AN INVESTIGATION INTO THE MECHANISMS OF VIRUS INDUCED WHEEZING IN AN EXPERIMENTAL ADULT MODEL

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by

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To Cristina, Tessa, Anna and Rose.
Abstract

Episodic wheezing associated with viral infections of the upper respiratory tract (URT) is a common problem in young children but also occurs in adults. This study investigated the mechanisms that underlie 'viral wheeze' by developing an adult model using an experimental infection with human coronavirus (HCoV), the second most prevalent common cold virus. Twenty-four adults with a history of episodic viral wheeze and 19 controls were inoculated with HCoV 229E and monitored for the development of symptoms, changes in airway physiology and inflammatory changes in the URT by nasal lavage and lower respiratory tract (LRT) by induced sputum. Ten subjects also underwent bronchoscopy to collect bronchial biopsies during the cold and to search for evidence of virus infection of the LRT. At baseline, viral wheezers were similar to controls in airway responsiveness, although they had lower forced expiratory volume in one second (FEV₁), and in the measured markers of inflammation (nasal lavage cell counts, sputum differential white cell counts, and nasal and sputum interleukin-8 (IL-8), IL-5, interferon-γ (IFN-γ), and eosinophilic cationic protein (ECP)). Nineteen viral wheezers and 11 controls developed colds. Viral wheezers with colds reported significantly more URT symptoms than controls. Sixteen viral wheezers and no controls reported LRT symptoms (wheeze, chest tightness and shortness of breath). The viral wheezers with colds had small (3-4%) reductions in FEV₁ during the illness, but a progressive increase in airway responsiveness from baseline on days 2, 4 and 17 after inoculation, which affected both atopic and non-atopic subjects equally. A modest increase in nasal neutrophil count that correlated with nasal symptom scores was seen in both viral wheezers and controls with colds on day 4. A significant relative increase in sputum differential neutrophil count was seen on day 4 that correlated with LRT symptom scores in the viral wheeze group with a cold but not in controls. Change in sputum differential neutrophil counts for viral wheezers correlated with change in airway responsiveness. IL-8 increased in both URT and LRT in both viral wheezers and controls with colds, the largest change being seen on day 4 in the sputum of viral wheezers. Only modest changes were seen in IFN-γ and no changes in IL-5 or ECP. None of the results were influenced by atopic status. Conclusions. Viral wheeze is characterised by increased airway responsiveness, neutrophilic inflammation in both URT and LRT without eosinophilia. IL-8 is likely to be an important chemokine in this process.
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<td>URTI</td>
<td>Upper respiratory tract infection.</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assays.</td>
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<td>PIV</td>
<td>Parainfluenza virus.</td>
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<td>RSV</td>
<td>Respiratory syncytial virus.</td>
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<tr>
<td>HRV</td>
<td>Human rhinovirus.</td>
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<tr>
<td>HCoV</td>
<td>Human coronavirus.</td>
</tr>
<tr>
<td>PIV</td>
<td>Parainfluenza virus.</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction.</td>
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<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness.</td>
</tr>
<tr>
<td>PEF</td>
<td>Peak expiratory flow.</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in 1 second.</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide.</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon γ.</td>
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<tr>
<td>LRT</td>
<td>Lower respiratory tract.</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage.</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8.</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T-cell expressed and presumably excreted.</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage colony-stimulating factor.</td>
</tr>
<tr>
<td>IL-11</td>
<td>Interleukin-11.</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6.</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor-kappa B.</td>
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<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1.</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α.</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1.</td>
</tr>
<tr>
<td>LTB₄</td>
<td>Leukotriene B₄.</td>
</tr>
<tr>
<td>IFNα</td>
<td>Interferon α.</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon.</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophilic cationic protein.</td>
</tr>
<tr>
<td>Th</td>
<td>T helper.</td>
</tr>
<tr>
<td>Te</td>
<td>T cytotoxic.</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial.</td>
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<tr>
<td>SD</td>
<td>Standard deviation.</td>
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<tr>
<td>CI</td>
<td>Confidence intervals.</td>
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<td>ICC</td>
<td>Intraclass correlation coefficients.</td>
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<tr>
<td>LTC₄</td>
<td>Leukotriene C₄.</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin.</td>
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<tr>
<td>TCI₅₀</td>
<td>Tissue culture infective dose that causes cytopathic effect in 50% of cell cultures.</td>
</tr>
<tr>
<td>HEL</td>
<td>Human embryonic lung cells.</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol.</td>
</tr>
<tr>
<td>TCC</td>
<td>Total cell count.</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline.</td>
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<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate.</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Tween in phosphate buffered saline.</td>
</tr>
<tr>
<td>PBS-BT</td>
<td>1% Bovine serum albumin + Tween in phosphate buffered saline.</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy DNA.</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukaemia virus.</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density.</td>
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RIA .......................................................... radio-immunoassay.
EIA .......................................................... enzyme-immunoassay.
dATP .......................................................... 2'-deoxyadenosine-5' triphosphate.
SDS .......................................................... sodium dodecyl sulphate.
RT .......................................................... Reverse transcription.
PART I - INTRODUCTION
Chapter 1  Introduction

This thesis is concerned with the association between viral upper respiratory tract infection (URTI) and wheezing and the biological mechanisms that link the two together. This introduction will therefore review in turn wheezing and its clinical background in children and adults, the virology related to studies of the common cold and wheezing disorders, and finally, the current understanding of host-organism interactions that underlie viral wheeze.

1.1  Clinical background

1.1.1  The different phenotypes of asthma

Wheeze is a musical sound generated in the large airways. Forgacs' classic text on lung sounds describes wheeze as breath sounds generated by turbulent flow in the large airways with their intensity varying directly with the flow rate. Large airways have a smaller total cross-sectional area than small airways and when narrowing occurs, an increase in flow velocity of gas through them results in oscillation of the bronchial wall. This oscillation results in wheezing either reported as a symptom or detected as a sign on auscultation.

The study of wheezing disorders has been plagued by the difficulty in defining clearly the population being studied. There are two major considerations. Firstly, to a physician wheeze is a characteristic noise with musical quality heard on auscultation whereas wheeze to a patient may mean something entirely different. In a recent questionnaire to parents of wheezing children, some thought it a sound such as whistling, squeaking or rasping, others defined it as a different rate, style or timbre of breathing, and some thought it was the same as coughing. This is an important reminder to those studying and treating wheeze that 'all that wheezes might not be wheezing after
all'. Secondly, if wheeze is a sign or symptom produced by the physical narrowing of the airways, then like hemiplegia can have several different causes, so airway narrowing is an end result of several different pathological processes. These intrinsic complexities create difficulty when collecting data that is based solely on patient questionnaire or doctor diagnosis. Therefore the approach most widely used in surveys is to assume that all that wheezes is asthma with the implication that asthma is a single disorder characterised phenotypically by wheeze. This circumvents the variations and inconsistencies in doctor diagnoses and allows a symptom-based approach that can explore illness severity. It is however based on a false assumption, that asthma is a single phenotype disorder.

i) The impact of wheeze

Strachan has eloquently described the health problems associated with wheeze as a "mountain of morbidity" which creates a "shadow of sickness" seen in the country's GP surgeries and children's hospitals. There are several studies, reviewed by Strachan, which demonstrate a marked increase in the prevalence of wheezing. General practitioner diagnoses for asthma and bronchitis have risen dramatically over the last two decades, with the increase being most marked in the under-fives (a fivefold increase). Age-specific time trends for hospital admission show an upward trend in each age group but this is most dramatic for the under-fives (see Figure 1.1). These changes are not explained by the transfer of alternative diagnostic labels or due to a shift in the threshold of admission towards accepting milder cases for inpatient care. This increase in incidence of children with wheeze is mirrored by a similar increase in hospital admission rates and an explosion of prescribed medication for wheezing placing a huge burden on health services around the world. Not only is there significant morbidity suffered by children, there are many days
of work missed by parents, many consultations of healthcare workers and many admissions to hospital.

ii) Epidemiology of viral wheeze

Over the past 10-15 years the assertion that all that wheezes is asthma has increasingly been called into question. There are three major epidemiological studies that form the basis of this change of thinking. Firstly, Cogswell and colleagues demonstrated the importance of early exposure to Der p I in a small cohort of infants at high risk of developing asthma\textsuperscript{5,6}. Their data showed that early exposure, in the first year of life, was critical to the subsequent development of asthma; and that the higher environmental levels of Der p I in the first year, the earlier the onset of asthma and the more severe its manifestations. These conclusions, however, applied only to asthma at school age. In their population the odds ratio for atopic asthma was not greater in those children who wheezed in the first two years of life. Nor, in retrospect, were those children who were subsequently shown to be atopic at the age of 11 more likely to have wheezed in the first two years of life. These data suggested, for the first time, that wheezing disorders of young children and childhood atopic asthma were completely independent disorders.

The second major epidemiological evidence supporting different phenotypes of childhood wheezing comes from the cohort followed in Tucson, Arizona\textsuperscript{7}. In this study over 800 infants have been followed from birth with reports published to the age of six years. At least two different prognostic categories of pre-school wheeze with distinctive risk factors have been identified. One group ("persistent wheezers") initially suffered wheeze during viral infections, but wheezing persisted into school age in association with risk factors characteristic of classical, atopic asthma (elevated cord blood IgE levels and maternal history of asthma). Another group of children
transient wheezers) also suffered wheeze during viral infections, but were not associated with early markers of atopy. Interestingly, the transient wheezers had reduced lung function measured in the first year of life whereas the persistent wheezers did not. This strongly suggests some pre-existing condition, associated with reduced lung function in infancy, predisposes a child to viral wheeze.

The third evidence comes from a cohort of 2,511 children in Aberdeen enrolled at primary school and followed for 25 years. Three groups were selected based on a clinical diagnosis of asthma (n = 121), wheeze only in the presence of viral infection (n = 167) and controls (n = 167). Around 80% in each group were followed up for 25 years when current symptoms, atopic status, treatments, lung function and airway responsiveness were measured. Both asthmatics and viral wheezers were more likely to have current wheeze than controls (odds ratios (95% CI) of 14.4 (6.9 – 30.3), 3.8 (1.9 – 7.6) and 1, respectively). More asthmatics were on bronchodilators compared to viral wheezers and controls (42%, 12% and 6%, respectively), and more asthmatics took inhaled steroids compared with viral wheezers and controls (15%, 3%, 2%, respectively). There was no difference between the viral wheezers and controls in airway responsiveness but the asthmatics displayed the characteristic hyperresponsiveness to an inhaled challenge. This was the first study to clearly demonstrate physiological as well as symptomatic differences between adults with a history of viral wheeze and atopic asthma in childhood.

These studies have clearly demonstrated that there are different phenotypes of wheezing disorders and rather than there being ‘typical asthma’, asthma includes a spectrum of disorders. Indeed, there are several recognised causes of wheeze in childhood (Table 1.1). In addition to this, there is
evidence that not only is there a separate illness of viral wheeze, but this illness does not respond to inhaled corticosteroid therapy in the same way as asthma.

1.1.2 Corticosteroids and viral wheeze

Treatment of wheeze has followed the assumption that all that wheezes is asthma and consequently most children's doctors have used 'asthma' therapies in treating children with episodic wheeze in association with a viral URTI. The mainstay of asthma therapy has been the use of corticosteroids, both acutely during exacerbations and chronically as prophylaxis using low dose inhaled therapy.

A systematic review of studies into maintenance (prophylactic) low-dose corticosteroids and high-dose episodic corticosteroids for viral wheeze in childhood was conducted by the author in association with the Cochrane Collaboration. Only 5 papers, two dealing with prophylactic/maintenance inhaled corticosteroids, one in pre-school and the other in school children, and three dealing with episodic high dose inhaled corticosteroids in pre-school children, were identified. The paucity of literature on viral wheeze is in itself an important observation, highlighting the denial or poor awareness of this important phenotype.

The meta-analysis showed that acute high-dose corticosteroids were favoured by parents and reduced the need for rescue oral corticosteroids (Figure 1.2). Benefits were also seen with reductions in severity and duration of symptoms reported in the individual studies (a meta-analysis of symptoms was not possible due to the lack of a standardised symptom diary). No such benefits were seen with the low-dose maintenance corticosteroids.
The review adds weight to the assertion that episodic viral wheeze is a distinct disorder. The lack of chronic inflammation seen in those with viral wheeze compared to atopic asthma (discussed in chapter 3) may explain the lack of response to maintenance inhaled corticosteroids. It may be that entirely different mechanisms are responsible for chronic daily asthma symptoms and acute exacerbations, as has been suggested by a study of ‘classical’ adult asthmatics where maintenance low dose inhaled corticosteroids improved daily symptoms and peak flow variability but did not reduce the frequency or severity of exacerbations with URTIs.

1.1.3 Viral wheeze is a separate disorder

The fact that there are different phenotypes of asthma in childhood should not surprise us. This has long been the case in adults where ‘intrinsic’ asthma, occupational (allergic and non-allergic) asthma and atopic asthma are well defined. Paediatricians will recall that in the past ‘wheezy bronchitis’ was frequently the diagnosis used for children who wheezed only during colds. The evidence from studies of epidemiology and therapeutic intervention strongly suggest that viral wheeze is a separate disorder from atopic asthma. This is a major problem in preschool children where many are admitted to hospitals during epidemics of the common cold, however, it is not limited to the preschool years. The study of Doull and colleagues has shown that school children also suffer from this problem, although for most it is mild and dealt with in the community. The study of Godden and colleagues has shown that the problem can persist into adult life where viral wheezers are less likely to take inhaled corticosteroids than asthmatics and have normal airway responsiveness, but some will use bronchodilators. However, viral wheeze shares many common features with allergic asthma as both conditions encompass wheezing in the presence of an URTI. The key features that could differentiate viral wheeze from allergic asthma are:
**VIRAL WHEEZE**

- Wheeze only in the presence of an URTI
- Well in between episodes
- Normal airway responsiveness
- No interval airway inflammation

**ALLERGIC ASTHMA**

- Wheeze with a variety of stimuli*
- Chronic/daily symptoms
- Airway hyperresponsiveness off steroids
- Chronic airway inflammation
  (eosinophils and mast cells)

*allergens, exercise, cold air, pollen, dust, smoke etc.

There is no documented evidence of viral wheezers have variable airflow obstruction, one of the cardinal features of allergic asthma, as the main studies into this condition have been in young children. It seems unlikely that significant variable airflow obstruction is a feature of viral wheeze as there are no interval symptoms, but as this has not been studied this should not be relied on as a defining feature of viral wheeze.

With the increasing insights in the different phenotypes of wheeze the agenda has now been set for researchers of childhood wheeze to attempt to investigate the underlying pathophysiology in the hope of developing more effective therapies.

### 1.2 Virology background

#### 1.2.1 Epidemiology of viruses and wheeze

Important issues regarding the epidemiology of viruses that cause wheezing include a historical perspective on laboratory techniques and an appreciation of the spectrum of viruses identified in different populations, age-groups and illnesses. Studies of respiratory virus epidemiology have
been limited by the diagnostic methods available at the time. Early diagnostic methods used direct cell/organ culture to detect live virus in nasal secretions or immunological techniques to detect virus in smears on slides. Indirect immunological techniques were used to detect serum antibodies by fluorescence and enzyme-linked immunosorbent assay (ELISA).

These classical methods are relatively successful for the diagnosis of respiratory viral infections such as influenza, parainfluenza (PIV), respiratory syncytial virus (RSV) and adenovirus. However, rhinovirus (HRV) and human coronavirus (HCoV) do not grow well on standard cell lines and antibody responses are not easily detected, meaning that these two viruses were frequently not sought in epidemiology surveys, and if they were, suboptimal tests may have underestimated their prevalence. Even with the most exhaustive testing, including serology and organ culture, under-diagnosis was likely, with infectious agents being demonstrated in only 60% of common colds. Although believed to be highly specific, these tests are clearly not very sensitive.

Recent advances in molecular biology have permitted the sequencing of large parts of the genome of infectious agents including the common respiratory viruses. This has permitted the development of polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR) assays that provide sensitive and specific detection. The results of previous epidemiology HRV should therefore be interpreted in the light of this knowledge.

These techniques have increased our understanding of the range of viruses capable of causing upper and lower respiratory tract illnesses. The common cold is a frequent illness of childhood, its prevalence decreasing with age probably as a result of increased immunity and reduced
opportunity for spread. Despite there being many different virus types, there is a general predictability of seasonal occurrence with peaks being from late autumn to early spring. Prevalence rates vary considerably depending on age, time of year, and on the population being studied with additional variation from year to year. Many of these common respiratory viruses are associated with wheezing illnesses. Two recent surveys have demonstrated a clear link between respiratory viruses and exacerbations of asthma. Both studies used standard enzyme immunoassays on paired sera for identification of common respiratory viruses, including HCoV. Both HRV and HCoV were also detected by RT-PCR on nasal samples. These surveys therefore give the most up to date information on the virology of virus-induced episodes of asthma. In the first, Nicholson and colleagues studied 138 adults who had 315 exacerbations of asthma and found 80% of episodes were associated with a symptomatic viral URTI. Approximately 50% of samples had a confirmed cause, with HRV accounting for 64% and HCoV for 30%. In the second, Johnston and colleagues studied 108 children aged 9-11 years with asthma who had 292 reported exacerbations. Viruses were detected in 77% of episodes with HRV accounting for 65% and HCoV for 17% of these.

Different viruses are associated with wheezing illnesses at different ages (Table 1.1). In infancy, RSV and PIV cause bronchiolitis, a condition characterised by wheeze and the presence of crepitations on auscultation. This condition is distinct from viral induced wheeze and asthma, which are recurrent and persist beyond infancy. Infants with reduced lung function and/or exposure to tobacco smoke are at increased risk of developing wheeze with RSV infection, but only a subset of those who develop wheeze with RSV go on to develop recurrent wheezing. Beyond infancy, RSV and PIV are associated with wheezing episodes indistinguishable from those caused
by HRV and HCoV. It therefore seems probable that wheeze is a host response and not specifically
determined by virus type.

HCoV is of particular interest in regard to childhood wheezing. In a study using serology based on
haemagglutination to identify viruses, 42% of 139 exacerbations of wheeze in 32 pre-school
children had a positive viral diagnosis. HCoV accounted for one third of the positive isolates
but HRV was not tested for. Another study of sibling pairs under 6 years of age, one of the pair
suffering from recurrent viral wheezing, used ELISA to look for HCoV in nasal secretions. HCoV
was found in 30% of 108 URTIs in the index group and 29% of 51 infections in the sibling
controls. 'Wheezy bronchitis' occurred in 30% of the index group with a positive HCoV infection
but in none of the controls. In a similar study of 54 pre-school children with 'wheezy bronchitis',
viral pathogens were identified in 39% (30/76) of wheezing episodes with HCoV (9/30) and HRV
(8/30) most prominent. Therefore, HCoV is a common virus associated with childhood
wheezing disorders and is believed to be second in prevalence only to HRV.

1.2.2 Human coronavirus

The first report of HCoV was in 1965 when Tyrrell and Bynoe isolated a virus from nasal
washings that had been collected 5 years earlier from a male child. This child had typical
symptoms and signs of a cold and the washings were able to induce a common cold in volunteers
challenged intranasally. The term 'coronavirus' that describes the characteristic morphology of
these agents was accepted in 1968 (Figure 1.3i).

The coronaviruses are RNA-containing viruses with at least 10 genes, which transcribe for
polymerase, surface, membrane and nucleocapsid proteins. Two main serotypes cause common
colds, HCoV 229E and OC 43. Coronaviruses are pleomorphic, enveloped particles, approximately 100 nm in diameter with a characteristic ‘fringe’ of 20-nm long surface projections that are round or petal-shaped (Figure 1.3ii).

A range of techniques can be used to detect HCoV, including organ, mouse brain and cell culture, haemadsorption, electron microscopy and ELISA. Many of these techniques have proved technically demanding and time consuming. Cell culture, commonly performed for many respiratory viruses, is hampered by the failure to find an ideal cell line in which to grow HCoV and by the fact that the cytopathic effects are not easily recognised making identification difficult. However, with the advent of molecular biology, RT-PCR using gene amplification based on ‘nested’ priming have been shown to be a sensitive and specific means of identifying both 229E and OC43 strains. The assay is highly specific, relying on specific primers against the nucleocapsid genes, and is also at least as sensitive as a combination of cell culture and serology. This is borne out in the epidemiological survey of 292 reported virus induce exacerbations of asthma suffered by 108 children aged 9-11 years. RT-PCR identified HCoV in 21% of 80 available samples compared to 5% and 7% of 292 samples by culture and ELISA, respectively.

HCoVs have received relatively little attention by researchers interested in the common cold and its associated illnesses. Most of the published common cold research has focused on three clinical situations: healthy volunteers with natural colds, asthmatic volunteers with natural colds and experimental infections. The latter has usually involved infection, usually with a strain of HRV in healthy subjects, those with allergic disease such as asthma or allergic rhinitis. Because different viruses trigger wheezing the host response rather than virus...
type is likely to be a key feature in pathogenesis of wheeze. It is important to study other viruses, such as HCoV, to search for targets for therapeutic intervention effective over a range of viruses.

1.3 Mechanisms of viral wheeze.

1.3.1 Viruses and airway physiology

Airway obstruction is a term used to describe the limitation of airflow through the airways usually due to some form of narrowing. This narrowing may be fixed and irreversible, as in structural defects such as tracheal stenosis, or reversible as in asthma and viral wheeze. Reversible airways obstruction is one of the cardinal clinical features of asthma and classically the degree of obstruction is the major determinant of severity. Another feature that has come to play a central role in the theory and management of asthma is airway hyperresponsiveness (AHR). In simple terms airway responsiveness reflects the degree of airflow obstruction which is induced by a standardised stimulus. Techniques that quantify the degree of response to a set stimulus allow us to compare individuals and populations. Untreated asthmatics have a high level of response to such stimuli and are designated as having AHR. There is considerable evidence linking virus infection to changes in airway physiology.

i) The effects of respiratory viral infection on airway obstruction

The measurements of peak expiratory flow (PEF) and forced expiratory volume in 1-second (FEV$_1$) are used to represent the changes in large airway diameter that are seen during obstruction. There is only one study that has measured changes in lung function during episodes of viral wheeze (as distinct from chronic asthma). Doull and colleagues studied the effect of prophylactic inhaled corticosteroids or placebo in 52 children aged 7-9 years who had minimal or no chronic symptoms of asthma but wheezed with viral URTIs $^{10}$. Similar small reductions in PEF
manoeuvres (average reduction about 20%) lasting for an average of 13 days were seen during exacerbations in both treatment and placebo groups.

To gain further insight into the measurement of lung function during viral induced exacerbations of wheeze, we must look at studies of healthy and asthmatic adults during natural and experimental infections. One study of 20 healthy volunteers with 'wild' colds, three due to coronavirus, demonstrated a small but significant decrease in FEV₁ (mean reduction 0.19 L) along with increased airway responsiveness. The clinical significance of such a small fall in FEV₁ is questionable, but the “amplifying effect” on airway hyperresponsiveness may be relevant. In the many experimental infections of allergic and asthmatic individuals the same pattern is seen with only minor if any changes in FEV₁ and PEF during infection. This differs from common clinical observations in asthma exacerbations, when the majority (due to viral URTI) are associated with often large changes. There are several potential reasons for this: experimental infections use a virus that could be attenuated during passage in cell culture; virus is applied in an artificial manner; subjects usually suffer mild asthma. Another possibility is that greater changes are present in the peripheral airways and are not detected by change in FEV₁ and PEF, which are mainly determined by large airway function.

Therefore, although experimental models are extremely useful tools, for practical and ethical reasons, they usually examine mild illness that inevitably results in minor changes in symptoms and airway physiology. Another drawback of such measurements is that they may miss important wheezing and obstruction, for instance during a secondary trigger such as exercise. A more sensitive way to study the airway’s propensity to obstruct during infection is to measure airway responsiveness, the “airways amplifier”.

31
ii) The effect of respiratory viral infection on airway responsiveness

Viral infections of the respiratory tract can cause transient increased airway responsiveness in humans and in animals. The use of experimental infections with HRV has enabled the longitudinal study of airway responsiveness before, during and after infection. Cheung and colleagues inoculated 14 asthmatic subjects with either HRV 16 or placebo and measured airway responsiveness by inhalation challenge with methacholine, before, during and 2 weeks after inoculation. Those with virus infections had an increase in airway responsiveness by 2 days post inoculation that persisted beyond symptoms to 2 weeks post inoculation, whereas there was no change in the placebo group. Thus, HRV 16 is able to enhance the reactivity of the lower airways to inhaled contractile substances in asthma.

Similar results have been demonstrated in studies of adults with allergic rhinitis infected with HRV 16, asthmatics and healthy subjects infected with HRV 16 and in healthy subjects infected with influenza A. However, experimental infections with HRV-Hanks and HRV produced no changes in airway responsiveness. It would seem that not all experimental strains of virus have the same propensity to alter lower airway function, possibly because of laboratory attenuation of virus during culture or strain variation in cytopathicity.

The precise mechanisms of increased airway responsiveness are unknown but are likely to involve: airway structure, neural control, cytopathic effects of virus and inflammation. One factor is airway wall structure, which affects airway geometry, and which can amplify the effects of airway narrowing. With reduced airway caliber a standard dose of constricting agent will have a more pronounced effect on airflow. In those experimental studies in which HRV caused an increase in
airway responsiveness there was no associated decrease in FEV₁, suggesting that mechanisms other than geometry were involved, although FEV₁ alone is a rather insensitive measure of airway geometry.

iii) Alteration of neural control of the airways

Much of our detailed understanding of the neural control of airways comes from animal studies as definitive experiments often require the disruption of neural tissue. Viral infection could potentially cause bronchoconstriction and increased airway responsiveness by enhancing parasympathetic pathways, by stimulating sensory C fibres resulting in neural reflex bronchoconstriction, or by interfering with nonadrenergic, noncholinergic neurones that produce the potent bronchodilator, nitric oxide (NO) (Figure 1.4).

*Parasympathetic effects.* Altered neural control during viral URTI in human airways was first suggested in healthy subjects by the observation that inhalation of atropine before and after bronchial challenge prevented and reversed, respectively, the enhanced airway responsiveness suggesting that post-ganglionic cholinergic neurones were involved. This has been supported by a study of rodents infected with PIV in which the typical increase in airway responsiveness was blocked by atropine. Further studies in guinea pigs have demonstrated that the M₃ muscarinic receptors function normally in viral infection whereas M₂ muscarinic receptors on the vagal nerve endings (Figure 1.4), which normally inhibit acetylcholine release, are markedly dysfunctional during viral infection. This leads to a substantial increase in acetylcholine release and enhanced reflex bronchoconstriction. Potential mechanisms causing dysfunction of M₂ receptors include: viral neuraminidase deglycosilating the M₂ receptor thereby reducing its affinity for acetylcholine; release of IFN-γ with associated reduction in the expression of the M₂ receptor gene; and, in
atopies, the production of eosinophilic major basic protein which can bind to and block the M2 receptor.

The hypothesis that M2 receptor dysfunction accounts for the virus associated bronchospasm seen in asthmatics and those with viral wheeze is an attractive one but may not fully explain the prolonged changes in airway responsiveness demonstrated in some experimental infections. In rats infected with Sendai virus (similar to PIV), M2 receptor dysfunction is transient whereas virus-induced parasympathetic hyperresponsiveness continues for at least 2 weeks. The role of M2 receptor dysfunction during viral infection in humans is unclear but it is likely that there are other unidentified factors involved in parasympathetic hyperresponsiveness.

**Sensory C fibres.** Airway sensory C fibres (which form afferent fibres in the vagus nerve) act by initiating a bronchoconstrictor reflex in the brainstem and also at a local level by the release of neuropeptides such as substance P. Such neurogenic stimulation has been shown in animal studies to induce smooth muscle contraction and airway oedema, leukotriene synthesis, activation of mast cells and the facilitation of parasympathetic output. There is evidence that some of these mechanisms may be induced during viral infection with increased airway contractility to neuropeptides being observed in influenza A infected ferret tracheas and in guinea pigs infected with PIV. There is still no clear support for this in human models, although, a study of patients with mild asthma demonstrated a tachyphylaxis to repeated challenges with inhaled bradykinin and that inoculation with HRV16 abolished this tachyphylaxis.
Nonadrenergic, noncholinergic effects. Finally, experiments on viral infected rodents suggest there is a loss of nonadrenergic, noncholinergic activity that usually serves to oppose airway smooth muscle contraction\textsuperscript{76}, possibly by a reduction in the release of NO, a potent bronchodilator\textsuperscript{77}.

iv) Changes in small airway function

Much of the research on airway function during viral infection has concentrated on the proximal (large) airways. Changes in small airway structure and function are also known to contribute to the hyperinflation seen in severe asthma and could contribute to viral wheeze. PIV and RSV in rats and guinea pigs are known to produce bronchiolar wall oedema and inflammation\textsuperscript{78,79}. However, these viruses may behave in a very different way to HRV and HCoV, which in humans are the most prevalent causes of acute wheezy episodes. Little is known about small airway changes in viral exacerbations of asthma or viral wheeze.

1.3.2 Do viruses that trigger wheeze infect the lower airways?

There is no doubt that some types of virus can infect and replicate in the lower respiratory tract (LRT), particularly adenovirus, RSV and PIV. The ability of HRV and HCoV, the most prevalent common cold viruses, to infect and replicate in the LRT is controversial. HRV and HCoV have optimal replicating temperatures of 33°C\textsuperscript{27}, as occurs in the cooler nasal passages\textsuperscript{80}. The warmer temperature of the LRT is believed to be less conducive to virus replication leading to the argument that these viruses do not replicate in the LRT. If this is the case then there must be some 'indirect' link between the URT, where typical common cold symptoms are generated from the infected mucosa, and the LRT in those predisposed to wheeze during colds.
The assertion that HRV does not replicate at warmer temperatures has recently been refuted by a study in which several serotypes of laboratory cultured HRV and wild-type HRV infected bronchial cell cultures at 33 and 37°C. Although replication was generally better at 33°C, several strains of HRV were able to infect and replicate at 37°C. It is also important to note that during viral URTI when the nasal passages are congested and blocked, subjects mouth breathe and consequently lose the warming effect the nose has on inspired air. The effect is to reduce tracheal temperature and therefore to create the conditions conducive to viral replication in the lungs.

What evidence of *in vivo* infection of the LRT with HRV or HCoV exists? Johnston has described observations made during bronchoscopy of volunteers infected with HRV-16 in which there was redness of the trachea and patchy erythema around and beyond the carina. However, redness of the airways implies inflammation but not necessarily infection. Gem and colleagues have detected HRV by RT-PCR in cell pellets from BAL taken from volunteers with allergic rhinitis infected with HRV16. Contamination of BAL samples with HRV as the bronchoscope passed through the upper airway could not be excluded.

In order to overcome these criticisms it is necessary to prove infection by *in situ* hybridisation, identifying viral genome within bronchial cells of a biopsy. Papadopoulos and colleagues have recently demonstrated this. In bronchial biopsies taken from 10 subjects (3 asthmatic and 7 normal) during an experimental infection with HRV16, both genomic and replicative virus RNA was detected in epithelial cells of four subjects (2 asthmatic and 2 normal) 4 days after inoculation, the height of symptoms. No virus was detected in baseline biopsies, but 2 biopsies were still positive 6-8 weeks after infection. It was speculated that this was due to wild infection, although
there was no data to confirm the disappearance of virus from the URT. A second possibility is the persistence of genome in the LRT.

Taking all these data together there does appear to be strong evidence that HRV infects and replicates in the LRT. However, the degree of replication and the relationship to symptoms remains unclear. It at least seems plausible that ‘direct’ infection of the LRT occurs and therefore could be important in triggering LRT inflammation and wheezing. Whether this applies to HCoV and whether this occurs in isolation or combination with ‘indirect’ influences on the LRT from the URT has not been established. If virus LRT infection is involved in wheezing disorders it’s role in other diseases such as pneumonia, bronchitis and cystic fibrosis should also be investigated.

1.3.3 Viruses and airway inflammation

Many studies have established that respiratory viruses cause inflammation in the respiratory tract. The strong association between inflammation and symptoms strongly suggests that the study of inflammation and the immune response to virus infection may provide the key to understanding the mechanisms of viral wheeze and perhaps lead to the development of new therapeutic interventions. The host response to a viral URTI can be divided into two broad areas: (1) early innate responses mediated through epithelial and other cells and (2) adaptive immune responses mediated through T cells.

i) Epithelial cells

Epithelial cells have two key roles in the airway response to viral infection: they are the principal site of viral replication and they respond to infection by the release of chemical mediators important in initiating an inflammatory response. Evidence of damage to respiratory epithelium during infection with viruses such as HRV and HCoV comes largely from the URT, with
surprisingly little known about the LRT. Different viruses produce different degrees of cell
damage, RSV and influenza virus producing a more marked cytopathic effect than HRV.88,89.

Damage to the epithelium could have several direct effects on the airway. The epithelium acts as a
barrier and disruption may expose the underlying tissues to virus, viral antigens or other inhaled
agents. The ciliated surface is functionally important for the homeostatic mechanisms of the
respiratory tract allowing clearance of debris and mucus and its disruption undoubtedly leads to
the development of nasal symptoms. If epithelial damage occurs in a similar way in the LRT this
too may contribute to LRT symptoms that develop in susceptible individuals. Alternatively, URT
epithelial damage could be the primary initiating event leading to secondary effects on the LRT.

Epithelial cells respond to virus by producing a wide range of pro-inflammatory cytokines,
chemokines and adhesion molecules. For example, interleukin-8 (IL-8) is a potent neutrophil
chemoattractant and is elevated in nasal secretions of children with virus-induced asthma.90 Both
RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), a
chemoattractant for eosinophils, and granulocyte/macrophage colony-stimulating factor (GM-
CSF), a potent activator of eosinophils, are released by epithelial cells.91,92 RANTES secretion is
produced by RSV and influenza A infection of epithelial cell cultures91,93 and levels are increased
in nasal aspirates from children with virus-induced exacerbations of asthma.94 GM-CSF was not
elevated in non-atopic adults infected with HCoV92 but certainly plays a role in allergic airways95
and it has been proposed as a candidate molecule involved in virus-induced asthma.84 Another
chemokine for eosinophils, eotaxin, has also been shown to be elevated in nasal secretions of
atopic individuals during an experimental rhinovirus infection96. Interleukin-11 (IL-11) is secreted
in large amounts by epithelial cells infected with HRV, parainfluenza virus and RSV in vitro.97
and has been implicated in airway obstruction in mice. The activation of the genes of these cytokines is also an area of interest. The transcription factor nuclear factor-kappa B (NFkB) has been linked to the transcription of interleukin-6 (IL-6), a pro-inflammatory cytokine, and IL-8 in cell cultures infected with HRV and may be an important step in the mechanisms of virus-induced inflammation.

Cell recruitment to the site of infection is governed not only by cytokines and chemokines, but also by the expression of molecules on the surface of epithelial cells. HRV infection of epithelial cell cultures induces its own receptor, intercellular adhesion molecule-1 (ICAM-1). ICAM-1 may be involved in the retention and activation of mucosal lymphocytes and eosinophils and its upregulation is also promoted by NFkB. Other adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1), a molecule upregulated during inflammation, may also be involved. It has now been confirmed *in vitro* that HRV increases the expression of ICAM-1 and VCAM-1 on respiratory epithelial cells via the transcription factor NFkB, with the transcription factor of the GATA family also involved in the upregulation of VCAM-1. A recent *in vivo* study of asthmatics inoculated with HRV16 has also found evidence of ICAM-1 expression in bronchial biopsies. Such adhesion molecules and transcription factors could be potential therapeutic targets at reducing virus-induced inflammation, although as with VCAM-1, targeting a single transcription factor may not be sufficient to block an inflammatory stimulus.

A further function of epithelial cells is the synthesis of nitric oxide, a potent smooth muscle relaxant. The production of nitric oxide is reduced in guinea pig airways after infection with PIV and this is associated with AHR. Reduced nitric oxide production in the URT could have an ‘indirect’ effect on LRT airway physiology via inspired air.
ii) *Endothelial cells*

The endothelium is also likely to contribute to the recruitment of inflammatory cells and consequently airway dysfunction during viral infection. Serum transudation occurs 2-4 days after HCoV and HRV infection as shown by increased levels of proteins such as fibrinogen and albumin in nasal lavage suggesting altered endothelial permeability \(^{105,106}\). This leads to nasal congestion and secretions. The endothelium is also known to play a key role in the recruitment of eosinophils to the airways during asthma by the expression of adhesion molecules \(^{107}\) and is likely to be involved in cell recruitment from the circulation during virus-induced respiratory tract inflammation.

iii) *Macrophages and monocytes*

Airway macrophages are a mixed pool of cells derived from circulating monocytes that form one of the first lines of defences in the pulmonary immune system. They are likely to be involved in the early immune response to respiratory viruses through the secretion of mediators and cytokines with antiviral properties and/or proinflammatory effects. RSV and influenza virus are known to stimulate the release of cytokines such as tumour necrosis factor \(\alpha\) (TNF\(\alpha\)), interleukin-1 (IL-1), IL-6 and IL-8 from macrophages infected *in vitro* \(^{108,109}\). HRV can enter macrophages and induce the production of IL-8 \(^{110}\), but is thought not to replicate in these cells \(^{111}\). It is not known whether HCoV can infect macrophages.

Cytokines such as IL-1 and TNF\(\alpha\) can increase cell recruitment to the airways leading to the inflammatory response characteristic of viral infection. Furthermore, a group studying alveolar macrophages taken from BAL samples from wheezy and non-wheezy infants found a 5.6 fold
increase in the spontaneous release of TNFα in severely wheezy infants compared to controls\textsuperscript{112}. This group also looked at arachidonic acid metabolites in alveolar macrophages from wheezy and non-wheezy infants and found increased release of proinflammatory eicosanoids thromboxane A\textsubscript{2} and leukotriene B\textsubscript{4} (LT B\textsubscript{4}) in the wheezers\textsuperscript{113}.

Macrophages are also a source of interferon (IFN) and this can be induced by virus infection\textsuperscript{114}. Interferons are proteins and glycoproteins that protect other cells against infection. There are two major types: interferon α (IFNα), part of the innate immune system and produced by lymphocytes as well as macrophages, and IFNγ, part of the adaptive immune system and produced mainly by activated T cells. Both types are present in nasal lavages of subjects with common colds\textsuperscript{92,115}. Indeed IFNα has been used with some limited success as a nasal spray to prevent common colds\textsuperscript{116}.

Macrophages also act as antigen presenting cells and may also have a role in developing a specific immune response to viruses. The role of macrophages in the different phenotypes of wheezing is still unclear, but as functionally important cells that are present in large numbers in the respiratory tract, it seems likely they may be involved in viral wheeze.

\textit{iv) Neutrophils}

The relatively minor damage to epithelium seen in early histopathological studies of virus-infected nasal mucosa led to the search for other changes that could account for the symptoms of a common cold. Further studies observed an influx of neutrophils into the nasal mucosa and nasal secretions that was not seen in asymptomatic infections or uninfected subjects\textsuperscript{33,34,117}. These studies have established that neutrophils are the main cell recruited into the upper respiratory tract (URT)
during the acute stages of an URTI. The strong temporal relationship between symptoms and 
neutrophil influx suggest that these cells play some role in the pathogenesis of the URT illness.

The role of the neutrophil in LRT inflammation during respiratory virus infections is less clear. A 
neutrophilia has been demonstrated in two studies of ‘wild’ type virus exacerbations of asthma 
31;32. In a study of 18 subjects with acute severe asthma attacks, 50% of which were thought to be 
due to colds, 86% of sputum cells were found to be neutrophils when the exacerbation was 
associated with an URTI, whereas in exacerbations without an URTI, 57 % were neutrophils 31. 
Another study of 5 asthmatics with virus-induced asthma exacerbations showed a modest sputum 
eutrophilia of 58% during the illness compared to 52% twenty-one days later 32. Neutrophils are 
also recruited to the lungs of infants infected with RSV bronchiolitis but there is no knowledge 
of their involvement in viral wheeze.

The relationship of neutrophils in the LRT to symptoms such as wheeze is far from clear. A study 
of children with common cold symptoms limited to the URT found significantly more neutrophils 
as well as lymphocytes in BAL fluid than in asymptomatic children 119. Whether it is simply 
degree of neutrophil influx or a more complex mechanism depending on the interaction of other 
host factors with neutrophils that separates wheezers from non-wheezers needs to be established.

Neutrophils are likely to be recruited to the airways by chemotactic factors such as IL-8 and LTB4 
90;120. IL-8 is a potent chemokine for neutrophils produced by epithelial cells, macrophages, 
lymphocytes and neutrophils 121. IL-8 has been found in the media supernates from HRV and 
influenza-A virus-infected cell cultures 93;122;123, in nasal secretions of HRV-infected volunteers 
35;39;55;124 and in nasal secretions of children with virus-induced asthma and bronchiolitis 90;125;126.
Not only is nasal IL-8 associated with neutrophil influx to the URT, it has also shown to correlate with nasal symptoms in healthy adults infected with rhinovirus\textsuperscript{127} and with airway responsiveness in asthmatic adults infected with rhinovirus\textsuperscript{35}.

The primary cell believed to be infected by viruses such as HRV and HCoV is the epithelial cell. In bronchial epithelial cell cultures, rhinovirus can stimulate IL-8 production by infection of the cells and by binding of inactive rhinovirus to its receptor\textsuperscript{123}. It seems possible that the epithelium, either by viral infection or virus-antigen stimulation could initiate the migration of neutrophils into the airways and play an important role in the pathogenesis of LRT symptoms.

How might neutrophils within airway tissues be involved in the pathogenesis of LRT symptoms such as wheeze? Traditionally it has been proposed that neutrophils limit viral infection of the URT, however, these cells may cause tissue damage through the release of cytotoxic granule-derived proteins such as elastase and myeloperoxidase, oxygen metabolites and lipid-derived mediators such as LTB\textsubscript{4}. An animal study has demonstrated the involvement of neutrophils in tissue damage by the administration of anti-IL-8 antibodies in rabbits with lung reperfusion injury resulting in a reduced neutrophil influx associated with decreased lung damage\textsuperscript{128}. If neutrophils do cause LRT tissue damage in those who wheeze one could speculate that such damage could have secondary effects, such as tissue swelling and a reduction in the protective epithelial barrier, which in turn could facilitate the activation of mechanisms leading to bronchospasm and heightened airway responsiveness.

\textit{v) Eosinophils}
Eosinophils are a key cell found in greater numbers in atopic individuals and believed to be involved in the pathogenesis of lung inflammation in allergic asthma. Several studies point to a role for eosinophils in viral exacerbations of asthma. Eosinophilic cationic protein (ECP), a granular protein released by activated eosinophils, is found in increased concentration in the sputum of adult asthmatics infected with HRV 16. Two recent studies of wild URTIs produced evidence of eosinophil influx in sputum expectorated by asthmatics. In 18 asthmatics, half of whom had exacerbations triggered by colds, 3 subjects had markedly increased eosinophils whereas 10 had markedly elevated neutrophils. In another study, 5 asthmatics suffering a virus-induced exacerbation were found to have high levels of ECP despite relatively low levels of eosinophils suggesting activation of eosinophils in the epithelium and/or submucosa. An experimental infection has demonstrated the involvement of eosinophils along with lymphocytes in LRT submucosa during HRV colds in asthmatics and healthy subjects, the increased eosinophils persisting for 6 to 10 weeks in the asthmatics.

