 normalised to wet left lung weight (TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>) isolated from guinea pig left lung 1, 2, 4, 7, 25 and 40 S.E.M of mean titres of intrapulmonary replicating PIV-3. \* denotes the significant differences in viral titres in PIV-3 compared with virus-free inoculated guinea pigs as determined by ANOVA, followed by Dunnet's test.

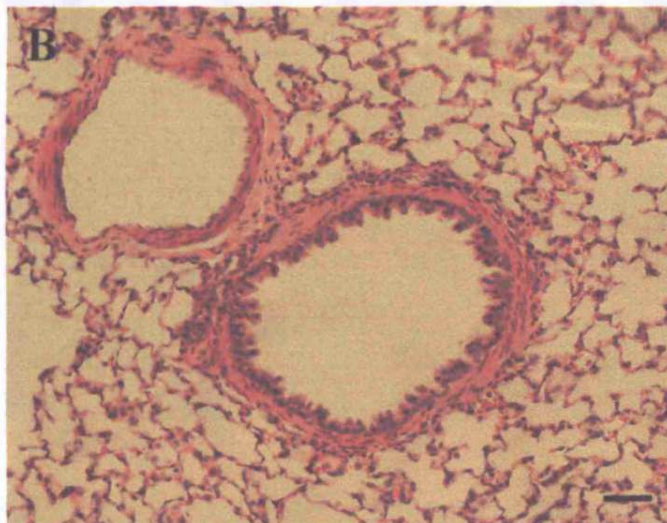
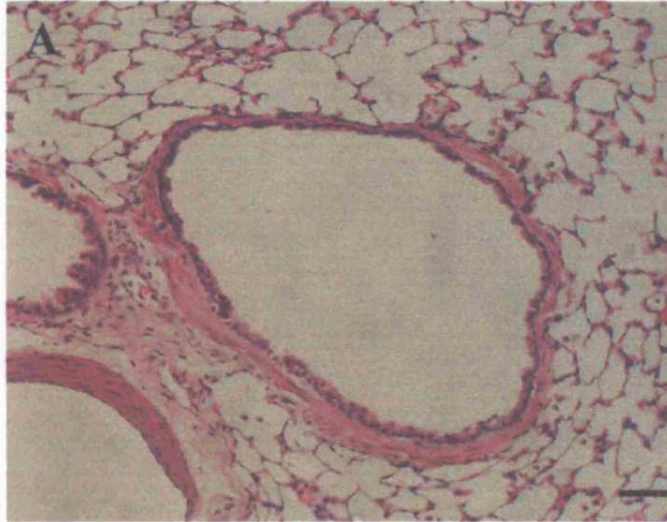
**Figure 4.11**

Histological appearance of lung tissue from virus-free medium (A) and PIV-3 (B) infected guinea pigs post inoculation day 1. Tissues were stained with haematoxylin and eosin for general morphology (Internal scale bar = 50 $\mu$ m).



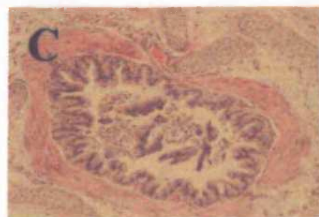
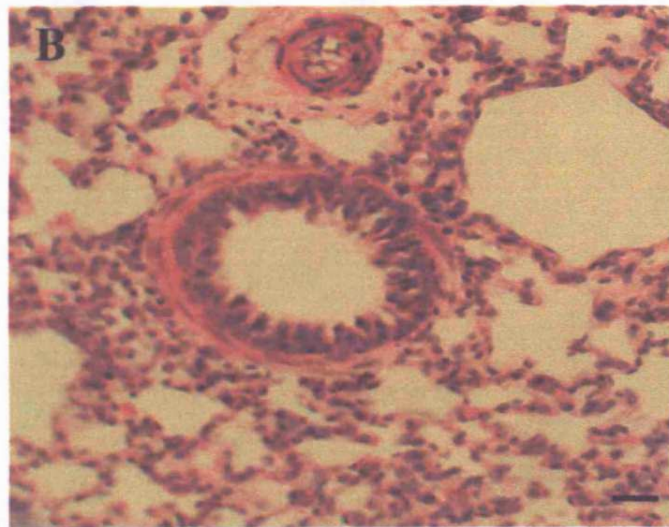
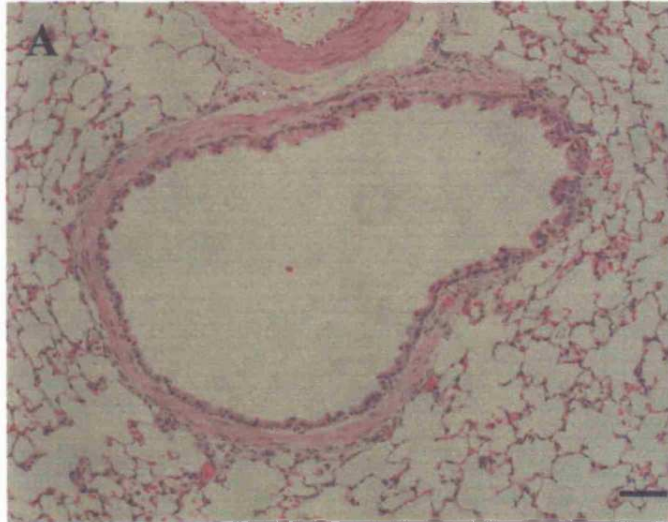
**Figure 4.12**

Histological appearance of lung tissue from virus-free medium (A) and PIV-3 (B) infected guinea pigs post inoculation day 2. Tissues were stained with haematoxylin and eosin for general morphology (Internal scale bar = 50 $\mu$ m).



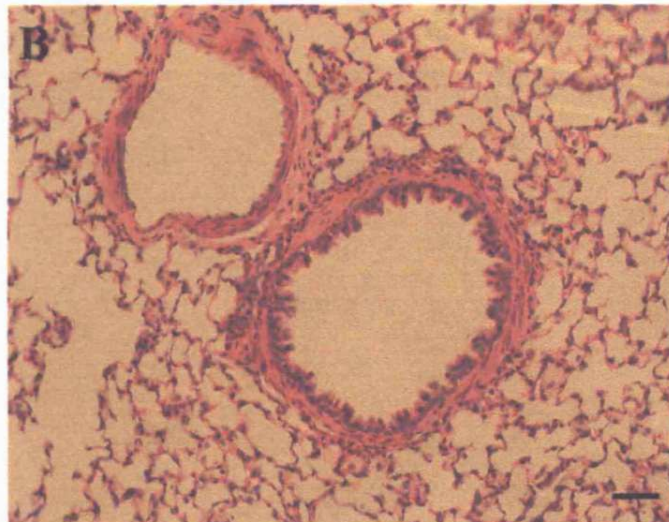
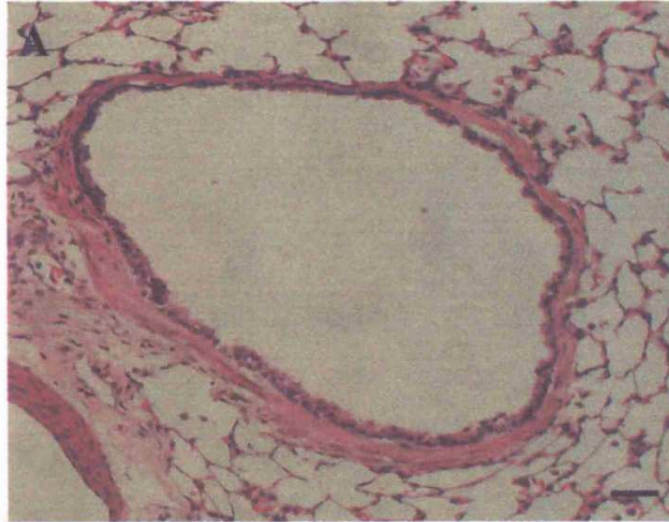
**Figure 4.13**

Histological appearance of lung tissue from virus-free medium (A) and PIV-3 (B) infected guinea pigs post inoculation day 4 C/D Occluded airways. Tissues were stained with haematoxylin and eosin for general morphology (Internal scale bar = 50µm).



**Figure 4.14**

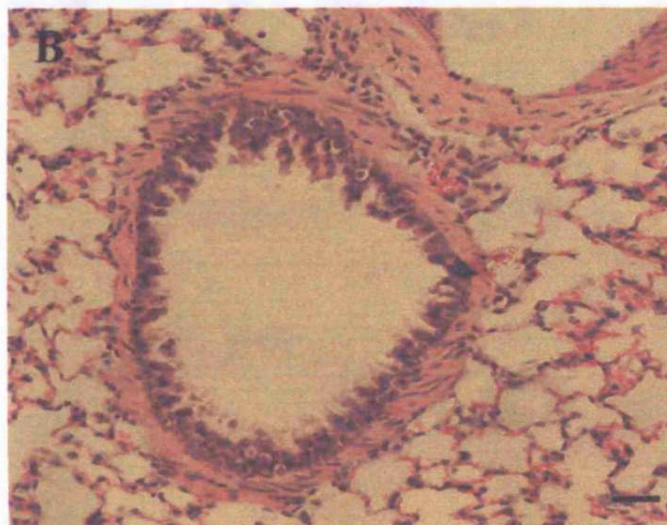
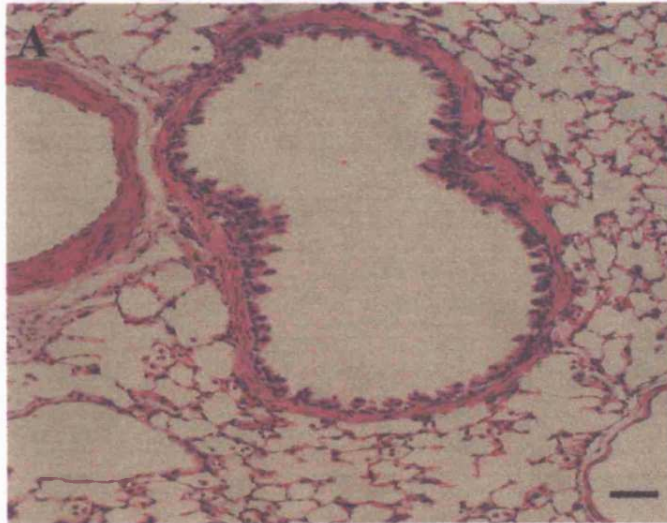
Histological appearance of lung tissue from virus-free medium **(A)** and PIV-3 **(B)** infected guinea pigs post inoculation day 7. Tissues were stained with haematoxylin and eosin for general morphology (Internal scale bar = 50µm).





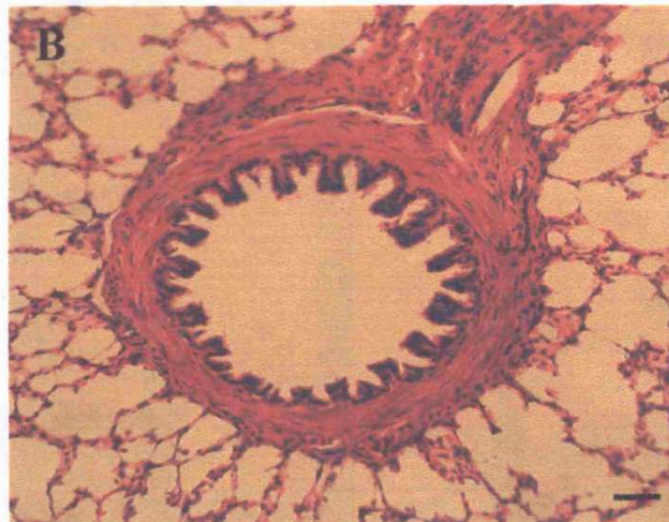
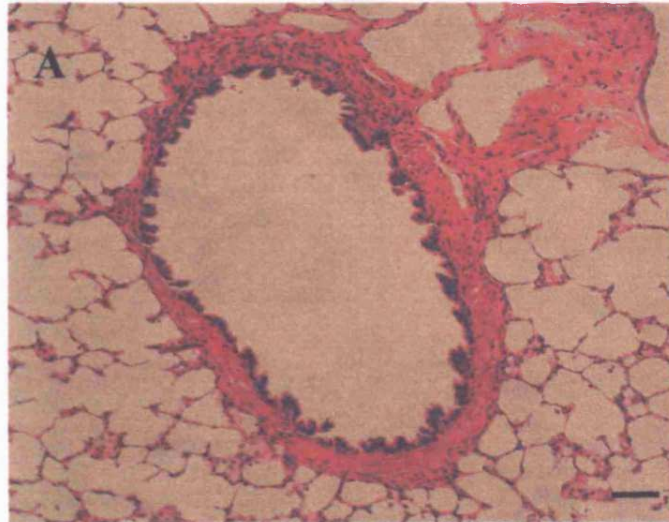
**Figure 4.15**

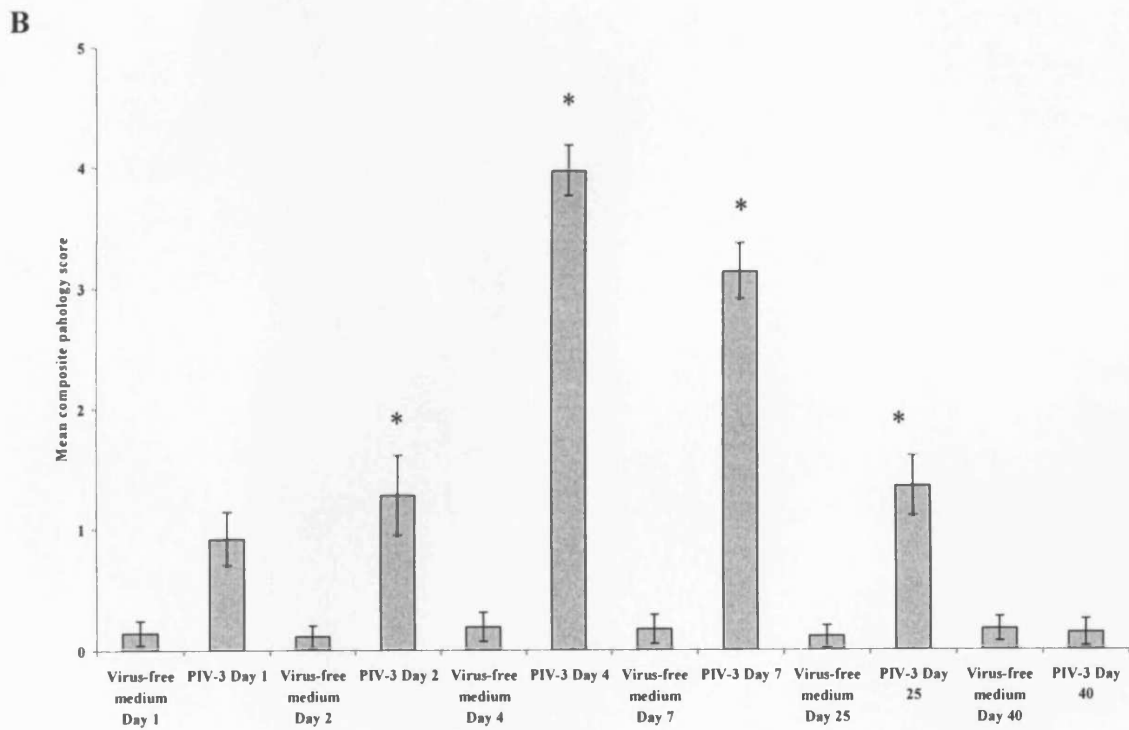
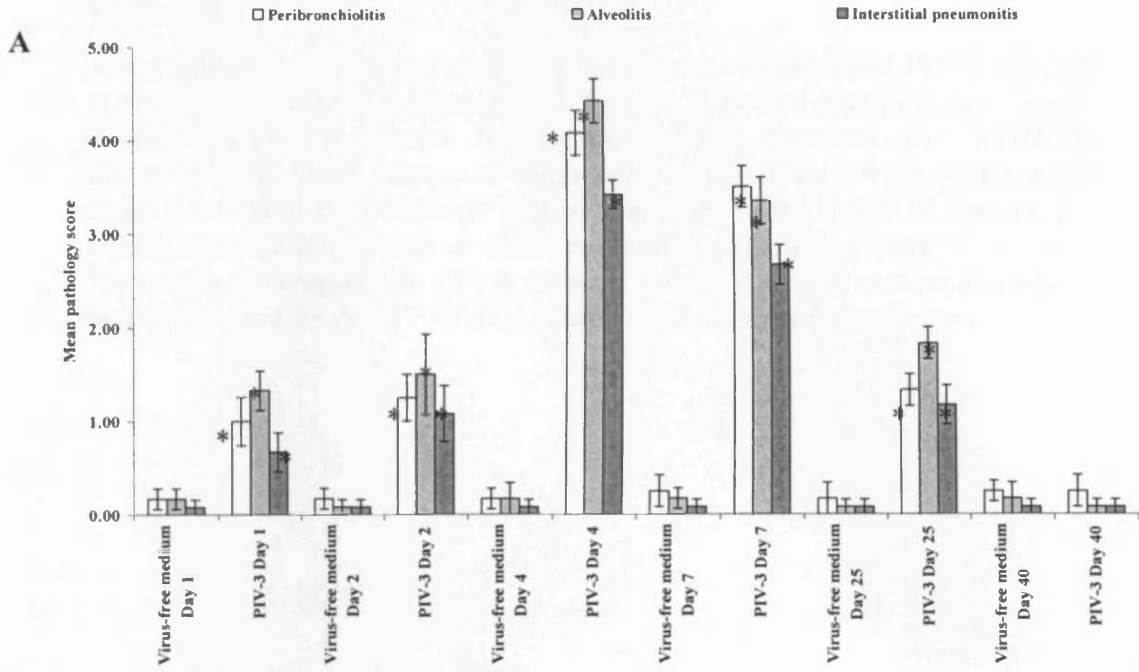
Histological appearance of lung tissue from virus-free medium (A) and PIV-3 (B) infected guinea pigs post inoculation day 25. Tissues were stained with haematoxylin and eosin for general morphology (Internal scale bar = 50µm).



**Figure 4.16**

Histological appearance of lung tissue from virus-free medium (A) and PIV-3 (B) infected guinea pigs post inoculation day 40. Tissues were stained with haematoxylin and eosin for general morphology (Internal scale bar = 50µm).





**Figure 4.17**

Mean pulmonary pathology scores from virus-free medium and PIV-3 infected guinea pigs as defined in Chapter 2, 1, 2, 4, 7, 25 and 40 days post inoculation. **A** Mean pulmonary scores for peribronchiolitis, alveolitis and interstitial pneumonitis **B** Mean composite pulmonary scores. Each point represents the mean  $\pm$  S.E.M of the pathology scores (range 1-5) (n=6). \* P<0.05 denotes the significance of differences in mean pulmonary pathology scores from virus-free medium compared to PIV-3 infected guinea pigs. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

A

		Virus-free medium					
		Day 1 (n=6)	Day 2 (n=6)	Day 4 (n=6)	Day 7 (n=6)	Day 25 (n=6)	Day 40 (n=6)
<b>Body weight (g)</b>		5.0±0.3	7.2±0.2	15.3±0.7	26.1±0.9	29.3±1.9	91.3±1.3
<b>Rectal temp (°C)</b>		38.6±0.2	38.9±0.2	38.9±0.1	39.2±0.1	39.0±0.1	38.6±0.2
<b>Lung function (s<sup>-1</sup>cm of H<sub>2</sub>O<sup>-1</sup>)</b>		0.1±3.0	-2.0±.4	-0.5±3.0	1.1±2.4	-2.0±3.8	1.5±2.3
<b>Lung function before histamine exposure (s<sup>-1</sup>cm of H<sub>2</sub>O<sup>-1</sup>)</b>		-2.2±2.1	-1.5±0.7	1.0±0.5	-2.0±0.7	-2.4±0.3	1.9±1.3
<b>Lung function after histamine exposure (s<sup>-1</sup>cm of H<sub>2</sub>O<sup>-1</sup>)</b>		-1.0±1.9	1.0±0.8	-2.6±1.1	-1.3±2.2	-1.5±0.7	0.7±1.1
<b>Total Cells (10<sup>6</sup> cells ml<sup>-1</sup>)</b>		2.0±0.1	2.2±0.1	2.0±0.1	2.1±0.1	2.2±0.1	2.0±0.2
<b>Macrophages (10<sup>6</sup> cells ml<sup>-1</sup>)</b>		1.8±0.1	2.0±0.1	1.8±0.1	1.9±0.1	2.0±0.1	1.7±0.2
<b>Eosinophils (10<sup>6</sup> cells ml<sup>-1</sup>)</b>		0.2±0.03	0.2±0.01	0.2±0.01	0.2±0.02	0.2±0.02	0.2±0.03
<b>Neutrophils (10<sup>6</sup> cells ml<sup>-1</sup>)</b>		0.03±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.03±0.01
<b>Respiratory rate (breaths min<sup>-1</sup>)</b>		77.0±5.5	72.0±7.0	72.0±7.0	62.0±6.5	82.0±5.7	63.0±4.3
<b>Wet lung weights (g kg<sup>-1</sup>)</b>		7.8±0.2	7.7±0.3	6.7±0.3	6.4±0.1	5.4±0.2	5.1±0.1
<b>Total nitrate and nitrite (µM)</b>		50.1±1.6	48.4±2.0	44.2±2.6	53.3±5.6	54.6±4.6	51.7±2.5
<b>Protein levels (µg ml<sup>-1</sup>)</b>		1074.4±42.6	1121.8±36.6	992.7±35.9	1143.7±31.3	1011.5±35.3	1019.8±32.8
<b>Isolation in BAL fluid</b>		0/6	0/6	0/6	0/6	0/6	0/6
<b>Isolation in right lung</b>		0/6	0/6	0/6	0/6	0/6	0/6
<b>Viral titre (TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>)</b>		Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
<b>Mean pulmonary score</b>	<b>Peribronchiolitis</b>	0.17±0.11	0.17±0.11	0.17±0.11	0.25±0.17	0.17±0.17	0.25±0.11
	<b>Alveolitis</b>	0.17±0.11	0.08±0.08	0.17±0.17	0.17±0.11	0.08±0.08	0.17±0.7
	<b>Interstitial pneumonia</b>	0.08±0.08	0.08±0.08	0.08±0.08	0.08±0.08	0.08±0.08	0.08±0.08
<b>Mean composite pulmonary score</b>		0.14±0.10	0.11±0.09	0.19±0.12	0.17±0.12	0.11±0.09	0.17±0.10

**B**

		PIV-3					
		Day 1 (n=6)	Day 2 (n=6)	Day 4 (n=6)	Day 7 (n=6)	Day 25 (n=6)	Day 40 (n=6)
Body weight (g)		5.7±0.2	7.6±0.3	16.2±1.2	23.2±0.9	77.7±1.2	80.8±2.6
Rectal temp (°C)		39.0±0.2	38.7±0.1	39.1±0.1	39.1±0.1	39.0±0.1	38.6±0.1
Lung function (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )		3.7±5.0	2.4±3.2	2.1±3.7	-4.8±4.2	2.5±1.7	-3.6±1.8
Lung function before histamine exposure (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )		1.0±2.1	-1.2±1.8	-2.0±1.5	0.8±0.7	-2.5±1.8	-0.7±1.7
Lung function after histamine exposure (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )		-9.4±3.2	-15.8±2.3	-40.5±5.4	-33.0±2.5	-22.4±2.8	-8.8±3.3
Total Cells (10 <sup>6</sup> cells ml <sup>-1</sup> )		3.0±0.2	3.5±0.2	8.1±0.3	6.1±0.2	4.0±0.2	3.0±0.2
Macrophages (10 <sup>6</sup> cells ml <sup>-1</sup> )		2.6±0.1	2.9±0.2	6.4±0.2	5.2±0.2	4.0±0.2	2.8±0.1
Eosinophils (10 <sup>6</sup> cells ml <sup>-1</sup> )		0.3±0.03	0.5±0.02	1.4±0.1	0.7±0.03	0.4±0.03	0.3±0.02
Neutrophils (10 <sup>6</sup> cells ml <sup>-1</sup> )		0.09±0.01	0.10±0.01	0.30±0.05	0.3±0.02	0.15±0.01	0.04±0.01
Respiratory rate (breaths min <sup>-1</sup> )		109.0±5.5	126.0±6.8	160.0±6.0	132.0±3.5	114.0±4.9	75.0±2.8
Wet lung weights (g kg <sup>-1</sup> )		9.3±0.1	11.1±0.3	11.8±0.3	9.2±0.2	6.4±0.1	5.6±0.1
Total nitrate and nitrite (µM)		61.5±2.5	71.3±2.8	93.3±3.3	80.0±2.2	61.7±3.2	48.3±2.4
Protein levels (µg ml <sup>-1</sup> )		1131.9±61.2	1314.7±48.9	1793.6±50.9	1483.4±55.2	1243.9±36.5	956.1±51.0
Isolation in BAL fluid		0/6	6/6	6/6	6/6	6/6	0/6
Isolation in right lung		4/6	6/6	6/6	6/6	6/6	2/6
Viral titre (TCID <sub>50</sub> ml <sup>-1</sup> g <sup>-1</sup> )		2.0x10 <sup>2</sup> ±9.5x10 <sup>1</sup>	3.5x10 <sup>4</sup> ±1.3x10 <sup>4</sup>	4.2 x10 <sup>7</sup> ±7.8x10 <sup>6</sup>	6.6x10 <sup>5</sup> ±2.5x10 <sup>5</sup>	6.3x10 <sup>2</sup> ±2.1x10 <sup>2</sup>	2.4 ±0.6
Mean pulmonary score	Peribronchiolitis	1.0±0.3	1.3±0.3	4.08±0.24	3.5±0.2	1.3±0.2	0.3±0.2
	Alveolitis	1.3±0.2	1.5±0.4	4.4±0.2	3.3±0.3	1.8±0.2	0.08±0.08
	Interstitial pneumonia	0.7±0.2	1.1±0.3	3.4±0.2	2.7±0.2	1.2±0.2	0.08±0.08
Mean composite pulmonary score		0.9±0.2	1.3±0.3	4.0±0.2	3.1±0.2	1.4±0.2	0.1±0.1

**Table 4.2**

Summary of results in virus-free medium (A) and PIV-3 (B) infected guinea pigs. For each parameter the value given is that recorded or determined on the final day in groups of animals that were examined for 1, 2, 4, 7, 25 and 40 days post-inoculation.

## CHAPTER 5

### EFFECT OF DEXAMETHASONE PRETREATMENT ON PIV-3 INFECTED GUINEA PIGS

#### INTRODUCTION

Despite decades of research, there are no licensed agents with proven clinical efficacy against PIV infections (Henrickson; 2003). In recent years, there has been a realisation that the therapy of infectious diseases needs to target both the infectious agent and host inflammation (Ottolini *et al*; 2002). Glucocorticoids have powerful anti-inflammatory activity and have traditionally represented the mainstay of anti-inflammatory therapy of the respiratory tract because they have broad-spectrum and far reaching effects that limit the activities of pro-inflammatory leucocytes and signal transduction pathways (Cash & Hoffma; 1996). Thus, glucocorticoids could possibly be used to treat viral infections of the airways. This approach to the treatment of viral infections has been controversial as some previous *in vitro* studies report up-regulation of viral yield (Domachowske *et al*; 2001), while others report the opposite effect on viral yield depending on the method used (Ottolini *et al*; 2003, Moreno *et al*; 2003)). However, it has been suggested that one consequence of these actions in the treatment of viral infections is that they may carry the hazard to suppress the necessary protective responses and decrease the essential process, which may lead to increased viral shedding and thus increased viral yield.

Studies using glucocorticoids to treat viral infections are surrounded by controversy, although there are several reports indicating beneficial effects in glucocorticoid treatment of some infections. The four main types of glucocorticoids which have been investigated to treat viral infections *in vivo* and *in vitro* have included triamcinolone acetonide, (Prince *et al*; 2000, Ottolini *et al*; 2002 & 2003, Eichelberger *et al*; 2004), methylprednisolone (Kimsey *et al*; 1989, Ottolini *et al*; 2002), dexamethasone (Sheth *et al*; 1994, Ottolini *et al*; 2002, Moreno *et al*; 2003, Toward *et al*; 2005) and hydrocortisone (Bonville *et al*; 2001, Domachowske *et al*; 2001). The types and doses of corticosteroids have been sufficiently varied to make interpretation of results extremely difficult.

Treatment of tracheobronchitis (predominantly caused by PIV-1) with glucocorticoids has been shown to decrease the severity of symptoms, reduce the need for hospitalisations or time spent in the emergency room, and reduce the need for further pharmacologic intervention (Ausejo *et al*; 1999). In addition, several animal studies have shown the beneficial effect of glucocorticoids on the respiratory symptoms of virus infections (Ottolini *et al*; 2002, Moreno *et al*; 2003, Toward *et al*; 2005). In direct contrast, clinical studies suggest that glucocorticoid administration has only marginal, if any, benefit in the treatment of viral infections (Springer *et al*; 1990, Roosevelt *et al*; 1996, De Boeck *et al*; 1997).

In view of this, the aim of the present study was to evaluate the effect of dexamethasone pre-treatment on PIV-3 infection in guinea pigs. The glucocorticoid, dexamethasone was selected based on its efficacy in other PIV infections. Studies by Moreno *et al* (2003) demonstrated a reduction in viral replication in cotton rats infected with PIV-1. In addition, studies by Toward and co-workers (2005) demonstrated that dexamethasone prevented AHR and cellular infiltration in PIV-3 infected guinea pigs.



The parameters investigated included airway function, airway reactivity to histamine, cellular infiltration, wet lung weight, inflammatory markers (NO and total protein levels) in BAL fluid, pulmonary histopathology and recovery of virus from the lung tissue. This investigation is novel as no other investigation has ascertained and correlated the level of virus recovery in the lung tissue, histological analysis or wet lung weights in PIV-3 infected guinea pigs with regard to dexamethasone treatment.

### **EXPERIMENTAL PROTOCOL**

The following protocols used to perform this study have all been previously described in chapter 2.

To determine the effect of the synthetic glucocorticoid, dexamethasone, in PIV-3 inoculated guinea pigs post inoculation day 4, the following experimental groups were as follows.

<b>GROUPS (n=6)</b>	
1	Virus-free medium infected animals pre-treated with vehicle assessed post inoculation day 4
2	Virus-free infected animals pre-treated with dexamethasone assessed post inoculation day 4,
3	PIV-3 virus infected animals pre-treated with vehicle assessed post inoculation day 4.
4	PIV-3 virus infected animals pre-treated with dexamethasone assessed post inoculation day 4,

From the results of the time course study in chapter 4 it was decided that the effect of dexamethasone in PIV-3 infected guinea pigs would be investigated up to post inoculation day 4, regarded as the peak response to infection due to the significant increase in cellular infiltration, AHR and viral detection in BAL fluid and lung tissue compared to the other study days.

On day -1 guinea pigs were assessed for airway reactivity to histamine and dexamethasone ( $20 \text{ mg kg}^{-1}$ ) or vehicle (50% DMSO/50% saline) was injected via the intraperitoneal cavity as a pre-treatment 24 hours before viral inoculation. On study day 0 guinea pigs were inoculated with virus-free medium or PIV-3 by intranasal instillation. Thereafter, guinea pigs received daily injections of vehicle or dexamethasone 30 minutes up to day 4 post inoculation. At the end of each study, 24 hours after the last dose of dexamethasone the animals were reassessed for airway reactivity to histamine and within 20 minutes were sacrificed and BAL performed and lung tissue removed. BAL fluid specimens were analysed for PIV-3 induced changes by comparison of total and differential cell counts, NO and total protein levels and viral titre determination. Lung tissue specimens were used for total viral content per gram of lung and pulmonary histopathology. PIV-3 was detected in BAL fluid and lung tissue samples by using standard culture techniques and indirect immunofluorescence as described in chapter 2. Throughout the study each animal was weighed and rectal temperature recorded on appropriate study days. Animals were also examined daily for general signs of illness including hair loss and decreased locomotor activity.

## RESULTS

### *Effect of PIV-3 on body weight gain with or without dexamethasone*

PIV-3 guinea pigs treated with dexamethasone failed to significantly ( $P>0.05$ ) alter body weight gain throughout the 4 day study period compared to PIV-3 guinea pigs treated with vehicle (Figure 5.1B). No significant differences ( $P>0.05$ ) were observed between virus-free medium infected guinea pigs treated with dexamethasone or vehicle (Figure 5.1A)

***Effect of PIV-3 on rectal temperature with or without dexamethasone***

PIV-3 guinea pigs treated with dexamethasone failed to significantly ( $P>0.05$ ) alter rectal temperature throughout the 4 day study period compared to PIV-3 guinea pigs treated with vehicle (Figure 5.2B). No significant differences ( $P>0.05$ ) were observed between virus-free medium infected guinea pigs treated with dexamethasone or vehicle (Figure 5.2A)

***Effect of PIV-3 on airway function with or without dexamethasone treatment.***

PIV-3 guinea pigs treated with dexamethasone failed to significantly ( $P>0.05$ ) alter lung function throughout the 4 day study period compared to PIV-3 guinea pigs treated with vehicle (Figure 5.3B). No significant differences ( $P>0.05$ ) were observed between virus-free medium infected guinea pigs treated with dexamethasone or vehicle (Figure 5.3A)

***Effect of PIV-3 on airway reactivity to inhaled histamine with or without dexamethasone.***

Inhalation of histamine (1mM) for 20 seconds 24 hours before intranasal inoculation of virus-free medium and PIV-3 treated with vehicle or dexamethasone did not produce any significant ( $P>0.05$ ) bronchoconstriction and failed to cause any significant deviation from baseline  $sG_{aw}$  values (Figure 5.4). This confirms that this was a sub-threshold dose of histamine.

Prior to inoculation, virus-free medium infected guinea pigs treated with dexamethasone were subjected to a higher dose of inhaled histamine (3mM, 20 seconds) resulting in a significant bronchoconstriction ( $-32.7\pm 3.2\% \text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ) which remained increased on subsequent challenge to histamine assessed post inoculation day 4 ( $-28.0\pm 2.0\% \text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ). These values were not significantly different ( $P<0.05$ ). T

hus, no statistically significant ( $P>0.05$ ) differences were observed between starting baseline values of  $sG_{aw}$  for any of the groups studied. In PIV-3 infected guinea pigs treated with dexamethasone post inoculation day 4, inhalation of histamine did not produce any significant bronchoconstriction (Before:  $1.4\pm 0.8\%$ , After:  $-2.7\pm 1.2\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ) and failed to cause any significant deviation from baseline  $sG_{aw}$  values compared with before inoculation. In contrast PIV-3 infected animals treated with vehicle post inoculation day 4 produced a significant bronchoconstriction with peak reductions in  $sG_{aw}$  (Before:  $-1.7\pm 2.1\%$ , After:  $-41.3\pm 2.5\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ). This indicates AHR to histamine. Thus, dexamethasone treatment inhibited PIV-3 induced AHR.

***Effect of PIV-3 on airways infiltration of leucocytes with or without dexamethasone.***

Treating PIV-3 infected guinea pigs with dexamethasone significantly decreased ( $P<0.05$ ) airways infiltration of total cells ( $2.6\pm 0.2 \text{ cells ml}^{-1}$ ) including macrophages ( $2.2\pm 0.2 \times 10^6 \text{ cells ml}^{-1}$ ), eosinophils ( $0.4\pm 0.1 \times 10^6 \text{ cells ml}^{-1}$ ) and neutrophils ( $0.03\pm 0.1 \times 10^6 \text{ cells ml}^{-1}$ ) compared to PIV-3 infected guinea pigs treated with vehicle total cell count ( $8.2\pm 0.3 \times 10^6 \text{ cells ml}^{-1}$ ) and infiltration of macrophages ( $6.5\pm 0.3 \times 10^6 \text{ cells ml}^{-1}$ ), eosinophils ( $1.4\pm 0.1 \times 10^6 \text{ cells ml}^{-1}$ ) and neutrophils ( $0.3\pm 0.1 \times 10^6 \text{ cells ml}^{-1}$ ) in the airways. Thus, dexamethasone treatment inhibited cellular infiltration of inflammatory cells into the lungs.

Virus-free medium infected guinea pigs treated with vehicle or dexamethasone failed to significantly ( $P>0.05$ ) alter total ( $2.1\pm 0.1, 2.3\pm 0.1 \times 10^6 \text{ cells ml}^{-1}$ ) and differential cell counts including macrophages ( $1.9\pm 0.1, 2.1\pm 0.1 \times 10^6 \text{ cells ml}^{-1}$ ), eosinophils ( $0.2\pm 0.1, 0.2\pm 0.1 \times 10^6 \text{ cells ml}^{-1}$ ) and neutrophils ( $0.02\pm 0.1, 0.02\pm 0.1 \times 10^6 \text{ cells ml}^{-1}$ ) in the airways (Figure 5.5).

***Effect of PIV-3 on respiratory rate with or without dexamethasone.***

A significant decrease ( $P < 0.05$ ) in respiratory rate was observed in PIV-3 infected guinea pigs treated with dexamethasone ( $81.0 \pm 4.0$  breaths  $\text{min}^{-1}$ ) compared to PIV-3 infected animals treated with vehicle ( $150.0 \pm 4.4$  breaths  $\text{min}^{-1}$ ) (Figure 7.6B). Treating virus-free medium infected guinea pigs with vehicle ( $75.0 \pm 5.1$  breaths  $\text{min}^{-1}$ ) or dexamethasone ( $72.0 \pm 3.5$  breaths  $\text{min}^{-1}$ ) failed to cause any significant difference ( $P > 0.05$ ) in respiratory rates (Figure 7.6A). Intranasal inoculation of virus-free medium animals treated with vehicle or rolipram failed to significantly ( $P > 0.05$ ) alter respiratory rate (Figure 7.6A). Treating PIV-3 inoculated guinea pigs with rolipram significantly decreased respiratory rate on days 2-4 compared to PIV-3 inoculated guinea pigs treated with vehicle (Figure 7.6B).

***Effects of PIV-3 on wet lung weights with or without dexamethasone.***

Treating PIV-3 infected guinea pigs with dexamethasone significantly decreased ( $P < 0.05$ ) wet lung weights ( $8.2 \pm 0.32$  g  $\text{kg}^{-1}$ ) compared to PIV-3 infected animals treated with vehicle ( $12.1 \pm 0.63$  g  $\text{kg}^{-1}$ ) (Figure 5.7). No significant differences ( $P > 0.05$ ) were observed between virus-free medium guinea pigs treated with dexamethasone ( $8.5 \pm 0.34$  g  $\text{kg}^{-1}$ ) compared to virus-free medium inoculated animals treated with vehicle ( $7.7 \pm 0.27$  g  $\text{kg}^{-1}$ ) (Figure 5.7).

***Effect of PIV-3 on airways NO with or without dexamethasone..***

In PIV-3 infected guinea pigs treated with dexamethasone, a significant decrease ( $P < 0.05$ ) in combined NO levels in BAL fluid was observed ( $49.1 \pm 3.0$   $\mu\text{M}$ ) compared to PIV-3 infected animals treated with vehicle ( $80.3 \pm 4.9$   $\mu\text{M}$ ) (Figure 5.8). Treating virus-free medium infected guinea pigs with vehicle ( $51.4 \pm 2.6$   $\mu\text{M}$ ) or dexamethasone ( $47.3 \pm 4.3$   $\mu\text{M}$ ) failed to cause any significant difference ( $P > 0.05$ ) in nitric oxide levels (Figure 5.8).

***Effect of PIV-3 on total protein levels with or without rolipram.***

A significant decrease ( $P < 0.05$ ) in total protein levels in BAL fluid was observed in PIV-3 infected guinea pigs treated with rolipram ( $905.6 \pm 72.2 \mu\text{g ml}^{-1}$ ) compared to PIV-3 infected animals treated with vehicle ( $1642.1 \pm 72.5 \mu\text{g ml}^{-1}$ ). Treating virus-free medium infected guinea pigs with vehicle ( $863.5 \pm 50.0 \mu\text{g ml}^{-1}$ ) or dexamethasone ( $774.4 \pm 48.0 \mu\text{g ml}^{-1}$ ) failed to cause any significant difference ( $P > 0.05$ ) in total protein levels (Figure 5.9).

***Isolation of PIV-3 virus in BAL fluid and right lung tissue with or without dexamethasone.***

Replicating virus was isolated in all PIV-3 infected animals treated with vehicle or dexamethasone post inoculation day 4 (Table 5.1) as measured by CPE of BAL fluid and homogenised left lung tissue on Vero cells and a positive reaction by indirect immunofluorescence using an antibody against PIV-3. Titres of intrapulmonary replicating PIV-3 were determined in left lung tissue samples treated with vehicle or dexamethasone 4 days post inoculation and were normalised to left lung tissue weight ( $\text{TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ) (Figure 5.10). Treating PIV-3 infected guinea pigs with dexamethasone significantly reduced ( $P > 0.05$ ) viral titres ( $2.1 \times 10^4 \pm 1.5 \times 10^4 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ) compared to PIV-3 infected guinea pigs treated with vehicle ( $7.6 \times 10^7 \pm 2.6 \times 10^7 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ). Thus, dexamethasone treatment inhibited PIV-3 replication in left lung tissue samples.

***Effect of PIV-3 on mean pathology scores in pulmonary histopathology with or without dexamethasone.***

PIV-3 infected guinea pigs treated with dexamethasone revealed a dramatic reduction in inflammatory cells compared with the levels observed in PIV-3 infected guinea pigs treated with vehicle, which demonstrated evidence of peribronchiolitis, interstitial pneumonia and alveolitis (Figure 5.11). This indicates near complete reversal of the pathologic changes.

Treating PIV-3 infected guinea pigs with dexamethasone resulted in significantly reduced ( $P < 0.05$ ) mean composite pulmonary scores ( $1.5 \pm 0.2$ ) compared with PIV-3 infected guinea pigs treated with vehicle ( $4.2 \pm 0.2$ ). No significant differences ( $P > 0.05$ ) were observed in virus-free medium infected guinea pigs treated with dexamethasone ( $0.4 \pm 0.1$ ) compared to vehicle ( $0.4 \pm 0.1$ ) (Figure 5.12).

The data for all parameters recorded on day 4 post inoculation is summarised in Table 5.2.

## DISCUSSION

This study investigated the effect of the glucocorticoid, dexamethasone in PIV-3 infected guinea pigs. The parameters investigated were body weight gain, rectal temperature, airway function, airway reactivity to histamine, respiratory rate, infiltration of inflammatory cells, wet lung weights, viral titre, nitric oxide and total protein levels and histological analysis. The effects of PIV-3 inoculation in guinea pigs have been extensively discussed in chapter 4 of this thesis and will not be duplicated in this current chapter. During the investigation, no significant differences were observed in rectal temperature, body weight, or lung function following PIV-3 inoculation whether dexamethasone was present or not, which has been consistent throughout this thesis.

A significant reduction in respiratory rate to basal levels was demonstrated in this study, although no effect on  $sG_{aw}$  was evident. Therefore, the inflammation and pulmonary histopathology had some detrimental effect on respiratory function although this was not evident from conductance.

In the current study, dexamethasone administration in PIV-3 infected guinea pigs resulted in the complete ablation of AHR to histamine. The prevention of AHR could not be due to functionally antagonistic bronchodilation (i.e increase in  $sG_{aw}$ ) by dexamethasone since treatment of virus-free medium infected guinea pigs with dexamethasone failed to attenuate the responses to a higher bronchoconstricting dose of inhaled histamine (3mM). Therefore, the inhibition of PIV-3-induced AHR was independent of a residual bronchodilation or an anti-histamine mechanism. The prevention of airway hyperreactivity as suggested in this study and by others (Van Oosterhout *et al*; 1995, Folkerts *et al*; 1998, Ikemura *et al*; 2001) may be due to a causative association between pro-inflammatory mediators, eosinophilic PIV-3 induced inflammation and AHR. Thus, protection from PIV-3 induced AHR using dexamethasone treatment arising from its anti-inflammatory activity.