Clearly eosinophils are involved in viral exacerbations of asthma in adults but what of their role in children? There is evidence from several studies that eosinophils are involved in childhood asthma exacerbations in a similar way as in adults. As viruses are the main cause of childhood asthma exacerbations it seems likely that eosinophils are an important cell. There is, however, little evidence of the role of eosinophils in pre-school episodic viral wheeze. Indeed the only study looking at lower airways found no evidence of eosinophilic inflammation. In this study, non-bronchoscopic BAL was conducted in asymptomatic pre-school children with atopic asthma, episodic viral wheeze and healthy controls as they underwent routine minor surgery. Those with atopic asthma had increased numbers of eosinophils and mast cells compared to viral wheezers who were similar to controls. These results give the first cellular evidence that there are different
underlying mechanisms behind viral wheeze and atopic asthma. There does seem to be a chronic inflammatory process in asthma but this is not the case in viral wheeze. There is still no data on the biological mechanisms during viral exacerbations of asthma or viral wheeze as it is ethically difficult to collect LRT samples in these circumstances from young children.

vi) T cells

T cells are of particular importance in the adaptive immune response by promoting antibody production, mediating inflammation and encoding the long-term immune memory. Evidence supporting the involvement of T cells in viral infections will be discussed in terms of T cell migration and activation, and the different responses seen in different groups of people.

*T cell migration.*

The greatest body of information regarding T cell migration comes from experimental infections with HRV. Increased numbers of lymphocytes as well as neutrophils have been demonstrated in nasal secretions 3-4 days following infection with HRV. Most but not all studies have found a coincident peripheral blood lymphopenia. The latter study failed to demonstrate lymphopenia but showed an increase in T cells 23 days post-inoculation. There is also evidence of lymphocyte recruitment to the lower airways during HRV infections. Fraenkel and colleagues found a significant increase in CD3+ lymphocytes in the submucosa of bronchial biopsies taken from volunteers 4 days after inoculation with HRV returning to normal levels 6 weeks later. They also found trends of increased CD4+ and CD8+ lymphocytes infiltrating the submucosa during colds, which correlated positively with the subsequent level of seroconversion. These findings were present in healthy volunteers as well as atopic asthmatics. It seems likely that T cells leave the blood stream during viral URTIs and migrate into the airways.
There is limited information in the literature regarding HCoV and T cells. Callow and colleagues have demonstrated a peripheral blood lymphopenia during HCoV 229E infection suggesting that perhaps responses similar to those found in HRV infections can be expected. Peripheral blood mononuclear cells have been shown to be inversely proportional to airway responsiveness during experimental HRV infections.

These studies suggest that HRV mobilises T cells from the circulating pool of lymphocytes into both the upper and lower airway and may participate in some way to the development of symptoms and bronchial hyperresponsiveness.

_T cell - virus interaction._

**Cytotoxic T Cell (CTL) recognition of virus-infected cells.** The key to cellular recognition by CD8+ T lymphocytes is expression of major histocompatibility complex (MHC) class I molecules. In humans these are known as human leukocyte antigen (HLA) class I and are divided into HLA-A, B and C. During viral infection any cell expressing MHC class I molecules is able to present viral antigens to the host immune system via a ubiquitous antigen processing system that starts in the endoplasmic reticulum. Here the peptide antigens are bound into the groove of the MHC class I molecule and stable complexes are then transported to the cell surface through the trans-golgi where they are displayed to the host immune system. In an infected cell there will be some MHC class I molecules with virus antigen attached but many more with host-derived peptides. It is the job of the CD8+ T lymphocytes to recognise these viral peptide-MHC complexes via a specific T cell receptor (TCR) that is unique to each T cell clone. Once recognised, cell signalling via phosphorylated molecules activates the CD8+ cell via the activation of kinases that
lead to: proliferation; secretion of cytokines; and release lytic granules that are able to kill target cells.

**Respiratory virus-T cell interaction.** Viral recognition is a key step in the development of immunity. HCoV is thought to enter ciliated epithelial cells by a specific receptor, aminopeptidase N in the case of 229E virus. For HCoV the events which follow this and lead to the immune response remain unclear. Again HRV studies are more advanced and may give some insight into potential mechanisms for HCoV. Animal studies have demonstrated HRV binding to T, B and dendritic cells. Following this the virus is internalised and processed so that antigen can be presented on the cell surface, by mechanisms that are likely to be similar to those given above. Such antigen presentation may account for the HRV specific T cell response in peripheral blood seen in several studies. Gern and colleagues have also shown in-vitro direct activation without antigen presentation of HRV-specific T cells by a soluble factor released from peripheral monocytes after binding HRV. The response of T cells is not serotype-specific suggesting HRV has some shared epitopes.

**Activation effects.**

**Type 1 and Type 2 cells.** There is much current interest in the functional polarisation of lymphocytes in asthma, the polarity being determined by different cytokine products. Different patterns of cytokine products are seen in both CD4+ T helper cells CD8+ T cytotoxic cells. The patterns were originally identified by analysis of murine CD4+ T helper clones, with 'Th1' defined as producing IFNγ, TNF and lymphotoxin, but not IL-4, IL-5, or IL-13. 'Th2' cells were defined as producing IL-4, IL-5, IL-9, IL-10, and IL-13 but not IFNγ. While many cell populations conform to this paradigm in terms of non-overlapping patterns of cytokine expression,
under physiological conditions the paradigm is more applicable to populations with polarisation evident from the quantity of cytokine expression. There are several features other than cytokine expression that distinguish Th1 and Th2 cell populations. Among these are cytokine, chemokine and adhesion receptors, which presumably influence the migration and homing of differentiated T-cells and their response to their immediate environment. The IL-12 receptor β-chain, the IFNγ receptor β-chains, and the chemokine receptors CXCR3 and CCR5 are preferentially expressed on Th1 cells, whilst CCR3 (the eotaxin receptor), and the chemokine receptors CCR4 and CCR8 are preferentially expressed on Th2 cells. Th1 and Th2 cells are also distinguished by their different expression of certain transcription factors (ERM and T-bet in Th1 cells, GATA-3 and cMaf in Th2 cells) that are important in maintaining the differential expression of cytokines.

The importance of the later with regard to respiratory virus infection is not yet known.

The role of CTL during viral infection. Much of the understanding of the role of CTL in viral infection stems from murine models experimentally infected with viruses. CTL arise early after infection and process surface markers that enable them to migrate towards infected tissue. There they encounter infected cells which they lyse. The role of CD8+ cells is believed to be supported by CD4+ cells and neutralising antibodies from B cells.

T cell function in respiratory virus infection. Respiratory virus infection usually promotes CD4+ Th 1 and CD8+ T cytotoxic (Tc) 1 cells characterised by the production of IFNγ and IL-2 which have direct antiviral activity by the production of IFNγ. Respiratory virus infection also promotes the proliferation of natural killer cells which also possess cytotoxic activity. Th2 and Tc2 responses are thought to be important in asthmagenesis by releasing IL-4, which promotes the production of IgE, and IL-5, which attracts eosinophils. A recent experimental infection with
HRV-16 has shown that predominance of a type-1 reaction according to sputum IFNγ/IL-5 ratio is associated with reduced symptoms and more rapid clearance of virus when compared with the predominance of a type-2 reaction. This suggests that the immunophenotype of an individual is involved in the response to virus URTI. Whether this predisposes to wheeze as yet is unclear. The balance of 'type-1' / 'type-2' (i.e. Th1/Th2 and Tc1/Tc) activation and its importance in episodic viral wheeze is unknown.

Again little is known of the interaction of HCoV with lymphocytes. HCoV 229E has been shown to be a good inducer of IFN in natural leukocyte cultures from healthy children. For further insight into potential mechanisms we must look to HRV again. A study of T cells in tonsils removed from children and then infected with HRV showed a CD4-positive T cell proliferation. The subsequent production of IL-2 and IFN-γ demonstrates a Th1 reaction resulting from the viral activation.

Differences in physiological responses to colds between atopic and non-atopic individuals have been well documented. Likewise adults with allergic rhinitis develop a different T cell response in their blood to controls, developing an increased helper/suppressor T cell ratio (23 days post-HRV inoculation) and increased natural killer cells (7 and 23 days post HRV inoculation). HRV is also known to have different effects in asthmatic lungs compared with healthy controls. The eosinophilic inflammation together with the increased bronchial hyperresponsiveness is well described. However there is a paucity of information regarding T cell activation by viruses in the lung. The study by Fraenkel and colleagues looking at bronchial biopsies in asthmatics failed to demonstrate any difference in the T cell recruitment between asthmatics and controls although they only had a sample size of 6 asthmatics. The largest body of work on T cell activation during
viruses comes from experimental work on mice infected with influenza virus\textsuperscript{153,154}. This work has demonstrated the involvement of CD8\textsuperscript{+} T lymphocytes during infection in both mediastinal lymph nodes and lung tissue.

It is still unclear what role T cells play in viral wheeze but T cells are known to be activated by viruses and may play some role in LRT illness. There is a large amount of literature on the role of CD8\textsuperscript{+} T cells in the acute and convalescent phases of virus infections based upon experiments using tetramers. Most of this work is on the HIV virus, but despite the paucity of work on respiratory viruses important and relevant lessons on the behaviour of cytotoxic T cells are apparent.

\textit{Tetramer stimulation of CD8\textsuperscript{+} lymphocytes}

Understanding the interactions between a host and a pathogen relies crucially on quantitative measurements of the immune responses. Until recently measurements of the levels of cellular immune responses, ie/ those mediated by CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocytes have depended largely on culture \textit{in vitro} with the subsequent measurement of specific functions. This is because the interaction between TCR and single MHC-anitgen complexes is weak. More recently, new technologies based around tetrameric class I peptide complexes (tetramers) have allowed immunologists to measure CD8\textsuperscript{+} cytotoxic T lymphocyte (CTL) levels directly \textit{ex vivo}. Tetramers are laboratory manufactured complexes of 4 MHC class I – antigen complexes bound to streptavidin\textsuperscript{137}. Tetramers bind successfully to TCR thus allowing the identification and quantification of CD8\textsuperscript{+} t lymphocytes\textsuperscript{155}, the phenotyping of CTL by additional assays with antibodies to cell markers, and the separation of specific T cell clones\textsuperscript{137}.
Tetramers in non-respiratory virus work. Tetramers have been used to study various viruses including HIV, CMV, EBV and others. The initial description of tetramers included work on CTL against HIV, which were present in large numbers in asymptomatic patients, a finding also seen in ex vivo functional assays. Tetramers have been used to assess viral load in HIV and assess response to antiretroviral treatment. Tetramers have been used to identify the presence of chronically maintained CTL populations against CMV and the expansion specific CTL populations (HLA-B8 positive) during acute EBV infection.

Tetramers in respiratory viruses. There is now increasing interest in the use of tetramers to identify and study CTL responses in respiratory viruses. CTL clones specific to influenza A have been identified in human peripheral blood and using the haemagglutinin peptide in a MHC classII tetramer, clonal T helper cells (CD3+, CD4+, CD8-, CD25+) have recently been identified in influenza immune individuals. Tetramers have been used to investigate the maturation of dendritic cells, a major antigen presenting cell for CTL. In one study human monocytes (precursors of dendritic cells), immature dendritic cells and mature dendritic cells were inoculated with influenza A virus and tetramers used to quantify the MHC class I-virus antigen response on added resting memory lymphocytes. It was found that only mature dendritic cells could expand and differentiate CTL precursor cells into cytotoxic effector cells over a 7 day period. Tetramers have also recently been used to study the CTL response to experimental RSV infection in mice. In this interesting study the tetramer included the peptide to the fusion protein of RSV (known to antigenically active). CD8+ cells were assayed for in harvested lungs during the acute infection of RSV naïve mice and mice previously exposed to RSV. A peak of 4.8% of pulmonary CD8+ cells on day 8 were bound the tetramer in RSV naïve mice whereas a peak of 50% were bound in RSV-
immune mice. These findings give some potential insight into the role of CTL in respiratory virus infection, although there is much more to understand.

It seems highly likely that CTL are key cells in the early immune response to a respiratory virus and, although present in low levels in the memory pool of lymphocytes, the study of the dynamics of these cells with technology such as tetramers could be of great importance.

1.3.4 Interaction of respiratory viruses and allergic disease

The role of environmental factors in relation to asthma and allergy has become increasingly topical through the 1990s. Concerns regarding outdoor pollution and aeroallergens, and indoor factors such as dust, pet allergens, tobacco smoke and their relationship with allergy receive much prominence. Despite the known relationship between these factors and allergic disease they do not explain the 'epidemic' of allergic conditions sweeping the developed world. One hypothesis, the hygiene hypothesis, attempts to explain this 'epidemic' by a decrease in childhood infections and the subsequent effect this has on the developing immune system. There are 3 strands of evidence supporting this hypothesis.

i) Family size/day-care attendance. In 1989 Strachan reported some fascinating observations regarding hayfever and household size from a national survey of 17,414 British children born in 1958 and followed up at 23 years old. Of 16 perinatal, social, and environmental factors studied the most striking associations with hay fever were those for family size (prevalence of hayfever = 20% for 0 siblings and 6.5% for 4+ siblings) and the position in the household in childhood, with those with older siblings more likely to develop hayfever than those with younger siblings. Strachan suggested that these observation "could be explained if allergic diseases were prevented
by infection in early childhood, transmitted by unhygienic contact with older siblings, or acquired prenatally from an infection mother by contact with here older children”166. This ‘hygiene hypothesis’ suggested that declining family size, improvements in household amenities and higher standards of personal cleanliness have reduced the opportunity for cross infection in young families and this in some way increased the clinical expression of allergic disease.

Further indirect evidence of the hygiene hypothesis comes from the study of allergy in twins and of the affect of daycare attendance on subsequent allergic disease. A survey of Swedish army conscripts reported in 1998 that the prevalence of asthma was lower in twins than in singletons (4.9% versus 5.9%) 167. A similar and more striking finding was identified in a survey of hospital admission data in children during 1981-4 that identified that twins had a reduced risk of admission for asthma by more than half when compared with singletons 168. Despite the criticisms that one hospital admission for one twin could lead to the more expedient treatment of the other in the community, these data do fit with the protective effect towards allergy of a large family. Young children attending day-care are known to have more frequent infections than those who remain at home 169. However, the results of studies examining the affect of the attendance at day-care on the development of allergic disease are conflicting. In one study, day-care attendance during infancy was found to protect against the development of asthma, hay fever and skin reactivity amongst children with few siblings 170, but in three other studies no association was found between day-care attendance and the risk of allergic disease 171-173. The cohort of 1035 children in Tuscon found the relationship between wheezing/asthma and exposure to older children or day-care to be more complex. Children exposed to older siblings or attending day-care were more likely to have wheezing at 2 years than children with little or no exposure (adjusted relative risk (95% CI) 1.4 (1.1-1.8), p = 0.01), but were less likely to have wheezing at the age of 6 (adjusted relative risk
(95% CI) 0.8 (0.6-1.0), \( p = 0.03 \) through to 13 years \( 6 \) (adjusted relative risk (95% CI) 0.3 (0.2-0.5, \( p<0.001 \)) 174. Those exposed to older siblings or attending day-care were less likely to have atopy defined by skin reactivity (adjusted relative risk (95% CI) 0.8(0.7-1.0), \( p = 0.03 \)) suggesting that exposure to children at a young age reduces the subsequent development of allergic conditions, such as asthma.

**ii) Changing populations.** The increase in prevalence of allergic disease over the past 30 years cannot be fully accounted for by genetic risk factors: genetically similar populations in East and West Germany had very different rates of asthma before unification, although the former East Germany is now catching up with the west 175. The increase in asthma and allergy prevalence in East Germany has gone along with improvement in the East German healthcare system and a reduction in childhood infections. This is seen as additional evidence favouring the hygiene hypothesis.

**iii) Childhood exposure to infections.** Data supporting the hygiene hypothesis more directly comes from epidemiology studies looking at the influence of various childhood infections and the subsequent development of allergy. A survey of 262 young adults in Africa found that 12.8% of 133 who had had measles infection were atopic by skin reactivity compared with 25.6% of 129 who had been vaccinated and not had measles (adjusted odds ratio (95%CI) 0.36 (0.17-0.78), \( p = 0.01 \)) 176. This suggested that measles infection in early childhood reduces the risk of allergen sensitisation. Some bacterial infections may have similar effects. Japanese school children who develop a strong positive tuberculin skin test after bacillus Calmette-Guerin (BCG) vaccination, possibly signifying exposure to tuberculosis, also have reduced rates of allergy and asthma 177.
A survey of 1659 Italian military students found that atopy by skin reactivity was less common in those with seroconversion to hepatitis A (21.9%) compared with those without seroconversion (30.2%) and that lifetime prevalence of allergic rhinitis or asthma, or both was also less common in those with seroconversion to hepatitis A (8.4%) compared with those without seroconversion (16.7%) 178.

In developed countries the incidence of measles, tuberculosis and hepatitis A is low but there is much interest between the relationship of common childhood respiratory infections and later childhood asthma. A recent retrospective survey, in which fever was assessed as a potential risk factor for developing asthma, showed that children who had experienced at least three episodes of fever as infants were more likely to develop asthma at school age 179. However those of the school-aged asthmatics that had more than 5 fever episodes as an infant were less likely to have atopy and bronchial hyperresponsiveness. These data suggest that children who experience frequent infections in infancy are more susceptible to non-atopic asthma at school age, but less likely to develop atopic asthma. These data are however retrospective and many children may well have had more mild viral URTIs which went unreported. It is estimated that common colds occur at a rate of 2 to 5 per person per year in adults 180,181 but is more likely to be in the region of 7-10 colds per year in children 20.

On the above evidence the 'hygiene hypothesis' has considerable merit. From these studies it appears that infections in early childhood could have a protective effect on the development of atopy and asthma. The immunological basis of this is believed to lie in the effects of infections on the developing immune system where viruses trigger a 'type 1' host response characterised by the production of IFNy 182. This type 1 response is more effective at eliminating virus than the
alternative 'type 2' response, characterised by the production of IL-4 and IL-5 and the development of atopy\textsuperscript{147,182}. There is evidence that shows that children are born with well-developed type 2 responses\textsuperscript{183} but in the early years mature their type 1 response, with children born to atopic parents slower to do this that those born to non-atopic parents\textsuperscript{184}. Understanding the effect of early childhood infections, including viral URTIs, on the developing immune system has potential therapeutic implications for reducing allergic disease.

### 1.3.5 Respiratory Syncytial Virus and childhood wheeze

Respiratory Syncytial Virus (RSV) is a paramyxovirus that commonly infects infants causing and URTI and in some the clinical syndrome, bronchiolitis. Indeed virtually 100% of young children are infected with RSV in the first few years of life\textsuperscript{185}. There are intercontinental differences in the definition of bronchiolitis. In Europe it is considered the features of LRT infection (increased work of breathing), associated with signs of an URTI and crucially crepitations with or without wheeze on auscultation. In the USA it is simply defined as the first episode of wheeze in infancy. RSV not only causes an URTI and bronchiolitis in infants but also infects older children and adults and is known to trigger acute episodes of wheeze in asthmatics\textsuperscript{19}. RSV is particularly interesting in regards to childhood wheeze from the debate surrounding the link between RSV infection and subsequent reactive airway disease (RAD) and the work surrounding the immunopathology of RSV. These are briefly reviewed.

i) The link between RSV and RAD

The association between LRTIs due to RSV in infancy and increased respiratory morbidity in early childhood has been evident for decades\textsuperscript{186}. Opinions are polarised between those who believe that symptomatic RSV LRTIs in early life induce or are a marker for an atopic asthma phenotype, and
those who believe that non-atopic mechanisms contribute to both the acute RSV illness and subsequent respiratory morbidity. There are four key epidemiological surveys that highlight this difference in opinions, two supporting each opinion.

Sims and colleagues were the first to study the long term effects of RSV bronchiolitis. They investigated 35 children who had been hospitalised during one RSV season and examined and tested their respiratory function at age 8 years. Compared with 35 age, sex and social class matched controls, the 35 index children had diminished lung function (lower PEFR and FEV_{1/VC}) However, atopy assessed by family and personal history did not seem to relate either to bronchiolitis or subsequent wheezing. More recently, Noble and colleagues followed up 61 index and 47 control children at the age of 10 years taken from a cohort of 73 index and 73 control children, the index children having been hospitalised for bronchiolitis. Coughing and wheezing were significantly increased, FEV_{1} was significantly lower, and the response to histamine challenge was slightly (but not significantly) greater in the post-bronchiolitic group. However, as with the Sims study the 2 groups remained similar with regards to family history of atopy and personal skin prick tests for atopy.

The original cohort studied by Noble and colleagues was first reported on by Murray and colleagues at the age of 6 years. At this age 42.5% of the post-bronchiolitis children wheezed during the preceding year, compared with 15% of the controls. Bronchial responsiveness to histamine was significantly elevated in the index group and although the family history of atopy was no different between groups, personal atopy was increased in the post-bronchiolitic children (37% versus 27%). However, by 10 years this affect on personal atopy was not seen. Sigurs and colleagues took this type of survey one step further and looked for evidence of IgE production in
serum as well as skin prick tests to signify allergic sensitisation \(^{192}\). They followed up 47 children who had been hospitalised for RSV bronchiolitis and 93 matched controls. At the age of 7 years the index group had an increased prevalence of asthma (at least 3 episodes of bronchial obstruction diagnosed by a physician) of 30% compared with 3% in the controls \(^{192}\). Allergic sensitisation by skin prick test and IgE levels to a host of allergens was found to be 41% in the post-bronchiolitic children and 22% in controls \(^{192}\). The authors concluded that RSV bronchiolitis influences the mechanisms involved in the development of asthma and atopy in children.

These interesting surveys highlight one certainty; RSV bronchiolitis of sufficient severity to warrant hospitalisation is associated with long term respiratory morbidity. What is debated is whether RSV specifically influences the development of atopy and whether RSV bronchiolitis simply occurs in children with a prior predisposition to develop LRT effects (i.e. acute bronchiolitis and future wheezing episodes). Why these surveys should lead to differing conclusions is not clear. Critics of the opinion that atopy is modulated by RSV bronchiolitis have suggested that the entry criteria used by Sigurs and colleagues included wheeze, which may have excluded many infants with what most would define as RSV bronchiolitis \(^{186}\). Stein and colleagues have recently reported the outcome from RSV bronchiolitis from the Tuscon cohort of 1246 children followed to 13 years \(^{193}\). Again they report an increased incidence of wheeze at 6 years but this was no longer significant by 13 years \(^{193}\). Also, despite those with a history of RSV bronchiolitis having lower FEV\(_1\) there was no difference in lung function post-bronchodilator \(^{193}\). They also found no difference in allergic sensitisation assessed by skin prick and serum total IgE \(^{194}\). This large and important prospective cohort study suggests that RSV bronchiolitis although leading to a transient susceptibility to childhood wheeze, does not modulate atopy.
ii) Immunopathology of RSV.

The association between RSV bronchiolitis and subsequent long term wheezing has prompted much investigation into pathophysiology. Such studies have been fueled by the controversial claim that RSV bronchiolitis might modulate the immune system and predispose an individual to atopy and its associated diseases. Valuable information regarding RSV bronchiolitis immunopathology has been learned from human and animal studies.

*Immunological studies of humans with RSV.* Inflammation during the acute bronchiolitic illness has been studied by analysis of broncho-alveolar lavage (BAL) samples in several studies. Everard and colleagues studied a group of infants ventilated for bronchiolitis, therefore representing the very severe end of the illness spectrum. Large numbers of inflammatory cells were obtained, of which neutrophils accounted for a median of 93% in the upper airway and 76% in the lower airway. The numbers of CD8 positive cells detected were small and consistently less than CD4 positive cells. This study was followed up by a study nasal lavages from 27 non-ventilated infants with RSV bronchiolitis that looked for evidence of neutrophil activation. When compared with control infants, the children with bronchiolitis had elevated levels of IL-8 (median (range) 1.53 (0-153) versus 0 (0-5.6) ng mL-1) Human Neutrophil Elastase (136 (32-694) versus 14 (0-516) ng mL-1) and elastase activity (4 (1-220) versus 1 (0-339) mU mL-1). These results suggest that neutrophils are attracted into the airways during acute bronchiolitis and are likely to contribute to the acute inflammatory process that accompanies the symptomatic illness. However, they do not support the concept that excessive lymphocyte mediated cytotoxic activity is responsible for the pathology in RSV infection.
Smyth and colleagues collected blood from 94 children with RSV bronchiolitis during their acute illness and 10 days or more late and compared these with age matched controls. Mediators of lymphocyte activity, including IL-4 soluble IL-2 receptor (sCD25), and soluble ICAM-1 (sICAM-1) were measured. They found that sCD25 was elevated during the illness independently of illness severity and interestingly this remained elevated during convalescence (for as long as 150 days). This provided evidence that there is a persistent inflammatory process in the lungs of children for much longer than was previously believed.

**Immunological studies of animal models.** More detailed information regarding the immunopathology of RSV bronchiolitis has been gained from studies of a mouse model. This allows invasive studies that would be unethical in human infants but has the major drawback when applying the research to human disease. Hussel and Oppenshaw have described a mouse model where 3 days after inoculation there is a sharp increase in the number of natural killer cells with associated IFNγ. These are rapidly replaced by CD4+ and CD8+ T lymphocytes, which predominate for up to 7 days and return to baseline by 21 days. It is hypothesised that it is the balance of the CD4+ and CD8+ response that determines future wheezing, as CD4+ cells in the absence of IFNγ (also secreted by CD8+ cells) default to produce IL-4 and IL-5, Th2 cytokines associated with lung eosinophilia. Further studies of BALBc mice with genetic knockouts give some insights into the potential ways in which the balance of immune response could influence subsequent immune development. Hussel and colleagues studied the effect of knockout of CD8α chain, β2-microglobulin, or transporter associated with antigen presentation (TAP-1) genes, all of which impair CD8+ function, on lung eosinophilia after RSV infection. Lung eosinophilia is not usually found during RSV infection of these mice but was found to develop in the knockout mice. This supports a critical role for CD8+ cells in regulating Th2-driven eosinophilia.
Delayed effects of RSV bronchiolitis. RSV infection seems likely to create T cell memory which could in part explain future responses to repeated RSV infection and perhaps other infectious agents. It is still to be clearly proved whether such an early severe infection could also affect subsequent responses to aeroallergens. It might be possible that by having a severe infection in early childhood, with the associated severe epithelial disruption, a feedback system could predispose the child to a severe immune response the next time an infectious agent is encountered. This in turn could lead to a vicious cycle of amplified responses that might cause chronic changes in the airways, such as airway remodeling. On the other hand, severe RSV bronchiolitis could simply reflect factors in an infant that already predispose them to developing a more severe LRT illness. These factors could be genetic and could involve the lung immune response to this common respiratory virus.

Conclusions. How might RSV bronchiolitis cause delayed effects? Clearly there are delayed effects with an increased predisposition to wheeze. It is debatable whether RSV bronchiolitis modulates atopy. From animal studies it seems likely that T lymphocyte response is important in the immunopathology of RSV bronchiolitis, however, the evidence for this is less clear in human studies. What is clear from human studies is that neutrophil influx and activation seems to be important in the acute phase of the illness. Clearly there is need to understand the interaction of neutrophils, cytotoxic T cells and immune memory during respiratory virus infections. Understanding the role of such immune cells may guide future research into therapeutic interventions in the acute illness. Also of great potential importance is understanding how respiratory virus infections might modulate immune responses and immune memory and perhaps
influence the development of future wheezing with repeated virus infections and also aeroallergens.

1.3.6 URT effects on the LRT

In contemplating the relationship between the upper and lower airways in wheezing illnesses, several questions arise: what effect does altered nasal function have on the LRT; is there a causal relationship between inflammation and/or symptoms in these two compartments; alternatively, are the two compartments independent of each other? There are two reciprocal hypotheses that encompass these possibilities and can be applied to viral wheezing, a condition with both upper and lower respiratory tract symptoms (Figure 1.5). Firstly, the mechanisms that lead to upper and lower respiratory tract symptoms are related with some 'indirect' link between the URT and LRT during this illness. Alternatively, there is a 'direct' cause of the LRT symptoms emanating from the LRT, namely direct infection of LRT epithelium. These two possibilities are not necessarily mutually exclusive as both 'direct' and 'indirect' mechanisms could be involved in viral wheeze. The evidence regarding direct viral infection of the LRT has already been presented (Chapter 3.2) but what evidence is there of interaction between the upper and lower respiratory tracts?

i) The nose as the air conditioner of the lower airways

The nose has two important respiratory functions: 1) filtration from air of allergens and polluting dust and 2)-humidification and heat exchange. The shape of the nose and the nasal hairs induce air turbulence, which facilitates the attachment of inhaled particles to the mucosa, thereby protecting the lower airways from exposure. It is known that up to 20% of inhaled dust particles are deposited in the nose with minimal deposition in the LRT \(^{198}\). Respiratory viruses are transmitted by various means, including close contact with infected fingers, lips, external nares and fomites but also by
aerosol of respiratory secretions containing virus particles \textsuperscript{199-201}. It is therefore believed that in protecting the LRT the nose traps aerosolised virus (either by direct contact with nasal mucosa or from the eye via the naso-lacrimal duct) and provides the primary site of infection. Interestingly, it has been shown that children have a lower degree of nasal particle deposition compared with adults \textsuperscript{202} but whether this leads to greater susceptibility to LRT infection is speculative.

The nose provides a moist environment enabling the humidification of inhaled dry air. It also has a highly sensitive blood supply enabling the warming of cool inhaled air. During a common cold where nasal blockage is common, mouth breathing will inevitably lead to the inhalation of cooler, drier air. Such conditions occur during hyperventilation seen in exercise challenges, which are known to induce bronchoconstriction in asthmatics \textsuperscript{203}. A further link between the upper and lower respiratory tract is suggested from experiments where nasal inhalation of cold and dry air (the colder the air the drier it becomes) increased lower airway responsiveness \textsuperscript{204}. This suggests a 'nasobronchial' reflex that was first proposed in Germany in 1952 as a reflex from the nose to the lungs via the trigeminal nerve, the "Wetter und Winderflex" \textsuperscript{205}. This is supported by a recent study in which cold stimulation (-15°C) to the nose of asthmatics lead to a significant increase in airway resistance and a mean fall in FEV\textsubscript{1} of 8\% \textsuperscript{83}.

Not only does the nose affect the heating and humidification of inspired air, it also produces NO, with a considerable part of NO production within the airways occurs in the paranasal sinuses \textsuperscript{206}. The exact role that NO plays in wheezing disorders is not yet clear. Exhaled NO is believed to reflect the degree of underlying airway inflammation in chronic asthma \textsuperscript{207} and in some infants with RSV bronchiolitis exhaled NO increases during the illness \textsuperscript{208}. If inhaled NO has the potential
to affect lower airways then it may be possible that inhalation of other active mediators present in an inflamed nasal mucosa during a viral URTI could contribute to the wheezing illness.

**ii) Neural interaction between upper and lower airways**

From the nose to the terminal bronchioles, sensory and autonomic nerve fibres densely innervate the airway. Their function is to maintain physiological homeostasis by reacting rapidly to changes of environment. Afferent fibres make up the majority of fibres in the URT and are capable of initiating reflexes resulting in increased nasal secretions and local blood vessel dilatation as well as more distal bronchoconstriction. The local effects are thought to be due to the release of neuropeptides, whereas the bronchoconstriction is thought to be a parasympathetic reflex. An experimental infection of guinea pigs with PIV found enhanced contractility of bronchial smooth muscle when stimulated by substance P, suggesting viral infections could potentiate neurogenic activity. This effect in rodents is believed to be mediated by reduced enzymatic activity of a neutral endopeptidase that normally degrades substance P. However, it is still unclear what role these reflexes play in human disease states.

**iii) Links between allergy in the upper and lower airways**

More evidence of links between the upper and lower respiratory tracts comes from the relationship between allergic rhinitis and atopic asthma. With a prevalence of asthma of 15-20% and of allergic rhinitis of up to 40%, it is not surprising that the two conditions frequently coexist, but the two conditions coexist more frequently (30-50%) than one would expect by chance. This seems hardly surprising as the origins of atopy lie in the production of a pool of progenitor stem cells within the bone marrow that move into the systemic circulation and home to a tissue site
stimulated with antigen. Both upper and lower respiratory tracts, despite the effects of nasal filtration on inhaled air are therefore targets for atopic inflammation.

This simplistic understanding of atopy is complicated by evidence of an interaction between nasal allergic inflammation and the lower airways. Studies of nasal allergen challenges in subjects with allergic rhinitis but without asthma have demonstrated increased bronchial responsiveness after the challenge. Whether this is an inflammatory response or a neural reflex is still not clear. Also, a study of children given nasal corticosteroids or placebo with allergic rhinitis and asthma demonstrated a reduction in airway responsiveness and a trend towards reduced LRT symptoms. More recently, two studies by Braunstahl and colleagues have examined the ‘cross-talk’ between the URT and LRT in subjects with allergic rhinitis by examining the responses in the URT and LRT to firstly segmental bronchial challenge and secondly nasal allergen challenge. Both demonstrated interactions between the URT and LRT. When the LRT was stimulated there was evidence of eosinophil activation in the nasal mucosa and when the URT was stimulated there was evidence of eosinophil influx in the LRT.

There is clearly the potential for ‘indirect’ effects on the LRT emanating from the URT, which is likely to be the primary site of common respiratory virus infection.

1.3.7 Summary of mechanisms

Episodic viral wheeze is a distinct disorder from classical atopic asthma but our current understanding of mechanisms underlying viral wheeze is either very basic or non-existent. We know from studies of virus-induced asthma that when wheeze occurs there is reversible airways obstruction with increased airway responsiveness. We know that inflammation of the URT occurs
during the common cold and that children with viral wheeze do not have the chronic eosinophilic inflammation between episodes seen in asthmatics. Much experimental work has been conducted into virus exacerbations of wheeze in the ‘classical’ asthmatic phenotype, with most experimental infections using HRV. From these studies and those in animals we know there are many potential mechanisms that could be involved in viral wheezing, indeed it may be that several mechanisms act together when causing this illness. These include neural reflexes initiated in the infected URT, alteration of the properties of inhaled air passing through an inflamed URT and activation of the immune system in the URT leading to LRT effects. Before such potential ‘indirect’ mechanisms are explored fundamental questions regarding the presence of LRT inflammation during viral wheeze and whether direct LRT infection is responsible for the wheezing illness need to be addressed (Figure 1.5). Virus is known to replicate in the URT, leading to inflammation and symptoms. If virus does replicate in the LRT and cause inflammation ‘directly’ then the response of bronchial epithelial cells to infection is likely to be of key importance. If virus does not replicate in the LRT then there must be an ‘indirect’ link between URT infection and LRT changes, either by producing inflammation or by some other mechanism. This thesis is directed at exploring the mechanisms of hitherto a poorly researched phenotype of wheeze.


1.4 **Aims and Objectives**

The aim of this thesis was to develop an adult model of viral wheeze that would enable several hypotheses to be tested. These are:

A. An inflammatory response in the LRT is involved in viral wheeze;

B. Direct infection of the LRT is not responsible for the LRT response, i.e. an ‘indirect’ link exists between the URT and LRT;

C. Inflammatory changes in the LRT reflect the changes in the URT.

We set about studying these overall hypotheses by developing several objectives.

1. To establish an experimental infection in adults with viral wheeze by monitoring symptoms with laboratory confirmation of infection.

2. To establish the physiological changes that occur during viral wheeze including any changes in airway responsiveness.

3. To measure the cellular inflammatory response to infection in both the upper and lower respiratory tracts.

4. To measure fluid phase markers of potential interest in viral wheeze in both upper and lower respiratory tracts.

5. To identify whether replication of virus occurs in the LRT.

We chose HCoV 229E as the virus to infect volunteers as this is the second most prevalent common respiratory virus to cause exacerbations of asthma, is known to cause wheeze in preschool children and has received relatively little attention compared with rhinovirus.
1.5 Figures and Tables

Figure 1.1 Hospital admission rates (per 10,000 per year) for asthma by age in England and Wales 1962-91. (From “Childhood asthma and other wheezing disorders”, Ed. Silverman, M. 1995, with permission).
Figure 1.2 - Schematic of odds ratios for episodic and maintenance corticosteroids in those outcomes meta-analysed in the Cochrane review. Those outcomes where steroids had a favourable effect compared to placebo are to the left of the centre line. Numbers in each group are given: T = treatment (i.e. corticosteroids); P = placebo (taken from Mckean and Ducharme [1058] with permission).

EPISODIC HIGH-DOSE STEROIDS

Peto Odds Ratio
(95% CI)

Exacerbations requiring oral steroids
x-over (T = 10/63; P = 19/63)
parallel (T = 14/26; P = 17/26)

Exacerbations requiring admissions
x-over (T = 5/63; P = 5/63)
parallel (T = 6/26; P = 2/26)

Parental preference for treatment
(T = 45/60; P = 29/63)

MAINTENANCE STEROIDS

Exacerbations requiring oral steroids
(T = 4/72; P = 5/73)

Exacerbations requiring admissions
(T = 0/70; P = 2/65)

.1 .2 1 5 10
Figure 1.3i – Electron microscopy of human coronavirus 229E used in this study.

Figure 1.3ii – The structural components of HCV 229E.

S = surface glycoprotein; M = membrane glycoprotein; sM = small membrane protein; N = nucleocapsid.

(Taken from: Viral and other infections of the human respiratory tract, Myint, S. and Taylor-Robinson, D. 1996 27 (with permission))
Figure 1.4 Effects of viral infection on the neural regulation of airway smooth muscle. Virus infection of epithelium exposes the sensory neurones, increasing their stimulation by inhaled particles and inflammatory mediators. Reflex bronchoconstriction occurs by two pathways: A – release of neuropeptides by sensory neurones and B – activation of parasympathetic fibres. Viruses and mediators can also block auto-inhibitory feedback of M2 receptors, enhancing acetylcholine release and potentiating bronchoconstriction.
A schematic of the potential mechanisms of viral wheeze. Filled arrows are interactions between virus and host that we know exists. Virus replicates in the URT, leading to inflammation and symptoms. It is still debated whether most common respiratory viruses replicate in the LRT and whether inflammation occurs as a result of this. The relationship between viral induced inflammation and altered lung function and wheeze is still unclear.
### Table 1.1  Outcome of respiratory viral infections

<table>
<thead>
<tr>
<th>Age group</th>
<th>Virus</th>
<th>Risk factors</th>
<th>Clinical effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants</td>
<td>RSV, PIV</td>
<td>None</td>
<td>URTI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ pulmonary function +passive smoking</td>
<td>Bronchiolitis and Pneumonia</td>
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<tr>
<td>Children + adults</td>
<td>HRV, HCoV &amp; others</td>
<td>None</td>
<td>URTI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asthma</td>
<td>Wheeze</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Previous viral wheeze</td>
<td>Wheeze</td>
</tr>
</tbody>
</table>

RSV = respiratory syncytial virus; PIV = parainfluenza virus; URTI = upper respiratory tract infection; HRV = human rhinovirus; HCoV = human coronavirus.
PART II - METHODS
Several factors are important in the design of an experimental common cold infection. Firstly, such experiments are labour intensive, time consuming and reliant on volunteers willing to attend repeated sessions in the study laboratory. Such experiments are relatively expensive, a factor that cannot be ignored at the design stages. Secondly, the design will be largely dependent on the success rate of the inoculum in inducing a common cold, as this will determine the number of subjects needed. Finally, sample schedule will depend on the type of measurement being made and the time course of the illness. Bearing in mind the second of these issues, a preliminary study was undertaken to establish the infectivity of the inoculum and method of instillation.

2.1 Inoculation by drops or spray

2.1.1 Introduction

The study of respiratory virus infection using an experimental infection has several advantages over studying wild-type infections. Subjects are inoculated with identical quantities of the same virus type without the heterogeneity of ‘wild’ viruses. The timing of the infection is known allowing sampling prior to the infection and at identical time-points during the study. Such experiments reduce the inevitable variability of wild infections and allow comparisons between different experimental viruses. There are, however, disadvantages. The experiment is dependent on virus grown in cultured cells in a laboratory and inoculated into the subject in an artificial manner. Both may affect the success of inoculation, the former by attenuating the infectivity of the virus, the latter by poor application of virus to the nose at a time of low natural susceptibility. As these
experiments are costly and time consuming, successful inoculation (i.e. a volunteer developing a cold) is crucial.

Several factors influence our ability to induce a common cold by nasal inoculation. These include the potency of the inoculum, the immune status of the volunteers and the method of administration of the inoculum.

1) Potency of the inoculum. In ideal circumstances there should be sufficiently high titre of live virus in an inoculum to cause not only viral replication and shedding but also clinical symptoms of a cold. The potency of the inoculum can be assessed by measuring the tissue culture infective dose that causes cytopathic effects in 50% of cell cultures (TCID$_{50}$). This is a standard microbiological technique based on the observations of d'Herelle who inoculated large numbers of identical cultures of bacterium with serial dilutions of phage viruses and observed that if the sample was diluted too far none of the cultures would lyse. At intermediate dilutions not all cultures would lyse, since not all received a virus particle. This method of applying serial dilutions of virus is used in standard cell cultures to quantify the potency of an inoculum with the TCID$_{50}$ quoted in terms of the dilution of the inoculum. For example a TCID$_{50}$ of 100 ml$^{-1}$ of inoculum (a dilution of 1:100) is twice as concentrated as a TCID$_{50}$ of 50 and therefore represents a stronger inoculum. As dilutions pass 1:1000 it is tradition to represent the TCID$_{50}$ as 10$^3$ or greater (e.g. 1:10,000 = 10$^4$).

The Reed and Muench method is a mathematical method for improved precision in estimating a titre that lies between 2 dilutions (e.g. 1:1000 and 1:10,000).

Table 2.1 shows inoculation information from several previous experimental infections using HRV and HCoV. It can be seen that the older HRV studies between 1986 and 1991 and all the
HCoV studies used relatively low titres of virus inoculum with reasonable success rates (67 - 100%) in terms of symptomatic colds. In the 1990s improvements in cell culture has enabled higher titres of virus to be grown and experimental infections with HRV have tended to use much larger titres (by a factor of 10 or 100) presumably to ensure successful infection although the table demonstrates that having high titres does not guarantee a superior infection rate. The drawback with such an approach is that one is delivering large amounts of virus and virus antigen in an artificial way to the nose. It is not inconceivable that such large doses of virus may produce an illness quite unlike a natural cold.

The ideal titre for an inoculum should be the minimal dose that causes a symptomatic infection in every inoculated individual. Perhaps the best illustration of this is the study of Bradburne and colleagues. They used several different dilutions of an inoculum of HCoV with TCID₅₀ of 4, 5, 16 and 32. The infectivity rate was 2/5, 1/6, 6/9, 4/6 respectively. Although based on low numbers, this suggests that a TCID₅₀ of 16 or 32 should be adequate for achieving infection rates of about 2/3. A TCID₅₀ of 100 ml⁻¹ now seems to be the accepted minimal titre, although care must be taken in comparing TCID₅₀ titres from different studies that use different viruses and different cell cultures.

ii) Immune status of the volunteers. Most of the information regarding the antibody response to HCoV comes from Callow and colleagues. They demonstrated that the presence of non-specific immunoglobulins and proteins as well as specific IgA in nasal washes protected an individual from inoculation with HCoV 229E. Not only was it more difficult to induce infection in individuals with detectable antibodies, but those who became infected had lower clinical scores and shortened periods of viral shedding. In 1990 the same group looked at
antibodies and experimental re-infection rates using HCoV 229E in 15 individuals 1 year after a controlled infection with the same virus. Eight out of 10 who had had low levels of antibody at the initial infection one-year earlier developed cold symptoms whereas none of those with high levels were symptomatic. Antibody levels reached a peak 2 weeks after infection and then slowly declined but in some individuals antibodies were still detectable 1 year later. At this stage the volunteers were successfully re-infected as judged by viral shedding but none became symptomatic and they had a shorter duration of viral shedding than at the initial infection. This pattern of antibody response is quite unlike that seen in HRV infections where the rise to peak is later (35 days) and there is no decline. This is believed to be due to repeated stimulation by related rhinoviruses. Since there are only two sub-types of clinically relevant HCoV, such stimulation would be less likely to occur. It is therefore apparent that HCoV has the ability to induce an antibody-mediated immune response but that the protection conferred from this wanes over a period of 12 to 18 months. Re-infection with the same virus can occur beyond this period.

**iii) Method of inoculation.** The exact mode of transmission of wild-type HCoV is not known. However if it behaves like HRV, transmission is likely to be by aerosol or fomites. The major target cell for infection is thought to be the ciliated epithelium of the URT. Therefore, in order to produce an active infection, the viral inoculum suspension should cover as much as possible of the nasal epithelium beyond the inferior turbinates, where the ciliated cells are found. Early experimental infections dating back to the 1960s used instillation of nasal drops to inoculate volunteers (Table 2.1). More recently, atomised nasal sprays have been introduced in addition to drops as they are believed to be more physiological and result in better coverage of the nasal lining with virus. There are no published papers comparing these different methods of inoculation on the Medline and Embase databases. We do know that the more recent studies that use atomised sprays
as well as high virus titres do have slightly better infection rates than those using drops alone with slightly lower virus titres (Table 2.1).

Bearing in mind that our ideal inoculum should be the minimal dose that causes a symptomatic infection, as a preliminary study we wished to know if a modest titre of inoculum was sufficient to cause a reasonable proportion of symptomatic infections and if atomised spray was superior to nasal drops.

The aims of this section are therefore:

1. to determine the potency of the inoculum.
2. to test the inoculum and determine if sufficient subjects caught a cold.
3. to compare atomised nasal spray to nasal drops as a method of inoculation.

2.1.2 Methods

i) Subjects and design

Twenty-three healthy volunteers aged 18 to 45 years were recruited by local advertisements. Subjects had no history of significant lung disease or nasal disease (including rhinitis), were non-smokers and had not suffered a cold for at least 3 months prior to the study. The numbers of volunteers required was based on a power calculation taken from Armitage and Berry. A comparison was made using previously reported proportions of symptomatic infections between nasal drops (based on unpublished pilot data from Leicester in which 2 out of 10 volunteers contracted a cold) and atomised spray (70% 55). Eleven volunteers in each group are required to detect a difference of 5 colds with a two sided 5% significance and a power of 90%, taking into account Fleiss and colleagues' correction. The study was approved by the Leicestershire Health
Authority Research Ethics Committee and written informed consent was obtained from all participants.

Subjects were randomised by selecting numbers out of a bag to receive either drops or spray. Eleven were randomised to receive drops and 12 spray. After inoculation subjects were instructed to complete a symptom diary and return it after 17 days.

ii) Viral techniques

Cell culture and virus propagation. The virus inoculum was prepared by Mrs Stephanie Euden under the supervision of Professor Steven Myint (University of Leicester). LP strain HCoV 229E (American Type Culture Collection, Rockville, Maryland, USA) was cultured according to standards of good laboratory practice in human embryonic lung (HEL) cells as previously described. HEL cells (Biowhittaker, Wokingham, UK) were grown in Eagle’s MEM medium (Gibco, UK) supplemented with 10% foetal bovine serum (Gibco), 2% of non-essential aminoacids (Life Science Technologies, Paisley, Scotland), 50 μg ml$^{-1}$ of penicillin and 50μg per ml$^{-1}$ of streptomycin and 1% amphotericin B (fungizone, Gibco). Cells were cultured at 37°C, in a 5% CO$_2$, 100% humidity atmosphere until confluent. Twenty-four hours before infection with HCoV 229E, the monolayers were washed with serum free medium. The medium was replaced with Eagle’s MEM supplemented with 0.4% Ultrocer G (Gibco Life Technologies, Paisley, UK) and 2% amino acids. Cells were infected with HCoV 229E at a concentration of $10^4$ TCID$_{50}$ ml$^{-1}$ and cultured at 33°C, in 5% CO$_2$. Virus was harvested from the cultures by filtration through a 4.5 micron filter and suspended in normal saline. After being aliquoted into 1 ml portions, inoculum was stored at −80 °C and later tested for safety according to the Gwaltney criteria.
**Determination of titre.** A TCID<sub>50</sub> quantification of the titre of virus in the inoculum was conducted based on the principles of d'Herelle using the mathematical model of Reed and Muench described above. This is a standard virology technique and not fully described here. Briefly, cultures of HEL cells were grown on flat bottomed 96 well titre plates, inoculated with serial dilutions (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup>) of inoculum and incubated at 33°C whilst covered with parafilm for 7 to 10 days. After pouring off excess medium plates were fixed and stained with crystal violet before enumerating those wells with greater than 50% cytopathic effect. The titre of the inoculum was found to be 200 TCID<sub>50</sub> ml<sup>-1</sup> and it was decided that this was a reasonable dose with which to inoculate our volunteers.

**Inoculation.** Inoculation was performed using 1 ml of HCoV 229E suspension (200 TCID<sub>50</sub> ml<sup>-1</sup>) instilled into each nostril. Those randomised to receive drops sat with their neck slightly extended whilst 2-3 drops were instilled at a time to alternate nostrils using a soft plastic pipette (Figure 2.1a). Those randomised to receive atomiser (Hoechst, Frankfurt, Ger) sat upright whilst 1 actuation was applied to alternate nostrils (Figure 2.1b). Subjects were instructed to sniff backwards only if the inoculum was dribbling anteriorly out of the nose. Subjects were also instructed not to blow their noses for at least 30 minutes after inoculation.

**iii) Characterisation of symptomatic colds**

This was a preliminary study aimed at determining the success rate of an inoculum in terms of symptomatic colds, hence outcomes were based entirely upon the symptom diary (appendix 2). The design and assessment of the symptom diary is discussed in detail in chapter 3.2.2.

**iv) Statistics**
As data is categorical the $\chi^2$ test was used for analysis using the software package SPSS 8.0 with a level of significance set at 0.05.

2.1.3 Results

All subjects completed the study satisfactorily without any significant illnesses other than a common cold. Of the 11 randomised to receive drops, 7 were male and of the 12 randomised to receive spray, 8 were male. Both groups were similar in age (mean (standard deviation) 23.5 (5.2) years and 23.6 (4.4) years for drops and spray, respectively). All were categorised as either having a definite cold or no cold. Seven and 8 developed symptomatic colds in the drops and atomiser groups, respectively (Figure 2.2) Four in each group did not develop colds. Virtually identical proportions of volunteers developed symptomatic HCoV colds when inoculated by drops (64%) or atomised spray (67%) with no significant difference between the groups ($\chi^2 p = 0.40$).

2.1.4 Discussion

This methodology study has achieved all 3 of the aims set out. Firstly, the potency of the inoculum provided was determined as a 200 TCID$_{50}$ ml$^{-1}$. This titre is slightly higher than previous HCoV experimental studies but is not as high as those used in recent HRV experiments (Table 2.1). It was decided that a slightly higher titre should be tried as a previous pilot in Leicester that had used a titre of 100 TCID$_{50}$ ml$^{-1}$ had achieved an infectivity rate of 2 out of 10 only. Secondly, an overall success rate of 15 colds out of 23 inoculations (65%) was deemed satisfactory for the present study, as this would allow us to include subjects without colds as controls.

Two recent studies have added a third method of inoculation using nasal nebulisers to inoculate high titres of virus $^{38,41}$, although the potential for nebulisation leading to large amounts of virus
entering the lungs may make this less physiological, especially when studying the LRT. The inoculation method used in this thesis avoided the use of very high titres delivered to the nose with the inevitable increased possibility of virus from the inoculum being aspirated either through post-nasal drip or inhalation, into the LRT, although this is still a possibility with a nasal atomiser. This is a key point when studying the LRT effects of an URT infection. Experimental infections should try and emulate natural infections as far as is possible and for HCoV and HRV it is believed that natural infection occurs by spread of virus via droplets or fomites to the URT.

This experiment also established that the experimental infection had a good safety record, with no significant problems other than a common cold reported. None of the subjects dropped out of the study and all reported they would be prepared to participate if asked again! The symptom diary that was designed specifically for this study was found to be easily understood and easy to use and interpret.

Finally, we were unable to find any difference in rates of symptomatic infection between the group inoculated by drops and the group inoculated by atomiser. It would seem that either technique is acceptable although there are some aspects of design that must qualify these results.

**Technical and design issues**

Several questions surrounding titre of virus and inoculation technique that remain unanswered. Firstly, this experiment has not addressed the issue of the optimal titre of virus. One could use the approach taken by several researching HRV and increase the titre of virus used massively but there are potential drawbacks as already mentioned. An experimental design based on that used by Bradburne and colleagues where infection rates at increasing titres of virus were used to explore
the relationship between titre and symptomatic infection could be used in titres beyond 32 TCID₅₀ ml⁻¹. The titre required to cause symptoms may vary between viruses and between preparations of inoculum, with laboratory storage and repeated passages in cell cultures potentially affecting virulence. To perform a detailed experiment to ascertain the optimal titre of virus prior to every experimental infection would be impractical and is not done. It was therefore decided to take a pragmatic approach based on previous experiments and use a modest titre of virus that should give a reasonable rate of infection, if not as high as seen in some experiments.

Secondly, there are likely to be inherent differences between HRV and HCoV making direct comparisons invalid. There are several hundred serotypes of HRV, hence most volunteers would be immunologically naive to a HRV inoculum. There are only 2 main serotypes of HCoV and although immunity wanes over 12 to 18 months, in a small group inoculated with HCoV there are likely to be a few volunteers who do not develop a cold. Therefore an experimental infection with HCoV is unlikely to achieve 100% symptomatic colds unless one tests for and excludes individuals with pre-existing neutralizing antibodies.

Finally, the design of this experiment has answered the question regarding inoculation by drops or spray, but most recent studies are now using a combination of the two methods, although there are no published data to support it. To investigate this would have increased the size of this preliminary experiment to an extent where it would have not been possible. It is still an important question to answer and by using a combined inoculation technique in the main study it will be possible to compare infection rates in a non-randomised manner. Also, recent experimental infections have inoculated individuals on two separate occasions separated by 24 hours. This could be a key step in increasing the inoculation rate in HRV infections but has not been
studied in HCoV infections. The drawbacks with this approach are an extra visit to the laboratory for subjects and loss of strict timing of subsequent measurements. For these reasons only one inoculation was used in this study.

If future experiments permit the study of inoculation techniques, atomised sprays are worth pursuing. The present study used the same device throughout the study and is the device used in the main study. No measurements of droplet size were made and should be considered so that more accurate comparisons between different studies can be made.

In conclusion, this preliminary methodological experiment proved very useful in the work-up to establishing an experimental model of viral wheeze. Confidence was gained in the ability of the inoculum to induce a common cold with a reasonable success rate. Two methods of inoculation were both found to be suitable, but we did not test the methods combined. Despite this, it was decided to combine these methods as has been done in recent experimental infections with HRV that have produced infection rates of 70% or more. If there is any benefit from combining the methods then this strategy should produce infection rates of greater than 67%.

2.2 Design of the main study

The main study was designed with the following in mind:

- samples must be collected before inoculation, during the illness and during recovery;
- samples taken during the illness must be timed in order to assess changes in cellular inflammation and fluid-phase mediators of inflammation, as well as enable the detection of virus in samples;
• techniques should be easily repeatable, minimally invasive and with minimal required visits so as to ensure adequate recruitment and compliance with the study.

A power calculation was not possible as this project was to establish this model for the first time. However, the many experimental infections that have preceded this one tended to have 10 to 20 subjects with symptomatic infections after inoculation (Table 2.1). With a rate of symptomatic colds of 65% we aimed to recruit 20 viral wheezers and 20 controls to give us an estimate of 13 symptomatic colds and 7 asymptomatic controls in each group. By designing the project so that changes in measurements were compared to baseline measurements meaningful comparisons could be made both within and between groups.

During the main experiment samples would be needed to confirm symptomatic colds with laboratory evidence of viral infection and to measure changes in inflammatory indices before, during and after the illness. The optimal time for diagnosing viral infection in the URT is believed to be 24 to 48 hours after virus inoculation. A previous experimental infection using HCoV demonstrated a highly significant rise in nasal inflammatory mediators 4 days after inoculation, coinciding with peak of symptoms. It was therefore decided to have samples taken 2 and 4 days after inoculation in order to identify virus and monitor the development of inflammation. The study protocol is illustrated here:

```
<table>
<thead>
<tr>
<th>Time (days)</th>
<th>PNSI</th>
<th>PNS</th>
<th>PNS</th>
<th>PNS</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>+17</td>
<td></td>
</tr>
</tbody>
</table>
```
The study involved four visits to the Clinical Trials Unit, Leicester Children’s Asthma Centre. Baseline physiological measurements (P) were carried out on day 0 immediately preceding nasal lavage (N) and sputum induction (S). After baseline measurements virus was inoculated (I) intranasally by nasal drops and atomised spray. Repeat measurements were made 2, 4 and 17 days after inoculation at the same time of morning (+/- 2 hours) and in the same order. Subjects completed daily symptom diaries and carried out electronic spirometry at home during the course of the study. As the principal aim was to study mechanisms underlying LRT symptoms, we categorised subjects based on their symptoms. Laboratory confirmation of viral infection was used as an adjunct to validate the model and assist categorising subjects with mild symptoms.
### 2.3 Figures and Tables

#### Table 2.1
Review of infection rates and inoculation methods in previous HCoV229E and HRV studies

<table>
<thead>
<tr>
<th>Name</th>
<th>Virus type</th>
<th>Inoculation method</th>
<th>No. of times inoculated</th>
<th>TCID50/ml</th>
<th>Symptomatic infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akerlund 1993</td>
<td>HCoV 229E</td>
<td>nasal drops</td>
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<td>100</td>
<td>10/20 (0.50)</td>
</tr>
<tr>
<td>Myint 1989</td>
<td>HCoV 229E</td>
<td>nasal drops</td>
<td>1</td>
<td>100</td>
<td>3/7 (0.43)</td>
</tr>
<tr>
<td>Bende 1989</td>
<td>HCoV 229E</td>
<td>nasal drops</td>
<td>1</td>
<td>100</td>
<td>13/24 (0.54)</td>
</tr>
<tr>
<td>Callow 1988</td>
<td>HCoV 229E</td>
<td>nasal drops</td>
<td>1</td>
<td>?</td>
<td>19/34 (0.56)</td>
</tr>
<tr>
<td>Bradburne 1967</td>
<td>HCoV 229E</td>
<td>nasal drops</td>
<td>1</td>
<td>4 - 32</td>
<td>4 - 2/5 (0.40) - 5 / 16 (0.17) - 16 - 6/9 (0.67) - 32 - 4/6 (0.67)</td>
</tr>
<tr>
<td>Greiff 1999</td>
<td>HRV 16</td>
<td>nasal drops + atomised spray</td>
<td>1</td>
<td>5 x 10³ - 5 x 10⁴ /ml</td>
<td>17/23 (0.74)</td>
</tr>
<tr>
<td>Fleming 1999</td>
<td>HRV 16</td>
<td>nasal drops</td>
<td>2</td>
<td>10³/ml</td>
<td>20/21 (0.95)</td>
</tr>
<tr>
<td>Turner 1998</td>
<td>HRV 23</td>
<td>nasal drops</td>
<td>1</td>
<td>500/ml</td>
<td></td>
</tr>
<tr>
<td>Grunberg 1997</td>
<td>HRV 16</td>
<td>nasal drops + atomised spray + nasal nebuliser</td>
<td>2</td>
<td>5 x 10³ - 10 x 10⁴ /ml</td>
<td>11/19 (0.58)</td>
</tr>
<tr>
<td>Fraenkel 1995</td>
<td>HRV 16</td>
<td>nasal drops + atomised spray</td>
<td>2</td>
<td>5 x 10³ - 10 x 10⁴ ml</td>
<td>11/17 (0.65)</td>
</tr>
<tr>
<td>Cheung 1995</td>
<td>HRV 16</td>
<td>nasal drops + atomised spray + nasal nebuliser</td>
<td>2</td>
<td>3 x 10⁴</td>
<td>5/7 (0.71)</td>
</tr>
<tr>
<td>Calhoun 1994</td>
<td>HRV 16</td>
<td>nasal drops + atomised spray</td>
<td>2</td>
<td>500 - 18 x 10³</td>
<td>12/12 (1.00)</td>
</tr>
<tr>
<td>Skoner 1993</td>
<td>HRV 39</td>
<td>nasal drops</td>
<td>1</td>
<td>100</td>
<td>32/38 (0.84)</td>
</tr>
<tr>
<td>Calhoun 1991</td>
<td>HRV 16</td>
<td>nasal drops + atomised spray</td>
<td>2</td>
<td>320 - 3 x 10³</td>
<td>8/8 (1.00)</td>
</tr>
<tr>
<td>Hsia 1990</td>
<td>HRV Hanks</td>
<td>nasal drops</td>
<td>1</td>
<td>200</td>
<td>7/9 (0.78)</td>
</tr>
<tr>
<td>Lemanske 1989</td>
<td>HRV 16</td>
<td>nasal drops + atomised spray</td>
<td>2</td>
<td>320 - 3 x 10³</td>
<td>10/10 (1.00)</td>
</tr>
<tr>
<td>Levandowski 1986</td>
<td>HRV 25</td>
<td>nasal drops</td>
<td>1</td>
<td>25</td>
<td>10/15 (0.67)</td>
</tr>
</tbody>
</table>
Figure 2.1 Different methods of inoculation:

(a) using a soft plastic pipette

(b) using an atomised nasal spray
Figure 2.2  The % of symptomatic colds in subjects nasally inoculated with drops or atomiser. n = the number of subjects.
Chapter 3 Clinical methods

3.1 Introduction

The objectives pertaining to clinical methods were:

1. To establish an experimental infection in adults with viral wheeze by monitoring symptoms (with laboratory confirmation of infection – see laboratory methods).
2. To measure the physiological changes that occur during viral wheeze, including any changes in airway responsiveness.
3. To obtain samples of cells and fluid-phase mediators from the upper and lower respiratory tracts.

Clinical methods required to achieve these objectives included: subject recruitment; measures of important group characteristics such as atopy and baseline lung function; measures of symptoms that develop during the illness; repeated measures of lung function during the illness; repeated measures of airway responsiveness during the illness; and methods to sample upper and lower airways secretions.

3.2 Subject recruitment

The first objective was to develop an experimental infection in adults. Inclusion criteria were:

- a history of episodic viral wheeze (at least 2 or more episodes of wheeze during an URTI in the last 2 years);
- an age range of 18 – 45 years (older adults excluded to reduce the effect of ageing and the possibility of complicating chronic obstructive pulmonary disease);
- no admission to hospital with a chest illness for at least 5 years (so as to study young and healthy people with minimal risk of a severe/life threatening wheezy episode);
• non-smokers that had never been smokers (to reduce the effect of inhaled tobacco smoke).

Exclusion criteria included:

• those who suffered exercise-induced wheeze, nocturnal or early morning wheeze or cough, allergen (dust, pollen, animal) induced wheeze, or wheeze in response to cold air (so as to exclude the 'typical' asthma phenotype);

• taking daily inhaled corticosteroids (so as to exclude the 'typical' asthma phenotype and reduce interference on outcomes from corticosteroids).

The aim was to recruit 20 healthy controls and 20 viral wheezers and to conduct a controlled infection safely. The inclusion and exclusion criteria would inevitably mean that the index group had a mild illness but his was felt necessary for reasons of safety. The study took place between May and November in 1997 and 1998 to try and limit interference from 'wild-type' URTIs.

The study of Godden and colleagues established that 12 % of adults with viral wheeze used inhaled bronchodilators during episodes 8, implying that they would be known to their general practitioner. Therefore, viral wheezers for this study were recruited from three local university health centres (University of Leicester, De Montfort University, Loughborough University) where the patient databases held records of such individuals. Approval was gained by the principal general practitioner in each health centre. Questionnaires were sent by the health centres to 610 students who had consulted a doctor with a history of wheezing during URTIs or who were known to have been prescribed inhaled bronchodilators but not corticosteroids. Of 111 replies expressing interest in the project, 25 subjects fulfilled the inclusion and exclusion criteria. All 25 were recruited to the project.
Healthy controls were recruited through local advertisements in Leicester. These included posters around the University of Leicester, an advertisement in a local newspaper (the Leicester Mercury) and a discussion about the project on a local radio station (Leicester Sound). Nineteen healthy adults were recruited (16 via posters, 2 via newspaper and 1 via the radio). None had any history of chest illness. All subjects received £200 compensation for their time and effort after the study was completed. The Leicestershire Health Authority Research Ethics Committee gave approval for the project.

3.3 Clinical techniques

3.2.1 Assessment of atopy

Atopic status was assessed by determining the skin prick test response to *Dermatophagoides pteronyssinus*, cat fur and six grass pollens compared with histamine positive and saline negative controls using lancets provided in the kits (Soluprick, ALK Abello, Reading, UK). Any past history of allergic disease (eczema, allergic rhinitis and allergy to animals) was also recorded but subjects were classified as atopic on the basis of one or more positive skin-prick tests, defined by a skin wheal greater than 2mm (the mean of 2 measurements) in diameter above negative control.

3.2.2 Symptoms

i) Background to symptom monitoring

Monitoring the symptoms of the common cold by some form of daily diary requires a detailed understanding of the types of symptom one would expect to develop. The hallmark of the common cold are nasal discharge, obstruction and sneezing. However, symptoms from all parts of the respiratory tract are seen, including paranasal sinuses, pharynx, larynx, and intrathoracic airways.
Indeed, it is now recognised that the 'simple' syndrome that is the common cold is not at all simple with many subtle differences in the prevalence of symptoms between different viruses. For example, HCoV seems more likely to cause a fever and headache (i.e. systemic symptoms) than HRV

Despite small differences in the prevalence of individual symptoms between viruses, it is generally accepted that there is a large overlap between most of the common cold viruses enabling the development of symptom diaries. Most studies have based their diaries on the work of Jackson and colleagues, who monitored eight symptoms (nasal discharge, nasal obstruction, sore throat, sneezing, malaise, chills, headache, and cough) after experimental infection with several viruses. These symptoms were found with each virus and were shown to peak between days 2 and 6 post-inoculation. Most of the reports of experimental infections shown in Table 2.1 have graded each of the main symptoms as: 0 = absent, 1 = mild, 2 = moderate, 3 = severe; total symptoms ≥ 12 are defined as severe colds, 7 – 11 as moderate colds, and < 7 as mild colds. Some studies have also found a daily count of the number of tissues used to be useful. None have reported an assessment of sensitivity or specificity of the diary used but this simple diary has become the main clinical assessment tool in common cold research.

Of those experimental studies of the LRT during common cold infections, only one prior to the start of the present study assessed LRT symptoms in a diary format along with the common cold symptoms described above. In that study breathlessness, wheeze and chest tightness were also recorded on a scale of 0 to 3 reflecting severity. Those with 'asthma' symptoms were found to have decreased lung function and increased airway responsiveness suggesting this simple method of scoring is sensitive.
ii) Symptom diary used

A symptom diary combining scores for upper and lower respiratory tracts was used for the present study. Common cold symptoms were based on the Jackson criteria \(^2\) and categorised as upper and lower respiratory symptoms, cough and systemic symptoms. URT symptoms included nasal discharge, nasal blockage, sneezing and sore throat. Systemic symptoms included fever, headache, chills and malaise. LRT symptoms were based upon the simple ‘asthma’ score of Cheung and colleagues \(^4\) and included wheeze, chest tightness and shortness of breath. Cough was also recorded but cough can be generated as part of either upper or lower respiratory tract illnesses, hence it was scored separately. Each symptom was graded from 0 (absent) to 3 (severe).

Because HCoV experimental infections have traditionally produced mild colds, difficulty was anticipated in defining the threshold for a mild cold. The simple definition of a total score < 7 being a mild cold could have been misleading as all subjects could have minor symptoms without necessarily suffering a cold. To overcome this problem symptom diaries were assessed blind by one of the project supervisors (MS). Symptomatic colds which were categorised as definite (URT scores ≥ 2 above a zero baseline on each of two consecutive days from day 2 to 6), possible (scores ≥ 1 above zero baseline, or scores ≥ 2 above a variable baseline on two consecutive days from day 2 to 6), and absent. The baseline was taken as the score on day 0. The symptom diary is shown in Table 3.1.

3.2.3 Physiology

Daily FEV\(_1\) and PEF manoeuvres were performed in the morning at home by the subjects using Vitalograph 2110 Spirometers (Vitalograph, Buckingham, UK) from day 0-17 after inoculation.
Subjects were instructed on spirometry and the best of two attempts within 0.2 litres of each other was recorded according to American Thoracic Society guidelines \(^{234}\), after a maximum of 3 attempts. Technique was reassessed at each visit to the laboratory.

### 3.2.4 Airway responsiveness

Bronchial challenge was performed with methacholine (Nova Laboratories, Leicester, UK). Methacholine is a muscarinic agonist that causes bronchoconstriction and is known to be a safe and sensitive agent for assessing airway responsiveness \(^{235}\). Methacholine was stored at 4°C and warmed to room temperature before nebulisation (Wright's nebuliser output 0.13 ml/min). After nebulisation of the normal saline diluent, serial doubling concentrations of methacholine ranging from 1 to 128 g/l were given by tidal breathing for 2 minutes at 5 minute intervals, with a nose clip in place, through a mouthpiece. The response was measured as FEVi. During the methacholine challenges, single measurements of FEVi were made 90 seconds after each dose, unless a manoeuvre was felt to be technically poor in which case it was repeated 30 seconds later. The tests were discontinued if FEVi decreased by more than 20% from baseline or when a methacholine concentration of 128 g/l had been administered, whichever was first. The PC\(_{20}\) was calculated by linear interpolation from the FEVi - log\(_{10}\) methacholine concentration curve. At the end of the tests, subjects inhaled 200 µg of salbutamol from a metered-dose inhaler using a Volumatic spacer (Glaxo-Wellcome, UK).

Quality of spirometry was maintained by daily calibration of the spirometer using a 2 litre Vitalograph calibrator, and weekly cleaning of the filter.
3.3 Nasal sampling

3.3.1 Review of existing techniques

The requirements for nasal sampling in this study are that sampling must:

- provide appropriate samples for cellular and fluid-phase analysis;
- be reproducible;
- be quick and simple;
- cause minimal discomfort;

Various techniques have been described for collecting nasal secretions but many of these have methodological flaws. These techniques include the direct collection of secretions (i.e. undiluted) or the collection of secretions mixed with lavage fluid.

Direct methods

There are several methods for directly collecting nasal secretions including: collecting drips sneezes or blown secretions; aspirating the nose; and absorbing secretions. The major advantage of these methods over lavage is that the problem of quantifying the degree of dilution does not arise. However in general, smaller amounts are obtained which is a particular problem when secretions are not copious such as in samples taken at baseline or after recovery from the illness.

Collecting drips as the secretions run out of the nose into a container avoids any interference with the nasal mucosa \(^{236}\). However adequate secretions can only be obtained after nasal methacholine challenge \(^{236}\). This method is time-consuming and may be more uncomfortable than a rapid but more invasive technique. Blowing the nose directly into a container has the main disadvantages of contamination with lacrimal secretions and volunteers cannot produce adequate volumes without a
saline nasal spray. Collecting secretions from sneezing is of no value as the majority of sneeze content is saliva unless the mouth is kept firmly closed which is extremely difficult to do in practice.

*Aspirating the nose* directly using a suction catheter can also be used but only if there are sufficient secretions. Induction of secretions with histamine has been used but this interferes with nasal physiology. Aspiration must be gentle so as not to cause trauma and discomfort. Contamination with blood could have profound effects on cellular and especially fluid phase measures.

*Absorbing secretions* onto material placed in the nose has been used in many studies. Different materials have been used including strips of filter paper, gauze rolls, cotton swabs, cellulose-acetate sponges, and filter-weave bags filled with powdered dextran. There are many drawbacks with the above methods. Some materials are left in the nose for a long time and some cause obvious discomfort. Material might have selective absorption properties that could influence the quality of secretions collected. Substances may also remain in the material after it is squeezed out to varying degrees as tenacious mucus may be retained in the swab. Material placed on the mucosa acts as an irritant and alters the nature of the secretions disturbing epithelial barrier function, with passage of subepithelial fluid and macromolecules into the lumen. Finally, the results obtained are only representative of a small area of mucosa and may not reflect inflammation throughout the nasal tract thus leading to inaccuracy. *Direct* methods of collecting nasal secretions were not chosen for this study.
Indirect method - nasal lavage

Nasal lavage is a technique whereby nasal secretions are obtained by washing out the nasal cavity. This technique is quick, relatively simple and cheap, and small amounts of secretions and nasal lining fluid can be obtained. The latter is particularly useful when studying a non-inflamed nose at baseline. However, the main disadvantage is that secretions are diluted by the wash solution so that accurate measurements require a method of estimating degree of dilution.

There is a need to clarify some of the terminology used in studies of nasal lavage. Nasal wash or nasal wash solution refers to the fluid before it is instilled into the nose. Nasal lavage fluid is the nasal wash mixed with the fluid contents recovered from the nasal cavity. The fluid recovered from the lavage consists of nasal lining fluid and nasal secretions although the two cannot be distinguished visually.

i) Instilling the nasal wash

Irrigation of the nose can be achieved using a pipette, catheter or syringe. Hilding and colleagues described the use of a Foley catheter with the balloon inflated in the vestibule of the nostril to instill and collect secretions, but when this was piloted for this study, subjects found it uncomfortable and sample was lost by dribbling. Holmes and colleagues described a method in which the posterior nasopharynx was sampled with the volunteer lying supine on a couch and hyperextending the neck. The fluid was introduced and ideally the volunteer allowed the fluid to stay in the nasal cavity for 1 minute before flexing forwards and allowing the lavage to flow into a receptacle. They found that with the head extended they obtained almost twice as much nasal secretions and a much higher cell count than their previous method in which the head was flexed forwards and small aliquots of wash introduced. When this was piloted for this study, subjects found it difficult to hold the wash in the nasal cavity for a minute.
Koren and colleagues developed a simple nasal wash technique in order to study the effects of air pollution \(^{251}\). This involved the volunteer sitting with the head tilted back to 45° and the use of a needleless syringe to instill warm wash into one nostril. During the process the volunteer occluded the palate by a Valsalva maneuver and the wash remained in the nasal cavity for 10 seconds before it was expelled into a receptacle. Aliquots were delivered to both nostrils until a total of 10 ml was used. Similar methods have been used during an experimental HRV study \(^{252}\). This method has the advantages of being quick, less invasive than other techniques and of allowing adequate sampling of most of the nasal cavity. One study reported a mean 6.6 ml return after instilling 10 ml which compares favorably with catheter devices \(^{253}\).

*Sniffing* up the wash has been tried, whereby subjects wore rubber gloves and sniffed a few drops of water from a cupped hand before forcefully expelling it into a container \(^{254}\). This method was not favoured because of the possibility of spillage.

*Devices* specifically designed for nasal wash have been developed. A rubber bulb filled with wash has been used successfully to collect samples from children in order to identify respiratory syncytial virus \(^{255}\). The “nasal pool” of Grieff and colleagues is a compressible bulb which again enables the introduction and collection of wash \(^{256}\). This device is used with the head tilted forwards and the authors report that the wash can be thus tolerated in situ for up to 15 minutes! This device was advocated for nasal challenges in allergic conditions but it might also have a place in nasal lavage fluid collection for cellular analysis.
ii) Recovery of the nasal lavage fluid

In order to minimise trauma the most effective method of collection for those who can cooperate is to allow the fluid to flow out into a sterile container followed by a forceful blow. In practice bleeding induced by forceful blowing is extremely uncommon. Fluid can be aspirated at the posterior nasopharynx or at the anterior nares, which is less invasive, by suction catheters but care must be taken to avoid trauma to the nasal mucosa. Fluid is usually aspirated from the same nostril in which it is instilled but the catheter can be placed at the posterior choana so that fluid instilled into one nostril is simultaneously aspirated from the back of the opposite nostril.

iii) Timing of the nasal wash

When deciding on the timing of repeated nasal washes it is important to be clear which measurements are to be made and the possible effects of repeated sampling. The design of the study (chapter 2.2) involved samples taken at day 0, day 2 (optimal time for virus identification), and day 4 (optimal time for measuring inflammatory response). Attention needs to be given to assessing the effects of repeated sampling on the nasal mucosa and the inherent repeatability of the technique.

"Washout" and "recruitment" are terms used to explain the interaction of the procedure on cell numbers. Washout refers to the physical process of washing cells out of the nasal cavity. Recruitment describes the reappearance, whatever the mechanism, of cells in the nasal airway. Washout may lead to lower subsequent cell counts or a reduction in any increase in subsequent cell counts. The washout of cells itself may induce an inflammatory response, leading to recruitment of cells into the mucosa. These mechanisms of cell recruitment may increase cell counts leading to repopulation following the lavage and there could be a rebound overshoot of cell counts. Hauser
and colleagues put forward the hypothesis that depending on the balance between cell washout and cell recruitment, cell counts in repeated lavages may have returned to baseline, remain below baseline or overshoot the baseline.\(^{259}\)

In a common cold experiment where large numbers of cells are likely to be present on the surface of an inflamed and hyperaemic mucosa the effects of wash-out and recruitment might be expected to be minimal. This is supported by previous studies. Akerlund and colleagues measured fibrinogen concentrations during an experimental infection with HCoV and demonstrated a highly significant rise on days 4 to 6, the time when symptoms were at a peak.\(^{105}\) Proud and colleagues demonstrated a marked increase in IL-1 in nasal secretions between days 2 and 4 in volunteers with an experimental HRV cold.\(^{260}\) Both studies used twice-daily lavages and despite this frequency a "wash-out" effect from repeated sampling did not seem to interfere with mucosal exudation as measured by fibrinogen levels or with concentrations of IL-1, a cytokine released by monocytes, macrophages and other cells. A washout effect seems unlikely.

But what of the possibility of a recruitment effect? A study of subjects without a cold found good agreement between cells in repeated nasal lavage samples when the interval between lavages was 2 and 3 days (intraclass correlation coefficients = 0.75 and 0.84, respectively)\(^{259}\). The authors suggest the slightly inferior repeatability at 2 days could be a result of a small recruitment or wash-out on the repeat nasal lavage, but generally repeatability was good. A major effect from recruitment also seems unlikely. Only by continuing to sample those volunteers who do not develop a cold can the degree of any of wash-out and recruitment be assessed.
iv) Choice of nasal wash solution

The ideal nasal wash solution should provide a physiological environment for cells to be suspended in so as to minimise degradation ex-vivo. It must also be non-toxic to the volunteer and induce minimal irritation so as not to provoke an inflammatory reaction. Smell and taste should not be unpleasant if repeated lavages are to be performed.

Several fluids have been used including distilled water, 0.9% sodium chloride, 10% sodium chloride solution, 0.1M Tris HCL with 0.85% sodium chloride solution, lactate Ringer’s solution, and phosphate buffered saline. It has been shown that physiological saline instilled in the nose does not induce significant nasal secretion of protein or IgA suggesting it is not very irritant. It is also well tolerated as far as smell and taste are concerned.

Temperature of the nasal wash solution is also an important factor. A cold solution causes changes in nasal vasculature and can lead to the release of inflammatory mediators, including cysteinyl-leukotrienes. During a small pilot of the nasal lavage technique used in this study volunteers found a warmed solution more comfortable and easily tolerated.

Volumes of nasal washes vary from study to study (from 3-5 drops to 14 ml). Factors such as size of nose will play a role in how much is tolerated before some is swallowed. The method of Hauser and colleagues successfully used a volume of 5 mls placed into each nostril. Others recommend repeated small aliquots as tolerated until the desired volume is reached. Again the key factor must surely be the volunteer’s comfort and ability to tolerate the desired volumes.
v) Estimation of dilution factor

No matter which lavage technique is used there is always some loss of the nasal wash, hence it cannot be established how much of the returned lavage fluid is wash solution and how much is mucosal lining fluid. As returned volumes vary from person to person the dilution of the nasal lining fluid may well vary and render comparisons of fluid phase mediators meaningless. In order to estimate how much of the lavage fluid is wash solution and how much is nasal secretion a ‘marker of dilution’ is required.

*Endogenous markers* are natural substances present in the nasal lining fluid and secretions. For a substance to be a useful marker it should have identical concentrations, under steady-state conditions, in blood and nasal secretions so that the dilution factor can be derived from comparison of the concentration of the substance in the nasal lavage fluid with the concentration in the blood. *Albumin* has been used in BAL work where it’s concentration in epithelial lining fluid is usually around 10% of that in plasma. However, albumin concentration is not in equilibrium with plasma and is greatly affected by capillary leakage and is therefore of questionable value as a marker of dilution. *Urea* is in equilibrium with plasma and has been used as an endogenous marker in BAL work. However it may diffuse too rapidly into the wash fluid during its residence in the nose, resulting in a falsely high estimate of the volume of secretions recovered. Urea is therefore not an ideal marker of dilution especially when during a cold there may be increased vascular permeability contributing to the rapid movement of urea.

*Exogenous markers* of dilution are substances added to the nasal wash. When the wash plus marker is instilled in the nose the nasal lining fluid will cause a dilution of the marker. If one can measure the concentration of the marker in the wash solution and the lavage fluid the dilution
The effect of the nasal lining fluid on the wash solution can be calculated. This enables the calculation of the proportion of nasal lavage fluid consisting of nasal lining fluid and hence takes into account a dilutional factor when measuring the constituents of the lining fluid. This technique must be reproducible, simple and involve a substance that is safe and well tolerated. The major is loss of the marker through absorption of the marker during its residence in the nose.

*Phenol red* and *methylene blue* are coloured dyes that have been used in nasal lavage and BAL work \(^{266,271,272}\). An advantage is that these dyes are safe, non-toxic and easily measured. A disadvantage is the simple practical issue of nasal lavage being a messy procedure in adults as well as children. Staining of face and clothing can occur. *Radioisotope markers*, such as tritiated water \(^{273}\) and Tc-albumin \(^{274}\), have been favoured by some because of the high degree of sensitivity of assays. One study showed that only 0.5% of Tc-albumin was absorbed during a 30 second nasal wash \(^{275}\). Another advantage is that the radioactivity of the whole sample is measured so that poor mixing between wash and nasal secretions does not affect the assay. The radiation hazards are thought to be low and even if a full 2 ml of Tc-albumin was swallowed the total body radiation dose would only be 1% of normal daily background radiation \(^{275}\). On the other hand when dealing with patients and volunteers one would have to spend considerable time explaining the hazards and some people may well be put off. A non-radioactive alternative both practically and ethically would be preferable.

*Chemical markers* such as lithium chloride \(^{276}\) and inulin \(^{253}\) have been used. Lithium has the disadvantage that it takes at least one hour for it to equilibrate between the gel and solute phase of the recovered mucous thus delaying valuable processing time and allowing more time for cell degradation. Lithium also has several toxic side effects, which makes it less attractive as a marker.
Inulin on the other hand is a chemically inert polysaccharide made up of fructose units with a molecular weight of 5200. It dissolves completely on warming, has a pH of 5.87 at 37°C, and has minimal effect on osmolarity. It is known to be safe and is commonly used to measure renal function. It is also inexpensive, readily available and easily assayed. Inulin was chosen as the marker of dilution for this study.

3.3.2 Technique used in this study

The technique used in this study was a nasal lavage based upon the method of Koren and colleagues for its simplicity, tolerability and that a lavage can sample the majority of the nasal cavity with minimal irritation of the mucosa. It involved the subjects sitting with their necks extended to 45° while warm phosphate buffered saline (PBS) containing 450μg/ml of inulin was introduced into one nostril. During the process the subject occluded the palate by positive oral pressure so that the wash remained in the nasal cavity for 10 seconds before being expelled into a sterile receptacle. A total of 10 ml in aliquots of 2.5 ml was inserted alternately in each nostril. One ml of mixed nasal lavage fluid was then removed and stored at –70°C for later viral detection by RT-PCR whilst the remainder was processed (section 4.3).

3.4 Sputum induction

3.4.1 Review of existing techniques

The requirements for LRT sampling in this study are that sampling must:

- provide appropriate samples for cellular and fluid-phase analysis;
- be reproducible;
- be quick and simple;
- cause minimal discomfort;
Whilst bronchial biopsy is required to identify whether HCoV infects the LRT, this is invasive, time consuming, expensive and not without discomfort for the subject. Sputum induction, on the other hand fulfils the above criteria.

i) Introduction

There is a long history of interest in the macro and microscopic appearance of sputum in asthma. In the later 19th century Charcot-Leyden crystals and Curshmann's spirals were identified in the sputum of asthmatics and eminent physicians theorised about their role in causing bronchospasm by mechanical irritation. The association of these crystals with sputum eosinophil turnover did not become clear until the latter half of this century. The more recent development of invasive techniques such as bronchial biopsy and BAL has lead to the recognition that even mild asthma is associated with evidence of airway mucosal inflammation. Consequently there has been renewed interest in the use of sputum to non-invasively assess airway inflammation.

In order to understand modern techniques of sputum collection it is necessary to understand developments in methodology and the application of sputum analysis in disease. Many of these developments are linked to progress in sputum processing and cell counting, hence these are reviewed in this sections rather than laboratory methods.

ii) Developments in Methodology

Sputum smears have been used to study people with chronic bronchitis, asthma and normal controls by removing plugs of sputum from surrounding saliva to minimise contamination. Quality was assessed by estimating the volume of sputum plugs and proportion of squamous cell...
contamination from saliva. After suspension in PBS or trypsin EDTA, in order to disperse the cells, total cell counts were performed in a Neubauer chamber after staining with May-Grunwald-Giemsa and toluidine blue. Cells were identified as basophils (>10 microns, lobulated nuclei), mast cells (>10 microns, round nuclei), small mast cells (<10 microns, round nuclei) or unclassified metachromatic cells. This is a previously established method and correlates well with electron microscopic classification. Total cell counts and eosinophils were reproducible between different plugs of sputum from the same sample when either PBS or trypsin was used and between different samples from the same patient taken 24 hours apart (intraclass correlation (reliability) coefficient $r > 0.7$ and $r = 0.94$, respectively). This reproducibility suggests that the distribution of cells from sputum that is carefully selected out from saliva is homogenous.

This technique was important in developing the credentials of sputum analysis, however, there were several problems. There can be difficulty in obtaining spontaneous sputum samples. Cell clumping on the smears often made counting very difficult and was also time consuming taking more than 2 hours for each sputum sample. Further developments came when collection followed saline inhalation and sputum was mixed with dithiothreitol (DTT) in order to improve cell dispersion.

*Saline-induced sputum.* The use of ultrasonic nebulised hypertonic saline has been shown to greatly facilitate sputum production. The most common complaint is of a salty taste and in early studies adequate sputum was obtained in 76 – 100% of healthy and asthmatic adults. There was also good repeatability of eosinophil, metachromatic, macrophage, and polymorphonuclear cell counts (reliability coefficient $R = 0.8, 0.7, 0.7, 0.73$ respectively).
Safety is an issue as inhaled hypertonic saline can cause bronchospasm. Significant reductions in FEV$_1$ during induction of sputum with hypertonic saline occur in 6 to 30% of subjects with asthma. One study demonstrated that those asthmatics with lower FEV$_1$ were more likely to bronchoconstrict with hypertonic saline, however, none suffered refractory bronchoconstriction and none required hospital admission. Although relatively safe there is a need for careful monitoring with spirometry and the availability of a bronchodilator for the emergency relief of obstruction if needed.