The anti-inflammatory mechanism of dexamethasone is well understood. Glucocorticoids are powerful anti-inflammatory agents and mediate their effects by binding to the glucocorticoid receptors (GR) localised in the cytoplasm. GR then dimerize and translocate to the nucleus, where they bind to glucocorticoid response elements (GRE) on glucocorticoid-responsive genes to directly or indirectly regulate the transcription of target genes. Several of the genes that are switched on by glucocorticoids have anti-inflammatory effects, including annexin-1 (lipocortin-1), secretory leukoprotease inhibitor (SLPI), IL-10 and the inhibitor of nuclear factor kappa-beta (NF-kB). Glucocorticoids also switch on the synthesis of two proteins that affect inflammatory signal transduction pathways, glucocorticoid-induced leucine zipper protein (GILZ), which inhibits both NF-kB and AP-1 (Mittelstadt & Ashwell, 2001) and MAP kinase phosphatase-1 (MKP-1), which inhibits p38 MAP kinase (Lasa *et al*; 2002).



However, it seems unlikely that the widespread anti-inflammatory actions of glucocorticoids could be entirely explained by increased transcription of small numbers of anti-inflammatory genes, particularly as high concentrations of glucocorticoids are usually required for this effect, whereas in clinical practice glucocorticoids are able to suppress inflammation at low concentrations (Barnes; 2006).

In controlling inflammation, the major effect of corticosteroids is to inhibit the synthesis of multiple inflammatory genes including cytokines, enzymes, receptors and adhesion molecules through suppression of the genes that encode them. This is an important determinant of glucocorticoid responsiveness and is a key mechanism whereby glucocorticoids exert their anti-inflammatory effect (Barnes; 1998). Glucocorticoids have direct inhibitory actions on many inflammatory and structural cells involved in inflammation including macrophages, eosinophils and neutrophils. Therefore, the inhibition of raised inflammatory cell influx into the lung observed here is caused by the above mechanisms. The inhibition of leucocyte influx into the BAL fluid by dexamethasone in this study has shown that the accumulation of inflammatory cells in lung tissue is prevented by dexamethasone treatment in concordance with studies by Toward *et al* (2005). However, in contrast, other studies involving pre-treatment with the glucocorticoid betamethasone in PIV-3 infected guinea pigs have shown that cellular infiltration into the lung was prevented although it failed to prevent AHR to histamine (Leusink-Muis *et al*; 1999). This suggests dissociation between AHR and the inflammatory response.

In this study, dexamethasone was shown to significantly reverse the increases in wet lung weights and protein levels in the lung, induced by PIV-3. These were used as an index for assessing oedema. The development, peak and recovery of leucocyte levels in the BAL fluid correlated temporally with wet lung weights and protein levels and were all susceptible to dexamethasone treatment.

It can be proposed that this was probably due to a consequence of a reduced leucocyte influx into the airways, inflammatory cell activity and a subsequent release of pro-inflammatory mediators capable of reducing oedema.

Dexamethasone, a known potent inhibitor of iNOS expression (Robbins *et al*; 1994) blocked the release of NO induced by PIV-3 post inoculation day 4. This effect has also been observed *in vivo* with PIV-3 (Toward *et al*; 2005) and *in vitro* using RSV (Kao *et al*; 2001). Studies have suggested that the induction of the inducible form of NO synthase (iNOS) is potentially inhibited by dexamethasone through decreasing the transcription of the inflammatory gene iNOS (Di Rosa *et al*; 1990). In cultured human pulmonary epithelial cells, pro-inflammatory cytokines result in increased expression of iNOS and increased NO formation due to increased transcription of the iNOS gene, which is inhibited by glucocorticoids (Robbins *et al*; 1994). Studies have ascertained that NF- $\kappa$ B is the most important transcription factor in regulating iNOS gene transcription (Xie *et al*; 1994). Since TNF- $\alpha$ , IL-1 $\beta$  and oxidants activate NF- $\kappa$ B in airway epithelial cells, and this accounts for their activation of iNOS expression. Dexamethasone may therefore prevent induction of iNOS by inhibiting NF- $\kappa$ B, thereby inhibiting transcription. However, this suppression of NO using glucocorticoids has not produced significant changes in the outcome of RSV-induced bronchiolitis in humans (Roosevelt *et al*; 1996).

A reduction of pulmonary lesions associated with inflammatory cell migration was also observed in PIV-3 infected guinea pigs treated with dexamethasone. In concordance, cotton rats infected with influenza virus demonstrated that high doses of glucocorticoid therapy can greatly reduce pulmonary lesions (Ottolini *et al*; 2003). Recent studies in murine models of viral pneumonia have shown significantly increased iNOS activity and mRNA expression within the lung tissue by viral replication. Inhibition of NO production improved the histologic changes and the survival of mice without affecting the propagation of viruses within the respiratory tract (Alder *et al*; 1997, Tanaka *et al*; 1997).

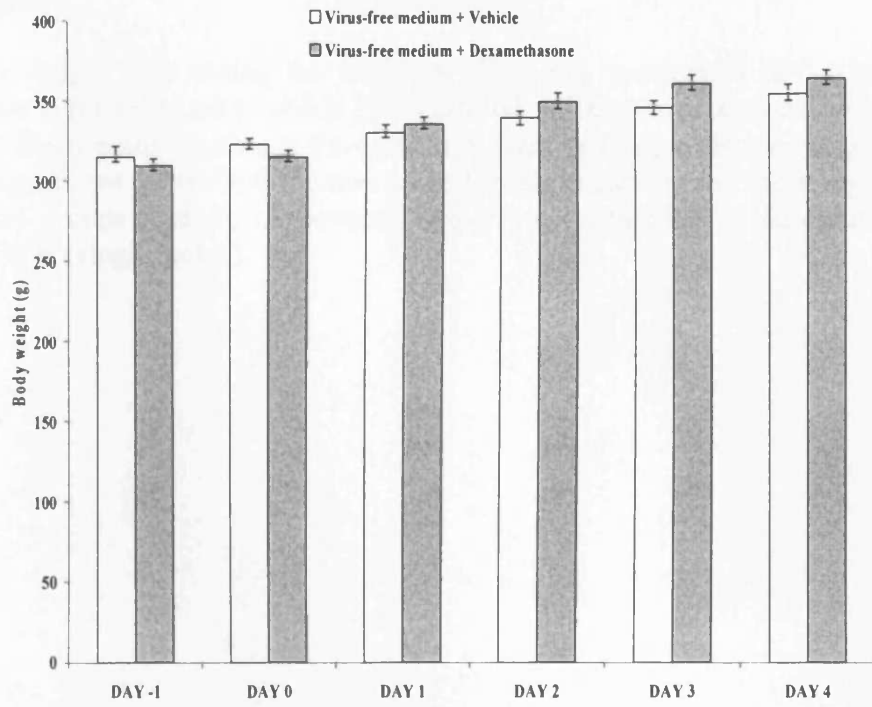
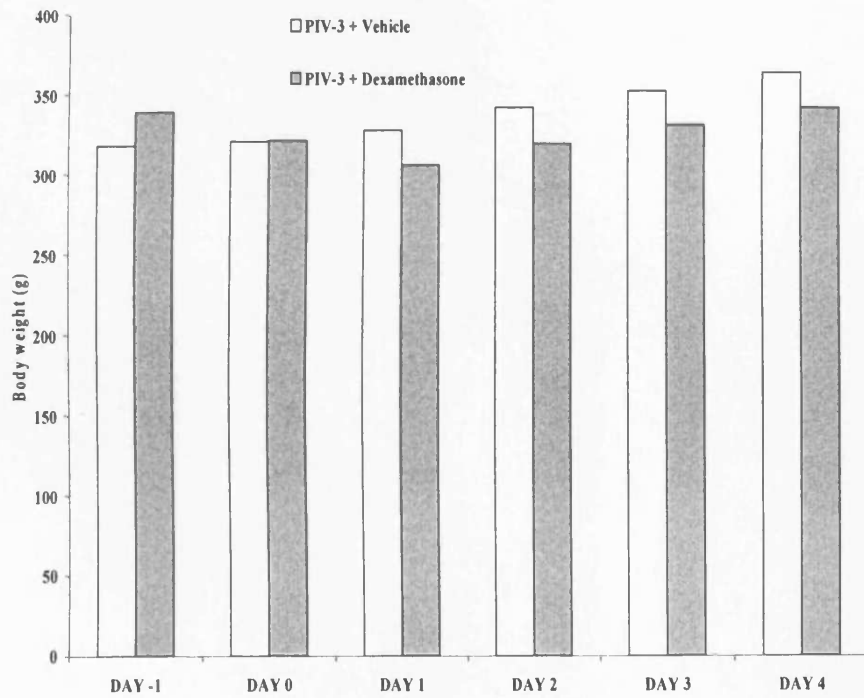
These studies have confirmed the importance of NO and other oxygen reactive species in the pathogenesis of viral diseases involving the respiratory tract. Therefore, pulmonary injury can be attenuated by administration of NO inhibitors regardless of viral propagation in the lung (Kao *et al*; 2001).

In this study, dexamethasone also reduced the viral titre recovered from the lung. Glucocorticoids usually exert immunosuppressive effects, leading to increased viral titres by suppressing the cell-mediated immunity. They act by inhibiting genes that code IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8 and IFN- $\gamma$ , the most important of which is the IL-2. While smaller cytokine production reduces the T cell proliferation, glucocorticoids also suppress the humoral immunity, causing B cells to express smaller amounts of IL-2 and IL-2 receptors. This diminishes both B cell clone expansion and antibody synthesis. The diminished amounts of IL-2 also causes T lymphocyte cells to be activated. Thus, the effect observed would appear to counteract the immunosuppressive actions of glucocorticoids. There have been few other reports of anti-viral effect using glucocorticoids. Kimsey and colleagues (1989) reported that pre-treating hamsters with steroids prevents subsequent infection with PIV. In addition, research by Morena *et al* (2003) also demonstrated anti-viral activity in PIV-1 infected guinea pigs treated with dexamethasone. However, although dexamethasone failed to completely clear the PIV-3 from the lung tissue, a longer incubation period and further injections of the glucocorticoid may be required for complete reversal.

The mechanism of antiviral effect is unknown; although it has been speculated by other authors that this may be due to decreased viral receptors on the epithelial cells (Kimsey *et al*; 1989). Decreased viral receptors account for the antiviral effects of steroids on infectability of cultured epithelial cells with rhinovirus (Suzuki *et al*; 2000). In order to replicate, PIV's attach to the host cell by the haemagglutinin, which binds to the host cell neuraminic acid receptor, which then penetrates the cell by fusion with the cell membrane (Plemer *et al*; 2003).

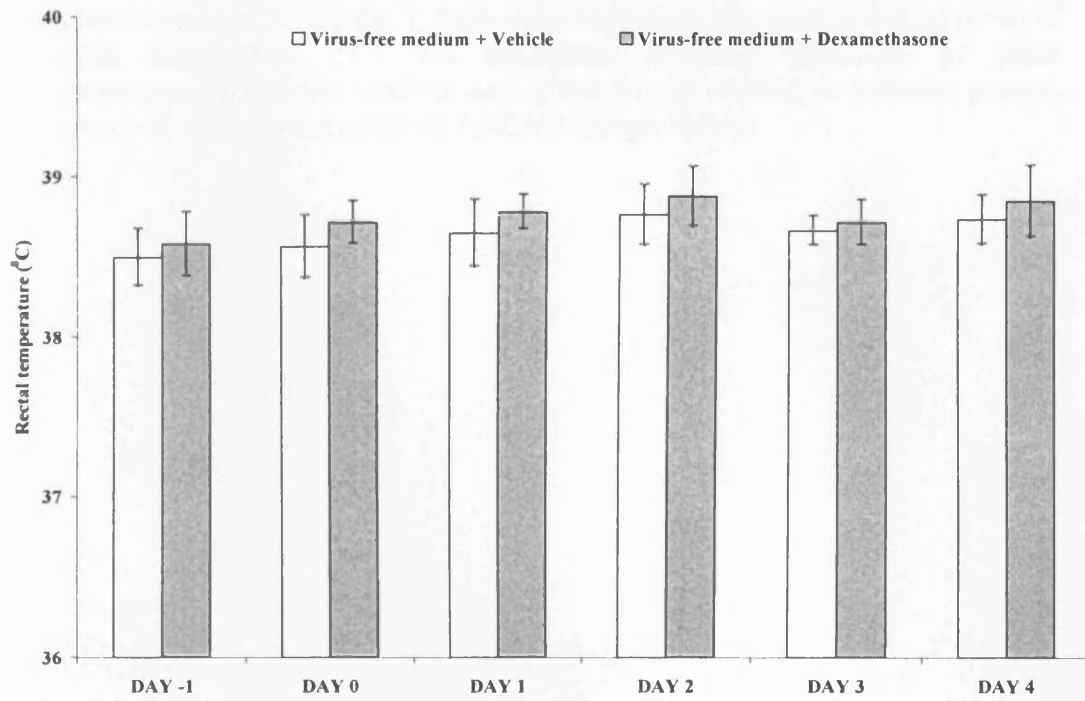
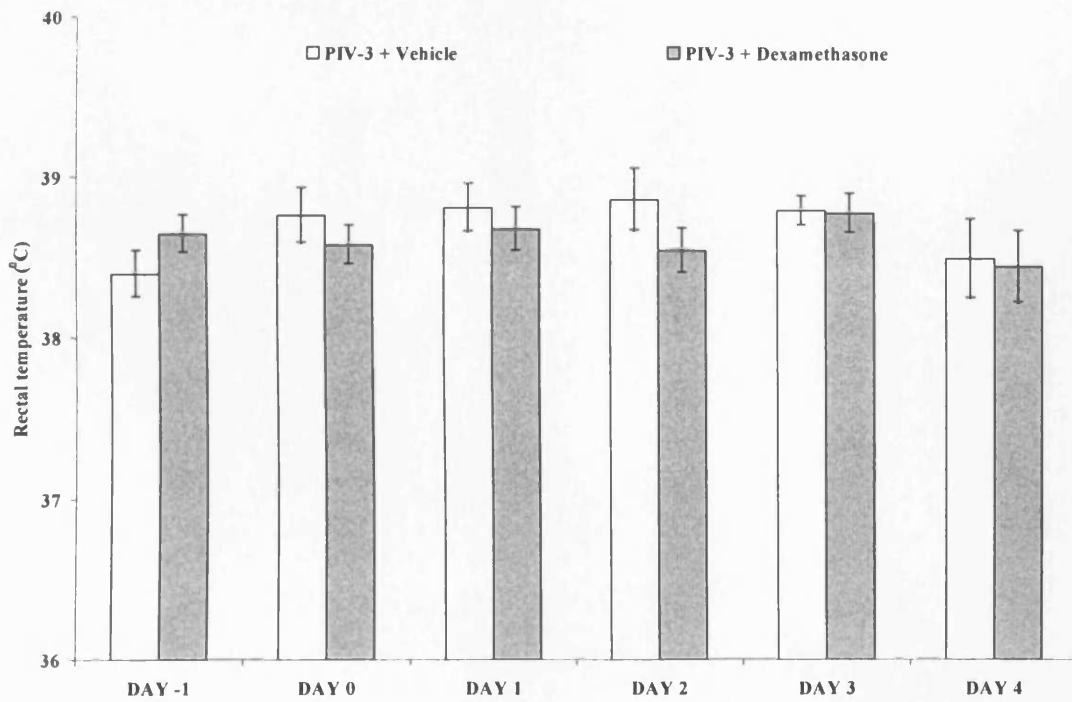
Expression of ICAM-1, the receptor for most RV infections was suppressed by steroid pre-treatment. In contrast, studies in mice infected with pneumonia virus treated with hydrocortisone produced elevated rates of viral replication in lung tissue (Domachowske *et al*; 2001). The results of this study suggest that hydrocortisone suppresses elements that are crucial to host defence against pneumonia virus infection i.e. the immunosuppressive action. In this study, dexamethasone treatment was given prior to infection (pre-treatment) and clinically it is difficult to give a patient treatment before they have contracted the virus. Therefore, it would be valuable in further studies to administer dexamethasone after infection to determine if glucocorticoid treatment had differential effects on the viral yield depending on the timing of the glucocorticoid treatment.

In conclusion, this study demonstrates that treating PIV-3 infected guinea pigs with dexamethasone inhibited the development of airways hyperreactivity, influx of inflammatory cells, respiratory rate, pulmonary oedema, NO and a reduction in lung lesions. Interestingly, dexamethasone also reduced viral replication in the lung. The findings of this study support a role for dexamethasone in the management of viral infections of the respiratory tract. This model gives justification for further study of anti-inflammatory therapy of PIV infections.

**A****B**

### **Figure 5.1**

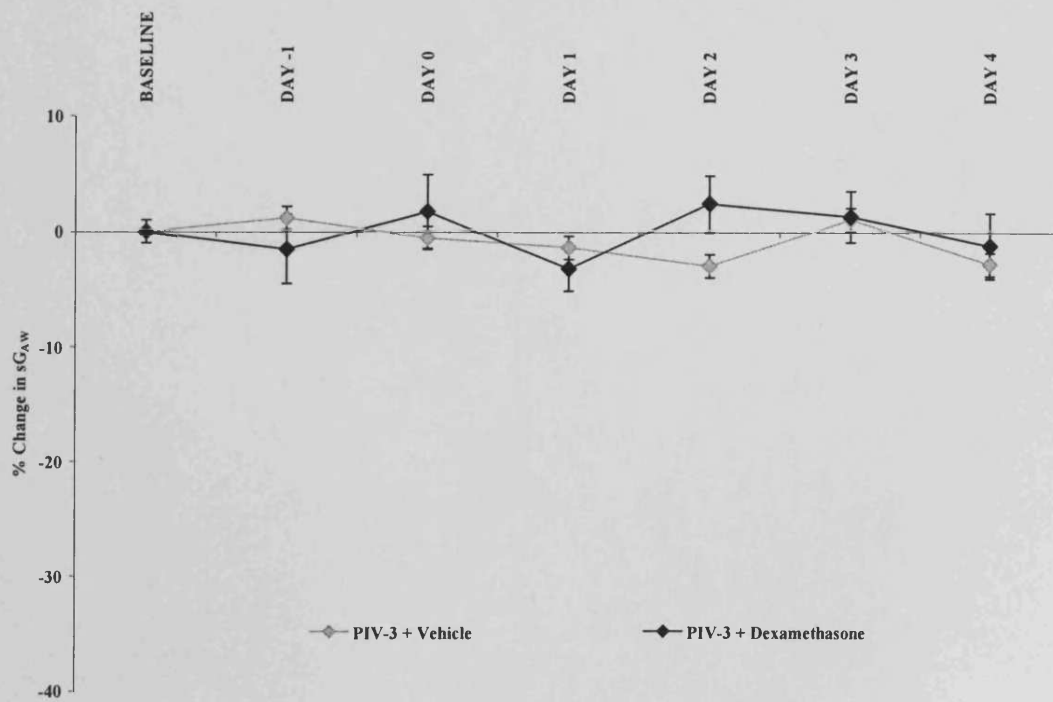
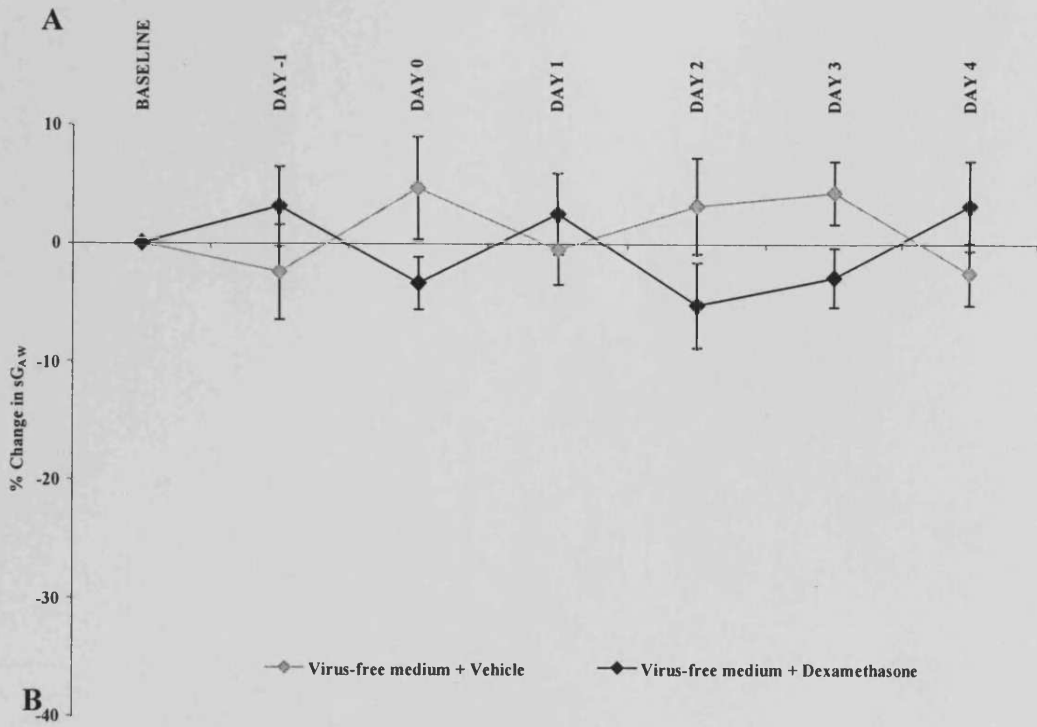
Body weight gain during the study. **A** Virus-free medium **B** PIV-3 infected guinea pigs treated with vehicle (50% DMSO:saline) or dexamethasone (20 mg kg<sup>-1</sup>). Each point represents the mean  $\pm$  S.E.M (n=6) of body weight gain (g). No significant (P>0.05) difference in body weight gain during the study in any of the groups studied, or between groups was observed as determined by ANOVA (single factor).

**A****B**

**Figure 5.2**

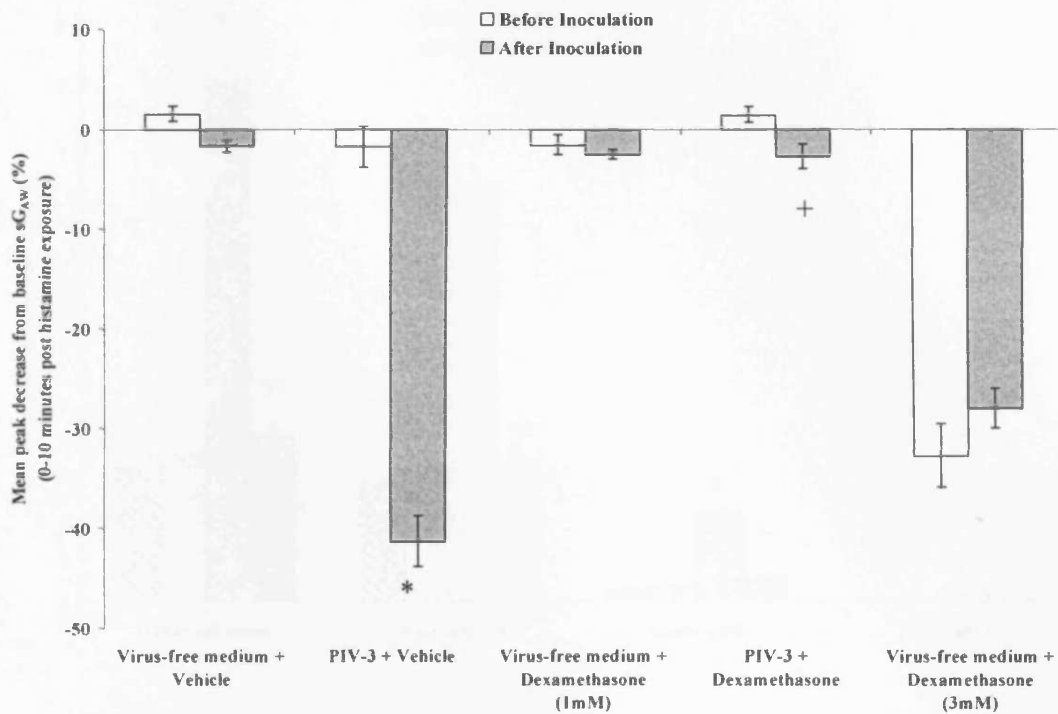
Rectal temperature during the study. **A** Virus-free medium **B** PIV-3 ( $5 \times 10^6$  ml<sup>-1</sup>) infected guinea pigs treated with vehicle (50% DMSO:saline) or dexamethasone ( $20 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M (n=6) of rectal temperature ( $^{\circ}\text{C}$ ). No significant ( $P > 0.05$ ) difference in rectal temperature during the study in any of the groups studied, or between groups, was observed as determined by ANOVA (single factor).





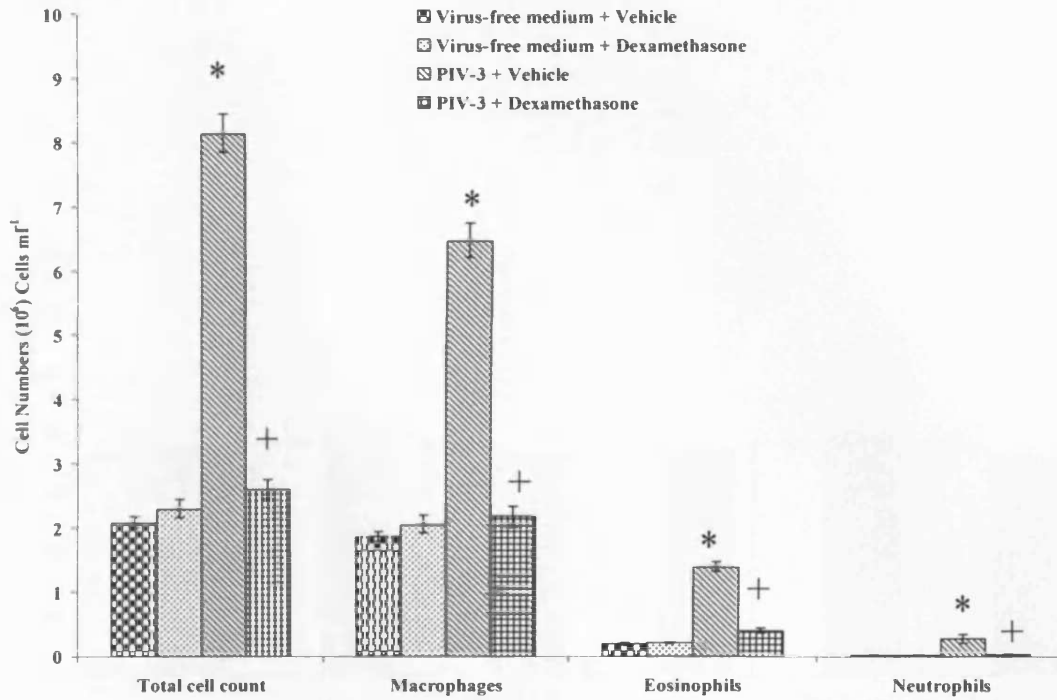
**Figure 5.3**

The effect on lung function with **A** Virus-free medium (Vero cell supernatant) **B** PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs treated with vehicle (50% DMSO: saline) dexamethasone ( $20 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M (n=6) change in  $sG_{aw}$  expressed as a percentage of the baseline (BL)  $sG_{aw}$  values (BL  $sG_{aw}$  ( $\text{s}^{-1}\text{cm}$  of  $\text{H}_2\text{O}^{-1}$ ). Negative values represent bronchoconstriction. No significant ( $P>0.05$ ) difference in airway function after inoculation, from baseline  $sG_{aw}$  values in any of the groups studied, or between groups was observed as determined by ANOVA (single factor).



**Figure 5.4**

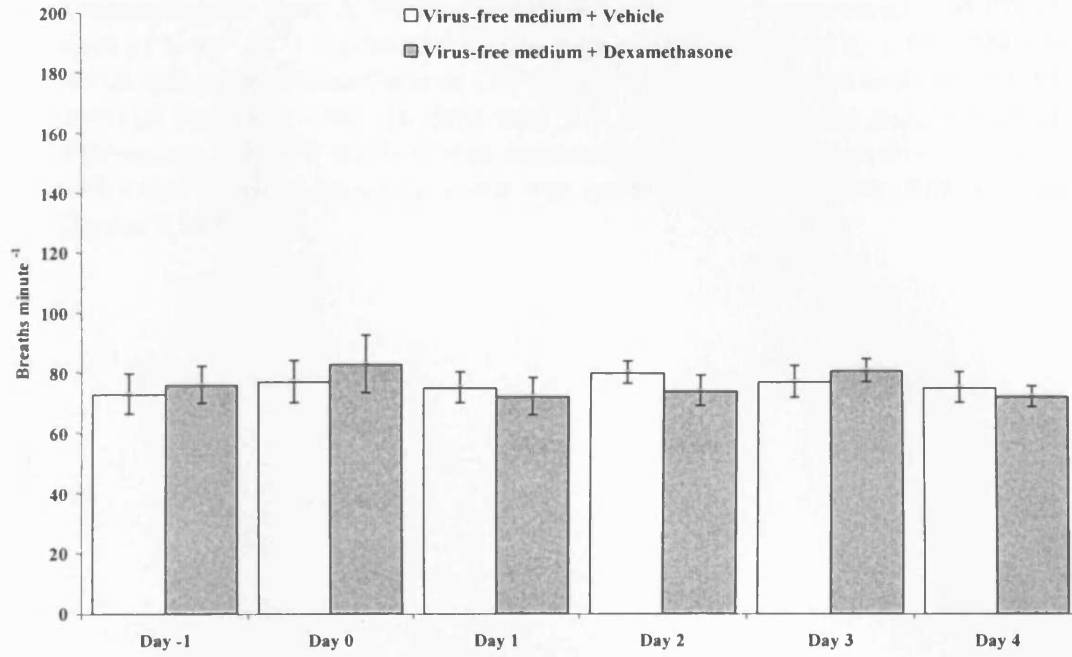
Peak percentage changes in the airway responses of conscious guinea pigs to a nose-only challenge with histamine (1mM, 20 seconds), 24 hours before the commencement of virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6$  ml<sup>-1</sup>) inoculation and post inoculation day 4. Also shown is the peak response to a bronchoconstrictor dose (3mM, 20 seconds) of histamine 24 hours and 4 days post inoculation with virus-free medium after treatment with dexamethasone (1mg kg<sup>-1</sup>). Animals were treated with vehicle (50% DMSO: 50% saline) or dexamethasone (20 mg kg<sup>-1</sup>). Each point represents the mean  $\pm$  S.E.M (n=6) of the peak change from baseline  $sG_{aw}$  (S<sup>-1</sup>cm of H<sub>2</sub>O<sup>-1</sup>), 0-10 minutes post histamine exposure. Negative values represent bronchoconstriction. \* P<0.05 denotes the significance of differences between 24 hours before inoculation and 4 days post inoculation. + P<0.05 denotes the significance of differences between post inoculation day 4 in PIV-3 treated with dexamethasone compared to vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



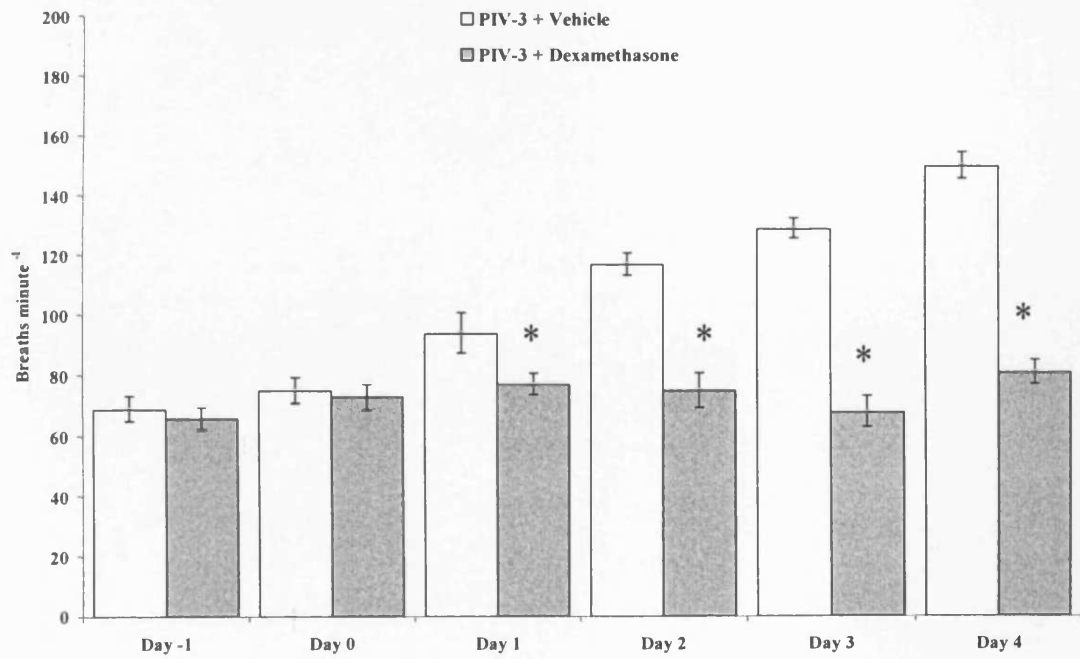
**Figure 5.5**

Total and differential cell (macrophage, eosinophil and neutrophil) counts of BAL fluid removed from virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) inoculated guinea pigs treated with vehicle (50% DMSO: sterile saline) or dexamethasone ( $20 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) cells per BAL fluid sample ( $\times 10^6$ ). \*  $P<0.05$  denotes the significance of differences in airway cells removed from PIV-3 compared to virus-free medium infected guinea pigs treated with vehicle. +  $P<0.05$  denotes the significance of differences in airway cells removed from PIV-3 virus treated with dexamethasone compared to vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

**A**

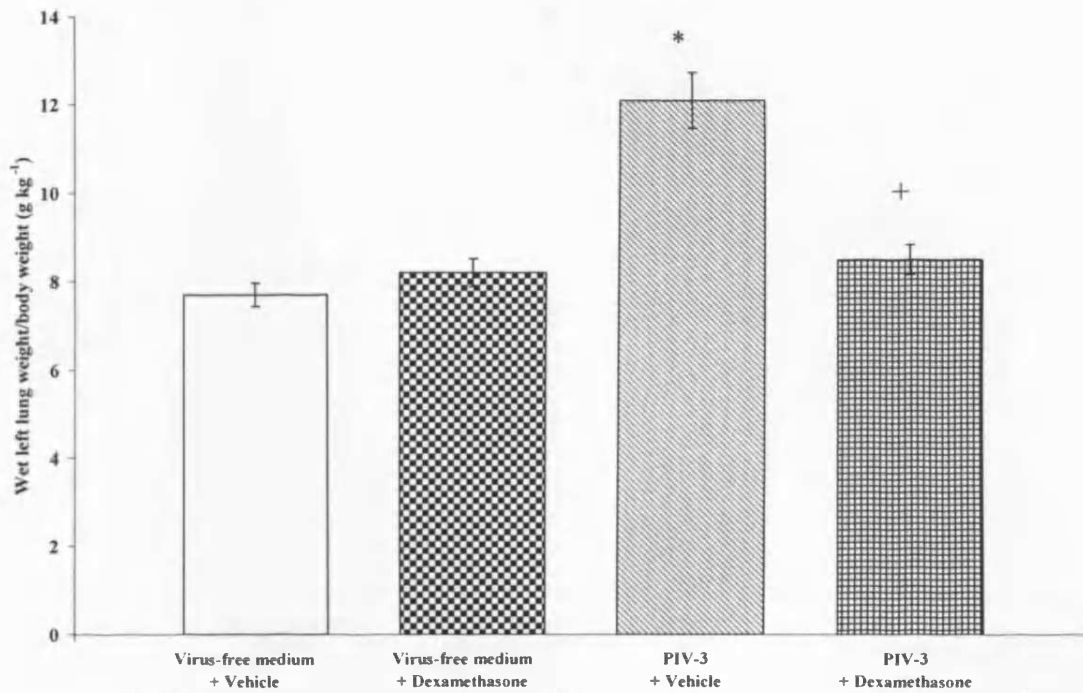


**B**



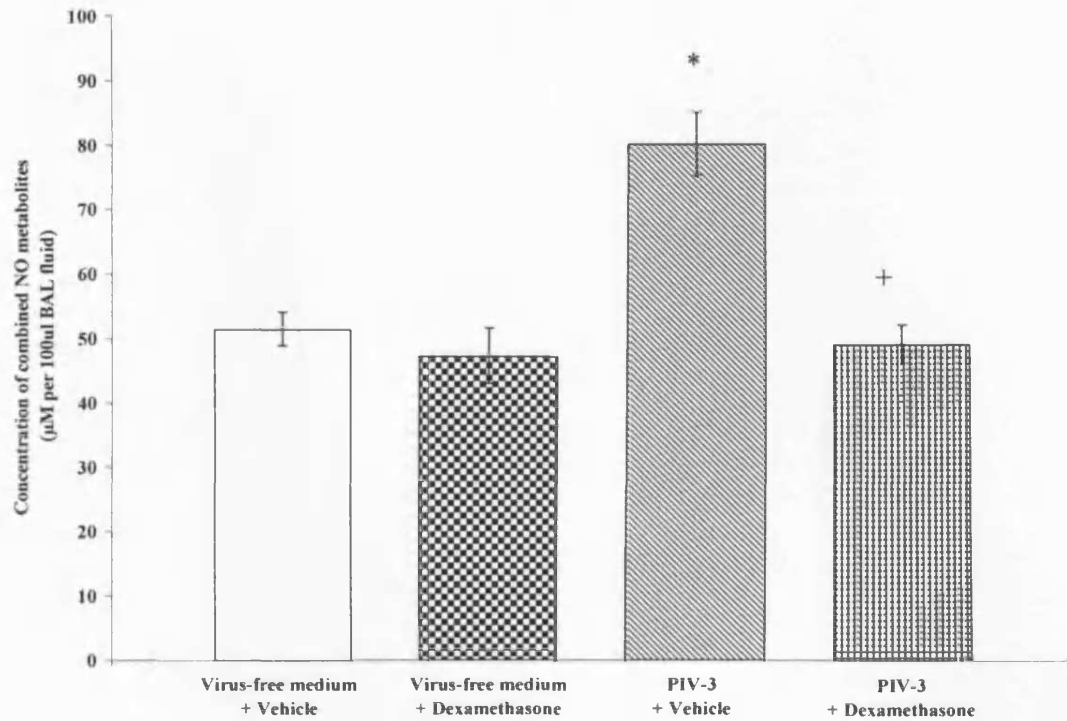
### **Figure 5.6**

Respiratory rate from **A** Virus-free medium (Vero cell supernatant) or **B** PIV-3 virus ( $5 \times 10^6 \text{ ml}^{-1}$ ) inoculated guinea pigs treated with vehicle (50% DMSO: sterile saline) or dexamethasone ( $20 \text{ mg kg}^{-1}$ ). Each point represents the S.E.M (n=6) of respiratory rate ( $\text{Breaths min}^{-1}$ ). \*  $P < 0.05$  denotes the significance of differences in PIV-3 treated with dexamethasone compared to PIV-3 treated with vehicle. All statistical analysis was determined by ANOVA, followed by Dunnet's test.



**Figure 5.7**

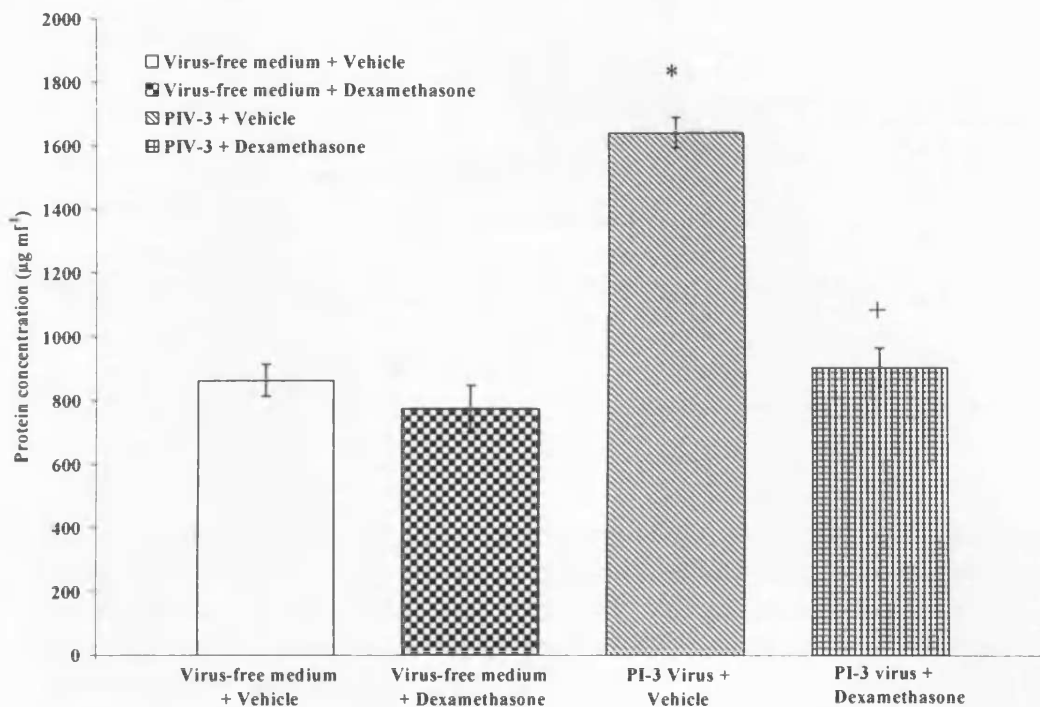
Wet left lung weights from virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6$  ml<sup>-1</sup>) treated with vehicle (50% DMSO: sterile saline) or dexamethasone (20 mg kg<sup>-1</sup>). Each point represents the mean  $\pm$  S.E.M (n=6) of wet left lung weights normalised to body weight (g kg<sup>-1</sup>). \* P<0.05 denotes the significance of differences in wet left lung weights from PIV-3 compared to virus-free medium infected guinea pigs with vehicle. + P<0.05 denotes the significance of differences in wet left lung weights from PIV-3 treated with dexamethasone compared to vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



**Figure 5.8**

Total nitric oxide levels in BAL fluid removed from virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) treated with vehicle (50% DMSO: saline) or dexamethasone ( $20 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of the total nitric oxide concentration per sample ( $\mu\text{M per } 100\mu\text{l}$ ). \*  $P < 0.05$  denotes the significant of differences in PIV-3 compared to virus-free medium treated with vehicle. +  $P < 0.05$  denotes the significance of differences from PIV-3 treated with dexamethasone compared to vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.