The mechanisms by which hypertonic saline induces sputum are not known. It may involve an osmotic effect, increased mucociliary clearance and stimulation of glandular secretions. But there has been concern that inhalation of hypertonic saline might interfere with the cellular or fluid-phase contents of sputum. Pizzichini and colleagues used this methodology to compare spontaneous and induced sputum. The sputum weights and total and differential cell counts were similar but induced sputum had less squamous cell contamination from the URT, produced cytospins (cells spun onto slides) of better quality, and had a higher proportion of viable cells (77% versus 47%). The latter assessment was by trypan blue exclusion, which stains non-viable cells. Poor cell viability (<50%) and high squamous cell counts (>20%) have been shown to lower inter-observer agreement when counting cells.

*Cell dispersion with DTT.* DTT is a sulphydryl reagent that produces mucolysis by opening disulphide bonds that crosslink glycoproteins responsible for maintaining the gel form of sputum. Comparisons of sputum plugs selected from saliva then mixed with DTT and sputum smears have shown that cell dispersion with DTT is highly effective, making cell counting easier, quicker and more reproducible. Cell definition is improved in DTT treated sputum, enabling
differential cell counts to be performed on cytospins stained with Wright’s stain without the need for further stains for eosinophils. A study of between-observer repeatability with this method has shown excellent repeatability for neutrophils, eosinophils and macrophages (intraclass correlation coefficients (ICC) = 0.89, 0.9, 0.9) but not for lymphocytes (ICC = 0.2) probably because of their low numbers.

There have also been several studies that have used these methods to examine the fluid-phase components of inflammation. Popov and colleagues have been able to identify EG2, GM-CSF, TNFα and IL-8. Keatings and colleagues found the method to be reproducible when looking at IL-8 and TNFα over a two-week period.

Overall, sputum induction by hypertonic saline is non-invasive, simple, safe and does not interfere with cellular proportions. When combined with DTT for cell dispersion, cell counting of cytospun sputum is highly repeatable. The only drawback is the dilution (possibly due to enhanced water influx into the airway lumen) of fluid-phase products as indicated by the lower levels of fibrinogen and ECP when compared to spontaneous sputum. This will not interfere with comparisons between different subjects when the same methods are used but must be borne in mind when comparing the fluid phase measurements with spontaneous sputa and bronchial biopsies.

Whole sputum versus plug selection. Two approaches to sputum induction, both incorporating the advances in methodology described above, are now commonly used. Fahy and colleagues have used whole specimens containing sputum and saliva. Their concern was that sputum plugs would not be a representative sample for LRT secretions for either cellular or fluid-phase components. However, the drawback with this approach is that sputum will inevitably be diluted.
by saliva with the extent of dilution very difficult to estimate. Pizzichini and colleagues compared
differential cell counts and fluid phase ECP in selected plugs with the residual portion that is
usually discarded. Differential cell counts did not differ but the quality of the cytospin was
better in the selected portion, principally because of less salivary squamous cell contamination.
More importantly the fluid-phase ECP concentration was 5-6 times higher in the selected rather
than the residual portions suggesting dilution does occur in the latter. This method of removing
all the more viscous and dense portions of sputum by blunt forceps, results in a median squamous
cell contamination of 1.6% so the contribution of saliva is likely to be minimal.

iii) Validity of induced sputum

A clinical technique is valid only if useful in the study and investigation of health and disease.
Normal ranges of cell differentials in healthy subjects have now been established (Table 3.2). The
comparison of healthy subjects with those with disease and also with the findings obtained by
different techniques, such as BAL and bronchial biopsy, has proven sputum induction to be a valid
method for the collection of inflammatory cells and mediators from the LRT.

*Induced sputum and cell analysis.* Induced sputum is now widely used to study inflammation in
asthmatic adults and children, chronic bronchitics and has recently been used to study natural and experimental virus exacerbations of asthma. The non-invasive nature of sputum induction has proved ideal to study eosinophilic inflammation in asthma. Recent studies have shown reasonably close correlation between differential eosinophil counts in induced sputum and BAL in normal and asthmatic subjects. The main difference between cell counts in induced sputum, BAL and bronchial wash is that induced sputum has a higher proportion of neutrophils and a reciprocally lower proportion of macrophages (Table 3.3). Fahy and
colleagues speculated that this phenomenon was due to differences in the site sampled by the different techniques, BAL and bronchial wash obtaining more distal secretions than sputum. This theory has been supported by a study in which lavage of the right main bronchus of normal subjects was compared to lavage of a subsegmental bronchus, with recovered fluid containing 28% and 10% neutrophils, respectively. The only other possible explanation for this phenomena is that the process of hypertonic saline induction of sputum actually causes an influx of neutrophils. This seems unlikely in a one off induction that may last only 15 minutes but could be problem where repeated inductions are performed.

**Induced sputum and neutrophils.** Studies of the effect of repeated sputum induction have shown that the differential neutrophil count and concentration of ECP increase significantly between inductions 24 hours apart, with neutrophils increasing by approximately 10-12% from the first to second induction. This effect does not persist to a period of one-week. Pavord has reviewed this problem and suggested that the effect may be related to the dose of hypertonic saline used as both studies over a 24 hour period used a high dose (51 ml at 1.7 ml/min and 67.5 ml at 4.5 ml/min). Sputum induction was equally successful when less than half this amount of hypertonic saline was given at a rate of 0.9 ml/min. In a study of asthmatics during exacerbations who had repeated sputum inductions 24 hours apart on 4 consecutive days no increase in neutrophils was seen. It is unlikely that repeated sputum inductions with intervals of 48 hours would have a significant effect on the inflammatory cells.

**Induced sputum and supernatant analysis.** Various cytokines have now been measured in induced sputum including IL-8, IL-5, IL-6 and TNFα. These products have been shown to reflect the inflammation status in disease. Higher levels of IL-8 have been found in the sputum of patients.
with cystic fibrosis compared with asthma but the asthmatics had higher levels of IL-6\textsuperscript{302}. IL-5 has been shown to correlate with eosinophil counts in some asthmatics\textsuperscript{294}. Products reflecting microvascular leakage, such as albumin and fibrinogen\textsuperscript{57}, and cell activation such as ECP\textsuperscript{303} have also been measured.

### 3.4.2 Technique used in this study

Hypertonic saline induction of sputum is safe, simple, cheap and repeatable and collects secretions from the major airways that can be analysed for cell and fluid-phase markers of inflammation. The protocol chosen for this study (Figure 3.1) was based upon the work of Pavord and colleagues\textsuperscript{300}.

Subjects inhaled 200 µg salbutamol by metered dose inhaler and volumetric spacer (Glaxo) 10 minutes before inhaling nebulised 4.5% saline for 3 periods of 7 min by ultrasonic nebuliser (Medix Ltd, Harlow, UK; output 0.9 mL/min; mass median diameter 5.6 µm). Sputum was expectorated after the subject had blown their nose and gargled to minimise nasal and oral contamination. Sputum was put on ice and processed within 1 hour (chapter 4.3).

### 3.5 Bronchial biopsy

#### 3.5.1 Introduction and aims

To be certain that virus actually infects the LRT epithelium it is necessary to look for virus within bronchial biopsies. This avoids the possibility of contamination of induced sputum as it is expectorated through the URT and of BAL as the bronchoscope is passed through the nasal passage. The aim of this section was to conduct a bronchial biopsy during the experimental infection in order to assess whether HCoV infects the LRT.
3.5.2 Methods

Subjects. Eleven of the subjects in the main study volunteered for the extra bronchoscopic study. Eight were females and ages ranged from 19 to 36 years. Eight were viral wheezers. One subject was excluded prior to bronchoscopy due to the detection of a heart murmur. The subjects received £150 compensation for their time and effort after the study was completed. The study was approved by the Leicestershire Health Authority Research Ethics Committee and written informed consent was obtained from all participants.

Study design. This section of the study was designed so as not to interfere with the measurement of physiology and the collection of nasal and sputum samples. The first bronchoscopy was performed on day 4 post-inoculation after all other samples and measurements had been made. Five volunteered for a follow up bronchoscopy was performed 6 to 8 weeks later in order to provide a comparison, when subjects were asymptomatic.

Fibreoptic bronchoscopy. Subjects fasted from 0800 hours. After measurement of airway physiology and collection of nasal and sputum samples subjects transferred to Glenfield General Hospital, Leicester for bronchoscopy between 1300 and 1400 hours. This procedure was carried out by Professor Andrew Wardlaw (see acknowledgements). Inhaled salbutamol (200-400 μg) was given prior to the procedure and all volunteers had an FEV₁ of greater than 80% predicted. Intravenous sedation (3-5 mg of midazolam) was given and lignocaine gel applied to the nasal mucosa. Eight to 10 ml of 2% lignocaine solution was applied to the vocal cords and airways before and an Olympus BF20 bronchoscope (Olympus Optical Co., Tokyo, Japan) was passed. Oxygen supplementation was mandatory and assessed with continuous pulse oximetry. Up to 4 bronchial biopsies were taken from the lower lobe subcarinae. A bronchial wash of the right
middle lobe using 50 ml of normal saline was also performed after the biopsies. Analyses of the latter samples are not reported here.
3.6 Figures and Tables

Table 3.1 The symptom diary used in the study

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Table 3.2  Reference ranges for induced sputum total cell count (TCC) and differentials from healthy non-asthmatic subjects \(^{299}\).

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<tr>
<th>Normal Range</th>
<th>Median</th>
<th>Interquartile range</th>
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<td>TCC (x10^6/ml)</td>
<td>3.1</td>
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<tr>
<td>Eosinophils (%)</td>
<td>0.5</td>
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<tr>
<td>Neutrophils (%)</td>
<td>24.1</td>
<td>26.8</td>
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<tr>
<td>Macrophages (%)</td>
<td>62.9</td>
<td>30.2</td>
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<tr>
<td>Lymphocytes (%)</td>
<td>1.3</td>
<td>0.6</td>
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Table 3.3  The percentages of cell types in induced sputa, BAL and bronchial washes in asthmatic subjects.

<table>
<thead>
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<th></th>
<th>Induced sputum</th>
<th>Bronchial wash</th>
<th>BAL</th>
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<tbody>
<tr>
<td>TCC x 10^5</td>
<td>4.6 (0.8-17.4)</td>
<td>1.6 (0.5-6.2)</td>
<td>2.8 (1.4-5.3)</td>
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<td>Epithelial cells %</td>
<td>1.1 (0.0-13.8)</td>
<td>4.2 (0.2-40.7)</td>
<td>0.3 (0.0-3.7)</td>
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<td>Macrophages %</td>
<td>60.0 (7.0-94.0)</td>
<td>90.0 (24.0-94.0)</td>
<td>93.0 (0.0-97.4)</td>
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<td>Neutrophils %</td>
<td>36.0 (0.0-83.0)</td>
<td>1.9 (0.2-46.0)</td>
<td>0.8 (0.2-7.6)</td>
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<tr>
<td>Eosinophils %</td>
<td>1.9 (0.2-13.8)</td>
<td>0.9 (0.1-3.5)</td>
<td>0.2 (0.0-4.6)</td>
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<tr>
<td>Lymphocytes %</td>
<td>0.4 (0.0-1.9)</td>
<td>1.8 (0.9-9.2)</td>
<td>2.9 (1.1-15.2)</td>
</tr>
</tbody>
</table>
Figure 3.1  Sputum Induction Protocol

Administer 200µg of salbutamol

↓

Measure FEV1

↓

Administer 4.5% saline using ultrasonic nebuliser
For 7 minutes at output 0.9 mL/min

↓

Measure FEV1

↓

If < 10% fall
If > 10% fall give salbutamol 200mcg and remeasure FEV1

↓

If recovered proceed
If still > 10% fall give salbutamol 2.5mg via nebuliser and remeasure FEV₁ and discontinue*

↓

Blow nose, rinse mouth and spit out water

↓

Expectorate sputum
Repeat x 3

* If > 20% fall in FEV₁ at any time discontinue and give salbutamol 2.5mg neb
If troublesome symptoms discontinue.
Chapter 4 Laboratory methods

4.1 Introduction

The objectives pertaining to laboratory methods are:

1. To establish an experimental infection with laboratory confirmation of infection and exclude other infections.
2. To measure the cellular inflammatory response to infection in both the upper and lower respiratory tracts.
3. To measure fluid phase markers of potential interest in viral wheeze in both upper and lower respiratory tracts, and where appropriate, in the urine.
4. To identify whether replication of virus occurs in the LRT.

The laboratory methods used to achieve these objectives include: RT-PCR for detection of viral genome; ELISA for detection of serum immune response to virus; cell processing to prepare slides by cytospin to assess inflammation; inulin assay to estimate the dilution factor of the nasal lavage; enzyme immunoassay and fluoroenzyme assay to measure fluid-phase markers of inflammation; and RNA extraction with RT-PCR followed by Southern blotting for the identification of virus genome in bronchial biopsies.

4.2 Confirmation of viral infection

Two methods were used to confirm whether virus infection occurred.

4.2.1 RT-PCR

Virus RNA was identified by RT-PCR in nasal lavage fluid, and throat and nose swabs taken on days 2 and 4-post inoculation. This method was derived from a nested RT-PCR described
previously 28. The throat swab was taken from the posterior pharynx and tonsil bed, and the nose swab from the inferior turbinate. Both were placed immediately into phosphate buffered saline containing RNAse inhibitor (10U/ml, Promega Life Sciences, Southampton, UK) to inhibit the action of RNAse that may have been present in the specimens, and stored at -70°C. The time from sample collection to storage was minimal as the freezer was in the laboratory in which the samples were collected, reducing the loss of RNA due to RNAse to a minimum. 1 ml of mixed nasal lavage was also stored at -70°C. For analysis, samples were rapidly thawed and RNA extractions immediately undertaken, again minimising losses.

All primers were designed under the supervision of Dr Pringle (see acknowledgements) from the genome of the nucleocapsid protein and checked against the EMBL database. The primers were manufactured by Sigma-Genosys Ltd (Cambridge, UK).

i) Extraction of HCoV 229E RNA

Prior to RNA extraction all Eppendorf tubes were treated with diethylpyrocarbonate (DEPC) (Sigma Aldrich, Dorset, UK) in order to reduce the possibility of RNAse contamination and subsequent degradation of extracted RNA (see appendix 2.1). RNAse/DNAse filter-guard tips were used in all manipulations.

Extraction was based upon a guanidium isothiocyanate method utilising RNAzol B™ (Biogenesis, Poole, UK) based on the method of Chomczynski and Sacchi 317. Essentially, each thawed aliquot of clinical specimen was added to 200μl of ice-cold RNAzol B™ in an RNAse free 1.5ml Eppendorf tubes and mixed by pipetting up and down several times. Due to their hazardous nature RNAzol B™ and chloroform, manipulations were performed in a fume-cupboard. 200μl of ice-cold chloroform (Fisons, Loughborough, UK) was then added to this
lysate and the new mixture vortexed for 15 seconds. The homogenate was then stored on ice for 5 minutes and centrifuged at 12000g for 15 minutes at 4°C (upon centrifugation the homogenate forms two phases, the proteins and DNA collecting at the interphase). The upper aqueous phase (containing all the extracted RNA) was then carefully removed, added to 300μl of ice-cold isopropanol (Fisons) and mixed by pipetting up and down several times. The mixture was then stored on ice for at least 30 minutes to precipitate the extracted RNA. After isopropanol incubation, the mixture was re-centrifuged at 12000g for 15 minutes at 4°C and the supernatant carefully removed (ensuring that any RNA pellet was not disturbed). 1 ml of ice-cold 70% ethanol was then added, the mixture briefly vortexed and then centrifuged at 7500g for 10 minutes at 4°C. After this final centrifugation stage, the supernatant was carefully removed (again ensuring that any RNA pellet was not disturbed), the Eppendorf tubes inverted onto tissue paper and the pellet left to dry at room temperature for approximately 15-30 minutes to remove ethanol that may interfere with later reverse transcription reactions. After the allotted time, the RNA was resuspended in 30μl of RNase free distilled H₂O (Appendix 2.1) containing 1μl of RNase inhibitor (20u/μl, Promega) and immediately utilised.

Both negative and positive controls were included in each batch of reverse transcriptions and subsequent PCRs. Negative controls consisted of diethylpyrocarbonate treated distilled H₂O (Appendix 2.1). Positive controls consisted of RNA extracted from HEL cells infected with HCoV 229E. Also, contaminating DNA was controlled for by running a water control alongside each and every sample undergoing PCR. Where contamination of the water control occurred, the whole PCR run was repeated.

ii) Reverse transcription of HCoV 229E
Reverse transcription is the method by which copy DNA (cDNA) is manufactured from the RNA extract. A standard reverse transcription methodology adapted from Sambrook and colleagues was used. Initially, a reverse transcription super-mix was prepared by multiplying the volume of a particular component required for an individual reverse transcription mix by the number of reverse transcription mixes required (one reverse transcription mix being required for each specimen to be reverse transcribed). Individual reverse transcription mixes comprised 2μl of 10x reverse transcription buffer (supplied free with the MMLV reverse transcriptase enzyme (Stratagene, Cambridge, UK)), 2μl of a 5mM equimolar mix of dNTPs (adenosine/cytosine/guanosine/thymidine deoxynucleotide triphosphates giving a final concentration of 0.5 mM), 0.5μl of 100mM DTT (2.5mM final concentration), 1μl of 200ng/μl downstream primer 0647 (1.25μM final concentration – see Appendix 2.2), 1μl of 10μg/ml gelatin and 3μl of RNAse free dH₂O.

When the supermix had been prepared, 9.5μl was pipetted into individually labelled RNAse free 0.5ml Eppendorf tubes and overlain with mineral oil (Sigma Aldrich). 10μl of extracted RNA was then added to the respective reverse transcriptase mix and then the resultant RNA/reverse transcriptase mix heated at 70°C for 5 minutes in order to unravel the structure of the RNA. The mixture was then placed on ice for 5 minutes after which time 0.5μl of monkey-murine leukaemia virus (MMLV) reverse transcriptase enzyme (50u/μl, Stratagene) was added to each mix. The complete mixtures (final volume 20μl) were then incubated at 37°C for 1 hour (in order to activate the MMLV reverse transcriptase enzyme), heated to 95°C for 5 minutes (in order to deactivate the MMLV reverse transcriptase enzyme) and then cooled to 4°C. cDNA mixes were then stored at −20°C until required.
iii) HCoV PCR

The PCR used was based upon the 2 round ‘nested’ PCR developed by Professor Myint and colleagues which had determined the cycling regime. Previous work within the department had determined the optimal concentrations of MgCl₂ and primer concentrations. Initial PCR experiments using the ‘nested’ method gave false positives in the negative controls. All samples used in these initial experiments were discarded. A simplified single round PCR was designed, avoiding such problems. This method was used for this study.

Each PCR reaction mix (total volume 50μl) comprised 32.1 μl dH₂O, 6μl of 25mM MgCl₂, 5μl 10x PCR buffer, 0.4μl of 5mM dNTP mix, 0.5μl of HCoV 229E upstream primer “7” and, 0.5μl of HCoV 229E downstream primer “8” (appendix 2.2). This PCR reaction mixture was initially made as a supermix before being aliquoted into individually labelled RNase free 0.5ml Eppendorf tubes (volumes of 44.5μl). 5μl of reverse transcribed cDNA, sterile dH₂O, negative control cDNA or positive control cDNA was added the their respective labelled PCR reaction mixes and overlain with mineral oil. At all times the positive control was processed in a fume cupboard at a separate bench. The reaction mixes were then heated to 95°C for 5 minutes on a thermal cycler (Hybaid, OmniGene, Cambridge, MA, USA) before the addition of 0.5μl of Thermus aquaticus DNA polymerase (Promega Life Sciences, Southampton, UK, No: M1665). Forty cycles at the following temperatures were conducted on the cycler: 94°C for 60 seconds, 62°C for 90 seconds, 72°C for 120 seconds. The temperature was then held at 72°C for 10 minutes before a thermal ramp to 4°C. Samples were stored at 4°C until required.

iv) cDNA detection
This was by the standard method of agarose gel electrophoresis using a 2% agarose gel. This was prepared by adding 2g of agarose (genetic technology grade, ICN Biochemicals Inc. Ohio, USA) to a heat resistant glass 250ml conical flask. To this was added 100ml of 1x Tris Borate EDTA buffer (10.6g/l Tris – HCl (ICN), 5.5g/l boric acid (Fisons), 0.93g/l sodium EDTA (Fisons) adjusted to pH 8.3 using 5M HCl (Fisons)) and swirled to mix. The agarose mixture was then heated in a microwave for 1 - 2 minutes (highest power setting) till just boiling whereupon the agarose mixture was allowed to cool for approximately 3 minutes in a fume-cupboard. The gel casting frame was assembled and 2μl of 500μg/ml ethidium bromide solution (Sigma Aldrich) was added to the cooled agarose. The agarose was then swirled to evenly mix the added ethidium bromide and poured into the assembled gel casting frame. The gel combs were then paced in their respective positions and the gel allowed to set within the fume-cupboard for approximately 30 minutes prior to use.

The agarose gel was removed from it's casting frame and completely immersed in an electrophoresis tank containing 1x Tris Borate EDTA buffer (see above). 2μl of 6x stock loading buffer (10ml Glycerol (Sigma Aldrich), 0.05g bromophenol blue (Sigma Aldrich), 0.05g xylene cyanol (Sigma Aldrich) and 10ml sterile dH2O) was then aliquoted into separate sterile 0.5ml Eppendorf tubes and 10μl of each PCR product to be investigated added. The solutions were then mixed by pipetting up and down several times before pipetting into an appropriate well of the agarose gel. A molecular weight marker (1μl 1 Kb DNA ladder (Gibco), 2μl 6x loading buffer and 9μl of dH2O) was also run.

Electrophoresis of the gel was then carried out at 120-130V until the dye front was approximately 3/4 of the way along the gel (approximately 1 hour) whereupon the gel was
removed from the electrophoresis tank and inspected under UV light (wavelength = 260nm) for the presence of specific PCR product.

4.2.2 ELISA

An ELISA method, modified from a previously published assay to detect HCoV 229E antibodies was used. This work was done by Melanie Leech under the supervision of Dr Colin Hewitt and the author (see acknowledgements). Virus was propagated in cell culture as described in chapter 2.1. HCoV infected cells were frozen and thawed and the cell debris pelleted at 2000g for 30 minutes at 4°C. Virus antigens were prepared from lysates containing $10^{4.2}$ TCID$_{50}$ml$^{-1}$ and were concentrated by centrifugation at 75000g for 3 hours at 4°C. Virus antigen concentrate was resuspended in 1ml aliquots of PBS. Control antigens were prepared by the same method using uninfected cell cultures. Aliquots were stored at -80°C.

Antigen and antibody dilutions used were empirically calculated by a checkerboard titration. Flat bottomed polystyrene ELISA plates (Nunc, Roskilde, Denmark), were coated with 50μl of viral antigen (2.55 μgml$^{-1}$) or control antigen (1.79 μgml$^{-1}$) diluted in 0.1M carbonate bicarbonate buffer (sodium bicarbonate/sodium carbonate pH9.6) and incubated overnight at 4°C. Plates were washed four times with PBS and shaken dry. The wells of the plates were blocked for 1 hour at 37°C in 100μl PBS containing 1% bovine serum albumin followed by washing as before in PBS and 0.05% Tween 20 (PBS-T). Sera (50μl) diluted 10-fold in PBS-T with 1% bovine serum albumin (PBS-BT) were added in triplicate to the wells and incubated for 2 hours at 37°C. After a further wash in PBS-T, rabbit anti-human IgG, labelled with a horse-raddish peroxidase-conjugate (Dako, Cambridge, UK), diluted to 1 in 4000 in PBS-BT was added to the wells in 50μl aliquots. The plates were incubated for a further 2 hours at
37°C. After an additional four washes with PBS-T, 50μl of tetramethylbenzinoate diluted in dimethylsulphoxide and 0.5M phosphate citrate buffer with perborate, pH 5.0 (Sigma Aldrich) was added to all wells. The optical density was read at a wavelength of 450nm after stopping the reaction with 2M H₂SO₄.

The ELISA procedure was validated using paired sera collected from healthy individuals 17 days apart. No individual had suffered a respiratory infection in four weeks previous to or during the experiment.

### 4.2.3 Assessment of other respiratory viruses

Throat and nasal swabs were collected on day 2 and 4 post-inoculation and stored in viral transport medium for transport. Samples were sent on the day of collection to the virology laboratory in the Leicester Royal Infirmary for the identification of the following viruses: rhinovirus, RSV, Influenza A + B, Parainfluenza viruses 1,2,3, Adenovirus. The methods for virus identification were taken from Lennette et al. and were as follows:

1. Swabs in viral transport medium (VTM) were vortexed for 30 seconds prior to inoculation into cell culture.

2. Cell lines used:
   - Rhesus monkey kidney cells (RMK),
   - Hep2 (Human epithelial carcinoma cell line),
   - MRC5 (Human embryonic lung fibroblasts).

   0.25ml of VTM was added to each culture tube.

3. After overnight incubation at 33°C the medium was changed.

4. Tubes were observed every 48 hours for 14 days (Hep2 and RMK cells) and 21 days (MRC5 cells) looking for cytopathic effect.
5. Haemadsorption test was performed on all RMK cell samples – for myxo and paramyxoviruses in RMK cells.

6. If cytopathic effect seen or haemadsorption positive, the following assays were conducted:
   - Immunofluorescence using monoclonal antibodies for RSV and adenovirus.
   - Immunofluorescence on haemadsorption positive RMK cells for myxo and paramyxoviruses.
   - Acid stability for rhinovirus.

### 4.3 Processing of nasal lavage and induced sputum

Cell processing was performed to assess the quality of sample (cell viability and for sputum, squamous contamination) and to perform differential cell counts, using standard methods. Pilot nasal lavage samples were found to be viscous with mucous resulting in clumped cells on slides that were difficult to count, therefore cell dispersion was used as with sputum. Sputum plugs were selected (Figure 4.1), weighed and dispersed using four times the volume of 0.1% DTT (Sigma Aldrich) giving a 0.08% solution. Nasal lavage fluid was weighed and mucus dispersed by adding 1% DTT, also to a concentration of 0.08%. DTT was stored as a 1% solution in distilled water and freshly diluted each day using PBS. Each cell suspension was rocked gently for 15 minutes, and the sputum (but not the nasal lavage sample as this was already diluted with nasal wash) diluted with a further four volumes of Dulbecco's PBS. The suspension was filtered through a 48-micron nylon gauze, centrifuged at 790g for 10 minutes and the supernatant removed, aliquoted and stored at -80°C. TCC was obtained using a Neubauer haemocytometer (Fisher Scientific, Loughborough, UK) and cell viability was assessed by the trypan blue exclusion method. The cell suspension was adjusted to 0.5 x 10⁶ cells/ml and 75 μl cell suspension was used to prepare cytospins at 450 rpm for 6 min using a
Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA). Cytospins were stained with the Diff Quick stain (Dade Behring, Switzerland). This involved 5 to 10 one second dips in Eosin G followed by a thiazine dye and finally methanol fixative. Cytospins were then coded by a third party. At least 200 cells were counted at low power (x40) in order to categorize squamous cells, bronchial or nasal ciliated epithelial cells and leukocytes, then where possible at least 300 leukocytes were counted at high power (x100). Supernatants were stored at -80°C.

Squamous cells, epithelial cells and leukocytes were expressed as a percentage of the cells counted at low power. Sputum leukocyte differentials are only reported where at least 200 leukocytes could be counted at high power. Nasal lavage fluid leukocyte differentials were multiplied by the TCC and adjusted for the dilutional effect of the nasal wash derived from the inulin concentrations pre and post-wash (section 4.5.3). This gave cell concentrations per ml of nasal secretions. Nasal lavage fluid cell counts where at least 200 leukocytes could not be counted were designated zero cells per ml.

4.4 Repeatability of cell counts

4.4.1 Introduction

The accuracy of cell counting is dependent on two factors: the ability of the person counting the cells and the quality of the slide preparation. Firstly, the ability of the human cell counter. The requirement for manual counting of slides inevitably introduces some degree of subjectivity when deciding which label to attach to which cell. It is therefore essential when conducting such a study to have some means of internal control assessing the possibility of bias during cell counting. In a study where repeated samples from the same individual are collected
it is advantageous to have all slides counted by the same, blinded individual. This will reduce inter-observer error. As a means of internal control, the ability of the cell counter (author) was assessed by looking at repeatability of cell counts on a blinded subset of sputum and nasal lavage cytospun slides.

Secondly, the quality of slide preparation. Markers of quality of cytospins include cell viability and for sputum, squamous cell contamination. Prior to the development of this project, there were no data on the effect of these markers on the variability of cell counts. It was not known if poor quality slides should be discounted from the study. Another factor with the potential to affect quality is the stain used. Slides are traditionally stained with May-Grunwald-Giemsa or Romanowsky stain, but these require access to a fume cupboard and are time-consuming (taking approximately 40 minutes). For this study, BAL and sputum were stained with Diff Quik, a stain that is based upon the May-Grunwald-Giemsa stain but which takes only 15 to 20 seconds to perform and does not require a fume cupboard. No published studies have directly compared Diff Quick to either of the standard stains used.

The aims of this methodology study were: to establish how the author compared to an experienced cell counter (Richard Ward – see acknowledgements), that is inter-observer repeatability; to establish the degree of in-built error associated with cell counting, that is the intra-observer repeatability; to assess how markers of slide quality affect the inter-observer repeatability; and to establish if Diff Quick was a reliable stain for sputum and nasal lavage cytospins.
4.4.2 Methods

i) Inter-observer variability. A random selection of 13 induced sputum slides were chosen and coded. Both cell counters counted all slides blindly. 300 non-squamous cells were counted at high power (x100) under oil emersion to identify macrophages/monocytes, neutrophils, lymphocytes, and eosinophils.

ii) Intra-observer variability. A random selection of 27 sputum and 17 nasal lavage slides were chosen and coded. For the assessment of intra-observer variability the same slides were counted twice at different occasions separated by 2 months in time. Sputum slides were stratified as “high quality” (viability >50% and squamous contamination <20%) and “low quality” (viability <50% and/or squamous contamination >20%). Nasal lavage slides were also stratified as high quality” (viability >50% and squamous contamination <50%) and “low quality” (viability <50% and/or squamous contamination >50%). A higher proportion of squamous cells was accepted in the nasal lavage slides as more of these cells were generally seen.

iii) Variability between stains. The Romanowsky stain was chosen for comparison with Diff Quick as there was local expertise in this stain. A selection of 25 pairs of sputa and 23 pairs of nasal lavage cytospins produced at the same time from the same sample were stained, one with Diff Quick and the other with Romanowsky stain. The same individual (author) counted both sets of slides in random order blinded to the identity of the slides.

Statistical methods. Cell counts were expressed as a percentage of the nonsquamous cells. Eosinophils were not normally distributed and were log-transformed prior to analysis. Inter and intra-observer repeatability was expressed as the intraclass correlation coefficient (ICC) and the
standard deviation (SD) of within and between-observer differences. The latter were also represented graphically (Bland and Altman plots). Comparisons of intra-observer repeatability were made to assess the effect of quality of slides.

4.4.3 Results

i) Inter-observer variability. The inter-observer repeatability in sputum cytospins was good for macrophages and neutrophils but less good for eosinophils and poor for lymphocytes (ICCs = 0.94, 0.97, 0.66, 0.12, respectively, Table 4.1 and Figure 4.3i).

ii) Intra-observer variability. The intra-observer repeatability for high quality sputum cytospins was good for macrophages and neutrophils, and also for eosinophils and lymphocytes (ICCs = 0.99, 0.99, 0.86, 0.81, respectively, Table 4.1 and Figure 4.3.ii). The low quality slides had comparable repeatability for macrophages, neutrophils and eosinophils, but very poor repeatability for lymphocytes (ICCs = 0.98, 0.98, 0.80, -0.1, respectively, Figure 4.3.ii). The intra-observer repeatability for nasal lavage fluid was good for monocytes, neutrophils and eosinophils, but unlike the sputum cytospins, lymphocyte repeatability was also good (ICCs = 0.84, 0.94, 0.91, 0.99, respectively, Figure 4.3ii). There were not sufficient nasal lavage slides of poor quality to perform statistical analysis, although a high squamous count may reduce the repeatability of eosinophils (Figure 4.3ii).

iii) Difference between stains. The variability of cell counts between two slides of cells taken from the same sample but stained with two different stains (Diff Quick and Romanowsky stains, Table 4.2, Figure 4.3iii) was more than seen when repeated counts were done of the same slide stained only with Diff Quick. However, there was still reasonable agreement between the two different stains for macrophages/monocytes, neutrophils and eosinophils in
both induced sputum (ICC = 0.67, 0.67, 0.73, respectively) and nasal lavage (ICC = 0.56, 0.82, 0.88, respectively). Again, there was marked variability in lymphocyte counts.

4.4.4 Discussion

This methodological study has demonstrated that the author produced similar cell counts to an experienced sputum cytologist. It has also demonstrated good repeatability for slides stained with Diff Quick. Diff Quick does not stain eosinophils as well as May-Grünwald-Giemsa or Romanowsky stains, but in practice when slides are counted at high power (x100) under oil immersion, eosinophils are easily recognised. Diff Quick was favoured for this study as immediate access to a fume cupboard was not available (as is required for May-Grünwald-Giemsa or Romanowsky stains) and it is much less time-consuming (15-20 seconds compared to 40 minutes for the Romanowsky stain), a major advantage in a study involving many measurements each day. Although slightly inferior at staining eosinophils, the choice of Diff Quick did not bias the results.

When 2 similar cytospins were stained with the two different stains, the correlation was worse than when the same cytospin was counted twice. There may have been increased variability because two different slides, albeit from the same sample, were being compared. A better comparison would have been to perform repeatability counts on the same slides stained with Romanowsky stain and then compare the ICC obtained with those obtained for Diff Quick stain. However, the Bland and Altman plots (Figure 4.4) do show there was no pattern to the differences between stains with all differences, except for lymphocytes, centering around a mean difference of 0.
This study has demonstrated that slides of lesser quality are still sufficiently clear to allow counting with good repeatability. This goes against the conclusions of Ward and colleagues who recently studied inter-observer repeatability in high quality induced sputum slides (viability >50% and squamous contamination <20%) compared to low quality slides with low viability (<50%) and low quality slides with high squamous cells (>20%)\textsuperscript{296}. Their results suggest that low viability has a minor reduction in repeatability of eosinophil counts (ICC = 0.77 compared to 0.90 for viability > 50%) with little effect on counts of macrophages and neutrophils (ICC for good and poor viabilities >0.90 for both macrophages and neutrophils), whereas high numbers of squamous cells seem to have a marked effect on macrophages and neutrophils counts (ICC = 0.25 and 0.55, respectively). The results from the current study suggest that slides of lower quality can be included but if slides are of poor quality, especially with high numbers of squamous cells, errors in interpreting the cell types are more likely to occur.

4.5 Determining the dilution factor of nasal lavage using inulin

4.5.1 Inulin assay background

Heyrovsky based an inulin assay on the finding that after the hydrolysis of inulin to fructose the addition of β-indolyl-acetic acid gave a qualitative purple colorimetric reaction with fructose in the presence of concentrated hydrochloric acid\textsuperscript{324}. The reaction has high specificity and sensitivity with only fructose and fructose containing saccharides giving comparable reactions\textsuperscript{324}. Interference by glucose is only of the order of 0.5-1.0% and this is important as a high proportion of nasal secretions in healthy people and those with infectious rhinitis contain glucose\textsuperscript{325}. Inulin assays of plasma which contain a relatively high protein content require prior deproteinisation with trichloracetic acid\textsuperscript{324}. This is not required with nasal secretions\textsuperscript{253}. 

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Although using inulin is safe and simple there are also various methodological problems.

i) Inulin is a relatively small molecule and can be absorbed into blood \(^{247}\). It is important to know if the marker is absorbed at a different rate to the diluent to avoid errors in the estimation of the volume of nasal secretion recovered.

ii) The amount of inulin recovered could be affected by the permeability of the nasal mucosa. This may vary from individual to individual and will also vary during a common cold infection. Again this might lead to inaccurate estimates of the volume at the peak of infection.

iii) Inulin is quickly digested by bacteria and so must be assayed or appropriately stored soon after collection.

iv) The use of inulin has been criticised by having a high coefficient of variation when its concentration is measured \(^{247}\). However, these criticisms are based on a study of renal clearance in 1949 which states that “significant variations in kidney function occur....” and that individual variations of kidney function lead to the high coefficient of variation. Use of inulin as an exogenous marker would not have this problem. Also, more recent, accurate and reliable methods of analysis have now been validated \(^{253}\).

v) Inulin has also been assessed as a marker of dilution in BAL. A study using rats found it to be “useless” as it gave some negative volumes of alveolar fluid when sampled immediately \(^{326}\). Allowing the inulin plus diluent to stay in the lung for 1 minute increased these negative estimates of fluid volume. The authors could not explain this phenomenon but suggested an influx of a sugar compound into the alveolar fluid was leading to an overestimation in the inulin fructose assay. This has not yet been proven and more recent developments in inulin assay have not encountered such a problem \(^{253}\). For inulin to lead to the estimate of a negative volume of lung secretions in BAL the concentration of inulin
must have increased (the authors do not give the raw data), that is the diluent was being rapidly absorbed at much faster rate than the inulin. Alternatively, these rather odd results could have resulted from a problem with the assay. A study comparing inulin with methylene blue in lung lavage of dogs and showed a high degree of correlation \((r=0.91)\) in secretion volume estimation without estimates of negative volumes \(^{272}\).

The exact extent to which inulin is absorbed is not known but two studies suggest that it is not likely to be as high as some suggest \(^{272,327}\). As already stated, a comparison of inulin with methylene blue in lung lavage of dogs showed a high degree of correlation \((r = 0.91)\) and pathological examination of the lungs showed that the dye had not been absorbed \(^{272}\). The effect of rapid breakdown by bacteria over different time scales has not been studied. However, this can be minimised by rapid cooling of the sample on ice and appropriate storage in a freezer prior to assay.

Balfour-Lynn compared inulin and urea as markers of dilution in nasal lavage finding that urea tended to underestimate the dilution by a factor of 2.9 with no correlation between the two methods \(^{327}\). Reliability was assessed by assaying for inulin in duplicate samples. The mean of the differences between samples was 1.9\% with a SEM of 0.2 \% showing a high degree of reliability \(^{253}\).

Inulin as a marker of dilution meets the requirements of the present study for the following reasons:

- it is a safe substance;
- it has a low absorption through distal airways in bronchial lavage (ie/ very little is lost);
- it is as reliable as methylene blue in BAL and more reliable than urea in nasal wash;
it is cheap and readily available;

the assay is highly repeatability.

4.5.2 Inulin assay

The inulin assay was based upon that used by Balfour-Lynn. The nasal wash solution was prepared by adding inulin biochemical reagent (BDH, Merck Ltd, Leicester, UK) to PBS at a concentration of 450 μg/ml. Fresh batches were made up every 2-3 weeks and stored at 4°C. The inulin concentration was measured in each individual batch after storage at the same time as the lavage fluid assay.

Inulin standard was made by dissolving 20 mg of inulin in 15 ml of distilled water, which was warmed until clear and then made up to 10 ml. Inulin is super saturated in water at room temperature hence the need for warming to produce a homogenous solution. A fresh stock solution was made for each assay and was diluted to working standards of 300, 250, 200, 150, 100, 50, and 25 μg/ml. (Inulin concentrations above 350 μg/ml produce a colour change above the spectrophotometer range).

0.5%(w/v) 4indol-3yl-acetic acid was prepared by adding 500 mg of the white crystalline form (BDH) to 100 ml of 96% ethyl alcohol (AnalaR), which was then vortex mixed until fully dissolved. This was stored at 4°C.

Aliquots of nasal lavage solution and nasal wash solution were removed from storage at -70°C and assayed in duplicate to account for the effects of any degradation during storage. Samples were diluted 3 fold by adding 200 μl to 400 μl of distilled water to bring the concentrations
within the range of the standards. Labelled screw-capped glass tubes were used to mix the solutions as followed: 250μl standard or dilute sample/wash + 50μl 0.5g% 4indol-4yl-acetic acid + 2mls concentrated hydrochloric acid (SG 1.18 AnalaR). Blanks were prepared as followed: 500μl distilled water + 50μl 0.5%(w/v) 4indol-3yl-acetic acid + 2mls concentrated hydrochloric acid. The tubes were vortex mixed then incubated at 37°C for 85 minutes before cooling in tap water for 30 minutes. One ml of sample or standard was transferred to a cuvette (Acryl-cuvette, Sarstedt) with 2 cuvettes for blanks. The optical density of standards and samples was then read at 530nm in a Unicam SP1800 UV spectrophotometer, which is first zeroed using the 2 blanks. The standards were read first to create a linear standard curve. The inulin concentrations of the samples and wash solutions were then read taking into account the initial 3 fold dilutions.

4.5.3 Calculating the dilution factor

The following were known from the assay results:

initial inulin concentration in nasal wash (i.e. pre-lavage);

inulin concentration in nasal lavage fluid (i.e. post-lavage);

concentration of the substance measured (e.g. interleukin).

By calculating the dilution of inulin by the nasal secretions, we can calculate the dilution factor (DF) of the substance measured (S) in the nasal lavage fluid (see Figure 4.2). We can thus calculate the true concentration of S in the undiluted nasal secretions using the following formula:

\[
\text{DF of inulin} = \frac{\text{concentration of inulin in nasal lavage fluid}}{\text{initial concentration of inulin in nasal wash}}
\]

\[
\text{Proportion of nasal lavage fluid consisting of secretions} = 1 - \text{DF}
\]
Concentration of S in nasal secretions = \frac{\text{Concentration of S in nasal lavage fluid}}{1 - DF}

4.6 Assays for fluid-phase markers

The following assays were all performed by laboratory technicians (Melanie Leech and Alyson Huntley, see acknowledgements) under the supervision of Dr Colin Hewitt and the author. All were based on standard commercial assays.

4.6.1 ELISA for IFN-\(\gamma\), IL-8, IL-5, TNF-\(\alpha\)

IL-8, IL-5, IFNy and TNF-\(\alpha\) were measured by enzyme immunoassay (OptEIA kits from PharMingen, San Diego, CA, USA) according to manufacturer's instructions. The assays were sandwich-ELISAs designed to specifically detect and quantify the concentration of soluble cytokine and chemokine proteins.

i) Assay technique. The basic sandwich ELISA method (Figure 4.4) makes use of purified anti-cytokine/chemokine antibodies (capture antibodies) that are non-covalently adsorbed ('coated' - primarily as a result of hydrophobic interactions) onto plastic microwell plates. After plate washings, the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples that were applied to the plate. After washing away unbound material, the captured cytokine/chemokine proteins were detected by biotin-conjugated anti-cytokine/chemokine antibodies (detection antibodies) followed by an enzyme-labeled avidin stage. Following the addition of a chromogenic substrate-containing solution, the level of coloured product generated by the bound, enzyme-linked detection reagents was measured spectrophotometrically using an ELISA-plate reader (Dynatech Revalation 2.0, Gemini
Scientific, Southampton, UK) at an appropriate optical density (OD). By including serial
dilutions of a standard cytokine protein solution of known concentration, standard curves were
calculated from which the concentrations of the cytokine/chemokine was interpolated. The line
of best fit was calculated for each standard curve using Microsoft Excel software and the
'goodness of fit' represented as the $r^2$ value (0 to 1.0, a value greater then 0.9 being
acceptable). The details of the method are shown in appendix 2.3. The dilution factor for nasal
measurements was taken into account so that all nasal fluid-phase measurements were
expressed per ml of nasal secretions.

The sensitivity of these tests were: 1 pg/ml, 2 pg/ml, 4 pg/ml, and 2 pg/ml for IFN-$\gamma$, IL-8, IL-5,
and TNF-$\alpha$ respectively.

**ii) Validation and precision.** This technique is a widely used for the quantification of
cytokines/chemokines in a wide range of biological materials. Additional validation was
required for the effect of adding the mucolytic compound DTT to the samples during
processing as DTT breaks disulphide bonds and it was possible for this to interfere with assays
of proteins. An assessment of precision of our results was required to account for intra-assay
and inter-assay variability.

*Intra-assay variability* was determined by comparison of duplicate samples. Samples were only
included if they lay within 10% of each other. The mean for the 2 samples was then used as the
result. Thirteen and 1 out of 159 possible samples assayed for IL-8 and IFN-$\gamma$, respectively, had
greater than 10% difference between duplicates. These were repeated in subsequent assays and
the difference was less than 10% in all. All duplicate assays of IL-5, and TNF-α were within 10%.

*Inter-assay variability* was accounted for by assays of different dilutions of cytokine/chemokine protein standards on each individual assay plate. The different dilutions were assayed in duplicate with the means of the duplicates used to construct a standard curve for each assay from which the results of the samples were read. In this way each sample results were related to the same dilutions of standard protein.

*DTT.* To determine whether DTT interfered with the assays recombinant cytokine with and without the addition of DTT were compared. The supernatant of 3 samples of nasal lavage were each spiked with the recombinant standard IFN-γ, IL-8, IL-5, and TNF-α proteins from the OptEIA kits to a concentration of 500pg/ml each. The supernatants were split and DTT added to half the samples to a concentration of 0.08%. The same volume of PBS was added to the other half of the samples and both were then assayed for each protein as described above. Standard curves were calculated for each protein assay (correlation coefficient \((r^2) = 0.99\) for IFN-γ, IL-8, IL-5, and TNF-α). The samples with added DTT resulted in slightly higher values of IL-8 and IFN-γ, and similar values of IL-5, and TNF-α (Table 4.3). These differences were consistent and trivial, therefore DTT was shown not to interfere significantly with the ELISA assay.

### 4.6.2 UniCAP enzyme immunoassay for ECP

ECP was measured by fluoroenzyme assay (UniCAP system, Pharmacia, Uppsala, Sweden) according to manufacturer’s instructions. The enzyme assay followed similar principles as the
ELISA described above. Anti-ECP immunoCAP antibody was added to samples where it bound ECP within the samples. After washing, enzyme labelled antibodies against ECP were added to form a complex. Bound complex was then incubated with a fluorescent developing agent. Fluorescence was measured on a UniCAP 100 automated reader with fluorescence being directly proportional to the concentration of ECP in the sample. The details of the method are shown in appendix 2.4.

The sensitivity of this assay is 2 pg/ml.

Validation and precision. This assay has been widely used and has been validated by its successful use in assessing ECP in health and diseases such as asthma\textsuperscript{38,303} infantile wheeze\textsuperscript{328} and allergic rhinitis\textsuperscript{329}. Assay precision and calibration was assessed according to the manufacturer's instructions (Appendix 2.5). All samples were assayed in duplicate and only included if they lay within 10\% of each other. The mean for the 2 samples was then used as the result.

4.7 Identification of virus in LRT samples

The aim was to investigate whether HCoV was capable of infecting the LRT and if so, if this accounted for those who wheezed with this common cold. Two different approaches were initially used. Firstly, an attempt was made to identify HCoV RNA within bronchial biopsy cells by \textit{in situ} hybridisation. Secondly, samples of induced sputum were analysed by RT-PCR for virus RNA. The attempt by \textit{in situ} hybridisation was unsuccessful and hence, RNA from the biopsies was extracted and identified using RT-PCR and Southern blotting.
4.7.1 Biopsy processing

Bronchial biopsies were processed according to published guidelines \(^{330,331}\). Biopsies were placed immediately into formol saline (containing 10% formalin, see appendix 2.7) for fixation. After 20 to 24 hours the formol saline was decanted and 99% industrial methylated spirit (Analar) was added. Biopsies were processed by standard methods into paraffin wax by the Department of Pathology, Glenfield Hospital, Leicester. The wax embedded tissue was stored at room temperature until required.

4.7.2 Methodology development

The initial aim was to identify HCoV RNA within biopsy cells by \textit{in situ} hybridisation. This requires adequate fixation and treatment with proteinase K to open the cells and allow access of probes that are designed specifically with the purpose of binding to HCoV RNA. The development of this methodology was done by the author under the supervision of Dr Howard Pringle (see acknowledgements) and involved the following steps:

- digoxigenin labelling and production of a ‘probe cocktail’ and testing of the ‘probe cocktail’;
- determining the optimal conditions for \textit{in situ} hybridisation;
- preparation and testing of a positive control sample.

It was necessary to achieve the above before attempting to identify HCoV by \textit{in situ} hybridisation in the bronchial biopsy samples.

\textit{i) Preparation and testing of a ‘probe cocktail’}

Four ‘oligo-probes’ were designed complimentary to the N-gene of HCoV 229E published on the NCBI database \(^{332,333}\). Primers were designed as detailed in appendix 2.2 and manufactured
commercially (Operon Technologies, CA, USA). Probe targets are given in appendix 2.2. A cocktail of the probe was produced to a concentration of 20ng/μl and stored in 200ng aliquots at −20°C until required (see appendix 2.7). The probes were labelled with digoxigenin (see appendix 2.7). The effectiveness of the labelling procedure was checked by performing a 'test strip' in which serial dilutions of digoxigenin-labelled probe were detected by an anti-digoxigenin-alkaline phosphatase antibody conjugate that produces a colour change when mixed with a detection system (NBT/BCIP = nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl-phosphate (Sigma-Aldrich) (see appendix 2.7).

The test strips showed adequate labelling by detecting the digoxigenin-labelled probes down to a concentration of 1 pg/μl.

ii) Determining the optimal conditions for in situ hybridisation.

The aim of this experiment was to determine the optimal concentration of proteinase K to treat the biopsies. Under-treatment would not allow probe access into the cells and over-treatment would mean tissue destruction. In order to achieve this in situ hybridisation was performed on slices of two bronchial biopsies using the mitochondrial RNA probe ‘C-21’.

Methods. C-21 probe was kindly donated by Dr Pringle at a concentration of 20ng/μl. The probe was and labelled with digoxigenin in the same way as the probe cocktail and a test strip run to determine its activity (see appendix 2.7). The probe was found to be active to a concentration of 1 pg/μl. Two processed bronchial biopsies were sliced to a thickness 5-micron and slide-mounted. Six slides from each biopsy were prepared. The slides were de-waxed by immersion in xylene for 5 minutes followed by alcohol dehydration (rinses with 99%, 99% and 95% industrial methylated spirit followed by DEPC H2O). The slides were transferred to a
'hybridisation box', a flat blackened perspex container in which slides can be mounted. The hybridisation box was kept moist by placing tissues soaked in TBS buffer (see appendix 2.7, footnote d) in the bottom. 100μl of proteinase K (diluted to 0.5, 1 and 2 μg/ml in 0.05M Tris pH 7.65) was pipetted onto the six slides from each biopsy so that two were each covered with a different concentration of proteinase K. The slides were then incubated at 37°C for 30 minutes. After 2 five minute washes with DEPC-treated water at 4°C the slides were covered with 100μl of 'Prehybridisation Solution' (see appendix 2.8) and incubated at 37°C for 1 hour. 30μl of C21 probe was added to one slide at each proteinase K concentration whilst the other was used as a ‘no hybridisation’ control. The slides were cover-slipped and incubated overnight at 37°C.

After overnight incubation with the probe the slides were treated with a series of ‘post-hybridisation’ washes prior to detection. Firstly the slides were washed for 10 minutes twice with 2x SSC (sodium citrate, sodium chloride) /30% formamide (see appendix 2.8) at 37°C. This was followed by a 10 minute wash with Blocking Solution (see appendix 2.7, footnote d) at room temperature. 100μl of anti-digoxigenin-alkaline phosphatase antibody conjugate (diluted 1:600 in Blocking Solution) was then pipetted onto the tissue and left for 30 minutes at room temperature. This was followed by 2 five minute washes with TBS buffer (see appendix 2.7, footnote d) and 1 five minute wash with sterile ultrapure H2O. Prior to detection ‘Substrate Buffer’ was added for 5 minutes (see appendix 2.7, footnote e). The slides were then incubated in the dark with 200μl of ‘Substrate Solution’ (see appendix 2.8, footnote f) for up to 2 hours.

**Results.** The slides were scored blindly by the author in terms of stain quality and tissue structure (Table 4.4). *In situ* hybridisation was successful at all three concentrations of
proteinase K tried, the best results being at 0.5 µg/ml. Tissue morphology was also marginally superior at the lower proteinase K. Figure 4.5 shows in situ hybridisation at a proteinase concentration of 0.5µg/ml.

**Conclusions.** Treatment of bronchial biopsies with proteinase K at 0.5 µg/ml for 30 minutes is satisfactory for in situ hybridisation.

**iii) Preparation and testing of a positive control sample.**

The aim of these experiments was to find a suitable positive control material that could: i) prove the probe cocktail was able to hybridise and generate satisfactory signal; ii) be used in in situ hybridisation assays of bronchial biopsies as a positive control to compare to. Cell cultures infected with HCoV fulfilled the requirement of having HCoV RNA within cells and were readily available. These were chosen as the positive controls.

**Methods.** HEL cells infected with HCoV (see Chapter 2.1) were harvested and cytocentrifuged into cytoblocks (Shandon) (see appendix 2.9). These blocks were processed in exactly the same way as the biopsies. Briefly, this included processing into wax blocks, preparation of 5-micron samples on slides, de-waxing, digestion with 0.5 µg/ml proteinase K, hybridisation with the digoxigenin-labelled oligo-probe cocktail (appendix 2.2, Figure 4.6) and detection.

**Results.** No signal could be obtained with the oligo-probe cocktail. A range of experiments was conducted to try and produce a successful assay. This included a range of proteinase K concentrations (0.5, 1.0 and 2.0 µg/ml), repeating experiments after checking the digoxigenin label (a repeat test strip gave signal between 1 and 10 pg/µl) and repeating the experiments
with freshly prepared probes (the repeat experiment was carried out by Dr Leon Hall – see acknowledgements). None of these steps resulted in a positive in situ signal in cells known to have large numbers of virus particles (the cell supernatant was titred to $10^4$ TCID$_{50}$ ml$^{-1}$).

Conclusions. Despite rigorous attempts to identify HCoV RNA within cells, no positive controls were established. This was an essential step in developing a HCoV in situ hybridisation assay and therefore this assay was not performed on the bronchial biopsies. An alternative strategy was pursued. This included the identification of HCoV RNA within induced sputum together with extraction of RNA from the biopsies allowing RT-PCR and Southern blotting to identify any HCoV RNA present.

4.7.3 Extraction of RNA from bronchial biopsies

The aim of this experiment was to extract sufficient RNA from slices of bronchial biopsies that had been processed into wax blocks in order to look for HCoV 229E RNA. These techniques were already established in Dr Pringle’s laboratory and performed by the author under his supervision (see acknowledgements).

Methods. The best biopsies from each volunteer were selected after staining a single slide-mounted 5-micron slice with Haematoxylin and Eosin (see appendix 2.10). Biopsies included the 10 subjects (8 viral wheezers and 2 controls) who were bronchoscoped on day 4 of the cold and the 5 subjects (4 viral wheezers and 1 control) who had a repeat bronchoscope 6-8 weeks later. The positive control was a cytoblock of cell cultures infected with HCoV (see 4.7.2 iii). Sterile Eppendorf tubes and pipette tips were used throughout.
Ten 5-micron slices of biopsy were placed into Eppendorf tubes and dewaxed as already described (see 4.7.2ii). The pellets were vacuum dried for 10-15 minutes, resuspended in 50mM Tris (pH 7.63) containing proteinase K at 0.5mg/ml. 10 µl of sodium dodecyl sulphate (SDS, Sigma Aldrich – L-4509) was added prior to incubation at 37°C overnight.

RNA extraction utilised a commercially available tri-reagent (Trizol, Sigma Aldrich Aldrich) system. After incubation, 1ml of tri-reagent was added to each Eppendorph and allowed to stand for 5 minutes at room temperature. 200µl chloroform was then added and vortex-mixed for 15 seconds before standing for 2-3 minutes at room temperature. Centrifugation followed at 13,000 rpm for 15 minutes at 4°C after which the samples separated into 3 phases. The bottom phase contained protein, the inter-phase contained DNA and the upper aqueous phase contained RNA. The upper phase was transferred to a clean Eppendorf to which was added 0.5ml of isopropanol. This mixture was incubated for 10 minutes at room temperature and then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was removed to reveal a small RNA pellet that was washed in 1-1.5ml of 75% ethanol/DEPC-treated H2O at room temperature. After centrifuging at 13,000 rpm for 15 minutes at 4°C the pellet was vacuum dried for 5-10 minutes and resuspended in 25µl of DEPC H2O. The suspension was stored at -20°C until required.

4.7.4 Identification of HCoV RNA in bronchial biopsies

The aim of this experiment was to assess whether any of the extracted RNA from the biopsies was from HCoV 229E, indicating cellular infection. As a control for any negative samples that could have represented poor RNA extraction, GAPDH (glyceraldehyde phosphate dehydrogenase), an enzyme ubiquitous to eukaryotic cells, was also assayed for by RT-PCR.
These techniques were already established in Dr Pringle’s laboratory and performed by the author under his supervision (see acknowledgements).

Methods.

Reverse priming. Prior to the reverse transcriptase reaction, total RNA was primed with the reverse primer (either HCoV or GAPDH, see appendix 2.2). A super-mix, prepared by multiplying the volume of a particular component required for an individual reverse transcription mix by the number of reverse transcription mixes required (one reverse transcription mix being required for each specimen to be reverse transcribed), containing 15 pmoles of reverse primer (1.5μl), 5μl RNA and 3.5μl DEPC H₂O per reaction was heated to 70°C for 5 minutes in a PCR machine (Hybaid, OmniGene). The samples were then cooled to room temperature prior to reverse transcription. Samples were run in duplicate and a positive sample consisting of RNA extracted from cell cultures infected with HCoV was included in each assay. At all times the positive control samples were processed at a separate bench to reduce the risk of contamination.

Reverse transcription (RT) reaction. Initially, a reverse transcription super-mix was made with the following components: 5μl AMV 5xRT buffer (10μg/ml, Promega, Southampton, UK), 2.5μl of 10mM dNTP’s, 0.62μl RNAsin (25 units), 6.38 μl DEPC H₂O and 0.5μl of either AMV-RT (5 units, Promega) or DEPC H₂O. Samples using the former super-mix were labelled as ‘RT+’ whereas those using the latter were labelled as ‘RT-’ and used as internal controls for contamination during the PCR. Each duplicate sample had both RT+ and RT- mixes. The total volume of mix per sample (15μl) was then added to the 10μl primed RNA (total volume 15μl)
and incubated at 42°C for 1 hour. This activated the RT enzyme to produce cDNA. The samples were stored at 4°C until required.

**PCR methodology development:**

A. **Single-round PCR.** A super-mix was made so that each reaction contained 48 μl consisting of: 5 μl of 10x AJ buffer (see appendix 2.12), 1 μl of forward primer for HCoV or GAPDH (see appendix 2.2), 1 μl of reverse primer for HCoV or GAPDH (see appendix 2.2) and 41 μl of ultrapure H2O. To Eppendorf tubes containing 48 μl of this super-mix was added 2 μl of cDNA in tubes labelled RT- and RT+ according to whether RT or H2O had been added earlier. A H2O negative control was used for both HCoV and GAPDH, and positive controls were RNA extracted from cell cultures infected with HCoV and tonsil tissue for the HCoV and GAPDH assays, respectively. 50 μl of mineral oil was added to each tube to seal the reaction and the tubes then underwent a 'hot-start' PCR. Cycles (Hybaid thermal cycler) consisted of: denaturation at 98°C for 3 minutes, holding at 60°C pending the addition of 1 μl Taq polymerase (diluted 1 in 5 in AJ buffer), and then 40 cycles of primer extension at 72°C followed by denaturation at 94°C and annealing at either 58°C (GAPDH) or 60°C (HCoV 229E) for 30 seconds each. Samples were then put on ice until agarose gel electrophoresis.

**Horizontal agarose gel electrophoresis.** cDNA detection was by agarose gel electrophoresis as described in chapter 4.2.1 iv.

No bands of cDNA corresponding to HCoV were detected using this 40 cycle PCR except for a strong band corresponding to RNA from infected cultured cells (Figure 4.7a). This indicated that the PCR worked but that this was not sufficiently sensitive to identify RNA in the biopsies.
RT-PCR for GAPDH in the biopsies revealed a clear band for the infected cultured cells supporting adequate RNA extraction. However, only one biopsy sample had a clear band (Figure 4.7b) suggesting that either small or no RNA was extracted from the biopsies.

In order to attempt to look for very small quantities of HCoV 229E RNA in the biopsies whilst maintaining a high degree of specificity, the methodology was developed further utilising a nested RT-PCR to multiply viral RNA followed by Southern blotting to confirm the origin of any RNA detected.

B. Hemi-nested PCR for HCoV 229E. Nested PCR involves two pairs of primers (one forward and one reverse primer in each pair) and a hemi-nested PCR describes a two-round PCR where there is a common primer in each round. The first round PCR uses the ‘external’ primer pair (primers ‘7 and 8’, see appendix 2.2) that produces a product on which the ‘internal’ pair (primers ‘5 and 8’, see appendix 2.2) can be used to increase the number of cycles and product. Specificity is maintained as more specific primers are used (in this case 3). The principle of this PCR is illustrated in figure 4.8.

The nested PCR used the stored cDNA already prepared. The first round was very similar to the single-round PCR. A super-mix was made so that each reaction contained 48 μl consisting of: 5μl of 10x AJ buffer (see appendix 2.12), 1μl of forward primer for HCoV (primer 7 - see appendix 2.2), 1μl of reverse primer for HCoV (primer 8 - see appendix 2.2) and 40μl of ultrapure H₂O. To Eppendorf tubes containing 48μl of this super-mix was added 3 μl of cDNA in tubes labelled RT- and RT+ according to whether RT or H₂O had been added earlier. 50μl of
mineral oil was added to each tube to seal the reaction and the tubes then underwent a 'hot-start' PCR. Cycles were slightly different to the single-round PCR and consisted of: denaturation at 98°C for 3 minutes, holding at 60°C pending the addition of 1μl Taq polymerase (diluted 1 in 5 in AJ buffer), and then 4 cycles of primer extension at 72°C (30 seconds) followed by denaturation at 94°C (1 minute) and annealing at either 60°C (30 seconds). Following this 25 cycles of primer extension at 72°C followed by denaturation at 94°C and annealing at 60°C each for 30 seconds completed the first round (total cycles = 30).

1μl of first-round product was carried over to the second round, which included the same reaction mixtures (the 2μl deficit in cDNA being made up by sterile ultrapure H2O). A further 30 cycles as described above were conducted (results are shown in the results section).

*Southern blotting for HCoV 229E*:

This method was used to prove the nested RT-PCR products were indeed derived from HCoV 229E. This process involved the transfer of PCR product from gel to nitrocellulose paper, labelling the PCR products with internal probes (optimised for stringency and concentration), and detection using the ultra-sensitive chemiluminescent kit (CDP-Star, Boehringer-Mannheim, Cat. No. 1685 627).

*A. Bonding of HCoV 229E nested RT-PCR products to Southern blot membranes.* The covalent attachment of DNA from the RT-PCR products to the Southern blot membrane and subsequent specific detection of bound HCoV 229E product was based upon the method detailed in the manufacturer’s information (Nylon Membranes, positively charged, Boehringer-Mannheim, Cat. No. 1209 272).
The agarose gel loaded with nested RT-PCR product was rinsed with sterile ultrapure H$_2$O then treated with 'Denaturation Solution' (1.5M NaCl, 0.5M NaOH) for 2 x 30 minutes on a plate shaker. After rinsing with sterile ultrapure H$_2$O the gel was treated with 'Neutralising Solution' (1.5M NaCl, 1M Tris pH 8.0) for 60 minutes on a plate shaker. This process fragments large DNA molecules facilitating transfer onto the membrane. After a further rinse in sterile ultrapure H$_2$O the gel was ready for blotting.

Blotting involved drawing 20 x SSC (see appendix 2.8) using a filter paper wick through the gel, carrying the DNA products onto nitrocellulose paper (Hybond N, Amersham). A Perspex sheet was placed across a large container holding 20 x SSC. Two sheets of 3MM filter paper were laid across the Perspex sheets with either ends soaked in the 20 x SSC. Care was taken to ensure no air-bubbles were trapped between the soaked filter-paper and the Perspex sheet. The filter paper was covered with Saran Wrap, air-bubbles were removed and a rectangle, the size of the gel was cut in the Saran. The gel was transferred onto the hole cut in the Saran. A rectangle of nitrocellulose paper was cut between two sheets of 3MM filter paper (to avoid contact with grease) to measure the same size as the gel and transferred using forceps onto the gel (corners of nitrocellulose and gel were cut so as to mark the orientation of the membrane). The two pieces of 3MM cut with the nitrocellulose were soaked in 20 x SSC and placed on top of the membrane. 1-3 inches of paper towels were placed upon this followed by a second perspex sheet and a 1 kg weight. Damp towels were changed every 5 minutes for the first 30 minutes and then every 15 minutes for 1½ hours and then left overnight. The nitrocellulose paper, with DNA transferred to the underside, was removed, placed between two further pieces of 3MM paper and baked at 65°C for 10 minutes. The membrane was Saran wrapped and the DNA cross-linked to the membrane by exposure to ultraviolet light for 30 seconds. The membrane, with protective filter paper, was stored at room temperature. To check for
successful transfer of DNA, the gel was stained with ethidium bromide and viewed under ultraviolet light.

B. Hybridisation with digoxigenin-labelled probes

i. Probe labelling. A cocktail of two internal probes (see appendix 2.2) was labelled with digoxigenin as described in section 4.7.2 and appendix 2.7. Efficacy of labelling was checked by running a test strip (appendix 2.7) with labelling detected down to 1pg/ml.

ii. Dot-blots to optimise stringency and probe concentration. The aims of this methodology experiment were to determine the concentration of formamide to add to the hybridisation mixture for optimal stringency and the optimal concentration of probe to use. Reagents used were according to manufacturer’s instructions (Nylon Membranes, positively charged, Boehringer-Mannheim, Cat. No. 1209 272). The principle of this experiment was to blot two dots of PCR product DNA, one being HCoV 229E the other being GAPDH, onto several pieces of 2cm² pieces of nitrocellulose membrane and subject the blots to different concentrations of probe cocktail at different concentrations of formamide.

Nitrocellulose blots were prepared as follows. RT-PCR product was taken from the cultured cells infected with HCoV 229E with the control taken from the RT-PCR for GAPDH on tonsil tissue. Both these samples gave strongly positive PCR signals. 5µl of each PCR product was spotted onto nitrocellulose (Hybond N) and air-dried. After baking at 65°C for 10 minutes and wrapping in Saran, the DNA was cross-linked to the nitrocellulose by exposure to ultraviolet light for 30 seconds.

Hybridisation with digoxigenin-labelled probes was as follows. Formamide was added to hybridisation buffer (see appendix 2.12) to make a 10%, 20% and 30% solution. 2 x 15 mls of
each were added to 6 well culture plates to which was added the HCoV 229E probe cocktail or a digoxigenin-labelled GAPDH internal probe (donated by Dr Pringle) to the following concentrations:

<table>
<thead>
<tr>
<th>HCoV 229E probe</th>
<th>GAPDH probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ng/ml</td>
<td>10ng/ml</td>
</tr>
<tr>
<td>10% formamide</td>
<td></td>
</tr>
<tr>
<td>20% formamide</td>
<td></td>
</tr>
<tr>
<td>30% formamide</td>
<td>30% formamide</td>
</tr>
</tbody>
</table>

Identical blots containing the two PCR product dots were placed in the different solutions (pre-warmed to 37°C) at 37°C for 1 hour after which the correct concentration of probe was added. The dot-blots were then ready for detection.

**Results - optimal stringency and probe concentration.** Optimal stringency for the HCoV probe cocktail was a 10% formamide solution with optimal probe concentration of 5ng/ml (see Figure 4.9).

*C. Detection of probe-labelled DNA bound to nitrocellulose.*
Detection followed the manufacturer’s instructions (Nylon Membranes, positively charged, Boehringer-Manheim, Cat. No. 1209 272). Briefly, the nitrocellulose filters were washed for 1 minute in buffer 1 (appendix 2.12) and incubated for 30 minutes in 100ml of buffer 2 (appendix 2.12). Anti-digoxigenin-alkaline phosphatase antibody was diluted to 150mU/ml in buffer 2 and pipetted on top of the filters where it was left to incubate for 30 minutes on a gentle rocker (a volume of ~20ml of dilute antibody was required for each filter). Unbound antibody was removed with 2 washes in 100 ml of buffer 1 for 15 minutes each. The membranes were then equilibrated in 20ml buffer 3 (appendix 2.12) for 2 minutes. Detection then followed using the ultra-sensitive chemiluminescent kit (CDP-Star, Boehringer-Mannheim, Cat. No. 1685 627). 10ml CDP-Star diluted 1:250 in buffer 3 was pipetted onto the membrane ensuring that the entire filter was covered. This remained for 5 minutes with care to avoid any drying out. Excess liquid was allowed to drip off and the moist membrane sealed in Saran wrap. The membrane was then exposed to x-ray film under conditions of total darkness for between 1 and 5 minutes and the film processed to depict the results.

4.7.5 Identification of HCoV RNA in induced sputum

The final method of identifying HCoV in LRT secretions was to look for virus RNA in the induced sputum. Small (5-10μl) aliquots of induced sputum plug were stored at −80°C. Aliquots were separated prior to sputum processing and stored immediately. RT-PCR for virus genome followed the method detailed in chapter 4.2.1. As such small samples were being analysed it was necessary to confirm successful RNA extraction by performing GAPDH positive control RT-PCR on all samples first (see chapter 4.7.4).
4.8 Figures and tables

Figure 4.1 An illustration of sputum plug selection.

Remains of induced sputum after plug selection consist mainly of saliva.

Selected plugs mixed together
Figure 4.2 An illustration of measurements taken for the estimation of inulin dilution.

\[ \text{Nasal mucosa} \quad \rightarrow \quad \text{Nasal lining fluid} \]

\( S \) = substance to be measured.
\( \{S\} \) = concentration of substance in nasal secretions.
\( \{\text{nw}\} \) = concentration of inulin in nasal wash.
\( \{S\} + \{\text{In}\}_{\text{NALF}} \) = concentration of substance and inulin in nasal lavage fluid

DF of inulin = \[ \frac{\{\text{In}\}_{\text{NALF}}}{\{\text{In}\}_{\text{nw}}} \]

Proportion of NALF consisting of secretions = \( 1 - \text{DF} \)

\[ \{S\} \text{ in nasal secretions} = \frac{\{S\}_{\text{NALF}}}{1 - \text{DF}} \]
Figure 4.3i Bland and Altman plots of inter-observer repeatability – showing the mean difference between cell differential counts (%) plotted against the mean. Mean difference (middle line) +/- 2 standard deviations (outer lines) are shown. Eosinophils are plotted on a log\(_{10}\) scale.
Figure 4.3ii Bland and Altman plots of intra-observer repeatability of sputum cell counts of the same Diff Quick stained slide counted twice by the author.

(a) Sputum

Slide quality: ■ = “high quality” (viability >50% and squamous contamination <20%)
□ = “low quality” (viability <50% and/or squamous contamination >20%).
Repeatability of nasal lavage cell counts of the same Diff Quick stained slide counted twice by the author.

Slide quality: ■ = "high quality" (viability >50% and squamous contamination <50%)
□ = "low quality" (viability <50% and/or squamous contamination >50%).
Figure 4.3iii  Bland and Altman plots of repeatability of cell counts from slides of the same sample stained with Diff Quick and Romanowsky stain.

(a) Sputum
Difference in lymphocyte differential counts %

Difference in eosinophil differential counts %

Difference in %monocyte differential counts %

Difference in neutrophil differential counts %

Mean lymphocyte differential count %

Mean eosinophil differential count %

Mean monocyte differential count %

Mean neutrophil differential count %
Figure 4.4  Basic stages of the sandwich ELISA for measuring soluble cytokine and chemokine protein levels.

1. CAPTURE ANTIBODY

Coat well with anti-creatine kinase capture antibody

2. BLOCKING

Block unoccupied well sites with protein

3. CYTOKINE/CHEMOKINE + SAMPLES

Add recombinant cytokine/chemokine protein or samples

4. DETECTOR ANTIBODY

Add biotinylated ant-cytokine/chemokine detector antibody.

5. ENZYME-AVIDIN THEN SUBSTRATE

a. Add enzyme-avidin.

b. Develop with substrate.

□ = blocking protein

○ = cytokine/chemokine protein or samples

■ = enzyme-avidin

\( \Rightarrow \) = antibody
Figure 4.5  A section of bronchial biopsy with the mitochondrial RNA probe ‘C21’ applied by *in situ* hybridisation after the biopsy was processed with proteinase K at a concentration of 0.5μg/ml.

Haematoxylin and Eosin stain of a bronchial biopsy.

*In situ* staining with the C21 probe
Figure 4.6 A schematic of probe positions used for \textit{in situ} hybridisation against the N-gene of HCoV 229E.

\begin{center}
\begin{tabular}{cccc}
911-930 & 968-987 & 991-1010 & 1087-1106 \\
A & B & C & D \\
\end{tabular}
\end{center}

N-gene \hfill 5' \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill \hfill 3'
Figure 4.7  Results of single-round RT-PCR for (a) HCoV 229E and (b) GAPDH I bronchial biopsies.

a. Half the biopsies are shown on this gel (RT+RT-in consecutive tracks). The positive control (RNA extracted from infected cultured cells) is positive for HCoV 229E. None of the biopsies were positive for HCoV 229E.

b. Half the biopsies are shown on this gel (RT+RT-in consecutive tracks). The positive control (RNA extracted from tonsil tissue) is positive for GAPDH. Only one (convalescent biopsy on volunteer 11) was positive for GAPDH.
Figure 4.8  A schematic of the hemi-nested RT-PCR used for the identification of HCoV 229E.

Virus RNA

PRIMER POSITION

5' ---------------------------------- 3'  
44 0647  1200-1220

dNTPs + Reverse Transcriptase

5' ---------------------------------- 3'  
3' ---------------------------------- 5'

1ST ROUND PCR

Denaturation

5' ---------------------------------- 3'  
3' ---------------------------------- 5'  
7 \rightarrow

482-501

Annealing

5' ---------------------------------- 3'  
3' ---------------------------------- 5'  

5' ---------------------------------- 3'  
3' ---------------------------------- 5'  

2ND ROUND PCR

5' ---------------------------------- 3'  
3' ---------------------------------- 5'  
7 \rightarrow

879-898

Annealing

5' ---------------------------------- 3'  
5 \rightarrow

646-665
Figure 4.9  Dot-blot Southern Blot to detect optimal stringency and probe concentration.

Each square of nitrocellulose was dotted with 2 PCR products. The top dot was PCR product from the RT-PCR for HCoV 229E derived from infected cell cultures. The bottom dot was PCR product from the RT-PCR for GAPDH derived from tonsil tissue.
Table 4.1 Inter and intra-observer repeatability

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Macrophages/Monocytes</th>
<th>Neutrophils</th>
<th>Eosinophils*</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inter-observer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum</td>
<td>13</td>
<td>SD 8.3</td>
<td>5.9</td>
<td>0.42</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICC 0.94</td>
<td>0.97</td>
<td>0.66</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Intra-observer</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum</td>
<td>19</td>
<td>SD 4.2</td>
<td>4.2</td>
<td>0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>- HQ</td>
<td></td>
<td>ICC 0.99</td>
<td>0.99</td>
<td>0.86</td>
<td>0.81</td>
</tr>
<tr>
<td>Sputum</td>
<td>8</td>
<td>SD 5.0</td>
<td>4.8</td>
<td>0.3</td>
<td>1.8</td>
</tr>
<tr>
<td>- LQ</td>
<td></td>
<td>ICC 0.98</td>
<td>0.98</td>
<td>0.80</td>
<td>-0.1</td>
</tr>
<tr>
<td>Nasal Lavage</td>
<td>17</td>
<td>SD 6.1</td>
<td>5.5</td>
<td>0.3</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICC 0.84</td>
<td>0.94</td>
<td>0.91</td>
<td>0.99</td>
</tr>
</tbody>
</table>

HQ = high quality (viability > 50% + squamous cells < 20%)
LQ = low quality (viability < 50% and/or squamous cells > 20%)
SD = Standard deviation of inter-observer and intra-observer differences
ICC = intraclass correlation coefficient
*Log_{10} Eosinophils
Table 4.2 Repeatability of cell counts of two cytospins taken from the same sample of induced sputum and nasal lavage when one is stained with Diff Quick and the other with Romanowsky stain

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Macrophages/ Monocytes</th>
<th>Neutrophils</th>
<th>Eosinophils*</th>
<th>Lymphocytes*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>25</td>
<td>SD 7.0</td>
<td>7.7</td>
<td>0.4</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICC 0.67</td>
<td>0.67</td>
<td>0.73</td>
<td>0.11</td>
</tr>
<tr>
<td>NAL</td>
<td>23</td>
<td>SD 8.4</td>
<td>8.5</td>
<td>0.33</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ICC 0.56</td>
<td>0.82</td>
<td>0.88</td>
<td>0.43</td>
</tr>
</tbody>
</table>

SD = Standard deviation of between-observer and within-observer differences  
ICC = intraclass correlation coefficient  
*Log₁₀ eosinophils/lymphocytes
Table 4.3 ELISA assays of cytokine/chemokine standards with and without DTT.

<table>
<thead>
<tr>
<th></th>
<th><strong>Sample 1</strong></th>
<th></th>
<th><strong>Sample 2</strong></th>
<th></th>
<th><strong>Sample 3</strong></th>
<th></th>
<th>Mean Diff (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DTT</td>
<td>PBS</td>
<td>Diff (%)</td>
<td>DTT</td>
<td>PBS</td>
<td>Diff (%)</td>
<td>DTT</td>
</tr>
<tr>
<td>IL-8  (pg/ml)</td>
<td>551</td>
<td>505</td>
<td>+ 8.3</td>
<td>535</td>
<td>497</td>
<td>+ 7.1</td>
<td>525</td>
</tr>
<tr>
<td>IFNγ (pg/ml)</td>
<td>531</td>
<td>499</td>
<td>+ 6.0</td>
<td>521</td>
<td>489</td>
<td>+ 6.3</td>
<td>511</td>
</tr>
<tr>
<td>IL-5 (pg/ml)</td>
<td>506</td>
<td>501</td>
<td>+ 1.0</td>
<td>501</td>
<td>510</td>
<td>- 1.7</td>
<td>507</td>
</tr>
<tr>
<td>TNFα (pg/ml)</td>
<td>487</td>
<td>492</td>
<td>- 1.0</td>
<td>490</td>
<td>506</td>
<td>- 3.2</td>
<td>490</td>
</tr>
</tbody>
</table>

DTT = Dithiothreitol
PBS = Phosphate buffered saline
Diff = Difference between DTT and PBS samples.
Table 4.3 ELISA assays of cytokine/chemokine standards with and without DTT.

<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th></th>
<th>Sample 2</th>
<th></th>
<th>Sample 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DTT</td>
<td>PBS</td>
<td>Diff (%)</td>
<td>DTT</td>
<td>PBS</td>
<td>Diff (%)</td>
<td>DTT</td>
</tr>
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<td>IL-8</td>
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<td>+ 8.3</td>
<td>535</td>
<td>497</td>
<td>+ 7.1</td>
<td>525</td>
</tr>
<tr>
<td>(pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>531</td>
<td>499</td>
<td>+ 6.0</td>
<td>521</td>
<td>489</td>
<td>+ 6.3</td>
<td>511</td>
</tr>
<tr>
<td>(pg/ml)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>506</td>
<td>501</td>
<td>+ 1.0</td>
<td>501</td>
<td>510</td>
<td>- 1.7</td>
<td>507</td>
</tr>
<tr>
<td>(pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>487</td>
<td>492</td>
<td>- 1.0</td>
<td>490</td>
<td>506</td>
<td>- 3.2</td>
<td>490</td>
</tr>
<tr>
<td>(pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DTT = Dithiothreitol
PBS = Phosphate buffered saline
Diff = Difference between DTT and PBS samples.
Table 4.4  Scores of in situ hybridisation with the mitochondrial ‘C21’ probe in biopsies treated with different concentrations of proteinase K.

<table>
<thead>
<tr>
<th>Bronchial biopsy</th>
<th>Proteinase K (µg/ml)</th>
<th>No hybridisation</th>
<th>C21 probe</th>
<th>Tissue morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>H259/98</td>
<td>0.5</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H2260/98</td>
<td>0.5</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Scoring system: +++ is good, ++ adequate, + poor, — no, probe signal/cell morphology.
Chapter 5 Statistics

All analyses other than the multilevel models were carried out by the author using the statistical package SPSS 8.0. Dr Paul Lambert (see acknowledgements) carried out multilevel modelling.

5.1 Symptoms and physiology

The distribution of subject characteristics (sex and atopy), symptomatic colds and laboratory proven colds between subject groups was analysed by $\chi^2$ test. An independent samples Student’s t-test was used to analyse the age difference between groups. Analysis of outcome data was based upon the categories of symptomatic colds defined as definite, possible, and no cold. Total symptom scores over 17 days and peak symptom scores were analysed non-parametrically with the Mann-Whitney U test and study day on which maximum symptoms occurred, which were normally distributed, by the Student’s t-test. A summary measure for each symptom, percentage of days with the symptom, was used to reflect duration of symptoms. Analysis of summary measures was by Mann-Whitney U test or the two samples Wilcoxon test, as required. The relationship between URT and LRT symptoms in those who developed LRT symptoms was assessed by Spearman’s correlation. The $\chi^2$ test was also used to assess the relationship between atopy and wheeze.

Normalised FEV$_1$ and PEF data were compared using the independent samples Student’s t-test. In order to assess the effect that the presence of each symptom had on PEF and FEV$_1$ multilevel models were used. Multilevel models take into account the correlation between repeated observations on the same subject by incorporating random effects into the linear model. A separate analysis was performed for each symptom that included terms for the mean FEV$_1$ and PEF in each group in the absence of symptoms and the change in FEV$_1$ and PEF
when the symptom was present. Controls did not suffer LRT symptoms, hence estimates could not be obtained.

Log$_2$ transformation of PC$_{20}$ methacholine measurements was used prior to analysis in order to represent changes as doubling doses. Student’s t-tests for related and independent groups were used for within and between group analysis, respectively. Association between change in log$_2$PC$_{20}$ and LRT symptom score was assessed by Spearman’s rank correlation. The PC$_{20}$ data were censored by designating those subjects unresponsive to 128 mg/ml as responsive at this concentration, for the purpose of analysis.

5.2 Nasal lavage, induced sputum measurements

Nasal lavage fluid volume and, after adjustment by inulin dilution, nasal lavage secretion volume were recorded. Cytospins of nasal lavage fluid were counted in order to analyse total cell counts (TCC = leukocytes + squamous cells + epithelial cells), total leukocyte counts (neutrophil + monocyte + eosinophil + lymphocyte), and individual cell counts. All these counts were expressed per ml of nasal secretion as this measure excludes the effects of dilution. Individual neutrophil cell counts were also analysed per ml of nasal lavage (returned) fluid in order to determine the effect of dilution on the results. The various outcomes are shown in this schematic:
Nasal lavage fluid cell counts where at least 200 leukocytes could not be counted were designated zero cells per ml.

Sputum plug weights were recorded prior to processing. Cytospins were counted in order to analyse total cell counts (TCC = leukocytes + squamous cells + epithelial cells), total leukocyte counts (neutrophil + monocyte + eosinophil + lymphocyte), and individual cell counts. All these counts were expressed per ml of sputum. The various outcomes are shown in this schematic:

<table>
<thead>
<tr>
<th>Neubauer count – 200 cells:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• %viability</td>
</tr>
<tr>
<td>• %squamous cells</td>
</tr>
<tr>
<td>• %non-squamous</td>
</tr>
<tr>
<td>→ TCC/ml of sputum</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Low power count – 200 cells:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• %squamous cells</td>
</tr>
<tr>
<td>• %ciliated cells</td>
</tr>
<tr>
<td>• %leukocytes</td>
</tr>
<tr>
<td>→ leukocytes/ml of sputum</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>High power count – 300 + cells:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• %monocytes</td>
</tr>
<tr>
<td>• %neutrophils</td>
</tr>
<tr>
<td>• %eosinophils</td>
</tr>
<tr>
<td>• %lymphocytes</td>
</tr>
<tr>
<td>• %non-identified</td>
</tr>
</tbody>
</table>

Squamous cells, epithelial cells and leukocytes were expressed as a percentage of total cells counted at low power. Sputum leukocyte differentials are only reported where at least 300 leukocytes could be counted at high power.

Cell counts were validated as described in section 4.4. Lavage fluid volumes with and without inulin adjustment were analysed by student’s unpaired t-test at baseline and during the symptomatic phase of the illness on day 4, for differences in those with and without colds.

The main outcomes were the leukocyte total and differential counts and soluble mediator concentrations. The sputum eosinophil and lymphocyte counts and all nasal lavage fluid cell counts were not normally distributed. \( \log_{10} \) transformation normalized the sputum eosinophils.
and lymphocytes prior to analysis at baseline. The mediators measured in sputum supernates were adjusted for the volume of supernatant and also for dilution during processing. Mediators in nasal lavage fluid were also adjusted for dilution during lavage (by inulin concentration) and processing. Levels below the level of detection of ELISA assays were designated as half the lower level of detection for all data.

Baseline comparison between groups (control and viral wheeze) of sputum differential counts were analysed by student’s paired t-test. Baseline comparisons between groups of nasal lavage fluid cell counts and all soluble mediators were analysed by Mann Whitney U test. The changes from baseline of sputum differential counts and sputum and nasal lavage fluid IL-8 concentrations on days 2, 4 and 17 were all normally distributed and analysed using a multivariate multilevel model. This is similar to performing a repeated measures MANOVA analysis, but does not assume that every subject has observations recorded on all days. The technique allows investigation of changes over time (‘time effect’) differences between groups (‘group effect’) and differences in the effect of time between group (‘time-group interaction’). Other soluble mediators (IFN-γ and ECP) and the neutrophil cell count in nasal lavage fluid were markedly skewed and were therefore analysed using the Mann-Whitney U test.

The summary statistics for changes in leukocytes and mediators are mean of differences (95% CI) for normal data and medians of differences (quartiles) for non-normalised data. The relationship between change in neutrophils and IL-8 and change in log$_2$ PC$_{20}$ methacholine (normally distributed) and symptom scores (not normally distributed) on days 2 and 4 (data reported previously (4)) were assessed by Pearson’s correlation and Spearman’s rank-correlation, respectively. The relationship between change in neutrophils and IL-8 was assessed
by Pearson’s correlation for the LRT (normally distributed) and Spearman’s rank-correlation for the URT (not normally distributed).
PART III – RESULTS
Chapter 6  Symptoms, confirmation of infection and physiology

6.1  Subject characteristics

All the viral wheezers gave a history of at least 2 episodes of wheeze during an URTI in the previous 2 years with 58% of them using inhaled bronchodilators during the episodes (Table 6.1). The viral wheeze group was slightly younger and had more females than the control group (Table 6.1). The greater proportion of atopic subjects in the viral-wheeze group did not reach statistical significance (Table 6.1). Only one non-atopic subject reported a history of allergic disease: “eczema as a baby”. Four of seven atopic controls and 6 of 15 atopic viral wheezers had a positive history of non-pulmonary allergic disease.