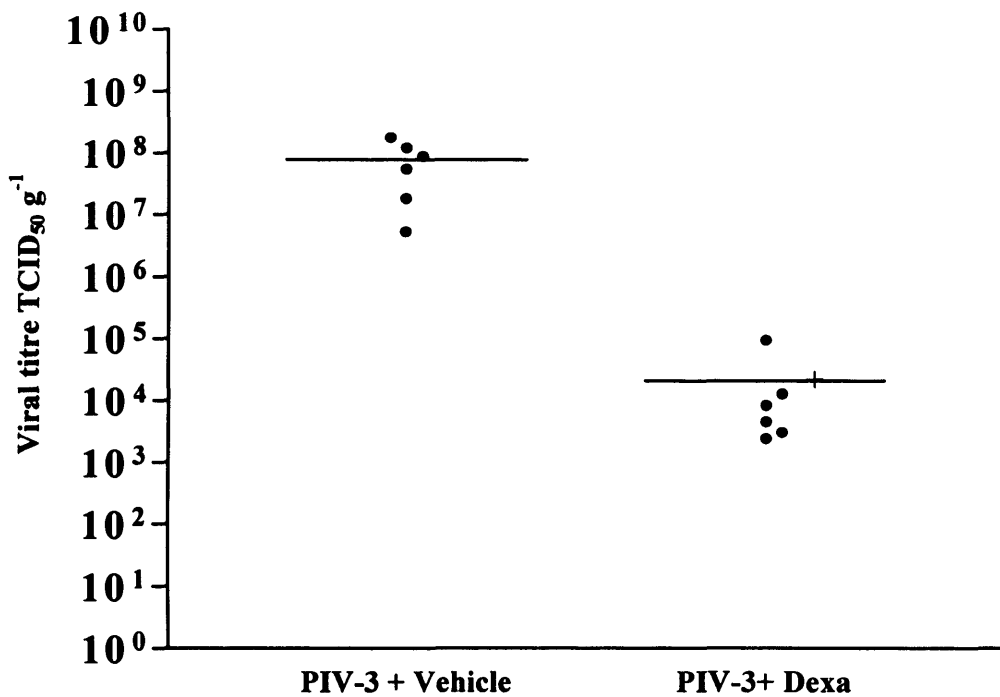
**Figure 5.9**

Total protein levels in BAL fluid removed from virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) treated with vehicle (50% DMSO: saline) or dexamethasone ( $20 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  standard S.E.M ( $n=6$ ) of the total protein levels per sample ( $\mu\text{g ml}^{-1}$ ). \*  $P < 0.05$  denotes the significance of differences in PIV-3 compared to virus-free medium treated with vehicle. +  $P < 0.05$  denotes the significance of differences in PIV-3 treated with vehicle compared to virus-free medium. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

		<b>Vehicle (n=6)</b>	<b>Dexamethasone (n=6)</b>
<b>BAL Fluid</b>	<b>Virus-free medium</b>	0/6	0/6
	<b>PIV-3</b>	6/6	6/6
<b>Right Lung Tissue</b>	<b>Virus-free medium</b>	0/6	0/6
	<b>PIV-3</b>	6/6	6/6

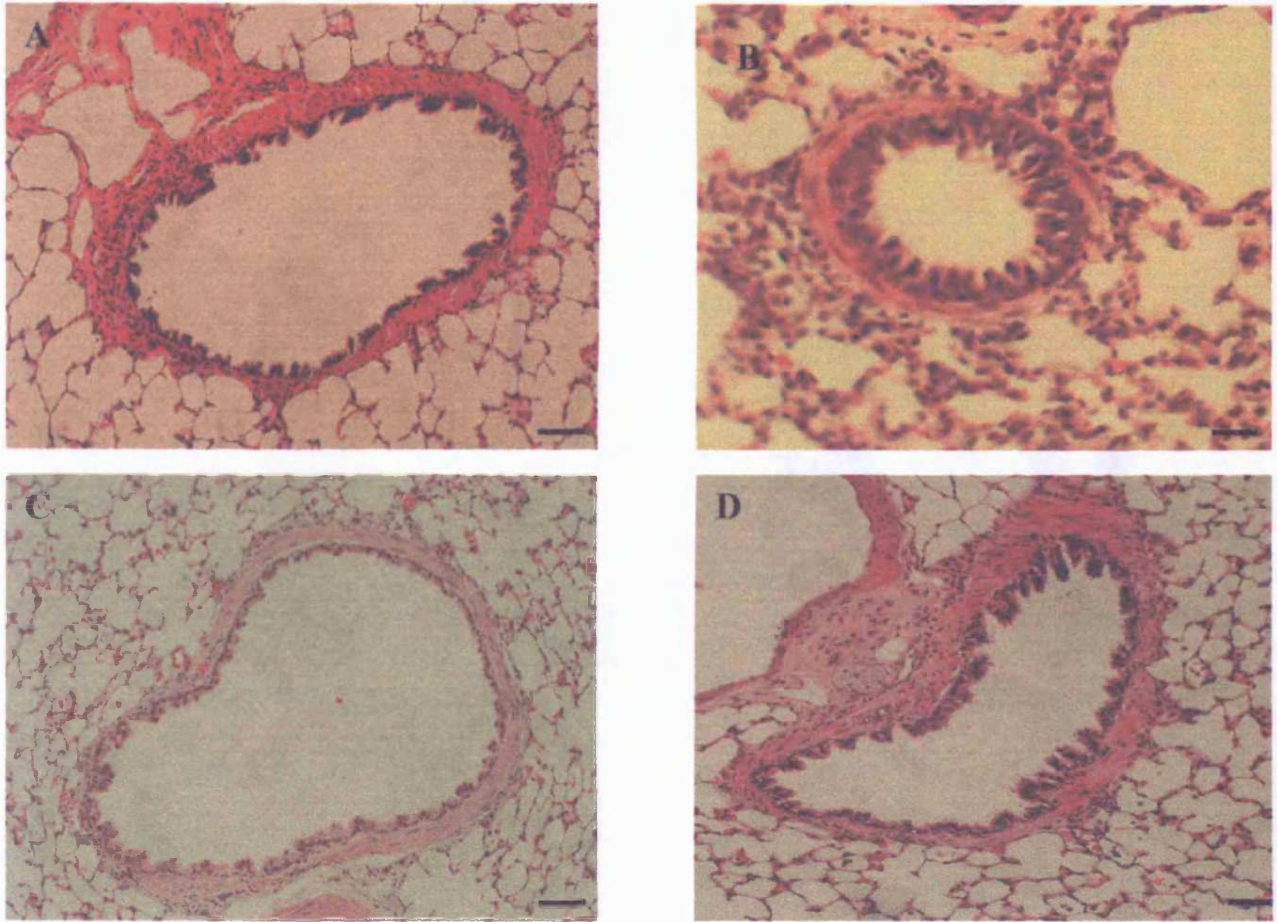
**Table 5.1**

The number of guinea pigs out of each group of six, which displayed evidence of PIV-3 growth on Vero cells using standard culture techniques and positive immunofluorescence in BAL fluid, and right lung tissue samples from virus-free medium and PIV-3 guinea pigs treated with vehicle (50% DMSO:saline) or dexamethasone (20 mg kg<sup>-1</sup>).



**Figure 5.10**

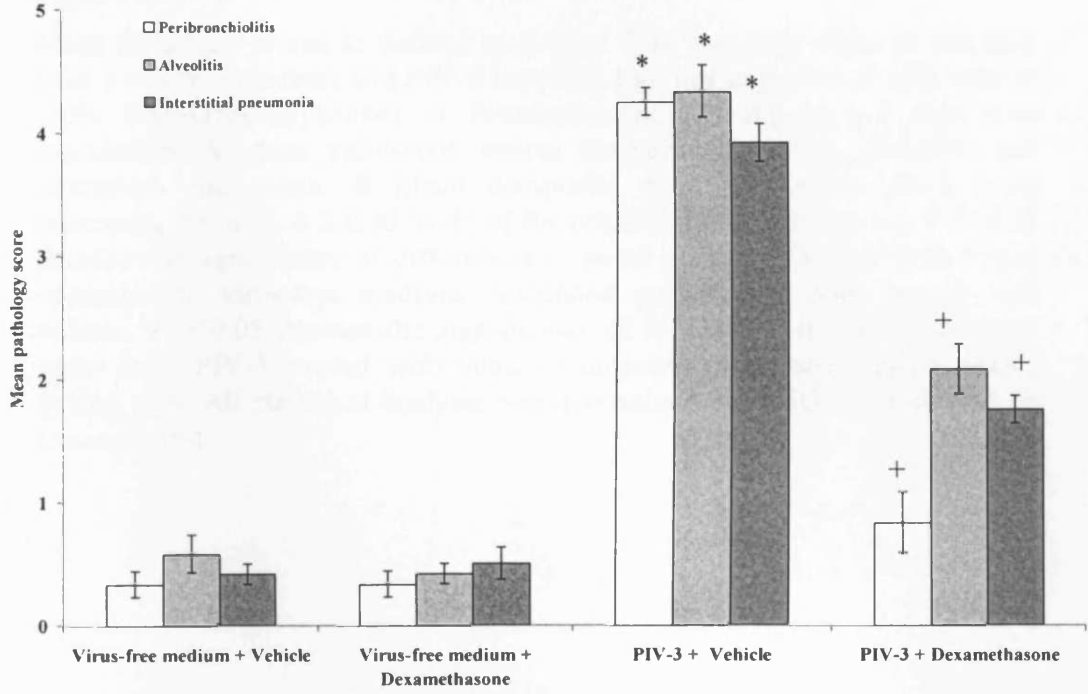
Titres of intrapulmonary replicating PIV-3 isolated from guinea pig right lung treated with vehicle (50% DMSO:saline) or dexamethasone (20 mg kg<sup>-1</sup>) post inoculation day 4. Each point is shown with the mean value shown as a horizontal line. Titres of intrapulmonary replicating PIV-3 normalised to wet left lung weight (TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>). + p<0.05 denotes the significance of differences in viral titre from PIV-3 treated with vehicle compared to dexamethasone treated guinea pigs as determined by ANOVA, followed by Dunnet's test.



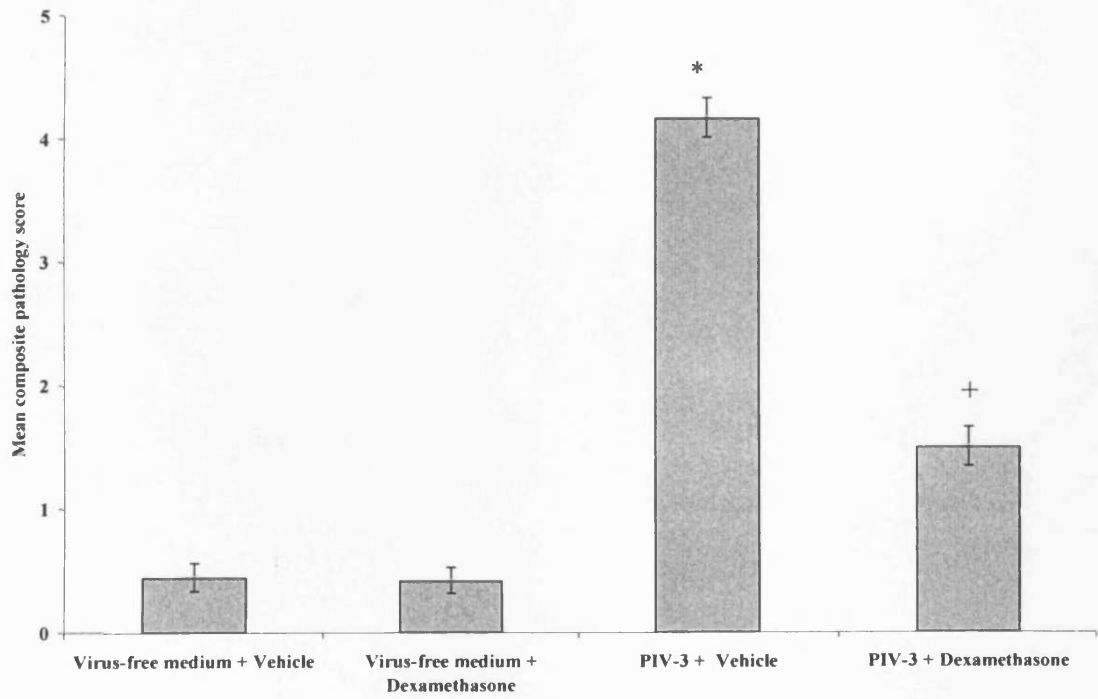
**Figure 5.11**

Histological appearance of lung tissue post inoculation day 4. Tissues were stained with haematoxylin and eosin for general morphology. **A.** Virus-free medium treated with vehicle (50% DMSO:saline) showing normal architecture. **B.** PIV-3 treated with vehicle (50% DMSO:saline) showing peribronchiolitis, interstitial pneumonia, and alveolitis as indicated by the increased density of inflammatory cells. **C.** Virus-free medium treated with dexamethasone ( $20 \text{ mg kg}^{-1}$ ). **D.** PIV-3 treated with dexamethasone ( $20 \text{ mg kg}^{-1}$ ). (Internal scale bar =  $50 \mu\text{m}$ ).

**A**



**B**



### **Figure 5.12**

Mean pathology scores as defined in chapter 2, in histology slides of left lung from virus-free medium and PIV-3 inoculated guinea pigs treated with vehicle (50% DMSO:sterile saline) or dexamethasone ( $20 \text{ mg kg}^{-1}$ ), 4 days post inoculation. **A** Mean pulmonary scores for peribronchiolitis, alveolitis and interstitial pneumonia. **B** Mean composite pulmonary score. Each point represents the mean  $\pm$  S.E.M (n=6) of the pathology scores range 1-5. \*  $P < 0.05$  denotes the significance of differences in mean pathology scores from PIV-3 compared to virus-free medium inoculated guinea pigs both treated with vehicle. +  $p < 0.05$  denotes the significance of differences in mean pathology score from PIV-3 treated with vehicle compared to dexamethasone treated guinea pigs. All statistical analysis was determined by ANOVA, followed by Dunnet's test.

	Virus-free medium + Vehicle (n=6)	Virus-free medium + dexamethasone (n=6)	PIV-3 + Vehicle (n=6)	PIV-3 + dexamethasone (n=6)	
Body weight (g)	11.9±1.5	-2.0±0.5	13.0±1.2	1.0±1.2	
Rectal temp (°C)	38.7±0.2	38.9±0.2	38.5±0.2	-2.8±1.5	
Lung function (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )	-2.5±3.8	3.2±2.7	-2.8±1.5	1.1±2.4	
Lung function before histamine exposure (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )	1.5±0.8	-1.6±1.0	-1.7±2.1	1.4±0.8	
Lung function after histamine exposure (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )	-1.7±0.6	-2.5±0.5	-41.3±2.5	-2.7±1.2	
Total Cells (10 <sup>6</sup> cells ml <sup>-1</sup> )	2.1±0.1	2.3±0.1	8.2±0.3	2.6±0.2	
Macrophages (10 <sup>6</sup> cells ml <sup>-1</sup> )	1.9±0.1	2.1±0.1	6.5±0.3	2.2±0.2	
Eosinophils (10 <sup>6</sup> cells ml <sup>-1</sup> )	0.2±0.02	0.2±0.01	1.4±0.08	0.4±0.04	
Neutrophils (10 <sup>6</sup> cells ml <sup>-1</sup> )	0.02±0.01	0.02±0.01	0.03±0.07	0.03±0.01	
Respiratory rate (breaths min <sup>-1</sup> )	75.0±5.1	72.0±3.5	150.0±4.4	81.0±4.0	
Wet lung weights (g kg <sup>-1</sup> )	7.7±0.3	8.2±0.3	12.1±0.6	8.5±0.3	
Total nitrate and nitrite (µM)	51.4±2.6	47.3±4.3	80.3±4.9	49.1±3.0	
Protein levels (µg ml <sup>-1</sup> )	863.5±50.0	774.4±48.0	1642.1±72.5	905.6±60.2	
Isolation in BAL fluid	0/6	0/6	0/6	0/6	
Isolation in Right lung	0/6	0/6	6/6	6/6	
Viral titre (TCID <sub>50</sub> ml <sup>-1</sup> )	Not Detected	Not Detected	7.6×10 <sup>7</sup> ±2.6×10 <sup>7</sup>	2.1×10 <sup>4</sup> ±1.5×10 <sup>4</sup>	
Mean pulmonary score	Peribronchiolitis	0.3±0.1	0.3±0.1	4.3±0.1	0.8±0.3
	Alveolitis	0.6±0.2	0.4±0.08	4.3±0.2	2.1±0.2
	Interstitial pneumonia	0.4±0.08	0.5±0.1	3.9±0.2	1.8±0.1
Mean composite pulmonary score	0.4±0.1	0.4±0.1	4.2±0.2	1.5±0.2	

**Table 5.2**

Summary of chapter 5 results from virus-free medium and PIV-3 infected guinea pigs treated with vehicle (50% DMSO: 50%saline) or dexamethasone (20 mg kg<sup>-1</sup>) post inoculation day 4.

## **CHAPTER 6**

### **EFFECT OF DEXAMETHASONE ON PIV-3 INFECTION IN CULTURED VERO CELLS**

#### **INTRODUCTION**

While glucocorticoids have been shown to be clearly effective in reducing the severity of even mild cases of PIV infection, the possibility of their anti-viral properties remains controversial and has not been well studied. Previous studies have investigated the effects of dexamethasone on RV-2 and RV-14 infection in cultured human tracheal epithelial cells (Suzuki *et al*; 2000). In this study, dexamethasone reduced the viral titres of supernatants of RV-14 infection in association with inhibition of cytokine production and ICAM-1 production. In contrast to RV-14 infection, dexamethasone did not alter viral titre in RV-2 infection. Studies by Bonville *et al* (2001) also reported no effect on viral titre in either PIV-1 or RSV infection treated with hydrocortisone.

Previous investigations in this thesis involved the effect of the glucocorticoid dexamethasone, in PIV-3 infected guinea pigs (Chapter 5). Studies revealed an inhibition of the inflammatory response and a significant reduction in viral titre. In view of this, the aim of this chapter was to determine whether the reduced viral titre is dependent or independent from the inflammatory response by determining the effect on viral load in response to dexamethasone in cultured vero cells infected with PIV-3.



## EXPERIMENTAL PROTOCOL

To determine the effects of dexamethasone on PIV-3 infection in Vero cells the following experiments were conducted to ascertain the following;

- To determine the effect of dexamethasone on viral titre in PIV-3 infected Vero cells
- To determine a concentration-response curve of dexamethasone
- To determine the minimum pre-incubation period required to cause inhibition.
- To determine whether viral replication recovered when dexamethasone treatment was removed.

The methods involving preparation of Vero cell culture, PIV-3 suspension, determination of TCID<sub>50</sub> values in cell supernatants are described in chapter 2.

### ***Determination of dexamethasone on viral titres in PIV-3 infected Vero cells***

In order to determine the effect of dexamethasone on viral titre in PIV-3, confluent Vero cells were incubated with medium containing dexamethasone (1µM) or vehicle (50% DMSO/50% saline) for 24 hours at 37<sup>0</sup>C with 5% CO<sub>2</sub>. A stock solution of dexamethasone was prepared (1mM) which was then diluted with medium A. The monolayers of Vero cells were then exposed to PIV-3 (5x10<sup>6</sup> TCID<sub>50</sub> ml<sup>-1</sup>) for 60 minutes before being rinsed with PBS and the addition of fresh medium 1 supplemented with either dexamethasone (1µM) or vehicle (50%DMSO/50%saline). Cells were incubated at 37<sup>0</sup>C in a humidified incubator containing 5% CO<sub>2</sub>. The whole volume of medium was taken for measurement of viral content at time points 0, 6, 12, 24, 48, 96 hours post inoculation. The PIV-3 titre was determined by TCID<sub>50</sub> assay.

Each time the culture medium was removed this was replaced by medium A containing either dexamethasone or vehicle.

***Determination of a concentration response effect with dexamethasone***

To determine a concentration-response effect with dexamethasone, confluent Vero cells were incubated with medium A containing various concentrations of dexamethasone ( $10^{-8}$ – $10^{-5}$ ) or vehicle (50% DMSO/50% saline) for 24 hours at 37<sup>0</sup>C with 5% CO<sub>2</sub>. The monolayers of Vero cells were exposed to PIV-3 ( $5 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) for 60 minutes before being rinsed with PBS and the addition of medium A supplemented with dexamethasone or vehicle. The whole volume of medium was taken for measurement of viral content 96 hours post inoculation. The PIV-3 titre was determined by TCID<sub>50</sub> assay.

***Determination of pre-incubation time with dexamethasone on PIV-3 titres***

To determine the minimum pre-incubation period required to cause inhibition of PIV-3 infection, Vero cells were incubated with medium A containing dexamethasone (1µM) or vehicle (50% DMSO/50% saline) for 0, 6, 12, 24, 48, 72, 96 hours at 37<sup>0</sup>C with 5% CO<sub>2</sub>. The monolayers of Vero cells were then exposed to PIV-3 ( $5 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) for 60 minutes before being rinsed with PBS and the addition of fresh medium A supplemented with dexamethasone or vehicle. The whole volume of medium was taken for measurement of viral content 96 hours post inoculation. Each time the medium was removed this was replaced by medium A containing either dexamethasone or vehicle. The PIV-3 titre was determined by TCID<sub>50</sub> assay.

***Determination of PIV-3 titres on the removal of dexamethasone***

To determine whether viral replication recovered when dexamethasone was removed, confluent Vero cells were incubated with medium A containing dexamethasone (1µM) for 24 hours at 37<sup>0</sup>C with 5% CO<sub>2</sub>.

The monolayers of Vero cells were then exposed to PIV-3 ( $5 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) for 60 minutes before being rinsed with PBS and the addition of fresh medium A supplemented with dexamethasone (1 μM) or vehicle (50%DMSO/50%saline). The whole volume of medium was taken for measurement of viral content at time points 0, 6, 12, 24, 48, 96 and 120 hours post inoculation. Each time the culture medium was removed this was replaced by culture medium A containing vehicle or dexamethasone. The PIV-3 titre was determined by TCID<sub>50</sub> assay.

## RESULTS

### *Effects of dexamethasone on viral titre in PIV-3 infected vero cells*

Exposing confluent Vero cell monolayers to PIV-3 treated with vehicle consistently led to infection. Collection of culture medium as early as 6 hours post inoculation detected the presence of PIV-3 ( $4.9 \pm 2.6$  TCID<sub>50</sub> ml<sup>-1</sup>). Evidence of continuous viral replication was obtained by demonstrating that the viral titres of supernatants collected 12, 24, 48, 72, 96 hours post infection ( $1.0 \times 10^2 \pm 7.9 \times 10^1$ ,  $2.8 \times 10^3 \pm 2.1 \times 10^3$ ,  $5.2 \times 10^4 \pm 3 \times 10^4$ ,  $1.4 \times 10^5 \pm 1.5 \times 10^4$ ,  $1.1 \times 10^7 \pm 8.0 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup> respectively) significantly increased with time. Treatment of Vero cells with dexamethasone (1 μM) significantly decreased ( $P < 0.05$ ) the viral titres of PIV-3 in the cell supernatants. Unlike PIV-3 cells treated with vehicle, dexamethasone treatment was able to delay the replication of PIV-3 until day 1 post infection, as viral titre was undetectable 6 and 12 hours after inoculation. A significant decrease in viral titre continued at day 24, 48, 72, 96 hours post infection ( $4.8 \pm 1.6$ ,  $3.9 \times 10^2 \pm 3.1 \times 10^2$ ,  $1.5 \times 10^3 \pm 6.0 \times 10^2$ ,  $5.8 \times 10^3 \pm 4.2 \times 10^3$  TCID<sub>50</sub> ml<sup>-1</sup>) respectively compared to supernatants treated with vehicle (Figure 6.1). Therefore, dexamethasone significantly decreased ( $P < 0.05$ ) the cell associated viral titres of PIV-3 virus *in vitro*.

### ***Concentration-response effects of dexamethasone on PIV-3 infection***

Collection of culture medium from PIV-3 exposed monolayers treated with vehicle failed to cause any significant ( $P>0.05$ ) concentration response in any of the vehicle concentrations equivalent to those using  $10^{-5}$ – $10^{-8}$  dexamethasone ( $2.4 \times 10^7 \pm 1.1 \times 10^6$ ,  $3.9 \times 10^7 \pm 6.2 \times 10^6$ ,  $3.2 \times 10^7 \pm 1.4 \times 10^7$ ,  $6.6 \times 10^7 \pm 4.6 \times 10^7$  TCID<sub>50</sub> ml<sup>-1</sup>) respectively. However, in supernatants from PIV-3 treated with dexamethasone a concentration response was observed. At dexamethasone concentration  $10^{-8}$  ( $1.8 \times 10^6 \pm 1.0 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) no significant ( $P>0.05$ ) reductions in viral titre were observed compared to vehicle. However, a significant reduction ( $P<0.05$ ) in viral titre was observed  $10^{-7}$  –  $10^{-5}$  ( $2.0 \times 10^7 \pm 1.2 \times 10^6$ ,  $3.0 \times 10^4 \pm 1.5 \times 10^4$ ,  $1.9 \times 10^4 \pm 1.2 \times 10^4$  TCID<sub>50</sub> ml<sup>-1</sup>) respectively. Thus, the maximum effect of dexamethasone was obtained at  $1 \mu\text{M}$  ( $10^{-6}$ ) (Figure 6.2).

### ***Effect of pre-incubation time with dexamethasone on viral titres.***

The inhibitory effects of dexamethasone ( $1 \mu\text{M}$ ) on PIV-3 infection were dependent on the pre-incubation period. Pre-incubation of dexamethasone for 0, 6 and 12 hours ( $4.7 \times 10^6 \pm 4.0 \times 10^6$ ,  $2.3 \times 10^6 \pm 1.2 \times 10^5$ ,  $3.0 \times 10^6 \pm 1.2 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) failed to significantly ( $P>0.05$ ) reduce viral titres in supernatants infected with PIV-3 treated with dexamethasone compared to PIV-3 treated with vehicle ( $3.3 \times 10^6 \pm 1.5 \times 10^6$ ,  $3.8 \times 10^5 \pm 1.7 \times 10^5$ ,  $2.4 \times 10^6 \pm 1.4 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>). However, after a pre-incubation period of 24 hours, ( $6.9 \times 10^3 \pm 5.1 \times 10^2$  TCID<sub>50</sub> ml<sup>-1</sup>), 48 ( $7.5 \times 10^3 \pm 6.2 \times 10^3$  TCID<sub>50</sub> ml<sup>-1</sup>) and 72 hours ( $2.1 \times 10^3 \pm 1.2 \times 10^3$  TCID<sub>50</sub> ml<sup>-1</sup>), viral titres were significantly reduced in PIV-3 treated with dexamethasone compared to PIV-3 treated with vehicle ( $4.7 \times 10^6 \pm 1.4 \times 10^6$ ,  $3.2 \times 10^6 \pm 1.5 \times 10^6$ ,  $4.1 \times 10^6 \pm 1.3 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>). Thus, the minimum pre-incubation time to cause inhibition of PIV-3 infection was 1 day (Figure 6.3).

### ***Effect of PIV-3 titres on the removal of dexamethasone***

Vero cells pre-treated with dexamethasone (1 $\mu$ M, 1 day) and thereafter continuously treated with medium containing dexamethasone produced significantly ( $P < 0.05$ ) decreasing virus titres 24-72 hours post inoculation ( $2.4 \times 10^3 \pm 1.5 \times 10^3$ ,  $5.3 \times 10^4 \pm 4.2 \times 10^4$ ,  $2.5 \times 10^6 \pm 1.5 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) compared to Vero cells pre-treated dexamethasone and thereafter continuously treated with vehicle ( $15.9 \pm 11.4$ ,  $2.9 \times 10^2 \pm 1.7 \times 10^2$ ,  $15 \times 10^5 \pm 1.2 \times 10^5$  TCID<sub>50</sub> ml<sup>-1</sup>). No significant difference was observed in cells post inoculation time 96 and 120 hours treated with either medium containing vehicle ( $2.7 \times 10^7 \pm 1.6 \times 10^7$ ,  $2.0 \times 10^7 \pm 1.2 \times 10^7$  TCID<sub>50</sub> ml<sup>-1</sup>) or dexamethasone ( $1.3 \times 10^7 \pm 1.0 \times 10^7$ ,  $2.2 \times 10^7 \pm 1.4 \times 10^7$  TCID<sub>50</sub> ml<sup>-1</sup>). Thus, when Vero cells were pre-treated with dexamethasone and dexamethasone then removed, the viral titres of PIV-3 in the supernatants became the same as those in the Vero cells pre-treated with vehicle 96 hours after infection (Figure 6.4).

## **DISCUSSION**

This present study suggests that dexamethasone inhibits PIV-3 infection in cultured Vero cells. These conclusions are based on the observation that dexamethasone reduces the titres of PIV-3 in the culture medium in a concentration-dependent manner and reduces cell associated viral titre. The results are summarised in Table 6.1.

Few studies have investigated the effect of dexamethasone on virus infection *in vitro*. However, these investigations are in concordance with other studies, which demonstrated that dexamethasone inhibited RV-14 infection in human tracheal epithelial cells (Suzuki *et al*; 2000). In this study it has been suggested that the mechanism by which dexamethasone inhibits RV-14 infection in human tracheal epithelial cells is most likely via reducing the expression of ICAM-1, a surface receptor for a major group of rhinovirus on the cells (Suzuki *et al*; 2000).

This is further supported by studies using RV-2 infection where dexamethasone does not reduce viral titres in human epithelial tracheal epithelial cells, as RV-2 does not use ICAM-1 as its receptor (Suzuki *et al*; 2000). Although, the effects of glucocorticoids on the replication of PIV-3 infection has not been well studied, this mechanism may be suggested for the inhibition of PIV-3 replication in this study.

Further evidence to support the above mechanism is provided when Vero cells were pre-incubated with dexamethasone (1day) and then dexamethasone was removed from the culture medium after PIV-3 infection to determine whether viral replication recovered. It was ascertained that viral titres in the cell supernatant became the same on day 4 after PIV-3 infection as the Vero cells treated with vehicle. Therefore, the mechanism by which dexamethasone treatment inhibits PIV-3 infection is most likely via reducing the expression of ICAM-1. Studies have also found that this was coincident with the pre-incubation time of dexamethasone decreasing ICAM-1 protein levels in monocytic U-937 cells (Van de Slope *et al*; 1993).

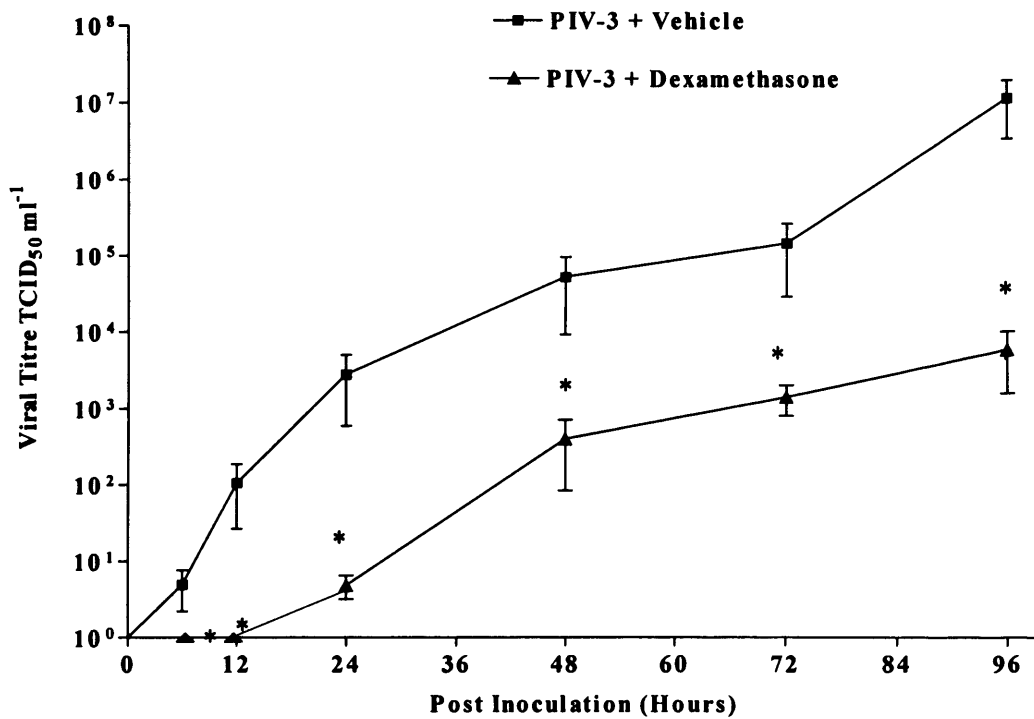
Epithelial cells in the human airway express ICAM-1 on their surface and studies by Gao *et al* (2000) have suggested that this is the possible site of attachment in PIV-3. Several studies have implicated ICAM-1 in inflammation during infection by viruses. ICAM-1 interacts physiologically with leucocyte function-associated antigen-1 expressed on leucocytes, and thus plays a vital role in the recruitment and migration of immune effector cells to sites of local inflammation. Recent investigations have shown that PIV-3 infection upregulates ICAM-1 expression on airway epithelial cells (Gao *et al*; 2000), which would facilitate viral cell attachment and entry. These studies have also shown that ICAM-1 induction was significantly reduced when virions were UV inactivated prior to PIV-3 infection, indicating that ICAM-1 induction was mostly viral replication dependent (Gao *et al*; 2000).

Culture supernatants of PIV-3 infected cells induced ICAM-1 at an extremely low level, indicating that virus-induced cytokines played only a minor role in the induction process. This data strongly indicates that PIV-3 induces ICAM-1 directly by viral antigens in a cytokine-independent manner; this induction may play a role in the inflammation during PIV-3 infection (Gao *et al*; 2000). However, further study is needed to examine the precise mechanisms and measure the levels of ICAM-1 in the supernatants.

Glucocorticoid therapy effectively relieves airway inflammation and inhibits inflammatory cytokine production in the cells of the lung (Torphy; 1998). Glucocorticoids activate their receptors that may then bind to activated NF- $\kappa$ B and prevent it from binding to  $\kappa$ B sites on genes that have a role in the inflammatory processes (Barnes & Karin; 1997). Therefore, in the present study dexamethasone might also reduce cytokine production after PIV-3 infection by inhibiting PIV-3 infection via the reduction of its receptor, ICAM-1. It is also possible that dexamethasone might further inhibit the production of cytokines and ICAM-1 induced by PIV-3 infection via its inhibitory effects on the production of cytokines and ICAM-1 (Barnes & Karin; 1997).

The effects of dexamethasone on viral titre in PIV-3 infection *in vivo* (chapter 5) and *in vitro* (current chapter) both support a role for the anti-viral effects of dexamethasone. Glucocorticoids have been found to be clearly effective in reducing the severity of PIV infection (Kairys *et al*; 1989, Geelhoed *et al*; 1996, Johnson *et al*; 1999, Klassen *et al*, 1998, Ausejo *et al*, 1999). In contrast, studies treating PIV-3 infected individuals with the glucocorticoid, hydrocortisone, failed to inhibit viral titre 3 days post inoculation (Bonville *et al*; 2001).

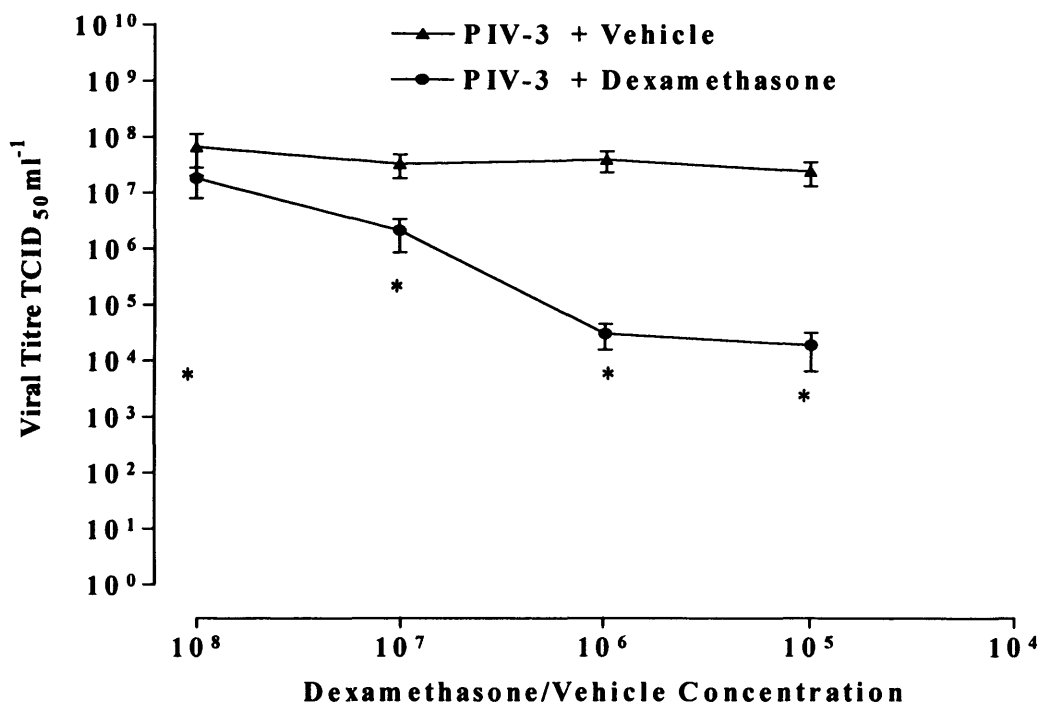
In conclusion, dexamethasone decreased viral replication in PIV-3 infection in the cultured supernatants of Vero cells. The most likely mechanism is via inhibition of ICAM-1 induction, although, further study is required to clarify an anti-viral action of dexamethasone in PIV-3 infection.



**Figure 6.1**

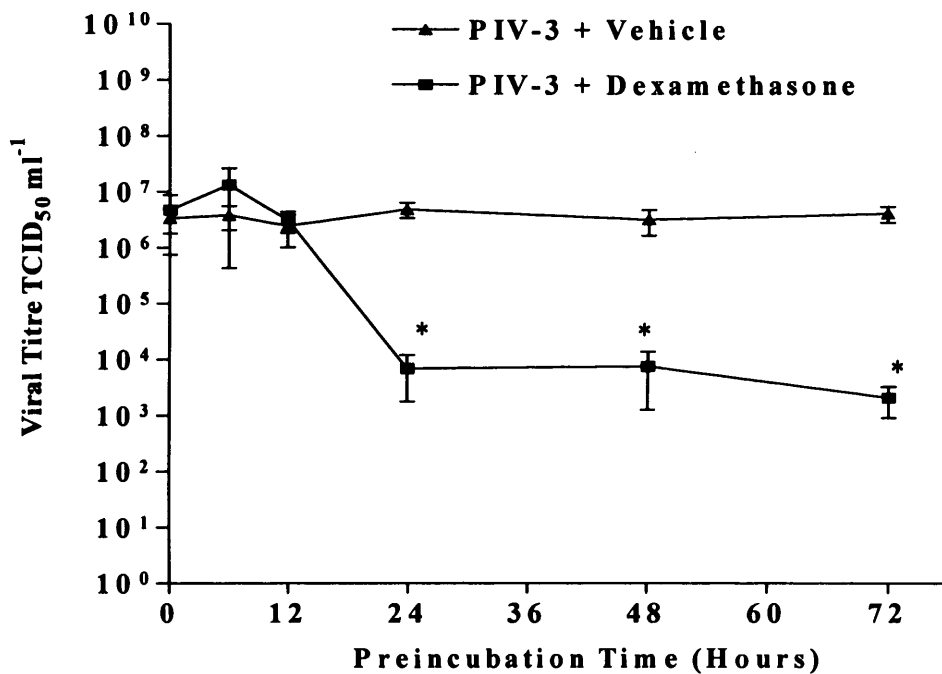
Viral titres in supernatants of Vero cells 0, 6, 12, 24, 48, 72 and 96 hours after exposure to  $10^6$  TCID<sub>50</sub> ml<sup>-1</sup> of PIV-3 required to infect 50% of cells treated with vehicle (50%DMSO/50%saline) or dexamethasone (1 $\mu$ M). Each point represents the mean  $\pm$  S.E.M titres of intrapulmonary replicating PIV-3 (n=6). \* P<0.05 denotes the significant differences in PIV-3 infection treated with dexamethasone compared to PIV-3 infection treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.





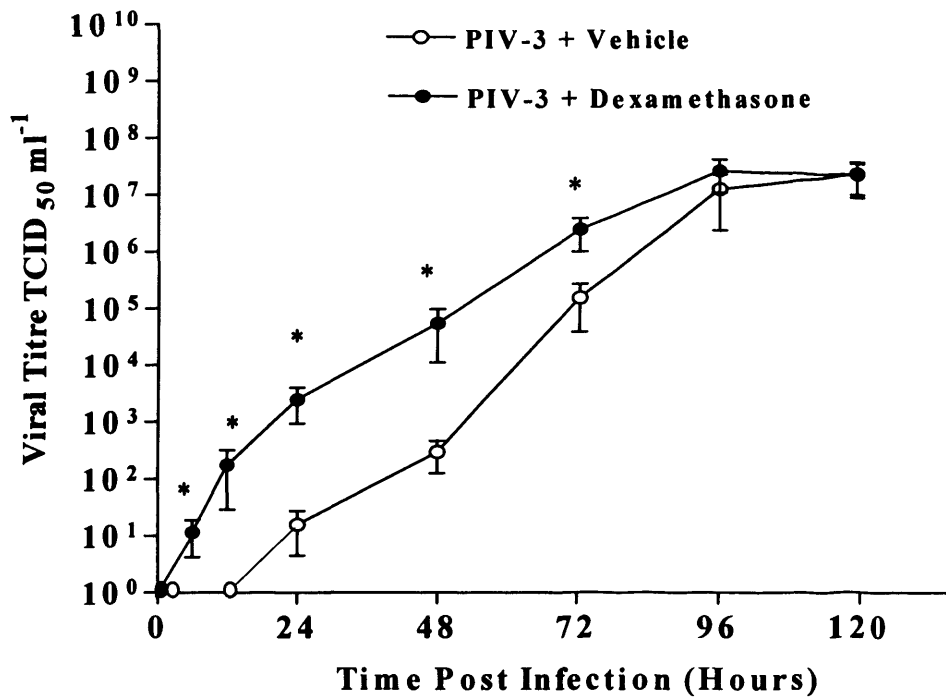
**Figure 6.2**

Concentration-response effects of dexamethasone ( $10^8$ - $10^5$ ) or vehicle (50%DMSO/50%saline) PIV-3 infection in Vero cells. Viral titres in supernatants were collected over 1-3 days after PIV-3 infection ( $10^6$  TCID<sub>50</sub> ml<sup>-1</sup>). Each point represents the mean  $\pm$  S.E.M titres of intrapulmonary replicating PIV-3 (n=6). \* P<0.05 denotes the significant differences in PIV-3 infection treated with dexamethasone compared to PIV-3 infection treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



**Figure 6.3**

Effects of pre-incubation time of dexamethasone (1 $\mu$ M) 6, 12, 24, 48, 72 hours on viral titres in supernatants collected 4 days post inoculation after exposure to PIV-3. Each point represents the mean  $\pm$  S.E.M titres of intrapulmonary replicating PIV-3 (n=6). \* P<0.05 denotes the significant differences in PIV-3 infection treated with dexamethasone compared to PIV-3 infection treated with vehicle (50%DMSO/50%saline). All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



**Figure 6.4**

Effects of viral titres after exposure to  $10^6$  TCID<sub>50</sub> ml<sup>-1</sup> of PIV-3 and dexamethasone (1 $\mu$ M, 1day). Cells were then cultured in medium containing vehicle for dexamethasone and viral titres in supernatants measured 0, 6, 24, 48, 72 and 96 hours after exposure. Each point represents the mean  $\pm$  S.E.M titres of replicating PIV-3 virus (n=6). \* P<0.05 denotes the significant differences in PIV-3 infection treated with dexamethasone compared to PIV-3 infection treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

**Table 6.1 Summary of chapter 6 results**

*Effects of dexamethasone on viral titre in PIV-3 infected Vero cells*

Time	Viral Titre (TCID <sub>50</sub> ml <sup>-1</sup> )						
	0 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs
PIV-3 + vehicle (n=6)	Not Detected	4.9±	1.1x10 <sup>2</sup> ±	2.8x10 <sup>3</sup> ±	5.2x10 <sup>4</sup> ±	1.4x10 <sup>5</sup> ±	1.1x10 <sup>7</sup> ±
	Detected	2.7	7.9x10 <sup>1</sup>	2.1x10 <sup>3</sup>	4.3x10 <sup>4</sup>	1.5x10 <sup>4</sup>	8.0x10 <sup>6</sup>
PIV-3 + dexamethasone (n=6)	Not Detected	Not Detected	Not Detected	4.8±	3.9x10 <sup>2</sup> ±	1.5x10 <sup>3</sup> ±	5.8x10 <sup>3</sup> ±
	Detected	Detected	Detected	1.6	3.1x10 <sup>2</sup>	6.0x10 <sup>2</sup>	4.2x10 <sup>3</sup>

*Concentration-response effects of dexamethasone on PIV-3 infection*

Time	Viral Titre (TCID <sub>50</sub> ml <sup>-1</sup> )			
	10 <sup>-8</sup>	10 <sup>-7</sup>	10 <sup>-6</sup>	10 <sup>-5</sup>
PIV-3 + vehicle (n=6)	6.6x10 <sup>7</sup> ±	3.2x10 <sup>7</sup> ±	3.9x10 <sup>7</sup> ±	2.4x10 <sup>7</sup> ±
	4.6x10 <sup>7</sup>	1.4x10 <sup>7</sup>	6.2x10 <sup>6</sup>	1.1x10 <sup>6</sup>
PIV-3 + dexamethasone (n=6)	1.9x10 <sup>4</sup> ±	3.0x10 <sup>4</sup> ±	2.0x10 <sup>7</sup> ±	1.8x10 <sup>6</sup> ±
	1.2x10 <sup>4</sup>	1.5x10 <sup>4</sup>	1.2x10 <sup>6</sup>	1.0x10 <sup>6</sup>

*Effect of pre-incubation time on viral titres with dexamethasone*

Time	Viral Titre (TCID <sub>50</sub> ml <sup>-1</sup> )					
	0 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs
PIV-3 + vehicle (n=6)	3.3x10 <sup>6</sup> ±	3.8x10 <sup>5</sup> ±	2.4x10 <sup>6</sup> ±	4.7x10 <sup>6</sup> ±	3.2x10 <sup>6</sup> ±	4.1x10 <sup>6</sup> ±
	1.5x10 <sup>6</sup>	1.7x10 <sup>5</sup>	1.4x10 <sup>6</sup>	1.4x10 <sup>6</sup>	1.5x10 <sup>6</sup>	1.3x10 <sup>6</sup>
PIV-3 + dexamethasone (n=6)	4.7x10 <sup>6</sup> ±	2.3x10 <sup>6</sup> ±	3.0x10 <sup>6</sup> ±	6.9x10 <sup>3</sup> ±	7.5x10 <sup>3</sup> ±	2.1x10 <sup>3</sup> ±
	4.0x10 <sup>6</sup>	1.2x10 <sup>5</sup>	1.2x10 <sup>6</sup>	5.1x10 <sup>2</sup>	6.2x10 <sup>3</sup>	1.2x10 <sup>3</sup>

*Effect of viral titres on removal of dexamethasone*

Time	Viral Titre (TCID <sub>50</sub> ml <sup>-1</sup> )							
	0 hrs	6 hrs	12 hrs	24 hrs	48 hrs	72 hrs	96 hrs	120 hrs
PIV-3 + dexamethasone (n=6)	Not Detected	Not Detected	Not detected	15.9±	2.9x10 <sup>2</sup>	1.5x10 <sup>5</sup> ±	1.3x10 <sup>7</sup> ±	2.2x10 <sup>7</sup> ±
	Detected	Detected	detected	11.4	±1.7x10 <sup>2</sup>	1.2x10 <sup>5</sup>	1.0x10 <sup>7</sup>	1.4x10 <sup>7</sup>
PIV-3 + vehicle (n=6)	Not Detected	11.5±	1.7x10 <sup>2</sup> ±	2.4x10 <sup>3</sup> ±	5.3x10 <sup>4</sup> ±	2.5x10 <sup>6</sup> ±	2.7x10 <sup>7</sup> ±	2.2x10 <sup>7</sup> ±
	Detected	7.3	1.4x10 <sup>2</sup>	1.5x10 <sup>3</sup>	4.2x10 <sup>4</sup>	1.5x10 <sup>6</sup>	1.6x10 <sup>7</sup>	1.2x10 <sup>7</sup>

## CHAPTER 7

### EFFECT OF ROLIPRAM PRE-TREATMENT ON PIV-3 INFECTED GUINEA PIGS

#### INTRODUCTION

Type 4 phosphodiesterases (PDE-4) inhibitors exhibit anti-asthma effects due in part to their bronchodilatory and anti-inflammatory actions (Torphy, 1998). PDE-4 is the major isotype in human (Dent *et al*; 1994; Hatzelmann *et al*; 1995) and guinea pig (Souness *et al*; 1991; Raeburn *et al*; 1994) eosinophils, therefore PDE-4 was an obvious target. Inhibiting PDE-4 causes a reduction in eosinophils chemotaxis and suppresses the activation and production of inflammatory cell derived pro-inflammatory mediators (Torphy *et al*; 1999). There have been several reports demonstrating inhibitory effects of PDE-4 inhibitors on AHR and lung eosinophilia in allergen induced airway obstruction in guinea pigs (Raeburn *et al*; 1994, Santing *et al*; 1995, Danahay and Broadley; 1997, Manabe *et al*; 1997). Other studies involving the PDE-3 inhibitor, milrinone have shown poor inhibitory activity (Ikemura *et al*; 2001). Studies by Toward *et al* (2005) have also indicated a potential for the PDE-4 inhibitor in the treatment of PIV-3 infected guinea pigs. It was therefore, decided to investigate the potential suppressive effect of the PDE-4 inhibitor, rolipram, on PIV-3 infected guinea pigs.

The parameters investigated included airway function, airway reactivity to histamine, cellular infiltration, wet lung weight, NO and total protein levels, protein levels in BAL fluid, histological analysis and recovery of viral load from the lung tissue.

This investigation is novel as no other investigation has ascertained the level of virus recovery in the lung tissue of PIV-3 infected guinea pigs treated with rolipram along with functional measurements.

### **EXPERIMENTAL PROTOCOL**

The following protocols used to perform this study have all been previously described in chapter 2.

To determine the effect of the PDE-4 inhibitor, rolipram pre-treatment in PIV-3 infected guinea pigs post inoculation day 4, the following experimental groups were as follows.

<b>GROUPS (n=6)</b>	
1	Virus-free medium infected animals pre-treated with vehicle assessed post inoculation day 4
2	Virus-free infected animals pre-treated with rolipram assessed post inoculation day 4,
3	PIV-3 virus infected animals pre-treated with vehicle assessed post inoculation day 4.
4	PIV-3 virus infected animals pre-treated with rolipram assessed post inoculation day 4,

From the results of the time course study (Chapter 4) it was decided that the effect of rolipram pre-treatment in PIV-3 infected guinea pigs would be investigated at post infection day 4 regarded as the peak response to infection due to the significant increase in cellular infiltration, AHR and viral detection in BAL fluid and lung tissue compared to the other study days. On day -1 guinea pigs were assessed for airway reactivity to histamine and then rolipram (1 mg kg<sup>-1</sup>) or vehicle (saline) was injected via the intraperitoneal cavity as a pre-treatment 24 hours before viral inoculation. On study day 0 guinea pigs were inoculated with virus-free medium or PIV-3 by intranasal instillation. Thereafter, guinea pigs received daily injections of vehicle or rolipram 30 minutes prior to viral inoculation until the end of the study.

At the end of each study, 24 hours after the last dose of rolipram the animals were reassessed for airway reactivity to histamine and within 20 minutes were sacrificed and BAL performed and lung tissue removed. BAL fluid specimens were analysed for virus-induced changes by comparison of total and differential cell counts, NO and total protein levels and viral titre determination. Lung tissue specimens were used for total viral content per g of lung and pulmonary histopathology. PIV-3 was detected in BAL fluid and lung tissue samples by using standard culture techniques and indirect immunofluorescence. Throughout the study each animal was weighed and rectal temperature recorded on appropriate study days. Animals were also examined daily for general signs of illness including hair loss and decreased activity.

## RESULTS

### ***Effect of PIV-3 on body weight gain with or without rolipram***

PIV-3 guinea pigs treated with dexamethasone failed to significantly ( $P>0.05$ ) alter body weight gain throughout the 4 day study period compared to PIV-3 guinea pigs treated with vehicle (Figure 7.1B). No significant differences ( $P>0.05$ ) were observed between virus-free medium infected guinea pigs treated with dexamethasone or vehicle (Figure 7.1A)

### ***Effect of PIV-3 on rectal temperature with or without rolipram.***

PIV-3 guinea pigs treated with dexamethasone failed to significantly ( $P>0.05$ ) alter rectal temperature throughout the 4 day study period compared to PIV-3 guinea pigs treated with vehicle (Figure 7.2B). No significant differences ( $P>0.05$ ) were observed between virus-free medium infected guinea pigs treated with dexamethasone or vehicle (Figure 7.2A)

***Effect of PIV-3 on airway function with or without rolipram.***

PIV-3 guinea pigs treated with dexamethasone failed to significantly ( $P>0.05$ ) alter lung function throughout the 4 day study period compared to PIV-3 guinea pigs treated with vehicle (Figure 7.3B). No significant differences ( $P>0.05$ ) were observed between virus-free medium infected guinea pigs treated with dexamethasone or vehicle (Figure 7.3A)

***Effect of PIV-3 on airway reactivity to inhaled histamine with or without dexamethasone.***

Inhalation of histamine (1mM) for 20 seconds 24 hours before intranasal inoculation of virus-free medium and PIV-3 treated with vehicle or rolipram did not produce any significant ( $P>0.05$ ) bronchoconstriction and failed to cause any significant deviation from baseline  $sG_{aw}$  values (Figure 7.4). This confirms that this was a sub-threshold dose of histamine.

Prior to inoculation, virus-free medium infected guinea pigs treated with rolipram were subjected to a higher dose of inhaled histamine (3mM, 20 seconds) resulting in a significant bronchoconstriction ( $-29.6\pm 1.9\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ) which remained increased on subsequent challenge to histamine assessed post inoculation day 4 ( $-32.4\pm 2.7\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ). These values were not significantly different ( $P<0.05$ ). Thus, no statistically significant ( $P>0.05$ ) differences were observed between starting baseline values of  $sG_{aw}$  for any of the groups studied. In PIV-3 infected guinea pigs treated with rolipram post inoculation day 4, inhalation of histamine did not produce any significant bronchoconstriction ( $P>0.05$ ) (Before:  $1.9\pm 0.9\%$ , After:  $-2.5\pm 1.8\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ) and failed to cause any significant deviation from baseline  $sG_{aw}$  values compared with before inoculation. In contrast PIV-3 infected animals treated with vehicle post inoculation day 4 produced a significant bronchoconstriction with peak reductions in  $sG_{aw}$  (Before:  $-2.7\pm 1.1\%$ , After:  $-36.3\pm 2.1\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ). This indicates AHR to histamine. Thus, rolipram treatment inhibited PIV-3 induced AHR.



***Effect of PIV-3 on airways infiltration of leucocytes with or without rolipram.***

Treating PIV-3 infected guinea pigs with rolipram significantly decreased ( $P < 0.05$ ) airways infiltration of total cells ( $1.9 \pm 0.2$  cells  $\text{ml}^{-1}$ ) including macrophages ( $1.6 \pm 0.2 \times 10^6$  cells  $\text{ml}^{-1}$ ), eosinophils ( $0.3 \pm 0.1 \times 10^6$  cells  $\text{ml}^{-1}$ ) and neutrophils ( $0.02 \pm 0.1 \times 10^6$  cells  $\text{ml}^{-1}$ ) compared to PIV-3 infected guinea pigs treated with vehicle total cell count ( $6.7 \pm 0.8 \times 10^6$  cells  $\text{ml}^{-1}$ ) and infiltration of macrophages ( $5.5 \pm 0.7 \times 10^6$  cells  $\text{ml}^{-1}$ ), eosinophils ( $1.0 \pm 0.2 \times 10^6$  cells  $\text{ml}^{-1}$ ) and neutrophils ( $0.2 \pm 0.1 \times 10^6$  cells  $\text{ml}^{-1}$ ) into the airways. Thus, rolipram treatment inhibited cellular infiltration of inflammatory cells into the lungs. Virus-free medium infected guinea pigs treated with vehicle or dexamethasone failed to significantly ( $P > 0.05$ ) alter total ( $2.1 \pm 0.4 \times 10^6$  cells  $\text{ml}^{-1}$ ) and differential cell counts including macrophages ( $1.9 \pm 0.3 \times 10^6$  cells  $\text{ml}^{-1}$ ), eosinophils ( $0.2 \pm 0.1 \times 10^6$  cells  $\text{ml}^{-1}$ ) and neutrophils ( $0.03 \pm 0.1 \times 10^6$  cells  $\text{ml}^{-1}$ ) in the airways (Figure 7.5).

***Effect of PIV-3 on respiratory rate with or without rolipram.***

A significant decrease ( $P < 0.05$ ) in respiratory rate was observed in PIV-3 infected guinea pigs treated with rolipram ( $85.0 \pm 5.0$  breaths  $\text{min}^{-1}$ ) compared to PIV-3 infected animals treated with vehicle ( $139.0 \pm 7.0$  breaths  $\text{min}^{-1}$ ) (Figure 7.6B). Treating virus-free medium infected guinea pigs with vehicle ( $81.0 \pm 4.0$  breaths  $\text{min}^{-1}$ ) or rolipram ( $70.0 \pm 4.0$  breaths  $\text{min}^{-1}$ ) failed to cause any significant difference ( $P > 0.05$ ) in respiratory rates (Figure 7.6A). Intranasal inoculation of virus-free medium animals treated with vehicle or rolipram failed to significantly ( $P > 0.05$ ) alter respiratory rate (Figure 7.6A). Treating PIV-3 inoculated guinea pigs with rolipram significantly decreased respiratory rate on days 2-4 compared to PIV-3 inoculated guinea pigs treated with vehicle (Figure 7.6B).

***Effects of PIV-3 on wet lung weights with or without rolipram.***

Treating PIV-3 infected guinea pigs with rolipram significantly decreased ( $P < 0.05$ ) wet lung weights ( $8.1 \pm 0.3 \text{ g kg}^{-1}$ ) compared to PIV-3 infected animals treated with vehicle ( $11.8 \pm 0.4 \text{ g kg}^{-1}$ ) (Figure 7.7B). No significant differences ( $P > 0.05$ ) were observed between virus-free medium guinea pigs treated with rolipram ( $7.2 \pm 0.2 \text{ g kg}^{-1}$ ) compared to virus-free medium inoculated animals treated with vehicle ( $6.5 \pm 0.4 \text{ g kg}^{-1}$ ) (Figure 7.7A).

***Effect of PIV-3 on airways NO with or without rolipram.***

In PIV-3 infected guinea pigs treated with rolipram, a significant decrease ( $P < 0.05$ ) in combined NO levels in BAL fluid was observed ( $50.8 \pm 3.3 \text{ } \mu\text{M}$ ) compared to PIV-3 infected animals treated with vehicle ( $76.3 \pm 4.5 \text{ } \mu\text{M}$ ) (Figure 7.8B). Treating virus-free medium infected guinea pigs with vehicle ( $48.1 \pm 2.9 \text{ } \mu\text{M}$ ) or rolipram ( $51.6 \pm 3.7 \text{ } \mu\text{M}$ ) failed to cause any significant difference ( $P > 0.05$ ) in nitric oxide levels (Figure 7.8A).

***Effect of PIV-3 on total protein levels with or without rolipram.***

A significant decrease ( $P < 0.05$ ) in total protein levels in BAL fluid was observed in PIV-3 infected guinea pigs treated with rolipram ( $856.4 \pm 69.6 \text{ } \mu\text{g ml}^{-1}$ ) compared to PIV-3 infected animals treated with vehicle ( $1528.8 \pm 62.7 \text{ } \mu\text{g ml}^{-1}$ ) (Figure 7.9B). Treating virus-free medium infected guinea pigs with vehicle ( $793.4 \pm 50.6 \text{ } \mu\text{g ml}^{-1}$ ) or rolipram ( $798.6 \pm 49.2 \text{ } \mu\text{g ml}^{-1}$ ) failed to cause any significant difference ( $P > 0.05$ ) in total protein levels (Figure 7.9A).

***Isolation of PIV-3 in BAL fluid and right lung tissue with or without rolipram.***

Replicating virus was isolated in all PIV-3 infected animals treated with vehicle or rolipram post inoculation day 4 (Table 7.1) as measured by CPE of BAL fluid and homogenised left lung tissue on Vero cells and a positive reaction by indirect immunofluorescence using an antibody against PIV-3.

Titres of intrapulmonary replicating PIV-3 were determined in left lung tissue samples treated with vehicle or rolipram 4 days post inoculation and were normalised to left lung tissue weight ( $\text{TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ) (Figure 7.10). Treating PIV-3 infected guinea pigs with rolipram failed to significantly alter ( $P>0.05$ ) the viral titre of PIV-3 ( $3.10 \times 10^8 \pm 2.17 \times 10^8 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ) compared to PIV-3 infected guinea pigs treated with vehicle ( $4.58 \times 10^7 \pm 1.25 \times 10^7 \text{ TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ). Thus, rolipram treatment failed to inhibit PIV-3 replication in left lung tissue samples.

***Effect of PIV-3 on mean pathology scores in pulmonary histopathology with or without rolipram.***

PIV-3 infected guinea pigs treated with rolipram revealed a dramatic reduction in inflammatory cells compared with the levels observed in PIV-3 infected guinea pigs treated with vehicle, which demonstrated evidence of peribronchiolitis, interstitial pneumonia and alveolitis (Figure 7.11). This indicates near complete reversal of the pathologic changes. Treating PIV-3 infected guinea pigs with rolipram resulted in significantly reduced ( $P<0.05$ ) mean composite pulmonary scores ( $1.6 \pm 0.3$ ) compared with PIV-3 infected guinea pigs treated with vehicle ( $3.9 \pm 0.2$ ). No significant differences ( $P>0.05$ ) were observed in virus-free medium infected guinea pigs treated with rolipram ( $0.4 \pm 0.1$ ) compared to vehicle ( $0.4 \pm 0.1$ ) (Figure 7.12).

## **DISCUSSION**

This study investigated the effect of the PDE-4 inhibitor, rolipram in PIV-3 inoculated guinea pigs. The parameters investigated included body weight gain, rectal temperature, airway function, airway reactivity to histamine, infiltration of inflammatory cells, wet lung weights, BAL fluid, NO and total protein levels, viral titre, and pulmonary histopathology. The results are summarised in Table 7.2.

The effects of PIV-3 inoculation in guinea pigs has been extensively discussed in chapter 4 of this thesis and will not be duplicated in this current chapter. The dose of rolipram used was based on PIV-3 studies by Toward *et al* (2005) and other guinea models of inflammation (Santing *et al*; 1995; Danahay and Broadley;1997).

During the investigation, no significant differences were observed in body weight gain, rectal temperature or lung function following PIV-3 inoculation whether rolipram was present or not, which has been consistent throughout this thesis. In the current study, rolipram administration in PIV-3 infected guinea pigs resulted in the complete ablation of AHR to histamine induced by PIV-3 and inhibition of the accompanying inflammatory cell influx to the lungs. The prevention of AHR could not be due to functionally antagonistic bronchodilation (i.e. increase in  $sG_{aw}$ ) by rolipram since treatment of virus-free medium infected guinea pigs with rolipram failed to display attenuated responses to a higher bronchoconstricting dose of inhaled histamine (3mM). Therefore, the inhibited PIV-3 induced AHR was independent of a residual bronchodilation or an anti-histamine mechanism. It is well established that PDE-4 inhibitors have a bronchodilatory effect in guinea pigs (Howell *et al*; 1992). The prevention of AHR as suggested in this study and by others (Torphy *et al*; 1998, Ikemura *et al*; 2001) may be due to a causative association between pro-inflammatory mediators, eosinophilic PIV-3 induced inflammation and airway hyperreactivity. This is further supported by studies in methacholine-induced bronchoconstriction in naïve mice (Ikemura *et al*; 2001). Under these conditions, the PDE-4 inhibitors failed to alter airway responsiveness following increasing concentrations of methacholine (Ikemura *et al*; 2001). Thus, protection from PIV-3 induced AHR using rolipram treatment arose from its anti-inflammatory activity (i.e inhibition of mast cell degranulation) rather than to a direct bronchodilatory effect.

The effects of PDE inhibitors in models of pulmonary inflammation have been evaluated in a large number of studies and have been shown to be active in a wide spectrum of pulmonary inflammation models. Rolipram inhibits the PDE4-selective degradation of intracellular cAMP, thus causing intra-cellular cAMP levels to rise (Torphy, 1998). The PDE-4 isotype has been located in guinea pig macrophages and is the predominant isoenzyme in eosinophils, although its presence has also been reported in epithelial cells, endothelial cells, lymphocytes, mast cells and neutrophils of other species (Raeburn *et al*; 1993). Evidence suggests that elevated cAMP levels from the inhibition of PDE-4, suppresses inflammatory cell activation and the release of leukotrienes, PAF, TNF $\alpha$ , cytokines (IL-2, IL-4, IL-5 and IL-8) and eicosanoids (Torphy *et al*; 1999).

The most impressive property of PDE-4 inhibitors in models of pulmonary inflammation is their ability to abolish eosinophil infiltration. This effect occurs in several animal species including guinea pig (Howell *et al*; 1993, Banner & Page; 1995), rat (Howell *et al*; 1995) and rabbits (Gozzard *et al*; 1996). In this study an explanation for this is that PDE-4 inhibitors ablate mast cell degranulation and consequently reduce the generation of chemotactic mediators that are responsible for eosinophils recruitment. Evidence suggests that PDE-4 inhibitors reduce eosinophils influx by two general mechanisms, one that involves an inhibition of mediator release and secondly one that involves a more generalised inhibitory effect on inflammatory cell trafficking (Torphy *et al*; 1998). In addition to their ability to suppress eosinophil infiltration, PDE-4 inhibitors also reduce the activation of these cells *in vivo* as assessed by decreased eosinophils peroxidase release into BAL fluid (Lagente *et al*; 1994). Thus, PDE-4 inhibitors can modify the role of the eosinophils in pathologic processes by suppressing the release of chemotactic mediators, inhibiting chemotaxis directly, reducing endothelial cell adhesion, inhibiting IL-5 secretion and inhibiting the generation and release of eosinophil-derived mediators and cytotoxic substances.

Thus, pleiotropic actions of PDE-4 inhibitors are not limited to effects on eosinophils but as shown in this study is applicable to all cells involved in airway inflammation (Torphy *et al*; 1998). Therefore, suppressing inflammatory cell activity and activation, with rolipram would reduce the release of further pro-inflammatory mediators from PIV-3-induced inflammatory cells responsible for exacerbating epithelial damage, activating exposed sensory nerves or ASM and promoting AHR. In concordance, studies by Ikemura *et al* (2001) have shown inhibition of AHR and eosinophils influx in mice induced by RSV infection using rolipram doses of 0.03 to 0.3 mg kg<sup>-1</sup>. Moreover, the results of the inhibitory effect of PDE-4 inhibitors on eosinophil infiltration support the previous reports in other antigen induced models (Underwood *et al*; 1994, Danahay and Broadley, 1997).

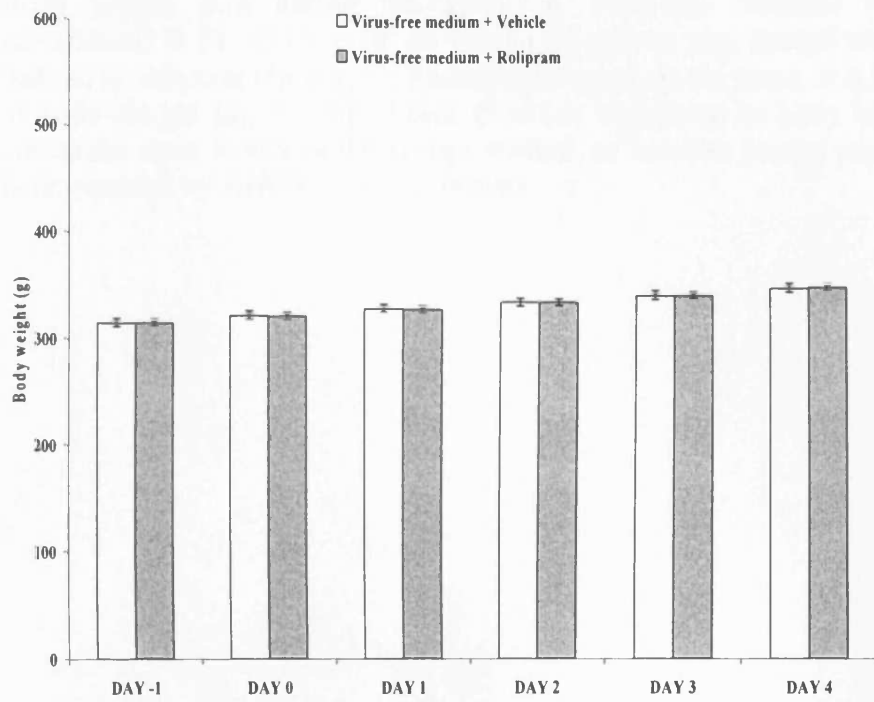
A significant reduction in respiratory rate was also observed from rolipram administration, although no effect was shown when measuring sG<sub>aw</sub>. Therefore, it may be suggested that inflammation and histology of the airways may have had some detrimental effect on respiratory function which induced an increase in respiratory rate, although this was not evident from conductance.

In this study, rolipram was shown to significantly reverse the increases in lung wet weights and protein levels in the lung induced by PIV-3. These were used as an index for assessing oedema associated with lung inflammation. The development, peak and recovery of leucocyte levels in the BAL fluid correlated temporally with wet lung weights and protein levels and all were attenuated by rolipram treatment. It can be suggested that this was most likely due to a reduction in inflammatory cell influx and a subsequent release of pro-inflammatory mediators, preventing oedema formation (Torphy; 1998). This is supported by a reduction in pulmonary lesions in PIV-3 infected guinea pigs treated with rolipram. Therefore, rolipram had a beneficial effect on pulmonary histopathology.

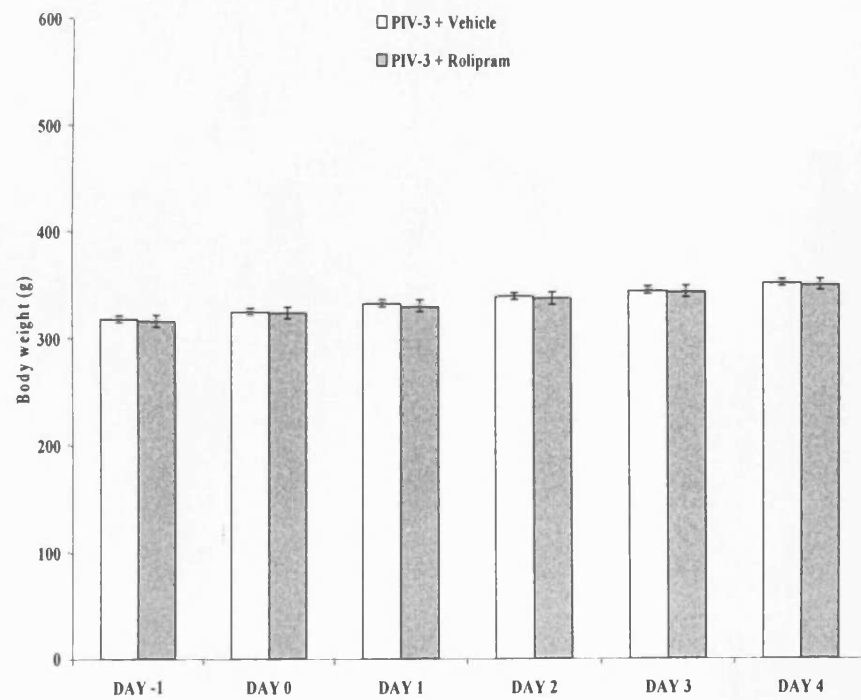
Treating PIV-3 infected guinea pigs with rolipram inhibited excess generation of airways NO. This is most likely related to their ability to suppress pro-inflammatory immune responses, since the source of the iNOS is presumably inflammation driven. Studies have shown that rolipram suppresses the pro-inflammatory inducers of iNOS gene activation (Torphy; 1998, Torphy *et al*; 1999). It can be suggested that rolipram inhibits respiratory burst activity and the release of superoxide (Torphy *et al*; 1999). Therefore, rolipram would reduce the formation of peroxynitrite from excess NO and superoxide, and thus attenuate any further peroxynitrite-induced exacerbation of the PIV-3 airway induced damage and AHR. In addition, PDE-4 inhibitors also suppress expression of the adhesion molecules, ICAM-1 and E-selectin responsible for leucocyte adhesion and facilitating recruitment into the airways (Torphy *et al*; 1999, Ikemura *et al*; 2001).

In conclusion, this study demonstrates that treating PIV-3 infected guinea pigs with rolipram inhibited the development of AHR, increased respiratory rate, influx of inflammatory cells, total protein and NO levels and pulmonary oedema and caused a reduction in lung lesions. However, it failed to significantly reduce viral replication in the lung. The findings of this study support a role for PDE-4 inhibitors in the management of the inflammatory consequences of viral infections of the respiratory tract. However, the failure to reduce viral titre and indeed promote a modest increase, may argue against their use.

**A**



**B**

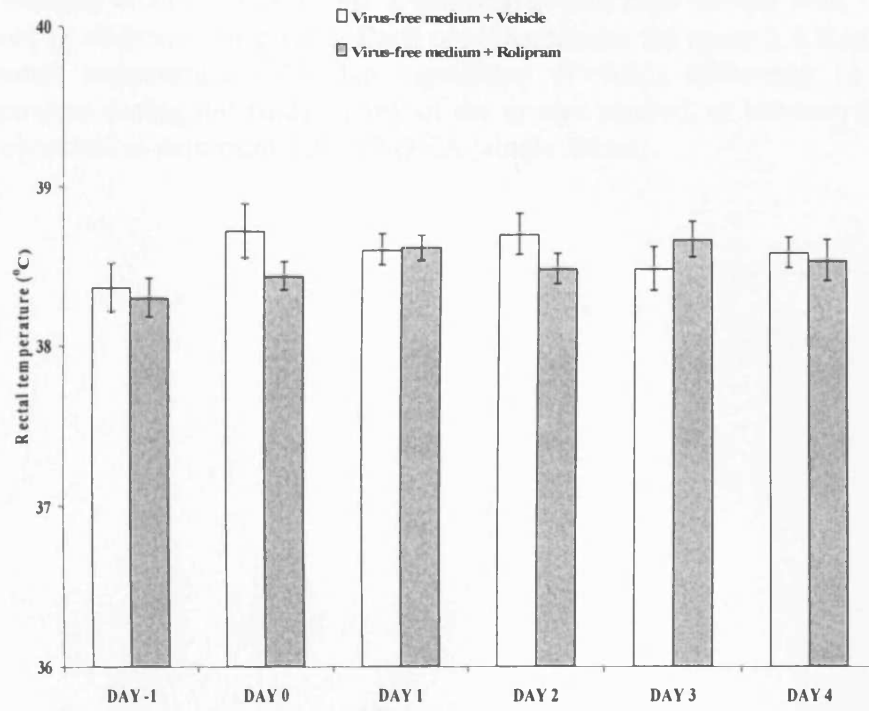




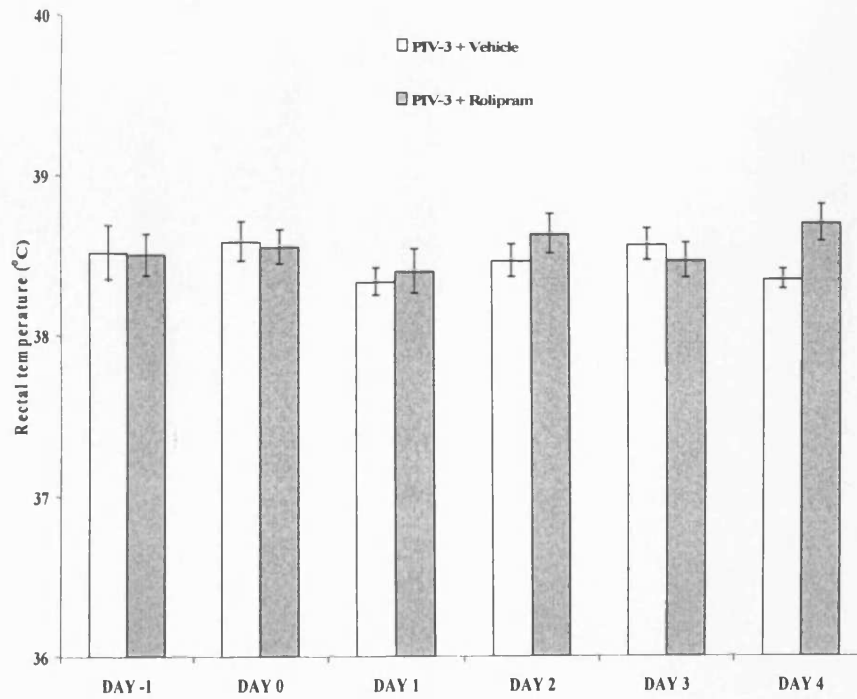
**Figure 7.1**

Body weight gain during the study. **A** Virus-free medium (Vero cell supernatant) **B** PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs treated with vehicle (saline) or rolipram ( $1 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M (n=6) of body weight (g). No significant ( $P > 0.05$ ) difference in body weight gain during the study in any of the groups studied, or between groups was observed as determined by ANOVA (single factor).

**A**



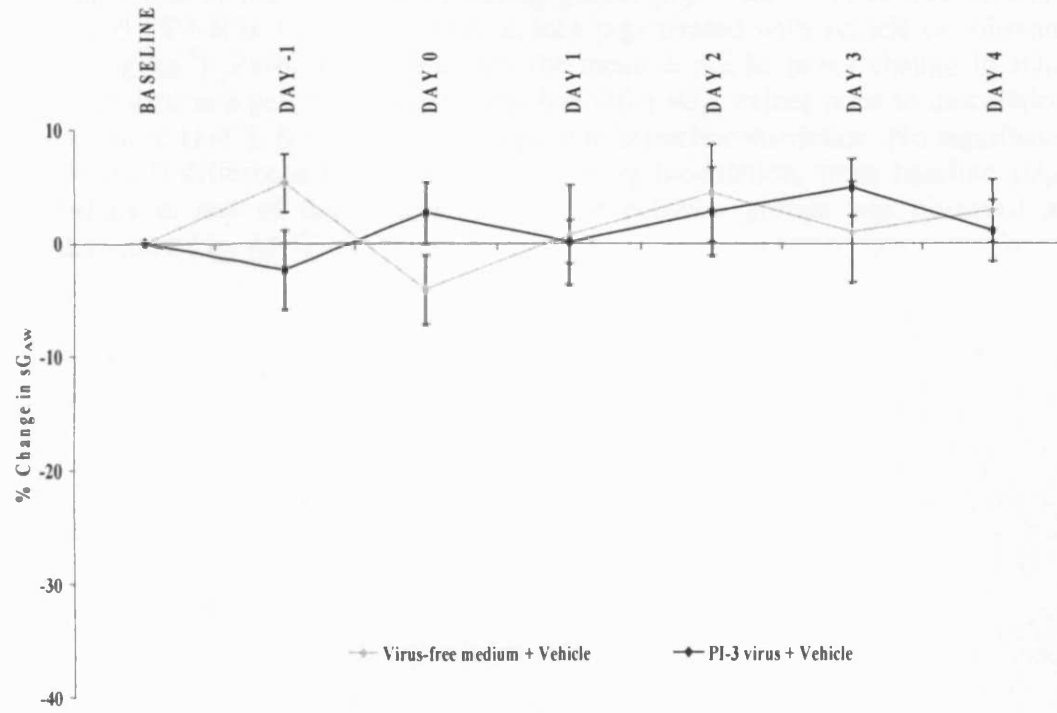
**B**



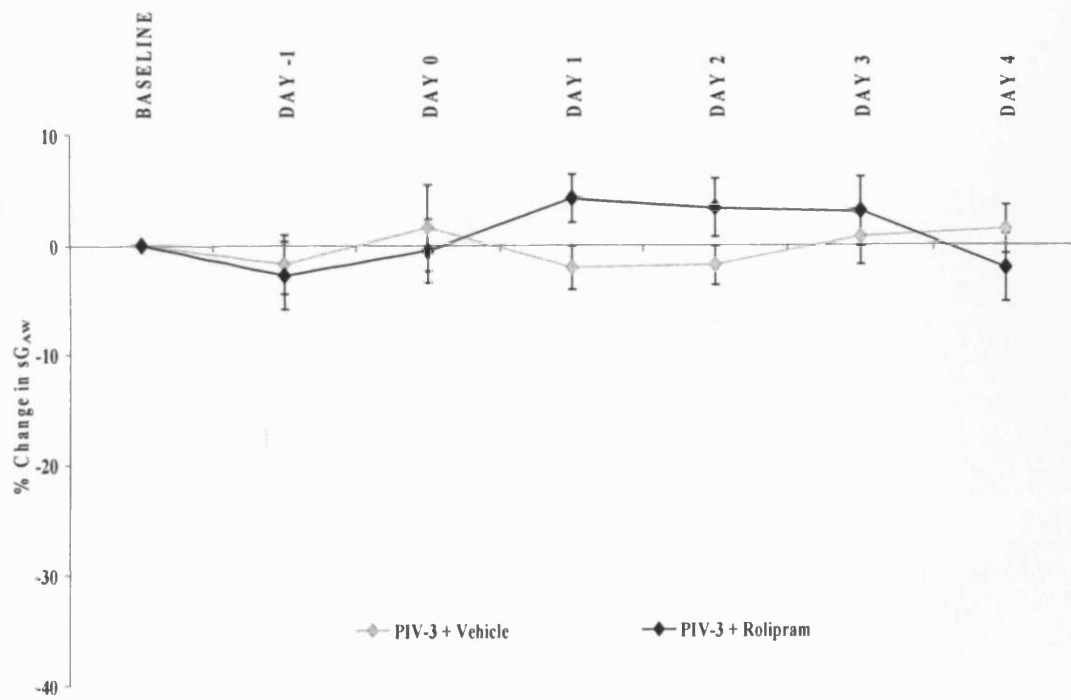
**Figure 7.2**

Rectal temperature during the study. **A** Virus-free medium (Vero cell supernatant) **B** PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs treated with vehicle (saline) or rolipram ( $1 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M (n=6) of rectal temperature ( $^{\circ}\text{C}$ ). No significant ( $P > 0.05$ ) difference in rectal temperature during the study in any of the groups studied, or between groups, was observed as determined by ANOVA (single factor).

A

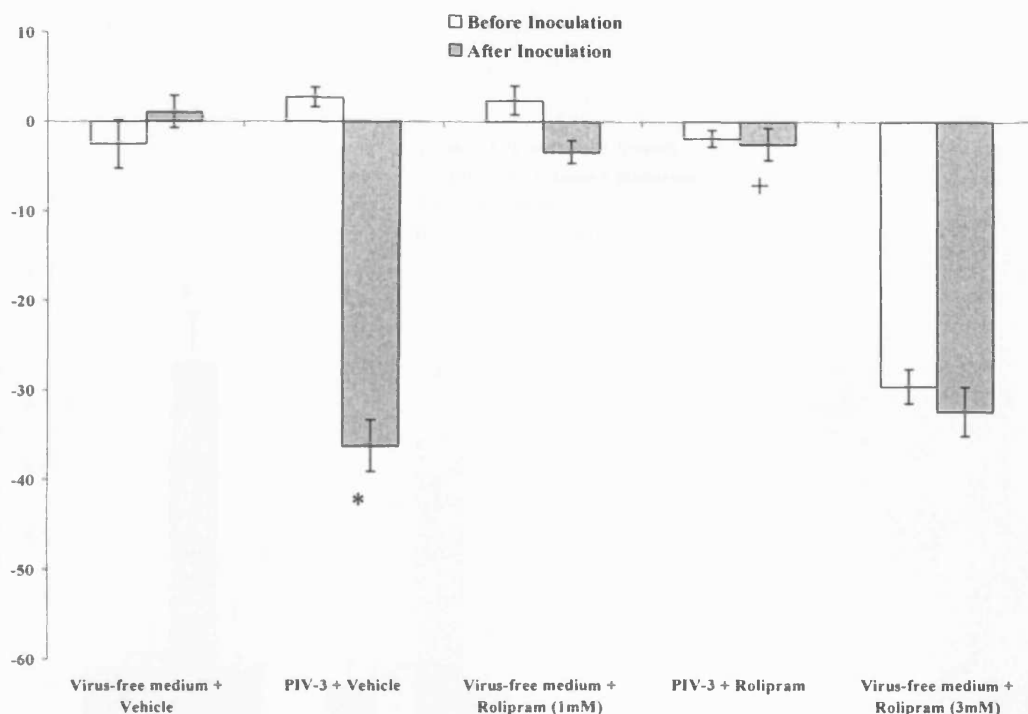


B



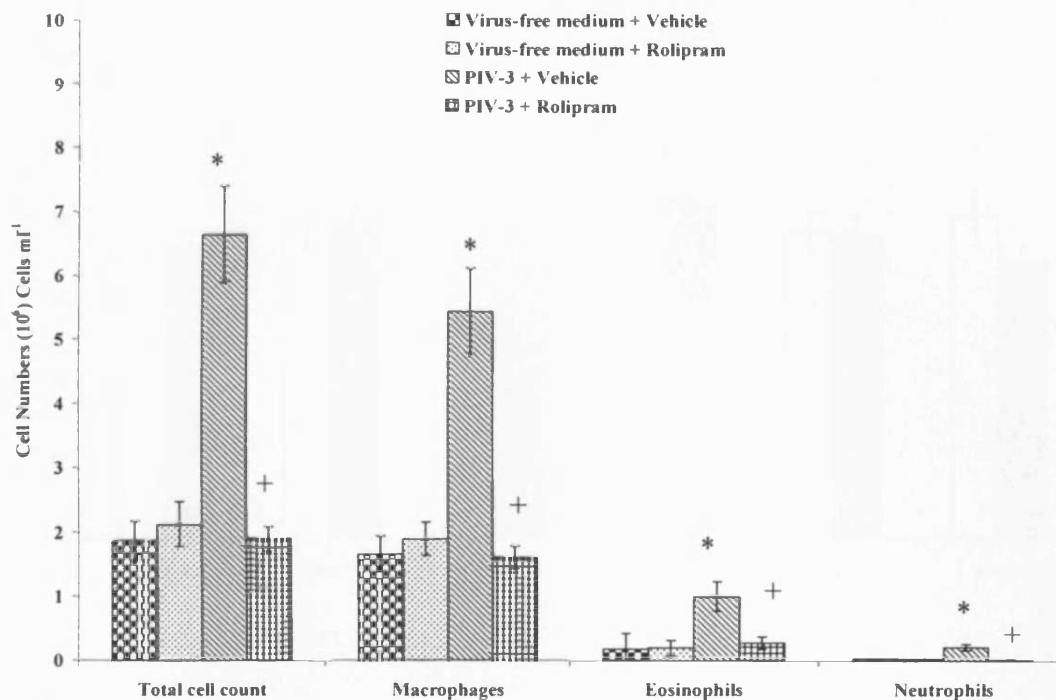
### Figure 7.3

The effect of intranasally inoculating guinea pigs with **A** Virus-free medium and **B** PIV-3( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs treated with vehicle or rolipram ( $1 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M (n=6) change in  $sG_{aw}$  expressed as a percentage of the baseline (BL)  $sG_{aw}$  values prior to inoculation ( $\text{s}^{-1} \text{cm of H}_2\text{O}^{-1}$ ). Negative values represent bronchoconstriction. No significant ( $P > 0.05$ ) difference in airway function after inoculation, from baseline  $sG_{aw}$  values in any of the groups studied, or between groups was observed as determined by ANOVA (single factor).