6.2  Exclusions and missing data

One viral wheeze subject was excluded because unreliable home monitoring precluded classification as a responder or non-responder to the experimental infection. Another failed to attend on day 2 but was included. Nine ‘wild’ colds were thought to have occurred in the latter half of the study hence diary data from the onset of symptoms attributed to the wild cold and the day 17 data from these subjects were excluded. One subject suffered a sporting injury to the nose and another to the neck between days 4 and 17. Diary data and physiology measurements from the date of the injury onwards were also excluded. The remainder completed the study satisfactorily.

6.3  Symptoms

Symptomatic colds were categorised according to URT symptoms. Systemic and LRT symptoms, and cough were also examined. Cough was examined separately as it was felt that cough could result from both upper and lower respiratory tract illness.
6.3.1 Upper respiratory tract

There were 27 'definite', 4 'possible' and 12 'no' colds (Table 6.2). There were 19 viral wheezers and 8 controls with definite colds defined on the time period 2 to 6 days post-inoculation (Table 6.2a). HCoV infection generated all URT symptoms included in the diary (Table 6.2b). The viral wheezers tended to report more severe symptoms. Three of the controls with possible colds had laboratory confirmation of infection (see section 6.4) and were thus included definite with the controls with colds. For analysis purposes, the 4 URT symptoms were summed in order to investigate whether there was a significant difference between viral wheezers and controls in the reported severity of colds. In order to account for any bias introduced by adding those with 'possible' colds and therefore milder symptoms to the control group the analysis was repeated with the controls with 'definite' colds only.

The viral wheeze group reported significantly more severe URT symptoms that were of slightly longer duration than the control group (Table 6.5a and Table 6.5b). The temporal pattern of URT symptoms was similar between groups (Figure 6.1), with the mean (SD) peak URT symptoms for controls and viral wheezers occurring at 3.4 (1.5) days and 3.6 (1.4) days, respectively (p = 0.67).

6.3.2 Systemic

HCoV infection was characterised by the presence of systemic symptoms, especially fever and headache (Table 6.3). The viral wheeze group reported significantly more severe systemic symptoms than the control group with definite and possible colds (Table 6.5a), but this did not reach statistical significance in those with definite colds only (Table 6.5b).
6.3.3 Lower respiratory tract

The diaries confirmed that the controls did not suffer LRT symptoms whereas 16 of the 19 viral wheezers suffered LRT symptoms. The majority of those who suffered LRT symptoms reported mild symptoms of wheeze, chest tightness and shortness of breath (Table 6.4). LRT symptoms were temporally related to the URT symptoms (Figure 6.2) with the onset of LRT symptoms following the onset of URT symptoms by 24 hours. Peak URT scores correlated with peak LRT scores ($r_s = 0.513$, 95% CI = -0.01 to 0.82, $p = 0.05$), but there was no correlation between total URT and LRT scores ($r_s = 0.16$, 95% CI = -0.38 to 0.61, $p = 0.56$) in those viral wheezers with LRT symptoms.

6.3.4 Cough

Both viral wheezers and controls with definite colds reported cough during the study (Table 6.4). This reinforced our prior concerns that cough should be treated differently from other ‘LRT’ symptoms as no controls reported any other LRT symptoms. There was no significant difference between viral wheezers and controls with colds in reported cough severity or duration of cough (Table 6.5a), although again the viral wheezers tended to report more severe cough.

6.3.5 Symptoms and atopy

Neither upper nor lower respiratory tract symptom severity was related to atopic status. For URT symptoms, median (quartile) total scores were 27 (22,37) and 21 (8,38) in non-atopics and atopics with colds respectively ($p = 0.22$). Among the 19 subjects in the viral wheeze group who had a definite cold, 9/12 atopics and all 7 of the non-atopics actually developed LRT symptoms ($p = 0.15$), and their LRT symptom severity was not related to atopic status.
Median (quartile) total LRT scores were 14 (5,24) and 5 (1,18) in non-atopics and atopics respectively (p = 0.27).

6.4 Confirmation of viral infection

None of the throat and nose swabs taken during the symptomatic stage of the experimental infection were positive for cultured viruses making wild-type contamination of the experiment unlikely. Laboratory confirmation of viral infection was by two methods, RT-PCR and serology using ELISA.

6.4.1 RT-PCR

All subjects had nasal lavage performed on days 2 and 4. Nose swabs and throat swabs were also taken after the first 12 subjects had participated. Not all samples were collected due to volunteer discomfort. The results show that RT-PCR confirmed clinical infection in 13 out of 19 viral wheezers and 8 out of 8 controls with definite colds (Table 6.6a and 6.6b). Not all samples (nasal lavage, nose and throat swab) were positive at the same time point. Nasal samples were more successful than throat samples at picking up evidence of infection. Two out of 5 viral wheezers and 1 out of 7 controls with no cold had at least one positive sample by RT-PCR (Table 6.6a and 6.6b). These were designated as asymptomatic infections and these subjects remained in the ‘no cold’ control groups as the primary outcomes of this study were the mechanisms leading to symptoms. Three out of 4 controls with ‘possible colds’ had positive RT-PCR samples (Table 6.6b).

6.4.2 ELISA

a) Non-inoculated controls.
The second method of confirmation was by seeking a rise in HCoV antibody titre in serum taken on days 0 and 17 using an anti-HCoV antibody ELISA based on a previously described method. A significant rise in antibody over this period was defined as greater than the upper 95% confidence interval for the mean ratio of antibody levels between 17 paired samples of sera taken from non-infected adults, 17 days apart.

All volunteers had detectable antibody levels at day 0. The mean (95% CI) of the ratios between paired antibody levels in 17 non-infected controls was 1.00 (0.75,1.25) absorbance values. Pre-existing antibody levels did not relate to symptomatic colds (mean (SD) absorption values at 1:10 serum dilution = 0.56 (0.15) and 0.61 (0.15) for colds and no colds, respectively) or to rise in antibody levels after experimental infection (mean (SD) absorption values at 1:10 serum dilution = 0.68(0.10) and 0.60 (0.18) for those with and without a significant antibody rise, respectively).

Laboratory confirmed infections. When both RT-PCR and ELISA are combined to support the diagnoses of colds we found 15/19 viral wheezers and 8/8 controls with ‘definite’ symptomatic colds had laboratory evidence of a cold. The three controls with mild URT symptoms between days 2 and 6 and laboratory evidence of infection were added to the ‘definite cold’ group when the study outcomes were analysed. Two out of 5 viral wheezers and 1/7 controls with no symptoms of a cold had laboratory evidence of a cold. Laboratory evidence therefore backed up the clinical categorisation of colds that were used to define groups for analysis. Groups were based upon symptomatic colds rather than laboratory proven infections as the aim was to investigate the mechanisms that underlie virus-induced wheeze. Symptom scores are well validated and it is widely accepted that it is not possible to identify virus in every symptomatic cold, hence all with definite colds were included. The groups were as follows:
6.5 Lung function tests

Viral wheezers had lower baseline normalised PEF and FEV₁ compared with controls, the later being statistically significant (Table 6.1).

FEV₁ tended to be lower in the viral wheeze group on symptom free days compared to controls (FEV₁ on days without nasal discharge, mean (95% CI) 83 % (78, 89) and 94 % (89, 105) of reference values, respectively). PEF measurements were similar in the two groups on symptom free days (PEF on days without nasal discharge, mean (95% CI) 105 % (99, 111) and 106 % (91, 122) of reference values, respectively). For all symptoms, on days when symptoms were present, there was a reduction in FEV₁ for the viral wheeze group (Table 6.6). The largest reductions in FEV₁ were associated with cough, shortness of breath and wheeze. The largest changes in PEF were associated with shortness of breath, sore throat, chest tightness and cough. The presence of symptoms in the control group made little difference to the levels of lower FEV₁ and PEF. However, due to the relatively small numbers when comparing the changes in the viral wheeze group to the controls, the only change that was formally significant was for the reduction in PEF in the presence of cough (p = 0.04).

6.6 Airway responsiveness

There was no significant difference in the log₂PC₂₀ between viral wheezers and controls at baseline (Table 6.1) and all were well outside the ‘asthmatic range’ (log₂PC₂₀ < 3 g/l) except
for three viral wheezers who were atopic \( (\log_2 PC_{20} = 2.9, 1.2, 2.3) \). Atopic viral wheezers had a significantly lower \( \log_2 PC_{20} \) at baseline than non-atopic viral wheezers (mean (SD) \( \log_2 PC_{20} = 4.5 (1.9) \) and 6.2 (1.4) g/l respectively, \( p = 0.027 \)).

There were no significant changes in \( \log_2 PC_{20} \) during the study in viral-wheezers without a cold or in controls with or without a cold (Figure 6.3). The viral wheeze group with a cold had a significant increase in bronchial responsiveness on days 2 and 4 shown by a decrease in \( PC_{20} \) by 0.82 and 1.35 doubling concentrations from baseline respectively (Figure 6.4). This persisted to day 17 where the \( PC_{20} \) had dropped by 1.82 doubling concentrations from baseline. The \( PC_{20} \) of the viral wheezers was progressively lower than the controls during the study and this was significantly lower on days 4 and 17 (Figure 6.4).

The total LRT score (excluding cough), reflecting severity of the LRT illness in the viral wheezers, was found to correlate with change in \( \log_2 PC_{20} \) on day 4 \( (r_s = 0.54, p = 0.02) \) but not on days 2 and 17 \( (r_s = 0.01 \) and 0.20, \( p = 0.98 \) and 0.46, respectively). The small decreases in \( FEV_1 \) and PEF seen during symptoms coincided with the decrease in \( PC_{20} \) on days 2 and 4 but symptoms, \( FEV_1 \) and PEF had resolved by day 17, when the change in \( PC_{20} \) was at its greatest.

In both atopic and non-atopic viral wheezers with a cold, \( PC_{20} \) decreased progressively during the study and the difference which had been present at baseline became less marked and was statistically insignificant by day 2 (Figure 3).
### 6.7 Figures and Tables

**Table 6.1** Entry characteristics of study subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Controls</th>
<th>Viral wheezers</th>
<th>Difference in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Proportions - %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(95% CI)</td>
</tr>
<tr>
<td><strong>Categorical data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>19</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Bronchodilator use (%)</td>
<td>0 (0)</td>
<td>14 (58)</td>
<td></td>
</tr>
<tr>
<td>Sex M/F (% M)</td>
<td>12 / 7 (63)</td>
<td>6 / 18 (25)</td>
<td>38 (8 to 68)*</td>
</tr>
<tr>
<td>Atopy (%)</td>
<td>7 (37)</td>
<td>15 (62)</td>
<td>25 (-4 to 56)</td>
</tr>
<tr>
<td><strong>Continuous data</strong></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>28 (5)</td>
<td>24 (5)</td>
<td>4 (1 to 7)**</td>
</tr>
<tr>
<td>Last URTI (months ago)</td>
<td>7 (3.8)</td>
<td>5 (3.7)</td>
<td>2 (-1 to 4)</td>
</tr>
<tr>
<td>FEV₁ (% predicted)</td>
<td>95.6 (13.2)</td>
<td>85.8 (11.4)</td>
<td>9.8 (2.3 to 17.3)*</td>
</tr>
<tr>
<td>PEF (% predicted)</td>
<td>118.8 (41.2)</td>
<td>104.3 (21.8)</td>
<td>14.4 (-6.9 to 34.2)</td>
</tr>
<tr>
<td>Log₂PC₂₀ (g/l)</td>
<td>5.8 (1.4)</td>
<td>5.1 (1.9)</td>
<td>0.7 (-0.4 to 1.7)</td>
</tr>
</tbody>
</table>

*p < 0.05; ** p < 0.01. Values are: numbers (%) for categorical data; mean (SD) for continuous data. URTI = upper respiratory tract infection; FEV₁ = forced expiratory volume in 1 second; PEF = peak expiratory flow.
Table 6.2a  Median (quartile) upper respiratory tract symptom scores from
days 2 to 6 (inclusive) during the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Clinical</th>
<th>Rhinorhoea</th>
<th>Blocked nose</th>
<th>Sneeze</th>
<th>Sore throat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral</td>
<td>19</td>
<td>Definite</td>
<td>4.0 (3.0,7.0)</td>
<td>5.0 (2.0, 8.0)</td>
<td>3.0 (1.0, 5.0)</td>
<td>4.0 (0.0, 7.0)</td>
</tr>
<tr>
<td>wheezers</td>
<td>5</td>
<td>No</td>
<td>0.0 (0.0, 3.0)</td>
<td>0.0 (0.0, 0.5)</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.0 (0.0, 0.0)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Definite</td>
<td>2.0 (1.2, 5.5)</td>
<td>3.5 (1.2, 6.5)</td>
<td>1.0 (0.0, 4.5)</td>
<td>1.0 (1.0, 3.0)</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>Possible</td>
<td>2.0 (0.5, 2.0)</td>
<td>0.5 (0.0, 1.7)</td>
<td>0.0 (0.0, 0.7)</td>
<td>0.0 (0.0, 0.0)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>No</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.0 (0.0, 1.0)</td>
</tr>
</tbody>
</table>

Table 6.2b  Median (quartile) upper respiratory tract symptom scores during the whole
study.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Clinical</th>
<th>Rhinorhoea</th>
<th>Blocked nose</th>
<th>Sneeze</th>
<th>Sore throat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral</td>
<td>19</td>
<td>Definite</td>
<td>4.0 (3.0,10.0)</td>
<td>9.0 (2.3, 9.7)</td>
<td>5.0 (2.0, 8.7)</td>
<td>5.0 (2.2,12.7)</td>
</tr>
<tr>
<td>wheezers</td>
<td>5</td>
<td>No</td>
<td>1.5 (0.2, 7.2)</td>
<td>0.0 (0.0, 1.5)</td>
<td>0.0 (0.0, 3.7)</td>
<td>0.0 (0.0, 0.0)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Definite</td>
<td>3.0 (0.5, 8.5)</td>
<td>4.0 (1.5, 9.5)</td>
<td>1.5 (0.0, 9.5)</td>
<td>1.0 (1.0, 6.0)</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>Possible</td>
<td>2.0 (0.0, 2.0)</td>
<td>4.0 (0.0, 4.0)</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.0 (0.0, 0.0)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>No</td>
<td>0.0 (0.0, 0.5)</td>
<td>0.0 (0.0, 0.5)</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.0 (0.0, 2.0)</td>
</tr>
</tbody>
</table>

URTI = upper respiratory tract infection
Table 6.3  Median (quartile) systemic symptom scores during the whole study.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Clinical</th>
<th>Fever</th>
<th>Chills</th>
<th>Malaise</th>
<th>Headache</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral</td>
<td>19</td>
<td>Definite</td>
<td>4.0 (0.0,7.0)</td>
<td>0.0 (0.0, 1.7)</td>
<td>0.0 (0.0, 3.0)</td>
<td>5.0 (1.2, 10.5)</td>
</tr>
<tr>
<td>wheezers</td>
<td>5</td>
<td>No</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.0 (0.0, 0.7)</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>Possible</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 1.0)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>No</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 1.0)</td>
</tr>
</tbody>
</table>

URTI = upper respiratory tract infection
Table 6.4  Median (quartile) lower respiratory tract symptom scores during the whole study.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Clinical URTI</th>
<th>Wheeze</th>
<th>Chest tightness</th>
<th>Shortness of breath</th>
<th>Cough</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral</td>
<td>19</td>
<td>Definite</td>
<td>2.0 (0.0,7.0)</td>
<td>3.5 (1.0, 8.2)</td>
<td>2.0 (1.0, 7.5)</td>
<td>4.5 (0.0, 15.0)</td>
</tr>
<tr>
<td>wheezers</td>
<td>5</td>
<td>No</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 1.5)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Definite</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>1.0 (0.0, 4.5)</td>
</tr>
<tr>
<td>Controls</td>
<td>4</td>
<td>Possible</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>No</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 0.0)</td>
<td>0.0 (0.0, 2.5)</td>
</tr>
</tbody>
</table>

URTI = upper respiratory tract infection
Table 6.5a  Symptomatic response to HCoV 229E inoculation

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Viral wheezers (n = 19)</th>
<th>Controls (n = 11)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (LQ, UQ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total scores URT</td>
<td>24 (10,37)</td>
<td>6 (4,15)</td>
<td>0.014</td>
</tr>
<tr>
<td>LRT</td>
<td>6 (2,19)</td>
<td>0 (0,0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systemic</td>
<td>9 (4,18)</td>
<td>1 (0,8)</td>
<td>0.048</td>
</tr>
<tr>
<td>Cough</td>
<td>4 (0,14)</td>
<td>0 (0,5)</td>
<td>0.134</td>
</tr>
<tr>
<td>Symptomatic days (%)*</td>
<td>23 (18,35)</td>
<td>18 (9,38)</td>
<td>0.32</td>
</tr>
<tr>
<td>Rhinorhoea</td>
<td>29 (10,41)</td>
<td>29 (18,62)</td>
<td>0.38</td>
</tr>
<tr>
<td>Blocked nose</td>
<td>26 (12,43)</td>
<td>12 (0,44)</td>
<td>0.24</td>
</tr>
<tr>
<td>Sneeze</td>
<td>26 (4,53)</td>
<td>18 (6,35)</td>
<td>0.39</td>
</tr>
<tr>
<td>Sore throat</td>
<td>22 (0,37)</td>
<td>0 (0,0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Wheeze</td>
<td>18 (4,43)</td>
<td>0 (0,0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tight chest</td>
<td>12 (4,35)</td>
<td>0 (0,3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Shortness of breath</td>
<td>23 (0,59)</td>
<td>18 (0,38)</td>
<td>0.36</td>
</tr>
</tbody>
</table>

LQ = lower quartile; UQ = upper quartile; URTI = upper respiratory tract infection; URT = upper respiratory tract; LRT = lower respiratory tract.

* Expressed as proportion of record period rather than total, to allow for missing data or data excluded due to wild colds (see text).
Table 6.5b  Symptomatic response to HCoV 229E inoculation

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>URT</th>
<th>LRT</th>
<th>Systemic</th>
<th>Cough</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total scores</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral wheezers (n = 19)</td>
<td>24 (10,37)</td>
<td>6 (2,19)</td>
<td>9 (4,18)</td>
<td>4 (0,14)</td>
</tr>
<tr>
<td>Controls (n = 8)</td>
<td>9 (6,19)</td>
<td>0 (0,0)</td>
<td>3 (0,12)</td>
<td>1 (0,8)</td>
</tr>
<tr>
<td>p value</td>
<td>0.045</td>
<td>&lt;0.001</td>
<td>0.06</td>
<td>0.28</td>
</tr>
</tbody>
</table>

LQ = lower quartile; UQ = upper quartile; URTI = upper respiratory tract infection; URT = upper respiratory tract; LRT = lower respiratory tract.
Table 6.6a  RT-PCR and ELISA positive samples in viral wheezers

<table>
<thead>
<tr>
<th>Cold Study No.</th>
<th>Viral wheezers D2 PCR samples</th>
<th>Viral wheezers D4 PCR samples</th>
<th>ELISA</th>
<th>POS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>NL</td>
<td>TS</td>
<td>NS</td>
</tr>
<tr>
<td>1</td>
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<td>+</td>
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<tr>
<td>2</td>
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<tr>
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<td>11</td>
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<tr>
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<td>17</td>
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</tr>
<tr>
<td>Totals</td>
<td>5/13</td>
<td>4/16</td>
<td>2/10</td>
<td>5/10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cold Study No.</th>
<th>Viral wheezers D2 PCR samples</th>
<th>Viral wheezers D4 PCR samples</th>
<th>ELISA</th>
<th>POS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>NL</td>
<td>TS</td>
<td>NS</td>
</tr>
<tr>
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</tr>
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<td>Totals</td>
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<td>0/2</td>
<td>1/2</td>
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</table>

NS = nose swab; NL = nasal lavage; TS = throat swab; Blank spaces = no sample; POS = positive by either method; δ ab = change in antibody levels from day 0 to 17; Ab ratio = absorbency value at day 17/ absorbency value at day 0; positive ELISA samples have an antibody ratio > 1.25 (the upper 95% CI of the Ab ratio of the non-infected controls).
Table 6.6b  RT-PCR positive samples in controls

<table>
<thead>
<tr>
<th>Cold</th>
<th>Study No.</th>
<th>Controls D2 PCR samples</th>
<th>Controls D4 PCR samples</th>
<th>ELISA</th>
<th>Pos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>NL</td>
<td>TS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
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<tr>
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<td>3/7</td>
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<td>Total</td>
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</tr>
<tr>
<td>Total</td>
<td></td>
<td>0/1</td>
<td>1/6</td>
<td>0/1</td>
<td>1/7</td>
</tr>
</tbody>
</table>

NS = nose swab; NL = nasal lavage; TS = throat swab; Blank space = no sample; POS = positive by either method; Ab ratio = absorbency value at day 17/ absorbency value at day 0. δ ab = change in antibody over 17 days (OD values); positive ELISA samples have an antibody ratio > 1.25 (the upper 95% CI of the Ab ratio of the non-infected controls).
### Table 6.7: Difference in FEV$_1$ and PEF between days with and without symptoms

Values are mean difference $\Delta$ (95% CI) of % predicted values

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>$\Delta$ FEV$_1$ on days with symptoms</th>
<th>$\Delta$ PEF on days with symptoms</th>
<th>p*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Viral wheezers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Runny nose</td>
<td>-2 (-6, 2)</td>
<td>-1 (-4, 1)</td>
<td>0.86</td>
<td>2 (-2, 6)</td>
<td>-1 (-3,1)</td>
</tr>
<tr>
<td>Blocked nose</td>
<td>0 (-3, 3)</td>
<td>-1 (-3, 1)</td>
<td>0.46</td>
<td>0 (-3, 4)</td>
<td>0 (-2,3)</td>
</tr>
<tr>
<td>Sneezing</td>
<td>0 (-3, 3)</td>
<td>-2 (-4, 0)</td>
<td>0.31</td>
<td>2 (-1, 6)</td>
<td>-1 (-4,1)</td>
</tr>
<tr>
<td>Sore throat</td>
<td>-1 (-4,2)</td>
<td>-2 (-4, 0)</td>
<td>0.49</td>
<td>-1 (-5,2)</td>
<td>-4 (-6,-2)</td>
</tr>
<tr>
<td>Wheeze</td>
<td></td>
<td>-3 (-7, 1)</td>
<td></td>
<td></td>
<td>-3 (-5,0)</td>
</tr>
<tr>
<td>Chest tightness</td>
<td></td>
<td>-2 (-4, 0)</td>
<td></td>
<td></td>
<td>-4 (-6,-2)</td>
</tr>
<tr>
<td>Short of breath</td>
<td></td>
<td>-4 (-6,-1)</td>
<td></td>
<td></td>
<td>-4 (-7, -2)</td>
</tr>
<tr>
<td>Cough</td>
<td>0 (-4, 3)</td>
<td>-4 (-6,-2)</td>
<td>0.08</td>
<td>1 (-2, 5)</td>
<td>-3 (-6,-1)</td>
</tr>
</tbody>
</table>

p represents a test for the difference in $\Delta$FEV$_1$ and $\Delta$PEF between the two groups.
Figure 6.1

Time course of upper respiratory tract (URT) symptoms for viral wheezers (a) and control subjects (b). Boxes represent the median and 25th and 75th centiles, whiskers represent the minimum and maximum values.

Figure 6.2
Time course of (a) upper respiratory tract (URT) and (b) lower respiratory tract (LRT) symptoms for viral wheezers with colds. Boxes represent the median and 25th and 75th centiles, whiskers represent the minimum and maximum values.

**Figure 6.3** Mean log₂ PC₂₀ (95% CI) g/L in viral-wheezers (■) (n = 5) and control subjects (□) (n = 8) without colds during the study.
Log$_2$ PC$_{20}$ (95% CI) g/L

Days post inoculation

- d0
- d2
- d4
- d17
Figure 6.4  Mean log$_2$ PC$_{20}$ (95% CI) g/L in viral-wheezers (■) and control subjects (□) with colds during the study. The significant decrease from baseline in the viral wheeze group on days 2, 4 and 17 (p = 0.002, 0.002, <0.001) was not seen in the controls (p = 0.21, 0.20, 0.39). The viral wheezers had significantly lower log$_2$ PC$_{20}$ than controls on days 4 (p = 0.032) and 17 (p = 0.007) but not on day 2 (p = 0.156).
Figure 6.5 Mean log₂ PC₂₀ (95% CI) g/L in viral-wheezers with (■) and without (□) atopy. There was a significant difference at day 0 (p = 0.039) but not on days 2, 4, and 17 (p = 0.153, 0.475, 0.424, respectively).
Chapter 7  Cellular changes in the respiratory tract during experimental infection.

7.1 Exclusions and missing data

On 9 occasions in 5 subjects a sputum sample was not produced. Two further samples were missing due to injury and non-attendance. Six out of 161 sputum cytospins counted had less than 300 leukocytes and were excluded. On 3 occasions nasal lavage was not produced, 2 due to nasal injury and 1 to non-attendance. Together with the 9 sputum and nasal lavage samples excluded on day 17 because of ‘wild’ URTIs this gave 26 and 12 missing/excluded sputum and nasal lavage samples respectively, out of a possible 172 each. All the remaining samples produced satisfactory cytospins for cell counting.

In addition, 5 sputum samples where less than 200 cells were counted in the Neubauer chamber were also excluded.

7.2 Nasal lavage

The nasal lavage technique was quick and simple to perform, and was well tolerated. Minor discomfort was reported by most volunteers with 23/44 volunteers also reporting transient eye watering.

The measurements recorded from the nasal lavage samples are discussed in section 5.2. These include: lavage fluid volume and, after adjustment by inulin dilution, lavage secretion volume; TCC; total leukocyte count; and individual cell counts.
7.2.1 Nasal lavage fluid and secretion volumes.

The vast majority of nasal lavage samples obtained throughout the study produced return volumes of greater than 5 ml (50%). Those with colds tended to return more nasal lavage fluid on days 2 and 4 (Table 7.1a). Indeed, on day 4, during the symptomatic phase of the illness, those with colds returned significantly greater volumes of lavage fluid than those without colds (Table 7.1a). A similar finding was seen inulin-adjusted volumes of nasal secretion within the nasal lavage fluid, with a significant difference between those with and without colds on day 2 (Table 7.1a). This increase was not merely due to the increased volume of nasal lavage fluid. The proportion of nasal lavage fluid that was nasal secretion in those with colds tended to increase on days 2 and 4 (Table 7.1b).

There were no differences between viral wheeze and control groups with colds in the proportion of nasal secretions found in nasal lavage fluid, the mean volumes from both groups on all days lying between 48-58%.

7.2.2 Quality of samples

Viability of cells was generally good, although subjects without colds consistently scored lower than those with colds (ranges = 39-49% and 63-80% respectively) (Table 7.2). More squamous cells were generally seen in nasal samples compared with sputum samples (Tables 7.2 and 7.3).
7.2.3 Nasal cell counts

Total cell counts. The initial counts of cells using the Neubauer chamber did not reveal any large changes in TCC in those who developed colds during the study (Table 7.2). There appeared to be a small trend towards increased TCC on days 2 and 4 in viral wheezers with colds, but this was also seen in the control without colds but not in the controls with colds.

Squamous and ciliated cells. No obvious trends were seen in the proportion of ciliated and squamous cells (Table 7.2).

Total leukocyte count. Small increases in total leukocyte counts were seen in all groups on day 2 and/or 4 (Table 7.4). A significant increase from baseline was seen in the viral wheeze group with colds (Table 7.4).

Leukocyte counts. There were no significant changes or apparent trends in nasal cell counts in the control group without colds (data not shown). There was a small increase in nasal lavage neutrophil counts in both groups with colds during the study but no significant difference between the two groups (Table 7.5a). Neutrophils predominated in nasal lavage fluid. Very few other cells were found throughout the study. The changes in nasal lavage fluid neutrophils correlated poorly with URT symptom scores on days 2 and 4 ($r_s = 0.10, p = 0.54; r_s = 0.18, p = 0.45$ respectively). However, change in neutrophils on day 4 did correlate with the total URT symptom scores over the whole study period ($r_s = 0.38, p = 0.03$). There were no significant changes in eosinophils in the URT during the study in either atopic or non-atopic viral wheezers with colds.
Adjusting the nasal cell counts for the dilutional effect of the nasal lavage using the inulin dilution method did not appear to alter the final outcome of neutrophil cell counts (Table 7.5a), although the magnitude of change in neutrophil count was greater when dilution was taken into account.

7.3 Induced sputum

The technique of hypertonic saline induction of sputum is a well-established technique. Volunteers tolerated it well with complaints only of the salty taste. One volunteer suffered a drop in FEV₁ of 10% and required a dose of salbutamol.

The measurements recorded from sputum samples are discussed in section 5.2. These include: weight; viability; squamous contamination; TCC; total leukocyte count; and individual cell counts.

7.3.1 Weight and quality of samples

Large variability was seen in the weights of sputum plugs during the study. There was a non-significant trend towards the production of more sputum in both the viral wheezers and controls during colds (Table 7.3).

Viability of cells was generally good, although subjects without colds consistently scored lower than those with colds (ranges = 55-59% at 62-70% respectively) (Table 7.3). Finally, squamous contamination of sputum samples was generally < 20% (median 8.7%) (Table 7.3).

7.3.2 Sputum cell count
**Total cell counts.** A wide variability was seen in sputum TCC. There were no significant differences between groups, either with or without colds. A trend towards increased cell count was seen in both viral wheezers and controls with colds (Table 7.3).

**Ciliated cells.** Small numbers of ciliated cells were present throughout the study in all groups without any significant changes.

**Total leukocyte count.** Total leukocyte counts tended to increase in all groups with and without colds on days 2 and 4 of the study (Table 7.4). A significant rise was found in the viral wheeze group with colds on day 4 (Table 7.4).

**Leukocyte counts.** A significant increase in the proportion of sputum neutrophils was seen in the viral wheeze group with colds between days 0 and 4, returning towards baseline by day 17, with a reciprocal effect in the proportion of macrophages (Table 7.5b and Figure 7.1). There was no such effect in the control group with colds and the difference between groups was significant. No differences were seen in sputum lymphocytes or eosinophils during the study. Indeed, very low numbers of these cells were present.

There was a significant negative correlation between the change in sputum neutrophils and the change in log2 PC20 on day 4 (r = -0.36, p = 0.02) but not on day 2 (r = -0.01, p = 0.71). No such correlation was found between the change in sputum neutrophils and the LRT symptom score on day 4, the peak day for both scores (r_s = 0.24, p = 0.12). However, there was a significant correlation between the change in sputum neutrophils and the total LRT symptom scores over the whole study period (r_s = 0.39, p = 0.01).
### 7.4 Figures and Tables

#### Table 7.1a
Mean volume (95% confidence interval) of nasal lavage fluid and nasal secretion (by inulin dilution) returned.

<table>
<thead>
<tr>
<th>Day</th>
<th>No cold</th>
<th>Cold</th>
<th>No cold</th>
<th>Cold</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.9 (4.9, 6.8)</td>
<td>6.8 (6.3, 7.3)</td>
<td>2.9 (1.7, 4.1)</td>
<td>3.7 (3.3, 4.0)</td>
</tr>
<tr>
<td>2</td>
<td>6.2 (5.1, 7.3)</td>
<td>7.4 (6.6, 8.2)</td>
<td>2.7 (1.5, 3.9)</td>
<td>4.3 (3.6, 5.0)†</td>
</tr>
<tr>
<td>4</td>
<td>6.1 (5.1, 7.1)*</td>
<td>7.4 (6.9, 8.0)*</td>
<td>2.9 (1.6, 4.3)</td>
<td>4.4 (3.6, 5.1)</td>
</tr>
<tr>
<td>17</td>
<td>6.0 (4.7, 7.2)</td>
<td>7.2 (7.0, 7.7)</td>
<td>2.3 (0.1, 4.6)</td>
<td>3.6 (3.0, 4.3)</td>
</tr>
</tbody>
</table>

Comparison of colds vs no colds: * p = 0.02, †= 0.04.

#### Table 7.1b
Mean percentage (95% confidence interval) of nasal lavage fluid consisting of nasal secretions

<table>
<thead>
<tr>
<th>Day</th>
<th>No cold</th>
<th>Cold</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41.5 (22.5, 60.6)</td>
<td>52.0 (42.9, 61.1)</td>
</tr>
<tr>
<td>2</td>
<td>39.3 (38.2, 52.4)*</td>
<td>55.1 (48.2, 62.0)*</td>
</tr>
<tr>
<td>4</td>
<td>41.3 (23.3, 59.3)</td>
<td>58.0 (48.4, 67.6)</td>
</tr>
<tr>
<td>17</td>
<td>34.1 (6.1, 62.1)</td>
<td>48.1 (39.9, 56.3)</td>
</tr>
</tbody>
</table>

Comparison of colds vs no colds: * p = 0.04
Table 7.2  Total cell count and proportions (%) of cells in nasal secretions.

Values are medians (quartiles).

<table>
<thead>
<tr>
<th>Day</th>
<th>Control no cold</th>
<th>Control cold</th>
<th>VW cold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCC x 10^4/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.6 (3.5, 42.3)</td>
<td>7.0 (1.7, 25.2)</td>
<td>8.3 (2.6, 13.8)</td>
</tr>
<tr>
<td>2</td>
<td>7.9 (4.9, 22.5)</td>
<td>5.6 (2.7, 18.8)</td>
<td>12.2 (6.4, 24.0)</td>
</tr>
<tr>
<td>4</td>
<td>20.7 (9.0, 83.8)</td>
<td>6.2 (1.3, 19.9)</td>
<td>13.6 (6.4, 18.7)</td>
</tr>
<tr>
<td>17</td>
<td>8.4 (4.2, 26.8)</td>
<td>4.5 (1.5, 14.6)</td>
<td>8.6 (2.3, 18.1)</td>
</tr>
<tr>
<td></td>
<td>% viability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>41.3 (17.7, 72.7)</td>
<td>63.7 (48.1, 89.3)</td>
<td>79.6 (60.4, 91.4)</td>
</tr>
<tr>
<td>2</td>
<td>39.0 (14.5, 64.0)</td>
<td>62.9 (47.0, 75.2)</td>
<td>73.9 (58.2, 82.1)</td>
</tr>
<tr>
<td>4</td>
<td>45.4 (21.3, 77.6)</td>
<td>68.3 (65.1, 75.7)</td>
<td>73.5 (56.6, 83.3)</td>
</tr>
<tr>
<td>17</td>
<td>49.3 (30.5, 66.8)</td>
<td>74.4 (45.2, 90.9)</td>
<td>75.2 (58.5, 88.1)</td>
</tr>
<tr>
<td></td>
<td>% squamous cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24.3 (11.5, 47.8)</td>
<td>19.5 (10.5, 42.4)</td>
<td>23.3 (9.1, 37.8)</td>
</tr>
<tr>
<td>2</td>
<td>21.3 (08.5, 27.4)</td>
<td>33.5 (16.6, 55.4)</td>
<td>14.4 (9.1, 23.6)</td>
</tr>
<tr>
<td>4</td>
<td>27.2 (17.6, 39.7)</td>
<td>18.6 (12.4, 37.2)</td>
<td>18.2 (8.0, 22.7)</td>
</tr>
<tr>
<td>17</td>
<td>37.4 (21.4, 43.0)</td>
<td>32.8 (17.9, 61.9)</td>
<td>25.6 (7.9, 50.0)</td>
</tr>
<tr>
<td></td>
<td>% ciliated epithelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.1 (0.0, 27.3)</td>
<td>6.7 (0.0, 52.3)</td>
<td>3.3 (0.0, 21.4)</td>
</tr>
<tr>
<td>2</td>
<td>1.6 (0.0, 18.4)</td>
<td>2.5 (0.0, 37.4)</td>
<td>3.8 (0.0, 19.4)</td>
</tr>
<tr>
<td>4</td>
<td>0.0 (0.0, 21.5)</td>
<td>0.0 (0.0, 18.3)</td>
<td>3.1 (0.0, 15.0)</td>
</tr>
<tr>
<td>17</td>
<td>2.2 (0.0, 8.8)</td>
<td>1.2 (0.0, 2.3)</td>
<td>1.7 (0.0, 0.9)</td>
</tr>
<tr>
<td></td>
<td>% leukocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>43.2 (22.0, 61.2)</td>
<td>63.9 (8.0, 87.5)</td>
<td>60.5 (37.3, 87.6)</td>
</tr>
<tr>
<td>2</td>
<td>63.7 (00.0, 87.1)</td>
<td>47.1 (3.2, 71.1)</td>
<td>77.3 (59.7, 86.3)</td>
</tr>
<tr>
<td>4</td>
<td>63.7 (00.0, 87.1)</td>
<td>47.1 (3.2, 71.1)</td>
<td>77.3 (59.7, 86.3)</td>
</tr>
<tr>
<td>17</td>
<td>37.6 (11.0, 77.0)</td>
<td>41.5 (19.8, 59.7)</td>
<td>71.1 (44.5, 83.6)</td>
</tr>
</tbody>
</table>

TCC = total cell count per ml of nasal secretions
Table 7.3  Sputum weight, total cell count and proportions (%) of cells in induced sputum.

Values are medians (quartiles) unless otherwise stated.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control no cold</th>
<th>Control cold</th>
<th>VW cold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum wt. (mg)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>440 (212)</td>
<td>146 (073)</td>
<td>173 (062)</td>
</tr>
<tr>
<td>2</td>
<td>523 (272)</td>
<td>315 (247)</td>
<td>186 (077)</td>
</tr>
<tr>
<td>4</td>
<td>318 (096)</td>
<td>375 (336)</td>
<td>410 (352)</td>
</tr>
<tr>
<td>17</td>
<td>185 (098)</td>
<td>274 (137)</td>
<td>191 (096)</td>
</tr>
<tr>
<td>TCC x 10⁵/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>39.3 (26.8,66.0)</td>
<td>10.3 (5.8,20.6)</td>
<td>6.8 (5.3,17.7)</td>
</tr>
<tr>
<td>2</td>
<td>50.8 (19.2,107.9)</td>
<td>12.1 (5.3,20.6)</td>
<td>14.0 (5.2,30.1)</td>
</tr>
<tr>
<td>4</td>
<td>43.7 (20.7,73.8)</td>
<td>20.3 (10.1,52.8)</td>
<td>19.0 (12.1,38.0)</td>
</tr>
<tr>
<td>17</td>
<td>27.8 (11.5,82.9)</td>
<td>10.1 (3.0,58.6)</td>
<td>14.6 (4.4,51.6)</td>
</tr>
<tr>
<td>% viability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
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<td>69.0 (60.2,74.0)</td>
<td>62.6 (41.6,76.3)</td>
</tr>
<tr>
<td>2</td>
<td>58.5 (46.4,71.8)</td>
<td>62.8 (53.1,79.4)</td>
<td>65.6 (56.4,75.2)</td>
</tr>
<tr>
<td>4</td>
<td>57.2 (39.4,69.1)</td>
<td>70.3 (56.6,80.4)</td>
<td>70.4 (61.9,84.0)</td>
</tr>
<tr>
<td>17</td>
<td>56.4 (34.4,60.3)</td>
<td>61.7 (46.6,74.3)</td>
<td>62.3 (59.6,69.8)</td>
</tr>
<tr>
<td>% squamous cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24.8 (08.0,52.4)</td>
<td>09.8 (00.4,52.6)</td>
<td>10.1 (04.4,27.8)</td>
</tr>
<tr>
<td>2</td>
<td>09.8 (03.2,53.6)</td>
<td>05.4 (03.2,13.1)</td>
<td>05.8 (00.5,12.7)</td>
</tr>
<tr>
<td>4</td>
<td>13.1 (03.5,72.3)</td>
<td>01.1 (00.0,14.7)</td>
<td>09.1 (04.9,20.4)</td>
</tr>
<tr>
<td>17</td>
<td>06.5 (02.9,38.2)</td>
<td>03.9 (01.8,11.3)</td>
<td>09.4 (03.9,17.2)</td>
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<tr>
<td>% ciliated epithelial cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.5 (4.3,05.5)</td>
<td>3.8 (1.6,12.7)</td>
<td>2.8 (2.0,04.3)</td>
</tr>
<tr>
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<td>2.9 (1.5,05.3)</td>
<td>7.1 (2.9,08.8)</td>
<td>4.1 (0.9,06.6)</td>
</tr>
<tr>
<td>4</td>
<td>3.5 (0.2,06.7)</td>
<td>4.5 (2.4,05.3)</td>
<td>2.6 (1.4,18.1)</td>
</tr>
<tr>
<td>17</td>
<td>1.7 (1.4,12.9)</td>
<td>4.0 (2.3,34.4)</td>
<td>3.6 (1.8,10.7)</td>
</tr>
<tr>
<td>% leukocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>59.5 (42.2,83.5)</td>
<td>82.8 (41.6,92.1)</td>
<td>83.5 (68.5,92.4)</td>
</tr>
<tr>
<td>2</td>
<td>80.9 (46.4,94.5)</td>
<td>87.3 (73.7,90.9)</td>
<td>87.3 (66.8,97.3)</td>
</tr>
<tr>
<td>4</td>
<td>80.6 (25.9,83.4)</td>
<td>93.6 (41.1,97.1)</td>
<td>81.5 (61.4,93.0)</td>
</tr>
<tr>
<td>17</td>
<td>92.0 (33.7,95.7)</td>
<td>89.3 (57.3,93.6)</td>
<td>83.0 (69.5,93.0)</td>
</tr>
</tbody>
</table>

*mean (95%CI)
Table 7.4  Total leukocyte counts per ml of nasal secretions‡ or per ml of sputum. Values are median (quartile).

<table>
<thead>
<tr>
<th>Study Day</th>
<th>Leukocyte total cell count x 10⁵/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control no cold</td>
</tr>
<tr>
<td><strong>Nasal secretions</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.4 (2.7, 28.1)</td>
</tr>
<tr>
<td>2</td>
<td>6.3 (4.3, 22.0)</td>
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<tr>
<td>4</td>
<td>10.4 (6.7, 80.6)</td>
</tr>
<tr>
<td>17</td>
<td>0.0 (0.0, 13.0)</td>
</tr>
<tr>
<td><strong>Sputum plugs</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>31.1 (18.8,72.0)</td>
</tr>
<tr>
<td>2</td>
<td>47.9 (23.1,97.4)</td>
</tr>
<tr>
<td>4</td>
<td>39.9 (6.7,63.2)</td>
</tr>
<tr>
<td>17</td>
<td>25.1 (4.2,81.4)</td>
</tr>
</tbody>
</table>

VW = viral wheezers; change from baseline: † p = 0.07, †† p = 0.06, * p < 0.05, ** p < 0.01; ‡ i.e. corrected for dilution.
Special Note

Page missing from the original
### Table 7.5a
Change from baseline in neutrophil counts in nasal secretions and nasal lavage fluid in subjects with colds

<table>
<thead>
<tr>
<th>Viral wheezers</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td>Median difference (quartiles)</td>
</tr>
<tr>
<td>Nasal secretions (x 10⁴/ml)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.4 (-0.1, 11.6)</td>
</tr>
<tr>
<td>4</td>
<td>4.8 (0.0, 19.6)</td>
</tr>
<tr>
<td>17</td>
<td>0.9 (-0.1, 6.3)</td>
</tr>
<tr>
<td>Nasal lavage fluid (x 10⁴/ml)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.4 (-0.3, 6.2)</td>
</tr>
<tr>
<td>4</td>
<td>2.0 (0.0, 3.1)</td>
</tr>
<tr>
<td>17</td>
<td>0.2 (-0.5, 3.0)</td>
</tr>
</tbody>
</table>

Number of observations on days 2, 4 and 17, respectively: viral wheezers 19, 19, 16; controls 9, 9, 6.
Table 7.5b  Change from baseline in leukocyte differential counts in sputum in subjects with colds.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Day</th>
<th>Viral wheezers</th>
<th>Controls</th>
<th>Multivariate multilevel model analysis p-values</th>
</tr>
</thead>
<tbody>
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<td>Mean difference (95% CI)</td>
<td>T-G</td>
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<td>Macrophages</td>
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<td>-13.8 (-21.4, -6.2)</td>
<td>12.2 (-11.9, 36.3)</td>
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<tr>
<td></td>
<td>4</td>
<td>-25.7 (-35.0, -16.4)</td>
<td>4.5 (-16.1, 25.1)</td>
<td>0.97</td>
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<tr>
<td></td>
<td>17</td>
<td>-7.9 (-20.6, 1.4)</td>
<td>13.4 (10.0, 16.8)</td>
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<td>2</td>
<td>13.0 (5.5, 20.5)</td>
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<td>0.97</td>
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<tr>
<td></td>
<td>4</td>
<td>20.9 (12.3, 29.5)</td>
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<td>0.1 (-0.8, 1.0)</td>
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<td>0.17</td>
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<tr>
<td></td>
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<td>0.2 (-0.6, 1.0)</td>
<td>0.3 (-1.1, 1.7)</td>
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<td>Eosinophils</td>
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<td></td>
<td>4</td>
<td>-0.1 (-1.1, 0.9)</td>
<td>-2.0 (-4.8, 0.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>0.1 (-0.6, 0.8)</td>
<td>-0.5 (-1.7, 0.7)</td>
<td></td>
</tr>
</tbody>
</table>

Number of observations on days 2,4 and 17, respectively: viral wheezers 15, 17, 15; controls 8, 11, 5. T – time effect; G – group effect; T-G – time-group interaction.
Figure 7.1  Difference from baseline in mean (95%CI) sputum neutrophil differential count (%) in viral wheezers (■) and controls (□) with colds during the study.
Chapter 8 Changes in inflammatory mediators during experimental infection

All samples were stored at —80°C and analysed within one year of storage. Samples missing or excluded are described in section 7.1. All nasal mediators were expressed per ml of nasal secretion after correcting for the effects of dilution. Sputum mediator concentrations were expressed per ml of sputum.

There were no significant changes during the study in any mediators in the nasal lavage fluid or sputum of the controls without colds (n = 8). Data are therefore presented only from those with symptomatic colds.

8.1 ELISA for IFNγ, IL-8, IL-5, TNFα

Nasal samples. At baseline there was no significant difference between the viral wheeze and control groups in any measure from the URT (see Table 8.1). The viral wheezers with colds had a trend towards an increase in nasal IL-8 on day 4 returning towards baseline on day 17, which was not seen in the controls (Table 8.2a and Figure 8.1a). More than 25% of all subjects with colds had nasal lavage fluid IFNγ concentrations below the levels of detection. Although levels increased in some subjects during colds, the increases were modest with no significant difference between the groups (Table 8.2a). IL-5 was below the assay sensitivity (2pg/ml) for the majority of subjects in both groups at all time-points.

There was no significant correlation between change in IL-8 concentration and URT symptom scores on days 2 and 4 (rₜ = 0.23 (p = 0.21) and 0.21 (p = 0.21), respectively). However, the change in IL-8 concentration on day 4 was significantly correlated to the total URT score over
the whole study period ($r_s = 0.39$, $p = 0.04$). A weak but significant correlation was found between the changes in IL-8 and neutrophils from baseline on day 4 ($r = 0.3$, $p = 0.006$). There was thus a significant interrelationship between URT symptoms, neutrophil count and IL-8 during the illness.

*Sputum samples.* At baseline there was no significant difference between the viral wheeze and control groups in any measure from the LRT (see Table 8.1). In those with colds a significant increase in sputum IL-8 was seen in both viral wheezers and controls on day 4 and also on day 2 in the viral wheeze group, both groups returning towards baseline on day 17 (Table 8.2b, Figure 8.1b). There was no difference between groups in IL-8. More than 25% of all subjects with colds had IFNγ below the levels of detection and although some with colds had an increase, this was modest with no significant difference between the groups (Table 8.2b). Again, the majority of samples had levels of IL-5 below the level of detection of the assay.

Change in sputum IL-8 concentration from baseline on day 4 post-inoculation was weakly correlated to LRT neutrophil differential count ($r = 0.27$, $p = 0.013$) but not to LRT symptom score on day 4 ($r_s = -0.01$, $p = 0.94$) or to the total LRT symptom score ($r_s = 0.08$, $p = 0.63$).

8.2 UniCAP enzyme immunoassay for ECP

*Nasal samples.* There was no significant difference in baseline ECP between atopic (n = 10) and non-atopic (n = 7) viral wheezers in the URT (median (quartiles) = 9.0 (6.2, 13.8) and 11.7 (0.0, 32.6) pg/ml of nasal secretions, respectively, $p = 0.49$). There was no overall change in nasal ECP although a few subjects in both groups had modest increases on day 4. Change in ECP from baseline was not significant for viral wheezers or controls (median (quartiles) for days 0 and 4: 11.0 (3.1, 23.5) and 16.2 (0.0, 66.0) pg/ml, $p = 0.23$; and 5.6 (0.0, 19.8) and 14.1
(0.0, 20.3) pg/ml p = 0.67, respectively) and there was no difference between groups (Table 8.2a), or between atopic and non-atopic wheezers (median (quartiles) for day 4: 11.2 (0, 91.5) and 11.2 (0, 25.9) pg/ml p = 0.22, respectively).

Sputum samples. There was no significant difference in baseline ECP between atopic (n = 10) and non-atopic (n = 7) viral wheezers in LRT (median (quartiles) = 14.0 (4.6, 84.9) and 0.5 (0.0, 51.2) pg/ml of sputum supernatant, respectively, p = 0.43). There were no significant changes in sputum ECP in either group (Table 8.2b) and subgroup analysis of atopic viral wheezers with colds also showed no significant change from baseline (median (quartile) ECP at day 0 and 4 = 7.7 (0.0, 10.9) and 10.5 (0.0, 12.3) ng/ml respectively, p =0.59).
8.3 Figures and Tables

Table 8.1 Comparison of baseline soluble mediators in all viral wheezers and controls. Values are expressed as medians (quartiles).

<table>
<thead>
<tr>
<th>Mediator</th>
<th>N</th>
<th>Viral wheezers</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 pg/ml</td>
<td>24</td>
<td>746 (221, 1429)</td>
<td>19 555 (230,1376)</td>
<td>0.97</td>
</tr>
<tr>
<td>URT IFN-γ pg/ml</td>
<td>24</td>
<td>3.6 (3.0, 15.6)</td>
<td>19 3.3 (2.9, 24.1)</td>
<td>0.88</td>
</tr>
<tr>
<td>ECP pg/ml</td>
<td>22</td>
<td>11.0 (3.1, 23.5)</td>
<td>17  5.6 (0.0, 19.8)</td>
<td>0.26</td>
</tr>
<tr>
<td>IL-5 pg/ml</td>
<td>24</td>
<td>&lt;2</td>
<td>19  &lt;2</td>
<td></td>
</tr>
</tbody>
</table>

| IL-8 pg/ml | 21  | 2,997 (1,038, 4,525) | 17 2,815 (500,5,407) | 0.67  |
| LRT IFN-γ pg/ml | 20  | 10 (0, 119) | 17  0 (0, 0) | 0.16  |
| ECP pg/ml | 17  | 6.2 (0, 28) | 12  3 (0,13) | 0.27  |
| IL-5 pg/ml | 11  | <2              | 11  <2          |       |

URT = upper respiratory tract - samples expressed / ml of nasal secretions; LRT = lower respiratory tract.
Table 8.2a Change from baseline in soluble mediators in nasal lavage fluid in subjects with colds

<table>
<thead>
<tr>
<th>Day</th>
<th>Viral wheezers</th>
<th>Controls</th>
<th>Multivariate analysis p-values</th>
</tr>
</thead>
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<tr>
<td></td>
<td>n</td>
<td>Mean difference (95% CI)</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8 pg/ml</td>
<td>2</td>
<td>15</td>
<td>62 (-364, 487)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16</td>
<td>698 (252, 1144)</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>14</td>
<td>-157 (-917, 603)</td>
</tr>
</tbody>
</table>

Median difference (quartiles) | Median difference (quartiles) | Mann-Whitney U test p-values |

| IFN-γ pg/ml | 2 | 15 | 1.3 (-0.0, 40.7) | 10 | 0.1 (-0.6, 54.1) | 0.70 |
|     | 4 | 16 | 1.3 (0.2, 357.5) | 10 | 13.7 (-1.9, 30.0) | 0.92 |
|     | 17 | 14 | 8.4 (0.3, 4.7) | 5 | 6.9 (-16.6, 22.0) | 0.96 |

ECP ng/ml | 4 | 16 | 1.8 (-0.9, 74.7) | 10 | 0.0 (-26.6, 7.6) | 0.31 |

Table 8.2b Change from baseline in soluble mediators in sputum in subjects with colds

<table>
<thead>
<tr>
<th>Day</th>
<th>Viral wheezers</th>
<th>Controls</th>
<th>Multivariate analysis p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean difference (95% CI)</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8 pg/ml</td>
<td>2</td>
<td>17</td>
<td>1470 (392, 2548)</td>
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<tr>
<td></td>
<td>4</td>
<td>19</td>
<td>1880 (185, 3575)</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>16</td>
<td>-866 (-2312, 580)</td>
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</tbody>
</table>

Median difference (quartiles) | Median difference (quartiles) | Mann-Whitney U test p-values |

| IFN-γ pg/ml | 2 | 13 | 00.0 (-45.0, 142.2) | 8 | 38.4 (0.0, 727.0) | 0.22 |
|     | 4 | 15 | 00.0 (0.0, 368.1) | 9 | 24.3 (0.0, 235.4) | 0.88 |
|     | 17 | 13 | 00.0 (-61.9, 000.0) | 3 | 99.0 (0.0, 645.3) | 0.10 |

ECP ng/ml | 4 | 13 | 3.9 (-0.5, 98.2) | 6 | 0.2 (-37.3, 23.1) | 0.35 |

T – time effect, G – group effect, T-G – time-group interaction
Figure 8.1  Difference in IL-8 (mean (95% CI)) from baseline during the study in viral-wheezeers (■) and control subjects (□) in the upper (8.1a) and lower (8.1b) respiratory tracts.

**Figure 8.1a**

![Graph showing IL-8 (pg/ml of nasal secretions) over days after inoculation.](image)

**Days after inoculation**

**Figure 8.1b**

![Graph showing IL-8 (pg/ml of sputum) over days after inoculation.](image)

**Days after inoculation**
Chapter 9  
Identification of human coronavirus in biopsies from the lower respiratory tract

The methodological work-up to attempting to identify HCoV in the LRT went through several stages, the results of which are given in section 4.7. These steps included:

A. In situ hybridisation of biopsies – conditions for successful hybridisation were established using the C21 probe, however, this method failed due to the lack of a satisfactory positive control demonstrating hybridisation with HCoV probes.

B. Single-round PCR for HCoV RNA – HCoV was not identified using the 40 cycle PCR despite good positive controls. This may have resulted from the RNA extraction from the bronchial biopsies as only small amounts of RNA were successfully extracted.

The final two methods used to identify HCoV RNA in the LRT included a nested RT-PCR and Southern blotting for HCoV RNA and RT-PCR for RNA in induced sputum.

9.1 Nested RT-PCR and Southern blotting for the identification of HCoV in the RNA extract

Horizontal gel electrophoresis showed one strong positive and 4 weak positive results (Figure 9.1). The positive controls (RNA extracted from infected cultured cells) were both strongly positive at two different dilutions and all negative controls were negative. The strongly positive result was from a viral wheeze subject on day 4 with a definite cold (Table 9.1) who also had lower respiratory symptoms. Weakly positive results were from a control without a cold on day 4 and two viral wheezers 6 weeks after having colds (Table 9.1). There were 10 samples positive for GAPDH and viral RNA was identified in 4 of these samples (Table 9.1).
The southern blots revealed positives in 2 viral wheezers and 2 controls on day 4 and one viral wheezer at 3 weeks (Figure 9.2 and Table 9.1). One positive viral wheezer on day 4 also had a positive signal in the RT-ve control suggesting cross-contamination during the RT-PCR process. One day 4 and two follow up biopsies with RT-PCR positive did not have corresponding positives in the Southern blot. Three Southern blot positives did not have corresponding RT-PCR positives. The discordance between these two assays and the poor quality of the Southern blot throws doubt on the validity of the Southern blot in particular.

9.2 RT-PCR for HCoV in sputum

The identification of HCoV RNA in the stored aliquotes of induced sputum involved two stages, confirmation of adequate RNA extraction using RT-PCR for GAPDH and RT-PCR for HCoV.

9.2.1 RT-PCR for GAPDH in extracted RNA

5-10μl aliquotes were stored at −80°C from 63 samples of sputum induced on either day 2 or 4 post-inoculation. RT-PCR for GAPDH was performed on these and RNA extracted from tonsil tissue that acted as a positive control. Again, each sample had an RT-ve negative control.

Results. 28/63 RNA extracts were positive for GAPDH. The tonsil was also positive and no RT-ve became positive.

9.2.2 RT-PCR for HCoV 229E RNA

Only one sample was positive for HCoV (Figure 9.3).
9.3 Conclusions

HCoV 229E virus RNA can be identified with bronchial biopsies 4 days and 6 weeks after being inoculated with laboratory prepared virus. Virus RNA can also be found in those without colds. RT-PCR for HCoV in induced sputum was not successful, although one sample was positive. It is unlikely that these results reflect contamination of samples as none of the negative controls or RT-negative controls became falsely positive through contamination and at all times the positive control samples were processed at a separate bench to reduce the risk of such contamination (see sections 4.2 and 4.7.2). These results will be discussed further.
### 9.4 Tables and figures

<table>
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<th>Bx No.</th>
<th>Stud No.</th>
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<th>LRT symptoms</th>
<th>Day/week</th>
<th>GAPDH RT-PCR</th>
<th>Nested RT-PCR</th>
<th>Southern blot</th>
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</table>

*C = contamination of RT-ve control. NB. Samples 12+13 = Surgically removed nasal turbinate with and without infection with HCoV were run as a potential controls for future work.*
Figure 9.1 Gel electrophoresis of nested RT-PCR of RNA extract from bronchial biopsies — 10 subjects at day 4 post inoculation and 5 at 6 weeks later.

a. Tracks are as follows: 100bp ladder, RT+ then RT-ve for 10 samples. Samples (with positives shown as bold) are:

L 1 - 2 - 3 - 4 - 5 - 6 - 7 - 8 - 9 - 10 -

b. Tracks are as follows: 100bp ladder (L), RT+ then RT-ve for 7 samples, +ve neat, +ve 1:10 dilute, -ve. Samples (with positives shown as bold) are:

L 11 - 12 - 13 - 14 - 15 - 16 - 17 - +ve +ve -ve

+ve = positive control (RNA extracted from cell cultures infected with HCoV 229E).
Figure 9.2  Southern blot of gel containing product from the nested RT-PCR on RNA extracted from bronchial biopsies.

All samples are run in duplicate track as RT+ then RT-. Biopsy numbers (with positives in bold) are as follows:

L 1 - 2 - 3 - 4 - 5 - 6 - 7 - 8 -* 9 - 10 -

* RT-ve track showing as positive – therefore contaminated.
Dashed lines indicate positive areas.
L = 100 kb ladder

Figure 9.3  Gel electrophoresis of product of RT-PCR for HCoV 229E on sputum samples.
Tracks contain individual sputum samples.
S = positive sputum sample
N = negative control
+ves = two positive controls (RNA extracted from cultured cells infected with HCoV 229E).
L = 100kb ladder
PART IV - DISCUSSION
Chapter 10   Discussion

Wheezing, a noise of musical quality due to a reduction in airway caliber, occurs in different individuals in response to a wide range of stimuli to the airways. Some of the major stimuli are URTIs with 'common cold' viruses. Many common cold virus infections can result in a wheezing response. HRV commonly causes exacerbations of asthma, as do adenovirus, parainfluenza virus, influenza virus, RSV and HCoV. These viruses have also been associated with exacerbations of wheeze in young children, many of whom do not have the typical asthma phenotype and do not go on to develop asthma. It is now widely accepted that there are different phenotypes of wheeze on the basis of clinical observations and epidemiology. Episodic viral wheeze occurs without typical allergic asthmatic interval symptoms and is an illness that affects young children predominantly, although there is evidence that it extends into adult life. Although some viruses seem to produce a more severe wheezing illness than others, the fact that a range of different viruses can lead to the same symptomatic response strongly suggests that it is the host response to the stimulus that is key to understanding why some wheeze and others do not.

To increase our understanding of episodic viral wheeze, invasive samples carefully timed with the onset of the illness are necessary. Invasive samples would not be ethical in young children and taking samples during wild-type infections would incur difficulties due to the variability of timing from illness onset. As it is the host response, rather than viral factors, that is important in this illness, an experimental infection in adults with a history of episodic viral wheeze was chosen as the method to investigate mechanisms behind this illness.
The experimental infection was successful at inducing URT and systemic symptoms, and in those with viral wheeze, LRT symptoms and increased airway responsiveness. It was possible to validate symptom scores with laboratory evidence of infection. The experimental infection was therefore successful and was a suitable tool for developing an adult model of viral wheeze with the aim of investigating the underlying mechanisms.

10.1 Mechanisms of viral wheeze — the contribution of this study

10.1.1 Physiology

We have demonstrated the potential for excessive airway narrowing in adults with viral wheeze. This is the first study to demonstrate this in any subject group with viral wheeze. A particularly interesting finding was that the increase in bronchial responsiveness persisted beyond LRT symptoms. This is also the first study to establish that infection with HCoV 229E can increase bronchial responsiveness in susceptible individuals. The precise mechanisms of increased airway responsiveness are unknown but are likely to involve a complex interaction of different factors. One factor is airway wall structure, which alters airway geometry causing differences in the host response to constrictor stimuli. In those experimental studies in which HRV caused an increase in airway responsiveness there was no accompanying decrease in FEV₁ suggesting that mechanisms other than geometric are involved, although FEV₁ alone is a rather crude measure of airway geometry. One study of 20 healthy volunteers with ‘wild’ colds, three of which were due to coronavirus, demonstrated a small but significant decrease in FEV₁ along with increased airway responsiveness. In the present study, the lower baseline FEV₁ in viral wheezers did not account for the difference in response to methacholine challenge between the two groups after viral inoculation. Indeed, at baseline there was no difference in PC₂₀ between viral wheezers and controls despite the difference in FEV₁. During
the symptomatic phase there was a small decrease in airway caliber associated with cough, shortness of breath and wheeze, but this too cannot account for the progressive decline in $PC_{20}$ at day 17, well after lung function and symptoms had returned to normal. We can conclude that the small changes in $FEV_1$ were not responsible for the change in airway responsiveness.

Whatever the mechanisms of the increased bronchial responsiveness, its occurrence in both atopic and non-atopic individuals, regardless of the small difference in baseline airway responsiveness, suggests that our subjects have a disorder which differs from classical atopic asthma.

The link between an URTI and increased bronchial responsiveness must involve subtle changes that can persist for some time. Several potential mechanisms have been suggested including LRT inflammation (reviewed by Folkerts et al \(^{335}\)), impairment of $\beta$-adrenoceptor and $M_2$ muscarinic receptors leading to smooth muscle constriction \(^{66}\), and persistence of virus in the LRT \(^{336}\). The latter was investigated in this study and is discussed in section 10.1.3. How these potential mechanisms relate to one another in the pathogenesis of LRT symptoms and changes in lung function during common respiratory virus infections, are still largely unknown.

### 10.1.2 Inflammatory changes in the upper and lower respiratory tracts

Inflammatory changes have been demonstrated in this experimental model. We have demonstrated for the first time an association between elevated nasal IL-8 levels and HCoV induced URT symptoms thus confirming similar findings for HRV \(^{34;150}\) and wild infections \(^{90}\). We have also established an association between neutrophilic inflammation and URT symptoms as has recently been demonstrated with HRV-16 \(^{150}\). IL-8 increased in both upper and lower respiratory tracts in both viral wheezers and controls with colds, the largest change being seen on day 4 in the sputum of viral wheezers. Only modest changes were seen in IFN-$\gamma$ and no changes in IL-5 or ECP. Viral wheeze is characterised by neutrophilic inflammation in
both upper and lower respiratory tracts without eosinophilia. IL-8 is likely to be an important chemokine in this process. The reason for low levels of other cytokines may reflect the inherent nature of that cytokine in lavage fluid and sputum. A recent experimental infection of adults with allergic rhinitis with HRV-16 managed to identify IFNγ and IL-5 with more success using RT-PCR for cytokine mRNA 150, something that should be borne in mind for future studies.

Neutrophils are clearly important in the 'common cold'. The role of neutrophils in LRT symptoms is less clear. Whilst we have demonstrated a correlation between increase in sputum neutrophils and both LRT symptoms and increased airway responsiveness, airway responsiveness persisted beyond the period of relative neutrophilia. No other study of viral URTI, with the exception of RSV bronchiolitis 118, has found a direct relationship between the LRT neutrophil cell differential count and LRT symptoms. How then do we interpret the results?

Traditionally it has been proposed that neutrophils limit the damage caused by viral respiratory tract infection, although they may also cause tissue damage through the release of cytotoxic granule-derived proteins such as elastase and myeloperoxidase, free radicals and lipid-derived mediators such as LTB₄. If neutrophils do cause LRT tissue damage in those who wheeze then clearly these cells could be involved in the pathogenesis of viral wheeze.

Stevenson and colleagues studied opportunistically collected bronchoalveolar lavage (BAL) fluid from young children with a history of viral wheeze and found no evidence of chronic inflammation during asymptomatic periods, in comparison with atopic asthmatics who had increased eosinophil and mast cell differential counts 131. We have confirmed the findings that viral wheeze is not characterised by chronic eosinophilic inflammation in the LRT, as is seen in
atopic asthma, and have also demonstrated that eosinophils are not involved in the acute illness, as appears to be the case in acute viral exacerbations of atopic asthma.\textsuperscript{31,38,42}

This is the first time that upper and lower respiratory tract inflammation has been studied in this manner during an experimental infection with HCoV, a virus known to be associated with wheezing illnesses. It is the second study to compare inflammatory changes in both upper and lower respiratory tracts in an experimental infection and the first to develop a model of episodic viral wheeze, as distinct from chronic atopic asthma. Flemming and colleagues inoculated 11 atopic asthmatics and 10 controls with HRV-16, but despite nearly all developing a symptomatic cold, only one asthmatic suffered significant LRT symptoms and it was the controls rather than the asthmatics that had a small increase in airway responsiveness.\textsuperscript{39} The authors of this study suggested their unexpected data could have resulted from methodological issues such as patient or virus selection, design or technical factors. They used a group of mild asthmatics and did not specifically assess whether viruses triggered their asthma. HRV-16 has been shown to cause LRT effects in several other studies.\textsuperscript{43,50,150,252} It is possible that attenuation of the virus occurs in laboratory preparation of the inoculum. These issues are discussed further in section 10.2.

\textbf{10.1.3 Direct versus indirect mechanisms}

The potential mechanisms that may lead to viral wheeze have been categorised as direct or indirect (see section 1.3.2). Direct implies infection of the LRT leading to local effects that in turn result in the release of inflammatory markers and alteration in physiology described in this model. If direct LRT infection occurs, the differences seen between viral wheezers and controls could result from:

- a qualitative difference in the site of infection – i.e. the viral wheezers are predisposed to direct infection of the LRT whereas the controls are not;
• a qualitative or quantitative difference between the two groups in response to direct infection of the LRT occurring in both groups;
• a quantitative difference between the two groups in the extent of infection of the LRT.

Our results are consistent with the findings of Papadopoulos and colleagues, and Gern and colleagues, by identifying virus genome in bronchial biopsies. Virus was found in both a viral wheezer (1 out of 6) and a control (1 out of 4) subject. Papadopoulos and Gern also discovered virus in controls. It therefore seems likely that the location of infection does not differentiate between the two groups and is unlikely to be the explanation why some wheeze and others do not.

The data presented here regarding the presence of viral infection in the LRT do not allow us to safely conclude that direct infection of the LRT is a key factor in the mechanism of viral wheeze. It was not possible to identify virus in situ in biopsies due to technical factors and in only 2/10 samples was viral genome identified, despite 8/10 having symptomatic colds. The technique used (nested RT-PCR) is highly sensitive as well as specific and will identify very small amounts of viral genome. Perhaps it is not surprising that small amounts of virus genome can be found in the LRT 4 days after large amounts of virus are instilled into the URT. Other factors too could lead to falsely positive results. These include contamination of samples from a bronchoscope passed through the URT, the lack of appropriate controls and the possibility of contamination in the laboratory. It remains uncertain whether LRT effects are due solely to LRT infection or whether some unknown indirect mechanism stemming from URT infection also play a role.

It is of interest that virus was also identified in biopsies of the LRT 6 weeks later in 2/5 subjects. Papadopoulos and colleagues also identified HRV several weeks after inoculation.
and concluded that a wild-type infection was responsible. An alternative interpretation might be the persistence of genome within the LRT or even the possibility of a chronic low-grade infection.

It may be that in viral wheezers, a greater degree of infection in the LRT is responsible for some of their features (e.g. bronchial hyperresponsiveness, neutrophilic inflammation and LRT symptoms). However, the techniques available for virus identification in the LRT did not allow quantification. How such differences could occur is not known but may involve a complex interaction of virus and host defense mechanisms. One potential explanation is the balance of Type-1 and Type-2 responses to infection. A recent experimental infection with HRV-16 in adults with allergic rhinitis has demonstrated that a predominantly Type-2 response (assessed by the ratio of IFNγ/IL-5 measured by RT-PCR of mRNA in sputum) was associated with greater URT symptoms. As Type-1 cytokines are associated with viral clearance, a weakness of this type of response could be associated with greater or prolonged viral infection. This potential explanation for viral wheeze requires investigation.

It is also possible that it is the host response to LRT infection that differentiates between the two groups, although we could not identify any major differences in the mediators measured in this study.

Alternatively, the response seen in viral wheezers may reflect a predisposition to a quantitatively greater degree of virus replication in the URT, and by implication, inflammation in the URT. Although RT-PCR of nasal samples in this study did not allow us to quantify the degree of virus replication, viral wheezers had significantly more severe URT symptoms than controls. Asthmatics have also been shown to report greater URT symptoms in experimental colds, and also greater nasal excretion HRV during wild-type infection when compared with
controls. Whether this apparent predisposition to a more severe URT illness in the viral wheeze group is related to the degree of epithelial infection or to an abnormal host response to a similar degree of infection in both the URT and LRT, is not known.

Whether or not LRT infection occurs, there may be indirect effects on the LRT from the infected and inflamed URT. Breathing through an inflamed URT is likely to alter the quality of inhaled air and may carry inflammatory products, such as cytokines and nitric oxide, to the LRT. If neural control of airway diameter is influenced by changes in the URT this too could result in LRT effects from an URTI. Finally, a systemic response to URT inflammation, such as T cell activation or chemokine release, could lead to LRT effects if such cells and mediators can ‘home’ to the LRT. There is little data supporting any of these indirect connections between upper and lower respiratory tracts but abnormal responses occurring in these routes could contribute to developing wheeze during viral URTI.

10.2 Methodological issues
10.2.1 Study design
Controls. This study was a parallel, non-placebo, controlled study. The vast majority of previous experimental infections have no control groups and compared changes during symptomatic infections with baseline, pre-inoculation measurements. This opens studies to criticism as one cannot attribute changes seen in illness groups to the illness without healthy controls and one cannot exclude the influence of a ‘placebo effect’. Although this study uses a healthy control group we cannot account for the possibility of a placebo effect. All volunteers were inoculated with active virus and it is entirely possible that the knowledge of this could have influenced various results. The inoculum also contains debris from the cell cultures used to grow the virus and there may be cell products that could exert some effect on the nasal
epithelium. The lack of a sham infected group leads to the criticism that the effects measured might not be totally due to virus infection.

A further potential problem is that asymptomatic controls could have had asymptomatic infections, indeed 1 of the 8 controls without colds had a positive RT-PCR nasal sample. This could have the effect of reducing quantitative differences between symptomatic and asymptomatic groups. On the other hand, grouping the 3 control subjects with ‘possible’ colds that had PCR positive results with the definite controls could have introduced a bias as there were no viral wheezers with ‘possible’ colds. This would inevitably act to increase the quantitative difference between groups as those with ‘possible’ colds would by definition have milder colds. Indeed this was the case for systemic symptoms as the difference between groups was no longer significant when the controls with ‘possible’ colds were excluded. However, the analysis with ‘possible’ colds were excluded did not change the overall findings that viral wheezers suffered more severe URT and LRT symptoms. Of course, it may be that viral wheezers do suffer more severe symptoms and therefore the controls are more likely to have minor symptoms and be classified as having ‘possible’ colds. The use of PCR to back up colds in these circumstances adds some weight to grouping definite and possible colds together, rather than lose important data.

A group infected blindly with a sham inoculum would have been ideal. The reasons for not including such a control include the expensive and time-consuming nature of experimental infections and also the difficulty in choosing an adequate sham inoculum. In a pilot study involving the author of this thesis in which the effects of HCoV 229E on nasal cilia structure and function were investigated, a small sham group were included. The sham chosen was ultra-violet inactivated virus so as to include all possible ‘by-products’ contained in the inoculum as a result of manufacture and storage. In contrast to the uniformly cytopathic effect
of active virus inoculum, inoculum containing ultra-violet inactivated virus did not have an
effect on nasal ciliary structure or function. It therefore seems unlikely that products in the
inoculum other than active virus were responsible for the effects seen. Sham controls are rarely
used in experimental common cold infections (the author could identify only 2 experimental
infections using a sham control group).  

Subject numbers. The aim of this project was to explore potential mechanisms of viral
wheeze and as such did not have an overriding primary outcome. A power calculation was
therefore not possible. From the methodology study of inoculation (section 2.1) it was expected
that each group would have 10 – 13 symptomatic infections. This would give three control
groups to compare with symptomatic viral wheezers: asymptomatic viral wheezers (in order to
identify the specific effects of virus infection in viral wheezers), symptomatic controls
(permitting the identification of symptoms, physiology and inflammation specific to viral
wheezers), and asymptomatic controls (controlling for random changes in outcome
measurements). The rate of symptomatic infection was greater in the viral wheeze group
(19/24) compared with the control group (11/19) leaving a small asymptomatic viral wheeze
group. Hence no meaningful comparisons could be made between symptomatic and
asymptomatic viral wheezers.

Duration. The study was run over a period of 17 days, with sampling on days 0, 2, 4, and 17.
The choice of days 0 (baseline), 2 and 4 has been discussed in the introduction (section 2.2
page 77). Sampling on day 17 was aimed at assessing the return of variables towards baseline
as most symptoms of a common cold are resolved by approximately 7 – 10 days. Day 17 fitted
in with the working week (days 0, 2, 4, 17 = Monday, Wednesday, Friday, and Thursday,
respectively). In hindsight it may have been preferable to extend the follow up beyond 17 days,
as lung physiology was still abnormal. This should be done in future studies.
10.2.2 Subjects

Subject recruitment. Subjects for this study were recruited from health centres attached to three local universities in order to obtain young and otherwise healthy adults. Patients known to wheeze but not on treatment with inhaled corticosteroids were approached by questionnaire, and those that reported typical asthma symptoms excluded. Subjects were not screened for the presence or absence of antibodies to HCoV 229E (this is discussed in section 10.2.2). As such, the subjects were biased only in that they were young, relatively healthy and reported mild illness. A 25-year follow up of school children with viral wheeze suggests that such subjects may continue to exhibit relatively mild symptoms \(^8\). There is no reliable evidence that subjects had viral wheeze as children, or that their current episodic symptoms mimic those of young children with episodic viral wheeze.

Subject characteristics – baseline data. Symptom scores were conducted from the day of inoculation until day 17 without the collection of a long baseline period. The subjects were screened by questionnaire and only those without daily symptoms included, however it might have been preferable to assess baseline observations over several weeks to establish with less subjectivity if viral wheezers did suffer any daily symptoms or changes in physiology. This could have been important as if there were daily or regular symptoms then differences between the groups during the cold could have been affected by starting from different baselines. The data from day 0 was collected prior to inoculation, and although very limited, there was no difference between groups in symptoms and airway responsiveness, although the viral wheezers did start with a lower FEV\(_1\) and PEF. In retrospect it would have been useful to measure baseline physiology for some time prior to the experiment in order to add weight to the assertion that our subjects with a history of episodic viral wheeze behaved differently from classical asthmatics (e.g. without PEF variability). One criticism may be that our group are
meerly mild asthmatics and any additional evidence to the histories and airway hyperresponsiveness would have been useful.

**Subject characteristics – FEV₁.** There was a small but significant difference in baseline FEV₁ that was not appreciated until data were analysed. This might suggest that the viral wheeze group simply contained mild asthmatics. However, there are several reasons to reject this. They wheezed only during URTIs and not in other circumstances (e.g. during exercise, with contact with dust); the vast majority had baseline bronchial responsiveness within the healthy range; only one had ever received a physician diagnosis of asthma. In retrospect it may have been useful to test for reversibility with a bronchodilator, and to use more sensitive lung function measurements (FEF₂₅, for example). An attempt was made to record FVC but this data was lost due to computer failure.

**Subject characteristics – Atopy.** The two groups were identical in most characteristics, although there were more atopics in the viral wheeze group. However, atopy and allergic rhinitis were found not to be factors in the development of increased hyperresponsiveness in this model, although atopy is thought to predispose an individual to a more severe URT illness in experimental HRV and HCoV 229E infection. In the current study, there was no difference between atopic and non-atopic subjects in either symptom severity or nasal neutrophil response to the infection. There was a trend towards a higher IL-8 response to HCoV 229E infection in atopic subjects suggesting that atopy could have some influence on the production of this cytokine, however there was a wide range of values and the difference was not significant. These results are largely in keeping with a recent study of nasal inflammatory mediators during naturally acquired colds in which there was little difference between atopic and non-atopic subjects during the acute phase, although non-atopics had higher levels of IL-10. Interestingly, only the atopic subjects had persistent elevation of
nasal cytokines beyond symptoms, but the significance of this is unclear. Atopy does not fully account for the inflammatory response seen in the viral wheezers.

Allergic rhinitis is known to predispose subjects to more severe URT symptoms \(^{45,150,229}\), but as there were only 4 subjects with a history of allergic rhinitis in each group, this also is unlikely to account for the inflammatory response seen.

10.2.3 The virus and its identification

**The inoculum.** This model used a virus passaged in cell culture and inoculated into the nose in high titre. It is theoretically possible that the use of a virus that may have been attenuated by laboratory passage and applied in an artificial way to the nose, might result in a different illness to that seen in wild infections. However, previous experimental inoculation studies have confirmed that the disease spectrum seen with HCV 229E inoculation is close to that of wild-type infection \(^{105,133}\). Such a model has the major advantage of avoiding virus heterogeneity and imprecise timing of the onset of infection that make the detailed study of the physiological and biological responses to 'wild' infections very difficult.

**Sample contamination.** An alternative explanation for the presence of virus in the samples of 3 subjects without symptoms of a cold is sample contamination at the time of collection or in the laboratory. The former is unlikely as two of these subjects were sampled in isolation making contamination at this stage highly unlikely. The later was an apparent problem when using the nested RT-PCR developed by Myint and colleagues \(^{28}\) however with the one-round RT-PCR none of the negative assay controls became positive during the assay, making contamination during the assay unlikely. It seems likely that the three did have asymptomatic colds, a phenomenon that has been described previously \(^{105,222,228,341}\) and which has been
demonstrated by the author in a study showing nasal epithelial cytopathic effects in asymptomatic individuals

**Virus identification.** When trying to understand the role of PCR and ELISA in laboratory diagnosis of an infection it is necessary to understand their advantages and disadvantages compared to older 'classical' techniques. Culture methods involve the inoculation of nasal specimens into cell culture and typical cytopathic effects seen on microscopy are used to detect virus. Such methods are relatively successful for the diagnosis or respiratory viral infections such as influenza, PIV, RSV and adenovirus. However, HRV and HCoV do not grow on standard cell lines and antibodies are not easily detected. It is generally accepted that cell cultures are unreliable for the primary isolation of HCoVs due to poor growth and the lack of easily recognisable cytopathic effects. This means that HCoV and HRV were frequently not reported in epidemiology surveys, and if they were, suboptimal tests may have underestimated their prevalence. Even with the most exhaustive testing, including serology and organ culture, under-diagnosis is evident, with infectious agents being demonstrated in only 60% of common colds. The advent of RT-PCR techniques has greatly improved our ability to identify viruses such as HRV and HCoV.

**RT-PCR.** The failure to identify virus genome in all subjects with colds is common to most experimental infections. Possible explanations include coincidental wild-type virus infection and false negative RT-PCR results. We found no evidence of the most common wild-type infections (rhinovirus, RSV, adenovirus, influenza A and B virus and PIV 1,2,3) by culture, although there was no means of differentiating coincidental wild-type HCoV 229E infection from our inoculated virus. There was however, a strong temporal relationship between inoculation and onset of symptoms suggesting strongly that infections were indeed due to our experimental virus. Considering the possibility of false negative RT-PCR results, three subjects
with definite colds had no laboratory confirmation. There are several possible reasons why RT-PCR might not identify virus in all those with symptomatic colds, the most likely being natural RNAse in nasal samples which degrades the viral genome. In addition, even with due care during the PCR process, RNAse contamination will reduce the positive rates.

**Differences between RT-PCR and ELISA.** The lower rate of ELISA positives compared with RT-PCR implies either a difference in the sensitivities of the assays, a lack of specificity of the RT-PCR assay (i.e. false positives), the presence of viral antigen without infection 2 to 4 days after inoculation or failure of the ELISA assay (i.e. false negatives). These possibilities are dealt with in turn.

**Difference in sensitivity of the assays.** RT-PCR is a powerful tool and is known to be very sensitive. Two recent surveys used standard enzyme immunoassays on paired sera for identification of common respiratory viruses, including HCoV \(^{19,20}\). The first also used RT-PCR to identify HRV and the second to identify both HRV and HCoV. Nicholson and colleagues studied 138 adults who had 315 exacerbations of asthma and found 80% of episodes were associated with a symptomatic URTI. Approximately 50% of samples had a confirmed viral infection, with HRV accounting for 64% and HCoV for 30%. They compared cell culture for HRV to RT-PCR. Eight percent of those tested by cell culture were positive with HRV cytopathic effect and 33%, including the 8% culture positives, were positive by RT-PCR. In the second, Johnston and colleagues studied 108 children aged 9-11 years with asthma who had 292 reported exacerbations. Viruses were detected in 77% of episodes with HRV accounting for 65% and HCoV for 17% of virus isolates. RT-PCR for HCoV picked up 21% of 80 samples analysed as positive compared to 5% and 7% of 292 for culture and ELISA, respectively. The current study is consistent with these studies in identifying more positives by RT-PCR than ELISA. RT-PCR is a highly sensitive and specific assay that is superior to serology for HCoV.
detection. It is certainly more sensitive than culture as HCoV does not grow on standard cell lines or produce easily recognisable cytopathic effects. It is for this latter reason that culture was not attempted in this study.

**False positive RT-PCR.** False-positives from contamination during the RT-PCR assays are unlikely as none were detected in the duplicate reverse transcriptase negative control samples. The RT-PCR assay for HCoV 229E is highly specific with primers designed specifically to target the N-gene of the virus.

**The persistence of viral antigen.** The persistence of viral RNA from the initial inoculum for 2 to 4 days is highly unlikely in an environment containing abundant RNAse. Our laboratory results back up the clinical data with 27/31 (87%) colds being laboratory positive compared to 3/12 (25%) with no symptomatic URTI.

**The failure of the ELISA assay.** There are several possible reasons for failure of the ELISA. Firstly, the ELISA is dependent on the quality of materials. Viral antigens were concentrated by ultra-centrifugation and the secondary antibody was a rabbit anti-human IgG. Non-specific binding may interfere with the detection of significant changes in antibody. Future improvements may come from the preparation of purified antigen and the development of a specific anti-coronavirus 229E antibody, which is not currently available. Secondly, the ELISA was conducted over a shorter period than reported previously and we may have missed some late but significant antibody rises. Finally the ELISA for HCoV 229E does not detect a 4-fold rise in antibody levels as occurs with other virus antibody assays. For these reasons the validity of the assay is questionable.
Validity of ELISA. Prior to the development of RT-PCR, ELISA was the most widely used method of detecting HCoVs. This technique was developed by Kraaijeveld and colleagues and most subsequent publications have based their methods on this. The tradition of looking for a 4-fold rise in antibody titres to signify infection seems not to apply to HCoVs. Kraaijeveld and colleagues assessed acute and convalescent serum 24 days apart and defined a significant rise as a difference of more than 2 standard deviations of log antibody units. This method has been used widely and reported in the recent epidemiological survey by Johnston and colleagues. Two other recent studies by Nicholson and colleagues and Trigg and colleagues used a similar ELISA but defined positive samples as those with a ratio > 1.3 between convalescent and acute samples. Looking further back, there have been reports of 4 fold rises but these were in neutralisation tests or haemaglutination tests. A more recent paper which reports a 4 fold rise in samples taken 3-4 weeks apart does not quote any figures on antibody levels yet quotes the Kraaijeveld paper as a methodology reference. This group used the Leicester laboratory to perform the ELISA and hence used similar standards to the current study. Correspondence with the author did not clarify the disparity. It is not usual to see a 4-fold rise in antibody with a HCoV infection. Because of this, we decided to define a significant rise in antibody in a more detailed manner than previously published.

We sampled 17 healthy volunteers 17 days apart (the same interval as for the main study) so as to ascertain the natural variance in antibody levels. The upper 95% CI of the ratios of antibody levels by ELISA (day 17/0) was 1.25, a level comparable to that quoted by Nicholson and colleagues and Trigg and colleagues (= 1.3). Although the ELISA assay may not be a very sensitive assay it was useful in validating the symptom scores with 13/24 and 0/4 subjects assayed with ‘definite’ and ‘no’ colds having a significant rise in antibody levels, respectively.
Season. The study was conducted outside winter months to reduce the possibility of interference from wild-type viruses. HCoV 229E accounts for approximately 50% of HCoV infections (the remainder are due to HCoV OC43). The RT-PCR we used is specific for 229E as is the ELISA and these will not identify OC43. The fact that 24/27 (16/19 viral wheezers + 8/8 controls) had positive identification of 229E suggests that interference with wild-type HCoV was unlikely. The problem of wild-type infections interfering with experimental infections is a criticism that can be made of all experimental infections, including those with HRV as well as HCoV. There is no simple method of differentiating wild-type HCoV from our laboratory grown virus but the chance of wild HCoV 229E virus interfering is extremely low given the clear temporal relationship between inoculation and onset of symptoms seen in all cases, the sporadic nature of wild infections and the season.