**Figure 7.4**

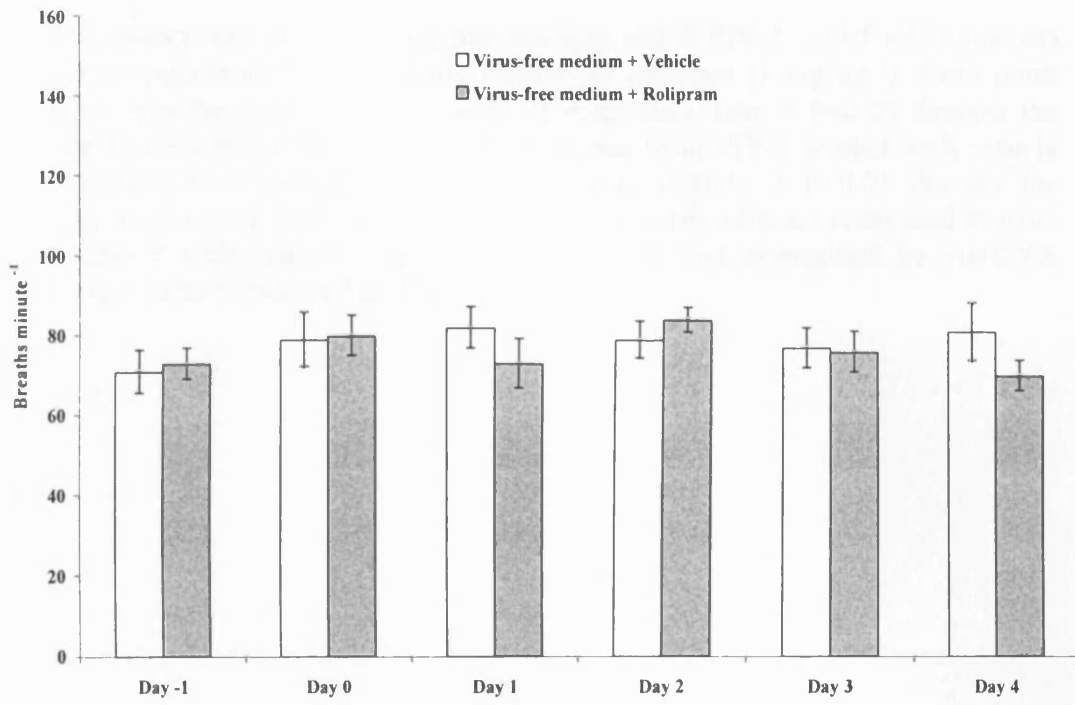
Peak percentage changes in the airway responses of conscious guinea pigs to a nose-only challenge with histamine (1mM, 20 seconds), 24 hours before the commencement of virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6$  ml<sup>-1</sup>) inoculation and 4 days post inoculation. Also shown is the peak response to a bronchoconstrictor dose (3mM, 20 seconds) of histamine 24 hours and 4 days post inoculation with virus-free medium after treatment with rolipram (1mg kg<sup>-1</sup>). Animals were treated with vehicle (saline) or rolipram (1 mg kg<sup>-1</sup>). Each point represents the mean  $\pm$  S.E.M (n=6) of the peak change from baseline  $sG_{aw}$  (s<sup>-1</sup>cm of H<sub>2</sub>O<sup>-1</sup>), 0-10 minutes post histamine exposure. Negative values represent bronchoconstriction. \* P<0.05 denotes the significance of differences between response to inhaled histamine, 24 hours before inoculation and 4 days post inoculation. + P<0.05 denotes the significance of differences between post inoculation day 4 in PIV-3 treated with rolipram compared to vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



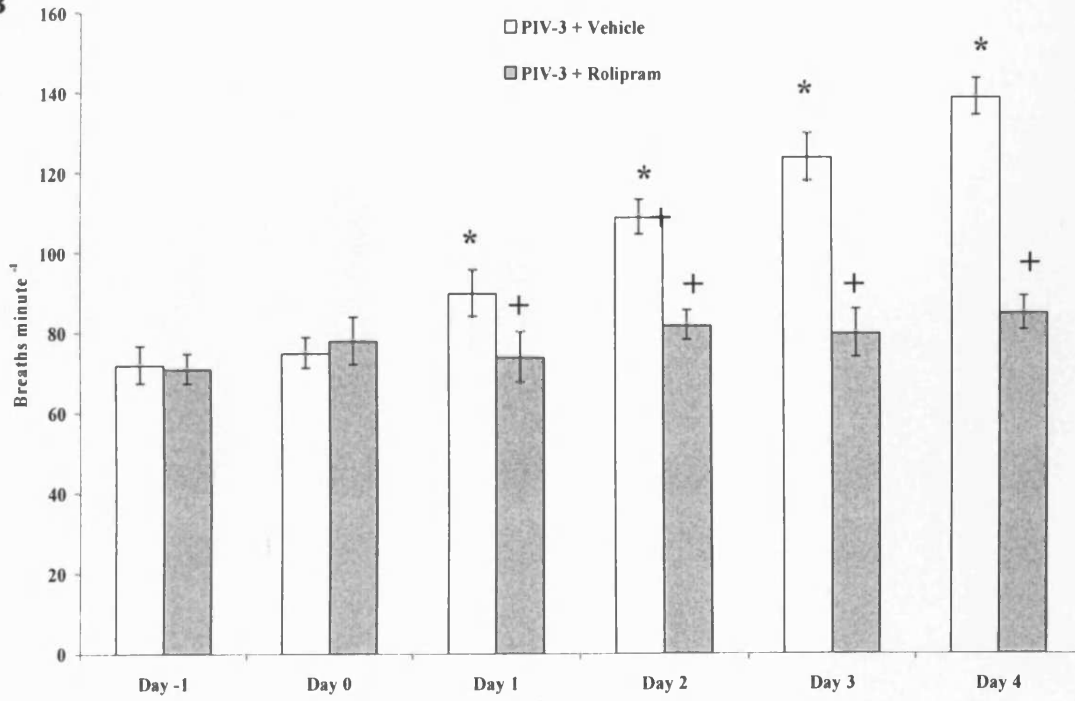
**Figure 7.5**

Total and differential cell (macrophage, eosinophil and neutrophil) counts in BAL fluid removed from virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 ml^{-1}$ ) infected guinea pigs treated with vehicle (saline) or rolipram ( $1 mg kg^{-1}$ ). Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of the cells per BAL fluid sample ( $\times 10^6$  cells  $ml^{-1}$ ). \*  $P < 0.05$  denotes the significance of differences in airway cell influx removed from PIV- treated with vehicle compared to virus-free medium treated with vehicle +  $P < 0.05$  denotes the significance of differences in airway cell influx removed from PIV-3 treated with rolipram compared to PIV-3 treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

A



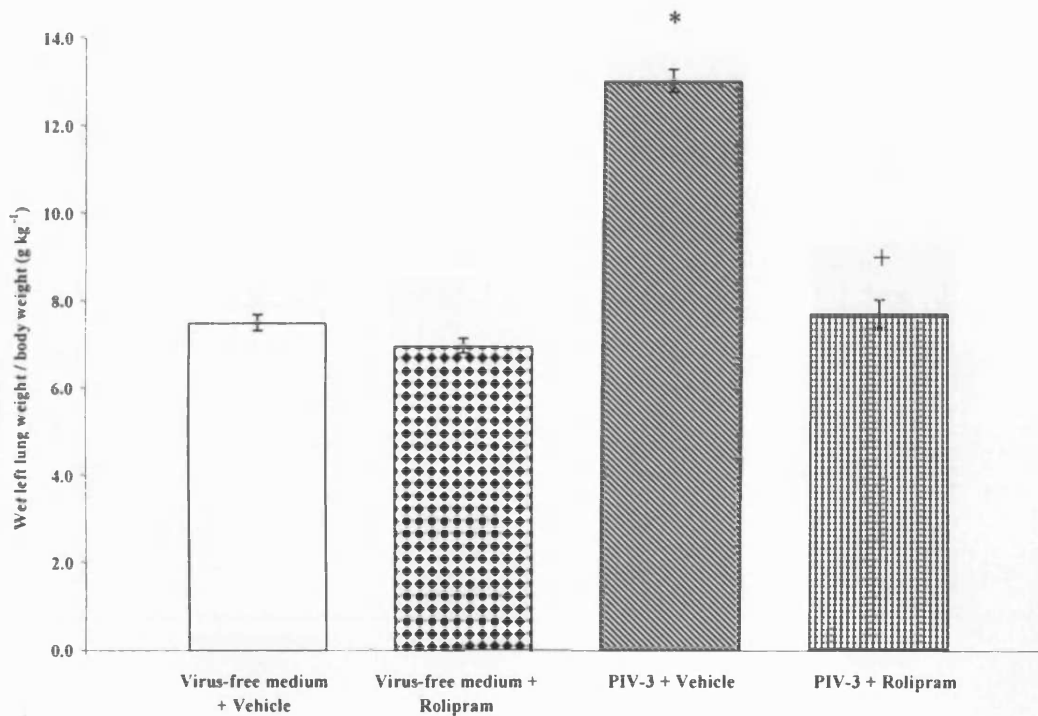
B





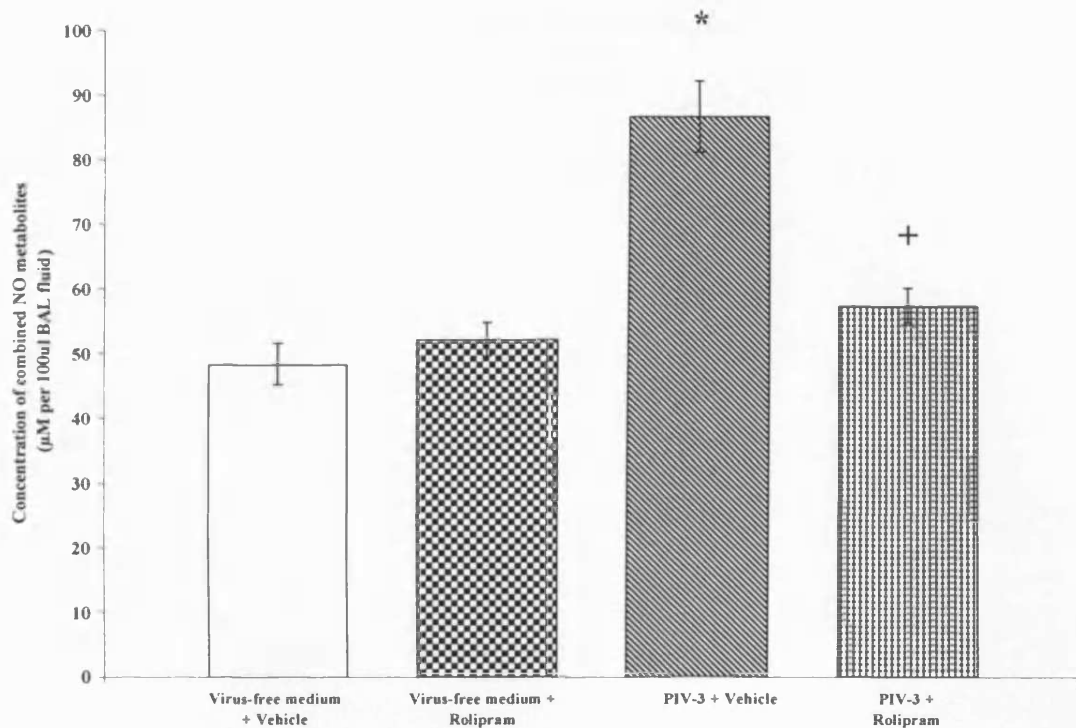
### **Figure 7.6**

Respiratory rate from **A** Virus-free medium and **B** PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs treated with vehicle (saline) or rolipram ( $1 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of respiratory rate. \*  $P < 0.05$  denotes the significance of differences in respiratory rate from PIV-3 treated with vehicle compared to virus-free medium treated with vehicle. +  $P < 0.05$  denotes the significance of differences from PIV-3 treated with rolipram compared to PIV-3 treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



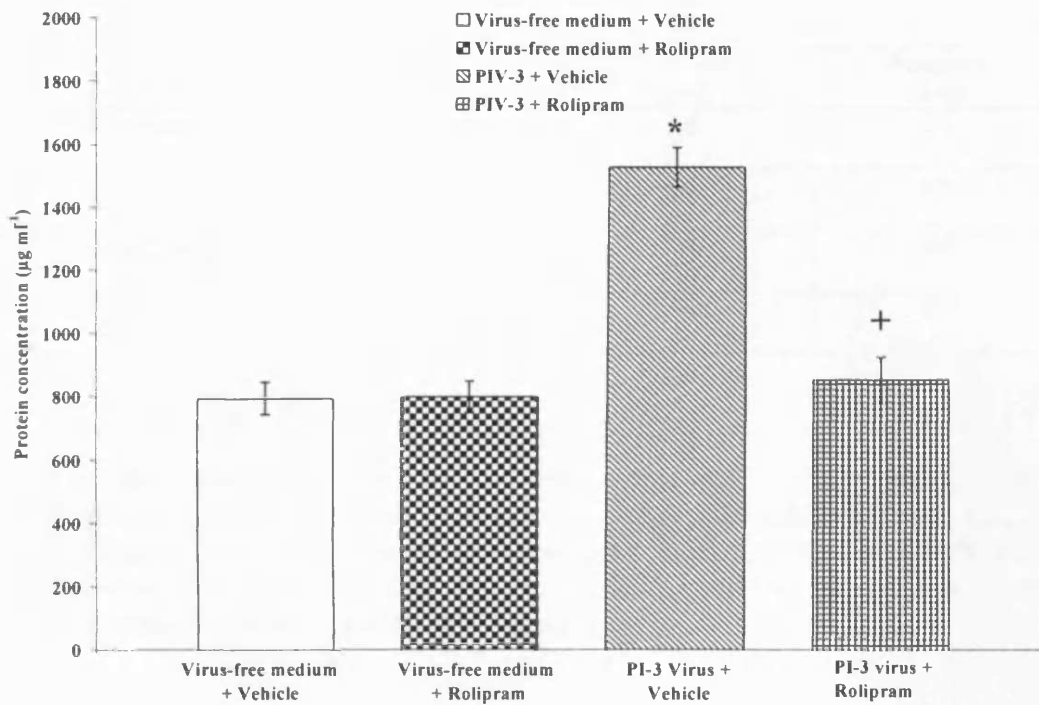
**Figure 7.7**

Wet left lung weights from virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs treated with vehicle (saline) or rolipram ( $1 \text{ mg kg}^{-1}$ ). Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of wet left lung weights normalised to body weight ( $\text{g kg}^{-1}$ ). \*  $P < 0.05$  denotes the significance of differences in PIV-3 treated with vehicle compared to virus-free medium infected guinea pigs treated with vehicle, +  $P < 0.05$  denotes the significance of differences in PIV-3 treated with rolipram compared to PIV-3 treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



**Figure 7.8**

Total nitric oxide concentration in BAL fluid removed from guinea pigs infected with virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6$  TCID<sub>50</sub> ml<sup>-1</sup>) treated with vehicle or rolipram (1 mg kg<sup>-1</sup>). Each point represents the mean  $\pm$  S.E.M (n=6) of the total nitric oxide concentration per sample of ( $\mu$ M). ). \* P<0.05 denotes the significance of differences in PIV-3 treated with vehicle compared to virus-free medium infected guinea pigs treated with vehicle, + P<0.05 denotes the significance of differences in PIV-3 treated with rolipram compared to PIV-3 treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



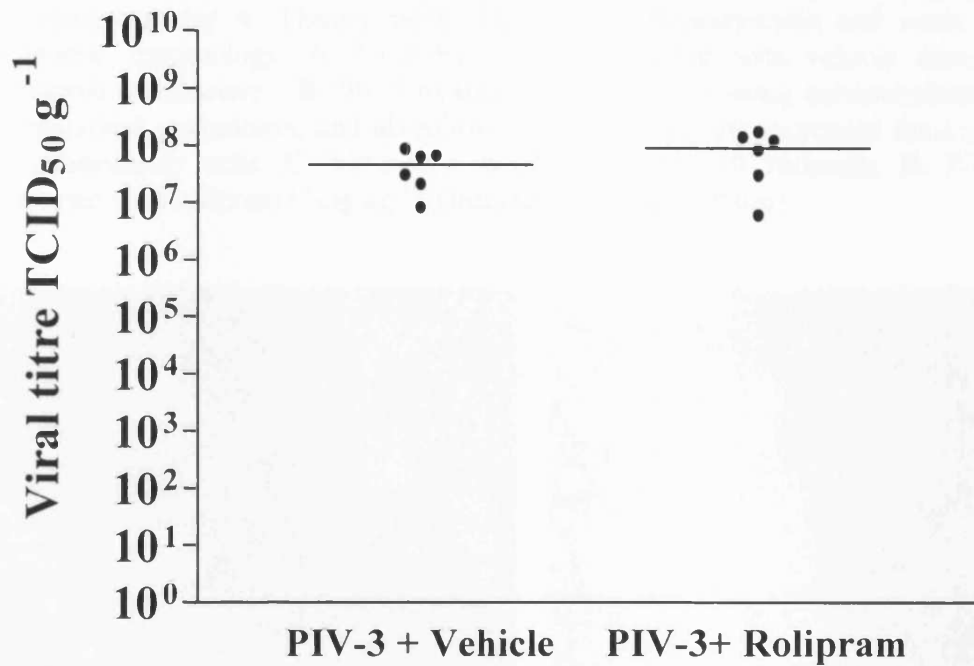
**Figure 7.9**

Total protein levels in BAL fluid removed from guinea pigs inoculated with virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) treated with vehicle (saline) or rolipram ( $1 \text{ mg kg}^{-1}$ ). Each bar represents the mean  $\pm$  S.E.M ( $n=6$ ) of the total protein levels per sample ( $\mu\text{g ml}^{-1}$ ). \*  $P < 0.05$  denotes the significance of differences in PIV-3 treated with vehicle compared to virus-free medium infected guinea pigs treated with vehicle, +  $P < 0.05$  denotes the significance of differences in PIV-3 treated with rolipram compared to PIV-3 treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

		<b>Vehicle (n=6)</b>	<b>Rolipram (n=6)</b>
<b>BAL Fluid</b>	<b>Virus-free medium</b>	0/6	0/6
	<b>PIV-3</b>	6/6	6/6
<b>Right Lung Tissue</b>	<b>Virus-free medium</b>	0/6	0/6
	<b>PIV-3</b>	6/6	6/6

**Table 7.1**

This demonstrates the number of guinea pigs out of each group, which displayed evidence of PIV-3 growth on Vero cells using standard culture techniques and positive immunofluorescence in BAL fluid, and right lung tissue samples from virus-free medium and PIV-3 infected guinea pigs treated with vehicle (saline) or rolipram (1mg kg<sup>-1</sup>).

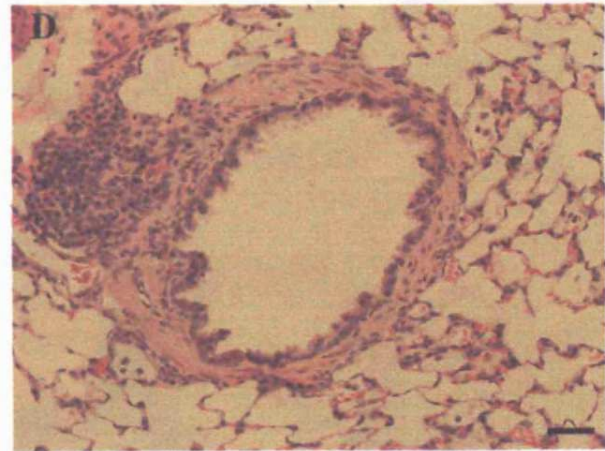
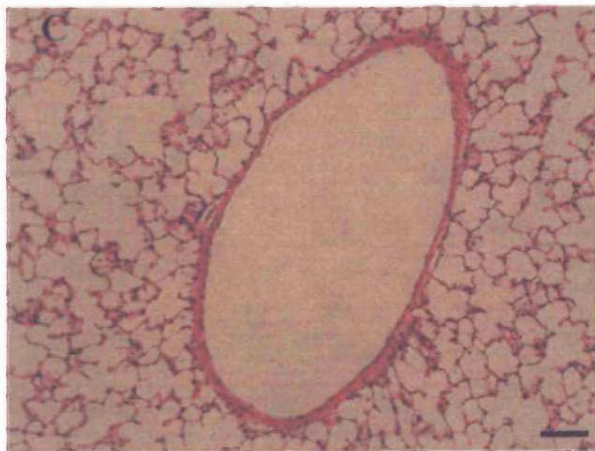
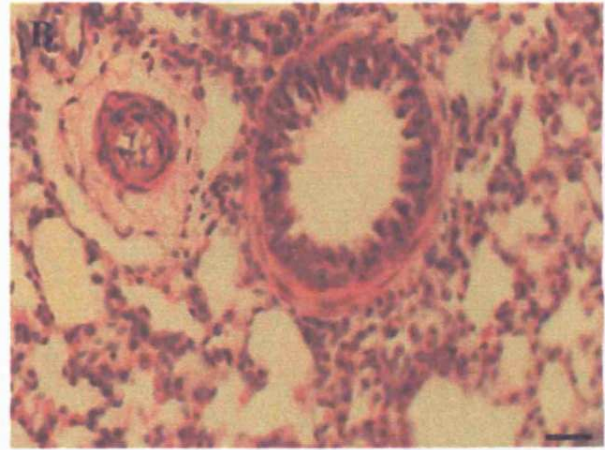
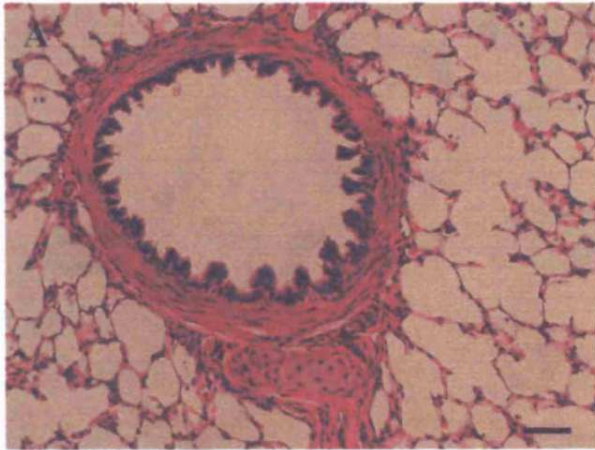


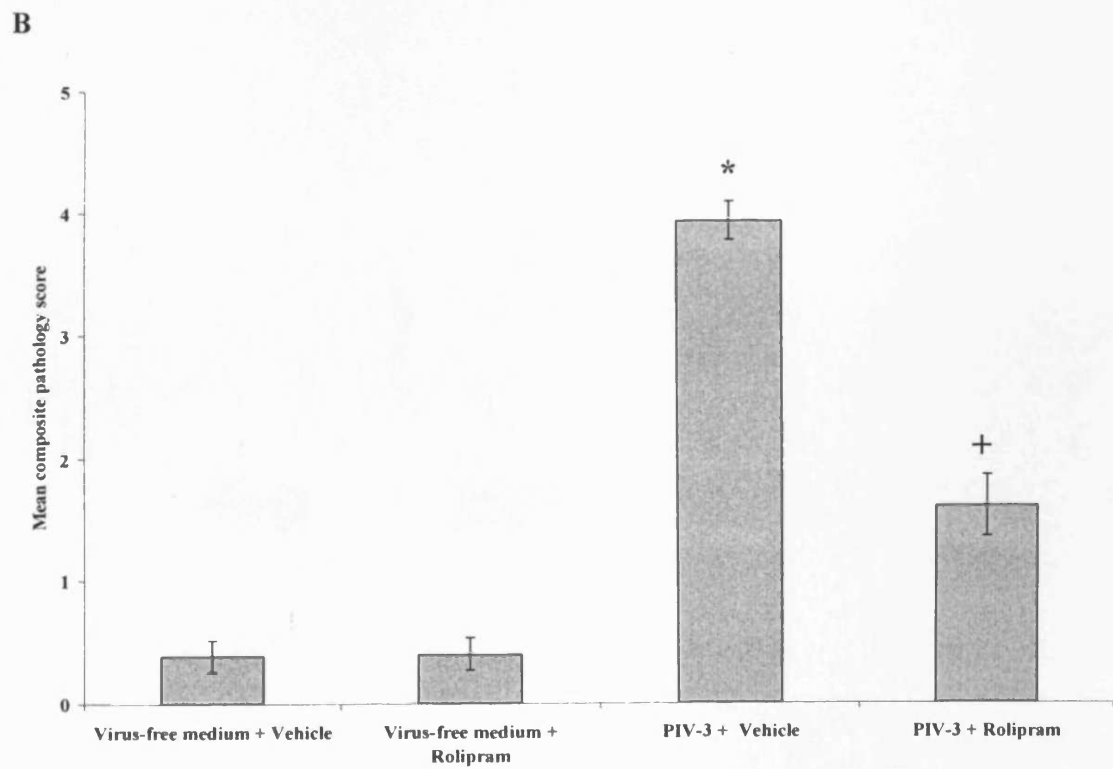
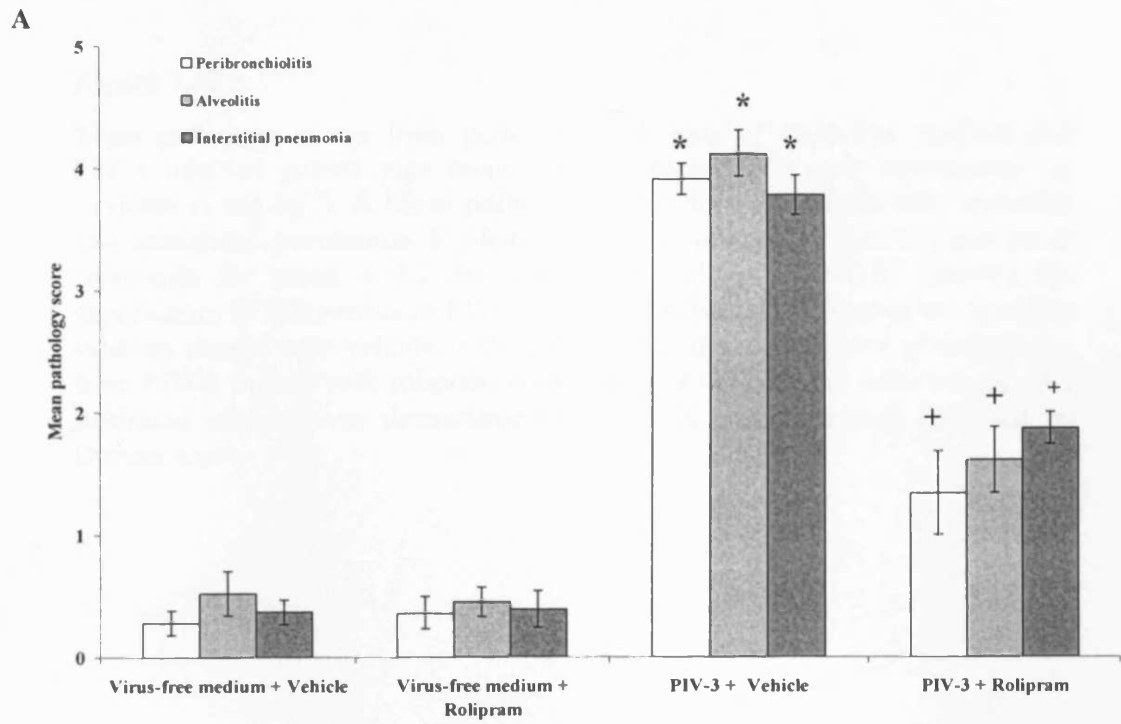
**Figure 7.10**

Titres of intrapulmonary replicating PIV-3 isolated from right lung in PIV-3 infected guinea pigs treated with vehicle or rolipram 4 days post inoculation. Each point represents the mean  $\pm$  S.E.M of titres of intrapulmonary replicating PIV-3 normalised to wet left lung weight (TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>). No significant ( $P > 0.05$ ) in viral titre (TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>) from PIV-3 infected guinea pigs treated with rolipram compared to vehicle as determined by ANOVA (single factor).

**Figure 7.11**

Histological appearance of lung tissue from PIV-3 infected guinea pigs post inoculation day 4. Tissues were stained with haematoxylin and eosin for general morphology. **A.** Virus-free medium treated with vehicle showing normal architecture. **B** PIV-3 treated with vehicle showing peribronchiolitis, interstitial pneumonia, and alveolitis as indicated by the increased density of inflammatory cells. **C.** Virus-free medium treated with rolipram. **D.** PIV-3 treated with rolipram ( $1\text{mg kg}^{-1}$ ). (Internal scale bar =  $50\mu\text{m}$ ).







**Figure 7.12**

Mean pathology scores from guinea pig left lung of virus-free medium and PIV-3 infected guinea pigs treated with vehicle (Vero cell supernatant) or rolipram ( $1 \text{ mg kg}^{-1}$ ). **A** Mean pulmonary score for peribronchiolitis, alveolitis and interstitial pneumonia **B** Mean composite pulmonary score. Each point represents the mean  $\pm$  S.E.M of mean pathology. \*  $P < 0.05$  denotes the significance of differences in PIV-3 treated with vehicle compared to virus-free medium treated with vehicle. +  $P < 0.05$  denotes the significance of differences from PIV-3 treated with rolipram compared to PIV-3 treated with vehicle. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

	Virus-free medium + Vehicle (n=6)	Virus-free medium + Rolipram (n=6)	PIV-3+ Vehicle (n=6)	PIV-3 + Rolipram (n=6)	
Body weight (g)	346.3±4.1	347.1±2.9	351.7±3.3	349.8±5.2	
Rectal temp (°C)	38.5±0.1	38.5±0.1	38.4±0.1	38.7±0.1	
Lung function (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )	2.9±2.7	1.1±2.7	1.3±2.2	-2.2±3.1	
Lung function before histamine exposure (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )	2.8±1.9	2.4±1.6	2.7±1.1	-1.9±0.9	
Lung function after histamine exposure (s <sup>-1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )	1.1±0.8	-3.4±1.3	-36.5±2.9	-2.5±1.8	
Total Cells (10 <sup>6</sup> cells ml <sup>-1</sup> )	1.9±0.3	2.1±0.34	6.7±0.8	1.9±0.2	
Macrophages (10 <sup>6</sup> cells ml <sup>-1</sup> )	1.7±0.3	1.9±0.3	5.5±0.7	1.6±0.2	
Eosinophils (10 <sup>6</sup> cells ml <sup>-1</sup> )	0.2±0.3	0.2±0.1	1.0±0.2	0.3±0.1	
Neutrophils (10 <sup>6</sup> cells ml <sup>-1</sup> )	0.03±0.1	0.03±0.01	0.2±0.1	0.02±0.1	
Respiratory rate (breaths min <sup>-1</sup> )	81.0±4.0	70.0±4.0	139.0±7.0	85.0±5.0	
Wet lung weights (g Kg <sup>-1</sup> )	6.5±0.4	7.2±0.2	11.8±0.39	8.1±0.30	
Total nitrate and nitrite (µM)	48.1±2.9	51.6±3.7	76.3±4.5	50.8±3.3	
Protein levels (µg ml <sup>-1</sup> )	793.4±50.6	798.6±49.2	1528.8±62.7	856.3±69.6	
Isolation in BAL fluid	0/6	0/6	6/6	6/6	
Isolation in Right lung	0/6	0/6	6/6	6/6	
Viral titre (TCID <sub>50</sub> ml <sup>-1</sup> g <sup>-1</sup> )	Not Detected	Not Detected	4.6×10 <sup>7</sup> ±1.5×10 <sup>7</sup>	3.10×10 <sup>8</sup> ±2.2×10 <sup>8</sup>	
Mean pulmonary score	Peribronchiolitis	0.3±0.1	0.4±0.1	3.9±0.1	1.3±0.3
	Alveolitis	0.5±0.2	0.5±0.1	4.1±0.2	1.6±0.3
	Interstitial pneumonia	0.4±0.1	0.4±0.2	3.8±0.2	1.9±0.1
Mean composite pulmonary score	0.4±0.1	0.4±0.1	3.9±0.2	1.6±0.3	

Table 7.2

Summary of chapter 7 results from virus-free medium and PIV-3 infected guinea pigs treated with vehicle (saline) or rolipram (1 mg kg<sup>-1</sup>) measured post inoculation day 4.

## **CHAPTER 8**

### **EFFECT OF ALLERGEN PRE-SENSITISATION FOLLOWING PIV-3 INFECTION IN GUINEA PIGS**

#### **INTRODUCTION**

Asthma is a chronic inflammatory lung disease associated with intermittent airflow obstruction, AHR and infiltration of the respiratory mucosa with inflammatory cells, mainly eosinophils (Frick *et al*; 1979). Multiple factors are involved in the pathogenesis of allergic asthma, but a critical element is the initiation of aberrant immune responsiveness against inhaled environmental respiratory allergens, characterised by the development of allergen-specific IgE and the presence of allergen-specific CD4<sup>+</sup> T cells producing IL-4, IL-5, and IL-13, but not IFN- $\gamma$  (Burrows *et al*; 1989, Martinez *et al*; 1995).

Both allergic and non-allergic individuals are exposed to environmental aeroallergens, but only allergic individuals develop allergen-specific Th2-biased immune responses to these allergens. Non-allergic individuals remain tolerant to allergen exposure because they lack allergen-specific IgE, lack functional immune reactivity to these allergens, or develop protective allergen specific Th1-biased responses. The development of asthma is linked not only to respiratory allergen sensitisation, but also to viral respiratory tract infections in early childhood (Burrows *et al*; 1989, Martinez *et al*; 1995).

Clinically, respiratory virus infection has been proposed as a common triggering factor in the development of allergy in children, and could also account for a large number of such episodes in adults (Frick *et al*; 1979, Gurwitz *et al*; 1981, Zweiman *et al*; 1971). It is well known that asthma symptoms such as wheezing worsen after respiratory viral infection (Frick *et al*; 1979). Eighty percent of wheezing episodes in school children and 76% of asthma exacerbations in adults were associated with respiratory infections (Johnson *et al*; 1995, Johnson; 1998, Wark *et al*; 2002). Among the respiratory viruses implicated in childhood asthma are RSV, RV, PIV-3 and coronavirus. (McIntosh *et al*, 1973).

Animal experiments support the hypothesis that there is a causal link between viral bronchiolitis and asthma. In guinea pigs, RSV infection causes increased sensitivity to inhaled histamine for at least 6 weeks, which is associated with viral persistence in the lung (Robinson *et al*; 1997). Brown Norway rats develop chronic episodic, and reversible airway obstruction after bronchiolitis. In this mode, a strong CD4+ T cell response is present, with reduced interferon  $\gamma$  production, persistent inflammation, fibrosis, and deposition of extracellular matrix material leading to airway remodelling (Kumar *et al*; 1997). Further mechanisms by which viral infections could effect later development of lung disease are virus chronicity, persistence or latency (Dakhama *et al*; 1997). Schwarze *et al* (1999) showed that in mice, inhalation of an antigen after RSV infection increased both airway responsiveness and eosinophil influx in the lung. There is also strong evidence from animal models that viral infections interact with inhaled allergens to promote the development of airway inflammation and atopy. Uninfected mice do not develop IgE antibody against inhaled antigen, but during acute influenza infection sensitisation takes place (Sakamoto *et al*; 1984). When antigen exposure coincides with influenza infection, airway responsiveness to inhaled methacholine increases with serum IgE rises; however, neither influenza A virus or ovalbumin alone causes these changes (Suzuki *et al*; 1998).

Inhaled nebulised ovalbumin induces systemic sensitisation if the protein is inhaled in the presence, but not in the absence, of acute viral infections. This sensitisation is sufficient to cause acute anaphylactic collapse during subsequent challenge with ovalbumin (O'Donnell & Openshaw 1998). This data suggest that viral inflammation allows inhaled antigen to penetrate the barrier of the respiratory mucosa, promoting systemic sensitisation. This suggests specific interactions between respiratory allergies and viral infections exist, where viral illnesses in early childhood may enhance the development of allergen sensitisation. However, this relationship remains controversial, as many other epidemiological studies suggest that viral and bacterial infections, in fact, protect against the development of asthma and allergy (Shaheen *et al*; 1996, Strachan; 1989, Martinez; 1994, Shirakawa *et al*; 1997).

This controversial idea, is termed the 'hygiene hypothesis' and states that the risk of developing allergies and asthma is inversely related to the number of children in the family (Strachen; 1989). This finding has led to speculation that infectious diseases, which are more likely to be transmitted in large families, could modulate the development of the immune system in such a way as to reduce the chances of developing allergies. This hypothesis implies that the immune system is skewed towards a Th2-like response pattern at birth (Prescott *et al*; 1999). According to the theory, each viral infection would provide a stimulus for the development and/or activation of Th1-like immune responses.

Overall, there is considerable controversy with regard to the potential role of viral respiratory tract infections, in allergic sensitisation. Thus, interactions of respiratory viruses with allergen sensitisation and allergic inflammation of the airways are of particular interest, since these constitute the underlying pathomechanisms of asthma.

The purpose of this study was to determine the effects of exposure to ovalbumin in pre-sensitised guinea pigs during the acute phase PIV-3 virus infection. The parameters investigated included airway function, airway reactivity to histamine, allergen exposure to ovalbumin, cellular infiltration, respiratory rates, wet lung weight, nitric oxide and total protein levels in BAL fluid, pulmonary histopathology and recovery of virus from the lung tissue. This study also assessed the effect of the mode of delivery of ovalbumin either by inhalation or intraperitoneal injection.

### EXPERIMENTAL PROTOCOL

The following protocols used to perform this study have all been previously described in chapter 2.

To examine the influence of exposure to ovalbumin in pre-sensitised guinea pigs during the acute phase PIV-3 infection the following experimental groups each including 6 animals were as follows.

GROUP	
1	Virus-free medium infected guinea pigs sham-sensitised with saline (inhalation) and challenged with saline.
2	PIV-3 infected guinea pigs sham-sensitised with saline and challenged (inhalation) with saline.
3	Virus-free medium infected guinea pigs sensitised with ovalbumin (inhalation) and challenged with ovalbumin (inhalation).
4	PIV-3 infected guinea pigs sensitised with ovalbumin (inhalation) and challenged with ovalbumin (inhalation).
5	Virus-free medium infected guinea pigs sham-sensitised with saline (intraperitoneal) and challenged with saline.
6	PIV-3 infected guinea pigs sham-sensitised with saline (intraperitoneal) and challenged with saline.
7	Virus-free medium infected guinea pigs sensitised with ovalbumin (intraperitoneal) and challenged with ovalbumin (inhalation).
8	PIV-3 infected guinea pigs sensitised with ovalbumin (intraperitoneal) and challenged with ovalbumin (inhalation).

On study day -1 guinea pigs were assessed for airway reactivity to histamine 24 hours before inoculation with virus. On study day 0 guinea pigs were inoculated with either virus-free medium (Vero cells) or PI-3 virus ( $5 \times 10^6$  TCID<sub>50</sub> units ml<sup>-1</sup>) by intranasal instillation. Four days later (day 4) guinea pigs were sensitised during the acute phase of infection (day 3-7) with ovalbumin either 0.5% inhaled or injected via the intra-peritoneal cavity. The inhalation challenge was for 3 minutes in a sealed chamber and a booster inhalation was administered 14 days later (day 18). The antigen sensitisation time points were chosen based on respiratory parameters observed in PI-3 virus inoculated guinea pigs by Riedel *et al* (1996). On day 24 guinea pigs were exposed to an aerosol of 0.01% ovalbumin or saline for 60 minutes. They were then monitored for specific airway conductance (sG<sub>aw</sub>) for regular intervals up to 24 hours.

Airway reactivity to histamine (1mM, 20 seconds) was again assessed on day 25 and within 20 minutes guinea pigs were sacrificed, BAL performed and lung tissue removed. BAL fluid specimens were analysed for virus-induced changes by comparison of total and differential cell counts and viral determination. Lung tissue specimens were used for total viral content per g of lung and histological analysis. BAL fluid specimens were used to determine nitric oxide and total protein concentrations. Throughout the study each animal was weighed and basal sG<sub>aw</sub> and rectal temperature recorded on appropriate study days. Animals were also examined daily for general physical signs of illness including hair loss and decreased motor activity.

## RESULTS

### ***Effect of PIV-3 on body weight gain in sensitised guinea pigs***

The body weight gain throughout the study did not significantly differ between guinea pigs that received intranasal inoculation with virus-free medium or PIV-3 or in guinea pigs sensitised with ovalbumin or saline (Inhalation or intraperitoneal) and challenged with either saline or 0.01% ovalbumin (Figure 8.1).

### ***Effect on rectal temperature in PIV-3 sensitised guinea pigs***

Rectal temperature did not alter significantly ( $P>0.05$ ) throughout the study between guinea pigs receiving intranasal inoculation with virus-free medium or PIV-3 and sensitised with ovalbumin or saline (Inhalation or intraperitoneal) and challenged with either saline or 0.01% ovalbumin (Figure 8.2).

### ***Effect of PIV-3 on lung function in sensitised guinea pigs***

Intranasal inoculation with virus-free medium or PIV-3 of guinea pigs sensitised with ovalbumin or saline (Inhalation or intraperitoneal) and challenged with either saline or 0.01% ovalbumin failed to significantly ( $P>0.05$ ) alter lung function during any time point investigated throughout the study (Figure 8.3).

### ***Effect of PIV-3 on airway reactivity to inhaled histamine in sensitised guinea pigs***

Inhalation of histamine (1mM) for 20 seconds 24 hours before intranasal inoculation of virus-free medium or PIV-3 did not produce any significant ( $P>0.05$ ) bronchoconstriction in any of the groups studied (Figure 8.4).



PIV-3 infected guinea pigs sensitised via inhalation with saline or ovalbumin and challenged with ovalbumin on day 25, produced a significant ( $P<0.05$ ) bronchoconstriction ( $-23.4\pm 2.4\%$ ,  $-36.0\pm 4.3\%$   $\text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ) after inoculation compared to virus-free medium infected guinea pigs sensitised via inhalation with saline or ovalbumin and challenged with ovalbumin ( $1.6\pm 2.7\%$ ,  $-11.7\pm 3.0\%$   $\text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ), thus indicating AHR.

AHR to histamine was also observed in PIV-3 infected guinea pigs sensitised via administration of either saline or ovalbumin via intra-peritoneal cavity and challenged with ovalbumin on day 25. A significant ( $P<0.05$ ) bronchoconstriction with peak reductions in  $sG_{aw}$  ( $-25.6\pm 2.09\%$ ,  $-18.93\pm 2.51\%$   $\text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ) compared to after inoculation in virus-free medium infected guinea pigs sensitised via the intraperitoneal cavity with saline or ovalbumin and challenged with aerosolised ovalbumin compared with before inoculation ( $-1.4\pm 2.7\%$ ,  $-9.7\pm 1.6\%$ ), thus indicating AHR. Inhalation of histamine produced a significantly greater bronchoconstriction ( $P<0.05$ ) in PIV-3 infected guinea pigs sensitised with via inhalation ( $-36.1\pm 4.3\%$   $\text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ) compared with intraperitoneal administration ( $-25.6\pm 2.1\%$   $\text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ). Histamine was also greater after PIV-3 compared with ovalbumin alone.

***Effect of allergen exposure on airway function in PIV-3 sensitised guinea pigs.***

Virus-free medium and PIV-3 infected guinea pigs sensitised with saline via inhalation and intraperitoneal administration and challenged with saline did not produce any significant ( $P>0.05$ ) bronchoconstriction and failed to cause any significant deviation from baseline  $sG_{aw}$  values throughout the 12 hours following challenge (Figure 8.5). However, in PIV-3 virus infected guinea pigs sensitised with ovalbumin (inhalation) and challenged with ovalbumin produced a sustained bronchoconstriction after ovalbumin challenge which remained for 12 hours with a mean peak fall in  $sG_{aw}$  from basal values ( $-56.2\pm 3.3\%$   $\text{H}_2\text{O}^{-1}\text{sec}^{-1}$ ).

These results are in comparison to virus-free medium inoculated guinea pigs sensitised with ovalbumin (inhalation) and challenged with saline which produced an immediate bronchoconstriction from basal values ( $-35.8 \pm 3.6\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ) after challenge. This early phase antigen-induced bronchoconstriction response (EAR) recovered to baseline values 5 hours later. A late phase antigen response (LAR) of bronchoconstriction followed between 6-8 hours with a mean peak fall in  $sG_{aw}$  ( $-22.6 \pm 3.8\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ). Airway function recovered to baseline values at 12 hours after ovalbumin exposure and was unaltered at 24 hours (Figure 8.5).

PIV-3 infected guinea pigs sensitised with ovalbumin (intraperitoneal) and challenged with ovalbumin produced an immediate bronchoconstriction from basal values ( $-33.2 \pm 3.0\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ) after challenge. This EAR recovered to baseline values 3 hours later. A LAR followed between 6-8 hours with mean falls in  $sG_{aw}$  ( $-20.6 \pm 2.3\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ). These results are in comparison to virus-free medium inoculated guinea pigs sensitised with ovalbumin and challenged with ovalbumin which produced an immediate bronchoconstriction from basal values ( $-25.6 \pm 3.39\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ) after challenge. This EAR recovered to baseline values 3 hours later followed by a LAR between 6-8 hours with a mean peak fall in  $sG_{aw}$  of ( $-8.3 \pm 1.7\% \text{ H}_2\text{O}^{-1}\text{sec}^{-1}$ ) (Figure 8.5)

***Effect of PIV-3 inoculation on airways infiltration of leucocytes in sensitised guinea pigs.***

In PIV-3 infected guinea pigs, sensitised (inhalation or intraperitoneal), with saline and challenged with saline (inhalation), a significant increase ( $P < 0.05$ ) in total cell number in the BAL fluid ( $4.2 \pm 0.4, 4.2 \pm 0.3 \times 10^6 \text{ cells ml}^{-1}$ ), was observed compared to virus-free medium, saline sensitised (inhalation or intraperitoneal) guinea pigs, challenged with saline (inhalation) ( $2.2 \pm 0.3, 2.3 \pm 0.2 \times 10^6 \text{ cells ml}^{-1}$ ).

The differential cell counts including macrophages, eosinophils and neutrophils were also significantly higher ( $P<0.05$ ) (Inhalation sensitisation:  $3.5\pm 0.3$ ,  $0.5\pm 0.05$ ,  $0.2\pm 0.03 \times 10^6 \text{ ml}^{-1}$ ); (Intraperitoneal sensitisation:  $3.6\pm 0.3$ ,  $0.4\pm 0.1$ ,  $0.1\pm 0.02 \times 10^6 \text{ ml}^{-1}$ ) compared to virus-free medium guinea pigs sensitised and challenged with saline (Inhalation sensitisation:  $1.9\pm 0.3$ ,  $0.3\pm 0.03$ ,  $0.03\pm 0.01 \times 10^6 \text{ ml}^{-1}$ ); (Intraperitoneal sensitisation:  $2.0\pm 0.2$ ,  $0.2\pm 0.04$ ,  $0.03\pm 0.01 \times 10^6 \text{ ml}^{-1}$ ) (Figure 8.7).

In PIV-3 infected guinea pigs sensitised (inhalation) and challenged with ovalbumin (inhalation), a significant increase ( $P<0.05$ ) in total cells ( $7.5\pm 0.2 \times 10^6 \text{ cells ml}^{-1}$ ), was observed compared to virus-free medium, ovalbumin sensitised (inhalation) guinea pigs, challenged with ovalbumin (inhalation) ( $3.6\pm 0.2 \text{ cells ml}^{-1}$ ) (Figure 8.7). The differential cell count including macrophages and eosinophils and neutrophils were also significantly higher ( $P<0.05$ ) (Inhalation sensitisation:  $6.1\pm 0.2$ ,  $1.1\pm 0.07$ ,  $0.3\pm 0.03 \text{ cells ml}^{-1}$ ) compared to virus-free medium guinea pigs sensitised and challenged with saline (Inhalation sensitisation:  $3.2\pm 0.2$ ,  $0.4\pm 0.04$ ,  $0.06\pm 0.01 \text{ cells ml}^{-1}$ ) (Figure 8.7).

In PIV-3 infected ovalbumin sensitised (intraperitoneal) guinea pigs challenged with ovalbumin (inhalation) a significance increase ( $P<0.05$ ) in total cells ( $6.0\pm 0.3 \text{ cells ml}^{-1}$ ), was observed compared to virus-free medium ovalbumin sensitised (intraperitoneal) guinea pigs, challenged with ovalbumin (inhalation) ( $3.3\pm 0.2 \text{ cells ml}^{-1}$ ) (Figure 8.7). The differential cell counts including macrophages, eosinophils and neutrophils were also significantly higher ( $P<0.05$ ) (Intraperitoneal sensitisation:  $4.9\pm 0.2$ ,  $0.9\pm 0.06$ ,  $0.2\pm 0.03 \text{ cells ml}^{-1}$ ) compared to virus-free medium guinea pigs sensitised and challenged with saline (Intraperitoneal sensitisation:  $2.9\pm 0.1$ ,  $0.30\pm 0.03$ ,  $0.07\pm 0.01 \text{ cells ml}^{-1}$ ) (Figure 8.7). Significantly more ( $P<0.05$ ) total and differential cell counts was observed in PIV-3 guinea pigs sensitised with ovalbumin (inhalation) compared to PIV-3 guinea pigs sensitised with ovalbumin (intraperitoneal).

***Effect of PIV-3 on respiratory rates in sensitised guinea pigs.***

Respiratory rates were measured in all groups at regular intervals throughout the study. In PIV-3 infected guinea pigs and saline sensitised (inhalation or intraperitoneal), challenged with saline (inhalation) a significant increase ( $P<0.05$ ) in respiratory rates ( $158.0\pm 4.3$ ,  $140.0\pm 4.6$  breaths  $\text{min}^{-1}$ ) compared to virus-free medium inoculated saline sensitised (inhalation or intraperitoneal) guinea pigs, challenged with saline (inhalation) ( $78.0\pm 5.1$ ,  $80.0\pm 4.7$  breaths  $\text{min}^{-1}$ ) (Figure 8.6).

In PIV-3 infected guinea pigs, sensitised (inhalation) with ovalbumin and challenged with ovalbumin (inhalation), a significant increase ( $P<0.05$ ) in respiratory rates was observed ( $190.0\pm 4.3$  breaths  $\text{min}^{-1}$ ) compared to virus-free medium inoculated ovalbumin sensitised (inhalation) guinea pigs, challenged with ovalbumin (inhalation) ( $80.0\pm 5.1$  breaths  $\text{min}^{-1}$ ) (Figure 8.6). A similar pattern was also produced in PIV-3 infected guinea pigs sensitised with ovalbumin (intraperitoneal), challenged with ovalbumin (inhalation) ( $160.0\pm 4.7$  breaths  $\text{min}^{-1}$ ) compared to virus-free medium infected guinea pigs sensitised to ovalbumin (intraperitoneal) and challenged with ovalbumin (inhalation) ( $70.0\pm 7.2$  breaths  $\text{min}^{-1}$ ). In addition, a significantly greater increase in respiratory rates ( $P<0.05$ ) was observed in PIV-3 infected guinea pigs sensitised with via ovalbumin inhalation ( $190.0\pm 4.3$  breaths  $\text{min}^{-1}$ ) compared with intraperitoneal administration ( $160.0\pm 4.6$  breaths  $\text{min}^{-1}$ ).

***Effects of PIV-3 on wet right lung weights in sensitised guinea pigs.***

In PIV-3 virus infected guinea pigs, sensitised (inhalation or intraperitoneal), and challenged with saline (inhalation), a significance increase ( $P<0.05$ ) in wet lung weights ( $8.5\pm 0.3$ ,  $7.4\pm 0.5$  g  $\text{kg}^{-1}$ ), was observed compared to virus-free medium saline sensitised (inhalation or intraperitoneal) guinea pigs, challenged with saline (inhalation) ( $5.0\pm 0.3$ ,  $4.6\pm 0.4$  g  $\text{kg}^{-1}$ ) (Figure 8.8).

In PIV-3 infected guinea pigs sensitised (inhalation) and challenged with ovalbumin (inhalation), a significance increase ( $P<0.05$ ) in wet lung weights ( $11.3\pm 0.5 \text{ g kg}^{-1}$ ), was observed compared to virus-free medium, ovalbumin sensitised (inhalation) guinea pigs, challenged with ovalbumin (inhalation) ( $5.5\pm 0.3 \text{ g kg}^{-1}$ ) (Figure 8.8). This observation was also reproduced in PIV-3 infected guinea pigs sensitised (intraperitoneal) and challenged with ovalbumin (inhalation) ( $9.1\pm 0.5 \text{ g kg}^{-1}$ ) compared to virus-free medium ovalbumin sensitised (intraperitoneal) guinea pigs, challenged with ovalbumin (inhalation) ( $5.3\pm 0.5 \text{ g kg}^{-1}$ ). A significant increase ( $P<0.05$ ) in lung wet weight was observed in PIV-3 infected guinea pigs sensitised with via inhalation ( $11.3\pm 0.5 \text{ g kg}^{-1}$ ) compared with intra-peritoneal administration ( $9.1\pm 0.5 \text{ g kg}^{-1}$ ).

#### ***Effects of PIV-3 on airway nitric oxide in sensitised guinea pigs***

In PIV-3 infected guinea pigs, sensitised (inhalation or intraperitoneal) and challenged with saline (inhalation), a significance increase ( $P<0.05$ ) in airway nitric oxide concentration ( $70.1\pm 3.3$ ,  $72.6\pm 3.9 \mu\text{M}$ ) was observed compared to virus-free medium, saline sensitised (inhalation or intraperitoneal) guinea pigs, challenged with saline (inhalation) ( $48.6\pm 2.1$ ,  $51.2\pm 3.6 \mu\text{M}$ ).

In PIV-3 infected guinea pigs sensitised (inhalation) and challenged with ovalbumin (inhalation), a significance increase ( $P<0.05$ ) in airway nitric oxide concentration ( $94.2\pm 3.9 \mu\text{M ml}^{-1}$ ), was observed compared to virus-free medium, ovalbumin sensitised (inhalation) guinea pigs, challenged with ovalbumin (inhalation) ( $65.2\pm 4.5 \mu\text{g ml}^{-1}$ ). This observation was also reproduced in PI-3 virus infected guinea pigs sensitised (intraperitoneal), and challenged with ovalbumin (inhalation) ( $82.6\pm 4.2 \mu\text{M ml}^{-1}$ ) compared to virus-free medium, ovalbumin sensitised (intraperitoneal) guinea pigs, challenged with ovalbumin (inhalation) ( $58.9\pm 2.8 \mu\text{M ml}^{-1}$ ). A significant increase ( $P<0.05$ ) in lung nitric oxide was observed in PIV-3 infected guinea pigs sensitised with via inhalation ( $94.2\pm 3.9 \mu\text{M ml}^{-1}$ ) compared with intraperitoneal administration ( $82.6\pm 4.2 \mu\text{M ml}^{-1}$ ) (Figure 8.9).

### ***Effects of PIV-3 on total protein levels in sensitised guinea pigs***

In PIV-3 infected guinea pigs, sensitised (inhalation or intraperitoneal), and challenged with saline (inhalation) a significance increase ( $P < 0.05$ ) in total protein levels ( $1287.4 \pm 36.5$ ,  $1251.7 \pm 32.6 \mu\text{g ml}^{-1}$ ), was observed compared to virus-free medium saline sensitised (inhalation or intraperitoneal) guinea pigs, challenged with saline (inhalation) ( $1062.6 \pm 34.2$ ,  $1033.2 \pm 41.8 \mu\text{g ml}^{-1}$ ).

In PIV-3 infected guinea pigs sensitised (inhalation) and challenged with ovalbumin (inhalation), a significance increase ( $P < 0.05$ ) in total protein levels ( $1834.2 \pm 48.3 \mu\text{g ml}^{-1}$ ), was observed compared to virus-free medium, ovalbumin sensitised (inhalation) guinea pigs, challenged with ovalbumin (inhalation) ( $1454.6 \pm 41.2 \mu\text{g ml}^{-1}$ ). This observation was also reproduced in PIV-3 infected guinea pigs sensitised (intraperitoneal) and challenged with ovalbumin (inhalation) ( $1613.7 \pm 46.3 \mu\text{g ml}^{-1}$ ) compared to virus-free medium, ovalbumin sensitised (intraperitoneal) guinea pigs, challenged with ovalbumin (inhalation) ( $1429.3 \pm 39.1 \mu\text{g ml}^{-1}$ ). A significant by greater increase ( $P < 0.05$ ) in protein was observed in PI-3 virus infected guinea pigs sensitised with via inhalation ( $1834.2 \pm 48.3 \mu\text{g ml}^{-1}$ ) compared with intra-peritoneal administration  $1613.7 \pm 46.3 \mu\text{g ml}^{-1}$  (Figure 8.10).

### ***Isolation of PIV-3 in BAL fluid and right lung tissue in sensitised guinea pigs***

Replicating virus was isolated in PIV-3 infected guinea pigs sensitised with saline or ovalbumin (inhalation or intraperitoneal) (Table 8.1) post inoculation day 25 measured by CPE of BAL fluid and homogenised left lung tissue on Vero cells and a positive reaction by indirect immunofluorescence using an antibody against PIV-3 (Day 25 post inoculation). Titres of intrapulmonary replicating PIV-3 virus were determined in left lung tissue from PIV-3 guinea pigs sensitised with ovalbumin or saline (inhalation or intraperitoneal) and challenged with either saline or ovalbumin were normalised to left lung tissue weight ( $\text{TCID}_{50} \text{ ml}^{-1} \text{ g}^{-1}$ ) (Figure 8.11).

PIV-3 was not detected in any the virus-free medium groups as determined by the absence of cytopathic effects on Vero cells and a negative reaction by indirect immunofluorescence.

Titres of PIV-3 were not significantly different in PIV-3 infected guinea pigs, sensitised by ovalbumin (inhalation) and challenged with ovalbumin ( $7.6 \times 10^2 \pm 2.1 \times 10^2$  TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>) compared with PIV-3 infected guinea pigs sensitised with saline (inhalation) and challenged with saline ( $7.5 \times 10^2 \pm 2.0 \times 10^2$  TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>). This observation was also reproduced in PIV-3 infected guinea pigs ovalbumin sensitised (intraperitoneal), challenged with ovalbumin (inhalation) ( $7.0 \times 10^2 \pm 4.0 \times 10^2$  TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>) compared to PIV-3 infected, saline sensitised (intraperitoneal) guinea pigs, challenged with saline (inhalation) ( $7.5 \times 10^2 \pm 2.5 \times 10^2$  TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>).

***Effect of PI-3 virus on histology of the airways and mean pulmonary score in sensitised guinea pigs.***

At the end of the lungs were assessed for peribronchiolitis, interstitial pneumonitis, alveolitis by using a scoring system (Figure 8.12). In PIV-3 infected guinea pigs, sensitised (inhalation or intraperitoneal) and challenged with saline (inhalation), a significance increase ( $P < 0.05$ ) in mean composite pulmonary scores ( $1.4 \pm 0.2$ ,  $1.4 \pm 0.2$ ) was evident ocompared to virus-free medium inoculated and saline sensitised (inhalation or intraperitoneal) guinea pigs, challenged with saline (inhalation) ( $0.11 \pm 0.09$ ,  $0.11 \pm 0.09$ ) (Figure 8.13). In PIV-3 infected guinea pigs sensitised (inhalation) and challenged with ovalbumin (inhalation), a significance increase ( $P < 0.05$ ) in mean pulmonary and composite scores ( $3.9 \pm 0.3$ ) was observed compared to virus-free medium inoculated ovalbumin sensitised (inhalation) guinea pigs, challenged with ovalbumin (inhalation) ( $1.0 \pm 0.2$ ). This observation was also reproduced in PIV-3 virus infected guinea pigs sensitised (intraperitoneal), challenged with ovalbumin (inhalation) ( $3.3 \pm 0.2$ ) compared to virus-free medium, ovalbumin sensitised (intraperitoneal) guinea pigs, challenged with ovalbumin (inhalation) ( $0.8 \pm 0.2$ ).

The scores seen in virus-free medium inoculated guinea pigs sensitised and challenged with ovalbumin were significantly greater ( $P < 0.05$ ) than in virus-free medium inoculated guinea pigs sensitised and challenged with saline.

The data for all parameters recorded is summarised in Table 8.2.

## DISCUSSION

Whether viral infections occurring during early childhood have been implicated in influencing their specific effects upon allergen sensitisation has been controversial. The objective of this study was to determine the effects of a respiratory allergen sensitisation on the development of PIV-3 virus infection. This issue was examined using ovalbumin as an allergen to induce airway inflammation in a guinea pig model during the acute phase of PIV-3 infection. To characterise these antigen-induced effects body weight, rectal temperature, airway function, airway reactivity to histamine, infiltration of inflammatory cells, respiratory rates, nitric oxide and total protein levels, viral titre and pulmonary histopathology were determined. Each experiment terminated in an inhalation challenge with either saline or ovalbumin, and the airways response was monitored at regular intervals.

In PIV-3 infected guinea pigs no changes in  $sG_{aw}$  and no significant change in body weight or rectal temperature was observed. However, there was a significant increase in respiratory rates during the study period, which peaked at day 5 and may be a useful parameter to monitor the progression of infection. This demonstrates that clinical symptoms do not have to be present in guinea pigs with PIV-3 infection.



In this study, antigen sensitisation by inhalation to ovalbumin resulted in enhanced allergic responses after exposure to PIV-3 during the acute stages of infection compared to virus-free medium ovalbumin treated guinea pigs demonstrated AHR accompanied by a macrophage, eosinophil and neutrophil driven influx into the lungs. In comparable investigations these observations were also reported in mice exposed to ovalbumin, infected with RSV (Schwarze *et al*; 1997) and influenza A virus (Tsitoura *et al*; 2000). Contrary to this study, Barends and co-workers (2002) observed a decrease in the influx of eosinophils in BAL fluid of antigen sensitised mice although their study investigated the influence of PIV-3 infection on pre-existing antigen sensitisation. The effects of the increased cellular influx were also further supported by significant increases in wet lung weights, nitric oxide and total protein levels and respiratory rates and demonstrated evidence of interstitial pneumonia, alveolitis and peribronchiolitis histological features in the lung, with the occlusion of many small airways.

The respiratory responses of virus-free medium infected guinea pigs sensitised with ovalbumin and challenged with aerosolised antigen are analogous to those elicited by allergic humans by specific allergen challenge with an immediate EAR that is often followed by a LAR between 6 and 9 hours after allergen provocation (Bousquet *et al*; 2000). Early phase reactions depend largely on mast cell with the mediated histamine release which mediates an immediate bronchoconstriction; later the airway responds to the initial mast cell release of the other mediators with eosinophil influx and airway hyperreactivity (Hutson *et al*; 1988; Iwana *et al*; 1991, Matsumoto *et al*; 1994). However, prior inoculation with PIV-3 guinea pigs sensitised by inhalation of ovalbumin resulted in an enhanced response to allergen challenge, which resulted in a significant bronchoconstriction for up to 12 hours before returning to baseline values with a significantly increased LAR. This finding is novel as no other study has monitored the effects of exposure to ovalbumin in pre-sensitised PIV-3 infected guinea pigs on  $sG_{aw}$ .

Probable mechanisms that have been suggested to be involved in the enhanced responses to ovalbumin sensitisation and challenge are increased permeability of the airway mucosa to allergens due to damage of the airway epithelium (Riedel *et al*; 1996, Kudlacz *et al*; 1996) and also increased recruitment of dendritic cells to the respiratory epithelium during acute viral infection (Yamamoto *et al*; 2001). Increased permeability of the respiratory epithelial barrier for inhaled allergens during virus infection could be the consequence of virus-induced inflammation and damage of the airway epithelium which allows ovalbumin to more easily enter the tissue (Freihorst *et al*; 1988). Investigations by Richardson *et al* (1981) have also described a chicken model with increased tracheal permeability after viral infection. This may also explain the reason for the enhanced allergic responses observed after inhalation sensitisation compared to intra-peritoneal sensitisation.

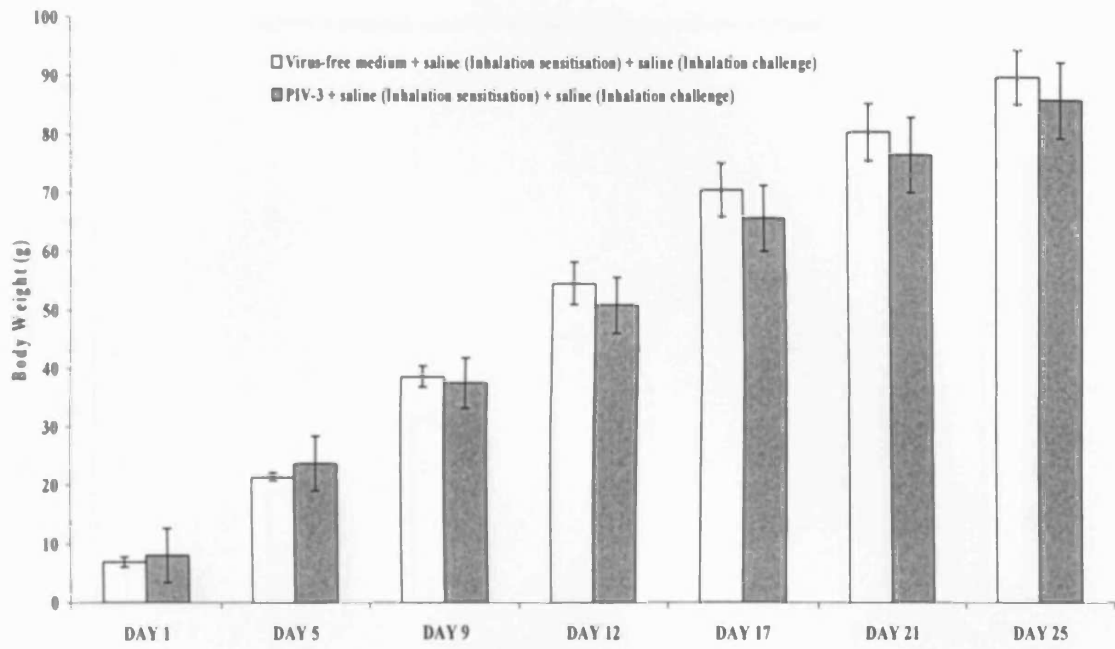
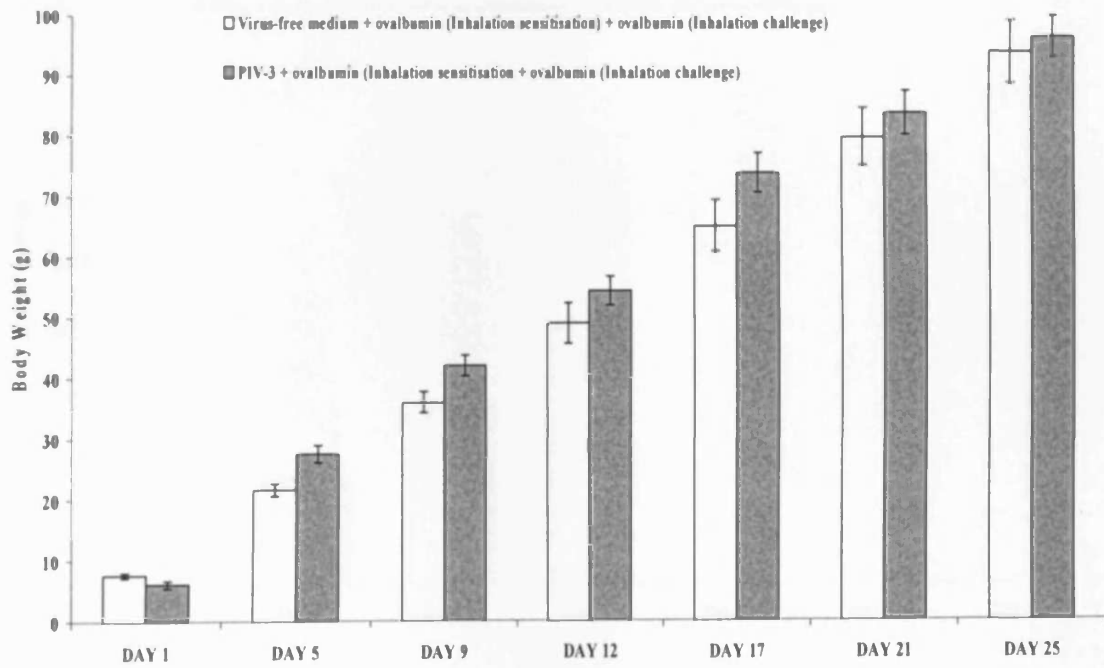
Riedel and co-workers (1996) investigated by ELISA the permeability of inhaled horseradish peroxidase (HRP), a molecule with a similar molecular weight to ovalbumin. HRP was inhaled the same day on which ovalbumin sensitisation was performed. Increased HRP concentrations were found in all PIV-3 infected animals, indicating increased permeability due to the preceding virus infection.

Dendritic cells (DC) have been shown to play a role in antigen presentation and induce a primary immune response to exogenous antigens. Studies involving influenza A virus during acute infection have shown increased migration of dendritic cells to the bronchial epithelium. Once the DC have captured antigens, they mature and their ability to capture antigens rapidly declines accompanied by the expression of assemble antigen MCH class II complexes on their cell surfaces (Banchereau & Steinman; 1988) upon maturation, the DC migrate to the lymphoid tissues. The DC complete their maturation and induce antigen specific immune responses by CD4<sup>+</sup> T cells which secrete IL-4, IL-5, and IL-13 and IFN- $\gamma$ .

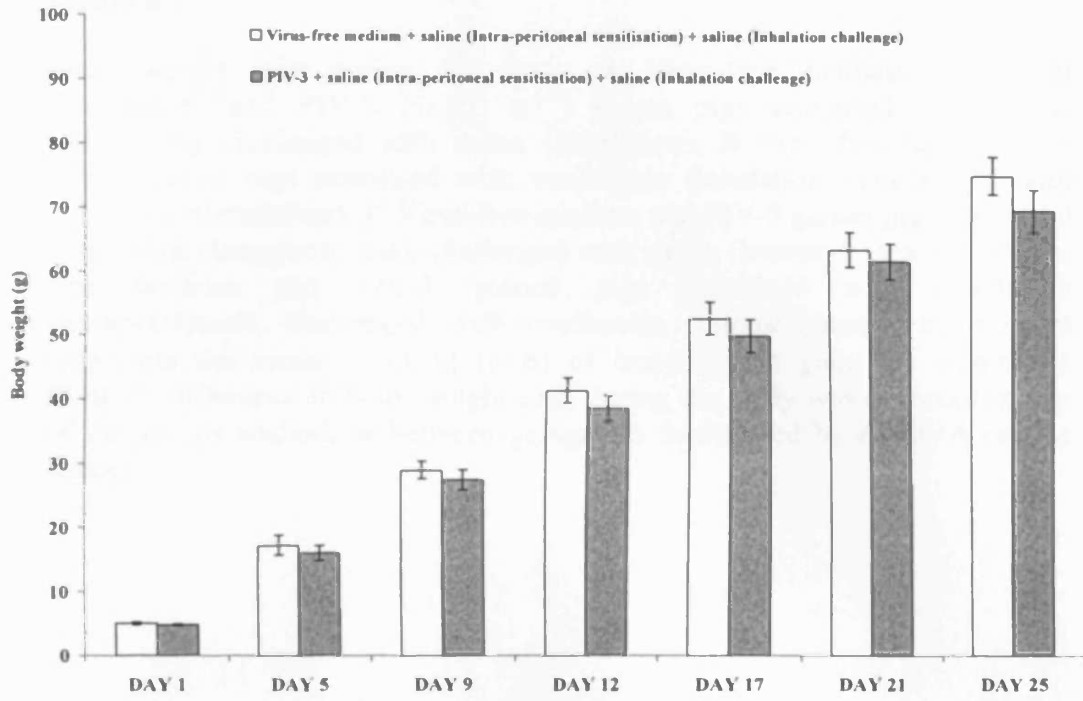
The recruitment of eosinophils in the airways has been associated with the two cytokines IL-4 and IL-5 (Hogan *et al*; 1998, Cohn *et al*; 1998). Studies in IL-5 deficient mice showed that RSV induced IL-5 production during the acute infection is essential for the influx of eosinophils in the lungs after challenge (Schwarze *et al*, 2000). It has also been reported that infection with PIV-1 increases the number of DCs and induces expression of MHC class II molecules on epithelial cells in rats (McWilliam *et al*; 1997). IL-13 has been shown to play a primary role in the induction of AHR in murine models of asthma (Grunig *et al*; 1998, Wills-Karp *et al*; 1998) and also play a role when mice are infected with influenza A infection.

In this study, the enhanced effects of allergic sensitisation occurs during the acute phase of PI-3 virus infection in a guinea pig model of asthma. Allergic sensitisation studies by Tsitoura and co-workers (2000) using influenza-A virus determined that the length of time between infection and exposure to ovalbumin was crucial. Their study determined that respiratory exposure to ovalbumin 3 days after influenza A infection resulted in allergic sensitisation, expansion of allergen-specific CD4+ T cells and was associated with the development of AHR and the production of ovalbumin IgE, IL-4, IL-5, IL-13, and IFN- $\gamma$ . However, respiratory exposure to allergen late after influenza A infection, during the recovery period from infection, disrupted tolerance induction, but promoted an IFN- $\gamma$ -predominant response which was associated with protection from the development of AHR. This may explain the conflicting results of clinical studies in humans demonstrating that respiratory viral infections either enhance the risk of, or prevent the development of asthma.

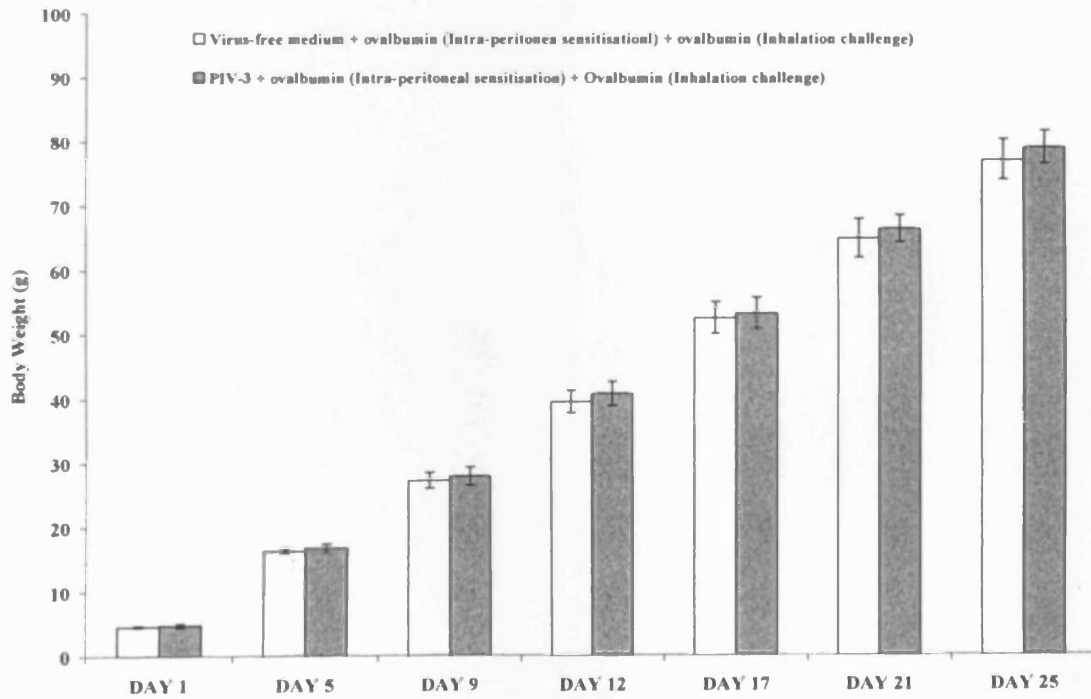
In conclusion this study demonstrates that pre-sensitisation during the acute phase of PIV-3 infection exacerbates the responses to allergen challenge of sensitised animals. Inhalation sensitisation appears to produce a greater effect possibly because the viral inoculation damages the bronchial epithelium and allows better penetration of the allergen than when it is given by intraperitoneal injection.

**A****B**

C

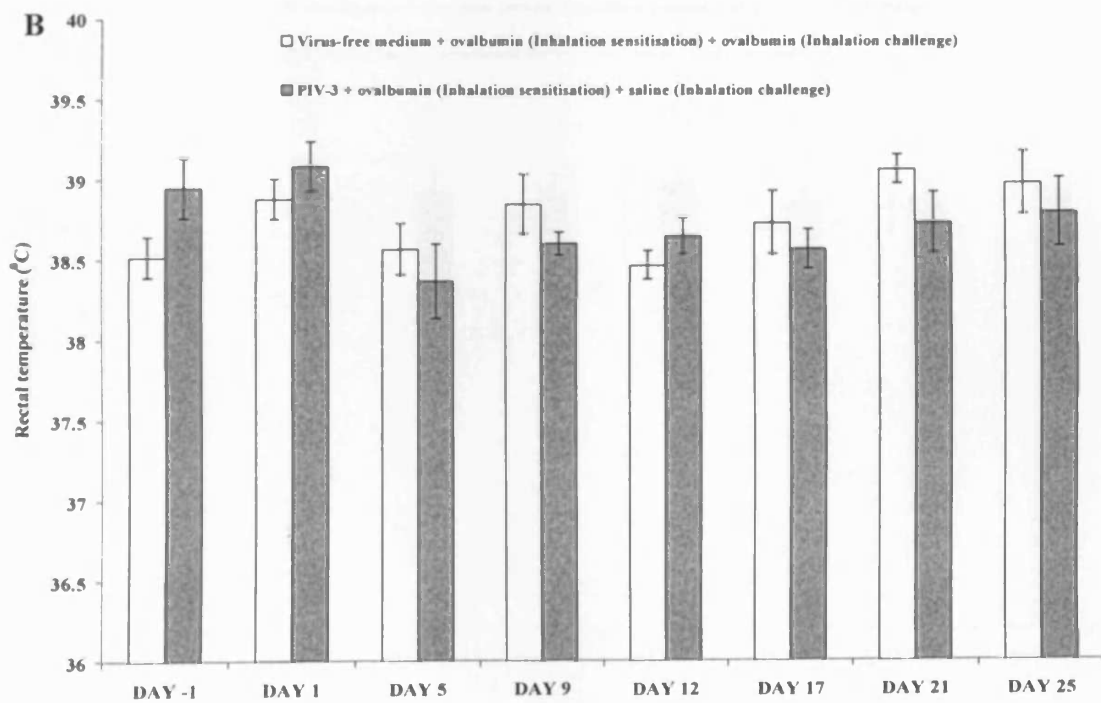
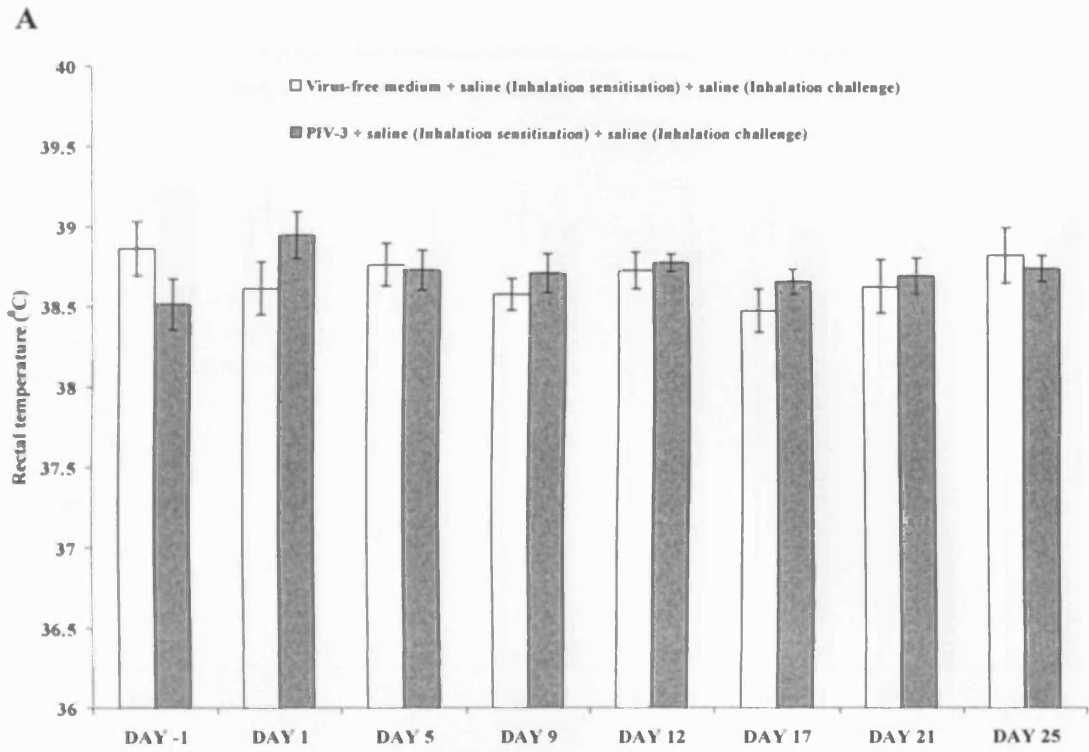


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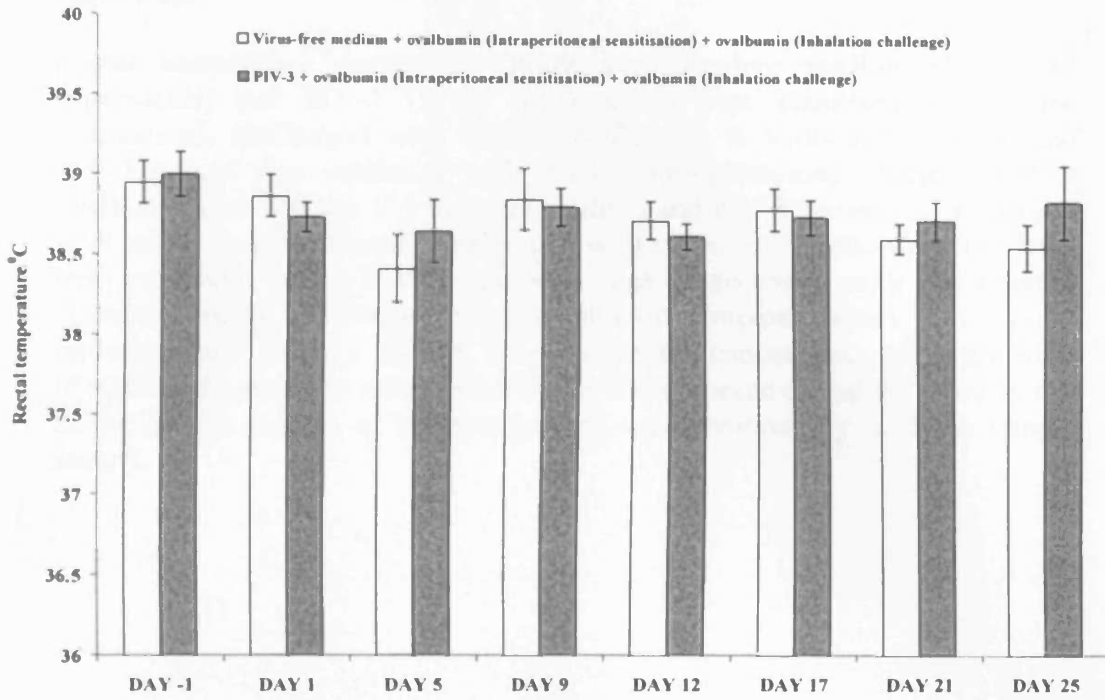
### **Figure 8.1**

Body weight gain during the study. **A** Virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) guinea pigs sensitised with saline (Inhalation), challenged with saline (Inhalation); **B** Virus-free medium and PIV-3 guinea pigs sensitised with ovalbumin (Inhalation), challenged with ovalbumin (Inhalation); **C** Virus-free medium and PIV-3 guinea pigs sensitised with saline (Intraperitoneal), challenged with saline (Intraperitoneal); **D** Virus-free medium and PIV-3 guinea pigs sensitised with ovalbumin (Intraperitoneal), challenged with ovalbumin (Intraperitoneal). Each point represents the mean  $\pm$  S.E.M (n=6) of body weight gain. No significant ( $P > 0.05$ ) difference in body weight gain during the study was observed in any of the groups studied, or between groups, as determined by ANOVA (single factor).