Data from the latter stages of the study in 9 subjects, 4 viral wheezers and 5 controls, were excluded on the grounds that they may have suffered a ‘wild-type’ infection after the experimental infection. This was based solely on the symptom diary scores where symptoms clearly fell after the initial infection before rising again. Subjects were at home during these stages of the study and samples could not be collected to confirm this.

10.2.4 Symptoms and physiology

Symptoms. This study categorised subjects into groups based upon clinical symptoms of a cold assessed as a score in a diary. The assessment is open to the criticism of subjectivity, but there is no other way to define “a cold”. The use of symptoms to identify a clinical cold goes back to the 1950s when common cold research was in its infancy. There are several ‘classical’ symptoms associated with a common cold and indeed the common symptoms associated with coronavirus common colds have been reported in several experimental infections. These common symptoms have formed the basis of symptom scores that are reported in all
recent experimental infections 41,50,56. Such scores have also been combined with scores of lower respiratory tract symptoms in the study of viral induced asthma 19,20, although the relevance of such scores is not clear. URT symptom scores, when assessed blindly along with a clear definition of a significant cold that is temporally related to the inoculation, are arguably as valid as relying on laboratory methods that have their own flaws. However, a problem does arise from the reporting of mild symptoms (low scores) and when asymptomatic infections might occur. Here laboratory confirmation of infection is vital in categorising colds. While there are still significant limitations in laboratory viral diagnosis, as there are with HCoV 229E, clinical assessment of symptoms is a useful and valid tool. Despite the occurrence of asymptomatic infections, only symptomatic colds were associated with wheeze and this is the group the study was ultimately interested in.

10.2.5 Sampling

Technical considerations surround nasal lavage and sputum induction. These have been discussed in detail in sections 3.3.1 and 3.4.1, respectively. Although recruitment and/or washout of cells could occur when nasal lavage is repeated, the fact that no such changes were seen in the asymptomatic controls makes this unlikely in lavages repeated over a 48-hour period. This is consistent with previous studies 105,259.

Concerns have been raised about repeated sputum induction irritating the airways and causing an increase in neutrophils and ECP (discussed in section 3.4.1). There have been no repeatability studies over a 48-hour period. The present study used a low dose of hypertonic saline given at a rate of 0.9 ml/min and no changes in neutrophils were seen in the sputum of controls, with or without colds, despite repeated inductions 48 hours apart. This strongly suggests no significant effect.
Repeatability of cell differentials was very good for macrophages and neutrophils. However, for samples with only a few eosinophils (0.3 – 2%) and very few lymphocytes (0-1%), when 300 random cells are counted on a cytospin, significant random error may arise, hence the lower levels of repeatability. The present study was not powered to detect changes in such small numbers that may in any case have doubtful clinical relevance.

The volume of sputum samples varied considerably, hence it is conventional to present cell differential counts rather than absolute cell counts. It is impossible to know whether a change in differential count is due to a relative or absolute change in cell numbers or (more probably) a combination of the two. In reality we cannot say whether viral infection caused an increase in neutrophils, a decrease in macrophages, a relatively larger increase in neutrophils than macrophages or a relatively larger decrease in macrophages than neutrophils. However, the presence of an increase in sputum IL-8 would suggest that there was an increase in neutrophils. Confirmation could have been sought by measuring a neutrophil activation enzyme, such as myeloperoxidase. Sputum is one step removed from the mucosa and submucosa where inflammatory changes may be closely linked to alterations in lung physiology. Indeed, the study of biopsies in atopic asthmatics has confirmed the influx of eosinophils and lymphocytes during a HRV cold 42. Although we found no evidence for a role of eosinophils, a more comprehensive study of bronchial biopsy material would enable a more detailed assessment of the role of these cells in viral wheeze.

The identification of IL-5 and IFNγ in sputum and nasal secretions proved difficult by ELISA. One of the reasons for this is believed to be the break-down of the certain cytokines by sputum proteases. In a recent paper sputum was processed with and without protease inhibitor and found improved recovery of IL-5 with minimal effects on cell counts and viability 345. This should be considered in future experiments.
10.2.6 Identification of virus in the LRT

This study used a methodical approach in attempting to determine whether HCoV 229E infects the LRT. The steps included:

- determining the optimal conditions for *in situ* hybridisation;
- preparation and testing of a positive control sample;
- identifying virus genome by RT-PCR in RNA extracted from bronchial biopsy material;
- confirmation of viral RNA identification by Southern blotting.

The initial aim was to identify virus *in situ* within biopsy material so as to avoid the criticism of sample contamination as the bronchoscope is passed through the URT. It was not possible in the time available to find an adequate positive control for the development and assessment of specific probes, so that *in situ* hybridisation was not possible. The finding of viral genome in extracted RNA from 2 samples during colds and 2 samples 6 weeks later is therefore open to the criticism of possible URT contamination.

10.3 Future studies

There is still a great deal to understand in the complex mechanisms that lead an individual to wheeze when they 'catch a cold'. Developing this experimental model could provide one method by which these mechanisms could be further studied. Areas that warrant further study included development of methodology, the immune and inflammatory response, and host and viral factors.

10.3.1 Methodology development
There are several lessons learnt from the present study that could be incorporated into future studies.

**Design.** The 'highest' level of evidence in clinical studies comes from a double blind, randomised placebo-controlled trial. This study was not blinded and there was no placebo. The reasons for this and the problems that could be associated with this are discussed in section 2.1. A sham infection has now been developed for HCoV 229E, and if incorporated into future studies would increase the relevance of any findings. This would, however, ultimately mean more subjects to be studied in what are lengthy and expensive experiments.

**Subjects.** Many questions remain as to the mechanisms that underlie episodic viral wheeze during childhood and adulthood. This model provides a useful tool for studying this illness. Some would argue that the subject group simply comprised mild asthmatics, but for many reasons discussed in the introduction and above, this is unlikely to be the case. A future study could include a group of atopic asthmatics as well as healthy controls, so as identify any differences in mechanisms between the two groups. The influence of atopy on the outcomes should also be taken into account. Ideally the viral wheeze group should comprise sufficient atopic and non-atopic subjects to allow differences in outcomes to be investigated. Atopy can be based upon skin prick testing, but IgE should also be measured.

Subjects should ideally be screened prior to recruitment for pre-existing neutralising antibodies to the experimental virus. This would undoubtedly improve the infection rate of the experiment, although practical considerations regarding expense and the time taken to screen may be an issue.
Duration. This study identified a prolonged increase in bronchial hyperresponsiveness lasting at least 17 days after inoculation. A future study should carry out additional follow-up measurements at a later stage than this so as to demonstrate the duration of any physiological effects.

Sampling technique. This study has used a simple technique of nasal lavage to collect samples for cellular and fluid-phase measurements. A dilutional effect was seen as a result of the lavage and this should be borne in mind when this technique is used again. Inulin dilution has been validated previously and was used successfully in this study and although only minor changes in the levels of cells and cytokines were seen when measurements were adjusted, this could prove important when looking for small differences.

Hypertonic saline-induced sputum collection proved safe, simple, non-invasive and acceptable to all the subjects. Twenty subjects were offered a bronchoscopy and 10 accepted. This also proved safe, if time consuming and invasive. Induced sputum is an excellent technique for collecting information on airway inflammation from a large group of individuals. It has the disadvantage that it measures cellular changes in what is a waste product of the LRT and may miss important information from the more distal LRT (where BAL would be of use) and from the mucosa and submucosa (where bronchial biopsies would be of use). These issues should be carefully considered when deciding the best technique to obtain samples of interest.

Outcome measures. As the mechanisms of viral wheeze become more clearly defined, studies should focus on specific virus-host interactions as their primary outcome measures. Studies should then be powered to fully explore these specific mechanisms. Based on the current level of understanding, studies will focus on outcome measures in the following areas:
**Duration.** This study identified a prolonged increase in bronchial hyperresponsiveness lasting at least 17 days after inoculation. A future study should carry out additional follow-up measurements at a later stage than this so as to demonstrate the duration of any physiological effects.

**Sampling technique.** This study has used a simple technique of nasal lavage to collect samples for cellular and fluid-phase measurements. A dilutional effect was seen as a result of the lavage and this should be borne in mind when this technique is used again. Inulin dilution has been validated previously and was used successfully in this study and although only minor changes in the levels of cells and cytokines were seen when measurements were adjusted, this could prove important when looking for small differences.

Hypertonic saline-induced sputum collection proved safe, simple, non-invasive and acceptable to all the subjects. Twenty subjects were offered a bronchoscopy and 10 accepted. This also proved safe, if time consuming and invasive. Induced sputum is an excellent technique for collecting information on airway inflammation from a large group of individuals. It has the disadvantage that it measures cellular changes in what is a waste product of the LRT and may miss important information from the more distal LRT (where BAL would be of use) and from the mucosa and submucosa (where bronchial biopsies would be of use). These issues should be carefully considered when deciding the best technique to obtain samples of interest.

**Outcome measures.** As the mechanisms of viral wheeze become more clearly defined, studies should focus on specific virus-host interactions as their primary outcome measures. Studies should then be powered to fully explore these specific mechanisms. Based on the current level of understanding, studies will focus on outcome measures in the following areas:
i) Symptoms and physiology. Groups should still be based upon URT and LRT symptoms as studies are ultimately seeking to understand the mechanisms leading to symptoms. Where laboratory diagnosis of virus infection is well established, these should also be performed so as to validate the symptoms. Ideally, groups should be formed based upon a combination of symptoms and viral diagnosis. Where this is not done, a sham infection will aid the validation of these symptoms (i.e. account for the possibility of a 'placebo effect'). Measures of large and small airway function should be included in future studies, as changes in small airway function could occur without significant changes in large airway function. A standard method of bronchial responsiveness testing should also be included.

ii) Cellular inflammation. The size of studies aimed at investigating cellular inflammation will depend on the cells of interest. The relationship between neutrophils and LRT symptoms could be investigated further using sputum induction and should be powered based upon the changes seen in the current study. It may be preferable to investigate further the eosinophilic and lymphocytic responses using bronchial biopsies so as to examine infiltration into the mucosa and submucosa.

iii) Fluid-phase mediators. Careful consideration of sampling technique is required prior to studying the cytokine or chemokine response to viral infection. The effect of dilution when using BAL may make this less desirable than induced sputum, although sometimes relatively small amounts of sputum are produced in subjects with mild disease.

10.3.2 The immune/inflammatory response

i) The role of neutrophils. There is undoubtedly an inflammatory response both in the upper and lower respiratory tracts during episodes of viral wheeze. The link between neutrophilic inflammation and URT symptoms is well established. More work is required to investigate the
role of LRT neutrophilic inflammation and the possible link to LRT symptoms and airway responsiveness. Of particular interest is the prolonged airway hyperresponsiveness beyond the resolution of the relative neutrophilia. Perhaps the neutrophils are altering the properties of the airways (e.g. damaging the epithelial layer) leading to secondary changes in the mucosa that in turn alter the airway responsiveness. A study of neutrophils in BAL as well as structural and cellular changes in bronchial biopsies during episodes of viral wheeze would improve the detail of the relationship of neutrophils to this disorder. At the same time, neutrophil proteases could be measured to demonstrate neutrophil activation.

ii) The role of eosinophils. Eosinophils are strongly associated with atopic asthma and have been associated with viral exacerbations of asthma. This study presents evidence suggesting these cells are not involved in episodes of viral wheeze. Again, a study looking for the presence of eosinophils in bronchial biopsies during episodes would enable these cells to be ruled out of the mechanisms of viral wheeze.

iii) The role of lymphocytes. There is much current interest in the interactions between lymphocytes and other inflammatory cells that are found in the respiratory tract. Lymphocytes are known to be activated during viral URT infections and lymphocytes are known to be involved in the pathogenesis of allergic asthma with T helper and cytotoxic cells having a typical Type-2 phenotype. It could be possible that these cells link URT infection to LRT inflammatory responses by responding to the local infection/inflammation in the nose and, via the circulation, home to the LRT where it’s effects are generated. This hypothesis remains to be tested.

iv) Immunodevelopment. There is also much interest as to why some young children with episodes of viral wheeze ‘grow out’ of their symptoms whereas others continue with this
episodic pattern of wheeze and others go on to develop the classical phenotype of allergic asthma. The interaction between viral infections in early life and the development of immune cells, such as T lymphocytes, is an area requiring further study. Evidence exists showing that children who develop bronchiolitis during RSV infection and adult asthmatics who wheeze during HRV infections have a relatively poor type-1 lymphocyte reaction. As type-1 cytokines (e.g. IFNγ) are important for clearing virus infection it could be that the persistence of virus infection plays a role in these illnesses.

**iv) Other areas of interest.** The role of cysteinyl leukotrienes and their antagonists in childhood wheeze is of much current interest as leukotriene receptor antagonists are now licensed for asthmatics to the age of 2 years. Young children with viral URTIs and associated wheeze have been shown to have increased cysteinyl leukotrienes in their nasal secretions during episodes. Unreported data from this study does not support a role for leukotriene receptor antagonists in episodic viral wheeze; no change in nasal, sputum or urinary cysteinyl leukotrienes was seen during the study. Whether this could be due to virus-specificity in triggering leukotrienes or the mild nature of the subject’s illness, is not clear.

If inflammation is a key component of the abnormal response to virus infection leading to wheeze, then blocking their actions or their release could have therapeutic possibilities. IL-8 is a chemokine for neutrophils and if blocked, perhaps by a receptor anagonsit, could reduce the influx of neutrophils. Whether this in turn would reduce symptoms needs to be proved. If other cytokines and chemokines are involved in the response to virus infections then blocking a single chemokine may not be effective. The link between virus infection and activation of cells via the transcription factor NFkB (see section 1.3.3) may also have therapeutic implications. This transcription factor is associated with the cellular production of IL-8, IL-6 and ICAM-1 (the primary receptor for HRV) and turning this off therapeutically could be of
benefit to those who wheeze with virus infections. This may be over simplified, as other transcription factors such as those in the GATA family of transcription factors are known to be activated during virus infections\textsuperscript{103}. It must be borne in mind that the cellular response to virus infections may have some beneficial functions. Neutrophils are involved in virus clearance and blocking their influx could be detrimental.

10.3.3 Host versus virus factors

Questions still remain in several areas: the role of LRT infection in generating LRT symptoms; the possibility of an indirect link from the infected URT to the changes seen in the LRT; the interaction between virus and immune response in those who wheeze compared with those who do not.

There is still uncertainty as to the role of LRT infection in viral wheeze and viral induced asthma. This is a vital question in our understanding the mechanisms (direct or indirect) that lead to wheeze and in focusing research attention in the search for therapeutic interventions. A future study should incorporate bronchial biopsies before and after inoculation. Probes for \textit{in situ} hybridisation should ideally be validated prior to the study. An alternative approach might be to use electron microscopy to identify infected cells. However, definitive proof of \textit{in situ} infection may be hampered by issues such as infection possibly occurring in a patchy distribution.

To further study the details of virus-host interaction at a molecular level it may be necessary to develop a system where a virus' genome can be altered so as to enable experiments where different epitopes of virus are manipulated and their effects \textit{in vitro} are studied. Such a system would be dependent upon developing a cloned virus. This could have many beneficial applications in terms of:
- safety for human studies;
- manipulation of the virus for in-vitro investigation of the molecular interactions of virus with a wide range of host cells.

There are significant concerns regarding the safety of inocula for human studies. Virus inocula are traditionally developed either directly from an infected human subject or from sources of unknown origin grown in virology laboratories and open to the significant risk of contamination with potentially harmful agents (e.g. other viruses from laboratory equipment or workers, or prions if grown in sera). Despite the application of stringent safety testing, it is not possible to provide an absolute guarantee that known or unknown infectious agents do not contaminate the inocula. A cloned virus, if it possesses similar pathogenic characteristics as wild-type viruses might provide a way of taking this work forward.

10.4 Conclusions

This is the first experimental infection carried out on a group of adults with episodic viral wheeze and not classical atopic asthma. Physiological changes were demonstrated with increased bronchial responsiveness occurring during common cold infections and persisting for some time afterwards. Inflammatory changes, specifically a relative neutrophilia, were present in the LRT of those with viral wheeze. The role of neutrophilic inflammation in episodic viral wheeze requires further study. Viral wheeze is distinct from atopic asthma in that subjects are not necessarily atopic, do not have baseline bronchial hyperresponsiveness and are not characterised by increased sputum eosinophils or eosinophil activation during infection. Although virus genome can be identified in the LRT its significance in terms of generating a LRT response is uncertain. This model has enabled the characterisation of physiological and inflammatory changes seen in acute episodes of viral wheeze. More detailed characterisation of the inflammatory and immune response will be possible in such a model.
Implications

This model of episodic viral wheeze in adults using an experimental infection with HCoV 229E has various restrictions when drawing implications for clinical illness. Episodic viral wheeze is a condition predominantly affecting young children and occurs in response to a range of different virus URT infections. The mechanisms that underlie LRT symptoms and changes in airway physiology may be different to those seen in atopic asthma, without eosinophil driven inflammation. This could in part explain why low dose maintenance corticosteroids are not effective in childhood episodic viral wheeze $^9$. The high prevalence and morbidity for children with this illness and the physiological and inflammatory differences outlined in this and other studies $^{13}$ strongly supports the need for future research, both in natural infections in children and adults and using experimental models such as this.
PART V
Declaration

I designed the study with the aid of my supervisors, Professors Silverman and Myint. I recruited all the subjects, taking their histories and consent. Inoculation was performed by myself in the ‘Leicester Children’s Asthma Centre Cell Biology Laboratory’. All samples in the main study (urine, blood, nasal lavages, induced sputum) were collected, processed and stored by myself. All data entry and simple analyses was performed by myself.

I processed nasal lavages and sputum to produce cell cytospins, staining them to assess morphology. I counted all slides.

I learned the technique of RT-PCR for the identification of HCoV 229E under the guidance of Stephanie Euden (Department of Microbiology, University of Leicester). Adjustments to the assay were made in conjunction with Dr Howard Pringle (Department of Pathology, University of Leicester). I performed the majority of RT-PCR assays.

Under the guidance of Dr Pringle and the MLSOs working in his laboratory (Mrs Linda Potter and Angie Gillies) I performed all the trials of in situ hybridisation, extracted RNA from the biopsies and performed RT-PCR for virus genome.

Dr Colin Hewitt (Department of Immunology, University of Leicester) and myself supervised Ms Melanie Leach (technician, Department of Child Health) in her work.

The following work was performed by other people:

- Preparation of virus inoculum – Mrs Stephanie Euden under the supervision of professor Myint.
- ELISA for HCoV antibody – Ms Melanie Leech.
- ELISA for cytokines – Ms Melanie leech and Alyson Huntly (postdoctoral research fellow, Department of Child Health).
- Inulin assay was kindly done by Dr Paul Whitakker (Senior Biochemist, Leicester Royal Infirmary)
- Bronchoscopy and bronchial biopsies – Professor Andrew Wardlaw (Department of Respiratory Medicine, University of Leicester).
- Biopsy processing was done by the Department of Pathology, Glenfield Hospital.
- Advanced statistical analysis (repeated measures and MANOVA) – Dr Paul Lambert (The Trent Institute).
- A double check of the in situ hybridisation technique using the 4-probe cocktail was performed by Dr Leon Hall (Department of Pathology, University of Leicester).
Acknowledgements

I would like to express my gratitude to the following people, without whom this thesis would not have been possible:

Professor Michael Silverman — for his warm support and constant encouragement throughout the project from design the writing of thesis. He has been and continues to be a source of inspiration.

Professor Steven Myint — for his kind support in all aspects of virology within the project.

Dr Colin Hewitt — for his frequent supportive advice on laboratory assays.

Mrs Stephanie Euden — for her kind support in Professor Myint’s laboratory and for preparing and safety testing the virus inoculum.

Melanie Leech and Alison Hunter – for their hard work in performing assays for HCoV antibodies and the ELISAs for cytokines.

Dr Paul Whitakker – for kindly performing the inulin assay.

Dr Ian Pavord and Mr Richard Ward – for teaching me how to perform sputum induction, and for Mr Ward for validating my ability as a cell counter.

Professor Andrew Wardlaw – for his support in performing the bronchial biopsies.

The nursing staff of the endoscopy suite, Glenfield Hospital – for their excellent care of the subjects undergoing bronchoscopy.

Dr Howard Pringle – for supervising the design of primers for RT-PCR and for his advice and support in identifying HCoV in the biopsies. Also to Mrs Linda Potter and Angie Gillies (MLSOs) working in Dr Pringle’s team for their kind and patient assistance whilst I did the in situ hybridisation and Southern blotting, and for their slicing and mounting sections of the biopsies.

Dr Jonathan Grigg – for his critical thoughts throughout the project and assistance with laboratory equipment.

Dr Paul Lambert – for his kind assistance with statistical analysis.

A special thanks to the subjects that volunteered.

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The National Asthma Campaign (UK) – for their extremely generous financial support.

Finally I would like to thank members of my family: my father for his constant support; my wife Cristina for listening with patience to endless words of excitement, frustration and boredom regarding viral wheeze (she also suffered more than her fair share of colds!); and
finally to my three daughters, Tessa, Anna and Rose, for cheering me up and taking my mind of work.
Appendices

Appendix 1  Publications and presentations resulting from this thesis

Publications:


Presentations/Posters


- An adult model of coronavirus-induced viral wheeze: changes in bronchial responsiveness. Mckean MC, Myint S., Silverman M.*
• An adult model of coronavirus-induced viral wheeze: symptomatic and functional response. Mckean MC, Myint S., Silverman M.*

• Experimental common cold - inoculation by drops or spray? Mckean MC, Myint S., Silverman M.*

• Downregulation of serum proteins during experimental coronavirus colds identified by proteome analysis. Grigg J., Mckean M.C., Silverman M., Myint S., Parekh R., Dwerk R.A., Heberstreit H.*

* Poster presentations accepted for the European Respiratory Society International Conference, Madrid 1999.

• Inhaled corticosteroids in episodic viral wheeze of childhood. Oral presentation at the Royal Society of Medicine, during the first Cochrane Airways Group International Conference, 1999.
Appendix 2 Laboratory work

Appendix 2.1 DEPC RNAse treatments.

All manipulations involving DEPC was performed in a fume cupboard wearing protective goggles and gloves.

**A. DEPC treatment of eppendorf tubes**

i) 6 litres of distilled water was decanted into a clean plastic receptacle of sufficient volume (e.g. a washing-up bowl) and 6ml of DEPC added. The correct quantity of 0.5ml or 1.5ml eppendorfs and their respective containers (e.g. clean coffee jars) were then fully immersed and left to soak overnight in the DEPC water.

ii) The next day, the eppendorfs and their containers were transferred to a fresh RNAse free washing-up bowl containing 6 litres of fresh distilled water and rinsed. The rinsed eppendorfs and containers were then transferred to another RNAse free washing-up bowl and rinsed again in 6 litres of fresh distilled water. This was repeated so that three rinses in distilled water were completed. Finally, the Eppendorfs and containers were transferred into an empty RNAse free washing-up bowl and drained of any excess distilled water.

iii) At this point, the DEPC treated containers were filled with their respective DEPC treated eppendorfs and autoclaved (B&T autoclave) at 120°C for 15 minutes. When cool the autoclaved containers were transferred to a hot air oven and heated at 120°C for 7 hours to sterilise and dry the eppendorfs. When cool the eppendorfs were ready for use.

**B) DEPC treatment of distilled water**

i) 1 litre of distilled water was decanted into a clean plastic receptacle of sufficient volume (e.g. a washing-up bowl) and 1 ml DEPC added. The lids from 30 x 3ml glass bijoux were then removed, and the bijoux plus lids immersed in the DEPC water and left to soak overnight.

ii) After overnight soaking, bijoux were filled with approximately 2ml of the DEPC treated water in which they had been soaking and their lids replaced. The bijoux were then autoclaved at 120°C for 15 minutes (B&T autoclave).

iii) After autoclaving, the bijoux were allowed to cool and then stored at room temperature until required. Each bijoux was opened once only and immediately discarded after use.
Appendix 2.2  Primers used.

Primers were designed with the following considerations:

- primers should be approximately 20 base pairs in length
- primer annealing temperatures should be between 50 and 64°C
- primers should not contain palindromic sequences
- neither termini of the primer should be complimentary
- primer pairs should no form 3' - 5' concentrates
- primers should have similar Tₘ values.

All primers/probes were designed complimentary to the N-gene of HCoV 229E published on the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/BLAST). Primers were checked using both the NCBI ‘BLAST’ search and the ‘FASTA’ search engine database from the EMBL outstation, European Bioinformatics Institute. No cross-reactivity was found to any of the primers or probes.

PCR primers against HCoV 229E
Manufactured by Sigma-Genosys Ltd, Cambridge, UK.

Primer 0647 = Downstream primer used for reverse transcription:
5’ TGC ACT AGG GTTAATGAAGAGG 3’ – position 1200 - 1222

Primer 8 = Downstream primer used for PCR:
5’ CCTGC ACTTCCAAGTTGTTG3’ – position 960 – 980

Primer 7 = Upstream primer used for PCR:
5’T GTT GAAGAACA CCTGACTCC 3’ – position 482 - 501

Primer 5 = Internal upstream primer used for 2nd round of nested PCR:
5’ CTCGGAATCCTTCAAGTGAC 3’ – position 646 - 665

PCR primers used against GAPDH
Manufactured by Sigma-Genosys Ltd, Cambridge, UK.

Forward primer = 5’ AGAAC ATCAT C C TGCTC 3’
Reverse primer = 5’ G CCAAATTCGTTGTCATA CC 3’

Oligo-probes for in situ hybridisation.
Oligo-probes were manufactured commercially and HPLC purified by Operon Technologies (CA, USA). The probes used were as follows:

A. 5’ ACT ACA CTG TAG ATT ACA GT 3’ – complimentary to ‘N-gene’ 911-930
B. 5’ ACC TTC ACG TCC ACA ACA CC 3’ - complimentary to ‘N-gene’ 968 - 987
C. 5’ TAC CAC AAT TTC GAT TTC CG 3’ - complimentary to ‘N-gene’ 991 – 1010
D. 5’ CGT TGT GAC ACC AGA ACT GA 3’ - complimentary to ‘N-gene’ 1087 –1106

Internal probes used for Southern blotting:
Manufactured by Sigma-Genosys Ltd, Cambridge, UK.

PCR product = 666 – 980

Probe A = 5’ ACT ACA CTG TAG ATT ACA GT 3’ – complimentary to ‘N-gene’ 911-930
Probe 0657 = TTT GGA AGT GCA GGT GTT GTG G 3’ - complimentary to ‘N-gene’ 885-906
Appendix 2.3 OptEIA™ set assay protocol (PharMingen, San Diego, CA, USA).

1) Microwells are coated with 100 µl per well of Capture Antibody diluted in Coating Buffer. Sealed plates and incubated overnight at room temperature.

2) Aspirate wells and wash 3 times with ≥300 µl/well Wash Buffer. After last wash, invert plate and blot on absorbent paper to remove any residual buffer.

3) Block plates with ≥200 µl/well Assay Diluent. Incubate at room temperature for 1 hr.

4) Repeat the aspiration/ wash as in step 2.

5) Prepare standard and sample dilutions in Assay Diluent or other appropriate diluent.

6) Pipette 100 µl of each standard, sample, and control into appropriate wells. Seal plate and incubate for 2 hr at room temperature.

7) Aspirate/ wash as in step 2, but with 5 total washes.

8) Add 100 µl of Working Detector, to each well (Note: Simultaneous incubation for Biotin/ Avidin reagents, saves a step). Seal plate and incubate for 1 hr at room temperature.

9) Aspirate/ wash as in step 2, but with 7 total washes. Soak wells for 30 seconds to 1 minute for each wash.

10) Add 100 µl of TMB Substrate Solution to each well. Incubate plate (without plate sealer) for 30 min at RT in the dark.

11) Add 50 µl of Stop Solution to each well.

12) Read absorbance at 450 nm within 30 min of stopping reaction.

Protocol Footnotes
a. 0.2 M Sodium Phosphate Buffer, pH 6.5 or 0.1 M Sodium Carbonate Buffer, pH 9.5.
b. PBS with 0.05% Tween-20.
c. 10% fetal bovine serum (Hyclone Cat #SH30088) in PBS, pH 7.0. PharMingen's Assay Diluent (Cat. #2641 1 E) recommended.
d. Overnight incubation for the following Sets: Mouse TCA3, Human sCD23.
e. Assay Diluent spiked with required volumes of Detection Antibody, Enzyme Reagent (for Biotin assays).
f. Tetramethylbenzidine and Hydrogen Peroxide. PharMingen's TMB Substrate Reagent Set (Cat. #2642KK) recommended.
g. 2M Sulfuric Acid or 1 M Phosphoric Acid.
Appendix 2.4 UniCAP enzyme immunoassay for ECP
Methodology taken from UniCAP, Pharmacia, Uppsala, Sweden

1. Aliquots of nasal lavage and sputum supernatants were defrosted.
2. Samples were tested undiluted, although if greater than 200μg/l they were diluted with the
diluent provided (UniCAP diluent) and repeated.
3. Subject samples and recombinant ECP standards were tested in duplicates. See appendix
2.5 for calibration of the assay.
4. The following were added to the UniCAP 100 (Pharmacia, Uppsala, Sweden) machine per
sample:
   • Sample 40μl
   • Enzyme-anti-ECP 50μl
   • Development solution 50μl
   • Stop solution 60μl

5. Fluorescence was read by the UniCAP 100, which automatically calculated the ECP
concentration from the calibration curve.
Appendix 2.5 Calibration of the UniCAP 100 assay for ECP.

1. Run 1:
- Full calibration curve (2, 5, 15, 100, 200 μg ECP/L, in duplicates - this is set as default in the software when a calibration curve is requested).
- Pharmacia ECP Control in four replicates
- At least six individual patient samples in duplicate

2. Criteria for acceptance of Run 1:
- The instrument software accepts the curve

3. Run 2 and 3:
- Curve control (two replicates only)
- Pharmacia ECP Control in four replicates
- At least six individual patient samples in duplicate

4. Criteria for acceptance of Runs 2 and 3:
- Curve control is within range, the run is accepted by the instrument software
- The mean value for Pharmacia ECP Control is within given range

1. Criteria for total acceptance of UniCAP ECP:
- Criteria for Runs 1 through 3 are fulfilled
- Pooled coefficient of variation (cv) within assay runs are ≤6% for Pharmacia ECP Control and patient samples
- Total cv (%) for Pharmacia ECP Control and patient samples are ≤10%

Calibration results.

i) Calculation of coefficient of variation (CV) with two replicates

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>M&lt;sub&gt;Tot&lt;/sub&gt;</th>
<th>V&lt;sub&gt;Within&lt;/sub&gt;</th>
<th>V&lt;sub&gt;Mean&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.92</td>
<td>2.40</td>
<td>2.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.79</td>
<td>2.47</td>
<td>2.35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2.86</td>
<td>2.43</td>
<td>2.32</td>
<td>2.54</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>Variance</td>
<td>0.008</td>
<td>0.002</td>
<td>0.004</td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Intra-assay CV (%) = 100(\(\frac{V_{Within}}{M_{Tot}}\))^{1/2} = 2.8%

Inter-assay CV (%) = 100(\(\frac{V_{Mean} + 0.5 \times V_{Within}}{M_{Tot}}\))^{1/2} = 9.3 %
Intra-assay CV (%) = \( \frac{100 \sqrt{V_{\text{within}}}}{M_{\text{Tot}}} \) = 4.9 %

Inter-assay CV (%) = \( \frac{100 \left( V_{\text{Mean}} + 0.75 \times V_{\text{within}} \right)^{1/2}}{M_{\text{Tot}}} \) = 4.9 %

\( V_{\text{within}} \) = Mean of the variances; \( V_{\text{Mean}} \) = Variance of the run means; \( M_{\text{Tot}} \) = Mean of the run means.

All duplicate results for the 6 subject samples were within 10% of each other. The assay was thus validated and samples analysed.
Appendix 2.6 10% Formol saline.

Methods taken from Leicester University Department of Pathology

1. Rinse out the empty carbuoy in the prep room with tap water.
2. Add 85g sodium chloride (analar) and start to fill up the carbuoy from the tap in the fume hood in the prep room.
3. Add 1 litre of formaldehyde (40%) and made the volume up to 10 litres with tap water.
4. Screw the lid on tightly and place the carbuoy on the worktop next to the fume hood.
Appendix 2.7 Preparation of probe cocktail and running of test strip

Digoxigenin labelling and production of a probe cocktail

1. A cocktail of the probe was produced by adding the following sterile eppendorphs:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1μg of oligo-probes (i.e. 0.334μg of each oligo-probe)</td>
<td>x = 8.6μl</td>
</tr>
<tr>
<td>5x terminal transferase (Tdt) buffer (Gibco)</td>
<td>4μl</td>
</tr>
<tr>
<td>25mM cobalt chloride (Boehringer-Mannheim)</td>
<td>4μl</td>
</tr>
<tr>
<td>1μM digoxigenin-11-dUTP (1nmol/μl, Boehringer-Mannheim)</td>
<td>1.7μl</td>
</tr>
<tr>
<td>5mM 2'-deoxyadenosine 5' triphosphate (dATP, Boehringer-Mannheim)</td>
<td>1.7μl</td>
</tr>
<tr>
<td>45-50U Tdt enzyme (Gibco)</td>
<td>y = 3.3μl</td>
</tr>
<tr>
<td>Sterile ultrapure water</td>
<td>w = 0.7μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>25μl</td>
</tr>
</tbody>
</table>

1. The tubes were whirlly mixed for 5 seconds and incubated for 15 minutes at 37°C on a Hybaid PCR machine.
2. 1μl of 0.5M EDTA pH8.0 buffer was added to all tubes to stop the Tdt activity.
3. 25μl of sterile ultrapure H2O was added to make a final concentration of probe cocktail of 20ng/μl.
4. Probe cocktail was stored in aliquots at −20°C.

Test strip

1. Diluent and serial dilutions of probe cocktail were prepared as follows:

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>sterile ultrapure H2O</td>
<td>1360μl</td>
</tr>
<tr>
<td>20 x SSCb</td>
<td>600μl</td>
</tr>
<tr>
<td>denatured ssDNAc</td>
<td>40μl</td>
</tr>
</tbody>
</table>

Serial dilutions -

| (1) | 1 ng of probe = 1μl of probe cocktail + 19μl diluent |
| (2) | 100pg , , , 5μl of (1) + 45μl , , , |
| (3) | 50pg , , , 25μl of (2) + 25μL , , , |
| (4) | 10pg , , , 10μl of (3) + 40μl , , , |
| (5) | 1pg , , , 5μl of (4) + 46μl , , , |
| (6) | 0.5pg , , , 25μl of (5) + 25μl , , , |

2. 1μl of each dilution was spotted onto a strip of nitrocellulose (Sartorius, Roche Diagnostics, Lewisham, UK).
3. The strip was baked at 80°C for 2 hours between 2 pieces of filter paper.
4. The strip was placed in a pre-warmed trough of Blocking Solution at 37°C for 20 minutes.
5. The strip was baked at 80°C for 20 minutes.
6. The test strip was placed in a trough of Blocking Solution at room temperature for 10 minutes.
7. The test strip was placed on a perspex surface and carefully covered for 30 minutes with anti-digoxigenin-alkaline phosphatase antibody conjugate (Boeringer-Mannheim) which was prior diluted 1:600 with Blocking Solution. Care was taken to make sure the strip did not dry out over this period.
8. The strip was then washed in TBS buffer for 5 minutes, twice followed by sterile ultrapure H2O for 5 minutes, twice.
9. The strip was then immersed in Substrate Buffer for 5 minutes.
10. The strip was then immersed in Substrate Solution for up to 2 hours.
11. When the colour reaction was judged to have reached its maximum (a dark purple colour) the strip was washed with sterile ultrapure H2O, air dried and covered in Saran wrap.
Footnotes:

a. 0.5M EDTA buffer: add 93.05g EDTA.2H2O (Sigma) to 400ml distilled H2O; stir vigorously; adjust pH to 8.0 with 10mM NaOH; aliquot and autoclave.

b. 20 x SSC (sodium citrate 0.3M (Fisons, Loughborough, UK)/sodium chloride 3M (Fisher, Loughborough, UK) pH7.0): dissolve 87.65g of NaCl and 44.1 of Na-citrate in 400ml sterile H2O; adjust pH to 7.0 with 10mM NaOH; adjust to 500ml with sterile H2O; aliquot and autoclave.

c. Denatured ssDNA (salmon sperm DNA (Sigma Aldrich)): warm ssDNA to 99°C for 5 minutes, then place on ice.

d. Blocking Solution: made up the day prior to the assay – 3% Bovine serum albumin + 0.1% TritonX (Sigma Aldrich) in TBS buffer (0.1M Tris-HCl (Sigma Aldrich) + 0.4M NaCl adjusted to pH 7.5); mixed with a magnetic stirrer, filtered and stored for up to 48 hours.

e. Substrate Buffer: 0.1M Tris-HCl + 0.1M NaCl + 0.05M MgCl2 (Sigma Aldrich) in DEPC H2O.

f. Substrate (detection) solution: 88μl nitroblue tetrazolium (NTB) (Sigma Aldrich) + 66μl 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) (Sigma Aldrich) + 20ml Substrate Buffer.
**Appendix 2.8 Solutions used during *in situ* hybridisation**

**Pre-hybridisation Solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.4ml</td>
</tr>
<tr>
<td>10 x PE</td>
<td>2ml</td>
</tr>
<tr>
<td>50% Dextran sulphate/DEPC</td>
<td>4ml</td>
</tr>
<tr>
<td>10mg/ml ss DNA</td>
<td>300μl</td>
</tr>
<tr>
<td>Formamide</td>
<td>6ml</td>
</tr>
<tr>
<td>DEPC water</td>
<td>5.3 ml</td>
</tr>
</tbody>
</table>

*Note:* stored at 4°C.

**Post-hybridisation washes**

2x SSC (sodium citrate. Sodium chloride)/30% formamide:
- Make up 20x SSC (see appendix 2.8 footnote b)
- Add 20ml 20x SSC to 120ml DEPC H₂O and 60ml formamide

Blocking solution – see appendix 2.7 footnote d

Anti-digoxigenin-alkaline phosphatase antibody conjugate (Roche Diagnostics, No 1093274)

TBS Buffer - see appendix 2.7 footnote d

Substrate Solution - see appendix 2.7 footnote f

Substrate Buffer - see appendix 2.7 footnote
Appendix 2.9  Preparation of cell cultures infected with HCoV 229E as positive controls for \textit{in situ} hybridisation.

1. HEL cells (Biowhittaker, Wokingham, UK.) were grown in Eagle's MEM (Minimum Essential Medium, Gibco Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum and twice the recommended concentration of aminoacids and vitamins (Gibco Life Technologies) (Hierholzer, 1976).

2. Cells were cultured at 37°C, in a 5% CO$_2$, 100% humidity atmosphere until confluent.

3. Twenty-four hours before infection with HCoV the monolayers were washed with serum free medium. The medium was replaced with EMEM supplemented with 0.4% ultroser G, 2% amino acids and vitamins.

4. Cells were infected with HCoV 229E (ATTC, Rockville, Maryland), at a concentration of 10$^4$ TCID$_{50}$ml$^{-1}$ and cultured at 33°C, in 5% CO$_2$. Virus was harvested from the cultures after 48 hours.
Appendix 2.10  Haematoxylin and Eosin staining.  
Taken from the Department of Pathology, University of Leicester.

For paraffin wax sections:
1. De-wax and rehydrate sections by passing the slide rack through a series of staining dishes containing the following solvents:
   Xylol 3 min
   Xylol 3 min
   99% I.M.S. 1 min
   99% I.M.S. 1 min
   95% I.M.S. 1 min
   Running tap water 1 min
2. Place slide rack in Haematoxylin 5 min
3. Rinse slides in tap water, then leave to 'blue', in running tap water for 1 - 2 minutes.
4. Place slides in Eosin 1 min
5. Rinse slides in running tap water 30 secs
* NB. Eosin is quickly removed by water. Prolonged immersion in 95% I.M.S. will also remove the stain.
6. Dehydrate and clear sections by passing slide rack through the series of staining dishes.
   95% I.M.S. 15 secs
   99% I.M.S. 1 min
   99% I.M.S. 1 min
   Xylol 3 min
   Xylol (for mounting)
1. Mount slides by placing DPX mountant onto each coverslip using an orange stick.
7. Lower slide onto coverslip with section downwards, allow DPX to spread out, then press gently to remove any air bubbles. Leave slides to dry in slide tray.
8. Write labels with block number, H&E. and date, using a fmc tipped waterproof pen. Store in numerical order in the H&E slide file, if required.

Expected Staining results:
Nuclei blue
Muscle dark pink
Collagen light pink
Red blood cells orange
Cytoplasm various shades of pink
Appendix 2.11  10 x Alec Jeffeys (AJ) Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris pH 8.8</td>
<td>900μl</td>
</tr>
<tr>
<td>1M (NH₄)₂SO₄</td>
<td>200μl</td>
</tr>
<tr>
<td>1M MgCl₂</td>
<td>90μl</td>
</tr>
<tr>
<td>100mM dNTP's</td>
<td>40μl each</td>
</tr>
<tr>
<td>20mg/ml bovine serum albumin</td>
<td>110μl</td>
</tr>
<tr>
<td>Neat (14.3M) βME (add last)</td>
<td>9.4μl</td>
</tr>
<tr>
<td>1mM EDTA (1:500 dilution with 0.5μ EDTA)</td>
<td>8.8μl</td>
</tr>
<tr>
<td>Sterile ultrapure water to 2ml</td>
<td>501.8μl</td>
</tr>
</tbody>
</table>

Mix well and aliquot in 10 eppendorphs of 200 μl each, stored at -20°C.
Appendix 2.12 Reagents used for southern blotting

All reagents were made according to manufacturer’s instructions (Nylon Membranes, positively charged, Boehringer-Mannheim, Cat. No. 1209 272).

Blocki ng stock sol ution: blocking reagent was dissolved in buffer 1 to a final concentration of 10% (w/v) with shaking and heating in a microwave oven. The stock solution was autoclaved and stored at 4°C.

Hybridisation buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Final conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 x SSC</td>
<td>7.5ml</td>
<td>5 x SSC</td>
</tr>
<tr>
<td>ssDNA (10mg/ml)</td>
<td>90μl</td>
<td>30μg/ml</td>
</tr>
<tr>
<td>30μl sarcosyly</td>
<td>30μl</td>
<td>0.1%</td>
</tr>
<tr>
<td>10% SDS</td>
<td>60μl</td>
<td>0.02% SDS</td>
</tr>
<tr>
<td>10% blocking solution 6 ml</td>
<td>x ml</td>
<td>2%</td>
</tr>
<tr>
<td>formamide</td>
<td>x ml</td>
<td>10/20/30%</td>
</tr>
<tr>
<td>sterile ultrapure H₂O yml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

20 x SSC—see appendix 2.8b
10% SDS – 50g sodium dodecyl sulphate (Sigma Aldrich, L-4509) in 450 ml DEPC H₂O.

Buffer 1: 0.1M maleic acid, 0.15M NaCl, pH 7.5 (NaOH), autoclaved.
Buffer 2: blocking stock solution, diluted 1:10 in buffer 1 (final concentration = 1% blocking reagent).
Buffer 3: 100mM Tris-HCl, 100mM NaCl, 50mM MgCl₂, pH9.5 (20°C).

References
References


interactions with peptide-MHC class I tetrameric complexes is temperature dependent. *Journal of Immunology* 163:4342-4348.


*Allergy, Supplement.* 54: 112-115.


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