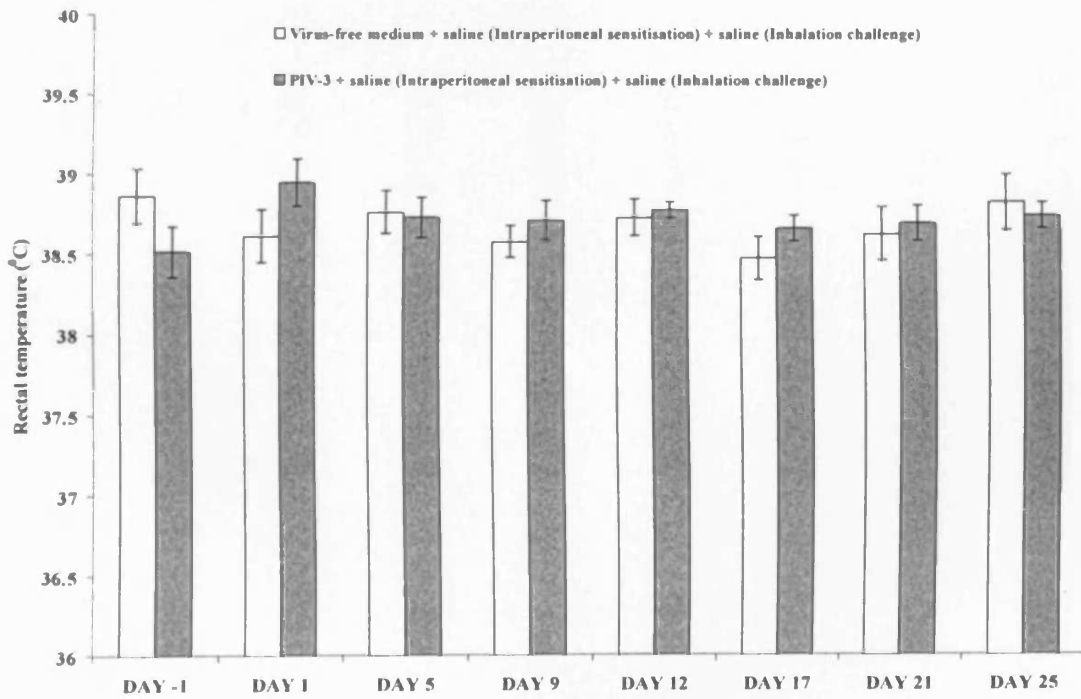




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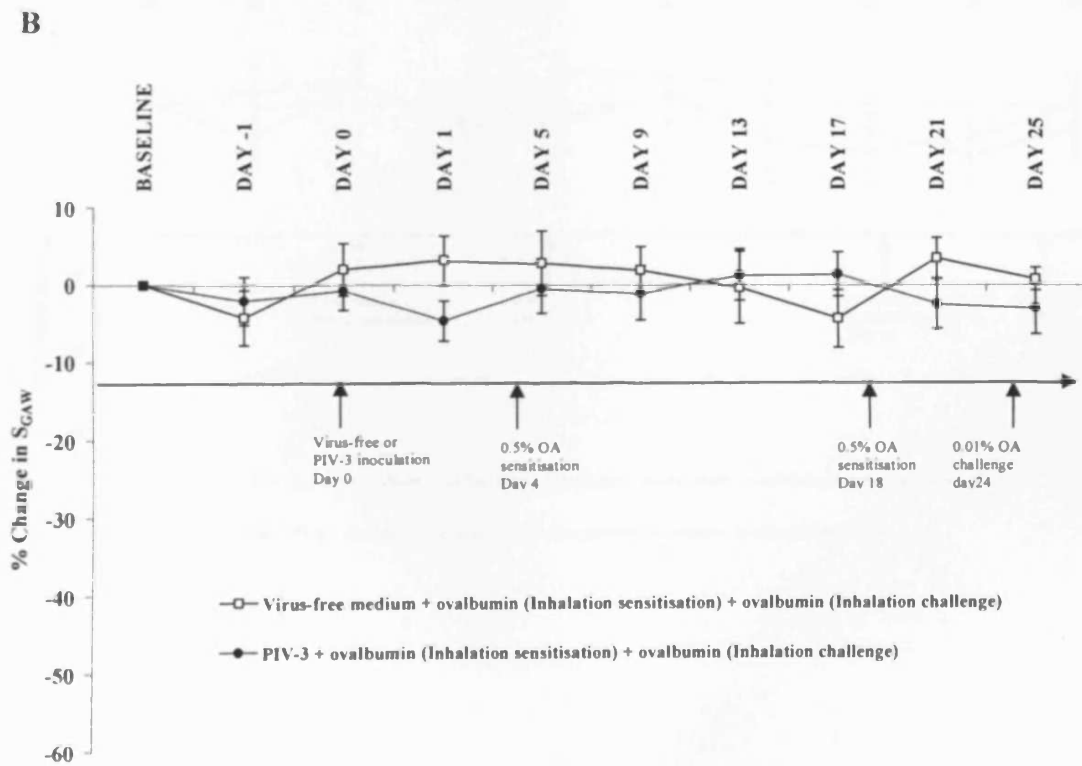
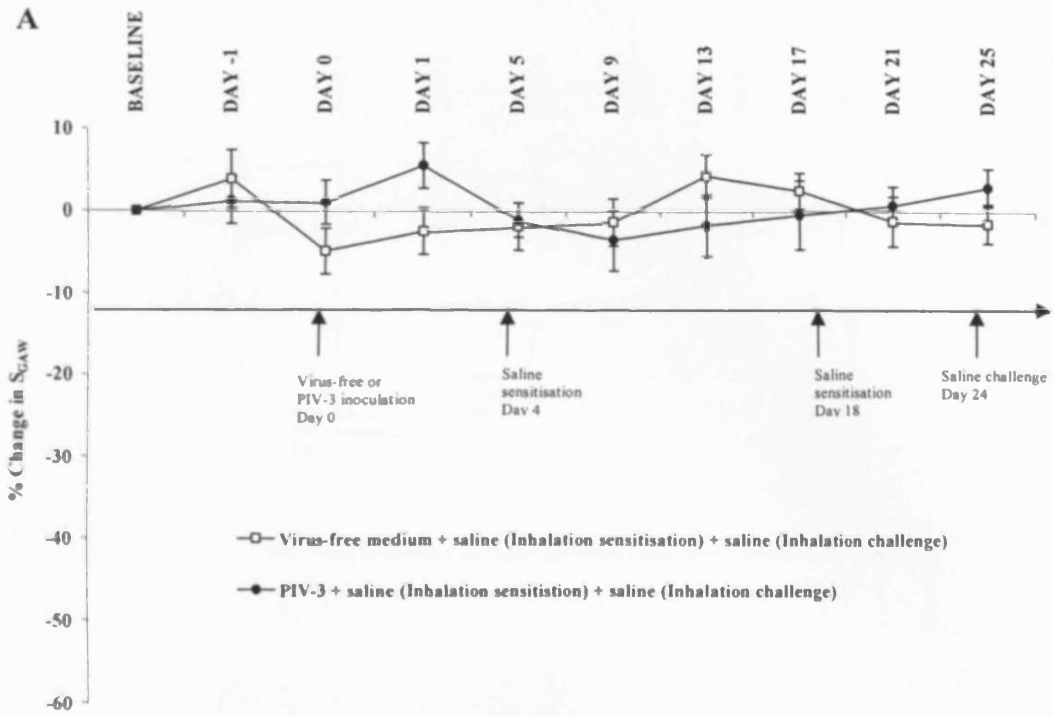


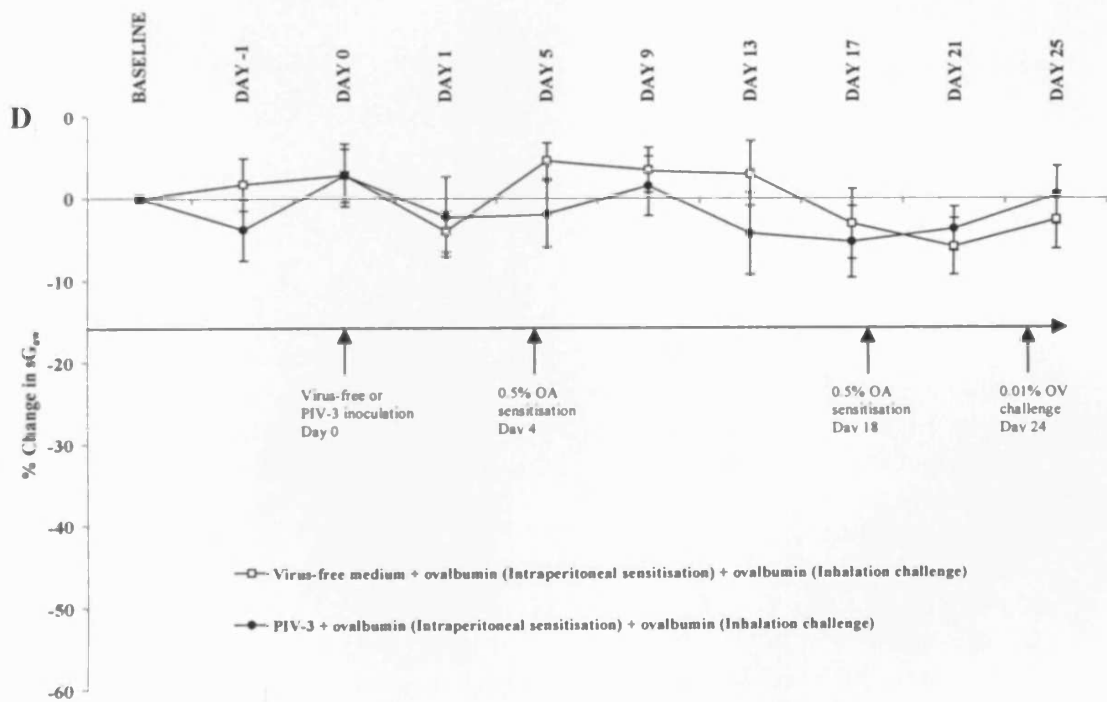
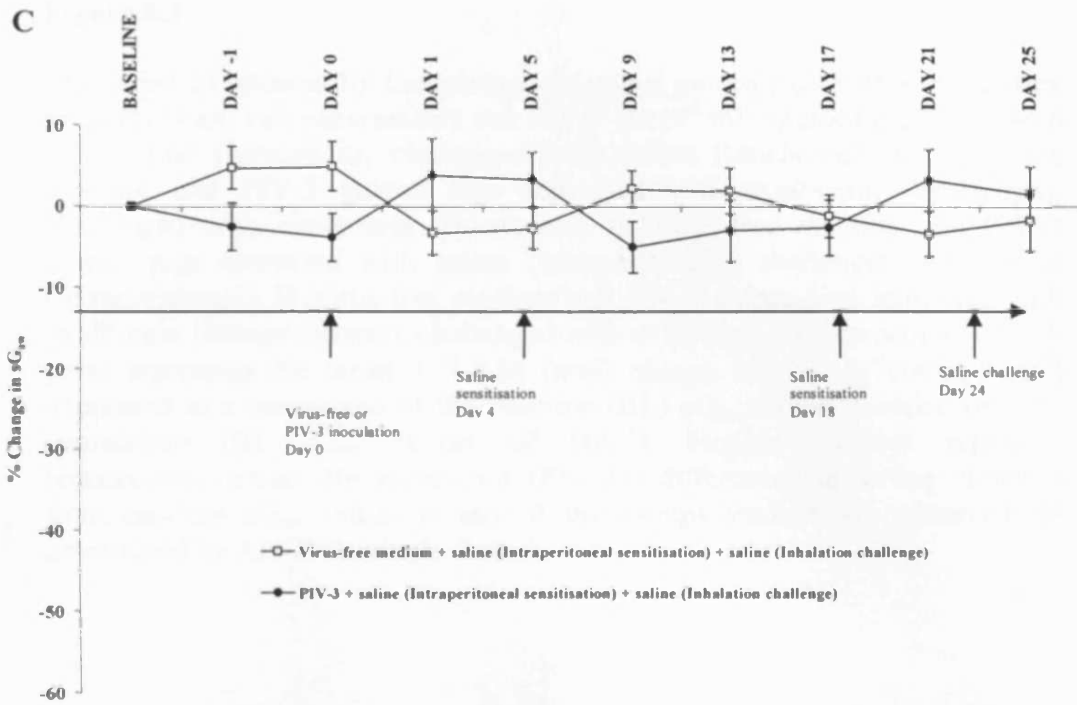
D



## Figure 8.2

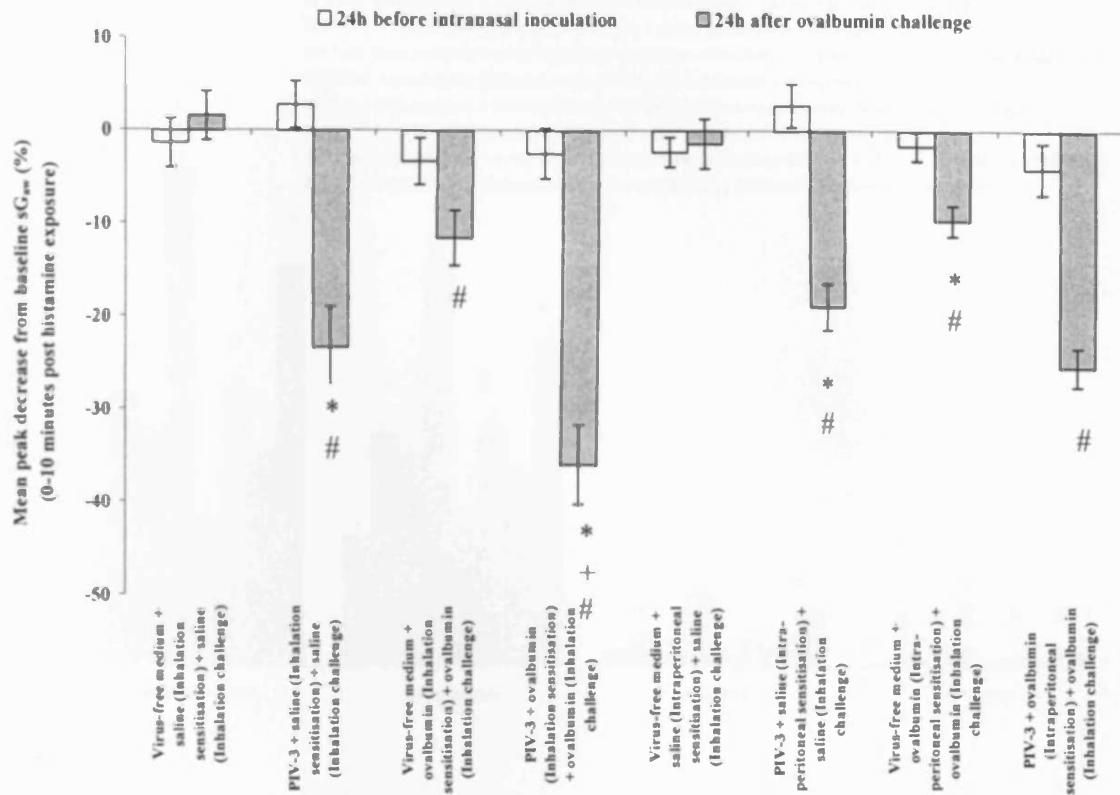
Rectal temperature during the study. **A** Virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) guinea pigs sensitised with saline (Inhalation), challenged with saline (Inhalation); **B** Virus-free medium and PIV-3 guinea pigs sensitised with ovalbumin (Inhalation), challenged with ovalbumin (Inhalation); **C** Virus-free medium and PIV-3 guinea pigs sensitised with saline (Intraperitoneal), challenged with saline (Intraperitoneal); **D** Virus-free medium and PIV-3 guinea pigs sensitised with ovalbumin (Intraperitoneal), challenged with ovalbumin (Intraperitoneal). Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of rectal temperature. No significant ( $P>0.05$ ) differences in rectal temperature was observed during the study in any of the groups studied, or between groups, as determined by ANOVA (single factor).





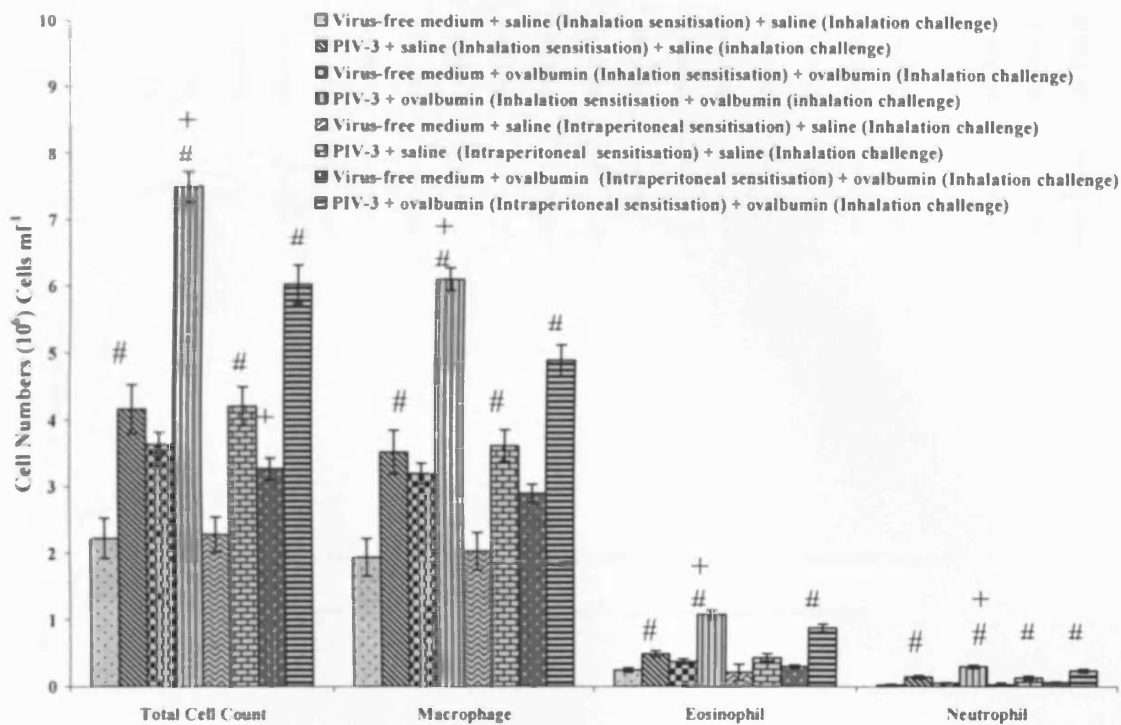
### Figure 8.3

The effect of intranasally inoculating conscious guinea pigs with **A** Virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) guinea pigs sensitised with saline (Inhalation), challenged with saline (Inhalation); **B** Virus-free medium and PIV-3 guinea pigs sensitised with ovalbumin (Inhalation), challenged with ovalbumin (Inhalation); **C** Virus-free medium and PIV-3 guinea pigs sensitised with saline (Intraperitoneal), challenged with saline (Intraperitoneal); **D** Virus-free medium and PIV-3 guinea pigs sensitised with ovalbumin (Intraperitoneal), challenged with ovalbumin (Intraperitoneal). Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) change in  $sG_{aw}$  ( $\text{s}^{-1}\text{cm}$  of  $\text{H}_2\text{O}^{-1}$ ) expressed as a percentage of the baseline (BL)  $sG_{aw}$  values recorded prior to inoculation (BL  $sG_{aw}$  ( $\text{s}^{-1}\text{cm}$  of  $\text{H}_2\text{O}^{-1}$ )). Negative values represent bronchoconstriction. No significant ( $P>0.05$ ) differences in airway function from baseline  $sG_{aw}$  values in any of the groups studied was observed, as determined by ANOVA (single factor).



**Figure 8.4**

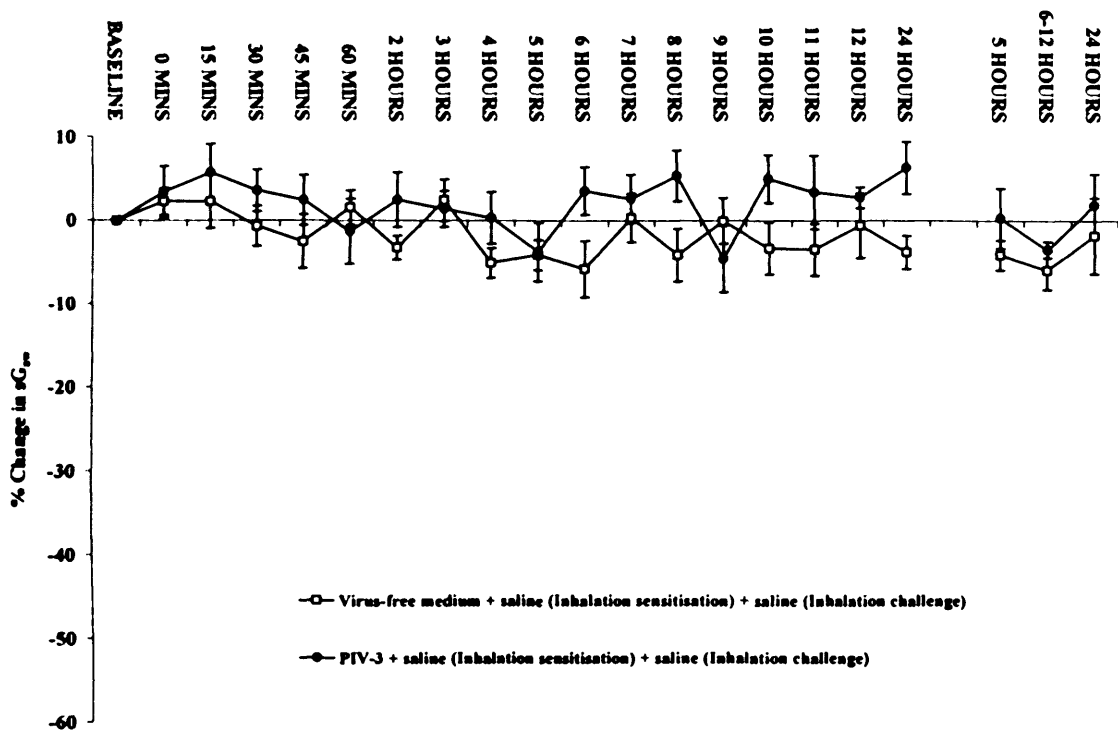
Peak percentage changes in the airway reactivity responses of conscious guinea pigs to a nose-only challenge with histamine (1mM, 20 seconds), 24 hours before the commencement of virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) guinea pigs sensitised with ovalbumin or saline (Inhalation or intraperitoneal) and challenged with 0.01% ovalbumin. Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of the peak change from baseline  $sG_{aw}$  ( $s^{-1} \text{cm of H}_2\text{O}^{-1}$ ), 0-10 minutes post histamine exposure. Negative values represent bronchoconstriction. \*  $P < 0.05$  denotes the significant of differences 24 hours before inoculation and days 25 post inoculation. +  $P < 0.05$  denotes the significant of differences in PIV-3 guinea pigs sensitised with ovalbumin by inhalation compared with intraperitoneal injection. #  $P < 0.05$  denotes the significant of difference in PIV-3 compared to corresponding virus-free medium groups. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



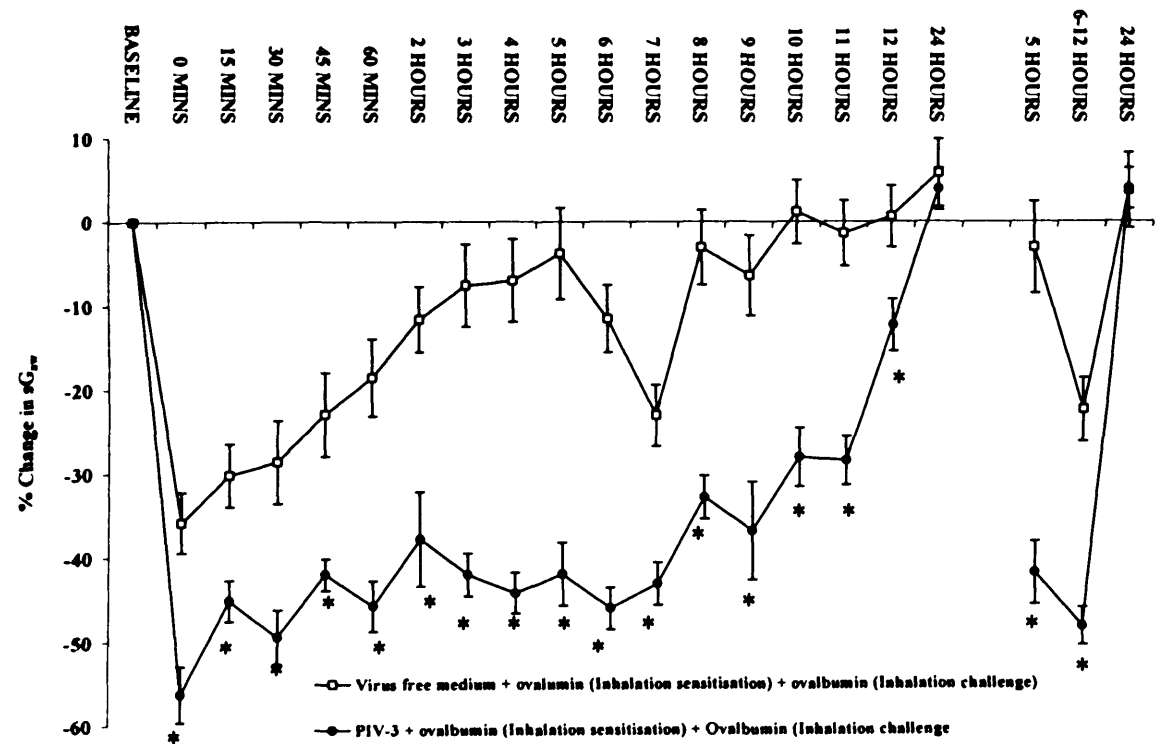
**Figure 8.5**

Total and differential cell (macrophage, eosinophil and neutrophil) counts of BAL fluid removed from virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs sensitised with saline or ovalbumin via inhalation or intra-peritoneal administration and challenged 0.01% ovalbumin. Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of the differential cells per BAL fluid sample ( $\times 10^6$ ). #  $P < 0.05$  denotes the significance of differences in respiratory rate from PIV-3 infected guinea pigs compared to virus-free medium infected guinea pigs. +  $P < 0.05$  denotes the significant of differences in PIV-3 guinea pigs sensitised with ovalbumin by inhalation compared with intraperitoneal sensitisation. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

**A**

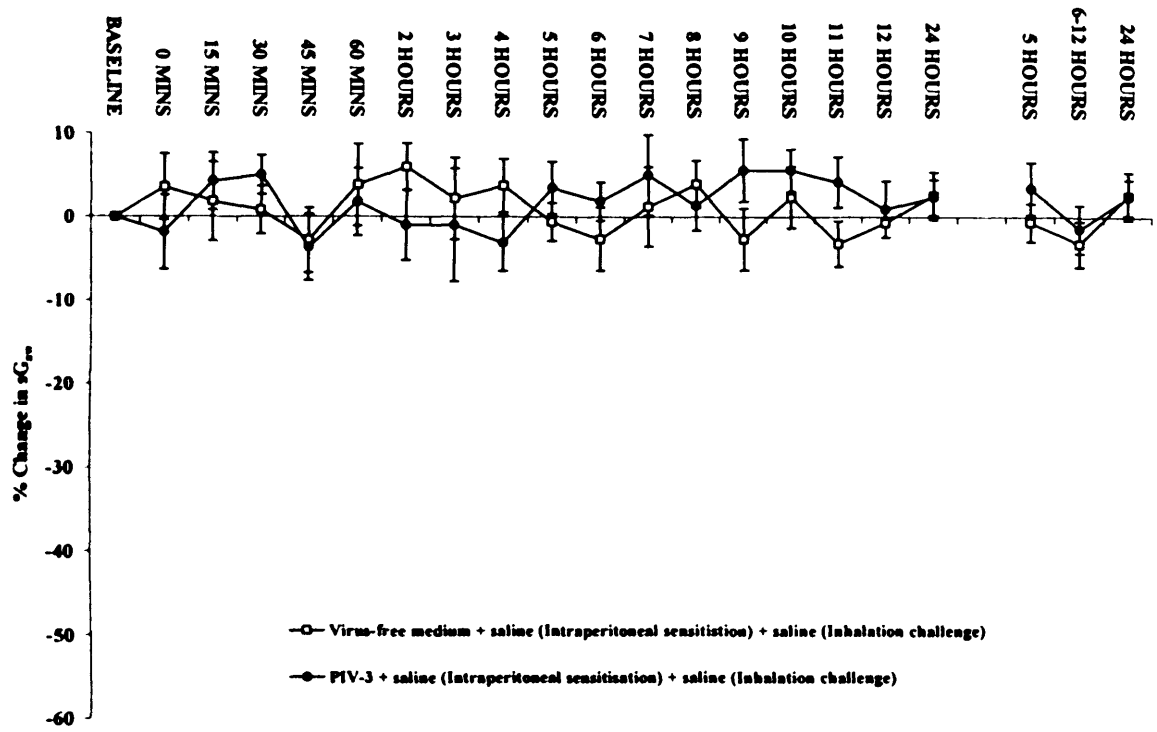


**B**

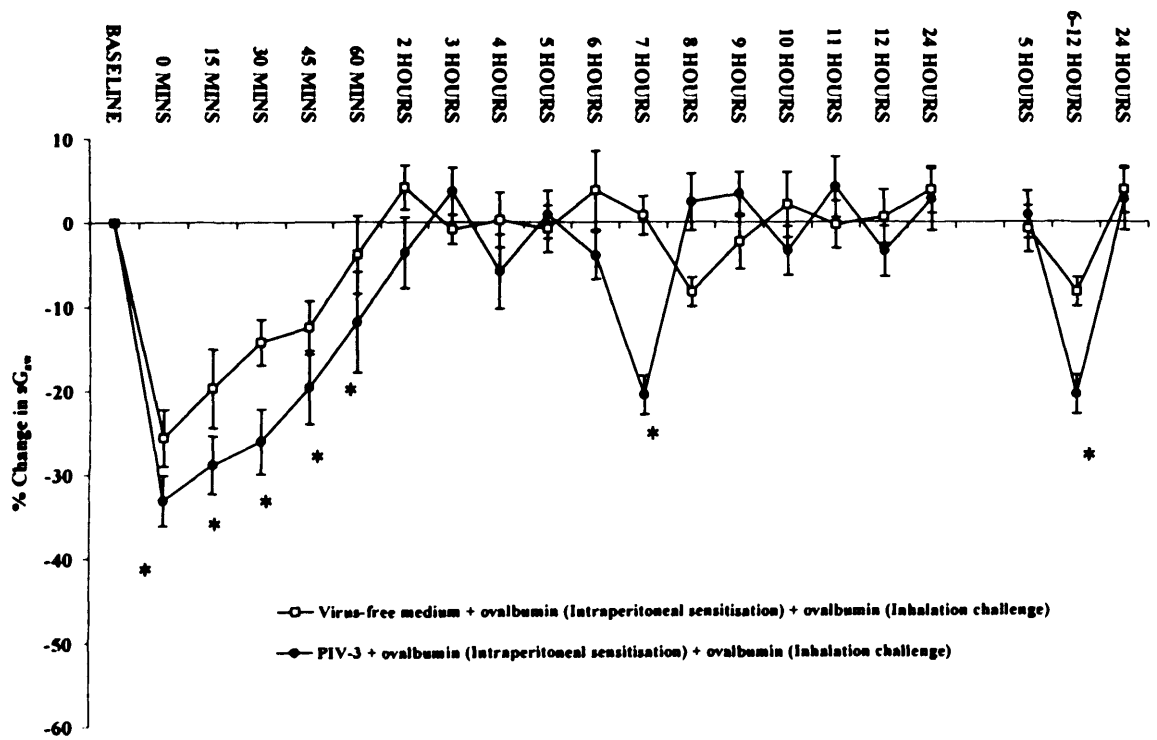




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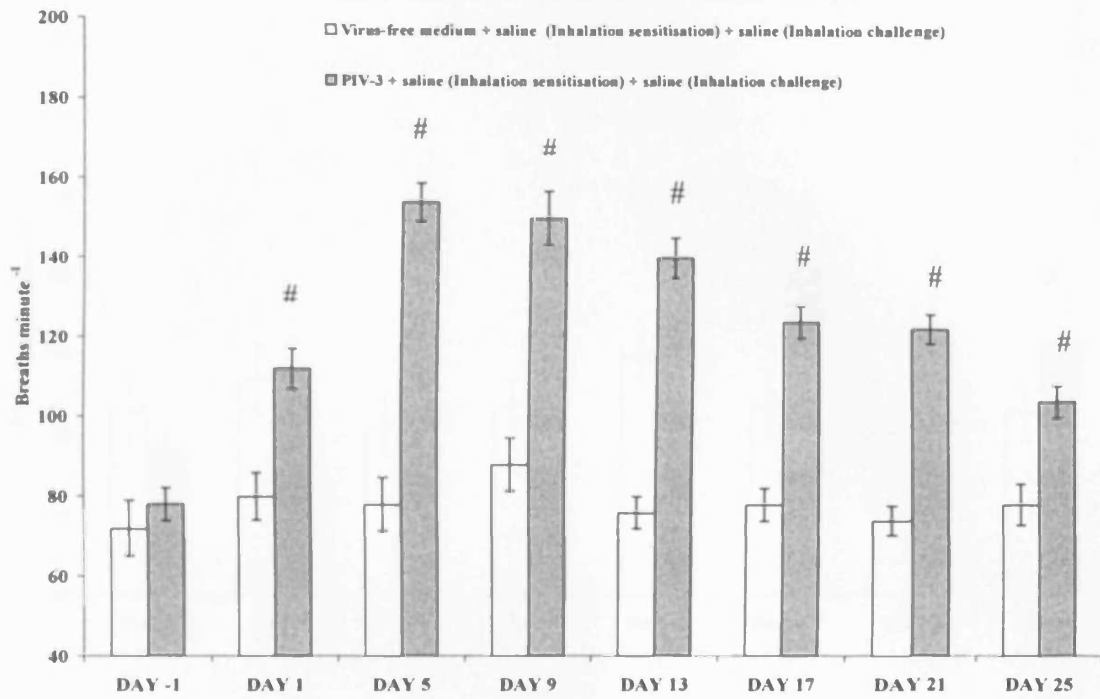
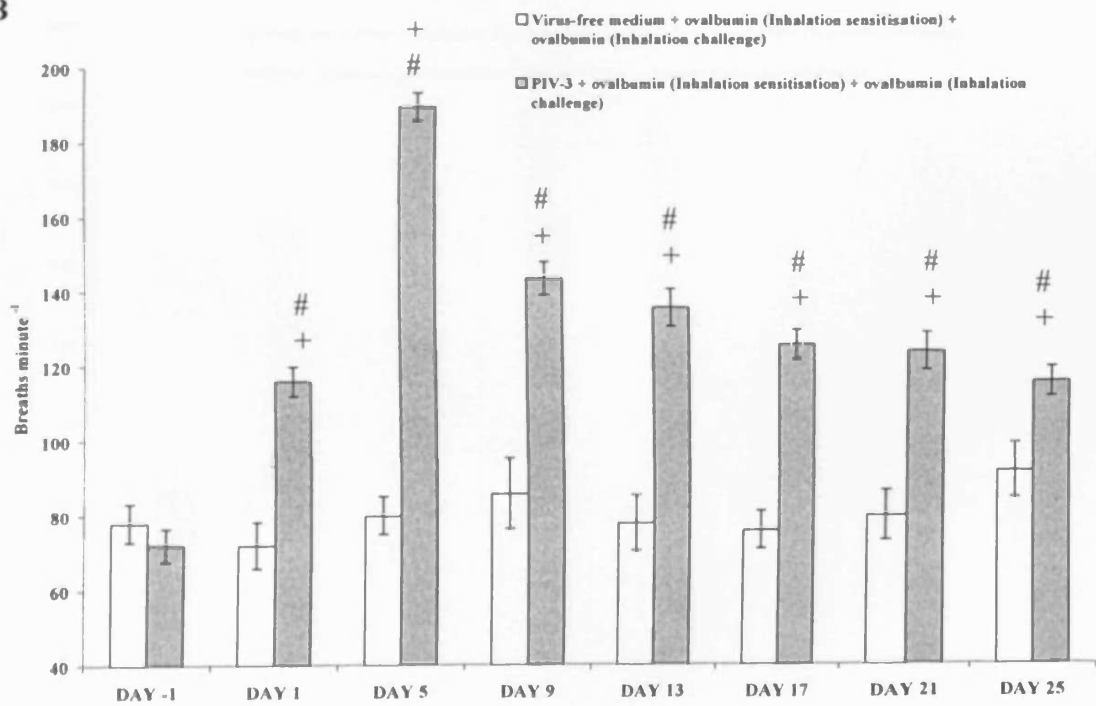


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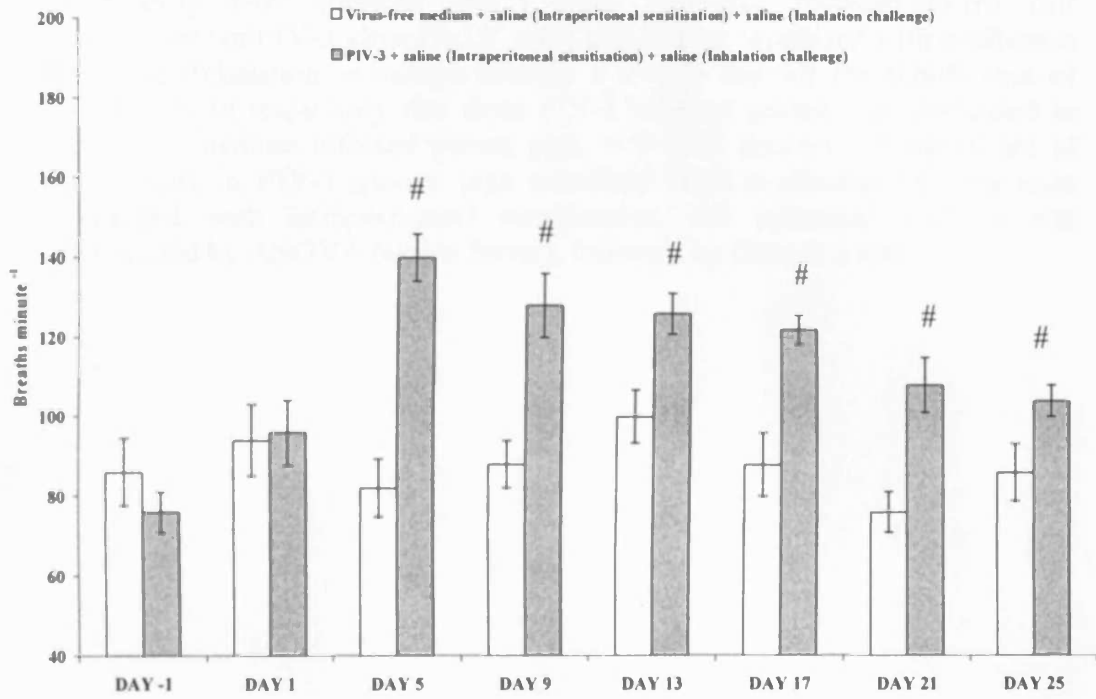


**Figure 8.6**

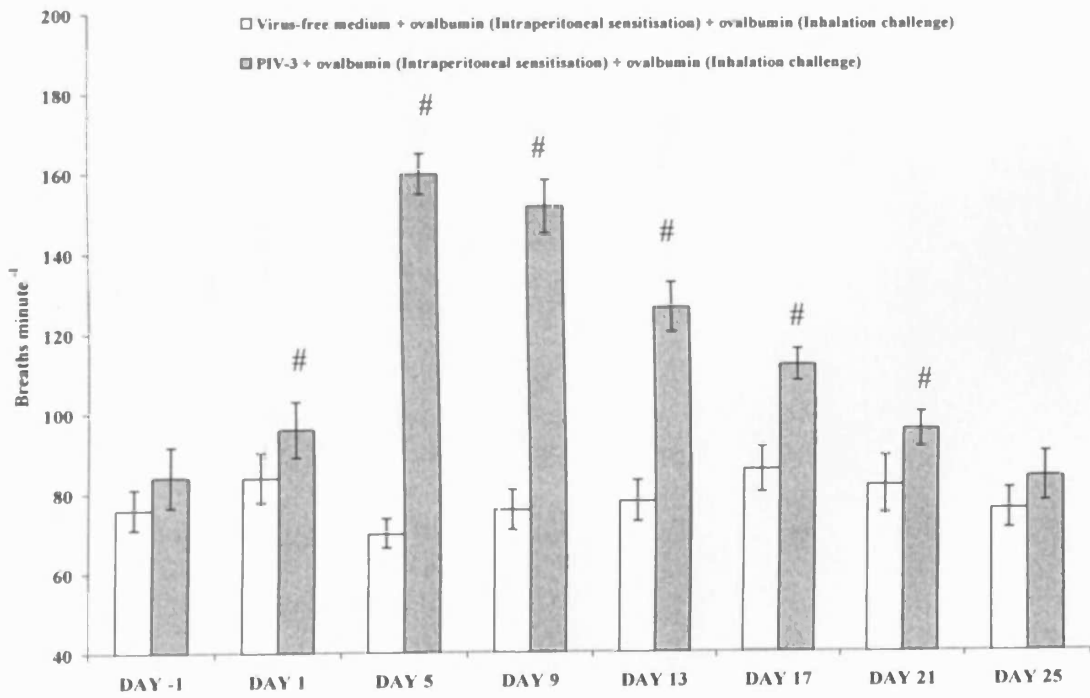
Allergen exposure on airway function in virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs sensitised with saline and ovalbumin (inhalation/intra-peritoneal). Each point represents the mean  $\pm$  S.E.M (n=6) of the change from baseline  $sG_{aw}$  ( $\text{s}^{-1} \text{cm}$  of  $\text{H}_2\text{O}^{-1}$ ). Also shown is the mean peak value occurring between 6 and 12 hours. Negative values represent bronchoconstriction. \*  $P < 0.05$  denotes the significance of differences between PIV-3 and the corresponding virus-free medium group. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

**A****B**

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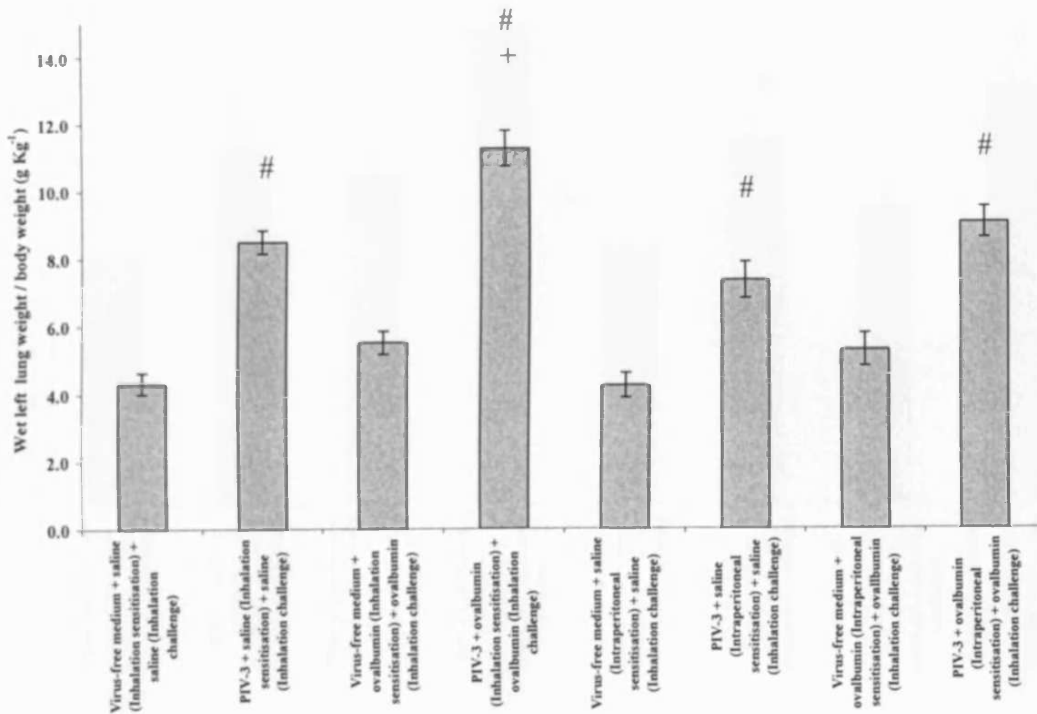


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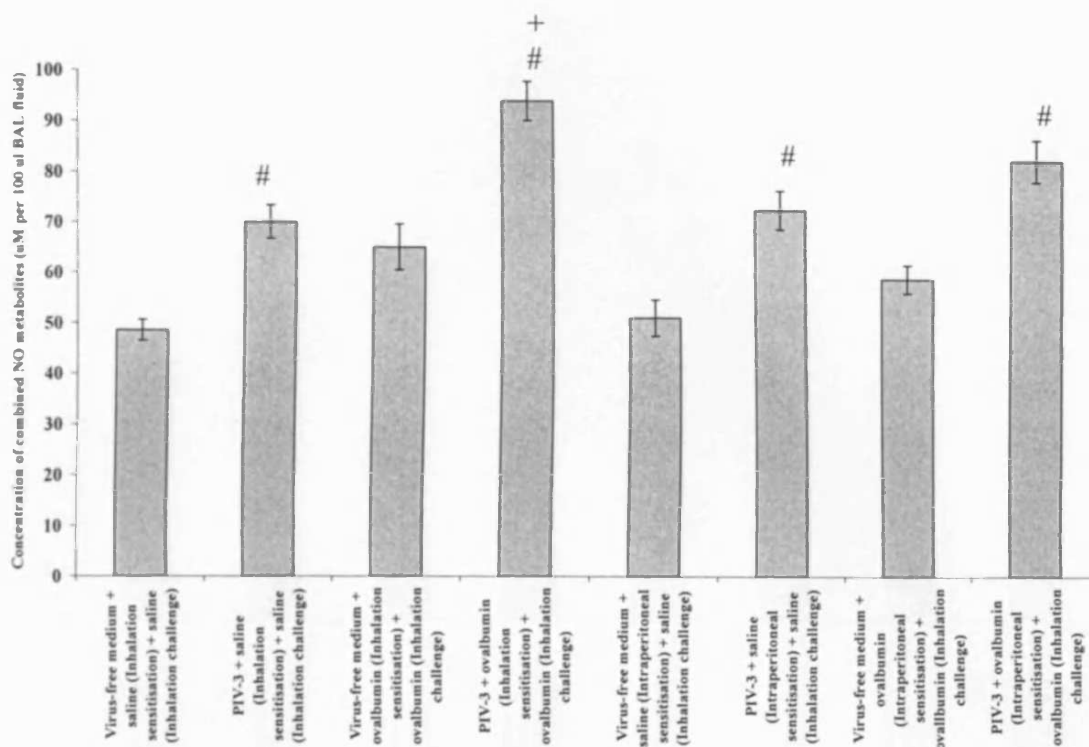
**Figure 8.7**

Respiratory rates (breaths  $\text{min}^{-1}$ ) from virus-free medium (Vero cell supernatant) or PIV-3 virus ( $5 \times 10^6 \text{ ml}^{-1}$ ) guinea pigs sensitised with ovalbumin or saline (Inhalation or intraperitoneal). #  $P < 0.05$  denotes the significance of differences in respiratory rate from PIV-3 infected guinea pigs compared to virus-free medium infected guinea pigs. +  $P < 0.05$  denotes the significant of differences in PIV-3 guinea pigs sensitised with ovalbumin by inhalation compared with intraperitoneal sensitisation. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



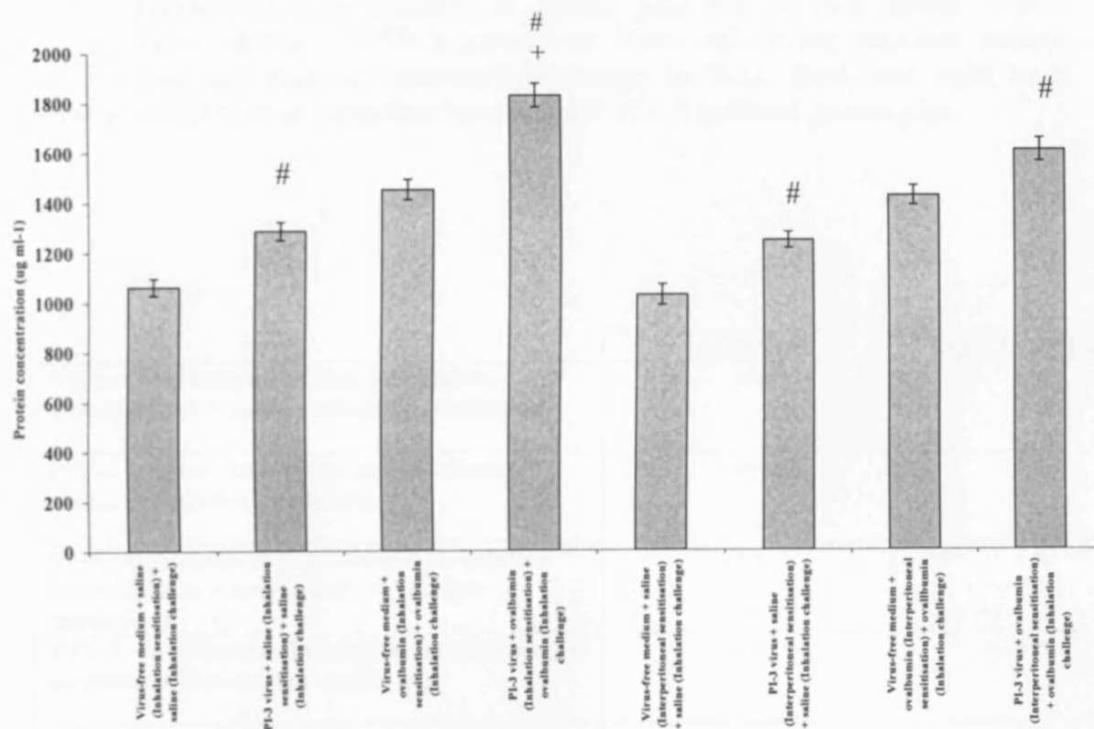
**Figure 8.8**

Wet left lung weights from virus-free medium (Vero cell supernatant) and PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs sensitised with saline or ovalbumin via inhalation or intraperitoneal administration and challenged 0.01% ovalbumin. Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of wet left lung weights normalised to body weight ( $\text{g kg}^{-1}$ ). #  $P < 0.05$  denotes significant of difference in PIV-3 compared to corresponding virus-free medium groups. +  $P < 0.05$  denotes the significant of differences in PIV-3 sensitised with ovalbumin by inhalation compared with intra-peritoneal injection of the corresponding group. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



**Figure 8.9**

Total nitric oxide concentrations in BAL fluid from virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) guinea pigs sensitised with ovalbumin or saline (Inhalation or intraperitoneal) and challenged with either saline or 0.01% ovalbumin. Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of the total nitric oxide concentration per sample ( $\mu\text{M}$ ). #  $P < 0.05$  denotes significant of difference in PIV-3 compared to corresponding virus-free medium groups. +  $P < 0.05$  denotes the significant of differences in PIV-3 sensitised with ovalbumin by inhalation compared with intraperitoneal injection. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



**Figure 8.10**

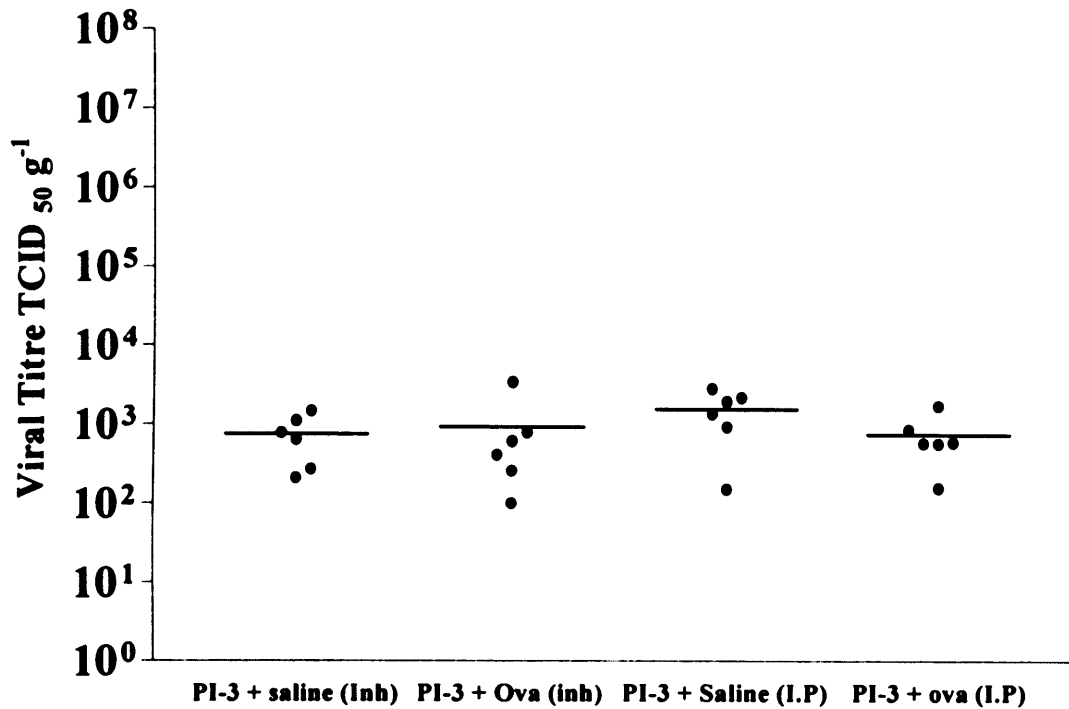
Total protein levels in BAL fluid from virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) guinea pigs sensitised with ovalbumin or saline (Inhalation or intraperitoneal) and challenged with either saline or 0.01% ovalbumin. Each point represents the mean  $\pm$  S.E.M ( $n=6$ ) of the total protein concentration per sample ( $\mu\text{g ml}^{-1}$ ). #  $P < 0.05$  denotes significant of difference in PIV-3 compared to corresponding virus-free medium groups. +  $P < 0.05$  denotes the significant of differences in PIV-3 sensitised with ovalbumin by inhalation compared with intraperitoneal injection. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.



**Table 8.1**

This demonstrates the number of guinea pigs out of each group, which displayed evidence of PIV-3 growth on Vero cells using standard culture techniques and positive immunofluorescence in BAL fluid, and right lung tissue samples from virus-free medium and PIV-3 infected guinea pigs.

	<b>BAL Fluid</b>	<b>Right Lung Tissue</b>
<b>Virus-free medium + saline (Inhalation sensitisation) + saline (Inhalation challenge)</b>	0/6	0/6
<b>PIV-3 + saline (Inhalation sensitisation) + saline (Inhalation challenge)</b>	6/6	6/6
<b>Virus-free medium + ovalbumin (Inhalation sensitisation) + ovalbumin (Inhalation challenge)</b>	0/6	0/6
<b>PIV-3 + ovalbumin (Inhalation sensitisation) + ovalbumin (Inhalation challenge)</b>	6/6	6/6
<b>Virus-free medium + saline (Intraperitoneal sensitisation) + saline (Inhalation challenge)</b>	0/6	0/6
<b>PIV-3 + saline (Intraperitoneal sensitisation) + saline (Inhalation challenge)</b>	6/6	6/6
<b>Virus-free medium + ovalbumin (Intraperitoneal sensitisation) + ovalbumin (Inhalation challenge)</b>	0/6	0/6
<b>PIV-3 + ovalbumin (Intraperitoneal sensitisation) + ovalbumin (Inhalation challenge)</b>	6/6	6/6

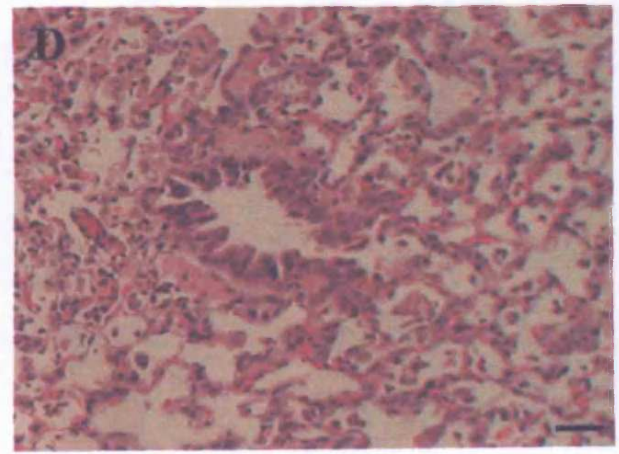
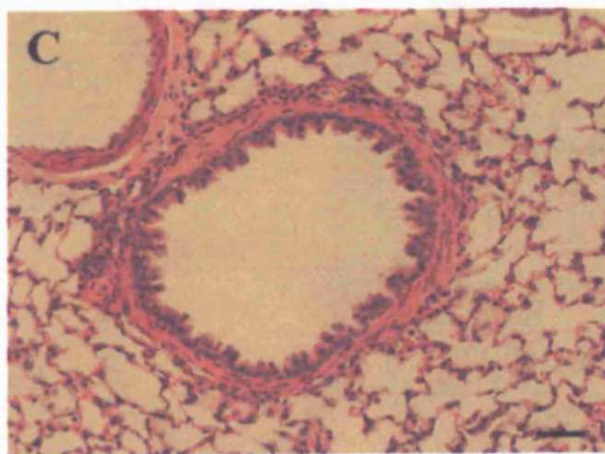
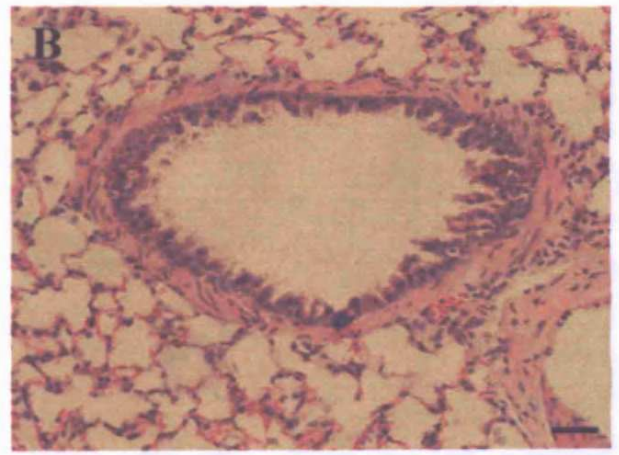
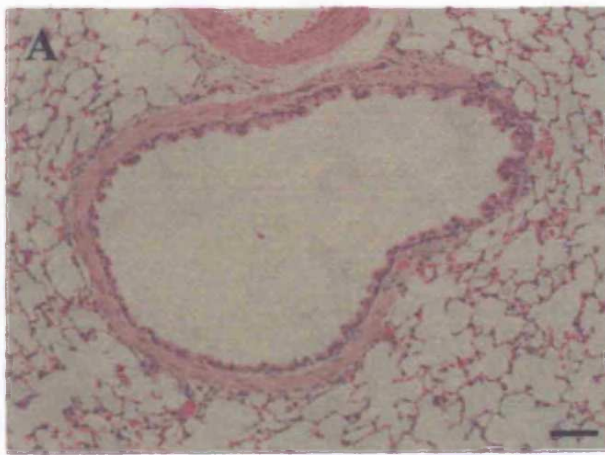


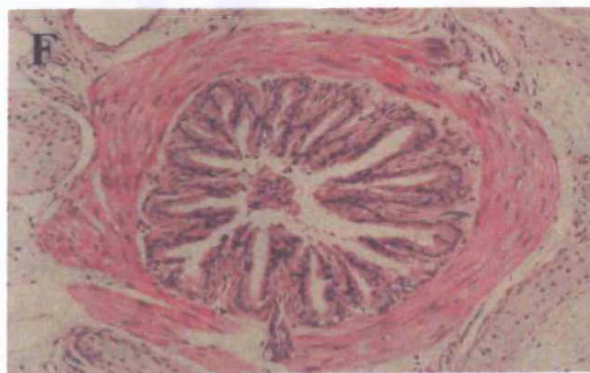
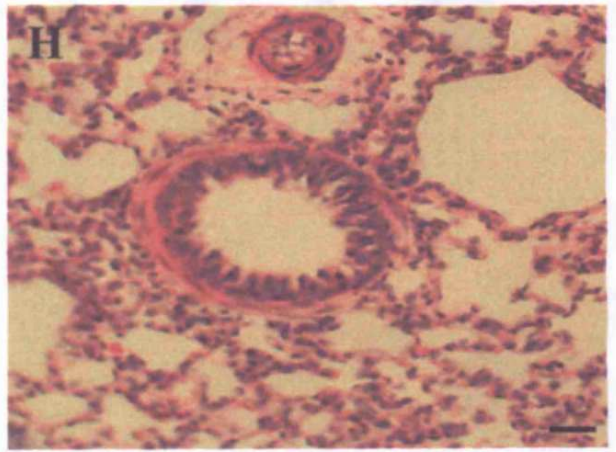
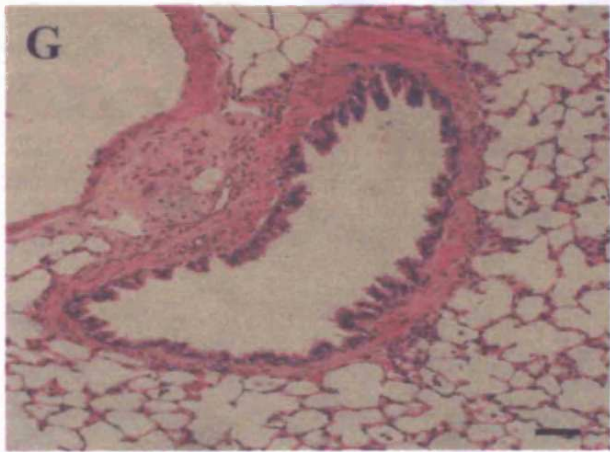
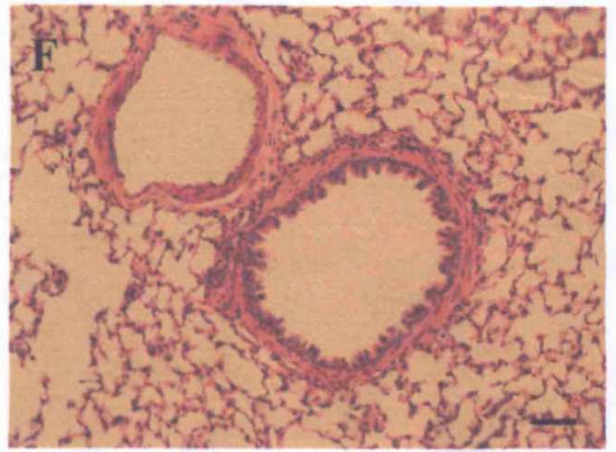
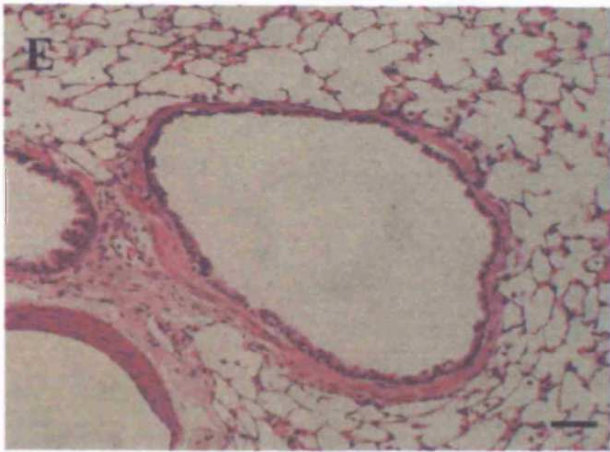
**Figure 8.11**

Titres of intrapulmonary replicating virus isolated from PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) infected guinea pigs sensitised with saline or ovalbumin by inhalation (Inh) or intra-peritoneal (I.P). Each point represents the mean  $\pm$  S.E.M of titres of intrapulmonary replicating PIV-3 normalised to wet left lung weight (TCID<sub>50</sub> ml<sup>-1</sup> g<sup>-1</sup>). No significant ( $P > 0.05$ ) differences in viral titre was observed during the study in any of the PIV-3 groups studied, or between groups, as determined by ANOVA (single factor).

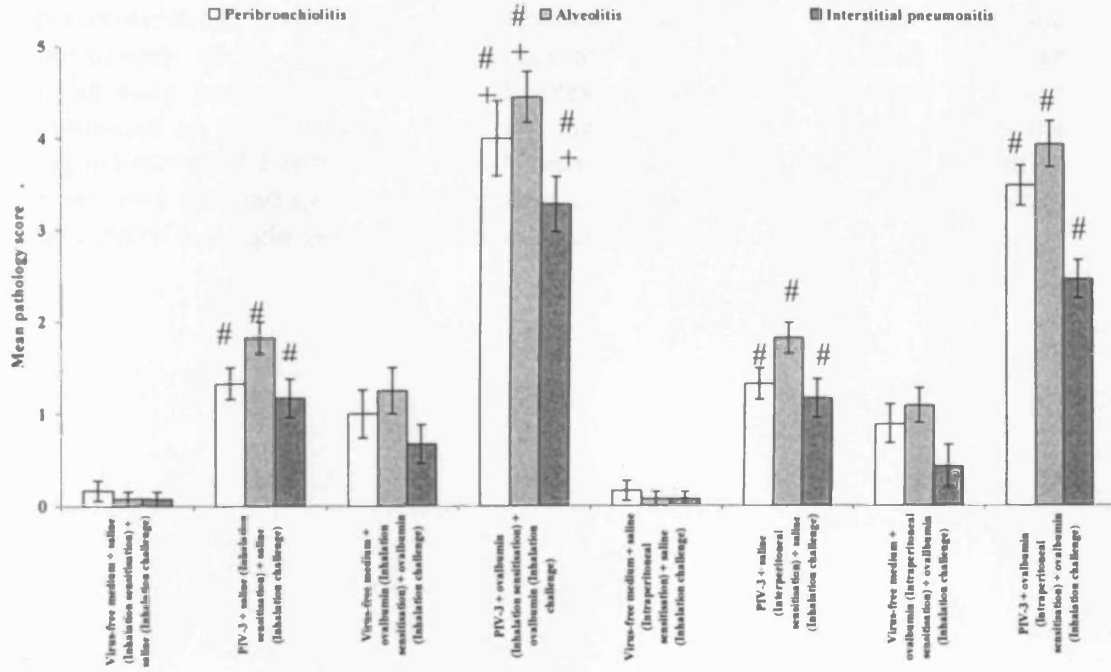
**Figure 8.12**

Histological appearance of lung tissue from (A) Virus-free medium + saline (Inhalation sensitisation) + saline (Inhalation challenge) (B) PIV-3 + saline (Inhalation sensitisation) + saline (Inhalation challenge) (C) Virus-free medium + Ovalbumin (Inhalation sensitisation) + Ovalbumin (Inhalation challenge) (D) PIV-3 + Ovalbumin (Inhalation sensitisation) + Ovalbumin (Inhalation challenge) (E) Virus-free medium + saline (Intraperitoneal sensitisation) + saline (Inhalation challenge) (F) PIV-3 + saline (Intraperitoneal sensitisation) + saline (Inhalation challenge) (G) Virus-free medium + ovalbumin (Intraperitoneal sensitisation) + ovalbumin (Inhalation challenge) (H) PIV-3 + ovalbumin (Intraperitoneal sensitisation) + ovalbumin (Inhalation challenge). Tissues were stained with haematoxylin and eosin for general morphology (Internal scale bar = 50µm).

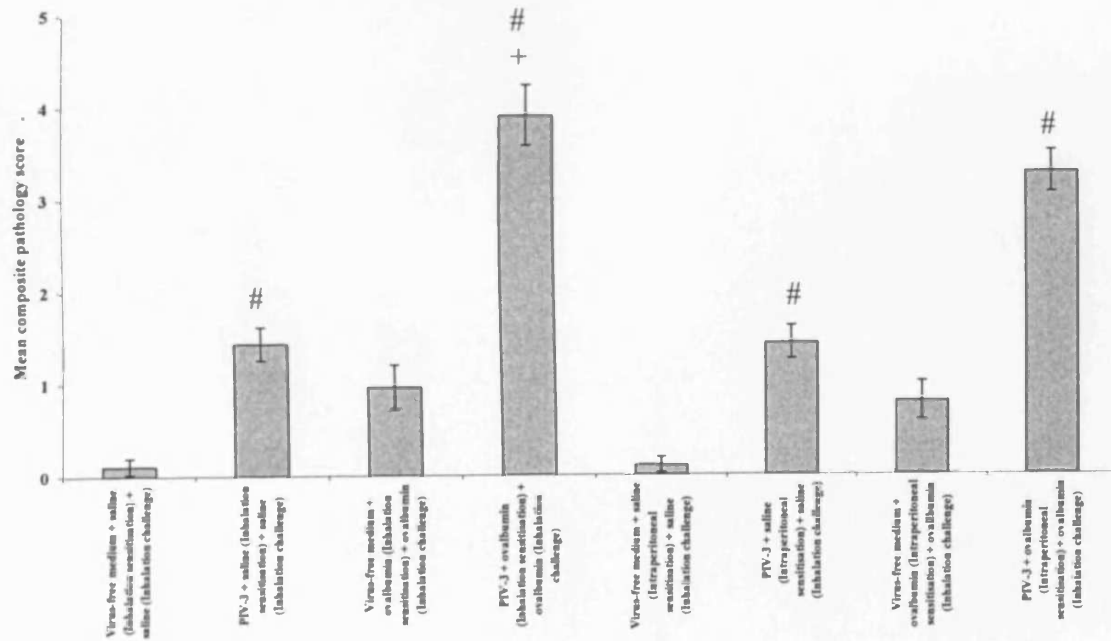




A



B



### **Figure 8.13**

Mean pulmonary pathology scores (as defined in chapter 2) from virus-free medium (Vero cell supernatant) or PIV-3 ( $5 \times 10^6 \text{ ml}^{-1}$ ) guinea pigs sensitised with ovalbumin or saline (Inhalation or intraperitoneal) and challenged with either saline or 0.01% ovalbumin. **A** Mean pulmonary scores for peribronchiolitis, alveolitis and interstitial pneumonitis **B** Mean composite pulmonary score. Each point represents the mean  $\pm$  (S.E.M) of mean pulmonary pathology. #  $P < 0.05$  denotes significant of difference in PIV-3 compared to corresponding virus-free medium groups. +  $P < 0.05$  denotes the significant of differences in PIV-3 sensitised with ovalbumin by inhalation compared with intraperitoneal injection. All statistical analysis was determined by ANOVA (single factor), followed by Dunnet's test.

Summary of results from virus-free medium (Vero cell supernatant) and PIV-3 (5x10<sup>6</sup> ml<sup>-1</sup>) guinea pigs sensitised with ovalbumin or saline (inhalation or intraperitoneal) and challenged with either saline or 0.01% ovalbumin.

Table 8.2

Treatment	Body weight (g)	Rectal temp (°C)	Lung function before exposure (s <sup>1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )	Lung function after exposure (s <sup>1</sup> cm of H <sub>2</sub> O <sup>-1</sup> )	Total Cells (10 <sup>6</sup> cells ml <sup>-1</sup> )	Macrophages (10 <sup>6</sup> cells ml <sup>-1</sup> )	Eosinophils (10 <sup>6</sup> cells ml <sup>-1</sup> )	Neutrophils (10 <sup>6</sup> cells ml <sup>-1</sup> )	Respiratory rate (breaths min <sup>-1</sup> )	Wet lung weights (g Kg <sup>-1</sup> )	Total nitrate and nitrite (µM)	Protein levels (µg ml <sup>-1</sup> )	Isolation in BAL fluid	Isolation in right lung	Viral titre (TCID <sub>50</sub> ml <sup>-1</sup> )	Mean pulmonary score			
																Peri bronchitis	Alveolitis	Interstitial pneumonia	
Virus-free medium + saline (inhalation sensitisation) + saline (inhalation challenge)	94.0±5.3	39.0±0.2	-1.5±2.4	2.8±2.5	4.2±0.4	3.5±0.3	0.5±0.05	0.2±0.03	158.0±4.3	8.5±0.3	70.1±3.3	1287.4±36.5	0/6	Not Detected	Not Detected	7.5x10 <sup>7</sup> ±2.0x10 <sup>7</sup>	0.7±0.1	1.8±0.2	1.2±0.2
PIV-3 + saline (inhalation sensitisation) + saline (inhalation challenge)	96.5±3.5	38.8±0.2	2.9±2.3	-3.4±2.5	3.6±0.2	3.2±0.2	0.4±0.04	0.06±0.01	80.0±5.1	5.5±0.3	65.2±4.5	1454.6±41.2	0/6	Not Detected	Not Detected	7.6x10 <sup>7</sup> ±2.1x10 <sup>7</sup>	1.0±0.2	1.3±0.3	0.7±0.2
Virus-free medium + ovalbumin (inhalation sensitisation) + ovalbumin (inhalation challenge)	90.0±4.6	38.8±0.2	0.7±1.5	-2.5±2.7	7.5±0.2	6.1±0.2	1.1±0.07	0.3±0.03	190.0±4.3	11.3±0.5	82.6±4.2	1834.2±048.3	6/6	Not Detected	Not Detected	7.6x10 <sup>7</sup> ±2.1x10 <sup>7</sup>	1.0±0.2	4.5±0.3	3.3±0.4
PIV-3 + ovalbumin (inhalation sensitisation) + ovalbumin (inhalation challenge)	86.0±6.5	38.7±0.1	-3.1±3.3	-2.3±1.6	2.3±0.2	2.0±0.2	0.2±0.04	0.03±0.01	80.0±4.7	4.6±0.4	51.2±3.6	1033.2±41.8	0/6	Not Detected	Not Detected	7.5x10 <sup>7</sup> ±2.5x10 <sup>7</sup>	0.1±0.09	0.08±0.08	0.08±0.08
Virus-free medium + saline (intraperitoneal sensitisation) + saline (inhalation challenge)	77.3±3.2	38.5±0.1	-1.8±3.8	2.8±2.3	4.0±0.3	3.6±0.3	0.4±0.1	0.1±0.02	140.0±4.7	7.4±0.5	72.6±3.9	1251.7±32.6	6/6	Not Detected	Not Detected	7.5x10 <sup>7</sup> ±2.5x10 <sup>7</sup>	0.2±0.1	1.8±0.2	1.2±0.2
PIV-3 + saline (intraperitoneal sensitisation) + saline (inhalation challenge)	79.2±2.6	38.8±0.2	1.4±3.5	-1.7±1.6	3.3±0.2	2.9±0.1	0.3±0.03	0.07±0.01	70.0±7.2	5.3±0.5	58.9±2.8	1429.3±39.1	0/6	Not Detected	Not Detected	7.0x10 <sup>7</sup> ±4.0x10 <sup>7</sup>	0.9±0.2	1.1±0.2	0.4±0.2
Virus-free medium + ovalbumin (intraperitoneal sensitisation) + ovalbumin inhalation challenge)	75.2±3.0	38.8±0.2	-2.7±3.6	-1.7±1.6	3.3±0.2	2.9±0.1	0.3±0.03	0.07±0.01	160.0±4.7	9.1±0.5	82.6±4.2	1613.7±46.3	6/6	Not Detected	Not Detected	7.0x10 <sup>7</sup> ±4.0x10 <sup>7</sup>	0.9±0.2	1.1±0.2	0.4±0.2
PIV-3 + ovalbumin (intraperitoneal sensitisation) + ovalbumin (inhalation challenge)	69.7±3.3	38.5±0.1	0.4±3.6	-4.1±2.8	6.0±0.3	4.9±0.2	0.9±0.06	0.2±0.03	160.0±4.7	9.1±0.5	82.6±4.2	1613.7±46.3	6/6	Not Detected	Not Detected	7.0x10 <sup>7</sup> ±4.0x10 <sup>7</sup>	0.9±0.2	1.1±0.2	0.4±0.2

## CHAPTER 9

### GENERAL DISCUSSION

*The ultimate aims of this work were to establish a time course of PIV-3 infection in guinea pigs, to determine the peak response to infection, and to further investigate the effect of pre-sensitisation with antigen on the responses to antigen in PIV-3 infected guinea pigs. Following this, the study also compared the anti-inflammatory and anti-viral effects of the glucocorticoid, dexamethasone and the PDE-4 inhibitor, rolipram.*

Viruses are the most common cause of lower respiratory tract disease in infants and young children and are a major public health problem in this age group (Van Woensel *et al*; 2004). Costs attributable to viral LRT infections in both outpatient and inpatients settings are an important burden on national healthcare budgets (Miedema *et al*; 2001). Human PIV's are second to RSV as a common cause of LRT disease in young children. Previous investigations in animals using PIV-3 virus infections have included both *in vitro* (Folkerts *et al*; 1992) and *in vivo* studies (Kudlacz *et al*; 1993). The maximal length of any *in vivo* study has been 19 days and therefore it was necessary to explore virus induced effects beyond this time point. Although some of the respiratory effects of PIV-3 infection have been examined by other investigators, a time course of PIV-3 infection was investigated in order to determine if there was a temporal relationship between the various parameters and therefore suggest if a causal relationship could be established.



Clinically, respiratory virus infection has been proposed as a common triggering factor in the development of allergy in children, and could also account for a large number of such episodes in adults (Frick *et al*; 1979, Zweiman *et al*; 1971). Schwarze *et al* (1997) showed that in mice, inhalation of an antigen after RSV infection increased both airway responsiveness and eosinophil influx in the lung. These studies suggest that specific interactions between respiratory allergies and viral infections exist, where viral illnesses in early childhood may enhance the development of allergen sensitisation. However, this relationship remains controversial, as many other epidemiological studies suggest that viral and bacterial infections, in fact, protect from the development of asthma and allergy (Strachan, 1989, Martinez, 1994, Shirakawa *et al*; 1997).

Despite decades of research, there are as yet no licensed agents with proven clinical efficacy against PIV infections (Henrickson; 2003). In recent years there has been a realisation that the therapy of infectious diseases needs to target both the infectious agent and host inflammation (Ottolini *et al*; 2002). Glucocorticoids have powerful anti-inflammatory activity and have traditionally represented the mainstay of anti-inflammatory therapy of the respiratory tract due to their far reaching effects that limit the activities of pro-inflammatory leucocytes and signal transduction pathways. The use of glucocorticoids as a therapy for infectious diseases is controversial, as they are immunosuppressant which may lead to reduced viral clearance causing an increased viral yield (Erlandsson *et al*; 2002). Recently, interest has focused on dexamethasone in the treatment of tracheobronchitis, (predominantly caused by PIV-1. It has been shown to decrease the severity of symptoms, and reduce the need for hospitalisations or time spent in out patient departments (Ausejo *et al*; 1999). Therefore, dexamethasone may reduce the severity of symptoms in PIV-3 infection.

In addition, research has also suggested the use of PDE-4 inhibitors in the treatment of viral infections. PDE-4, inhibitors exhibit anti-asthma effects due in part to their bronchodilatory and anti-inflammatory actions (Torphy, 1998). PDE-4 is the major isotype in guinea pig eosinophils (Raeburn *et al*; 1993, Souness *et al*; 1991) and was therefore an obvious choice in these studies.

### ***Time course model***

In the current studies, no signs of clinical infection (e.g. sneezing, panting, nasal discharge) were evident in the guinea pigs. Measurements of rectal temperature and body weight gain were not affected and therefore are poor indicators of viral-induced effects in this model. During the time course studies, AHR to the inhaled spasmogen was observed as early as day 1 post inoculation, reaching a peak response by day 4 and declining by day 40 where evidence of viral infection was minimal. This data is also consistent with clinical findings in patients, where hyperresponsiveness was observed in URT viral infections, during the infection and for several weeks after recovery (Empey *et al*; 1976). This prolonged AHR is likely to be caused by significant damage to the airway epithelium as exposed sensory nerves and airway smooth muscle contraction would allow easier permeability of spasmogens to airway smooth muscle and stimulate tachykinin-induced reflex bronchoconstriction from sub-epithelial sensory neurones (Folkerts *et al*; 1998). Thus in PIV-3 infected guinea pigs, damage to the airway epithelium, through viral replication, is a likely cause of the AHR (Folkerts *et al*; 1993). Respiratory rates of animals were also increased in infected guinea pigs, suggesting that inflammation and histology of the airways must have had a detrimental effect on respiratory function. However, this is not evident from airway conductance  $sG_{aw}$  measurements.

During the time course of PIV-3 infection, a rapid infiltration of inflammatory cell types including macrophages, eosinophils and neutrophils entered the guinea pig lungs which was also supported by the histological features exhibited in the lungs including, interstitial pneumonia, alveolitis, and peribronchiolitis. The majority of the inflammatory cells were alveolar macrophages, likely to be involved in the early immune response to viral infection. AHR in the study correlates with leucocyte infiltration, and it is likely that inflammatory cells are responsible for AHR. This is further supported by the anti-AHR effect of drugs that suppress inflammatory cell activity (e.g. dexamethasone and rolipram). The virus-induced inflammatory response was also reflected in the increased wet lung weights and protein levels in the BAL fluid indicating oedema, which may also contribute to the AHR and the increased respiratory rates.

Macrophages are also a potent source of iNOS (Barnes *et al*; 1999). The excess NO in these studies coincided with the duration of raised airway macrophages, and may implicate macrophages as a major contributor of NO overproduction in the airways, although eosinophils and neutrophils are capable of producing excess NO (Barnes *et al*; 1999). From the results of these guinea pig studies, an excess generation of iNOS-derived airways NO correlates with underlying inflammation condition in the airways.

Successful inoculation of the guinea pigs with PIV-3 infection was determined by measuring viral titres in the lung. Viral titres were shown to be detectable as early as day 1, reaching a peak titre 4 days post inoculation. Virus titres retrieved exceeded the initial inoculation titre and provides evidence that the virus was replicating effectively in the lung and levels were not due to the free virus remaining from the inoculation procedure.

### ***Sensitised model***

The objective of this part of the study was to determine the effects allergen sensitisation during the acute phase of PIV-3 infection by measuring the response to allergen challenges. As observed in the time course model, no significant changes in rectal temperature or body weight gain occurred in any of the groups studied. In this study exposure to PIV-3 enhanced the allergic responses of ovalbumin sensitised guinea pigs.

In this model the effects of allergen sensitisation during the acute phase of virus infection in PIV-3 guinea pigs sensitised by inhalation demonstrated an enhanced response of airway reactivity and inflammation. It failed to affect the viral titre 25 days post infection. However, other time points determining viral levels were not investigated in this study. The inflammation observed as a consequence of infection was also further supported by enhanced responses in wet lung weights, nitric oxide and total protein levels and respiratory rates in comparison to non-sensitised animals. One of the major changes observed in sensitised PIV-3 infected guinea pigs was the response to antigen challenge. Research has shown that inhalation of an antigen in atopic asthmatics has been shown to result in an early asthmatic response (EAR), reaching a maximum 15-30 min after challenge, followed by a late asthmatic reaction (LAR), 6-12 hours after challenge. However, in this model a significant bronchoconstriction for up to 12 hours was observed before returning to baseline levels and there was no clear distinction between early and late phase reactions.

Investigators have suggested that this effect may be due to increased permeability of the airway mucosa to allergens (Riedel *et al*; 1996; Kudlacz *et al*; 1995) and increased recruitment of dendritic cells to the respiratory epithelium during viral infection (Yamamoto *et al*; 2001). Therefore, damage to the respiratory epithelium would allow increased permeability of the antigen to ovalbumin, allowing more ovalbumin to enter the tissue, thus causing enhanced responses to airway reactivity and inflammatory parameters observed in this study.

However, studies have reported that the length of time between virus infection and exposure to allergen was crucial (Tsitoura *et al*; 2001). This may explain the conflicting results of clinical studies in humans demonstrating that respiratory viral infections either enhance the risk, or prevent the development of asthma.

### ***Drug treatment***

#### ***Dexamethasone***

In these studies, the effect of a glucocorticoid, dexamethasone (*in vivo* and *in vitro*) and a PDE-4 inhibitor, rolipram were evaluated in PI-3 virus infection. The effects of these treatments was determined at 4 days post inoculation, which was in concordance with the peak response of PIV-3. In these experiments, the dose-response data in the *in vitro* studies was not generated in these investigations, and the doses were selected based on dose-response findings of other investigators in guinea pigs (Dexamethasone: Whelan *et al*; 1995), (Rolipram: Danahay & Broadley, 1997).

Treating guinea pigs with dexamethasone (*in vivo*) provided significant anti-inflammatory activity, inhibiting AHR to histamine, significantly reducing respiratory rate, influx of inflammatory cells into the lung, pulmonary oedema (wet lung weights and total protein levels), pulmonary lesions and prevention of over production of airways NO. However, most importantly treatment with dexamethasone also reduced viral replication in the guinea pig lungs. This evidence of reduced viral replication, is further supported by observation *in vitro* using cultured Vero cells. Investigations revealed that by pre-incubation of the Vero cells with dexamethasone (1 day), which was then removed after PIV-3 infection, resulted in viral titres in the cell supernatants becoming the same on day 4 after PIV-3 infection as to the control. This data suggests that the inhibition of dexamethasone is reversible and secondly this shows that any effect of dexamethasone could not have been due to apoptosis or necrosis of the viral cells.

From this study, it can be suggested that dexamethasone may decrease viral replication in the lung via inhibition of ICAM-1 expression. Epithelial cells in the airways express ICAM-1 on their surface, which is the suggested site of attachment of PIV-3. ICAM-1 interacts physiologically with leucocyte function antigen-1 expressed on leucocytes, and thus plays a vital role in the recruitment and migration of immune effector cells to sites of local inflammation. Gao and co-workers (2000) have shown that ICAM-1 was significantly reduced when virions were UV inactivated prior to PIV-3 infection, indicating that ICAM-1 is mostly viral replication dependent. However, further study is required to examine the precise mechanisms and measure the levels of ICAM-1.

In this study, dexamethasone treatment was given prior to infection (pre-treatment) and clinically it is difficult to prescribe a patient treatment before they have contracted the virus. Therefore, it would be valuable in further studies to administer dexamethasone after infection to determine if this treatment has different effects on viral yield depending on the timing of infection and dosing.

### ***Rolipram***

Treating guinea pigs with rolipram also ameliorated the inflammatory response by inhibiting respiratory rate, significantly reducing AHR, respiratory rate, influx of inflammatory cells and pulmonary oedema and caused a reduction in lung lesions. However, rolipram failed to significantly affect viral replication in the lung. These findings support a role for PDE-4 inhibitors in the management of the inflammatory consequences of viral infections of the respiratory tract. However, the failure to reduce viral titre may argue against their use.

In conclusion, a temporal association was determined during the time course of PIV-3 infection of the parameters investigated, and PIV-3 infection enhances the effect of allergic sensitisation and challenge. The effects of dexamethasone on viral titre in PIV-3 infection *in vivo* and *in vitro* support a role for the anti-viral effects of dexamethasone. These studies have shown a clearly defined efficacy for the treatment of PIV infections, although further work is required to examine the anti-viral effect.

Further investigations of this nature are required in man to determine the validity of these findings in the guinea pig. However, any new treatment for viral infections will be well received, since it will reduce the burden on health care systems.

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# APPENDIX I

## PLETHYSMOGRAPHY THEORY

### *Airway Resistance*

Poiseuille's law describes the frictional resistance to a fluid flowing through a straight narrow tube. Assuming airflow to have the same properties as fluid, the equation can be adapted, such that the drop in pressure ( $\Delta P$ ), due to frictional resistance of laminar flow, equates to airways resistance in a smooth-walled tube.

$$(1) \quad \Delta P = 8\mu lv / \pi r^4$$

$\mu$  = Viscosity

$l$  = Length of tube

$r$  = Radius

$v$  = Flow rate

However, airways within the bronchial tree consist of many conduits and bends that are further subjected to dynamic changes, as bronchoconstriction and secretions reduce the airway calibre ( $r$ ). Therefore, as air flow increases velocity it becomes more turbulent and conversely, as air flow decreases and approaches zero velocity it becomes laminar. Flow through the airway is driven by the pressure difference ( $P_d$ ) between the upper airways pressure at the mouth ( $P_m$ ) and the peripheral airways pressure at the alveoli ( $P_{alv}$ ).

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

Airways 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**

Since conductance is the reciprocal of resistance, airways conductance ( $G_{aw}$ ) can be defined as:

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

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

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

## **Plethysmography**

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:

$$P_1 \cdot V_1 = P_2 \cdot V_2$$

Or

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

By multiplying the two brackets:

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

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

$$V \cdot \delta P = P \cdot \delta V$$

Inside a sealed plethysmography chamber, changes in the pressure differences ( $P_d$ ) between the upper airways pressure at the mouth and the peripheral airways pressure at the alveoli are reflected as changes in box pressure.

Box pressure changes result from the difference in chest volume ( $V_{chest}$ ) and respired air volume changes ( $V_{respir}$ ) at atmospheric pressure ( $P_{atm}$ ), corrected for saturated water vapour pressure ( $P_{svp}$ ). Therefore from Boyle's law:

$$(3) \quad \delta P_d \cdot TGV = \delta(V_{chest} - V_{respir}) \cdot (P_{atm} - P_{svp})$$

In order for Poiseuille's law to be true, flow must be laminar and therefore,  $v$  must tend towards zero, i.e. at flow manoeuvre reversal (end inspiration or end expiration).

Conventionally end expiration is frequently used since TGV approximates to the functional residual capacity. Hence any change in Pd must be small, i.e.  $\delta Pd$  which in turn will induce small changes in flow ( $\delta v$ ).

Therefore, substituting (2) into (3) gives:

$$(3) \quad sG_{aw} = \delta v / \delta(V_{chest} - V_{respir}) \bullet (P_{atm} - P_{svp})$$

Using computerised data acquisition, it is possible to measure  $\delta(V_{chest} - \delta r_{espir})$  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 ( $\delta v$ ).

$$P_{atm} = 1010 \text{ cmH}_2\text{O} \quad P_{svp} = 63 \text{ cmH}_2\text{O}$$

Therefore, by knowing  $\delta v$  and  $\delta(V_{chest} - \delta r_{espir})$  from the slopes of the flow and box volume traces respectively  $sG_{aw}$  can be calculated:

$$sG_{aw} = \delta [\text{flow}] / \delta [\text{box volume}] \bullet 947 \bullet cf s^{-1} \text{ cmH}_2\text{O}$$

Where  $cf$  is a correction factor to account for the volume of air displaced by the guinea pig ( $V_{gp}$ ). The density of a guinea pig ( $\rho_{gp}$ ) is  $1.07 \text{ kg}^{-1}$  and the volume of the plethysmography chamber ( $V_{box}$ ) is 5.5 litres. Thus:

$$V_{gp} (\text{Litres}) = W_{gp} / \rho_{gp}$$

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

$$V_n (\text{litre}) = V_{Box} - V_{gp}$$

Therefore:

$$cf = (V_{\text{Box}} - V_{\text{gp}}) / V_{\text{Box}}$$

$$cf = 1 - (V_{\text{gp}} / V_{\text{Box}})$$

$$cf = 1 - W_{\text{gp}} / \rho_{\text{gp}} \cdot V_{\text{Box}}$$

$$cf = 1 - W_{\text{gp}} / 5.88$$

